



**MEDICAL UNIVERSITY – PLOVDIV
FACULTY OF MEDICINE
DEPARTMENT OF CLINICAL LABORATORY**

Vesselina Stoyanova Koleva-Topova, MD

**COMPARATIVE EVALUATION OF THE CLINICAL
APPLICATION OF BIOMARKERS AND ALGORITHMS IN
ONCOGYNECOLOGY – CA125, HE4, ROMA AND CPH-I**

AUTHOR’S ABSTRACT
OF A DISSERTATION
FOR THE AWARD OF THE EDUCATIONAL AND SCIENTIFIC DEGREE
“DOCTOR” (PhD)

Supervisors:

Prof. Tanya Deneva, MD, PhD
Assoc. Prof. Pavel Bochev, MD, PhD

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The dissertation comprises 228 pages, 105 figures, and 53 tables. The bibliography includes 156 references, of which 1 is in Cyrillic and 155 in Latin script. Three publications and two scientific communications have been produced in connection with the dissertation.

The dissertation was discussed by the extended departmental council of the Department of Clinical Laboratory, Medical University – Plovdiv, and has been submitted for defense before a scientific jury composed of:

External members:

1. Prof. Krasimira Ikonomova-Shahova, MD, PhD
2. Prof. Adelaida Ruseva, MD, PhD
3. Assoc. Prof. Asya Konsulova-Kirova, MD, PhD
4. Prof. Daniela Gerova, MD, PhD

Internal members:

1. Assoc. Prof. Ivanka Nenova-Chilova, MD, PhD

Reserve members:

1. Assoc. Prof. Irena Gencheva-Angelova, MD, PhD
2. Assoc. Prof. Stoylka Mandadzhieva, MD, PhD

The public defense of the dissertation will take place on 11 May 2026 at 11:00 a.m. before the scientific jury in the Second Auditorium of the Auditorium Complex of the Medical University – Plovdiv, 15A Vasil Aprilov Blvd.

The materials for the defense are available at the Scientific Department of the Medical University – Plovdiv, 15A Vasil Aprilov Blvd., and are published on the website of the Medical University – Plovdiv: www.mu-plovdiv.bg

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LIST OF ABBREVIATIONS

In English

HE4 – Human Epididymis Protein 4
CA125 – Cancer Antigen 125
ROMA – Risk of Ovarian Malignancy Algorithm
CPH-I – Copenhagen Index
EOC – Epithelial Ovarian Carcinoma
WFDC2 – Whey acidic protein with Four Disulfide Core protein 2
CMIA – Chemiluminescent Microparticle Immunoassay
ECLIA – Electrochemiluminescent Immunoassay
LoD – Limit of detection
LLoQ – Lower limit of quantification
Se – Sensitivity
Sp – Specificity
AUC – Area Under the Curve
ROC – Receiver Operating Characteristic Curve
IQR – Interquartile Range
eGFR – Estimated glomerular filtration rate
 $\mu\text{mol/L}$ – micromoles per liter
 pmol/L – picomoles per liter
U/mL – units per milliliter

In Bulgarian

еОК – Епителен овариален карцином (Epithelial ovarian carcinoma)
ОК – Овариален карцином (Ovarian carcinoma)
ЕК – Ендометриален карцином (Endometrial carcinoma)
ДИ – Доверителен интервал (Confidence interval)
ДКЯ – Доброкачествена киста на яйчника (Benign ovarian cyst)
ДЧ – Диагностична чувствителност (Diagnostic sensitivity)
ДС – Диагностична специфичност (Diagnostic specificity)
ДЕ – Диагностична ефективност (Diagnostic efficiency)

INTRODUCTION

Gynaecological malignancies represent a significant medical and social problem due to their high incidence, unfavourable prognosis at advanced stages, and substantial impact on the quality of life of affected women. Among them, ovarian carcinoma (OC) is characterised by the highest mortality rate, which is mainly attributable to late diagnosis (more than 70% of patients are diagnosed at an advanced stage, when therapeutic options are limited and the 5-year overall survival rate is only 49%) and to the lack of effective screening strategies. One of the main reasons for delayed diagnosis is the presence of non-specific clinical symptoms in the early stages of the disease. This highlights the urgent need to identify reliable, highly sensitive and sufficiently specific biomarkers and algorithms that can be incorporated into the screening and diagnostic pathways for ovarian carcinoma.

In clinical practice, the diagnostic challenge most frequently arises during the evaluation of patients presenting with a pelvic mass, with the aim of achieving adequate preoperative triage and timely referral of high-risk patients to specialised gynaecological oncology centres. An incorrect assessment at this stage may result either in unnecessary aggressive surgical interventions in benign conditions or in a delay in appropriate oncological treatment.

The tumour marker CA125 is the longest-established serum biomarker used in ovarian carcinoma and, to date, remains the only one included in various international guidelines. Unfortunately, its diagnostic reliability is limited by low specificity, particularly in younger women and in the presence of benign conditions such as endometriosis, pelvic inflammatory disease, and pregnancy. This results in a high rate of false-positive results and complicates its interpretation as a standalone diagnostic tool. On the other hand, CA125 is expressed in only approximately 50% of early-stage ovarian carcinomas and shows marked variability depending on the histological characteristics of the tumour. These limitations necessitate the search for more specific and more sensitive biomarkers or the incorporation of additional diagnostic approaches, including combination with imaging modalities, in order to enhance its clinical applicability.

Human epididymis protein 4 (HE4) has been actively investigated since the beginning of the 21st century and, in 2010, was approved by the FDA (Food and Drug Administration, USA) as a marker for monitoring and detecting disease progression in patients with ovarian carcinoma. Accumulated evidence indicates that HE4 expression in healthy ovarian tissue is very low but increases significantly in malignant ovarian processes and in endometrial carcinoma, and considerably less frequently than CA125 in benign gynaecological diseases, including endometriosis. This makes HE4 a valuable biomarker, particularly in the premenopausal age group. Despite numerous published results from clinical trials and several meta-analyses in recent years, the data remain heterogeneous and inconclusive regarding to the clinical significance of HE4 when used as a standalone marker for the diagnosis and monitoring of patients with gynaecological malignancies. Additional limitations to its clinical use include its pronounced dependence on age and renal function, increased expression from the tracheal and bronchial mucosa (especially in smokers and in inflammatory respiratory diseases), as well as elevated levels in lung carcinomas. These factors necessitate the use of appropriate reference intervals and careful consideration of individual patient characteristics. To date, reliable information is lacking on how the use of reference values specific to the Bulgarian population would affect the clinical significance of CA125 and HE4.

The development of multimarker and multivariable models offers the potential to overcome the limitations associated with the standalone use of individual biomarkers; however, to date, no conclusive evidence has been published demonstrating the superiority of any specific model over others. Moreover, there is insufficient robust evidence supporting the use of these models for preoperative prognostication in terms of disease progression and survival. At present, the

only multimarker model implemented and used in routine clinical practice in Bulgaria is the Risk of Ovarian Malignancy Algorithm (ROMA), with different cut-off values applied depending on menopausal status and the analytical platform used. Despite its widespread adoption, ROMA has certain limitations related to the accurate definition of menopausal status, particularly in perimenopausal women, in those receiving hormonal therapy, or in women who have undergone hysterectomy.

The Copenhagen Index (CPH-I) represents an alternative multimarker model that incorporates HE4, CA125, and patient age, without the use of menopausal status. This approach has the potential to overcome some of the limitations of ROMA and to facilitate the practical application of the algorithm in heterogeneous clinical populations. Despite promising initial results, data on the clinical effectiveness of CPH-I remain limited, particularly outside the original populations in which the algorithm was developed and validated. The literature lacks established cut-off values for CPH-I that can be directly implemented in clinical practice, as reported thresholds vary widely among different authors. To test the hypothesis that the introduction of CPH-I into clinical practice would improve the early diagnosis of ovarian carcinoma and endometrial carcinoma, an analysis of the biomarkers CA125 and HE4 measured in the Bulgarian population is required, as well as an evaluation of their correlations when incorporated into mathematical algorithms such as ROMA and CPH-I. The application of CPH-I in laboratory practice using specific, population-adjusted predictive values could lead to the establishment of a simple and accessible method for the clinical selection of patients at high risk of ovarian or endometrial carcinoma who should be referred to specialised gynaecological oncology centres.

The use of HE4 as a prognostic and monitoring marker in ovarian carcinoma remains insufficiently studied, and the accumulation of data in this indication could fill an important diagnostic gap in patients who do not exhibit an increase in CA125 or in those in whom overall tumour burden and serum CA125 levels correlate with a low slope coefficient (usually reported as an increase in marker levels within reference ranges or as a delayed rise during disease progression). An increase in HE4, as well as in CA125, should not in itself prompt the initiation of treatment, but it does justify more intensive imaging investigations using more sensitive and specific modalities such as PET-CT and MRI. The identification of patients at higher risk at the time of diagnosis, followed by more targeted and rigorous monitoring, would offer significant clinical benefits. Determining the prognostic significance of these biomarkers with respect to disease progression and overall survival is expected to enable appropriate patient selection and identification for comprehensive oncological treatment.

The presented data substantiate the need for a comprehensive, systematic, and population-specific evaluation of the tumour markers CA125 and HE4 and of the algorithms ROMA and CPH-I. There is a lack of sufficient objective evidence regarding their application in the Bulgarian population, including their reference intervals, diagnostic performance in various clinical scenarios, and prognostic value in ovarian and endometrial carcinoma. The present doctoral dissertation aims to address these gaps by integrating diagnostic, prognostic, and monitoring approaches within a single comprehensive study.

AIM AND OBJECTIVES

Aim of the dissertation

The aim of the present doctoral dissertation is to perform a comparative evaluation of the clinical application of the tumour markers CA125 and HE4 and the multimarker algorithms ROMA and CPH-I in gynaecological oncology, with particular focus on their diagnostic, prognostic, and monitoring value in patients with ovarian and endometrial carcinoma, as well

as the potential for optimisation of preoperative triage and clinical interpretation through the use of population-specific reference intervals and cut-off values.

Objectives of the study

To achieve the stated aim, the following specific objectives were formulated:

1. **To establish reference intervals** for HE4, CA125, ROMA, and CPH-I specific to the Bulgarian population, and to evaluate the influence of age, menopausal status, pregnancy, and renal function on the values of these parameters.
2. **To assess the diagnostic performance** of HE4, CA125, ROMA, and CPH-I in differentiating ovarian carcinoma from benign ovarian cysts and from healthy women, both in the overall study population and after stratification according to menopausal status or tumour histological characteristics.
3. **To evaluate the prognostic significance** of preoperative marker levels and multimarker models with respect to overall survival.
4. **To assess the clinical applicability** of CPH-I, ROMA, and the standalone use of tumour markers in the diagnosis of patients with endometrial carcinoma.
5. **To evaluate the prognostic value** of HE4, CA125, ROMA, and CPH-I in patients with confirmed ovarian carcinoma regarding recurrence rate, time to progression, and overall survival, as well as the applicability and role of these markers in the monitoring of patients with ovarian carcinoma, including after the first and subsequent recurrences.

MATERIALS AND METHODS

Study design

Retrospective identification with prospective evaluation of time to recurrence, time to progression, and overall survival.

Study material

A retrospective analysis was performed of clinical data and results from tumour marker testing for CA125, HE4, and ROMA, as well as biochemical parameters used for the assessment of renal and hepatic function, available in the laboratory information system of the Diagnostic and Consultative Centre and University Hospital Acibadem City Clinic Tokuda for the period 2011–2023. In addition, the Copenhagen Index (CPH-I) was calculated.

Prospective data regarding time to recurrence, time to progression, and overall survival were collected until 31 July 2024.

Serum samples were collected, identified, processed, and stored prior to analysis in accordance with the approved standard operating procedures of the laboratory and in compliance with all requirements of the Medical Standard for Clinical Laboratory and the recommendations of the test manufacturers.

All patients signed a standard informed consent form and completed a questionnaire regarding menstrual status and previous medical history of gynaecological malignancy. These documents are part of the routine laboratory practice and the standard operating procedure for ROMA calculation and were not developed specifically for the purposes of the present study.

Inclusion criteria

The following inclusion criteria were applied to patients participating in the prospective part of the project:

- Patients with histologically confirmed epithelial ovarian carcinoma in whom CA125 and HE4 were measured at the time of diagnosis and who underwent radical hysterectomy with bilateral adnexectomy, without intraoperative evidence of residual disease (patients with residual disease or suboptimal cytoreduction were analysed in a separate group).
- Patients with recurrent or persistent ovarian carcinoma in whom HE4 and CA125 were measured or were being measured at the time of recurrence confirmation.
- Patients with isolated elevated levels of CA125 and/or HE4 prior to confirmation of recurrence.

Confirmation of recurrence was defined as either histological verification or unequivocal imaging findings. In cases with non-definitive imaging results, recurrence was accepted based on histological verification or documented radiological progression during follow-up. Patients with elevated tumour marker concentrations but negative computed tomography findings underwent FDG PET imaging. Patients with negative FDG PET/CT scans were classified as false-positive cases only after a minimum follow-up period of six months and exclusively in the absence of a rising trend in tumour marker levels. In cases of subsequent marker increase, repeat imaging evaluation was performed.

Exclusion criteria

- Patients with non-epithelial ovarian tumours.
- Patients without radical surgical removal of the uterus and ovaries.
- Patients with incomplete follow-up data.
- In patients in whom the absence of macroscopic disease was confirmed by FDG PET, treatment with metformin constituted an exclusion criterion.

Study population

A total of 1,647 women were included and distributed into the following groups:

- Healthy non-pregnant women – 246 (including 124 premenopausal);
- Healthy pregnant women – 52 (26 in the first trimester and 26 in the second trimester);
- Women without gynaecological disease and with varying stages of renal insufficiency – 94;
- Benign ovarian cysts – 942 (including 741 premenopausal);
- Epithelial ovarian carcinoma – 150;
- Endometrial carcinoma – 74;
- Other carcinomas or ovarian metastases – 47;
- Other non-gynaecological non-oncological diseases – 42.

Methods

Collection of clinical and demographic data

The required clinical and demographic data were obtained from the hospital information system. Data on overall survival were obtained through an official request to the National

Population Register (ESGRAON) of the Ministry of Regional Development and Public Works. Vital status (alive/deceased) was updated as of 1 August 2024.

Method for determination of HE4

In the present study, serum HE4 concentrations were measured using the Architect HE4 assay (Abbott Diagnostics), analysed on the integrated Architect ci4100 or Architect ci8200 systems. The analytical method is a chemiluminescent microparticle immunoassay (CMIA) that employs an acridinium-labelled monoclonal anti-HE4 antibody and anti-HE4 antibody-coated microparticles in phosphate-buffered saline (PBS) containing stabilised bovine protein.

Method for determination of CA125

Analytical determination of CA125 was performed using the Architect CA125 II assay (Abbott Diagnostics), based on chemiluminescent microparticle immunoassay (CMIA) technology.

Method for calculation of the ROMA algorithm

Calculation of ovarian malignancy risk is based on the concentrations of the two tumour markers in combination with the patient's menopausal status.

In the first step, the predictive index (PI) is calculated using one of the following equations:

Premenopausal patients

$$PI = -12 + 2.38 \times \text{LN}(\text{HE4}) + 0.0626 \times \text{LN}(\text{CA125})$$

Postmenopausal patients

$$PI = -8.09 + 1.04 \times \text{LN}(\text{HE4}) + 0.732 \times \text{LN}(\text{CA125})$$

Calculation of ROMA

$$\text{ROMA (\%)} = [\text{Exp}(\text{PI}) / (1 + \text{Exp}(\text{PI}))] \times 100, \text{ where } \text{Exp}(\text{PI}) = e^{\text{PI}}$$

The ROMA calculation formula is implemented as a software program integrated within the Architect/Alinity Abbott analytical platform. Results were reported as percentages.

Risk stratification into low- and high-risk groups for epithelial ovarian carcinoma was performed using cut-off values of 7.4% for premenopausal patients and 25.3% for postmenopausal patients, as recommended by the test manufacturer.

Method for calculation of the CPH-I algorithm

For calculation of the Copenhagen Index, the formula, described in the publication by Karlsen was applied:

$$\text{CPH-I} = -14.0647 + 1.0649 \times \log_2(\text{HE4}) + 0.6050 \times \log_2(\text{CA125}) + 0.2672 \times \text{age} / 10$$

$$\text{Predicted probability (PP)} = e^{(\text{CPH-I})} / [1 + e^{(\text{CPH-I})}] \times 100\%$$

For the purposes of the present study, dedicated software was developed in which the concentrations of both biomarkers and the patient's age were entered manually, and the program automatically calculated the CPH-I value. Results were reported as percentages.

Method for determination of serum creatinine

Serum creatinine was measured using a kinetic Jaffe method with alkaline picrate (Abbott Diagnostics reagents), calibrated with a calibrator traceable to the NIST SRM 967 WHO reference standard. For each creatinine result, estimated glomerular filtration rate (eGFR) expressed in mL/min was also reported, automatically calculated by the laboratory information system according to the CKD-EPI 2021 equation.

Statistical analysis

Statistical data processing was performed using the statistical software package SPSS version 25.0. A p -value < 0.05 was considered statistically significant.

Mean values of the analyzed variables are presented as mean \pm standard deviation (\pm SD).

The following statistical methods were applied:

- Descriptive analysis for determination of central tendency and dispersion parameters: arithmetic mean, median, mode, standard deviation, and interquartile range.
- Frequency analysis of variables grouped according to predefined factor variables.
- Analysis of empirical distribution type using the non-parametric Shapiro–Wilk and Kolmogorov–Smirnov tests.
- Correlation analysis using Spearman’s rho and Pearson’s correlation coefficients; interpretation of correlation strength was performed according to the rule of thumb.
- Regression analysis for continuous variables with distributions approximating normality and dichotomised regression analysis comparing high versus low values based on predefined cut-off thresholds.
- Non-parametric Kruskal–Wallis test for differences between independent samples.
- Graphical analysis including histograms for visualisation of empirical frequency distributions, scatter plots for assessment of relationships between continuous variables, and bar and pie charts for presentation of absolute and relative frequencies.

Ethical considerations

The study was approved by the Ethics Committee for Scientific Research at Acibadem City Clinic University Hospital Tokuda (Approval No. 58/15.02.2024).

RESULTS

1. Establishment of reference values for HE4, CA125, ROMA, and CPH-I specific to the Bulgarian population

For the derivation of reference intervals for the Bulgarian population for the parameters HE4, CA125, ROMA, and CPH-I, the cohort meeting the inclusion criteria of the present study consisted of 246 women aged between 20 and 82 years (mean \pm SD: 48.20 ± 13.04). The cohort was divided into two subgroups: premenopausal women ($n = 124$), aged 20–54 years (37.77 ± 8.13), and postmenopausal women ($n = 122$), aged 35–82 years (58.80 ± 7.26).

Statistical testing for normality of distribution, together with visual assessment of histograms and P–P plots, demonstrated the absence of a Gaussian distribution for HE4, CA125, ROMA, and CPH-I results within the reference groups. The Kolmogorov–Smirnov test rejected the null hypothesis of normal distribution for all four parameters ($p < 0.05$). Consequently, further statistical processing was performed after data transformation and using non-parametric analytical methods (significance level $\alpha = 0.05$). The derived upper reference limit corresponded to the 95th percentile with a 95% confidence interval.

The median and interquartile range (IQR) for HE4 in the entire cohort were 38.90 (33.50–47.43) pmol/L; in the premenopausal group 36.65 (31.70–42.35) pmol/L; and in the postmenopausal group 44.15 (37.20–54.33) pmol/L. The results of the Mann–Whitney test demonstrated significantly higher HE4 concentrations in postmenopausal women compared with premenopausal women ($U = 4391.5$, $p < 0.001$, $r = -0.362$). Based on these findings, reference intervals were established according to menopausal status. The upper reference limit

for HE4 in the entire cohort was 62.43 (59.02–65.45) pmol/L. The menopausal status–specific upper reference limits for HE4 were: premenopause < 53.22 (50.80–60.82) pmol/L and postmenopause < 65.31 (61.57–68.35) pmol/L.

The median and interquartile range for CA125 in the entire cohort were 12.00 (8.25–17.05) U/mL; in the premenopausal group 13.10 (9.20–19.58) U/mL; and in the postmenopausal group 9.60 (7.10–14.40) U/mL. The Mann–Whitney test demonstrated significantly lower CA125 concentrations in postmenopausal women compared with premenopausal women ($U = 3826.0$, $p < 0.001$, $r = -0.261$). The established upper reference limit for CA125 in the entire study population was 26.66 (24.66–28.73) U/mL. Menopausal status–specific upper reference limits for CA125 were: premenopause < 27.94 (25.30–30.99) U/mL and postmenopause < 22.42 (19.65–27.72) U/mL.

The median and interquartile range for ROMA in the entire cohort were 4.94% (3.27–7.39); in the premenopausal group 3.65% (2.64–5.14); and in the postmenopausal group 7.68% (5.14–9.70). The Mann–Whitney test demonstrated significantly higher ROMA values in postmenopausal women compared with premenopausal women ($U = 1681.0$, $p < 0.001$, $r = -0.589$). The upper reference limit of ROMA in the entire cohort was determined to be 12.45% (11.33–13.63). Menopausal status–specific reference limits for ROMA were: premenopause < 8.71% (7.98–10.80) and postmenopause < 13.70% (12.27–14.66).

The median and interquartile range for CPH-I in the entire cohort were 0.60% (0.39–1.00); in the premenopausal group 0.495% (0.353–0.783); and in the postmenopausal group 0.855% (0.513–1.438). The Mann–Whitney test demonstrated significantly higher CPH-I values in postmenopausal women compared with premenopausal women ($U = 3114.0$, $p < 0.001$, $r = -0.366$). The determined upper reference limit (95% CI) for CPH-I in the entire cohort was 2.869% (2.555–3.207). The menopausal status–specific upper reference limits (95% CI) for CPH-I were: premenopause < 2.332% (1.937–2.656) and postmenopause < 3.333% (2.869–3.699).

A summary of the data for the upper reference limits of each biomarker and algorithm in the two groups—premenopausal and postmenopausal women—is presented in the following figure (Figure 1).

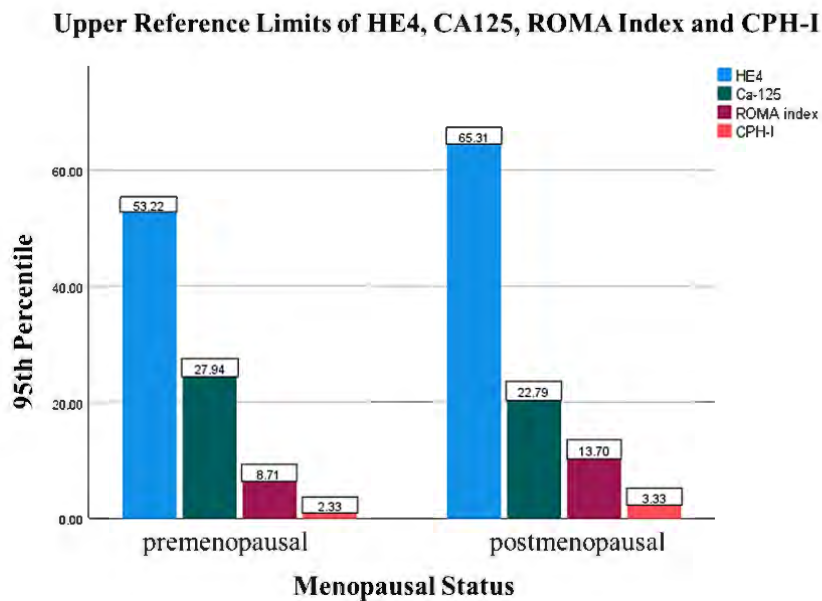


Figure 1. Upper reference limits of HE4, CA125, ROMA index, and CPH-I stratified by menopausal status

1.1. Assessment of the relationship between age and the values of HE4, CA125, ROMA, and CPH-I

To accomplish this subtask, the Spearman rank correlation coefficient (Spearman's rho) was used. Correlation analysis demonstrated statistically significant positive correlations between age and HE4, the ROMA index, and CPH-I ($\rho = 0.430$, $p < 0.001$; $\rho = 0.562$, $p < 0.001$; and $\rho = 0.498$, $p < 0.001$, respectively), as well as a statistically significant negative correlation with CA125 ($\rho = -0.244$, $p < 0.001$) (Figure 2).

To assess the strength of this association, the rule of Thumb was applied. A correlation coefficient of 0.43 at $p < 0.0001$ confirms the findings of the non-parametric analysis, indicating a positive correlation between HE4 concentration and patient age.

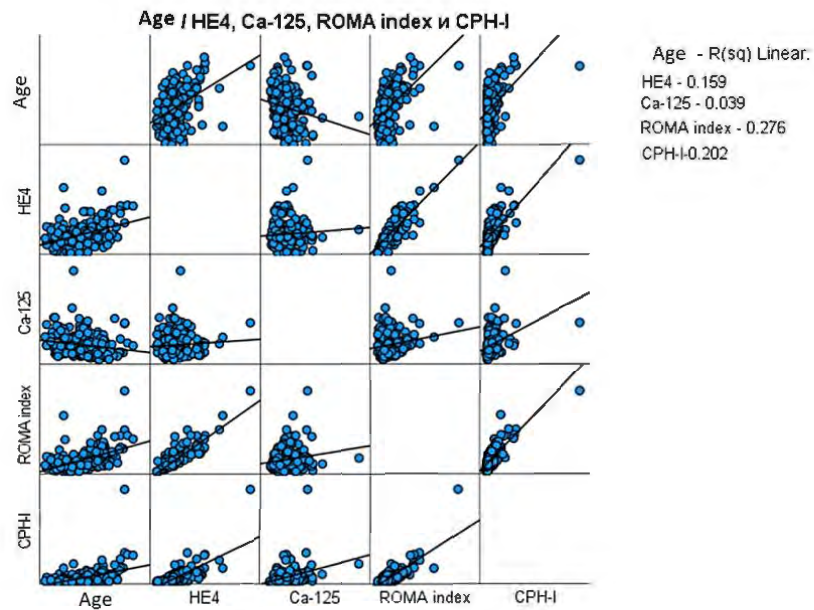


Figure 2. Age-related distribution of HE4, CA125, ROMA index, and CPH-I

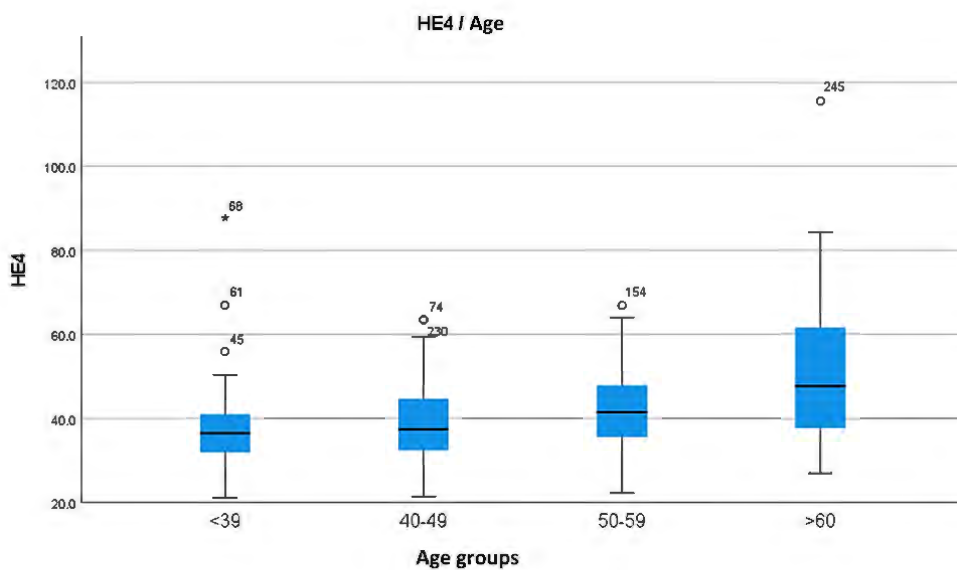


Figure 3. Distribution of HE4 concentrations across age groups

To address whether age-dependent reference values for HE4 are required, the cohort was divided into four age groups, irrespective of the menopausal status of the examined women: ≤ 39 years (N = 68), 40–49 years (N = 56), 50–59 years (N = 69), and ≥ 60 years (N = 52).

According to age, the median and interquartile range of HE4 were as follows: in individuals aged ≤ 39 years, 36.50 (31.83–41.00) pmol/L; in the 40–49-year age group, 37.35 (32.05–44.70) pmol/L; in the 50–59-year age decade, 41.40 (35.50–47.70) pmol/L; and in individuals ≥ 60 years, 47.70 (37.65–61.50) pmol/L.

The Kruskal–Wallis test demonstrated a statistically significant difference in HE4 results among the age-stratified patient subgroups ($\chi^2(3) = 33.51$, $p < 0.001$). Following post hoc testing, this difference was found to be statistically significant between the following group comparisons: <39 years versus 50–59 years ($U = 1582.5$, $p < 0.001$, $r = -0.281$), <39 years versus ≥ 60 years ($U = 830.0$, $p < 0.001$, $r = -0.462$), and 40–49 years versus ≥ 60 years ($U = 790.0$, $p < 0.001$, $r = -0.403$).

Age-stratified reference limits (95% CI) for HE4 were established as follows: <39 years – <54.86 (48.93–73.16) pmol/L; 40–49 years – <57.64 (51.47–60.82) pmol/L; 50–59 years – <60.85 (56.06–63.15) pmol/L; and ≥ 60 years – <68.84 (63.84–71.63) pmol/L (Figure 3).

According to age, the median and interquartile range of CA125 were as follows: in individuals ≤ 39 years, 13.20 (9.85–19.90) U/mL; in the 40–49-year age group, 11.90 (8.70–19.00) U/mL; in the 50–59-year age decade, 10.10 (7.40–14.80) U/mL; and in individuals ≥ 60 years, 9.40 (6.80–15.10) U/mL.

The Kruskal–Wallis test demonstrated a statistically significant difference in CA125 values among the age-differentiated patient subgroups ($\chi^2(3) = 11.02$, $p = 0.012$). Post hoc analysis demonstrated that this difference was statistically significant only for the comparison between the <39 -year and 50–59-year groups ($U = 1210.0$, $p = 0.005$, $r = -0.258$).

Age-stratified reference limits (95% CI) for CA125 were determined as follows: <39 years – <29.15 (24.66–33.87) U/mL; 40–49 years – <26.95 (23.73–29.35) U/mL; 50–59 years – <23.30 (20.45–28.73) U/mL; and ≥ 60 years – <27.72 (19.51–30.08) U/mL (Figure 4).

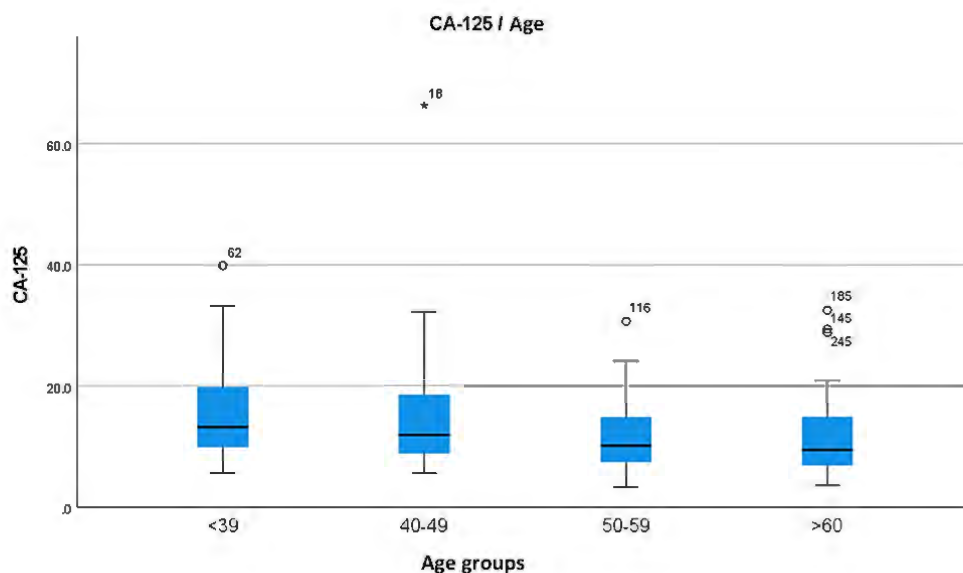


Figure 4. Distribution of CA125 results across age groups

The median and interquartile range of the ROMA index in the age-stratified groups were as follows: in individuals ≤ 39 years, 3.65% (2.62–4.76); in the 40–49-year age decade, 3.82% (2.73–6.08); in the 50–59-year age decade, 5.86% (4.75–8.29); and in individuals ≥ 60 years, 8.70% (5.96–11.51).

The Kruskal–Wallis test demonstrated a statistically significant difference in ROMA values among the age-differentiated patient subgroups ($\chi^2(3) = 67.66$, $p < 0.001$). Post hoc analysis demonstrated that this difference was found to be statistically significant for all intergroup comparisons except between the ≤ 39 -year and 40–49-year groups.

The Mann–Whitney test confirmed statistically significant differences in ROMA index values between the following groups: ≤ 39 years versus 50–59 years ($U = 722.5$, $p < 0.001$, $r = -0.498$), ≤ 39 years versus ≥ 60 years ($U = 294.0$, $p < 0.001$, $r = -0.638$), 40–49 years versus 50–59 years ($U = 730.5$, $p < 0.001$, $r = -0.413$), 40–49 years versus ≥ 60 years ($U = 277.0$, $p < 0.001$, $r = -0.623$), and 50–59 years versus ≥ 60 years ($U = 588.5$, $p = 0.002$, $r = -0.335$) (Figure 5).

Age-stratified reference limits (95% CI) for the ROMA index were determined as follows: < 39 years – < 9.84 (7.38–15.93); 40–49 years – < 9.38 (8.36–10.50); 50–59 years – < 12.28 (10.70–14.20); and ≥ 60 years – < 14.72 (13.10–15.29).

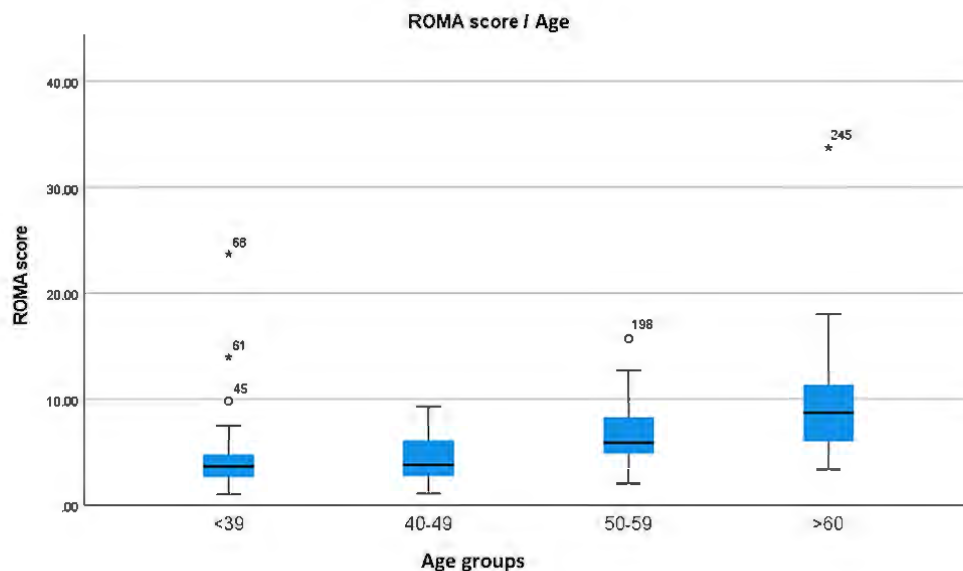


Figure 5. Distribution of ROMA score results across age groups

According to age, the median and interquartile range of CPH-I were as follows: in individuals aged ≤ 39 years, 0.460 (0.280–0.610); in the 40–49-year age group, 0.550 (0.400–0.910); in the 50–59-year age group, 0.790 (0.470–1.040); and in individuals ≥ 60 years, 1.405 (0.690–2.380). The Kruskal–Wallis test demonstrated a statistically significant difference in CPH-I values among the age-stratified patient subgroups ($\chi^2(3) = 49.48$, $p < 0.001$). Post hoc analysis demonstrated that this difference remained statistically significant for all intergroup comparisons except for the comparison between the 40–49- and 50–59-year groups ($p = 0.130$). CPH-I values in women aged < 39 years were significantly lower than those in the 40–49-, 50–59-, and ≥ 60 -year groups ($p = 0.004$, $p < 0.001$, and $p < 0.001$, respectively). CPH-I values in women aged 40–49 years were significantly lower compared with those in the ≥ 60 -year group, and CPH-I values in women aged 50–59 years were significantly lower compared with those in the ≥ 60 -year group ($p < 0.001$) (Figure 6).

Age-stratified reference limits (95% CI) for CPH-I were established as follows: <39 years – <2.096 (1.427–2.789); 40–49 years – <2.432 (1.938–3.174); 50–59 years – <2.555 (2.091–2.956); and ≥60 years – <3.728 (3.241–3.993) (Figure 7).

Across the individual age groups, the confidence intervals of the upper reference limits overlapped, rendering the derivation and application of reference intervals for HE4, CA125, ROMA, and CPH-I based solely on age unjustified.

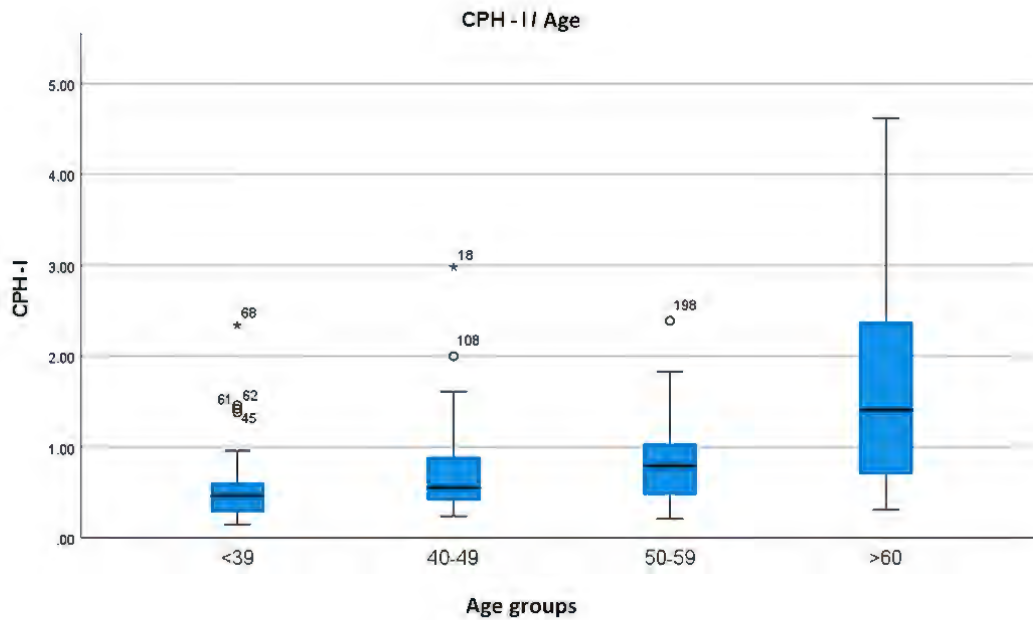


Figure 6. Age-related distribution of CPH-I values

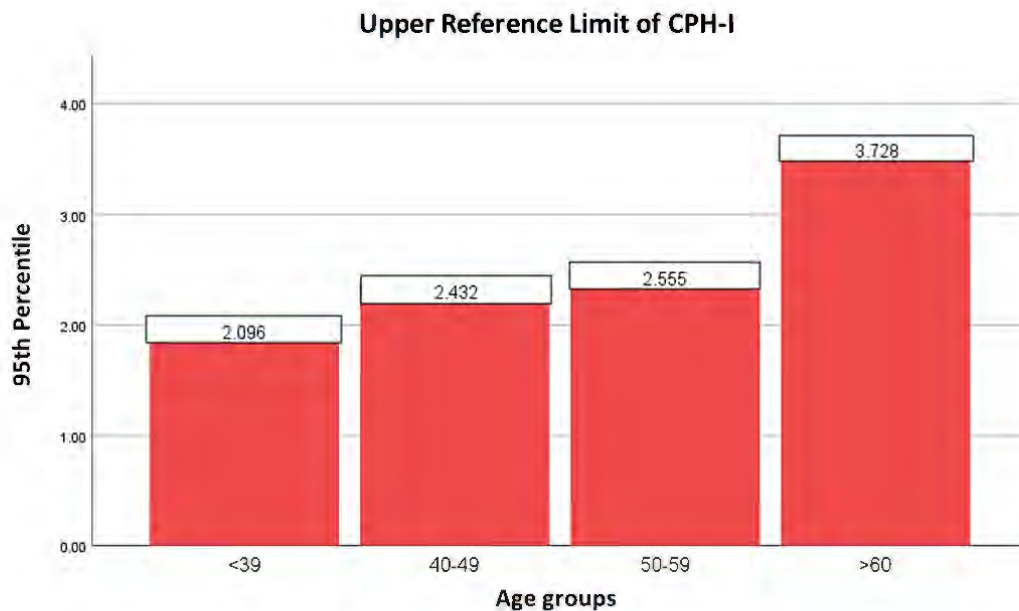


Figure 7. Upper reference limit of CPH-I across age groups

To investigate the combined influence of age and menopausal status on the values of HE4, CA125, ROMA, and CPH-I, the patients were stratified by age within each subgroup—premenopausal and postmenopausal.

In the premenopausal group, the age distribution was as follows: <39 years (N = 66), 40–49 years (N = 51), >50 years (N = 7), and >60 years (N = 0). Due to the small number of premenopausal women older than 50 years, it was not possible to define a separate subgroup; therefore, comparative analyses were performed between the <39-year and 40–49-year groups. In the postmenopausal group, the age distribution was as follows: <39 years (N = 2), 40–49 years (N = 5), 50–59 years (N = 62), and >60 years (N = 53). Owing to the limited number of postmenopausal women under 50 years of age, a separate subgroup could not be established, and comparative analyses were conducted between the 50–59-year and >60-year groups.

In the premenopausal group, Spearman’s rho correlation analysis demonstrated a statistically significant association between age and CPH-I ($\rho = 0.308$, $p < 0.001$), while no significant correlation was observed between age and HE4, CA125, or the ROMA index ($p > 0.05$).

In the postmenopausal group, statistically significant correlations were identified between age and HE4, the ROMA index, and CPH-I ($\rho = 0.316$, $p < 0.001$; $\rho = 0.338$, $p = 0.001$; and $\rho = 0.482$, $p < 0.001$, respectively).

Among premenopausal women, a statistically significant difference in CPH-I values was observed between the <39-year and 40–49-year subgroups ($U = 1241.0$, $p = 0.015$, $r = -0.225$), whereas no statistically significant differences were found for HE4, CA125, or the ROMA index ($p > 0.05$).

Among postmenopausal women, the Mann–Whitney test demonstrated statistically significant differences in HE4, ROMA index, and CPH-I values between the 50–59-year and >60-year subgroups ($U = 1195.0$, $p = 0.012$, $r = -0.235$; $U = 559.5$, $p = 0.010$, $r = -0.284$; and $U = 452.5$, $p < 0.001$, $r = -0.394$, respectively).

Based on these findings, age-dependent reference limits (95% CI) were established in the premenopausal group exclusively for CPH-I, as follows: <39 years <2.10% (1.478–2.791) and 40–49 years <2.488% (2.096–3.174).

Age-stratified reference limits in postmenopausal women were determined as follows:

HE4: 50–59 years <61.27 pmol/L (56.44–64.06) and >60 years <68.84 pmol/L (63.76–71.63);

ROMA index: 50–59 years <12.34% (11.12–14.20) and >60 years <14.72% (13.19–15.29);

CPH-I: 50–59 years <2.531% (2.033–2.956) and >60 years <3.728% (3.241–3.993) (Figure 8).

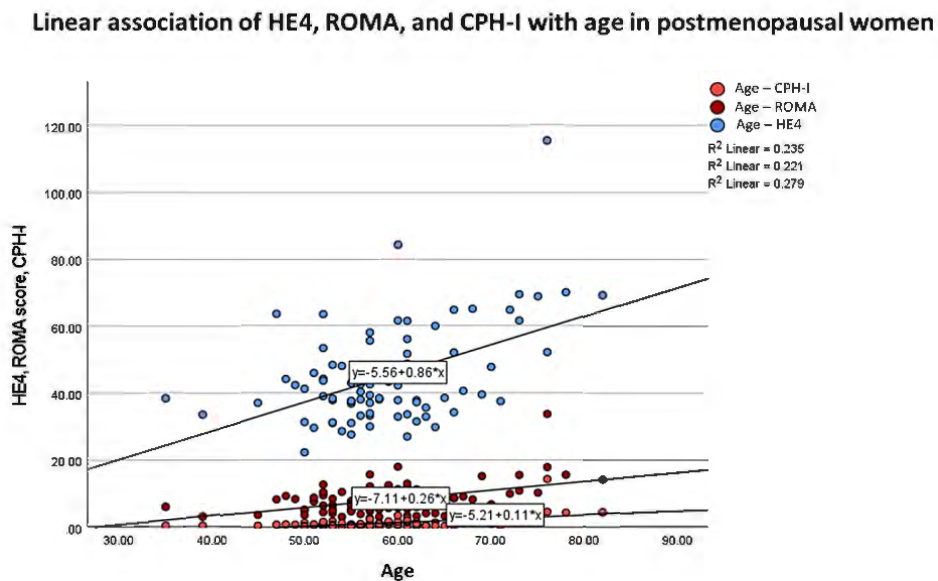


Figure 8. Linear association of HE4, ROMA, and CPH-I with age in postmenopausal women

The obtained results demonstrate that the values of HE4, CA125, ROMA, and CPH-I are dependent on menopausal status, with HE4, ROMA, and CPH-I values increasing with age, while CA125 levels decrease with age.

In the postmenopausal group, a statistically significant difference was observed in the values of HE4, ROMA, and CPH-I among the age-stratified subgroups. The determined confidence intervals of the upper reference limits for HE4, ROMA, and CPH-I did not overlap, thereby justifying the application of age-dependent upper reference limits for these parameters in postmenopausal women (Figure 9).

The reference intervals for HE4, CA125, ROMA, and CPH-I according to menopausal status, as established in the present study, are summarised in Table 1.

Table 1. Reference intervals for HE4, CA125, ROMA, and CPH-I according to menopausal status

Assay	Premenopausal		Postmenopausal		Units
	ГРГ (P95)	95% ДИ	ГРГ (P95)	95% ДИ	
HE4	53.22	50.80 – 60.82	65.31	61.57 – 68.35	pmol/L
CA-125	27.94	25.30 – 30.99	22.79	19.65 – 27.72	U/ml
ROMA	8.71	7.98 – 10.80	13.70	12.27 – 14.66	%
CPH-I	2.332	1.937 - 2.656	3.333	2.869 – 3.699	%

Upper reference limits of HE4, ROMA, and CPH-I in postmenopausal women

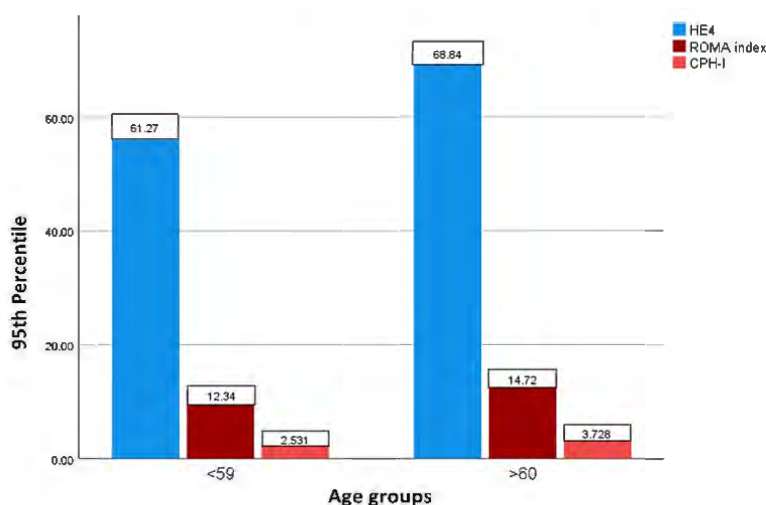


Figure 9. Upper reference limits of HE4, ROMA, and CPH-I in postmenopausal women

A statistically significant difference was identified in the values of HE4, ROMA, and CPH-I among age-stratified groups of postmenopausal women. The reference intervals determined according to menopausal status and age are summarised in Table 2.

Table 2. Age- and menopausal status-specific reference intervals

Assay	Premenopausal	Postmenopausal		Units
	P95 (95% ДИ)	<59 P95 (95% ДИ)	>59 P95 (95% ДИ)	
HE4	53.22 (50.80–60.82)	61.27(56.44–64.06)	68.84(63.76–71.63)	pmol/L
CA-125	27.94 (25.30–30.99)	22.79 (19.65–27.72)		U/ml

ROMA	8.71 (7.98–10.80)	12.34(11.12-14.20)	14.72(13.19-15.29)	%
CPH-I	2.332 (1.937-2.656)	2.531 (2.033-2.956)	3.728 (3.241-3.993)	%

CI – confidence interval

In the group of postmenopausal women, a statistically significant difference was observed between the 95th percentile of the overall group (65.31 pmol/L) and the cut-off value proposed by the manufacturer (140 pmol/L), as well as between the two age-defined subgroups—women aged ≤ 59 years and those aged >59 years (61.27 pmol/L versus 68.84 pmol/L).

In the analysis of the relationship between HE4 concentrations and age, in addition to the established linear association, non-linear relationships were also explored. The following graphs (Figure 10) illustrate quadratic and cubic relationships, demonstrating different rates of increase in marker concentration across the various age periods.

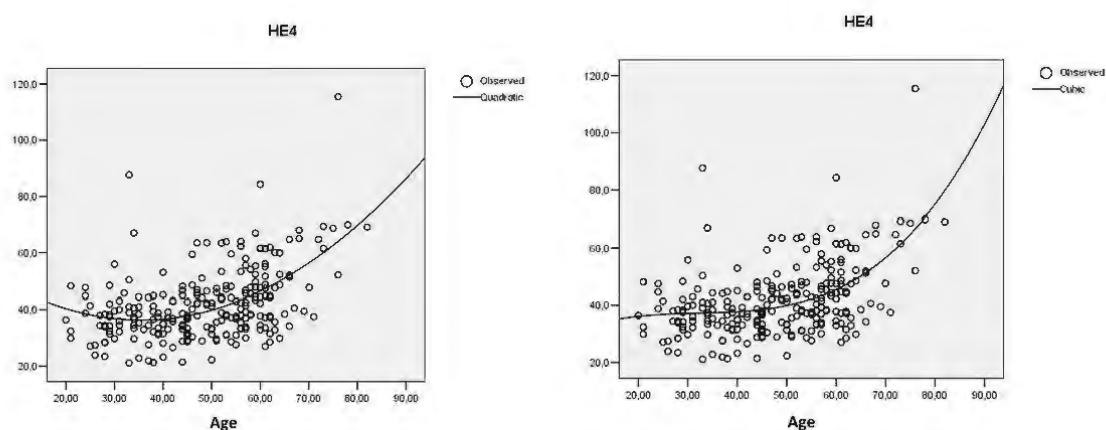


Figure 10. Non-linear relationships between HE4 concentration and age

Both graphs illustrating the non-linear relationship between HE4 concentration and age demonstrate a consistent trend—namely a relative plateau of HE4 values up to approximately 50 years of age, followed by a rapid increase thereafter, which is particularly pronounced in advanced age above 70 years. This pattern is further confirmed by the detailed decade-based comparative analysis presented above.

1.2. Establishment of reference intervals for HE4 in pregnant women

To accomplish this objective, the study included 52 healthy pregnant women in the first (N = 26) and second (N = 26) trimesters of pregnancy (up to the 26th gestational week). The mean age of the entire cohort was 30.98 ± 4.18 years (range 21–43 years); in the first-trimester subgroup, 31.69 ± 4.33 years (23–43 years); and in the second-trimester subgroup, 30.27 ± 3.99 years (21–37 years).

Statistical testing demonstrated a Gaussian distribution of HE4 results in the group of pregnant women; therefore parametric analytical methods were applied for data processing in this cohort (significance level $\alpha = 0.05$). The derived upper reference limit corresponded to the 95th percentile with a 95% confidence interval.

The median (IQR) and mean (\pm SD) HE4 concentrations in the entire group of healthy pregnant women were 35.65 (29.83–42.90) and 36.73 ± 8.20 pmol/L, respectively. In the first-trimester group, values were 38.10 (33.03–46.80) and 39.66 ± 8.95 pmol/L, while in the second-trimester group they were 33.65 (29.55–36.80) and 33.80 ± 6.26 pmol/L.

The established upper reference limit for HE4 in the entire pregnancy cohort was 50.90 pmol/L. The Student–Fisher t-test demonstrated a statistically significant difference in HE4 concentrations between the pregnancy stage–stratified subgroups ($t = 2.735$, $p = 0.009$). Gestational age–specific reference limits for HE4 were determined as follows: first trimester <57.73 pmol/L and second trimester <46.35 pmol/L (Figure 11).

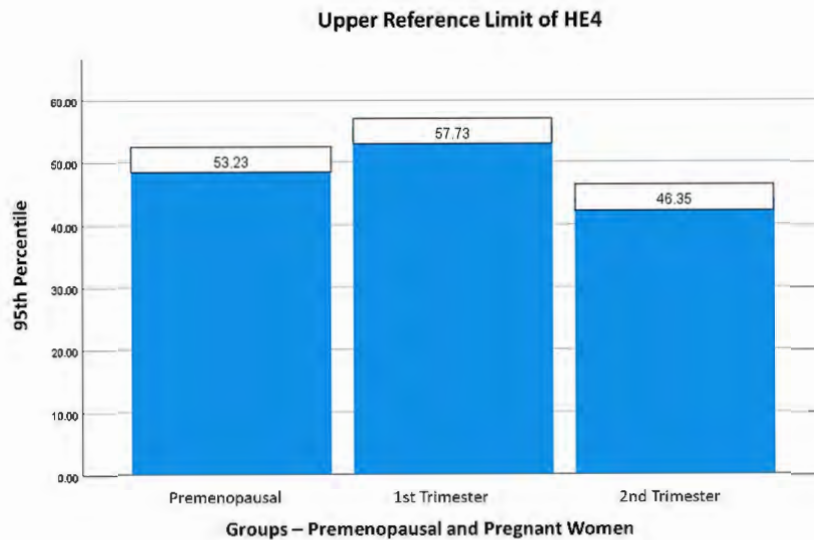


Figure 11. Upper reference limit of HE4 in premenopausal and pregnant women

To evaluate the effect of pregnancy on HE4 values, the results obtained in the cohort of pregnant women were compared with those of the entire group of premenopausal women and with healthy non-pregnant premenopausal women within the same age range.

Among the healthy non-pregnant premenopausal women, 82 were within the age range of 21–43 years (33.22 ± 5.92 years). The median (IQR) and mean (\pm SD) HE4 concentrations in this cohort were 35.50 (31.65–41.08) and 37.06 ± 9.83 pmol/L, respectively.

The Mann–Whitney test did not demonstrate a statistically significant difference in HE4 values between the entire group of pregnant women and non-pregnant premenopausal women ($p > 0.05$), nor between the entire group of pregnant women and age-matched premenopausal women ($p > 0.05$).

The Kruskal–Wallis test demonstrated that HE4 results differed significantly among the pregnancy stage–stratified subgroups and the group of age-matched premenopausal women ($\chi^2(2) = 6.579$, $p = 0.037$).

Post hoc analysis demonstrated that this difference reached statistical significance ($\alpha = 0.016$) exclusively between the first-trimester and second-trimester pregnancy groups ($p = 0.012$), but not in comparisons between the premenopausal group and the first-trimester pregnancy group ($p = 0.145$), nor between the premenopausal group and the second-trimester pregnancy group ($p = 0.084$) (Figure 12).

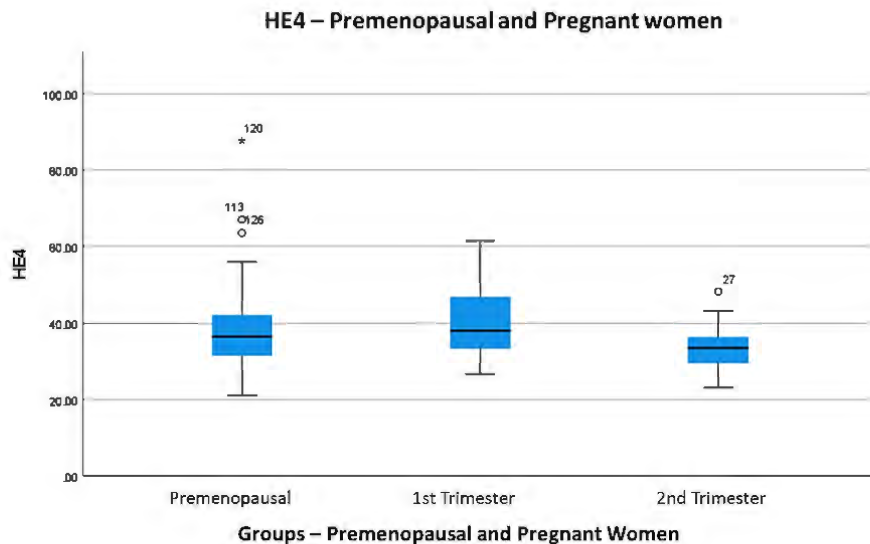


Figure 12. Distribution of HE4 results in premenopausal and pregnant women

In the present study, lower HE4 values were observed during the second trimester of pregnancy compared with those in non-pregnant women; however, this difference did not reach statistical significance. Additional data are required to adequately assess physiological changes in HE4 levels during pregnancy and, if necessary, to establish gestational age-specific reference intervals.

1.3. Evaluation of the relationship between serum HE4 concentration and impaired renal function, expressed as elevated serum creatinine or reduced estimated glomerular filtration rate (eGFR)

As the primary route of HE4 clearance is glomerular filtration, impairment of this process would be expected to result in increased serum HE4 concentrations, thereby posing a potential risk of misinterpretation regarding the presence of gynaecological malignancy.

To test this hypothesis, a cohort of 94 women (40 with elevated creatinine and 54 with creatinine values within the reference range) was analysed. Normality testing was performed, and coefficients of linear association between HE4 and creatinine concentrations were calculated. For all women included in this cohort, clinical data confirmed the absence of gynaecological and/or oncological diseases at the time of data analysis.

Measured HE4 concentrations ranged from 21.3 to 3782.4 pmol/L, while serum creatinine values ranged from 56 to 1060 $\mu\text{mol/L}$. None of the women with creatinine concentrations within the reference range exhibited elevated HE4 levels (according to a cut-off value of 70 pmol/L). Conversely, none of the patients with elevated creatinine demonstrated HE4 values below the 95th percentile for their respective age and menopausal status.

The correlation coefficient was 0.703 at $p < 0.0001$, indicating a positive linear relationship. This provides a basis for concluding that the observed linear association between HE4 concentration and serum creatinine at the time of data analysis is valid across the entire population.

When a non-parametric correlation analysis (Spearman's method) was applied to assess the coefficient of linear association, the results were consistent with those obtained from the parametric analysis. A positive correlation between HE4 concentration and serum creatinine concentration was confirmed, with a correlation coefficient of 0.703 ($p < 0.0001$) (Figure 13).

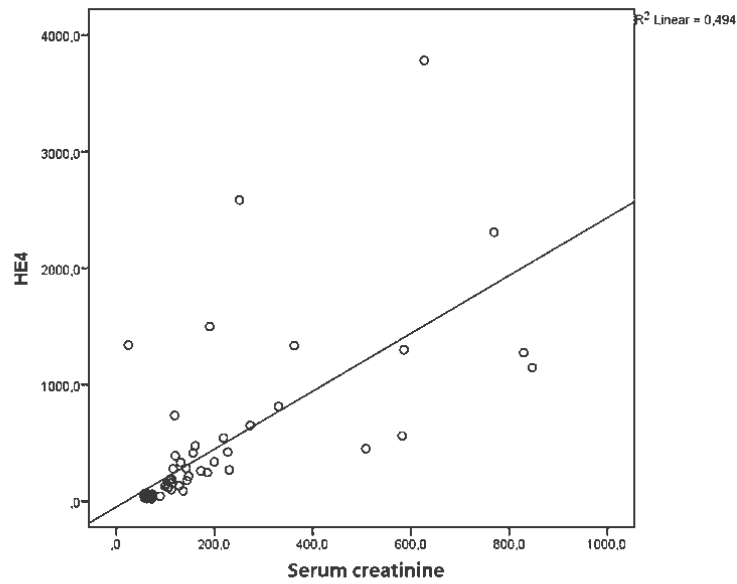


Figure 13. Linear association between HE4 concentration and serum creatinine

2. Evaluation of the diagnostic performance of HE4, CA125, ROMA, and CPH-I in differentiating ovarian carcinoma from benign ovarian cysts and from healthy women

To assess the diagnostic performance of HE4, CA125, ROMA, and CPH-I in differentiating pelvic space-occupying lesions, the present study included 942 women (741 premenopausal) with benign ovarian cysts and 150 women with histologically confirmed epithelial ovarian carcinoma (EOC). Their results were compared with data from 246 healthy women included in the control group.

An additional exploratory analysis addressing non-specific marker elevations was performed in 42 patients with non-oncological, non-gynaecological diseases and pelvic inflammatory conditions, as well as in 47 patients with other (non-gynaecological) malignancies, including primary peritoneal carcinoma, colorectal carcinoma, ovarian metastases from other primary tumours, among others.

The group with benign adnexal lesions comprised women with endometriosis (N = 157), mature cystic teratoma (N = 38), corpus luteum cysts and follicular cysts (N = 143), paraovarian cysts (N = 115), solid adnexal formations (N = 4), uterine leiomyoma (N = 63), and benign ovarian cysts without histological verification (N = 422) (Figure 14).

Statistical testing for normality of distribution for all four biomarkers demonstrated the absence of a Gaussian distribution. The Kolmogorov–Smirnov test rejected the null hypothesis of normal distribution ($p < 0.05$); therefore, subsequent statistical analyses were performed using non-parametric methods (significance level $\alpha = 0.05$).

Distribution of patients with benign ovarian cysts according to histological types

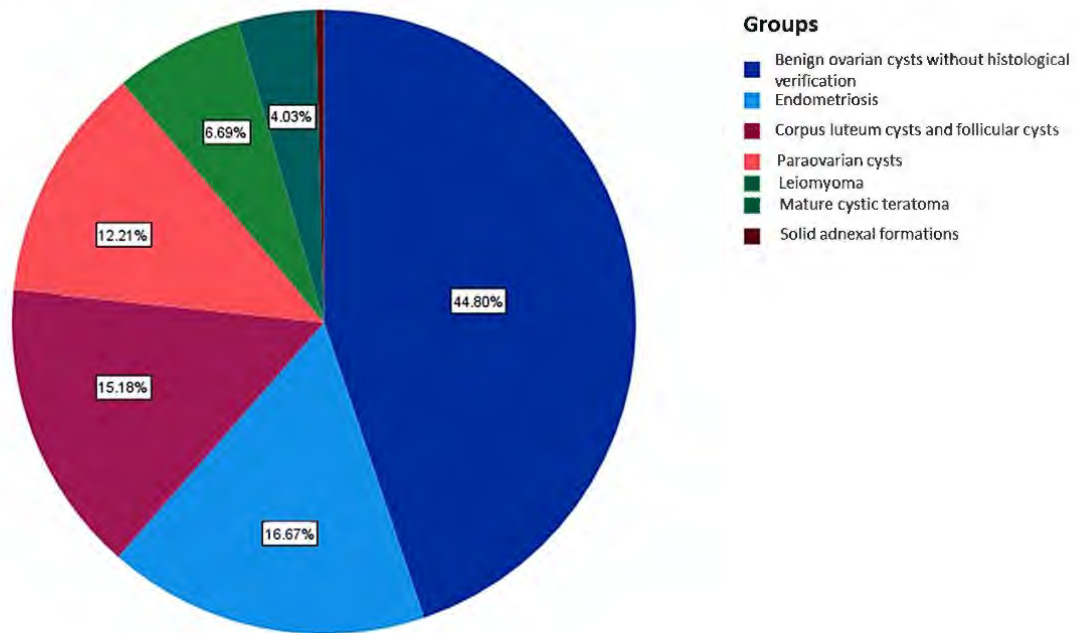


Figure 14. Distribution of patients with benign ovarian cysts according to histological types

2.1. Diagnostic performance of HE4, CA125, ROMA, and CPH-I in differentiating patients with benign ovarian cysts from healthy women

The results of the Mann–Whitney test demonstrated significantly higher median values and interquartile ranges (IQR) of HE4, ROMA, and CPH-I in postmenopausal women with benign ovarian lesions compared with those in premenopausal women. In contrast, CA125 values were higher in premenopausal women than in postmenopausal women.

When compared with the control group, patients with benign ovarian cysts exhibited significantly higher values for all four parameters. This difference remains both in comparisons between the overall groups and in analyses stratified according to menopausal status (Figures 15, 16, 17, and 18).

Receiver operating characteristic (ROC) analysis demonstrated that the standalone application of HE4 and CA125, as well as their combination within the ROMA and CPH-I algorithms, showed poor diagnostic performance for identifying patients with benign ovarian cysts within a healthy population.

In view of their unsatisfactory diagnostic accuracy, no cut-off values were established for differentiating patients with benign ovarian cysts from healthy women.

HE4 distribution in healthy women and patients with benign ovarian cysts

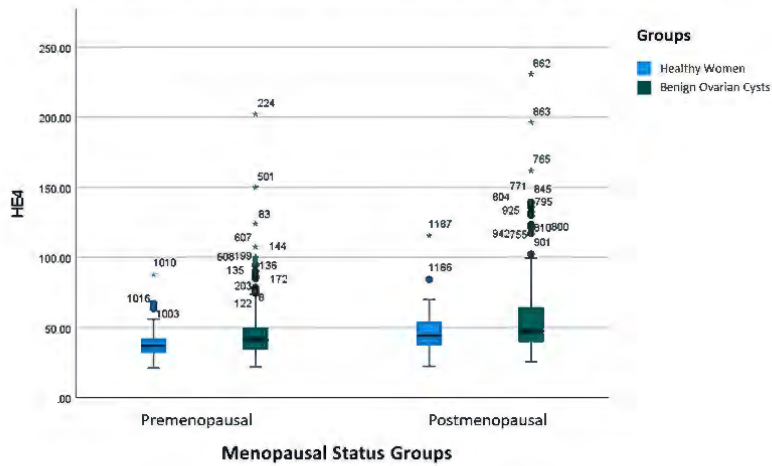


Figure 15. HE4 distribution in healthy women and patients with benign ovarian cysts

CA125 distribution in healthy women and patients with benign ovarian cysts

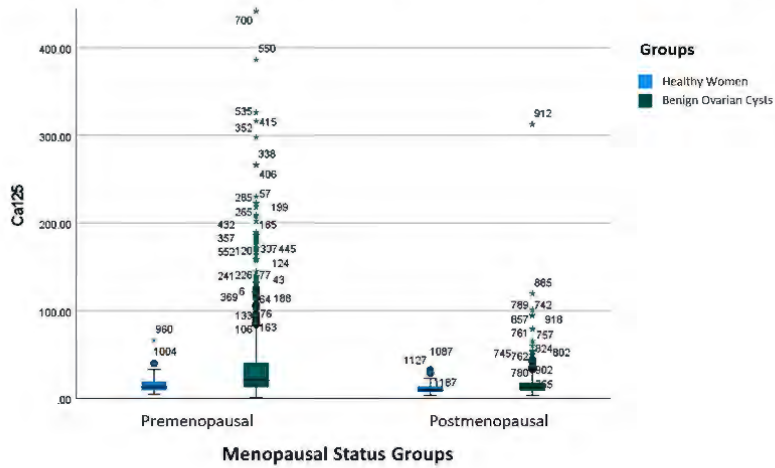


Figure 16. CA125 distribution in healthy women and patients with benign ovarian cysts

ROMA index distribution in controls and patients with benign ovarian cysts

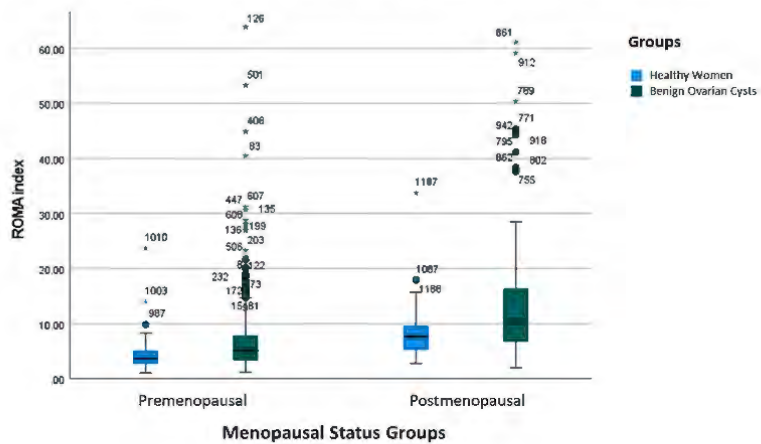


Figure 17. ROMA index distribution in controls and patients with benign ovarian cysts

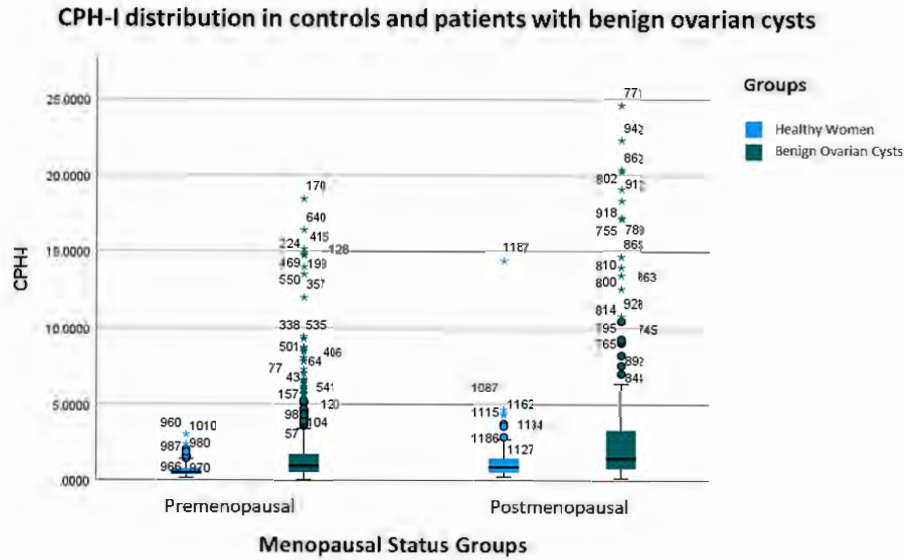


Figure 18. CPH-I distribution in controls and patients with benign ovarian cysts

Similar results were obtained from the subgroup statistical analysis based on the histological type of benign ovarian cysts. Overlap of the interquartile ranges (IQR) of the evaluated parameters was observed both among the histologically stratified groups and in comparison, with their respective control groups. In view of these findings, performing ROC analysis and defining cut-off values for differentiating patients with benign ovarian cysts according to specific histological diagnoses was considered unjustified.

One important observation should be highlighted. The Kruskal–Wallis test demonstrated a statistically significant difference in CA125 values among the histologically differentiated subgroups of patients with benign ovarian cysts ($\chi^2(6) = 285.33, p < 0.001$). Following post hoc analysis, this difference was found to be statistically significant between patients with endometriosis and all other histological groups (Table 3).

This trend remained consistent after stratification of patients according to menopausal status and comparison of the subgroups with their corresponding control groups.

Table 3. Median and interquartile range (IQR) of CA125 in histologically differentiated groups of ovarian cancer

Histological type	CA125								
	Total			Premenopausal			Postmenopausal		
	Median (IQR)	N	p	Median (IQR)	N	p	Median (IQR)	N	p
Controls	12.00 (8.80)	213	*	13.10 (10.38)	124	*	9.60 (7.30)	89	
Endometriosis	60.40 (95.10)	157	<0.001	59.70 (92.10)	153	<0.001	216.10 (495.90)	4	*
Teratoma	18.20 (15.20)	38	<0.001	23.40 (18.78)	32	<0.001	14.00 (3.30)	6	*

Corpus luteum cysts	14.10 (10.90)	143	<0.001	16.00 (12.85)	117	0.006	11.35 (7.18)	26	0.886
Paraovarian cysts	17.50 (22.00)	115	<0.001	20.70 (19.48)	58	<0.001	13.30 (24.00)	57	<0.001
Adnexial masses	24.75 (53.68)	4	*	*	1	*	*	3	*
Leiomyoma	15.60 (21.50)	63	<0.001	19.25 (25.65)	38	<0.001	14.20 (15.40)	25	0.067
Unknown	15.85 (14.07)	422	<0.001	16.85 (14.88)	342	<0.001	11.95 (7.75)	80	<0.001

ROC analysis demonstrated that CA125 has excellent diagnostic performance for the detection of patients with endometriosis within a group of healthy individuals, with an AUC-ROC of 0.943, and very good diagnostic performance for differentiating patients with endometriosis from healthy individuals and from patients with other benign ovarian tumours, with an AUC-ROC of 0.882 (Figure 19). At a CA125 cut-off value of 27.95 U/mL, the test showed a sensitivity and specificity of 82.8% and 81.1%, respectively; positive and negative likelihood ratios (LR+ and LR-) of 4.38 and 0.212; and positive and negative predictive values of 40.8% and 96.8%, respectively.

ROC curve of CA125 for differentiation of endometriosis from healthy individuals and other benign ovarian tumors

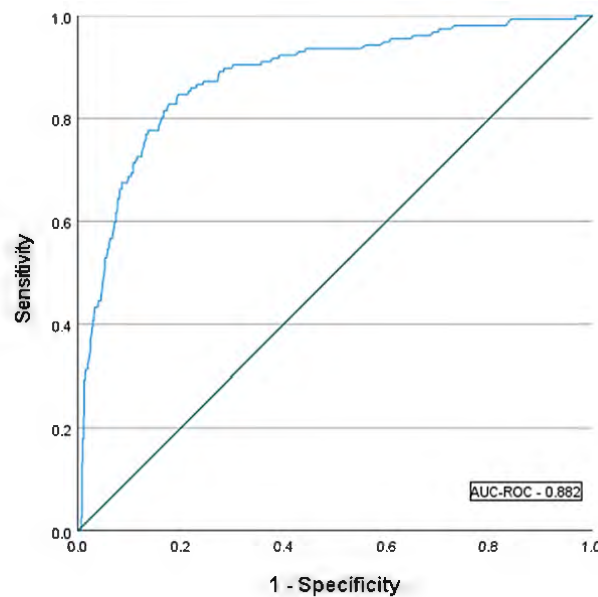


Figure 19. ROC curve of CA125 for differentiation of endometriosis from healthy individuals and other benign ovarian tumors

2.2 Diagnostic reliability of HE4 for differentiation of patients with ovarian cancer from patients with benign ovarian tumors or healthy women

The analysis for this objective included 150 patients with ovarian cancer, 246 healthy women, and 942 patients with benign ovarian tumours. The distribution according to histological type in the ovarian cancer group is presented in Figure 20. This cohort was divided into two

subgroups—premenopausal (N = 35) and postmenopausal (N = 115)—with an almost identical distribution of histological findings between the two groups.

According to the stage of the oncological disease, patients with ovarian cancer were classified into five subgroups: Stage I (N = 14), Stage II (N = 12), Stage III (N = 60), Stage IV (N = 27), and undetermined stage (N = 36).

Distribution of patients with ovarian cancer by histological type

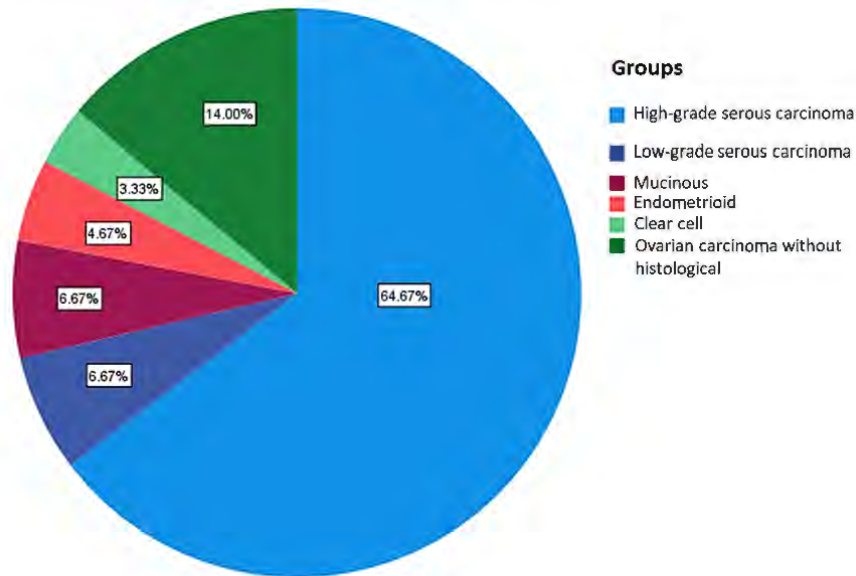


Figure 20. Distribution of patients with ovarian cancer

The median and interquartile range (IQR) of HE4 in the entire ovarian cancer cohort were 338.20 (131.65–862.78) pmol/L. In the premenopausal subgroup, the median HE4 level was 148.40 (58.60–421.60) pmol/L, while in the postmenopausal subgroup it was 353.90 (154.20–1060.10) pmol/L. HE4 levels differed significantly between the two subgroups ($U = 1209.50$, $p < 0.001$, $r = -0.292$).

Patients with ovarian cancer had significantly higher HE4 concentrations compared with the control group (338.20 vs 38.90 pmol/L; $U = 1255.50$, $p < 0.001$, $r = -0.780$) as well as compared with patients with benign ovarian tumours (338.20 vs 42.35 pmol/L; $U = 6778.00$, $p < 0.001$, $r = -0.539$) (Figure 21).

The observed significant differences in HE4 levels were also preserved in comparisons between subgroups stratified by menopausal status. Premenopausal patients with ovarian cancer had significantly higher HE4 levels compared both with the control group (148.40 vs 36.65 pmol/L; $U = 287.50$, $p < 0.001$, $r = -0.621$) and with patients with benign ovarian tumours (148.40 vs 41.10 pmol/L; $U = 2935.00$, $p < 0.001$, $r = -0.278$).

Similarly, postmenopausal patients with ovarian cancer exhibited significantly higher HE4 concentrations compared with the control group (353.90 vs 44.15 pmol/L; $U = 296.50$, $p < 0.001$, $r = -0.827$) and with patients with benign ovarian tumours (353.90 vs 47.40 pmol/L; $U = 999.00$, $p < 0.001$, $r = -0.763$) (Figure 22).

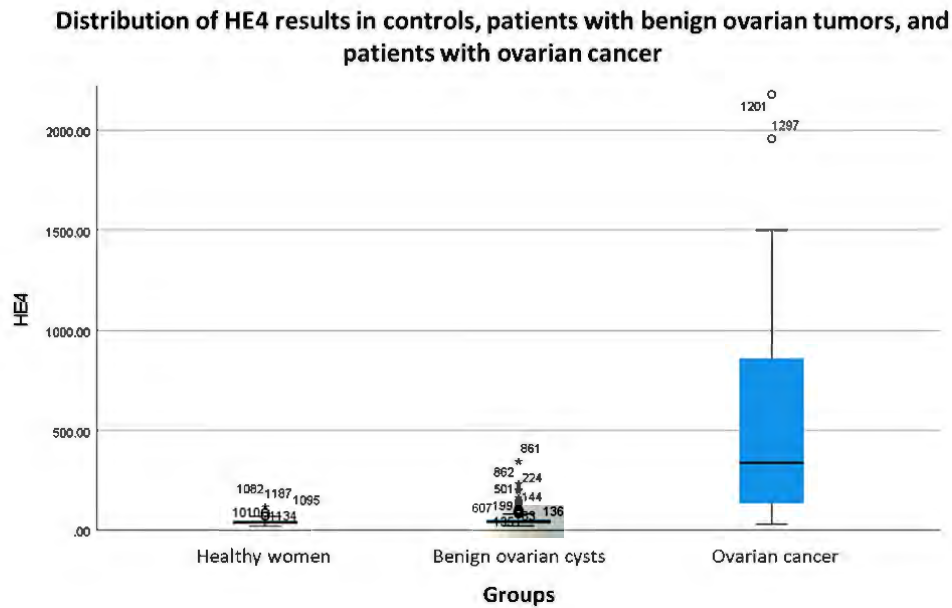


Figure 21. Distribution of HE4 results in controls, patients with benign ovarian tumors, and patients with ovarian cancer

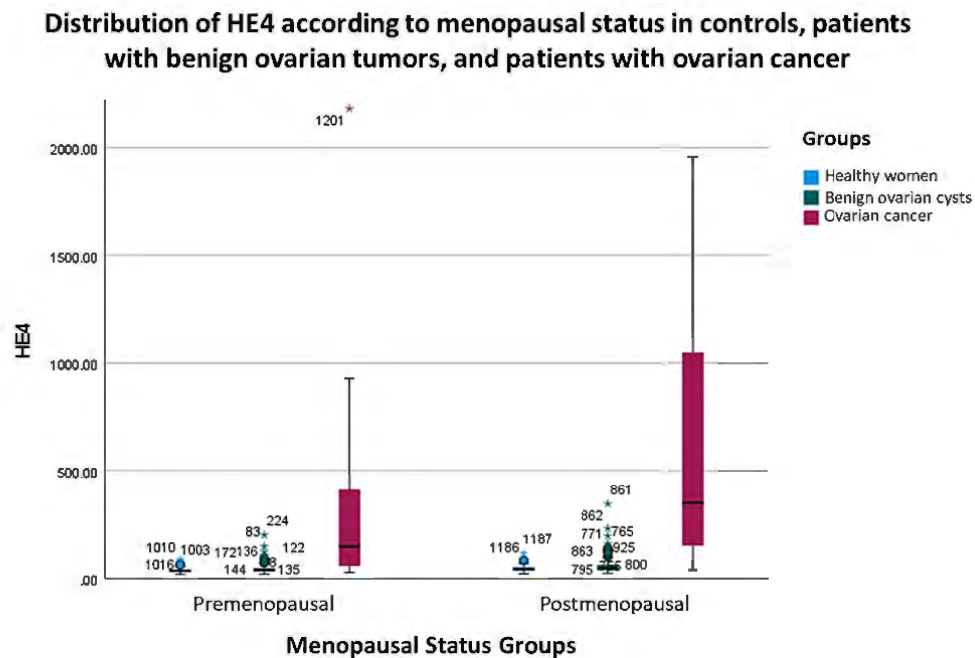


Figure 22. Distribution of HE4 according to menopausal status in controls, patients with benign ovarian tumors, and patients with ovarian cancer

To assess the clinical significance of HE4 as a triage test in the presence of a pelvic mass, the diagnostic reliability of the marker for differentiating ovarian cancer from benign ovarian tumours was evaluated. ROC analysis demonstrated that HE4 has excellent diagnostic performance for the detection of patients with ovarian cancer among patients with benign ovarian tumours, with an AUC-ROC of 0.952.

At an HE4 cut-off value of 83.95 pmol/L, the test showed a sensitivity of 86% and a specificity of 96.0%, with positive and negative likelihood ratios (LR+ and LR-) of 21.50 and 0.146,

respectively, and positive and negative predictive values of 77.2% and 97.7%, respectively (Figure 23, Table 4, Table 5).

ROC curve of HE4 for differentiation of ovarian cancer from benign ovarian tumors

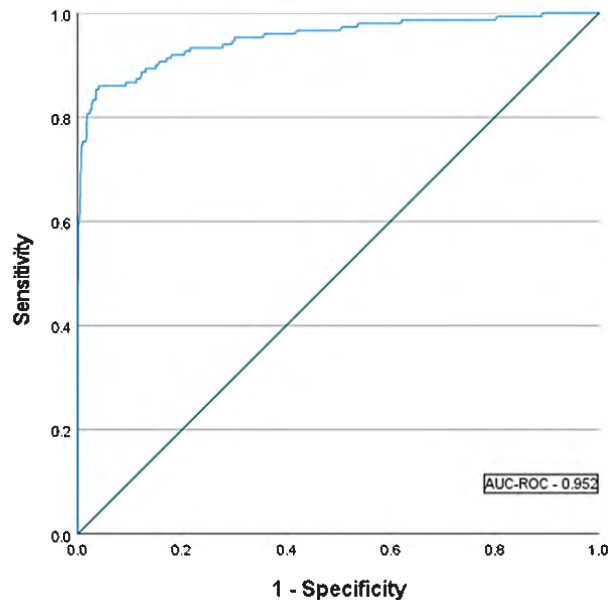


Figure 23. ROC curve of HE4 for differentiation of ovarian cancer from benign ovarian tumors

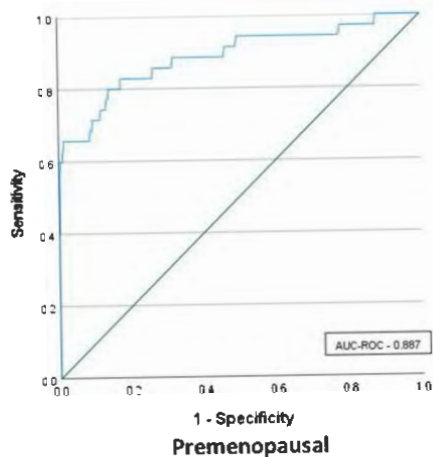
A repeat ROC analysis was performed after stratifying the ovarian cancer and benign ovarian tumor groups according to menopausal status, yielding the following results.

In the premenopausal group, HE4 demonstrated very good diagnostic performance for the detection of patients with ovarian cancer (N = 35) among women with benign ovarian tumours (N = 741), with an AUC-ROC of 0.887. At an HE4 cut-off value of 57.80 pmol/L, the test showed a sensitivity of 80% and a specificity of 86.1%, with positive and negative likelihood ratios (LR+ and LR-) of 5.76 and 0.232, respectively, and positive and negative predictive values of 21.4% and 98.9%, respectively, for differentiating ovarian cancer from benign ovarian tumours.

The low positive predictive value (PPV) can be explained by the low prevalence of ovarian cancer in the premenopausal group (ovarian cancer and benign ovarian tumors), which results in a higher number of false-positive results.

In the postmenopausal group, HE4 exhibited excellent diagnostic performance for the detection of patients with ovarian cancer (N = 115) among women with benign ovarian tumours (N = 201), with an AUC-ROC of 0.957. At an HE4 cut-off value of 92.80 pmol/L, the test showed a sensitivity and specificity of 89.6% and 91.0%, respectively, with positive and negative likelihood ratios (LR+ and LR-) of 9.96 and 0.114, respectively, and positive and negative predictive values of 85.1% and 93.8%, respectively, for differentiating ovarian cancer from benign ovarian tumours (Figure 24, Table 4, Table 5).

ROC curves of HE4 for differentiation of ovarian cancer from benign ovarian tumors



ROC curves of HE4 for differentiation of ovarian cancer from benign ovarian tumors

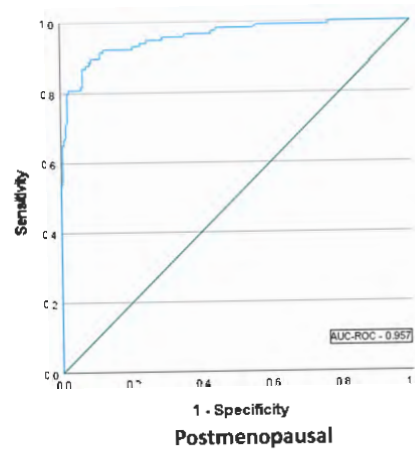


Figure 24. ROC curves of HE4 for differentiation of ovarian cancer from benign ovarian tumors in premenopausal and postmenopausal women

The medians and interquartile ranges (IQRs) of HE4 in the histologically differentiated groups were as follows: high-grade serous carcinoma – 421.60 (161.20–1218.40) pmol/L, low-grade serous carcinoma – 95.60 (53.53–161.38) pmol/L, mucinous carcinoma – 76.95 (43.58–153.28) pmol/L, endometrioid carcinoma – 181.50 (87.10–343.00) pmol/L, clear cell carcinoma – 66.50 (50.40–312.65) pmol/L, and ovarian carcinoma without histological identification – 283.60 (128.75–656.45) pmol/L.

Patients with high-grade serous carcinoma (N = 97) had significantly higher HE4 levels compared with the remaining patients with ovarian cancer (N = 53) (U = 1358.50, $p < 0.001$, $r = -0.389$). However, ROC analysis demonstrated that the standalone application of HE4 had unsatisfactory diagnostic performance for differentiating high-grade serous carcinoma from the other histological types of ovarian cancer, with an AUC-ROC of 0.736 (Table 4).

The medians and interquartile ranges (IQRs) of HE4 in subgroups stratified according to the stage of the oncological disease were as follows: Stage I – 65.70 (55.35–184.175) pmol/L, Stage II – 155.05 (98.95–333.13) pmol/L, Stage III – 478.80 (195.10–1303.65) pmol/L, Stage IV – 343.00 (165.50–1039.00) pmol/L, and undetermined stage – 233.75 (110.03–747.30) pmol/L (Figure 25).

A significant difference was observed between the results of patients with Stage I and Stage III disease (U = 161.50, $p < 0.001$, $r = -0.416$) and between Stage I and Stage IV disease (U = 57.50, $p < 0.001$, $r = -0.565$). Discriminant analysis showed that the standalone use of HE4 does not have sufficient diagnostic value for staging patients with ovarian cancer ($\lambda = 0.944$, $\chi^2 = 8.342$, $p = 0.08$, $\eta = 0.13$) (Table 4).

Distribution of HE4 results according to staging of patients with ovarian cancer

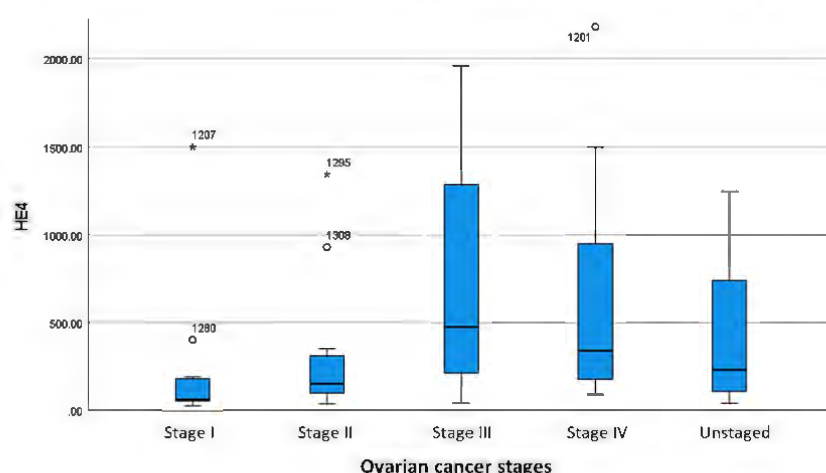


Figure 25. Distribution of HE4 results according to staging of patients with ovarian cancer

Table 4. Diagnostic reliability of HE4 in the diagnosis of ovarian cancer

Diagnostic efficiency	HE4 – common group		HE4 – premenopausal		HE4 – postmenopausal	
	cut-off	AUC-ROC	cut-off	AUC-ROC	cut-off	AUC-ROC
Distinguishing OC from benign cyst	83.95 pmol/L	0.952	57.80 pmol/L	0.887	92.80 pmol/L	0.957
Distinguishing healthy subjects from benign cyst and OC	There is not enough diagnostic efficiency					
Distinguishing of the histological type of OC	There is not enough diagnostic efficiency					
Staging of OC	There is not enough diagnostic efficiency					

Table 5. HE4 cut-off values derived in the present study

HE4	Group	cut-off	N	TP	TN	FP	FN	Se	Sp
Distinguishing OC from benign cyst	Common	83.95 pmol/L	109 2	129	904	38	21	86%	96%
Distinguishing OC from benign cyst	Premenopausal	57.80 pmol/L	776	28	638	103	7	80%	86%
Distinguishing OC from benign cyst	Postmenopausal	92.80 pmol/L	316	103	183	18	12	90%	91%

Abbreviations: TP – true positives, TN – true negatives, FP – false positives, FN – false negatives, Se – diagnostic sensitivity, Sp – diagnostic specificity

2.3 Diagnostic reliability of CA125 for differentiation of patients with ovarian cancer from patients with benign ovarian tumors or healthy women

The median (IQR) CA125 level in the ovarian cancer cohort was 364.35 (132.68–1000.00) U/mL. In the premenopausal subgroup, the median CA125 level was 332.40 (84.40–1000.00) U/mL, while in the postmenopausal subgroup it was 386.50 (141.80–1000.00) U/mL. CA125 levels in the two ovarian cancer subgroups stratified by menopausal status did not differ significantly ($p = 0.689$).

Patients with ovarian cancer had significantly higher CA125 levels compared both with the control group (364.35 vs 12.00 U/mL; $U = 728.00$, $p < 0.001$, $r = -0.813$) and with patients with benign ovarian tumours (364.35 vs 18.10 U/mL; $U = 9903.00$, $p < 0.001$, $r = -0.512$) (Figure 26).

This significant difference was also preserved in comparisons between subgroups stratified according to menopausal status. Premenopausal patients with ovarian cancer had significantly higher CA125 levels compared both with the control group (332.40 vs 13.10 U/mL; $U = 96.00$, $p < 0.001$, $r = -0.683$) and with patients with benign ovarian tumours (332.40 vs 20.50 U/mL; $U = 2029.00$, $p < 0.001$, $r = -0.302$).

Similarly, postmenopausal patients with ovarian cancer had significantly higher CA125 levels compared both with the control group (386.50 vs 9.60 U/mL; $U = 183.50$, $p < 0.001$, $r = -0.827$) and with patients with benign ovarian tumours (353.90 vs 12.90 U/mL; $U = 1062.00$, $p < 0.001$, $r = -0.756$) (Figure 27).

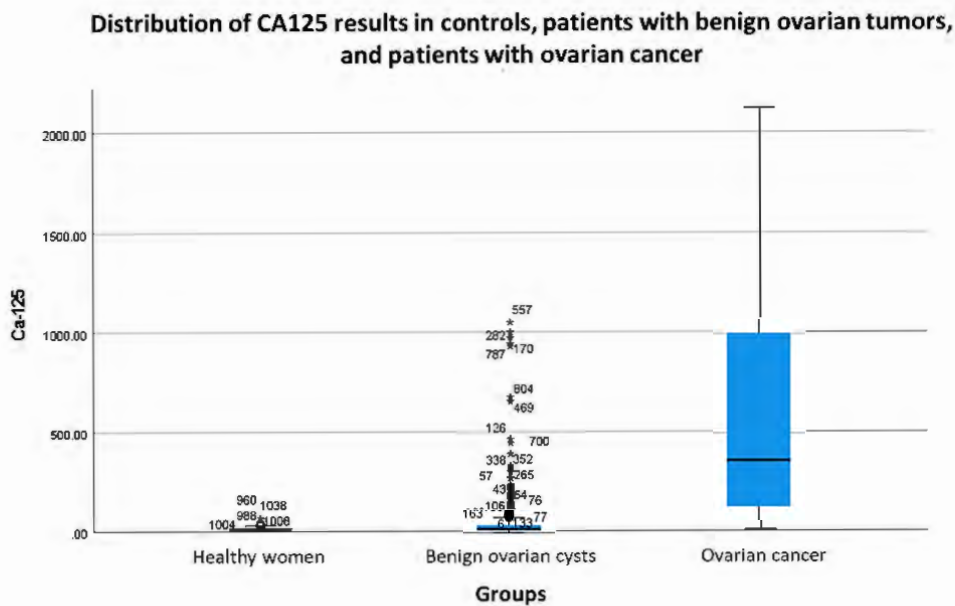


Figure 26. Distribution of CA125 results in controls, patients with benign ovarian tumors, and patients with ovarian cancer

Distribution of CA125 results according to menopausal status in controls, patients with benign ovarian tumors, and patients with ovarian cancer

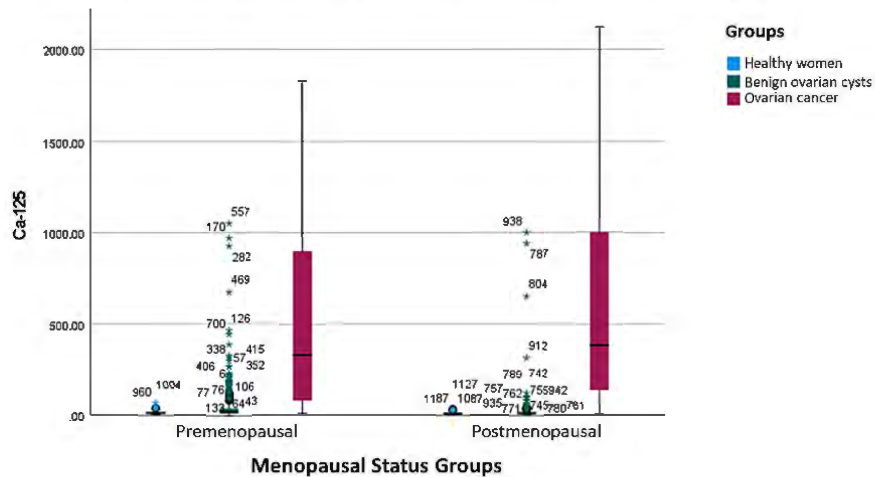


Figure 27. Distribution of CA125 results according to menopausal status in controls, patients with benign ovarian tumors, and patients with ovarian cancer

To assess the clinical significance of CA125 in differentiating a pelvic space-occupying process, the diagnostic reliability of the marker for distinguishing ovarian cancer from benign ovarian tumours was evaluated. ROC analysis demonstrated that the standalone application of CA125 has excellent diagnostic performance for detecting ovarian cancer among patients with benign ovarian tumours, with an AUC-ROC of 0.930.

At a CA125 cut-off value of 82.90 U/mL, the test showed a sensitivity of 82.7% and a specificity of 91.6%, with positive and negative likelihood ratios (LR+ and LR-) of 9.84 and 0.189, respectively, and positive and negative predictive values of 61.08% and 97.1%, respectively (Figure 28, Table 6, Table 7).

A repeat ROC analysis was performed in subgroups stratified by menopausal status. In the premenopausal group, CA125 demonstrated excellent diagnostic performance for detecting ovarian cancer among women with benign ovarian tumours, with an AUC-ROC of 0.922. At a CA125 cut-off value of 52.00 U/mL, the test showed a sensitivity and specificity of 91.4% and 81.0%, respectively, with LR+ and LR- values of 4.81 and 0.106, respectively, and positive and negative predictive values of 18.5% and 99.5%, respectively.

ROC analysis further demonstrated that in the postmenopausal group, CA125 has excellent diagnostic performance for differentiating ovarian cancer from benign ovarian tumours, with an AUC-ROC of 0.954. At a CA125 cut-off value of 37.75 U/mL, the test showed a sensitivity and specificity of 93.0% and 89.1%, respectively, with LR+ and LR- values of 8.53 and 0.08, respectively, and positive and negative predictive values of 82.9% and 95.7%, respectively (Figure 29, Table 6, Table 7).

ROC curve of CA125 for differentiation of ovarian cancer from benign ovarian tumors

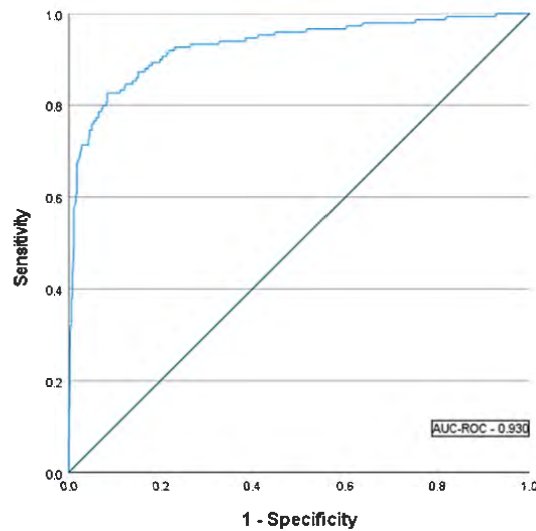
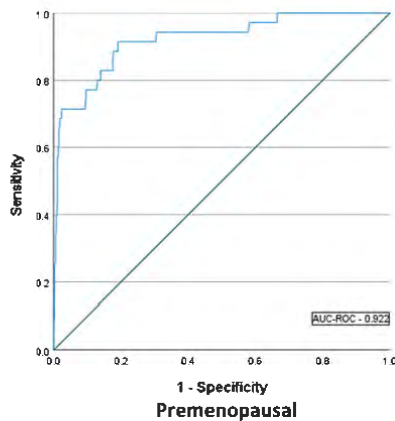


Figure 28. ROC curve of CA125 for differentiation of ovarian cancer from benign ovarian tumors

ROC curves of CA125 for differentiation of ovarian cancer from benign ovarian tumors



ROC curves of CA125 for differentiation of ovarian cancer from benign ovarian tumors

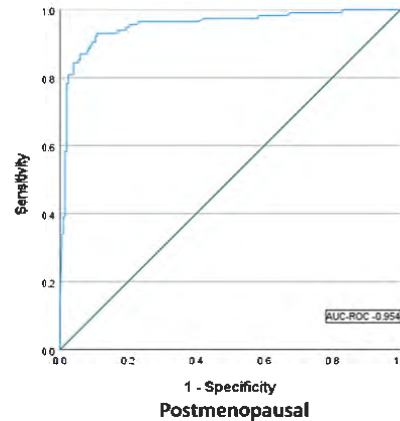


Figure 29. ROC curves of CA125 for differentiation of ovarian cancer from benign ovarian tumors in premenopausal and postmenopausal women

The medians (IQRs) of CA125 in the histologically differentiated groups were as follows: high-grade serous carcinoma – 519.40 (233.75–1000.00) U/mL, low-grade serous carcinoma – 423.15 (62.58–519.60) U/mL, mucinous carcinoma – 52.15 (24.43–86.65) U/mL, endometrioid carcinoma – 262.80 (127.70–951.50) U/mL, clear cell carcinoma – 63.70 (34.75–2955.75) U/mL, and ovarian carcinoma without histological identification – 332.40 (74.70–1000.00) U/mL.

Patients with serous carcinoma had significantly higher CA125 levels compared with the remaining patients with ovarian cancer ($U = 1485.50$, $p < 0.001$, $r = -0.277$). However, ROC analysis demonstrated that the standalone use of CA125 does not provide sufficient diagnostic performance for differentiating serous carcinoma from the other histological types of ovarian cancer, with an AUC-ROC of 0.677 (Table 6).

Table 6. Diagnostic reliability of CA125 in the diagnosis of ovarian cancer

Diagnostic efficiency	CA125 – common group		CA125–premenopausal		CA125–postmenopausal	
	cut-off	AUC-ROC	cut-off	AUC-ROC	cut-off	AUC-ROC
Distinguishing OC from benign cyst	82.90 U/ml	0.930	52.00 U/ml	0.922	37.75 U/ml	0.954
Distinguishing healthy subjects from benign cyst and OC	There is not enough diagnostic efficiency					
Distinguishing of the histological type of OC	There is not enough diagnostic efficiency					
Staging of OC	There is not enough diagnostic efficiency					

Table 7. CA125 cut-off values derived in the present study

CA125	Group	cut-off	N	TP	TN	FP	FN	Se	Sp
Distinguishing OC from benign cyst	Common	82.90 U/ml	1092	124	863	79	26	83%	92%
Distinguishing OC from benign cyst	Premenopausal	52.00 U/ml	776	32	600	141	3	91%	81%
Distinguishing OC from benign cyst	Postmenopausal	37.75 U/ml	316	107	179	22	8	93%	89%
Distinguishing of OC from endometriosis	Common	82.90 U/ml	307	124	102	55	26	83%	65%
Distinguishing of OC from endometriosis	Premenopausal	52.00 U/ml	188	30	67	86	3	91%	44%

Abbreviations: TP – true positives, TN – true negatives, FP – false positives, FN – false negatives, Se – diagnostic sensitivity, Sp – diagnostic specificity

The medians (IQRs) of CA125 in subgroups stratified according to the stage of the oncological disease were as follows: Stage I – 130.20 (32.28–540.88) U/mL, Stage II – 393.65 (123.15–1000.00) U/mL, Stage III – 519.75 (230.95–1000.00) U/mL, Stage IV – 360.70 (206.10–1000.00) U/mL, and undetermined stage – 330.00 (83.28–1000.00) U/mL.

These differences did not reach statistical significance in any of the comparisons between subgroups. Discriminant analysis demonstrated that the standalone use of CA125 does not have sufficient diagnostic value for staging patients with ovarian cancer ($\lambda = 0.995$, $\chi^2 = 0.666$, $p = 0.955$, $\eta = 0.126$) (Figure 30).

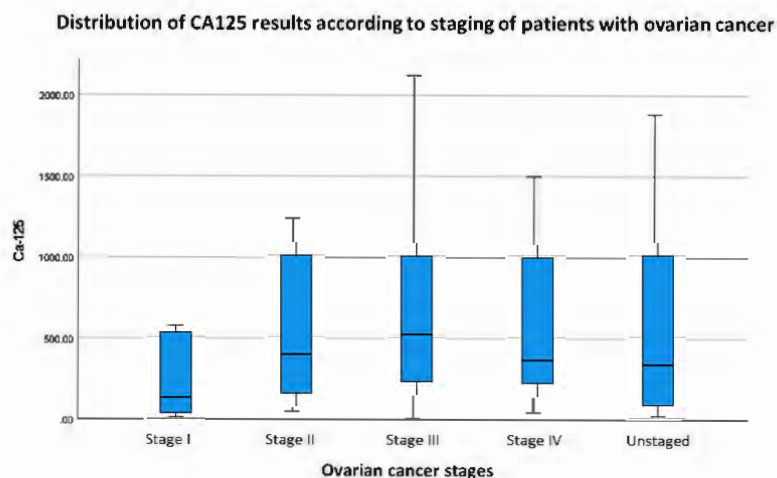


Figure 30. Distribution of CA125 results according to staging of patients with ovarian cancer

2.4 Diagnostic reliability of ROMA for differentiation of patients with ovarian cancer from patients with benign formations or healthy women

The median (IQR) ROMA value in the ovarian cancer group was 88.98 (64.59–97.76). In the premenopausal subgroup, the median ROMA value was 60.78 (13.64–95.70), while in the postmenopausal subgroup it was 90.40 (73.44–98.30). ROMA values in the two subgroups stratified according to menopausal status differed significantly ($U = 1285.00$, $p = 0.001$, $r = -0.269$).

Patients with ovarian cancer had significantly higher ROMA values compared both with the control group (88.98 vs 4.94; $U = 654.50$, $p < 0.001$, $r = -0.817$) and with patients with benign ovarian tumours (88.98 vs 5.76; $U = 4534.00$, $p < 0.001$, $r = -0.558$). The observed significant difference in ROMA values was also preserved in comparisons between groups stratified according to menopausal status (Figure 31, Figure 32).

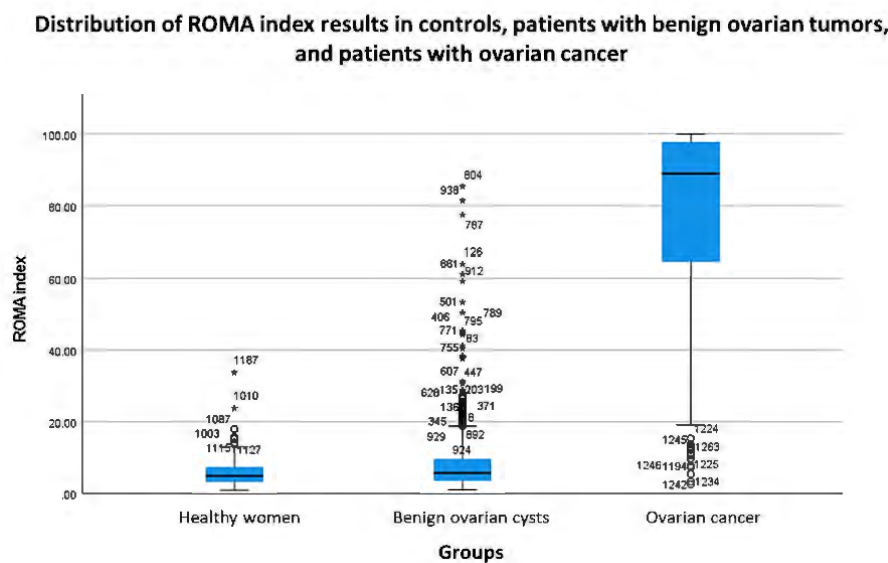


Figure 31. Distribution of ROMA index results in controls, patients with benign ovarian tumors, and patients with ovarian cancer

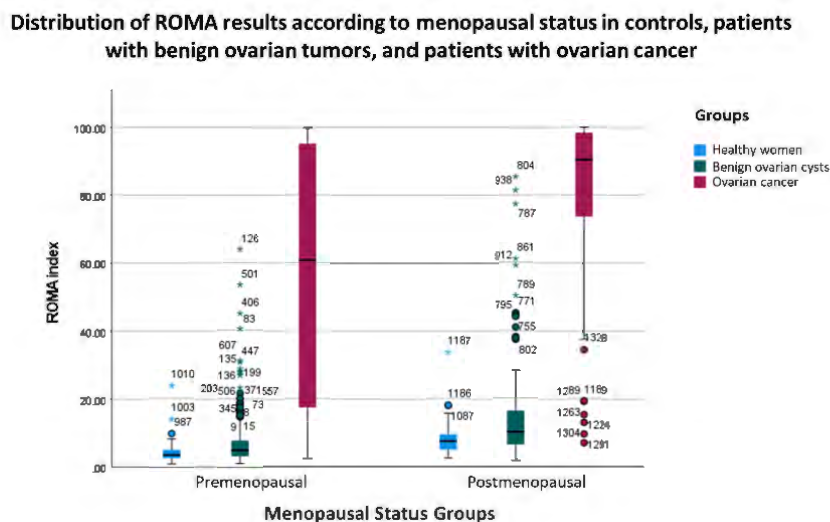


Figure 32. Distribution of ROMA results according to menopausal status in controls, patients with benign ovarian tumors, and patients with ovarian cancer

To assess the clinical significance of ROMA in differentiating a pelvic space-occupying process, the diagnostic reliability of the marker for distinguishing ovarian cancer from benign ovarian tumours was evaluated. ROC analysis demonstrated that the standalone application of ROMA has excellent diagnostic performance for differentiating patients with ovarian cancer from patients with benign ovarian tumours, with an AUC-ROC of 0.968.

At a ROMA cut-off value of 26.51, the test showed a sensitivity of 89.3% and a specificity of 96.9%, with positive and negative likelihood ratios (LR+ and LR-) of 28.80 and 0.110, respectively, and positive and negative predictive values of 82.1% and 98.2%, respectively (Figure 33, Table 8, Table 9).

ROC curve of the ROMA index for differentiation of ovarian cancer from benign ovarian tumors

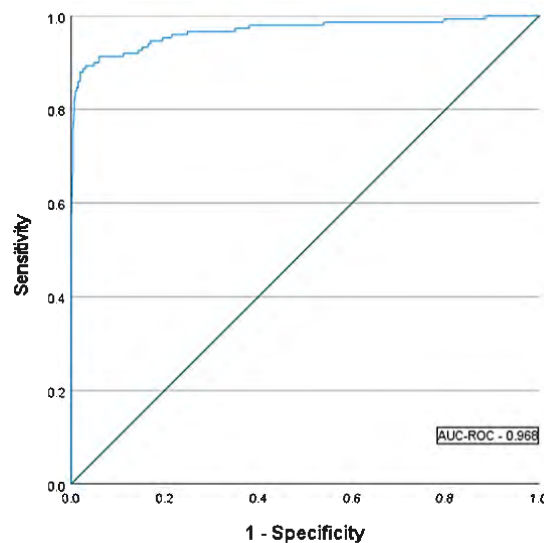


Figure 33. ROC curve of the ROMA index for differentiation of ovarian cancer from benign ovarian tumors

The ovarian cancer and benign ovarian tumour groups were stratified according to menopausal status, and a repeat ROC analysis was performed. ROC analysis demonstrated that in the premenopausal group, ROMA showed excellent diagnostic performance for differentiating patients with ovarian cancer (N = 35) from those with benign ovarian tumours (N = 741), with an AUC-ROC of 0.916. At a ROMA cut-off value of 10.55%, the test showed a sensitivity of 88.6% and a specificity of 85.7%, with positive and negative likelihood ratios (LR+ and LR-) of 6.20 and 0.133, respectively, and positive and negative predictive values of 22.6% and 99.4%, respectively.

ROC analysis further showed that in the postmenopausal group, ROMA has excellent diagnostic performance for differentiating patients with ovarian cancer from women with benign ovarian tumours, with an AUC-ROC of 0.974. At a ROMA index cut-off value of 47.19%, the test showed a sensitivity and specificity of 91.3% and 97.0%, respectively, with LR+ and LR- values of 30.43 and 0.089, respectively, and positive and negative predictive values of 94.6% and 95.1%, respectively (Figure 34, Table 8, Table 9).

The medians (IQRs) of ROMA in the histologically differentiated groups were as follows: high-grade serous carcinoma – 95.00 (80.04–98.78), low-grade serous carcinoma – 63.28 (12.14–81.26), mucinous carcinoma – 26.25 (8.65–45.69), endometrioid carcinoma – 71.70 (26.51–90.75), clear cell carcinoma – 76.50 (15.97–86.52), and ovarian carcinoma without histological identification – 88.85 (75.81–92.80).

Patients with high-grade serous carcinoma (N = 97) had significantly higher ROMA index values compared with the remaining patients with ovarian cancer (N = 53) (U = 1298.50, p < 0.001, r = -0.408). However, ROC analysis demonstrated that the standalone application of ROMA does not provide sufficient diagnostic performance for differentiating high-grade serous carcinoma from the other histological types of ovarian cancer, with an AUC-ROC of 0.747 (Table 8).

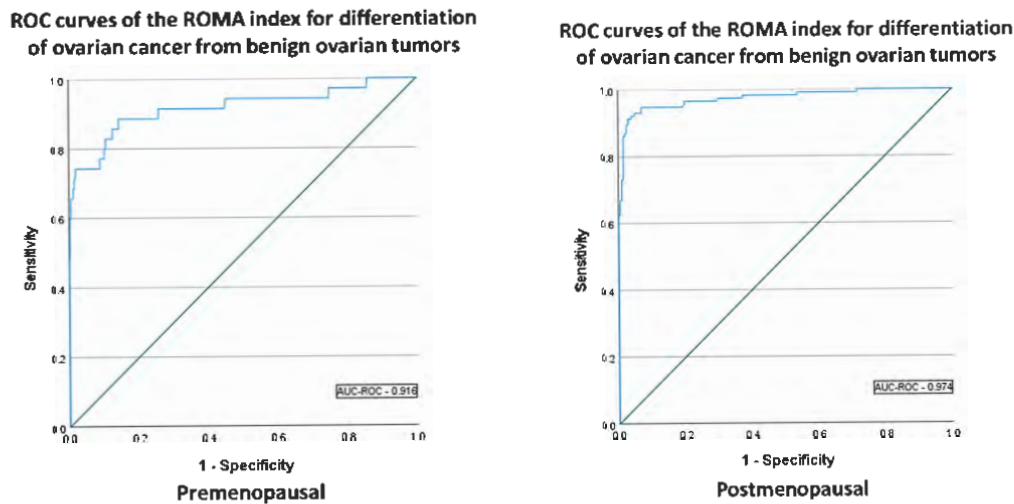


Figure 34. ROC curves of the ROMA index for differentiation of ovarian cancer from benign ovarian tumors in premenopausal and postmenopausal women

The medians (IQRs) of ROMA in subgroups stratified according to the stage of the oncological disease were as follows: Stage I – 36.12 (10.38–83.65), Stage II – 77.90 (42.08–95.81), Stage III – 94.95 (75.07–98.63), Stage IV – 89.90 (72.98–98.90), and undetermined stage – 87.40 (62.69–96.67) (Figure 35).

Following application of the Kruskal–Wallis test and subsequent post hoc analysis, a significant difference was observed between the results of patients with Stage I and Stage III disease (U = 169.00, p < 0.001, r = -0.403) and between Stage I and Stage IV disease (U = 71.00, p < 0.001, r = -0.507).

To assess the diagnostic value of ROMA in staging patients with ovarian cancer, discriminant analysis was performed. The analysis demonstrated that the standalone use of ROMA statistically significantly discriminates ovarian cancer stages ($\lambda = 0.850$, $\chi^2 = 23.608$, p < 0.001, $\eta = 0.230$) and explains 15.1% of the variance in patient group allocation.

The discriminant function was defined as: $D = -2.87 + (\text{ROMA index} \times 0.038)$ (Figure 35, Table 8).

Distribution of ROMA according to staging of patients with ovarian cancer

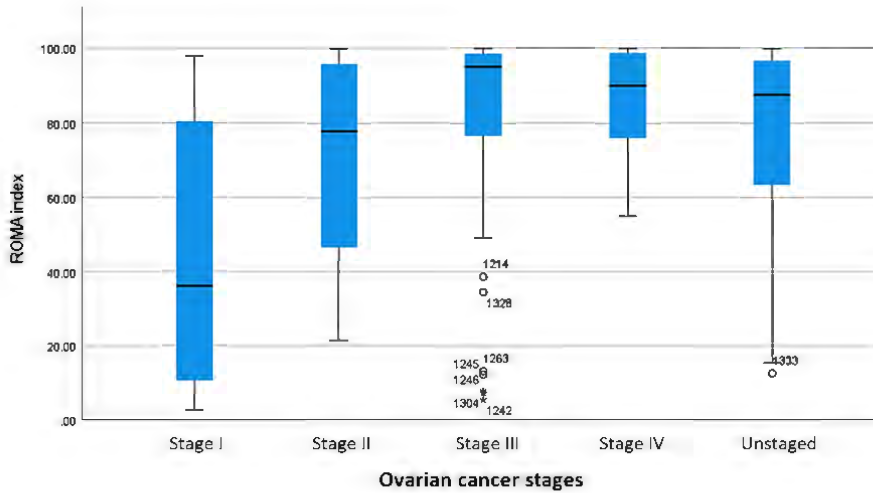


Figure 35. Distribution of ROMA according to staging of patients with ovarian cancer

Table 8. Diagnostic reliability of the ROMA index in the diagnosis of ovarian cancer

Diagnostic efficiency	ROMA – common group		ROMA – premenopausal		ROMA – postmenopausal	
	cut-off	AUC-ROC	cut-off	AUC-ROC	cut-off	AUC-ROC
Distinguishing OC from benign cyst	26.51	0.968	10.55	0.916	47.19	0.974
Distinguishing healthy subjects from benign cyst and OC	There is not enough diagnostic efficiency					
Distinguishing of the histological type of OC	There is not enough diagnostic efficiency					
Staging of OC	Reflects the progression of the disease – HR -1.03					

Table 9. ROMA index cut-off values derived in the present study

ROMA	Group	cut-off	N	TP	TN	FP	FN	Se	Sp
Distinguishing OC from benign cyst	Common	26.51	1092	133	913	29	17	89%	97%
Distinguishing OC from benign cyst	Premenopausal	10.55	776	31	635	106	4	89%	86%
Distinguishing OC from benign cyst	Postmenopausal	47.19	316	105	195	6	10	91%	97%

2.5 Diagnostic reliability of CPH-I for differentiation of patients with ovarian cancer from patients with benign formations or healthy women

The median (IQR) CPH-I value in the ovarian cancer cohort was 88.81 (44.28–97.06) %. In the premenopausal subgroup, the median CPH-I value was 53.04 (11.54–87.94) %, while in

the postmenopausal subgroup it was 83.46 (52.96–98.65) %. CPH-I values in the two ovarian cancer subgroups stratified according to menopausal status differed significantly ($U = 1202.00$, $p = 0.001$, $r = -0.294$).

Patients with ovarian cancer had significantly higher CPH-I values compared both with the control group (88.81 vs 0.600%; $U = 265.00$, $p < 0.001$, $r = -0.838$) and with patients with benign ovarian tumours (88.81 vs 1.030%; $U = 3578.50$, $p < 0.001$, $r = -0.566$) (Figure 36).

The observed significant difference in CPH-I values was also preserved in comparisons between groups stratified according to menopausal status (Figure 37).

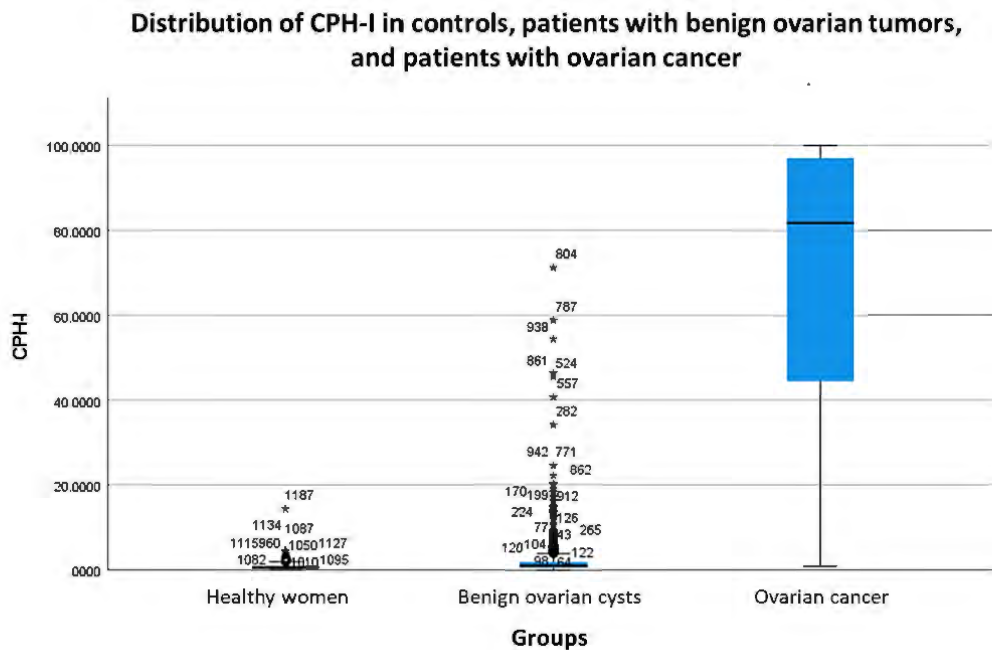


Figure 36. Distribution of CPH-I in controls, patients with benign ovarian tumors, and patients with ovarian cancer

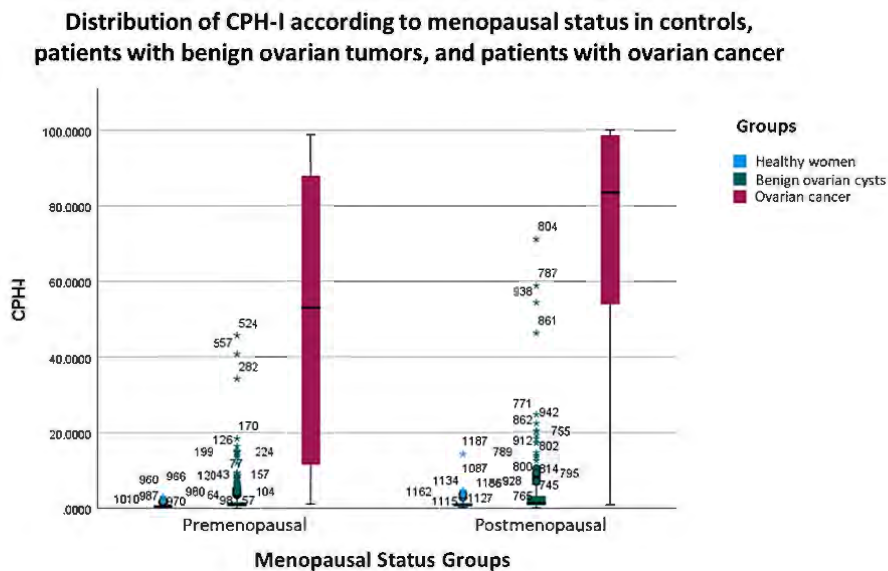


Figure 37. Distribution of CPH-I according to menopausal status in controls, patients with benign ovarian tumors, and patients with ovarian cancer

To assess the clinical significance of CPH-I in differentiating a pelvic space-occupying process, the diagnostic reliability of the marker for distinguishing ovarian cancer from benign ovarian tumours was evaluated. ROC analysis demonstrated that the standalone application of CPH-I has excellent diagnostic performance for differentiating patients with ovarian cancer from patients with benign ovarian tumours, with an AUC-ROC of 0.975.

At a CPH-I cut-off value of 11.14%, the test showed a sensitivity of 89.3% and a specificity of 97.0%, with positive and negative likelihood ratios (LR+ and LR-) of 29.77 and 0.110, respectively, and positive and negative predictive values of 82.7% and 98.3%, respectively (Figure 38, Table 10, Table 11).

ROC curve of CPH-I for differentiation of ovarian cancer from benign ovarian tumors

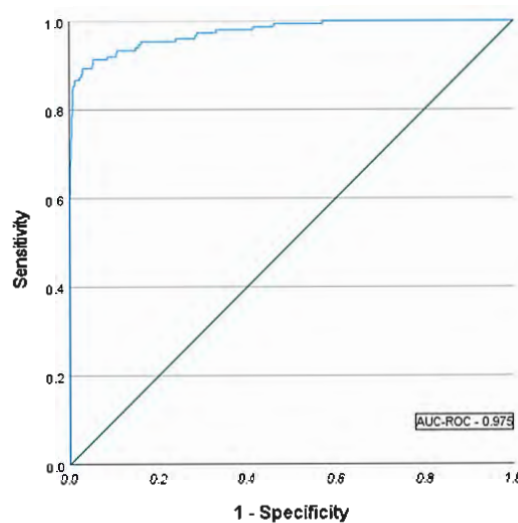


Figure 38. ROC curve of CPH-I for differentiation of ovarian cancer from benign ovarian tumors

The ovarian cancer and benign ovarian tumour groups were stratified according to menopausal status, and a repeat ROC analysis was performed. ROC analysis demonstrated that in the premenopausal group, CPH-I showed excellent diagnostic performance for differentiating patients with ovarian cancer from patients with benign ovarian tumours, with an AUC-ROC of 0.948. At a CPH-I cut-off value of 2.69%, the test showed a sensitivity of 88.6% and a specificity of 87.4%, with positive and negative likelihood ratios (LR+ and LR-) of 7.03 and 0.130, respectively, and positive and negative predictive values of 25.0% and 99.4%, respectively, for differentiating ovarian cancer from benign ovarian tumours.

ROC analysis further demonstrated that in the postmenopausal group, CPH-I has excellent diagnostic performance for differentiating patients with ovarian cancer from women with benign ovarian tumours, with an AUC-ROC of 0.976. At a CPH-I cut-off value of 22.49%, the test showed a sensitivity and specificity of 91.3% and 97.5%, respectively, with positive and negative likelihood ratios (LR+ and LR-) of 36.52 and 0.089, respectively, and positive and negative predictive values of 95.5% and 95.2%, respectively (Figure 39, Table 10, Table 11).

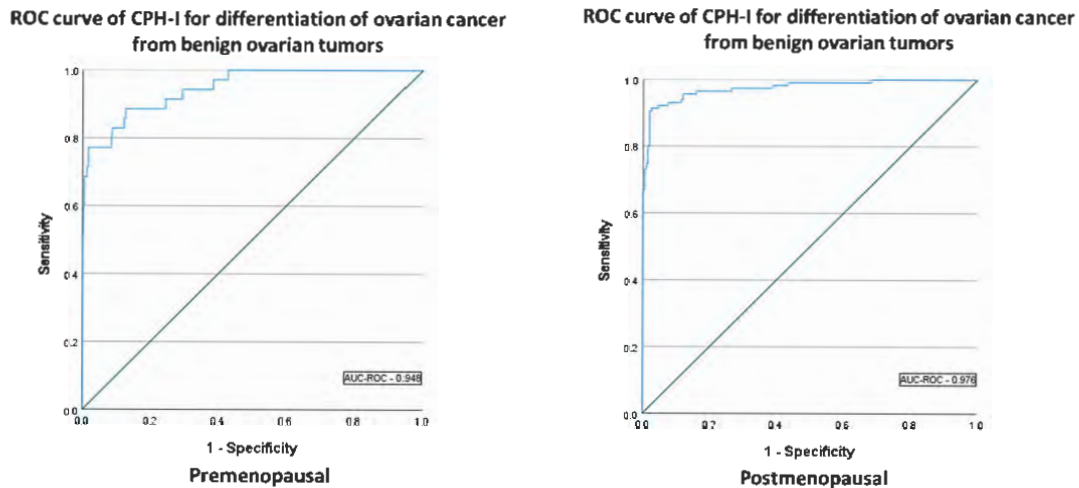


Figure 39. ROC curves of CPH-I for differentiation of ovarian cancer from benign ovarian tumors in premenopausal and postmenopausal women

The medians (IQRs) of CPH-I in the histologically differentiated groups were as follows: high-grade serous carcinoma – 91.75 (66.19–98.75)%, low-grade serous carcinoma – 50.86 (3.40–74.15)%, mucinous carcinoma – 6.97 (1.92–45.72)%, endometrioid carcinoma – 47.60 (26.32–86.81)%, clear cell carcinoma – 45.89 (2.25–68.76)%, and ovarian carcinoma without histological identification – 74.98 (60.56–88.25)%.

Patients with high-grade serous carcinoma had significantly higher CPH-I values compared with the remaining patients with ovarian cancer ($U = 1274.00$, $p < 0.001$, $r = -0.416$). However, ROC analysis demonstrated that the standalone application of CPH-I does not provide sufficient diagnostic performance for differentiating high-grade serous carcinoma from the other histological types of ovarian cancer, with an AUC-ROC of 0.752.

The medians (IQRs) of CPH-I in subgroups stratified according to the stage of the oncological disease were as follows: Stage I – 26.92 (1.92–78.82)%, Stage II – 49.42 (41.12–93.84)%, Stage III – 91.37 (61.58–98.84)%, Stage IV – 86.81 (59.46–98.58)%, and undetermined stage – 73.40 (37.59–94.90)%.

The Kruskal–Wallis test revealed a statistically significant difference in CPH-I values between subgroups stratified according to disease stage ($\chi^2(4) = 18.76$, $p < 0.001$). Subsequent post hoc analysis demonstrated that this difference was significant between patients with Stage I and Stage III disease ($U = 161.00$, $p < 0.001$, $r = -0.416$) and between Stage I and Stage IV disease ($U = 64.00$, $p < 0.001$, $r = -0.537$).

Discriminant analysis showed that the standalone use of CPH-I statistically significantly discriminates ovarian cancer stages ($\lambda = 0.861$, $\chi^2 = 21.782$, $p < 0.001$, $\eta = 0.245$) and explains 13.9% of the variance in patient group allocation. The discriminant function was defined as: $D = -2.20 + (\text{CPH-I} \times 0.032)$. This model successfully predicted the group allocation of 22.8% of patients with ovarian cancer according to disease stage (Figure 40, Table 11).

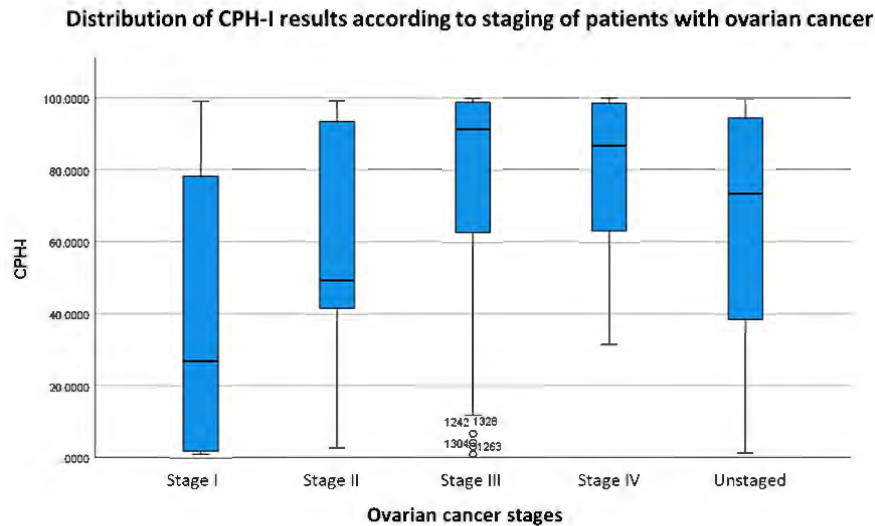


Figure 40. Distribution of CPH-I results according to staging of patients with ovarian cancer

Table 10. Diagnostic reliability of CPH-I in the diagnosis of ovarian cancer

Diagnostic efficiency	CPH-I – common group		CPH-I – premenopausal		CPH-I – postmenopausal	
	cut-off	AUC-ROC	cut-off	AUC-ROC	cut-off	AUC-ROC
Distinguishing OC from benign cyst	11.14%	0.975	2.69%	0.948	22.49%	0.976
Distinguishing healthy subjects from benign cyst and OC	There is not enough diagnostic efficiency					
Distinguishing of the histological type of OC	There is not enough diagnostic efficiency					
Staging of OC	Reflects the progression of the disease – HR -1.03					

Table 11. CPH-I cut-off values derived in the present study

CPH-I	Group	cut-off	N	TP	TN	FP	FN	Se	Sp
Distinguishing OC from benign cyst	Common	11.14%	1092	134	914	28	16	89%	97%
Distinguishing OC from benign cyst	Premenopausal	2.69%	776	31	648	93	4	89%	87%
Distinguishing OC from benign cyst	Postmenopausal	22.49%	316	105	196	5	10	91%	98%

A summary of the diagnostic reliability of HE4, CA125, ROMA, and CPH-I in the diagnosis of ovarian cancer is presented in the following figures and tables (Figure 41, Figure 42, and Table 12).

ROC curves of HE4, CA125, ROMA, and CPH-I for differentiation of benign ovarian tumors from ovarian cancer

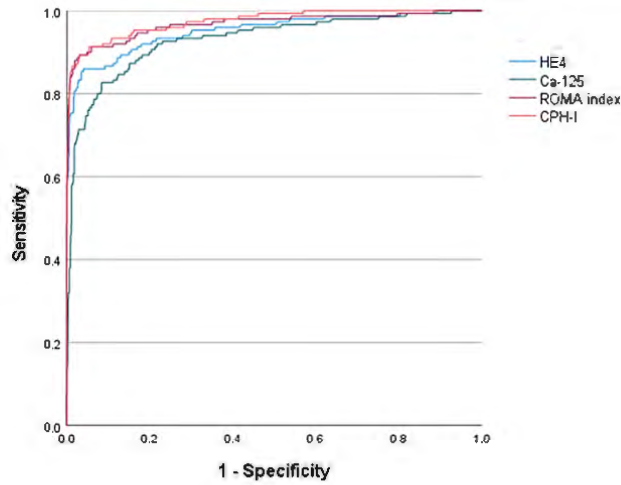


Figure 41. ROC curves of HE4, CA125, ROMA, and CPH-I for differentiation of benign ovarian tumors from ovarian cancer

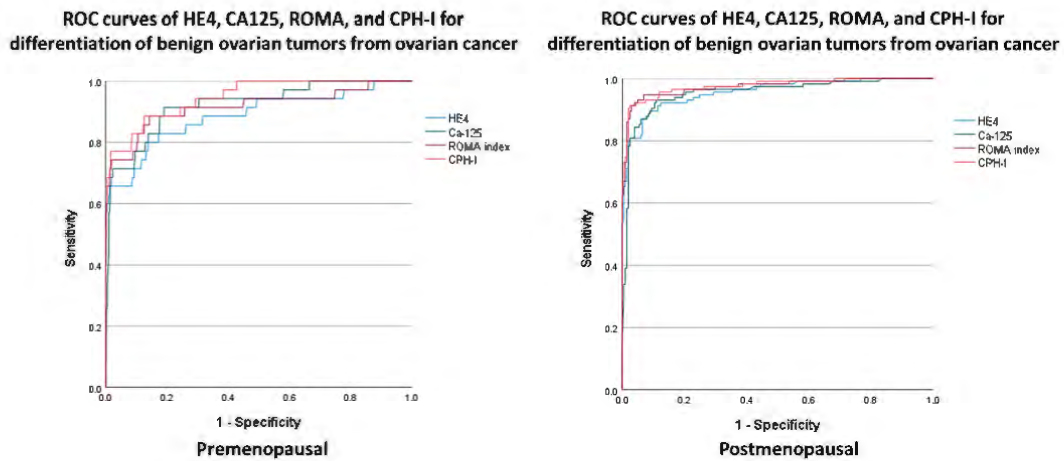


Figure 42. ROC curves of HE4, CA125, the ROMA index, and CPH-I for differentiation of benign ovarian tumors from ovarian cancer in premenopausal and postmenopausal women

Table 12. Diagnostic efficiency of HE4, CA125, ROMA, and CPH-I in the diagnosis of ovarian cancer

OC versus benign masses	HE4		CA125		ROMA index		CPH - I	
	cut-off	AUC-ROC (CI 95)	cut-off	AUC-ROC (CI 95)	cut-off	AUC-ROC (CI 95)	cut-off	AUC-ROC (CI 95)
Total	83.95 pmol/L	0.952 (0.930-0.974)	82.90 U/ml	0.930 (0.904-0.956)	26.51 %	0.968 (0.949-0.987)	11.14 %	0.975 (0.961-0.988)

Premenopausal	57.80 pmol/L	0.887 (0.815- 0.959)	52.00 U/ml	0.922 (0.871- 0.973)	10.55 %	0.916 (0.850- 0.982)	2.69%	0.948 (0.911- 0.985)
Postmenopausal	92.80 pmol/L	0.957 (0.934- 0.980)	37.75 U/ml	0.954 (0.929- 0.980)	47.19 %	0.974 (0.955- 0.992)	22.49 %	0.976 (0.958- 0.993)

The data indicate that ROMA and CPH-I demonstrate the highest diagnostic reliability for differentiating ovarian cancer from benign ovarian tumours, with diagnostic performance as follows: overall group – 96% / 96%, premenopausal group – 86% / 88%, and postmenopausal group – 95% / 95% (Table 13). ROMA and CPH-I also correlate with disease progression and stage.

Table 13. Diagnostic efficiency of ROMA and CPH-I in the diagnosis of ovarian cancer

Score	Group	cut-off	DSe	DSp	DE
ROMA	Total	26.51	89%	97%	96%
	Premenopausal	10.55	89%	86%	86%
	Postmenopausal	47.19	91%	97%	95%
CPH-I	Total	11.14%	89%	97%	96%
	Premenopausal	2.69%	89%	87%	88%
	Postmenopausal	22.49%	91%	98%	95%

2.6 Assessment of potential interferences and non-specific elevations of HE4 due to other benign non-gynecological diseases

To assess potential interferences related to elevated HE4 levels resulting from other benign non-gynaecological conditions (such as pelvic inflammatory processes, liver disorders, etc.), the 95th percentile of HE4 was determined in a group of 43 patients, yielding a value of 348.12 pmol/L (95% CI: 175.7–424.2). Patients were not stratified according to menopausal status, and the data analysis was performed for the entire cohort.

These results support the need for caution when interpreting HE4 levels in the presence of concomitant inflammatory or chronic diseases.

2.7 Assessment of non-specific expression of HE4 in non-gynecological malignancies

To evaluate the non-specific expression of tumour markers in the presence of non-gynaecological malignancies, a group of 47 patients was analyzed. The results were as follows: for HE4, the 95th percentile was 909.3 pmol/L (95% CI: 349.3–1744.6); for CA125, 1786.3 U/mL (95% CI: 747.7–7987); and for ROMA, 99.1% (95% CI: 95.5–99.9) (Table 14).

These findings indicate that the investigated tumor markers are not specific exclusively to neoplasms of gynaecological origin and may also be elevated in malignancies of other primary sites, as well as in cases of ovarian metastases from tumors with a different primary localization.

Table 14. Concentration of the markers in other non-gynecological neoplasms

HE4 other non-gynecological neoplasms	95 percentile	95% CI	
		Low limit	Upper limit
	909,34	349,31 ^b	1744,60 ^b
		95% CI	

CA125	95 percentile	Low limit	Upper limit
other non-gynecological neoplasms	1786.3	747.73	7987,00
		95% CI	
ROMAscore	95 percentile	Low limit	Upper limit
other non-gynecological neoplasms	99,10	95,52	99,90

3. Evaluation of the prognostic significance of preoperative marker levels and multimarker algorithms with respect to overall survival

The analytical approach in this section of the project included a univariate Cox proportional hazards model with independent assessment of the effect of each factor, followed by a multivariate model evaluating the combined impact of several factors, such as age, histological type, and disease stage.

To assess the prognostic value of HE4, CA125, ROMA, and CPH-I in relation to overall survival in patients with ovarian cancer, survival data from 150 women with ovarian cancer were analyzed. The mean age at diagnosis was 61 years (range: 30–92 years).

Patients were followed for a period of 144 months, with an overall mortality rate of 71% (N = 106) during the observation period. The median overall survival for all patients was 42 months (95% CI: 29–55 months). The one-year and three-year survival rates were 70% and 55%, respectively. Among patients with a fatal outcome, the median survival was significantly shorter—17 months (95% CI: 8–26 months).

The Kaplan–Meier analysis showed that patients with different histological subtypes of ovarian cancer had different prognoses; however, the differences in survival between the groups did not reach statistical significance (Chi-square = 3.821, $p = 0.051$). Nevertheless, the poorest prognosis was observed in patients with high-grade serous carcinoma, with a mortality rate of 80% (N = 78) during the observation period, a median overall survival of 24 months (95% CI: 8–40 months), and one-year and three-year survival rates of 62% and 43%, respectively (Table 15, Figure 43).

Table 1. Overall survival in the different histological groups OC

Histology	Number	OS-144months.	Median of the survival in months	1-year survival	3-years survival
Serous- high grade	97	80 %	24	62 %	43 %
Serous- low grade	10	50 %	92	90 %	70 %
Mucinous	10	50 %	98	90 %	80 %
Endometrioid	7	57 %	140	86 %	86 %
Creal cell	5	60 %	144	80 %	80 %
Unknown	21	48 %	87	71 %	71 %

Survival curve according to histological outcome in ovarian cancer

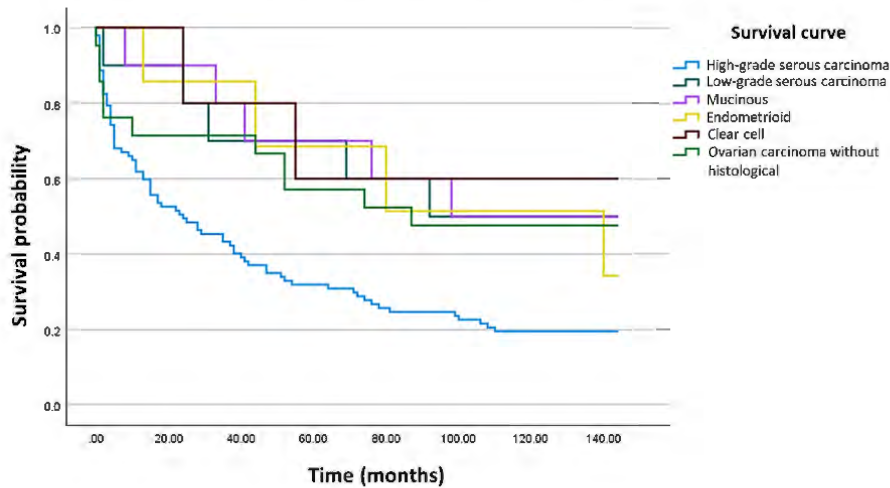


Figure 43. Survival curve according to histological outcome in ovarian cancer

The results of the Log-rank (Kaplan–Meier) test showed a significant difference in survival between patients at different stages of ovarian cancer (Chi-square = 25.322, df = 4, $p < 0.001$). The differences were most pronounced when Stage IV was compared with all other groups ($p < 0.001$, $p = 0.013$, $p = 0.019$, $p < 0.001$), as well as between Stages I and II and Stages I and III ($p = 0.040$, $p = 0.006$).

The poorest prognosis was observed in patients with Stage IV disease, in whom a mortality rate of 89% (N = 25) was recorded during the observation period, with a median overall survival of 5 months (95% CI: 0–16 months) and one-year and three-year survival rates of 46% and 29%, respectively (Table 16, Figure 44).

Survival curve according to stage in ovarian cancer

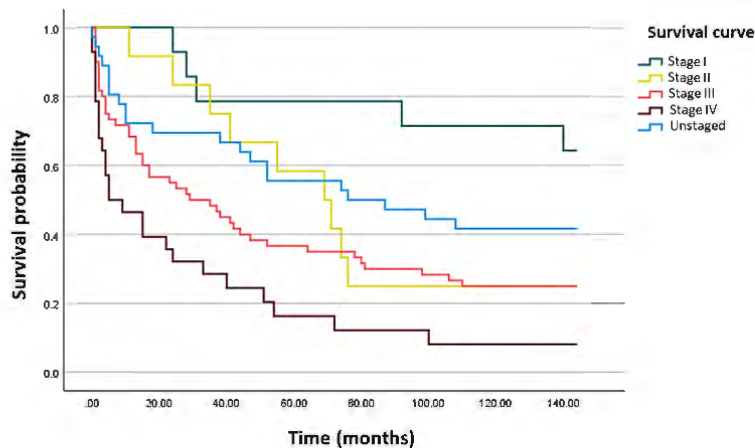


Figure 44. Survival curve according to stage in ovarian cancer

Table 16. Survival according to stage in ovarian cancer

Stage	Number	OS144months.	Median of the survival in months	1-year survival	3-years survival
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Stage I	14	36 %	144	100 %	79 %
Stage II	12	75 %	69	92 %	75 %
Stage III	60	75 %	29	68 %	48 %
Stage IV	28	89 %	5	46 %	29 %
Unknown	36	42 %	76	72 %	69 %

To assess the relationship between the biomarkers (HE4, CA125) and the combined prognostic indices (ROMA, CPH-I) with overall survival, the values of these parameters at the time of diagnosis were analyzed.

The median (IQR) HE4 level in the entire ovarian cancer cohort was 338.20 (131.65–862.78) pmol/L; in surviving patients it was 154.20 (62.05–463.40) pmol/L, and in patients with a fatal outcome it was 364.00 (156.45–1090.35) pmol/L. The results of the Mann–Whitney test showed significantly higher HE4 values in patients with a fatal outcome compared with surviving patients ($U = 1509.0$, $p < 0.001$, $r = -0.286$).

The median (IQR) CA125 level in the entire ovarian cancer cohort was 364.35 (132.68–1000.00) U/mL; in surviving patients it was 330.20 (79.30–919.75) U/mL, and in patients with a fatal outcome it was 386.50 (155.10–1000.00) U/mL. No significant difference was observed between these groups ($p = 0.302$).

The median (IQR) ROMA value in the entire ovarian cancer cohort was 88.98 (64.59–97.76); in surviving patients it was 79.94 (30.25–93.64), and in patients with a fatal outcome it was 90.44 (72.79–98.59). The results of the Mann–Whitney test showed significantly higher ROMA values in patients with a fatal outcome compared with surviving patients ($U = 1679.0$, $p = 0.005$, $r = -0.229$).

The median (IQR) CPH-I value in the entire ovarian cancer cohort was 81.81 (44.28–97.06)%; in surviving patients it was 61.66 (11.63–87.26)%, and in patients with a fatal outcome it was 85.24 (53.01–98.62)%. The results of the Mann–Whitney test showed significantly higher CPH-I values in patients with a fatal outcome compared with surviving patients ($U = 1546.5$, $p < 0.001$, $r = -0.273$) (Figure 45, Figure 46).

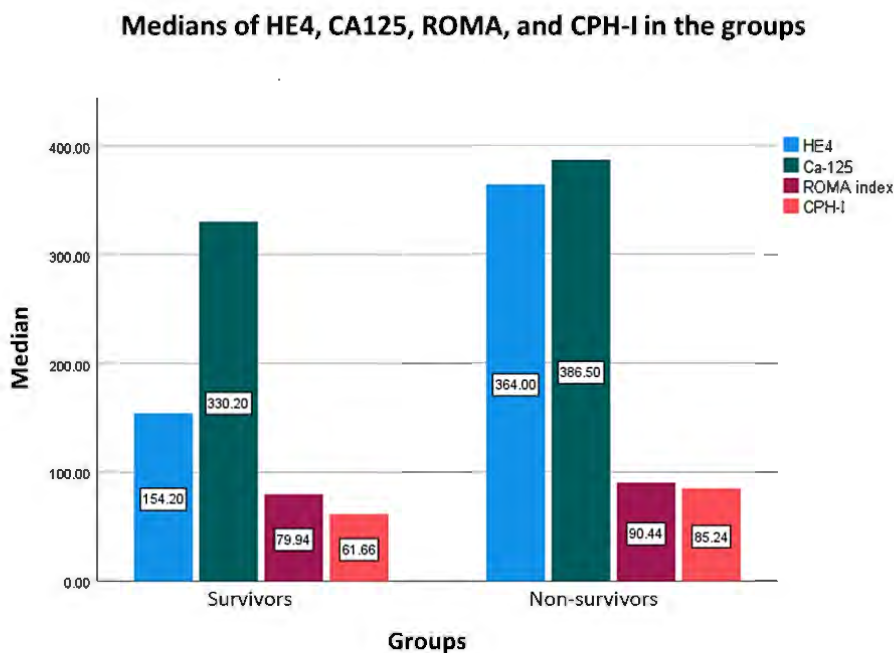


Figure 45. Medians of HE4, CA125, ROMA, and CPH-I in the groups

Correlation between HE4, CA125, ROMA, and CPH-I and overall survival

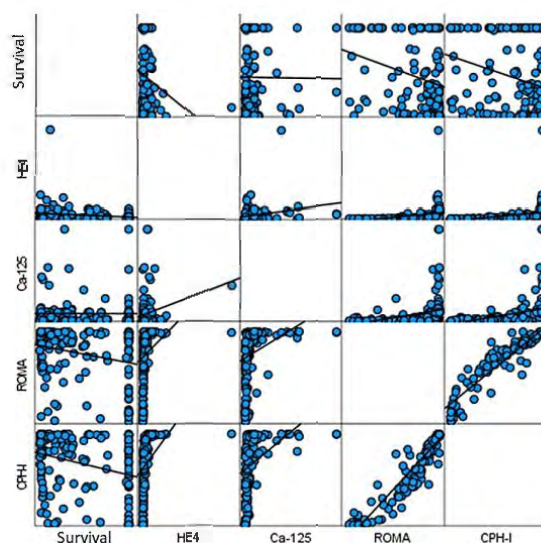


Figure 46. Correlation between HE4, CA125, ROMA, and CPH-I and overall survival

The correlation analysis (Spearman’s rho) showed a significant negative correlation between survival and HE4, ROMA, CPH-I, and age ($\rho = -0.281$, $p < 0.001$; $\rho = -0.222$, $p = 0.006$; $\rho = -0.279$, $p < 0.001$; and $\rho = -0.433$, $p < 0.001$, respectively).

Cox regression analysis demonstrated that HE4 was a statistically significant predictor of survival in the univariate model (Chi-square = 7.210, $p = 0.007$). However, the value of $\text{Exp}(B) = 1.000$ (95% CI: 1.000–1.000) indicates that an increase in HE4 does not lead to a meaningful change in the risk of the event (death). After inclusion of the variables disease stage, histological subtype, and age at diagnosis, the p-value for HE4 changed to 0.354, indicating that the marker does not retain statistical significance in the context of the multivariate model. Kaplan–Meier analysis based on an HE4 cut-off value of 143.05 pmol/L showed a sensitivity of 81.9% and a specificity of 48.9% for differentiating patients with a fatal outcome. In the group with $\text{HE4} \leq 143.05$ pmol/L ($N = 41$), overall mortality was 46%, one-year survival was 81%, and three-year survival was 66%. In the group with $\text{HE4} > 143.05$ pmol/L ($N = 109$), overall mortality was 79%, one-year survival was 66%, and three-year survival was 51%.

The Log-rank test showed a statistically significant difference in survival between the two groups (Chi-square = 12.299, $df = 1$, $p < 0.001$), with higher HE4 values associated with an increased risk of a fatal outcome (Figure 47).

Survival curve in patients with ovarian cancer according to HE4

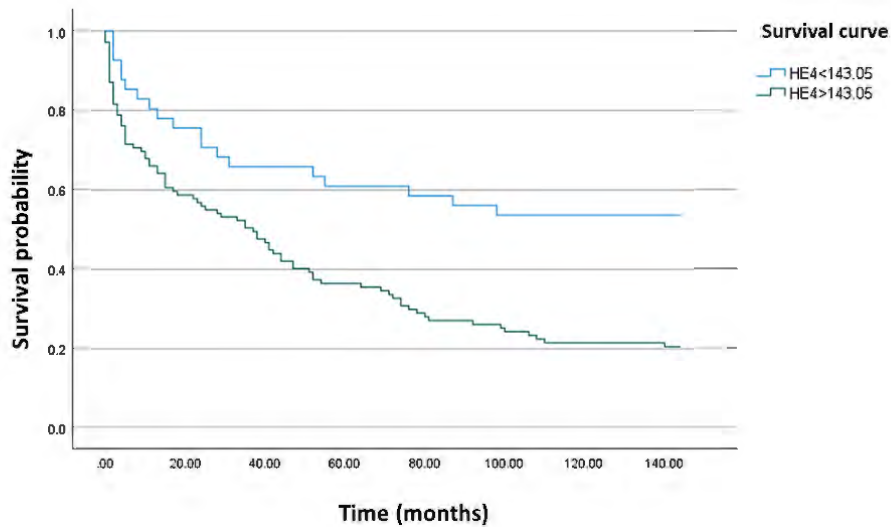


Figure 47. Survival curve in patients with ovarian cancer according to HE4

However, when patients were stratified by histological subtype, the difference in survival between the groups did not reach statistical significance (Log-rank test: Chi-square = 3.588, df = 1, p = 0.058), supporting the hypothesis that HE4 alone is not a sufficiently reliable predictor of survival in the context of a specific histological subtype.

Cox regression analysis examining the association between CA125 and survival in patients with ovarian cancer showed that CA125 is not a significant predictor of survival. The values of p = 0.984 and Exp(B) = 1.000 (95% CI: 1.000–1.000) indicate that the marker is not predictive of survival.

Kaplan–Meier analysis based on a CA125 cut-off value of 594.40 U/mL showed a sensitivity of 42.9% and a specificity of 81.1% for differentiating patients with a fatal outcome. In the group with CA125 ≤ 594.40 U/mL (N = 92), overall mortality was 65%, one-year survival was 72%, and three-year survival was 54%. In the group with CA125 > 594.40 U/mL (N = 58), overall mortality was 78%, one-year survival was 67%, and three-year survival was 52%. The Log-rank test did not demonstrate a statistically significant difference in survival between the two groups (Chi-square = 2.494, df = 1, p = 0.114) (Figure 48).

Survival curve in patients with ovarian cancer according to CA125

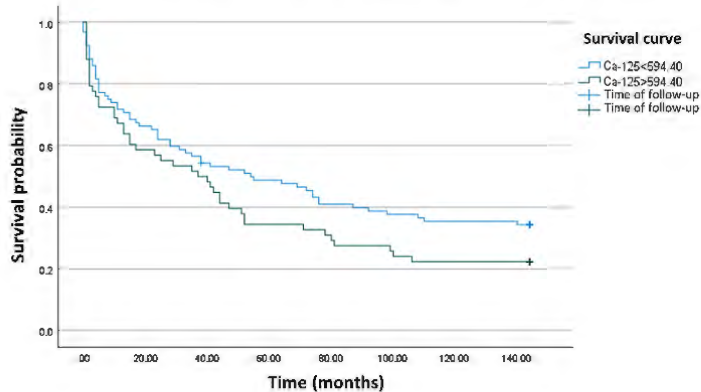


Figure 48. Survival curve in patients with ovarian cancer according to CA125

Cox regression analysis showed that ROMA is a statistically significant predictor of survival in the univariate model (Chi-square = 9.251, $p = 0.002$). The value of the coefficient $\text{Exp}(B) = 1.012$ (95% CI: 1.004–1.020) indicates that with each one-unit increase in ROMA, the risk of an adverse outcome (death) increases by 1.2%. However, in the multivariate model, ROMA loses its statistical significance and cannot be considered an independent predictor of survival. Kaplan–Meier analysis showed that in the group with ROMA index ≤ 39.06 ($N = 22$), overall mortality was 32%, one-year survival was 96%, and three-year survival was 77%. In the group with ROMA > 39.06 ($N = 128$), overall mortality was 77%, one-year survival was 66%, and three-year survival was 51%. The Log-rank test showed a statistically significant difference in survival between the two groups (Chi-square = 13.447, $df = 1$, $p < 0.001$), with higher ROMA values associated with an increased risk of a fatal outcome (Figure 49).

When the histological subtype is considered, the prognostic value of ROMA for survival is limited, highlighting the need to include additional clinical and biological parameters.

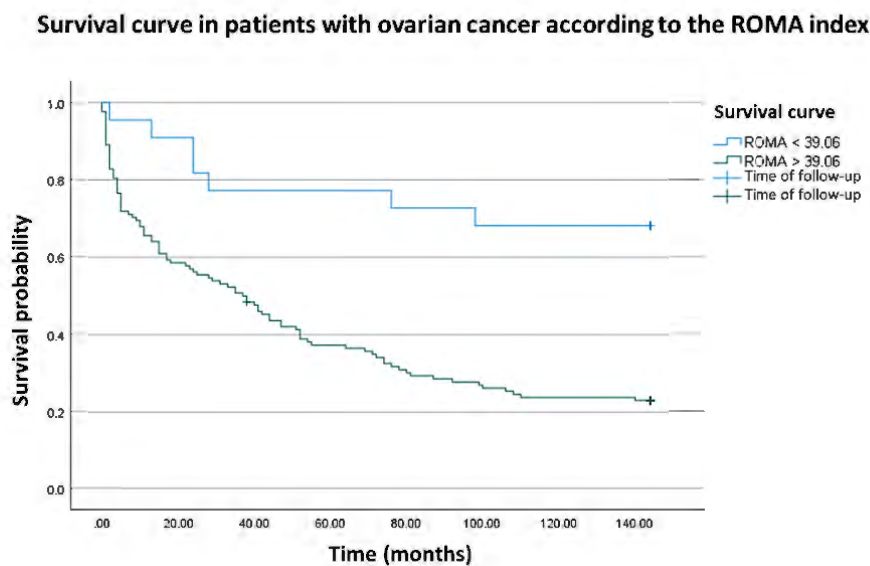


Figure 49. Survival curve in patients with ovarian cancer according to the ROMA index

Cox regression analysis showed that CPH-I is a statistically significant predictor of survival in the univariate model (Chi-square = 11.358, $df = 1$, $p < 0.001$), with $\text{Exp}(B) = 1.011$ (95% CI: 1.004–1.018). In the multivariate model, however, CPH-I loses its statistical significance and cannot be considered an independent predictor of survival.

Kaplan–Meier analysis based on a CPH-I cut-off value of 87.82% showed a sensitivity of 49.5% and a specificity of 77.8% for differentiating patients with a fatal outcome. In the group with CPH-I $\leq 87.82\%$ ($N = 88$), overall mortality was 60%, one-year survival was 75%, and three-year survival was 61%. In the group with CPH-I $> 87.82\%$ ($N = 62$), overall mortality was 84%, one-year survival was 63%, and three-year survival was 45%. The Log-rank test showed a statistically significant difference in survival between the two groups (Chi-square = 10.810, $df = 1$, $p < 0.001$), with higher CPH-I values associated with an increased risk of a fatal outcome (Figure 50).

When patients were stratified by histological subtype, the difference in survival between the groups based on CPH-I values (87.82%) was reduced, further supporting the hypothesis that CPH-I alone is not a sufficiently reliable predictor of survival in the context of a specific histological subtype.

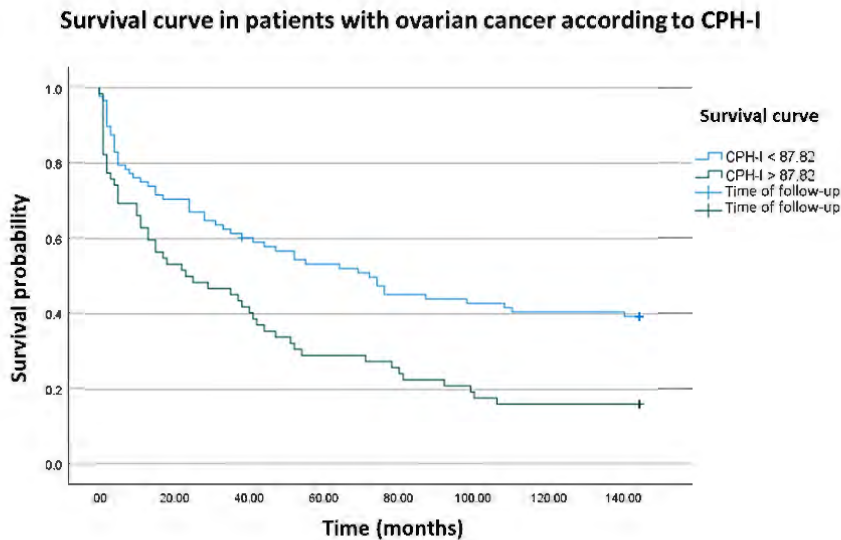


Figure 50. Survival curve in patients with ovarian cancer according to CPH-I

Summary data on the prognostic significance of baseline marker and algorithm values with respect to overall survival are presented in Table 17.

Table 17. Diagnostic reliability of HE4, CA125, ROMA, and CPH-I in the assessment of survival in patients with ovarian cancer – Cox analysis

Test	Univariate analysis (p-value)	Exp(B) (95% CI)	Multivariate analysis (p-value)	Exp(B) (95% CI)
HE 4	p = 0.007	1.000 (1.000-1.000)	p = 0.354	1.000 (1.000-1.000)
CA 125	p = 0.984	1.000 (1.000-1.000)	p = 0.638	1.000 (1.000-1.000)
ROMA	p = 0.002	1.012 (1.004-1.020)	p = 0.849	0.999 (0.989-1.009)
CPH-I	p < 0.001	1.011 (1.004-1.018)	p = 0.877	1.001 (0.993-1.008)

The multivariate Cox analysis, stratified by histology and examining the relationship between HE4, CA125, ROMA, CPH-I, disease stage, and age with survival in patients with ovarian cancer, showed that the model is statistically significant (Chi-square = 44.525, $p < 0.001$). However, when the individual variables in the equation were examined, the biomarkers did not reach statistical significance ($p > 0.05$), indicating that they do not predict survival when controlled for other factors in the model.

A mediation analysis, stratified by histology, was performed to assess the influence of interactions between the biomarkers and disease stage on survival. The analysis showed that, among the four investigated biomarkers, HE4, ROMA, and CPH-I demonstrated significant indirect effects on survival mediated by disease stage. CPH-I was the only marker to demonstrate a significant direct effect on survival independent of stage ($B = -0.3056$, $p = 0.0431$). CA125 did not show significant direct or indirect effects on survival, suggesting limited prognostic value in this context.

The Decision Tree algorithm showed that, in predicting a fatal outcome, the values of Independent Variable Importance for ROMA, CPH-I, HE4, and disease histology were 0.092, 0.067, 0.060, and 0.027, respectively. Analysis of the derived algorithm revealed that ROMA is a key predictor, separating patients into subgroups based on a threshold value of 39.06 (diagnostic sensitivity 93.3% and specificity 33.3%). Patients with lower ROMA values

(<39.06%) showed a higher survival rate (68.2%) compared with those with higher values (>39.06%), in whom a fatal outcome predominated (76.6%). Histology and CPH-I also contributed to subgroup stratification. Patients with certain histological subtypes (high-grade serous carcinoma) and high CPH-I values (>92.55%) demonstrated a significantly more unfavorable outcome (Figure 51).

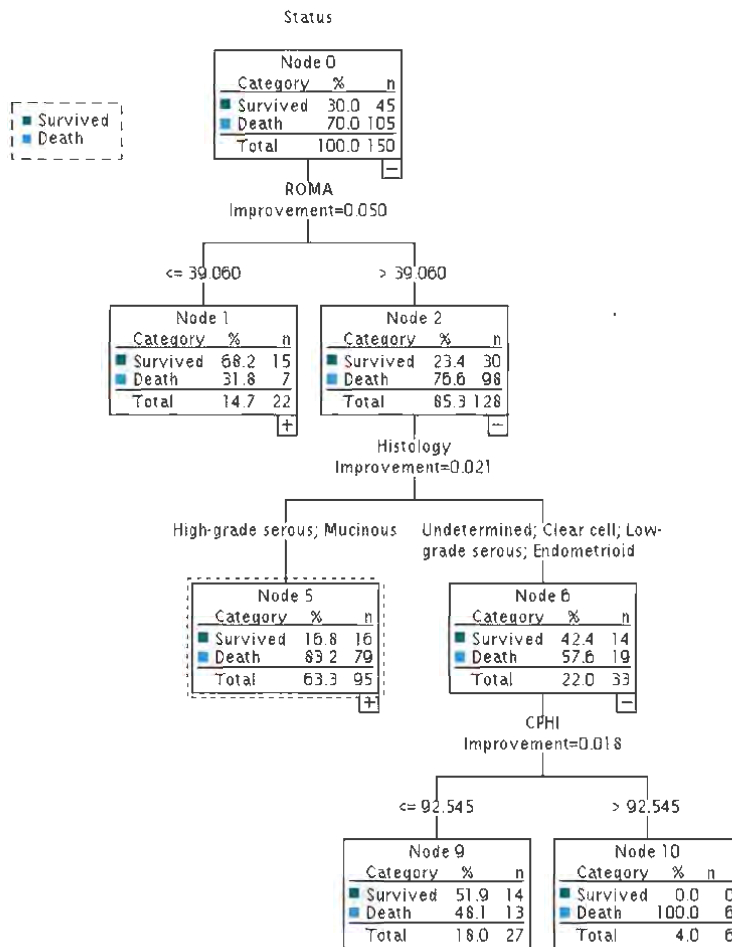


Figure 51. Decision tree algorithm for predicting fatal outcome in patients with ovarian cancer using ROMA, CPH-I, and histology

Additional analyses were performed regarding the prognostic significance of the markers in patients with other oncological localizations outside ovarian cancer. Three patient cohorts were included: patients with ovarian cancer, endometrial cancer, and other non-gynaecological malignancies (breast carcinoma, colorectal carcinoma, primary peritoneal carcinoma, and lung carcinoma). The total number of patients included in the survival variation analysis was 160. They were distributed as follows: 86 patients with ovarian cancer (53.8%), 44 patients with endometrial cancer (27.5%), and 30 patients with other oncological localizations (18.7%). Since this additional analysis included patients with non-gynaecological malignancies, the evaluation was performed only with respect to the independent prognostic value of CA125 and HE4, but not the algorithms, which are validated exclusively in oncogynaecological diseases. The results of the performed univariate Cox regression analysis are presented in summary Table 18 and can be summarized as follows:

- HE4 shows the highest prognostic value with respect to overall survival in the combined group. Patients with a baseline marker concentration > 143 pmol/L have a 2.94-fold higher risk of reduced survival (HR = 2.94, 95% CI 1.706–5.076, p < 0.0001). CA125 shows similar results (Figure 52, Figure 53).
- In the analysis of the univariate Cox regression data by individual localization: in patients with ovarian cancer, the histological type of carcinoma has the highest prognostic value—high-grade tumors are highly predictive of reduced survival (HR = 3.337, 95% CI 1.48–7.511, p = 0.004). Among the biomarkers, HE4 has the best prognostic value. Marker values > 1049.6 pmol/L are associated with HR = 2.988 (95% CI 1.593–7.511, p = 0.001). CA125 has weak prognostic power in patients with ovarian cancer (Figure 54, Figure 55).
- In the group of patients with endometrial cancer, HE4 again has the greatest prognostic significance. Marker values > 298.3 pmol/L are associated with significantly worse survival (HR = 9.853, 95% CI 2.53–38.3, p < 0.001). Once again, the standalone determination of CA125 has weaker prognostic value (Figure 56, Figure 57).

Table 18. Summary results on the prognostic significance of the markers with respect to overall survival

Independent variance	Cut-off	Risk category	Reference category	HR Hazard Ratio	CI 95%	CI 95%	P-value
OS in the common group							
HE4	143,4	2=143,4+	1<143,4	2,943	1,706	5,076	<0,0001
CA125	103,9	2=104,0+	1=<= 103,9	2,915	1,727	4,922	<0,0001
Стадий_2		4	0	2,623	1,451	4,743	0,001
OS in the group with OC							
HE4	1049,6	2=1049,6+	1=<1049,6	2,988	1,593	5,604	0,001
CA125	345,7	2=345,7+	1=<345,7	2,264	1,211	4,234	0,010
Histology_2		1	0	3,337	1,483	7,511	0,004
Stage 2		4	0	2,119	1,070	4,195	0,031
OS in the group with EC							
HE	289,3	2=289,3+	1=<289,3	9,853	2,533	38,333	0,001
CA125	81,1	2=81,1+	1=<81,1	4,678	1,480	14,791	0,009
Stage_2		4	0	3,995	1,186	13,458	0,025

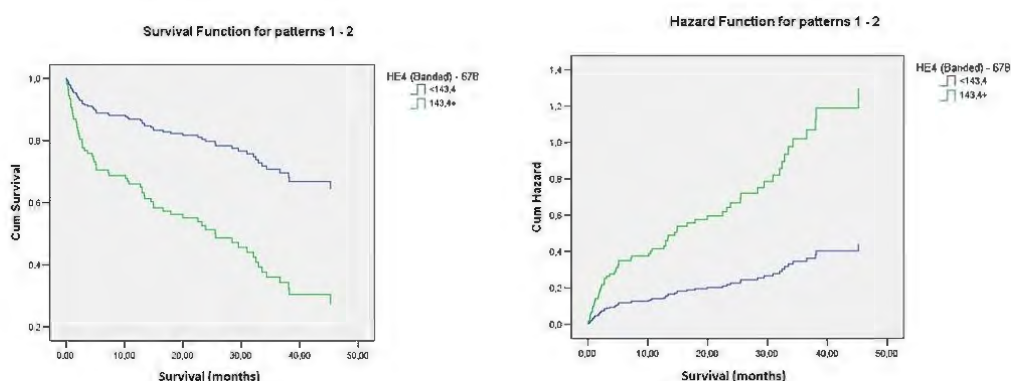


Figure 52. Regression analysis for overall survival: HE4 – combined group

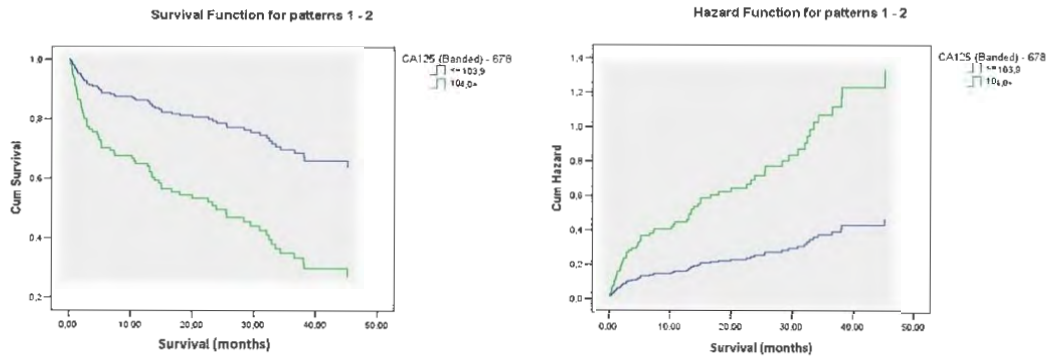


Figure 53. Regression analysis for overall survival: CA 125– combined group

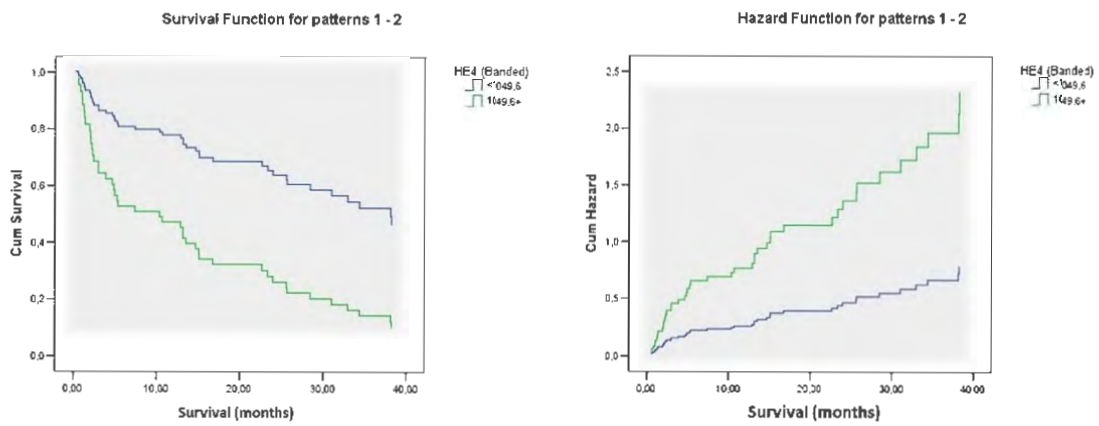


Figure 54. Regression analysis for overall survival: HE4– patients with OC

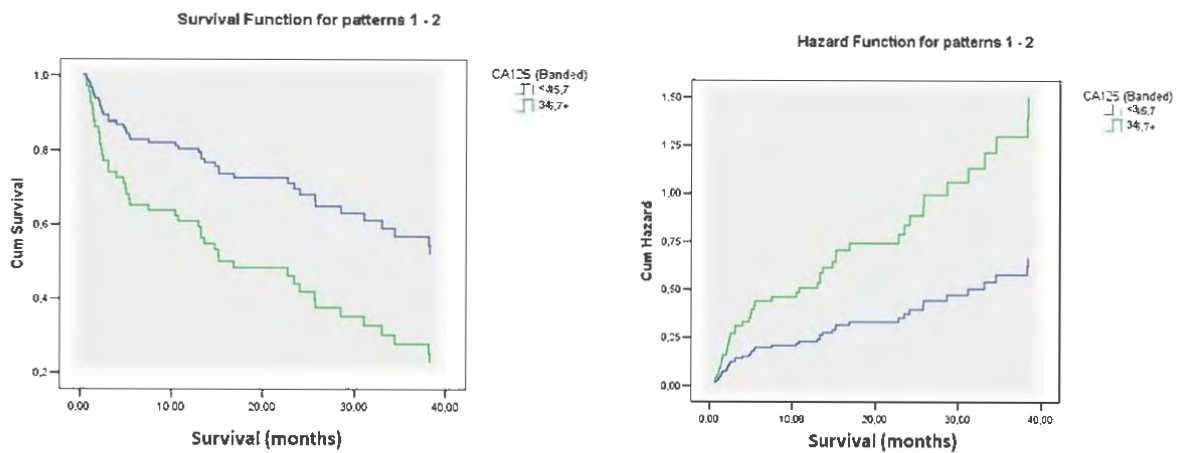


Figure 55. Regression analysis for overall survival: CA 125– OC group

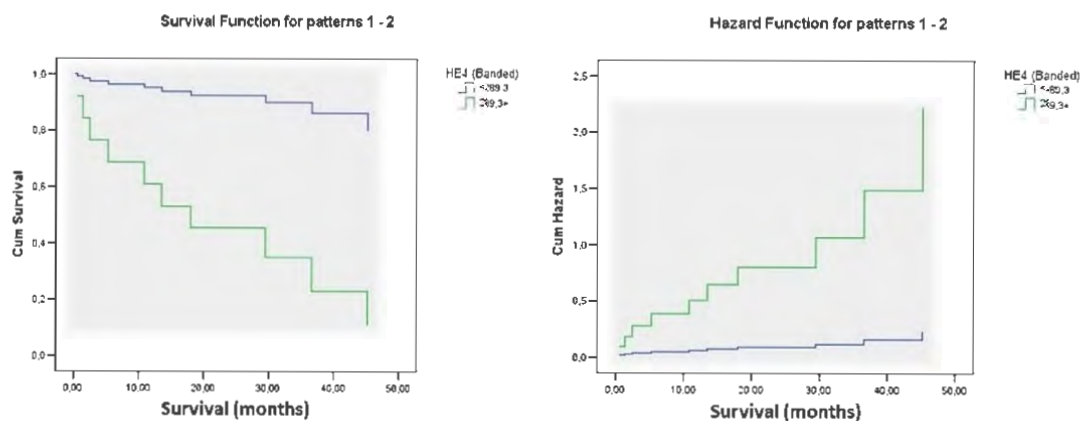


Figure 56. Regression analysis for overall survival: HE4– patients with EC

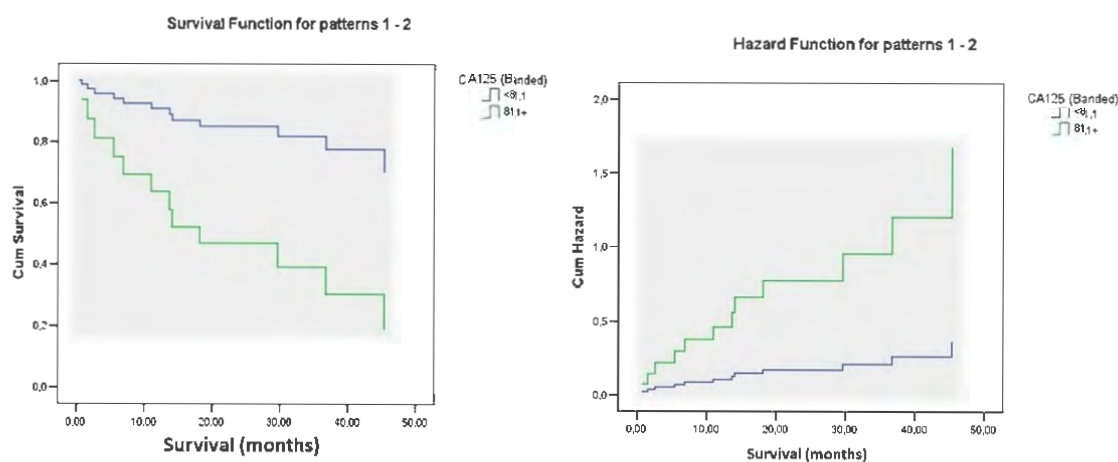


Figure 57. Regression analysis for overall survival: CA 125– patients with EC

4. To evaluate the clinical applicability of CPH-I, ROMA, and the standalone use of the tumor markers CA125 and HE4 in patients with endometrial cancer

To accomplish this objective, 74 women with histologically confirmed uterine carcinoma were included in the study. Statistical testing for normal distribution of HE4, CA125, ROMA, and CPH-I values showed non-Gaussian distribution, and subsequent analyses were therefore performed using non-parametric methods (significance level $\alpha = 0.05$).

Due to the small number of patients in some histological subgroups, the cohort was divided into two main groups: endometrioid carcinoma (N = 52) and other histological types of uterine carcinoma (N = 22). Subgroup analysis according to menopausal status was not performed, as only 8 women were in the premenopausal group. According to disease stage, the cohort was divided into two groups: Stages I–II (N = 38) and Stages III–IV (N = 12).

The median and interquartile range (IQR) of HE4 in the entire cohort with uterine carcinoma were 98.80 (51.68–195.98) pmol/L. In the subgroup with endometrioid carcinoma, the values were 72.35 (47.90–160.85) pmol/L, and in the subgroup with other histological types of uterine carcinoma, they were 127.75 (95.95–415.70) pmol/L. The differences in HE4 values between

the two groups of patients with uterine carcinoma, stratified according to histological result, were significant ($U = 348.00$, $p = 0.023$, $r = -0.332$). This is due to the higher HE4 levels observed in patients with serous carcinoma of the uterus (Table 19).

Patients with uterine carcinoma had significantly higher HE4 values compared with the control group (98.80 vs 38.90 pmol/L; $U = 2745.00$, $p < 0.001$, $r = -0.509$) and compared with patients with benign diseases (98.80 vs 42.35 pmol/L; $U = 12855.00$, $p < 0.001$, $r = -0.284$). However, HE4 values in patients with uterine carcinoma were significantly lower than those observed in patients with ovarian cancer (98.80 vs 338.20 pmol/L; $U = 2748.00$, $p < 0.001$, $r = -0.410$) (Figure 58).

Table 19. Median and IQR of HE4, CA125, ROMA, and CPH-I in histologically differentiated groups with uterine carcinoma

Histology	HE4 (pmol/L)		CA 125 (U/ml)	ROMA index	CPH-I (%)
	N	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
Controls	246	38.90 (13.93)	12.00 (8.8)	4.94 (4.13)	0.60 (0.61)
Endometrioid	52	72.35 (112.95)	23.90 (30.43)	22.02 (33.97)	5.02 (26.22)
Serous	5	542.70 (1162.0)	429.70 (933.70)	95.80 (41.43)	94.07 (68.37)
Clear Cell	2	29.10 *	11.75 *	3.27 *	0.40 *
Squamous	5	170.50 (195.30)	48.60 (48.35)	52.30 (23.27)	27.09 (36.80)
Unknown	6	261.55 (349.30)	28.00 (102.75)	61.90 (44.93)	37.33 (39.68)

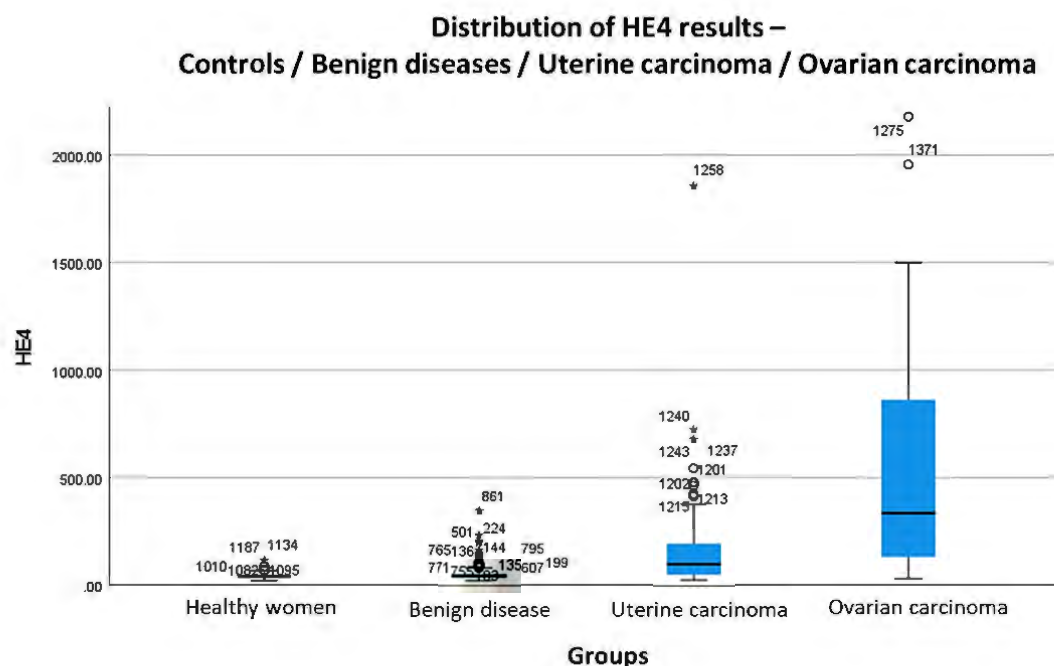


Figure 58. Distribution of HE4 results – Controls / Benign diseases / Uterine carcinoma / Ovarian carcinoma

The standalone use of HE4 demonstrated very good diagnostic performance for detecting patients with uterine carcinoma among patients with benign diseases (AUC-ROC = 0.816, 95% CI: 0.750–0.881). At an HE4 cut-off value of 69.90 pmol/L, the test showed a sensitivity of 64.9% and a specificity of 91.7%, with positive and negative likelihood ratios of $LR+ = 7.82$ and $LR- = 0.382$, as well as positive and negative predictive values of 38.1% and 97.1%, respectively, for differentiating patients with uterine carcinoma from those with benign diseases (Figure 62).

The median (IQR) HE4 values in subgroups stratified according to stage of uterine carcinoma were as follows: Stages I–II: 80.90 (54.63–118.33) pmol/L and Stages III–IV: 312.50 (135.95–522.60) pmol/L (Table 20). The results of the Mann–Whitney test showed significantly higher HE4 values in patients with Stages III–IV compared with those with Stages I–II ($U = 51.00$, $p < 0.001$, $r = -0.569$).

Table 20. Median and IQR of HE4, CA125, ROMA, and CPH-I in groups stratified according to stage – uterine carcinoma

Stage	HE4 (pmol/L)		CA 125 (U/ml)	ROMA index	CPH-I (%)
	N	Median	Median	Median	Median
		IQR	IQR	IQR	IQR
Stage I	36	72.35 (70.90)	29.85 (31.85)	22.96 (27.57)	5.48 (18.54)
Stage II	2	97.30 *	44.05 *	30.97 *	8.94 *

Stage III	6	387.95 (721.72)	236.00 (625.72)	76.75 (28.23)	64.39 (51.13)
Stage IV	6	242.50 (333.85)	58.45 (392.30)	69.91 (40.24)	42.46 (53.09)
Unknown	20	114.35 (175.50)	29.35 (32.95)	29.35 (32.95)	15.98 (40.59)

The median (IQR) CA125 level in the entire cohort of patients with uterine carcinoma was 30.25 (16.20–57.15) U/mL. In the subgroup with endometrioid carcinoma, the values were 29.85 (16.45–49.28) U/mL, whereas in the subgroup with other histological types of uterine carcinoma, they were 45.45 (21.90–218.03) U/mL. The differences in CA125 values between the two groups of patients with uterine carcinoma, stratified according to histological result, were significant ($U = 336.00$, $p = 0.015$, $r = -0.290$). This is due to the higher CA125 levels observed in patients with serous carcinoma of the uterus (Table 19).

Patients with uterine carcinoma had significantly higher CA125 values compared with the control group (30.25 vs 12.00 U/mL; $U = 2550.00$, $p < 0.001$, $r = -0.512$) and compared with patients with benign diseases (30.25 vs 18.10 U/mL; $U = 25023.00$, $p < 0.001$, $r = -0.127$). However, CA125 values in patients with uterine carcinoma were significantly lower than those observed in patients with ovarian cancer (30.25 vs 364.35 U/mL; $U = 1339.50$, $p < 0.001$, $r = -0.617$) (Figure 59).

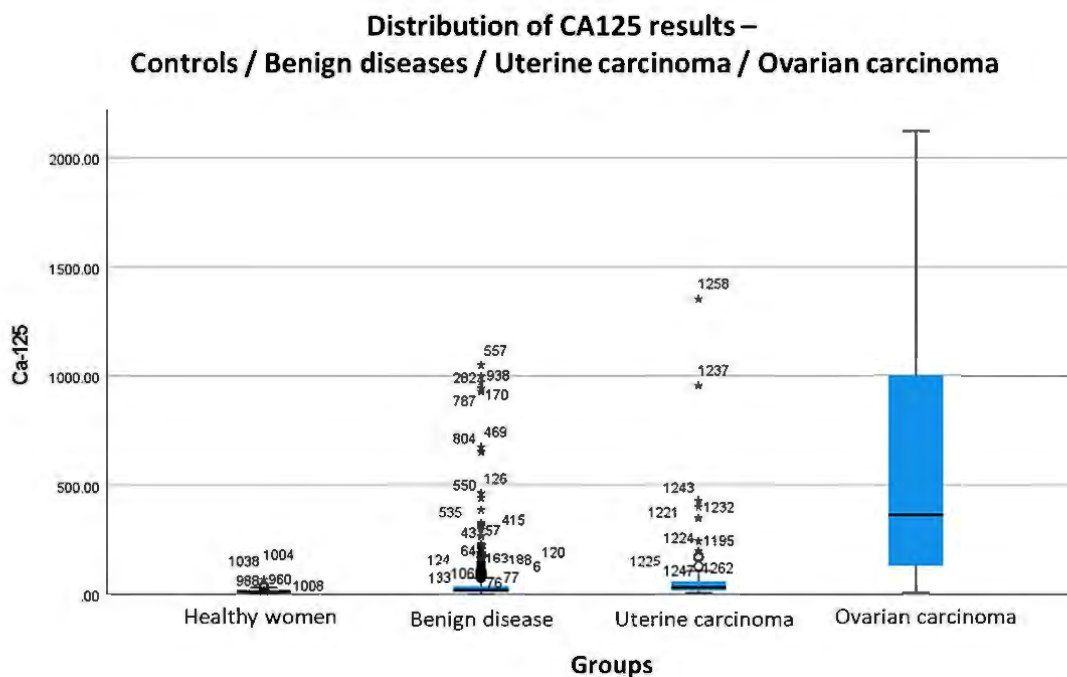


Figure 59. Distribution of CA125 results – Controls / Benign diseases / Uterine carcinoma / Ovarian carcinoma

ROC analysis showed that CA125 has unsatisfactory diagnostic performance for differentiating healthy individuals from those with benign diseases and uterine carcinoma (AUC-ROC =

0.712). The marker is also insufficiently effective for detecting patients with uterine carcinoma among those with benign diseases (AUC-ROC = 0.641) (Figure 62).

The median (IQR) CA125 values in subgroups stratified according to the stage of uterine carcinoma were as follows: Stages I–II: 29.85 (15.85–49.93) U/mL and Stages III–IV: 103.40 (32.90–409.43) U/mL (Table 20). The results of the Mann–Whitney test showed significantly higher CA125 values in patients with Stages III–IV compared with those with Stages I–II ($U = 91.00, p = 0.002, r = -0.440$).

The median (IQR) ROMA index in the entire cohort of patients with uterine carcinoma was 29.53 (13.99–57.90). In the subgroup with endometrioid carcinoma, the values were 23.25 (13.58–50.09), whereas in the subgroup with other histological types of uterine carcinoma, they were 55.65 (31.99–74.19). The differences in ROMA values between the two groups of patients with uterine carcinoma, stratified according to histological result, were significant ($U = 289.00, p = 0.004, r = -0.346$). This is due to the higher ROMA levels observed in patients with serous carcinoma of the uterus (Table 19).

Patients with uterine carcinoma had significantly higher ROMA values compared with the control group (29.53 vs 4.94; $U = 1393.50, p < 0.001, r = -0.623$) and compared with patients with benign diseases (29.53 vs 5.76; $U = 9148.00, p < 0.001, r = -0.332$). However, ROMA index values in patients with uterine carcinoma were significantly lower than those observed in patients with ovarian cancer (29.53 vs 88.98; $U = 1846.00, p < 0.001, r = -0.542$) (Figure 60).

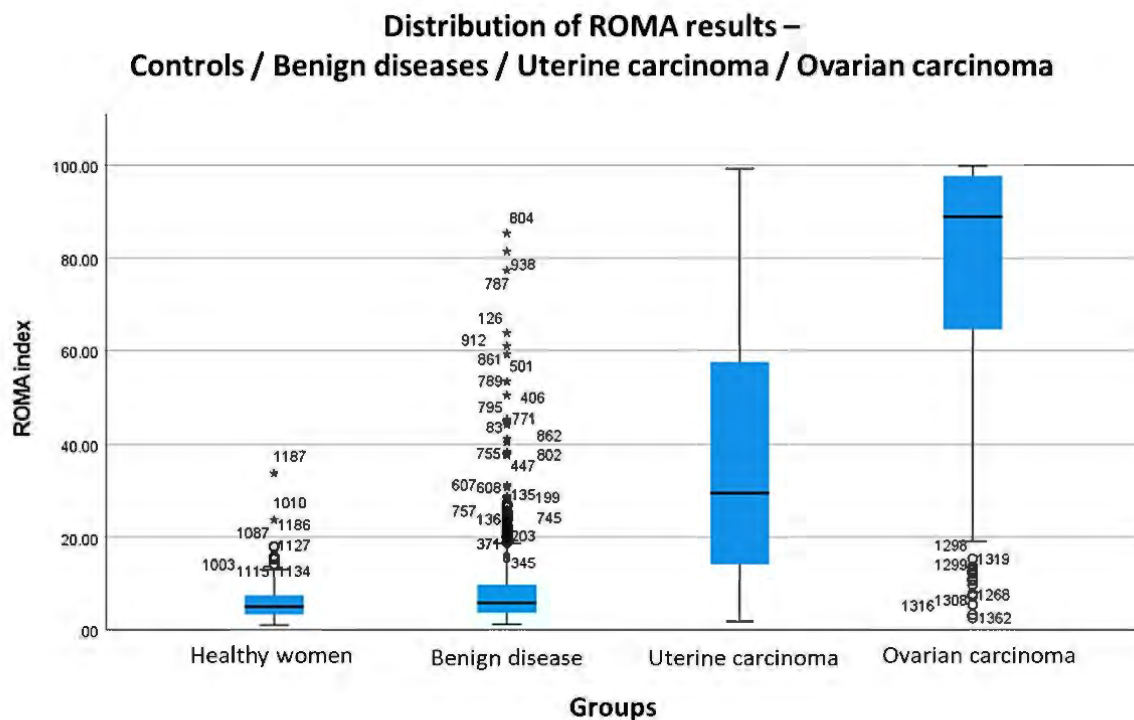


Figure 60. Distribution of ROMA results – Controls / Benign diseases / Uterine carcinoma / Ovarian carcinoma

ROC analysis showed that the standalone use of ROMA demonstrates very good diagnostic performance for detecting patients with uterine carcinoma among patients with benign diseases (AUC-ROC = 0.869, 95% CI: 0.818–0.920). At a ROMA cut-off value of 13.70 (corresponding to the high-risk threshold in postmenopausal women), the test showed a diagnostic sensitivity of 75.7% and a specificity of 85.7%, with positive and negative likelihood ratios of $LR+ = 5.29$

and $LR^- = 0.284$, as well as positive and negative predictive values of 29.3% and 97.8%, respectively, for differentiating patients with uterine carcinoma from those with benign diseases (Figure 62).

The median (IQR) ROMA values in subgroups stratified according to stage of uterine carcinoma were as follows: Stages I–II: 22.96 (14.02–41.83) and Stages III–IV: 72.81 (61.24–91.78) (Table 20). The results of the Mann–Whitney test showed significantly higher ROMA values in patients with Stages III–IV compared with those with Stages I–II ($U = 37.00$, $p < 0.001$, $r = -0.614$).

The median (IQR) CPH-I value in the entire cohort with uterine carcinoma was 8.81 (2.46–36.02)%. In the subgroup with endometrioid carcinoma, the values were 5.48 (2.38–29.97)%, whereas in the subgroup with other histological types of uterine carcinoma, they were 31.39 (11.57–51.02)%. The differences in CPH-I values between the two groups of patients with uterine carcinoma, stratified according to histological result, were significant ($U = 313.00$, $p = 0.007$, $r = -0.325$) (Table 19).

Patients with uterine carcinoma had significantly higher CPH-I values compared with the control group (8.81 vs 0.60%; $U = 1760.00$, $p < 0.001$, $r = -0.587$) and compared with patients with benign diseases (8.81 vs 1.03%; $U = 12399.00$, $p < 0.001$, $r = -0.290$). However, CPH-I values in patients with uterine carcinoma were significantly lower than those observed in patients with ovarian cancer (8.81 vs 81.81%; $U = 1717.00$, $p < 0.001$, $r = -0.561$) (Figure 61).

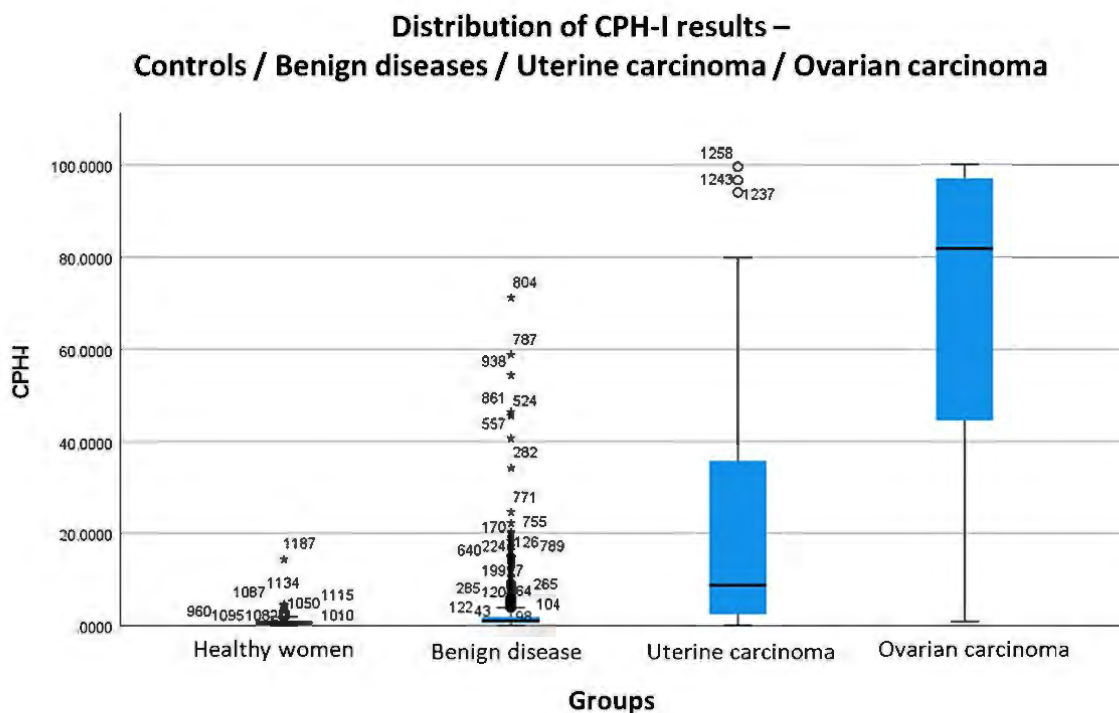


Figure 61. Distribution of CPH-I results – Controls / Benign diseases / Uterine carcinoma / Ovarian carcinoma

ROC analysis showed that the standalone use of CPH-I demonstrates very good diagnostic performance for detecting patients with uterine carcinoma among patients with benign diseases (AUC-ROC = 0.822, 95% CI: 0.757–0.887). At a CPH-I cut-off value of 3.33% (corresponding to the high-risk threshold in postmenopausal women), the test showed a diagnostic sensitivity of 67.6% and a specificity of 86.6%, with positive and negative likelihood ratios of $LR^+ = 5.05$ and $LR^- = 0.374$, as well as positive and negative predictive values of 28.4% and 97.1%,

respectively, for differentiating patients with uterine carcinoma from those with benign diseases (Figure 62).

The median and interquartile range (IQR) of CPH-I in subgroups stratified according to stage of uterine carcinoma were as follows: Stages I–II: 5.48 (2.61–20.98)% and Stages III–IV: 52.58 (40.02–87.95)% (Table 20). The results of the Mann–Whitney test showed significantly higher CPH-I values in patients with Stages III–IV compared with those with Stages I–II ($U = 36.00$, $p < 0.001$, $r = -0.617$).

A summary of the diagnostic reliability of HE4, CA125, ROMA, and CPH-I in the diagnosis of uterine carcinoma is presented in the following graphs and tables (Table 42, Table 43, Figure 62, Figure 63).

ROC curves of HE4, CA125, ROMA index, and CPH-I for differentiation between benign diseases and uterine carcinoma

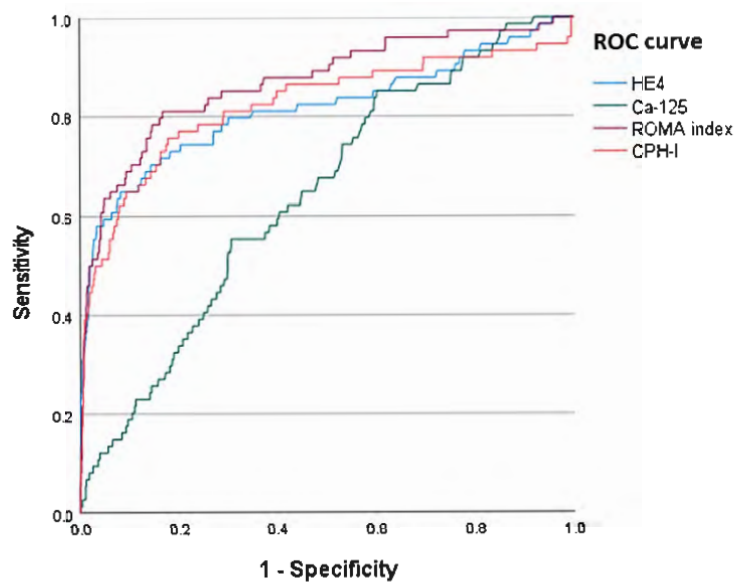


Figure 62. ROC curves of HE4, CA125, ROMA index, and CPH-I for differentiation between benign diseases and uterine carcinoma

ROC curves of HE4, CA125, ROMA index, and CPH-I for differentiation between uterine carcinoma and ovarian carcinoma

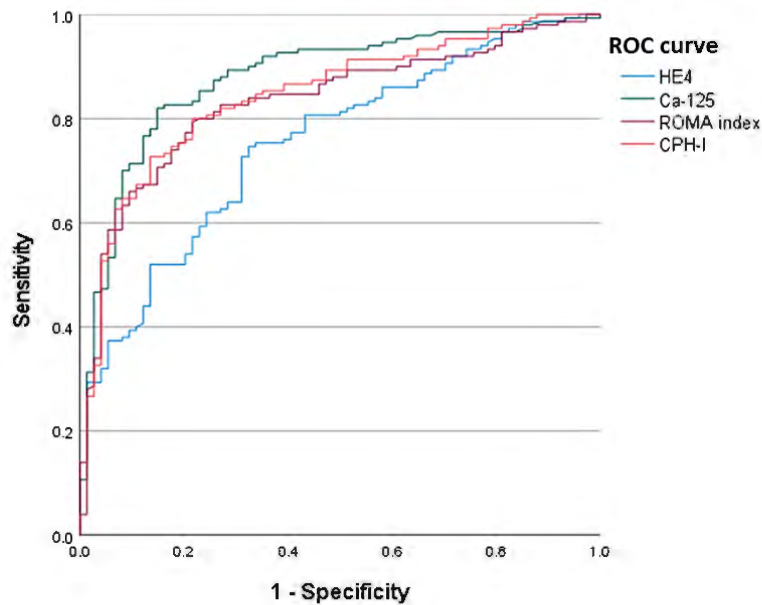


Figure 63. ROC curves of HE4, CA125, ROMA index, and CPH-I for differentiation between uterine carcinoma and ovarian carcinoma

Table 21. Diagnostic reliability of HE4, CA125, ROMA, and CPH-I in the diagnosis of uterine carcinoma

Diagnostic efficiency	HE-4		CA 125		ROMA		CPH-I	
	cut-off	AUC-ROC (CI 95)	cut-off	AUC-ROC (CI 95)	cut-off	AUC-ROC (CI 95)	cut-off	AUC-ROC (CI 95)
Distinguishing of EC from BD	69.90 pmol/L	0.816 (0.750-0.881)	Няма достатъчна ДЕ		13.70	0.869 (0.818-0.920)	3.33 %	0.822 (0.757-0.887)
Distinguishing of EC from OC	Not enough DE		83.75 U/ml	0.879 (0.831-0.927)	59.13	0.834 (0.780-0.888)	47.49 %	0.845 (0.792-0.898)
Distinguishing of healthy from EC and BD	Not enough DE		Not enough DE		Not enough DE		Not enough DE	
Distinguishing according to histology of the EC	Not enough DE		Not enough DE		Not enough DE		Not enough DE	
Staging of EC - Stages I/II versus Stages III/IV	HR – 1.007		HR – 1.014		HR – 1.080		HR – 1.071	

Abbreviations: UC – uterine carcinoma; BD – benign diseases; OC – ovarian carcinoma; DE – diagnostic efficiency

Table 22. Diagnostic reliability of HE4, CA125, ROMA, and CPH-I in the diagnosis of uterine carcinoma

Marker	Criteria	cut-off	N	TP	TN	FP	FN	Se	Sp
HE4	Distinguishing of EC from BD	69.90 pmol/L	1016	48	864	78	26	65%	92%
CA 125	Distinguishing of EC from BD	83.75 U/ml	224	123	63	11	27	82%	85%
ROMA index	Distinguishing of EC from BD	13.70	1016	56	807	135	18	76%	86%
ROMA index	Distinguishing of EC from OC	59.13	224	119	58	16	31	79%	78%
CPH-I	Distinguishing of EC from BD	3.33%	1016	50	816	126	24	68%	87%
CPH-I	Distinguishing of EC from OC	47.49%	224	109	64	10	41	73%	87%

Abbreviations: UC – uterine carcinoma; BD – benign diseases; OC – ovarian carcinoma; TP – true positive; TN – true negative; FP – false positive; FN – false negative; Se – diagnostic sensitivity; Sp – diagnostic specificity

The data show that ROMA and CPH-I have the best diagnostic efficiency in the diagnosis of uterine carcinoma. The diagnostic efficiency of ROMA and CPH-I in differentiating uterine carcinoma from benign diseases was 85% / 85%. The diagnostic efficiency of ROMA and CPH-I in differentiating uterine carcinoma from ovarian carcinoma was 79% / 77% (Table 22).

5. To evaluate the prognostic value of HE4, CA125, ROMA, and CPH-I in patients with confirmed ovarian cancer with respect to recurrence rate, time to progression, and overall survival, as well as the applicability and role of these markers in monitoring patients with ovarian cancer, including after the first and subsequent recurrences

Following the initial data review, 110 patients were included in the analysis. The minimum follow-up period was 33 months, and the maximum was 144 months. Patients were divided into three groups, defined as follows:

Group 1 – No response achieved: 36 patients (primary endpoint reached – death related to the oncological disease). In patients without response, time to progression was equated to the time of the first recorded oncological event—in this case, death.

Group 2 – Response achieved: “progression”: 46 patients. All cases with a documented therapeutic response of “Progressive disease” according to RECIST (Response Evaluation Criteria in Solid Tumors), dated at the time of diagnostic evaluation, were classified as progression. Progression also included cases adjudicated by positive PET/CT in the presence of elevated tumor marker levels and negative CT findings, as well as cases with previously achieved remission and subsequent recurrence, defined as a combined assessment of elevated CA125 and imaging evidence of recurrence from imaging modalities other than CT and PET/CT that were not evaluated according to RECIST.

Group 3 – Response achieved: “remission”: 28 patients with achieved remission (imaging and biochemical), followed for at least 30 months (33–144 months). In five patients, death from non-oncological causes was recorded during remission of ovarian cancer.

The median age at diagnosis for all patients was 62 years (95% CI: 59–64). By group, the median age was: 70 years (95% CI: 67.5–75) in the non-response group, 59 years (95% CI: 56.5–62) in the progression group, and 57 years (95% CI: 49–62) in the remission group.

For progression-free survival (PFS) and overall survival (OS), the median values (months) were calculated for the respective groups (Table 23).

Table 23. Median PFS and OS in months

Group	Number	Median PFS (months)	95% CI	MedianOS (months)	95% CI
Common	110	15.0	11.0 -24.0	32.0	15.0- 47.0
No response	36	1.94	1.18 - 2.79	1.94	1.18 – 2.79
Progression	46	18.5	12.0 -23.0	38.0	25.0 - 47.0
Remission (CR)	28	119.34	98.96 -138.97	119.34	98.96 - 138.97

The results, as expected, show the shortest survival in the group without response or with the first event being death. All patients in this group had advanced disease at the time of diagnosis (Stages III and IV) and, in the majority of cases, there was no possibility to initiate treatment or sufficient time to assess its effect. As expected, this group also included the oldest patients. The Kruskal–Wallis test showed the following results: PFS: $\chi^2 = 54.8$, $p \approx 1.2 \times 10^{-12} \rightarrow$ significant difference between groups; OS: $\chi^2 = 57.7$, $p \approx 3.0 \times 10^{-13} \rightarrow$ significant difference between groups.

Thus, the medians for both time to progression and overall survival differ statistically among the three patient groups.

The medians of the baseline values of the tumor markers HE4, CA125, as well as the algorithms ROMA and CPH-I are presented in Table 24.

Table 24. Median values of biomarkers and algorithms at the time of diagnosis

Group	Number of patients	HE4 pmol/L (median, IQR)	CA125 U/ml (median IQR)	ROMA % (median IQR)	CPH-I % (median, IQR)
Common	110	347.8 (120.8 – 915.2)	389.6 (158.2 – 1000.0)	90.4 (65.0 – 98.3)	82.8 (44.9 – 97.7)
No response	36	383.2 (145.4 – 1214.2)	359.9 (158.3 – 1000.0)	89.1 (72.1 – 98.5)	85.3 (58.4 – 98.9)
Progression	46	399.4 (216.2 – 926.0)	443.8 (219.5 – 1000.0)	91.4 (73.9 – 98.6)	87.8 (54.9 – 98.4)
Remission (CR)	28	109.1 (62.8 – 538.5)	375.2 (122.6 – 1000.0)	75.1 (27.1 – 95.9)	48.8 (11.7 – 92.2)

To determine whether the medians of the markers (HE4, CA125) and the algorithms (ROMA, CPH-I) differ statistically among the three groups (No response, Progression, Remission), the Kruskal–Wallis test was applied. In cases where $p < 0.05$, a post-hoc analysis using Mann–Whitney U tests was performed for each pair of groups (Progression–Remission, Progression–No response, Remission–No response), and Bonferroni correction was applied to the p-values. The results are summarized in Table 25, Table 26, and Figure 64 and show that only HE4 and CPH-I differ significantly between the groups.

HE4 was higher in the progression and no response groups compared with the remission group, with significant differences observed between Progression–Remission ($p = 0.014$) and Remission–No response ($p = 0.021$). No difference was observed between Progression–No response. CPH-I also showed a significant difference between Remission and No response (p

= 0.049), with a trend toward significance for Progression–Remission ($p = 0.072$). ROMA demonstrated only a tendency toward lower values in the remission group, but the differences did not reach statistical significance ($p \approx 0.09–0.18$). No significant differences were observed for CA125 between the groups ($p > 0.9$).

The Dunn’s post-hoc analysis, applied with Bonferroni correction, allowed the identification of the specific pairs of groups between which significant differences existed. In conclusion, HE4 and CPH-I best discriminate between the groups with respect to prognosis, whereas CA125 appeared to be a weaker marker and ROMA showed borderline significance.

The statistical data from the sample, although significant for HE4, do not by themselves have a substantial practical prognostic value, but they at least define the need for increased attention in patients with initially high absolute HE4 concentrations and support the need for subsequent monitoring. The levels of CA125 remain without prognostic significance with respect to initial absolute values.

Table 25. Comparison of marker medians between groups (Kruskal–Wallis + Mann–Whitney U post-hoc)

Marker	Kruskal–Wallis H	Kruskal–Wallis p	MW p (progression–remission)	MW p (progression–no response)	MW p (remission–no response)
HE4	9.7120	0.0078	0.0141	1.0	0.0214
CA125	0.5098	0.7750	1.0	1.0	1.0
ROMA	5.0919	0.0784	0.091	1.0	0.1801
CPH-I (%)	6.8353	0.0328	0.072	1.0	0.0489

Table 26. Dunn’s post-hoc analysis with Bonferroni correction

Comparison between 2 groups	Z	raw_p	p_Bonf	Marker
Progression- No response	0.2418	0.8090	1.0000	HE4
Progression- Remission	2.9339	0.0033	0.0100	
No response- Remission	2.5774	0.0100	0.0299	
Progression- No response	0.5814	0.5610	1.0000	CA125
Progression- Remission	0.6129	0.5399	1.0000	
No response- Remission	0.0696	0.9445	1.0000	
Progression- No response	0.3625	0.7170	1.0000	ROMA
Progression= Remission	2.1811	0.0292	0.0875	
No response- Remission	1.7546	0.0793	0.2380	
Progression- No response	0.0191	0.9848	1.0000	

Progression- Remission	2.3796	0.0173	0.0520	CPH-I
No response- Remission	2.2804	0.0226	0.0677	

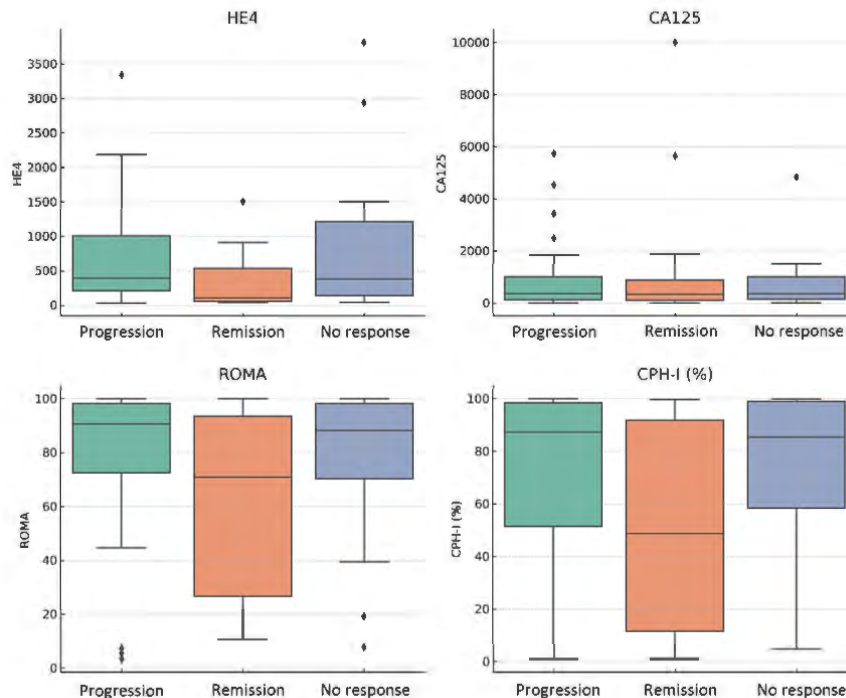


Figure 64. Distribution of marker and algorithm levels across the three groups

Correlation between preoperative serum concentrations of HE4, CA125, ROMA, and CPH-I and time to progression or death: Spearman analysis showed no significant correlations for any of the markers with respect to either progression-free survival (PFS) or overall survival (OS). Although the observed correlations were negative (as expected), their magnitude was within the range of random variation and cannot be considered prognostic for time to progression. The summarized data are presented in Table 27.

Table 27. Spearman correlations of biomarkers with progression-free survival (PFS) and overall survival (OS)

Marker	PFS (ρ , p)	OS (ρ , p)	Interpretation
HE4	ρ -0.017, p=0.878	ρ -0.022, p=0.846	No prognostic value of HE4
CPH-I	ρ -0.067, p=0.536	ρ -0.052, p=0.637	No prognostic value of CPH-I
ROMA	ρ -0.019, p=0.864	ρ -0.003, p=0.975	No prognostic value of ROMA
CA125	ρ -0.027, p=0.807	ρ 0.015, p=0.895	No prognostic value of CA125

The Spearman correlation analysis performed within the individual groups demonstrated the limited prognostic value of baseline marker levels when considered independently within each subgroup.

Two approaches were applied for Cox regression analysis of the prognostic value of the markers and algorithms: a dichotomized approach (median split—high vs. low values according to the median) and a continuous approach (log₂ transformation with estimation of

the hazard ratio for each doubling of the marker or a 10% increase for the algorithms). Univariate models were calculated for each factor separately, including both clinical factors and biomarkers/algorithms. The clinical factors analyzed were: age at diagnosis, tumor grade, FIGO stage, presence of lymph node involvement (N1), and presence of distant metastases (M1). The results demonstrated a prognostic effect only for the presence of distant metastases: HR = 2.21 (95% CI: 1.40–3.51, $p < 0.001$) for OS and HR = 1.61 (95% CI: 1.04–2.48, $p = 0.03$) for PFS. Among the remaining factors, only age showed a weak but significant association with worse OS and PFS, while FIGO stage showed only a trend towards this (**Table 28**). In the multivariable clinical models (GRADE, FIGO, M1, N1), M1 remained at the threshold of statistical significance (**Figure 65, Figure 66**).

Table 28. Cox univariate models (for OS) for clinical factors and markers.

Factor	N	HR	95% CI	p
Age	84	1.025	1.005 - 1.046	0.0156
HE4	84	1.000	0.999 - 1.000	0.6495
CA125	84	0.999	0.999 - 1.000	0.4671
ROMA	84	1.002	0.993 - 1.010	0.6822
CPH-I	84	1.000	0.993 - 1.007	0.9643
Grade	42	1.370	0.876 - 2.142	0.1677
FIGO stage	40	1.436	0.983 - 2.098	0.0614
Distant metastases	84	2.216	1.398 - 3.513	0.0007
Lymph node involvement	84	1.055	0.681 - 1.633	0.8101

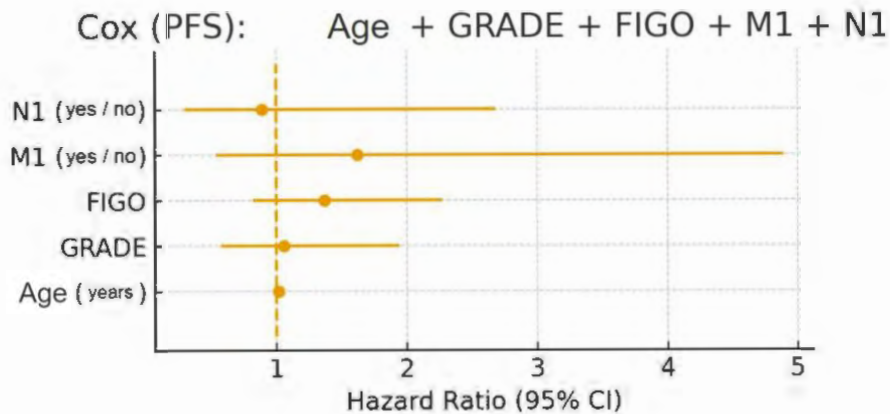


Figure 65. Forest plot – multivariable Cox model for PFS (clinical factors).

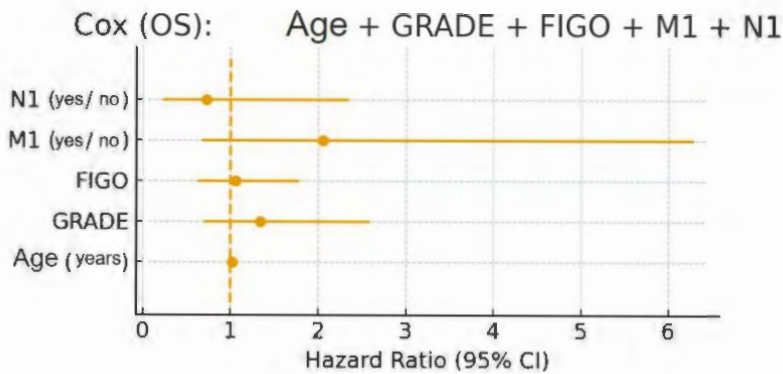


Figure 66. Forest plot – multivariable Cox model for OS (clinical factors).

The focus of the analysis was on the results from multivariable models combining clinical covariates with biochemical markers (log-transformations and median split). The results indicate that HE4 and CPH-I were significant in univariate analysis, but their effects weakened in the multivariable model, suggesting that clinical factors explain a greater proportion of the variance. CA125 and ROMA did not demonstrate independent prognostic value. The calculated HR(t) for 12/24/60 months showed that in some models the effect of M1 and/or age decreased or increased over time, which is biologically plausible given subsequent lines of therapy, delayed events, and the selection of higher-risk populations over time. Since the results of the analysis of the prognostic value of the markers with respect to PFS demonstrated a lack of any association—both statistical and clinical—and this finding contradicts the currently published literature, we applied a landmark approach: to assess the prognostic value of the markers, the group of patients with “no response” was excluded.

Table 29. Log-rank comparisons (PFS).

Comparison	Z	p-стойност
N1 (1 vs 0)	3.714	0.0002
M1 (1 vs 0)	3.401	0.0007
Age ≥ 58.5 vs < 58.5	2.375	0.0175
GRADE 3 vs < 3	2.612	0.0090
FIGO III–IV vs I–II	0.000	1.0000
HE4 high vs low	3.342	0.0008
CA125 high vs low	1.421	0.1553
ROMA high vs low	2.981	0.0029
CPH-I% high vs low	2.301	0.0214

The log-rank test analysis demonstrated clear differences among the individual clinical and biomarker predictors with respect to progression-free survival (PFS) (Table 29). These results highlight the increasing role of HE4 and the combined algorithms ROMA and CPH-I as reliable prognostic tools in oncogynaecology, expanding upon traditional clinical factors and providing potential for more accurate individualized prognosis.

An exponential model was used to calculate Hazard Ratios (HR) and 95% confidence intervals for all dichotomized predictors (clinical and biomarker variables). The results indicate the direction and magnitude of the effect, consistent with the previously performed log-rank tests (Table 30).

Table 30. Hazard Ratios (exponential model)

Comparison	HR	95% CI низ	95% CI връх
N1 (1 vs 0)	4.695	2.634	8.369
M1 (1 vs 0)	3.896	2.124	7.147
Age ≥ 58.5 vs < 58.5	2.44	1.357	4.389
GRADE 3 vs < 3	4.508	2.103	9.662
FIGO III-IV vs I-II	2.733	1.001	7.461
HE4 high vs low	7.183	2.576	20.032
CA125 high vs low	1.532	0.859	2.731
ROMA high vs low	9.519	2.308	39.265
CPH-I% high vs low	3.252	1.517	6.972

Summary data on the prognostic significance of the biomarkers and algorithms, as well as their comparison with the classical clinical determinants, are presented in Figure 67.

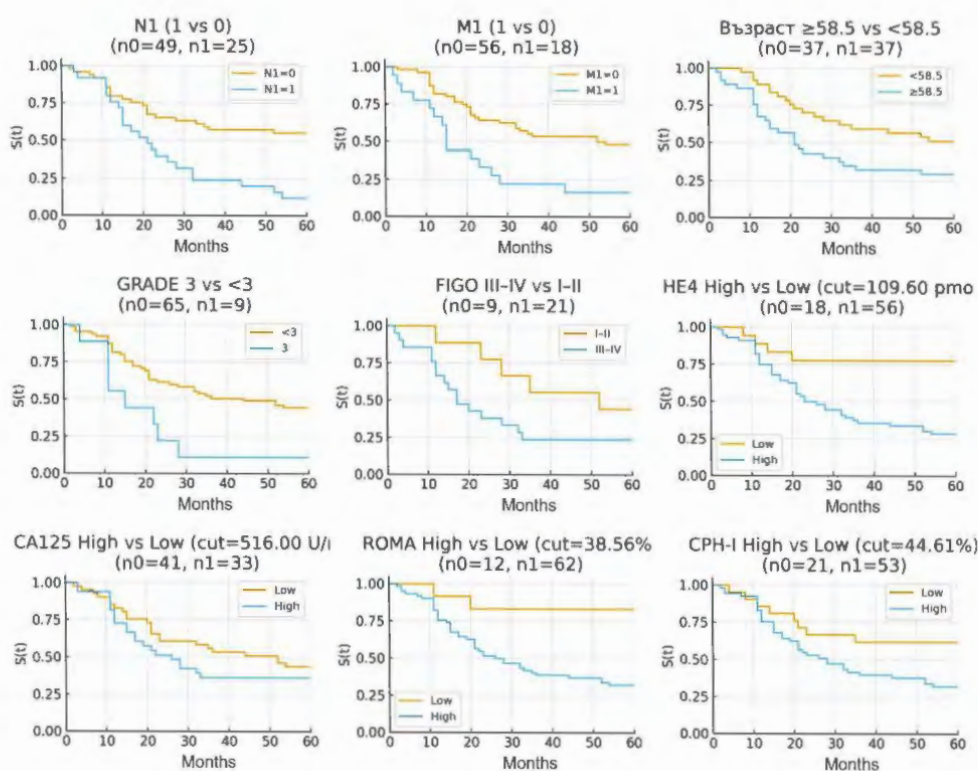


Figure 67. Kaplan–Meier curves of PFS with dichotomization by the optimal ROC-derived cut-off value (Comparisons: N1, M1, age ≥ 58.5 , grade 3, FIGO III–IV, HE4, CA125, ROMA, CPH-I (cut-off by Youden). X-axis: 0–60 months).

In the entire cohort, the continuous Cox models (\log_2) showed that HE4 and CPH-I were the most reliable prognostic biomarkers for PFS and OS; ROMA demonstrated more moderate prognostic value (particularly for PFS), whereas CA125 did not show an independent prognostic role. Dichotomization by the median reproduced the same hierarchy but with reduced statistical power.

The practical implication of this analysis is that HE4 and CPH-I can be used for risk stratification, monitoring, and the discussion of follow-up intensity. CA125 remains useful for monitoring treatment response, but not as a standalone prognostic marker in this cohort.

Significance of the markers in patient follow-up and for early detection of recurrence or progression.

When comparing marker levels at the time of primary diagnosis (defined as baseline concentrations) with those measured at the time of recurrence (although the number of such patients was small), no statistically significant difference was observed. The limited number of patients monitored with both markers after first-line therapy did not allow for formal statistical analysis; therefore, the results are presented for descriptive purposes only.

VII. DISCUSSION

Reference values and population-specific characteristics of CA125, HE4, ROMA, and CPH-I: One of the central achievements of the present dissertation is the definition and critical evaluation of reference limits and threshold values for the tumor markers HE4 and CA125, as well as for the multivariate algorithms ROMA and CPH-I, specific to the Bulgarian population. The data clearly demonstrate that direct application of manufacturer-recommended reference limits, derived from cohorts in other geographic and ethnic populations, carries a risk of inaccurate interpretation and compromised diagnostic performance.

The upper reference limits for HE4 obtained in this study are substantially lower than those stated in manufacturers' package inserts and differ from values reported in several international studies. These differences may be explained by both population characteristics and analytical factors, including the analytical platform and antibodies used. The comparative analysis between the Abbott and Roche platforms confirms the presence of a systematic shift toward higher values in measurements performed with Roche, which should be considered when interpreting results and when translating published cut-off values across different laboratory systems.

Age was established as a leading determinant of serum HE4 concentration, with a statistically significant, albeit moderate, association. At the same time, the analysis shows that age has a greater impact than menopausal status, calling into question the practice of using binary reference limits based solely on menopause. The observed overlap of confidence intervals across certain age subgroups does not support the introduction of strictly age-stratified reference limits but rather necessitates careful interpretation at borderline values and during longitudinal follow-up.

The findings regarding pregnancy indicate that HE4 does not increase significantly in pregnant women compared with non-pregnant women of the same age group, which is of considerable clinical importance, especially in contrast to the well-known physiological increase of CA125 during pregnancy. This allows the use of HE4 without correction in pregnant patients and expands its diagnostic applicability in clinical situations where interpretation of CA125 is limited.

A particularly strong relationship was observed between HE4 and renal function, with a very strong positive correlation with serum creatinine and a strong negative correlation with eGFR. These data confirm renal clearance as a major factor influencing serum marker concentrations and necessitate the mandatory consideration of renal function when interpreting results. Attempts at universal correction of HE4 according to eGFR remain speculative and cannot currently be recommended for routine clinical practice.

Regarding ROMA and CPH-I, the present study demonstrates that cut-off values recommended in the literature and by manufacturers are not optimal for the Bulgarian population. The identified lower reference limits and the differences between premenopausal and

postmenopausal women support the need for local validation of the algorithms and the adjustment of cut-off values to improve specificity.

Diagnostic value in ovarian carcinoma and preoperative triage: The main clinical application of HE4, CA125, ROMA and CPH-I remains the preoperative triage of patients with pelvic masses and the differentiation between benign and malignant processes. The present study, including a large and well-balanced cohort, confirms that the standalone use of serum markers is not suitable for population screening due to substantial overlap of values between healthy women and patients with benign cystic formations.

The independence of HE4 from endometriosis is confirmed in this investigation as well, providing an advantage in the premenopausal population, where CA125 is often falsely elevated. Nevertheless, the highest diagnostic performance is achieved through the multivariate algorithms ROMA and CPH-I, which outperform individual marker measurements.

Of greatest clinical relevance are the results of the comparison between patients with benign cysts and those with ovarian carcinoma. All four indicators demonstrate excellent diagnostic performance, with CPH-I and ROMA achieving the highest AUC values and surpassing HE4 and CA125. The obtained results are fully comparable with data from large meta-analyses and contribute original information regarding the diagnostic value of CPH-I, which remains less extensively represented in the literature.

Analysis by histological subtype and stage shows that none of the markers or algorithms have sufficient accuracy to reliably distinguish histological variants of ovarian carcinoma or to stage the disease, which corresponds to the established role of biomarkers as tools for risk stratification rather than replacements for histological and imaging diagnostics.

Prognostic significance of baseline marker and algorithm values with respect to overall survival in ovarian and endometrial carcinoma: Evaluation of the prognostic value of baseline concentrations of HE4, CA125, ROMA and CPH-I shows that the markers have a limited but clinically meaningful role, particularly in univariate analysis. HE4 emerges as the marker with the highest prognostic value for overall survival, whereas CA125 does not demonstrate consistent prognostic strength.

In multivariate analysis, including classical clinical covariates such as age, stage and histological grade, the prognostic value of the markers and algorithms decreases to a lack of independent significance. This confirms the leading role of clinical factors and supports the concept of using biomarkers as complementary rather than determining prognostic tools.

Of particular interest are the findings in endometrial carcinoma, where HE4 demonstrates clear superiority over CA125 in both diagnostic and prognostic value. HE4 and algorithms based on it show good correlation with disease stage and potential for preoperative prognostic assessment, which has practical significance in treatment planning and therapeutic decision-making.

Clinical significance of HE4, CA125, ROMA and CPH-I in endometrial carcinoma: The results of the present dissertation extend the clinical evaluation of HE4, CA125 and the multivariate algorithms ROMA and CPH-I beyond their classical role in ovarian carcinoma and provide a systematic analysis of their significance in endometrial carcinoma. The data show that HE4 has significantly higher diagnostic value compared with CA125 in this disease entity, in line with the growing body of international publications, but validated for the first time in a Bulgarian population.

When comparing patients with endometrial carcinoma and women with benign gynecological diseases, HE4 demonstrates higher specificity and better discriminative capacity, with less overlap of serum concentrations between groups. CA125, in turn, shows limited diagnostic value, particularly in early disease stages, confirming its lower sensitivity and specificity in uterine carcinoma.

The algorithms ROMA and CPH-I, although originally developed for assessing the risk of ovarian carcinoma, demonstrate additional diagnostic utility in endometrial carcinoma as well. However, their performance is strongly dependent on the choice of cut-off values, as the use of standard thresholds results in lower specificity. Optimization of cut-off values through ROC analysis improves their diagnostic performance, supporting the need for population- and disease-specific adaptation of the algorithms.

Particularly important is the analysis of serum HE4 concentrations and ROMA and CPH-I values in relation to the stage of endometrial carcinoma. A trend toward progressively increasing HE4 levels in advanced stages is observed, suggesting a potential role of the marker in preoperative risk assessment and patient stratification. This association is considerably weaker for CA125, further emphasizing its limited applicability in this disease.

Monitoring and clinical limitations: Analysis of monitoring and progression data highlights significant practical limitations related to the lack of standardized follow-up protocols and additional structural issues such as the absence of a functioning national cancer registry. This restricts the ability to perform reliable analyses of PFS and DFS and directs interpretation primarily toward overall survival.

Despite these limitations, the results indicate that HE4 and CPH-I have better discriminatory capacity in distinguishing patients with different risks of progression compared with CA125 and ROMA. This supports the concept of a multimarker approach and the leading role of HE4 and CPH-I in dynamic risk assessment.

The findings regarding the role of HE4 and the algorithms in endometrial carcinoma are particularly valuable. HE4 shows the highest clinical significance in endometrial carcinoma in both diagnostic and prognostic terms, while ROMA and CPH-I may have an additional role within a carefully selected diagnostic context. These findings expand the scope of application of HE4 and multivariate biomarker algorithms in oncogynaecology and emphasize the need for further prospective studies aimed at optimizing diagnostic strategies for uterine carcinoma.

In summary, the present study provides a systematic and critical evaluation of the diagnostic and prognostic roles of HE4, CA125, ROMA, and CPH-I, highlighting the need for population-specific validation, careful selection of cut-off values, and integration of biomarkers into comprehensive clinical assessment. The results support the expanded use of HE4 and CPH-I in oncogynaecology while clearly defining the limits of their clinical utility.

VIII. CONCLUSIONS

1. There is a statistically significant positive correlation between serum HE4 concentration, ROMA and CPH-I values, and patient age, as well as a negative correlation between CA125 and age.
2. Up to 50 years of age, the 95% confidence intervals for the upper reference limit of HE4 overlap and do not necessitate the introduction of age-stratified reference values. Above 50 years of age, the use of different HE4 reference limits for age groups below and above 59 years is justified.
3. Patient age has a greater impact on HE4 elevation than hormonal (menopausal) status.
4. The statistically significant differences in ROMA values observed in both premenopausal and postmenopausal women allow the derivation and recommendation of new population-specific upper reference limits for clinical practice.
5. The use of specific HE4 reference limits during pregnancy is not required.
6. In the preoperative triage of women with an adnexal mass, the combined algorithms ROMA and CPH-I demonstrate the highest diagnostic performance, surpassing the standalone use of HE4 and CA125.

7. When used individually, HE4 is more specific and less affected by endometriosis than CA125, making it particularly useful in premenopausal patients.
8. The investigated markers and algorithms are not suitable for distinguishing benign formations from healthy women and should not be used for population screening, but they have a clearly defined role in clinical preoperative triage.
9. None of the markers or algorithms shows sufficient performance for independent determination of histological subtype or stage of ovarian carcinoma.
10. Age, disease stage, and high-grade histology remain the leading independent clinical determinants of prognosis in patients with ovarian carcinoma.
11. HE4 demonstrates the highest prognostic value with respect to overall survival in both ovarian and endometrial carcinoma.
12. HE4 is a reliable biomarker in endometrial carcinoma, with higher specificity than CA125 and less influence from benign gynecological conditions.
13. CA125 does not demonstrate prognostic value and should be used primarily as a monitoring marker during post-treatment follow-up.
14. CA125 has no diagnostic value in endometrial carcinoma when used alone; however, its inclusion with HE4 in the ROMA and CPH-I algorithms improves diagnostic performance.

IX. CONTRIBUTIONS

Original scientific contributions

1. For the first time, reference intervals for HE4, CA125, ROMA, and CPH-I specific to the Bulgarian population have been defined.
2. For the first time, a systematic evaluation of the clinical significance of CPH-I in the diagnosis of endometrial carcinoma has been performed.
3. For the first time, the prognostic significance of CPH-I has been evaluated in patients with ovarian and endometrial carcinoma.

Theoretical and scientific-applied contributions

4. The importance of HE4 and HE4-based multimarker algorithms (ROMA and CPH-I) in the preoperative triage of patients with pelvic tumor formations has been confirmed.
5. The biological characteristics of HE4 expression—dependence on age and renal function and the absence of significant influence of pregnancy—have been confirmed for the Bulgarian population.
6. It has been confirmed that HE4 is not elevated in endometriosis and demonstrates superior specificity compared with CA125 in the evaluation of premenopausal patients.
7. The diagnostic superiority of HE4 over CA125 in endometrial carcinoma has been confirmed.
8. The present study demonstrates the non-inferiority of CPH-I compared with ROMA in the assessment of pelvic masses for early detection of the risk of oncogynaecological disease.
9. The present study establishes cut-off values for CPH-I and proposes its inclusion in routine clinical practice as an alternative to ROMA, with the advantages of independence from hormonal status and the risk of incorrect menopausal classification, as well as a more reliable reflection of the age-related effect on HE4 values.

X. SCIENTIFIC PUBLICATIONS

1. Koleva-Topova V., Shefket S. Multimarker models and risk stratification algorithms for ovarian cancer in women with adnexal masses. *Scripta Scientifica Medica*, 2024; 56(2):14–19. ISSN 1314-6408.
2. Koleva V., Shefket S., Stoencheva S. Clinical significance of human epididymis protein 4 (HE4), cancer antigen 125 (CA125), the risk of ovarian malignancy algorithm (ROMA), and Copenhagen index (CPH-I) for the diagnosis of endometrial carcinoma. *Folia Medica*, 2025; 67(1). ISSN 0204-8043. DOI: 10.3897/folmed.67.e143849.
3. Koleva-Topova V., Shefket S. Reference values of human epididymis protein 4 in the Bulgarian population – assessment of the influence of age, menopause, pregnancy, and renal function. *Folia Medica*, 2025; 67(4): e155013. DOI: 10.3897/folmed.67.e155013.

Participation in scientific forums related to the dissertation

1. Koleva V. *How beneficial are multi-marker panels for ovarian cancer diagnosis?* *Balkan Journal of Clinical Laboratory*, XXVI, 18(1); oral presentation at the 26th Congress of the Balkan Federation of Clinical Laboratory, 2018.
2. Koleva V. *Can we improve the early diagnosis of ovarian carcinoma using different multimarker models?* Plenary lecture, National Conference on Clinical Laboratory Medicine, Plovdiv, September 2022.

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