Korelacija pre-operativnih serumskih razina CA-125 te ekspresije p53 s kliničkopatološkim faktorima i preživljenjem pacijentica oboljelih od seroznog karcinoma jajnika visokog gradusa

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Summary (Abstract)

Background: Ovarian cancer (OC) it the 7th most common cancer among women worldwide, while its 5-year survival rate in Croatia amounts to 39%. Epithelial ovarian cancer (EOC) represents 90% of all ovarian cancers, while high-grade serous ovarian cancer (HGSOC) is the most common EOC histological subtype and accounts for 75% of all EOCs. Its lethality is attributable to the majority of patients being diagnosed at an advanced stage. There are discrepancies between studies that investigated pre-operative serum CA-125 levels as an outcome predictor, while the *TP53* gene is ubiquitously mutated in HGSOC. Based on these findings, I have decided to evaluate pre-treatment serum CA-125 levels together with p53 mutation status and pattern in HGSOC, as well as their correlation. Pre-treatment serum CA-125 was also correlated with clinicopathological variables, while their impact on patient overall survival was also examined.

Methods: A retrospective cohort study was undertaken on 28 HGSOC patients diagnosed between July 2016 and December 2018 at the Gynecology and Obstetrics Clinic in Rijeka (Croatia). Pre-treatment serum CA-125 levels were assessed for their correlation with FIGO stage, p53 mutation pattern, residual tumor (RT) size and type of cytoreductive surgery. Furthermore, pre-treatment serum CA-125, RT, type of cytoreductive surgery, chemotherapy regimen and FIGO stage were examined as potential prognostic factors.

Results: Pre-treatment CA-125 serum levels did not significicantly correlate with FIGO stage, p53 mutation, RT nor the type of cytoreductive surgery. The pattern of p53 mutation also had no significant association with FIGO stage. While pre-treatment CA-125 serum levels, FIGO stage and RT size were not classified as significant survival predictors, reduced survival was noted for patients who underwent interval cytoreduction ($p = 0.002$) and neoadjuvant chemotherapy ($p < 0.0001$).

Conclusion: Despite pre-treatment CA-125 being abnormal in the majority of HGSOC patients, it showed no prognostic value, nor significant association with clinicopathological factors. Similarly, an aberrant p53 is indeed a characteristic of this HGSOC cohort.

Keywords: ovarian cancer, epithelial ovarian cancer, high-grade serous ovarian cancer, CA-125, p53, prognosis, clinicopathological factors

Sažetak

Povod i značaj: Karcinom jajnika sedmi je najčešći karcinom kod žena u svijetu, a petogodišnje preživljenje Hrvatica iznosi 39%. Epitelni karcinom jajnika predstavlja 90% svih slučajeva karcinoma jajnika, dok serozni karcinom visokog gradusa čini najčešći histološki podtip epitelnog karcinoma jajnika (75% svih slučajeva). Stopa smrtnosti vrlo je visoka zbog toga što se najčešće dijagnosticira u uznapredovalim stadijima. Prijašnje studije pokušale su evaluirati prognostički značaj serumskih razina tumorskog markera CA-125 prije ikakvih liječničkih tretmana i zahvata, ali se njihovi rezultati ne podudaraju. Međutim, mutacije tumor supresorskog gena *TP53* karakteristika su seroznog karcinoma jajnika visokog gradusa. Bazirajući se na rezultatima prijašnjih istraživanja, u ovom diplomskom radu evaluirane su pre-operativne razine serumskog CA-125 te je analizirana ekspresija proteina p53 u slučajevima seroznog karcinoma jajnika visokog gradusa. Osim toga, analizirana je i korelacija ovih dviju varijabli. Nadalje, pre-operativna razina serumskog CA-125 korelirana je i s kliničkopatološkim varijablama te je razmatran njihov učinak na sveukupno preživljenje pacijentica.

Metode: Provedeno je retrospektivno kohortno istraživanje, koje je uključivalo 28 pacijentica s dijagnozom seroznog karcinoma jajnika visokog gradusa koje su liječene između srpnja 2016. i prosinca 2018.godine u Klinici za ginekologiju i opstetriciju, KBC-a Rijeka. Razmatrana je statistička korelacija pre-operativnih razina CA-125 s FIGO stadijem, vrstom mutacije p53, veličinom rezidualnog tumora te s vrstom citoreduktivnog zahvata. Također, analiziran je i značaj pre-operativnih razina CA-125, veličine rezidualnog tumora, vrste citoreduktivnog zahvata te kemoterapijskog režima u prognozi bolesnica.

Rezultati: Pre-operativne razine CA-125 nisu značajno korelirale s FIGO stadijem, vrstom p53 mutacije, veličinom rezidualnog tumora ni s vrstom citoreduktivnog zahvata. Vrsta p53 mutacije također nije pokazala značajnu korelaciju s FIGO stadijem. Dok pre-operativne razine CA-125, FIGO stadij i veličina rezidualnog tumora nisu svrstani kao značajne prediktorske varijable, indikacija je suprotna za vrstu citoreduktivnog zahvata ($p = 0.002$) te za kemoterapijski režim ($p < 0.0001$).

Zaključak: Unatoč tome što je pre-operativna CA-125 razina abnormalno povišena u većini pacijentica sa seroznim karcinomom jajnika visokog gradusa, nema prognostički značaj niti značajnu korelaciju s kliničkopatološkim varijablama. Slično, p53 je abnormalno eksprimiran u većini pacijentica te se radi o potvrđenoj karakteristici ovog histološkog podtipa.

Ključne riječi: karcinom jajnika, epitelni karcinom jajnika, serozni karcinom jajnika visokog gradusa, CA-125, p53, prognoza, preživljenje, kliničkopatološki faktori

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1.Introduction

1.1.Prevalence, prognosis and risk factors

Ovarian cancer has a relatively low prevalence when compared to other cancers, but it makes up for it with its high mortality rate. It is the 7th most common cancer among women worldwide and the most lethal gynaecological malignancy. Every year, 314 000 women from all around the world are diagnosed with ovarian cancer and 207 000 succumb to it. According to the Registry for Cancer at the Croatian Institute of Public Health, 400-500 women are diagnosed with ovarian cancer per year, with more than 300 dying as a result. In more developed countries, the five-year survival rate ranges from 36% to 46%, while the one in Croatia is around 39% [1,2].

Epithelial ovarian cancer (EOC), originating from epithelial cells which underwent malignant transformation, represents 90% of all ovarian cancers. High-grade serous ovarian cancer (HGSOC) is the most common histological subtype, accounting for 75% of all EOCs[3,4]. HGSOC is also responsible for 90% of deaths from ovarian cancer[5,6]. HGSOC is usually detected when it has progressed to FIGO stage IIIC, for which the 5-year survival amounts to a very low 29% [5,7]. Since HGSOC is the most prevalent and aggressive histological subtype of EOC, it will be the main subject of this thesis.

A vast number of risk factors are linked to EOC. Increasing age poses a significant risk of being diagnosed with EOC. Consequently, it commonly manifests in postmenopausal women, while it's rare to occur in premenopausal ones[8,9]. The 'incessant ovulation' theory claims that ovulation contributes to the malignant transformation of the ovarian surface epithelial cells, as scar tissue forms every time an egg is expelled from the ovary[5,9]. Therefore, pro-inflammatory mediators are released and ROS induce genotoxic stress, which is why those cells gain cancerous

features overtime. Pregnancy, breastfeeding, and the use of oral contraceptives all inhibit ovulation, and these factors have been demonstrated to reduce the incidence of EOC. Similarly, multiple pregnancies also diminish this risk. Hormone replacement therapy (contains oestrogen) is used to treat menopausal symptoms, but it significantly elevates the risk of EOC onset in postmenopausal women[8,10]. Smoking, diabetes, obesity and diet are potential risk factors connected to the lifestyle of an individual. Apart from environmental and lifestyle risk factors, those with family history of breast and/or ovarian cancer are at a higher risk due to genetics. Furthermore, if a woman is diagnosed with EOC below the age of 50, the risk of passing it onto her daughter is three-fold higher. Mutations in genes involved in the homologous DNA repair, such as BRCA, are to blame for an augmented risk of HGSOC[8,9,10].

1.2.Diagnosis and staging

The majority of women harboring EOC are diagnosed at an advanced stage. Because the symptoms are non-specific and often related to other disease processes, the possibility of EOC is often dismissed at an early stage. However, at an advanced stage, symptoms become more apparent and/or severe. Constipation and/or bowel obstruction, diarrhea, vomiting, nausea, gastrointestinal reflux, abdominal bloating, abdominal and/or pelvic pain, fatigue, shortness of breath, back pain and change in bowel movements are some of the presenting symptoms[9,10].

After identifying presenting EOC symptoms, diagnostic procedures such as pelvic and rectovaginal examination, radiographic imaging (transvaginal ultrasonography, abdominal ultranosonography, CT, MRI and/or PET) are performed. The mentioned diagnostic procedures help approximate the size, location, spread and complexity of what is hypothesized to be ovarian cancer[9,10,11]. Pre-treatment serum levels of the cancer antigen 125 (CA-125) are also measured to supplement those diagnostic procedures. However, the prognostic ability of pre-treatment CA-125 remains

controversial[12,13,14,15]. CA-125 is a tumor marker commonly elevated in most EOC histological subtypes, but it has no obvious difference in expression level between benign and malignant ovarian masses. Furthermore, it also has a low specificity for the diagnosis of an early-stage EOC. Since CA-125 levels are generally elevated during pregnancy and menstruation, there is no surprise that its positive predictive value is higher in post-menopausal women rather than in pre-menopausal women[9,11,16]. As a result, researchers are investigating different tumor biomarkers to aid in the early diagnosis of EOC. For example, the human epididymis protein 4 (HE4) has been introduced as a biomarker of high differential specificity. The risk of ovarian malignancy algorithm (ROMA), on the other hand, mathematically combines HE4, CA-125 and menopausal status. According to recent studies, the combined use of ROMA and HE4 might be useful in the detection of early-stage EOC in the future[11,17]. Finally, a biopsy of the tumor growth is obtained through laparoscopic surgery or primary cytoreductive surgery, which is crucial for staging the disease. EOC is staged using Roman numerals (I-IV) and letters (A, B, C) according to the International Federation of Gynecology and Obstetrics (FIGO)[9,10,11]. The location of the tumor is determined by Roman numerals I-IV, whereas its extent is defined by the letters A, B, C. The currently valid FIGO classification from 2014 is displayed in Table 1. Staging is a very important step for the assignment of the right treatment protocol to a patient. Pathologists also prepare the tissue sample for immunohistochemistry, where the expression of tumor-promoting biomarkers, such as p53, is assessed[9,11,18].

Table 1 FIGO stages for EOC (classification from 2014). Adapted from [10]

FIGO stage	Description
I	The cancer is confined only to the ovaries or fallopian tubes
IA	The cancer is only inside 1 ovary (with an intact ovarian capsule) or fallopian tube; no cancer is found on the ovarian or fallopian tube surface or in the abdomen
IB	The cancer is in both ovaries (with an intact ovarian capsule) or fallopian tubes; no cancer is found on the ovarian or fallopian tube surface or in the abdomen
IC	The cancer is in 1 or both ovaries or fallopian tubes
IC1	Intraoperative surgical spill of the cancer
IC2	The cancer wall ruptures before surgery or cancer on the surface of the ovary or fallopian tube
IC3	Cancerous cells are found in fluid buildup of the abdominal cavity (ascites) or peritoneal washings
II	The cancer involves one or both ovaries or fallopian tubes and has spread below the pelvis
IIA	The cancer has spread to the uterus and/or fallopian tubes and/or ovaries
IIB	The cancer has spread to other tissues within the pelvis
III	The cancer involves one or both ovaries or fallopian tubes; it has spread to the peritoneum outside the pelvis and/or to lymph nodes in the retroperitoneum behind the abdomen
IIIA1	The cancer has spread to the retroperitoneal lymph nodes, but not to the peritoneal surfaces
IIIA1(I)	Metastases are 10 mm or smaller
IIIA1(II)	Metastases are larger than 10 mm
IIIA2	The cancer has spread microscopically from the pelvis to the abdomen; cancer may or may not have spread to retroperitoneal lymph nodes
IIIB	Macroscopic peritoneal metastasis of 2 cm or smaller forms beyond the pelvis and spreads to the abdomen, with or without metastasis to the retroperitoneal lymph nodes

IIIC	Macroscopic peritoneal metastasis larger than 2 cm found in the abdomen, with or without metastasis to the retroperitoneal lymph nodes
IV	The cancer has spread to organs outside the abdominal area (distant metastasis)
IVA	The cancer has spread to the fluid around the lungs (pleural effusion contains cancerous cells)
IVB	The cancer has spread to the liver or spleen or to the organs beyond the abdomen (including lymph nodes in the groin and those outside of the abdominal cavity)

1.3.Histopathological architecture and cytological features

EOC is a disease comprised of multiple histological subtypes, out of which HGSOC is has the highest frequency and mortality rate. The 'high-grade' in its terminology means that this histological subtype is given a Grade 3. Grade 3 defines poorly differentiated cancerous tissues that are characterized by abnormal cells, severe nuclear atypia, high nuclear-to-cytoplasmic ratio and abundant mitoses (Figure 1C)[5,10]. In addition, HGSOC are typically recognized by a solid cellular mass (Figure 1B), but papillary architecture can also be present in some areas (Figure 1A)[5,10]. Figure 1 represents HGSOC tissue stained with hematoxylin and eosin (H&E staining).

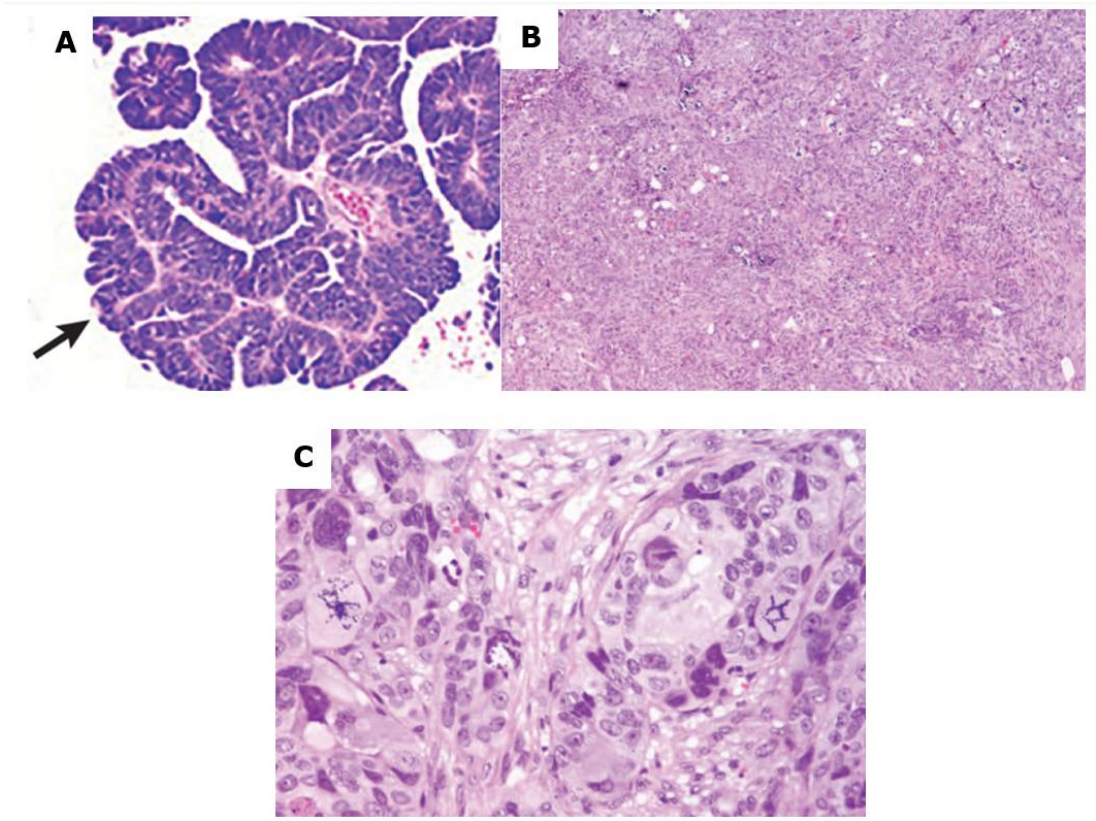

Figure 1 Histopathological architecture and cytological features features of HGSOC. **A**, papillary architecture (arrow); **B**, solid architecture; **C**, abnormal cells, severe nuclear atypia, high nuclear-to-cytoplasmic ratio, abundant mitoses. Adapted from [5] and [10]

1.4.p53 immunohistochemistry

Apart from its distinct histopathological architecture and cytological features, HGSOC is also very genetically unstable since it ubiquitously harbors *TP53* mutations[19,20,21]. The *TP53* gene expresses the p53 gene, which undergoes IHC to help encounter *TP53* mutations. The p53 protein is a tumor-suppressor that is activated in response to cellular stress through damage-activated kinases ATM and Chk2[22]. Upon phosphorylation and activation, p53 acts as a transcription factor in the nucleus, whereby it promotes cell cycle inhibition, apoptosis, senescence, DNA repair, autophagy and the opposition of oncogenic metabolic programming. However, without the onset of cellular stress, the E3 ubiquitin ligase MDM2 marks p53 for proteasomal degradation by polyubiquitination[22].

Missense mutations in the *TP53* gene lead to the expression of a mutant p53 protein, which loses its role as a transcription factor as it fails to recognize DNA response elements in the nucleus. Similarly, MDM2 can't polyubiquitinate mutant p53, which then then results in a massive nuclear accumulation of this mutant protein[23,24]. Together with the loss of wild-type function, the accumulated mutant p53 proteins display the gain-of-function (GOF) mechanism through which cancer progression and drug resistance are promoted[25]. In IHC, this is called a diffuse strong positive expression if at least 80% of tumor cell nuclei contain a mutant p53 overexpression (Figure 2A). This mutation pattern is the most frequent one in HGSOC, as it occurs in around 66% of cases[19].

In addition to the diffuse strong positive expression pattern, there could be a complete absence of the p53 protein in the nuclei of cancer cells. This is called a null-type mutant pattern, which is identified when there is no staining in cancer nuclei (Figure 2B). Indels, stopgains and splicing mutations in the *TP53* gene result in a deficient p53 mRNA, and ultimately in its destruction and complete absence of the corresponding protein. This mutation pattern occurs in around 25% of HGSOCs[19,26].

When the overexpression and null-type mutant patterns are present in the same HGSOC tissue sample, it's called a combination or heterogeneous staining (Figure 2C). Since this phenomenon has been observed in less than 3% of HGSOCs, the blame could be put on delayed fixation during IHC staining as well as on a splicing mutation that affects the expression of p53 differently in every cancer cell nuclei[19,26].

A focal positive expression is, in essence, a wild-type expression of p53 that accounts for 5% of HGSOCs (Figure 2D). However, this could be contributed to antigen degradation and weak staining during the IHC process, since it is considered that HGSOCs ubiquitously harbor *TP53* mutations[19,26].

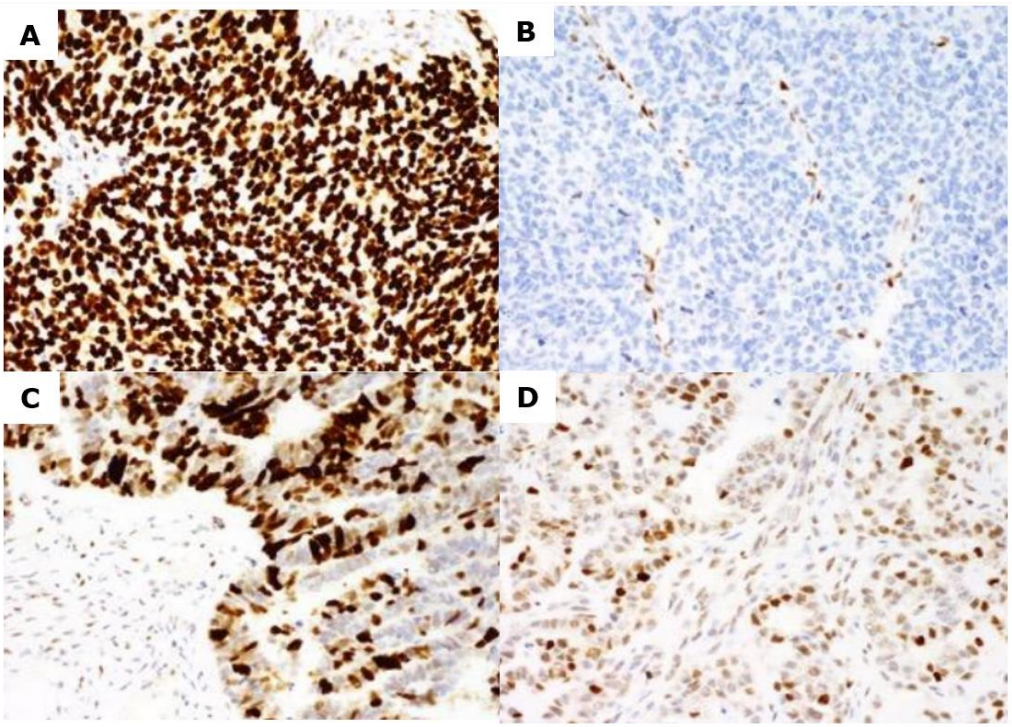

Figure 2 Types of p53 expression in HGSOC. **A**, diffuse strong positive (overexpression); **B**, null-type (absence of p53 expression); **C**, combination of the diffuse strong positive and null-type pattern; **D**, focal positive (wild-type expression). Adapted from [19]

1.5.Current treatment protocol

After the physical examination and the approximation of the size, location, spread and complexity together with the identification of the EOC histological subtype, a treatment protocol is created. When the physical exam and imaging results (CT scan of the abdomen and pelvis) reveal that a complete cytoreduction can be performed, total abdominal hysterectomy, a bilateral salpingo-oophorectomy and an omentectomy follow. This type of surgery is called primary cytoreduction. Chemotherapy given after primary cytoreductive surgery is called adjuvant chemotherapy and it is administered through six cycles. When a complete cytoreduction cannot be achieved at the time of diagnosis, which is often the case for advanced EOC, the histological subtype is determined via a laparoscopic biopsy and neoadjuvant chemotherapy is administered in three cycles to diminish it. Interval debulking surgery is the next step after neoadjuvant chemotherapy

and it is performed only if the cancerous bulk can be reduced to less than 1 cm in size. Both neoadjuvant and adjuvant chemotherapy for HGSOC include intravenously administered carboplatin and paclitaxel with or without the VEGF inhibitor bevacizumab. In addition, olaparib, a PARP inhibitor, can be added to the chemotherapy cocktail if HGSOC contains a BRCA mutation[9,27].

Recurrence and response to chemotherapy are monitored with physical examinations, including the analysis of post-treatment CA-125. The recurrent cancer is either classified as platinum-resistant (platinum-free interval is less than 6 months) or platinum-sensitive (platinum-free interval is more than 6 months). The platinum-free interval is defined as the interval between the date of the last platinum dose and the date of relapse detection. Platinum-resistant cancer is usually treated with an alternative single agent chemotherapeutic such as topotecan or PLD with or without bevacizumab. Platinum-sensitive cancer, on the other hand, could undergo secondary cytoreduction if complete resection is possible. This is again followed by platinum-based chemotherapy, with or without other options like bevacizumab and olaparib[27].

In the case of secondary and further recurrences, a tertiary cytoreduction may be considered before olaparib and bevacizumab monotherapies if BRCA mutated or if the recurrence is platinum-resistant, respectively. Otherwise, patients can consider participation in clinical trials or can be made comfortable through palliative systemic treatment[27].

2.Aim of paper

Aberrant expression of p53 is a common characteristic found in cancer and it has previously been correlated to the emergence of high-grade serous ovarian cancer (HGSOC)[28]. HGSOC is a poorly differentiated and most frequent histopathologic OC type that is often diagnosed at an advanced stage, which contributes to its high mortality rate. Pre-treatment CA-125 serum levels are a gold standard screening test for epithelial ovarian cancer, including HGSOC. Interestingly, certain research papers assigned them a prognostic value, while others dismissed their link to patient survival[12,13,14,15].

The main aim of this study was to evaluate pre-treatment CA-125 serum levels and p53 expression in a cohort of HGSOC patients admitted to the CHCR. I wanted to confirm that an aberrant p53 is indeed the hallmark of HGSOC of my cohort, while I also explored the correlation of p53 mutation pattern with FIGO stage and pre-treatment CA-125 serum levels. The latter investigation was performed in hopes of finding an association between HGSOC progression and p53 mutation pattern. In search for a link between pre-treatment CA-125 serum level and prognosis, the former was correlated to clinicopathological variables and overall survival.

3.Materials and methods

3.1.Study population

This retrospective cohort study included 28 patients from the Gynecology and Obstetrics Clinic (work unit at the Clinical Hospital Center of Rijeka, CHCR) diagnosed with high-grade serous ovarian cancer (HGSOC) between July 2016 and December 2018. There were two main inclusion criteria: (1) available data on pre-treatment serum CA-125 levels and (2) p53 expression in tissue samples. After the Ethical Committee of the Clinical Hospital Center of Rijeka gave their authorization, the study was carried out in partnership with the Clinical Department of Laboratory Diagnostics and the Clinical Department of Pathology and Cytology. Data on clinicopathological characteristics (age at diagnosis, menstrual status, FIGO stage, type of cytoreductive surgery, residual tumor, chemotherapy, biological therapy) were retrieved from the Gynecology and Obstetrics Clinic database.

3.2.Evaluation of pre-treatment serum CA-125 levels

After the collection of preoperative blood samples at the Clinical Department for Laboratory Diagnostics, serum CA-125 levels are usually determined by the chemiluminescence immunoassay analyzer Cobas 6000 (Roche Diagnostics). In clinical practice, every value above 35 U/mL is considered a high CA-125 concentration. For the purpose of this research paper, CA-125 concentrations of all 28 patients were retrieved from the Gynecology and Obstetrics Clinic database.

3.3.Immunohistochemical analysis of p53 expression

Immunohistochemical p53 staining is performed at the Department of Pathology and Cytology prior to a confirmed diagnosis of HGSOC. Obtained tissues are fixed in formalin at room temperature, embedded in paraffin and cut into 3 μm tissue sections. Deparaffinization, rehydration, and

epitope retrieval are performed next, followed by anti-p53 monoclonal antibody (Clone D0-7, Dako A/S Glostrup, Denmark) incubation of tissue sections according to the manufacturer's instructions. The Department of Pathology and Cytology database was used to obtain p53 expression data for further analysis in this study. p53 immunoreactivity was categorized as diffuse strong positive (1), null-type (2), focal positive/wild-type (3) and combination of diffuse strong positive and null-type tumor cells (4).

3.4.Statistical analysis

The Kruskal-Wallis test, together with Spearman's and Kendall's tau-b rank correlation, were used to evaluate any association between the continuous variable pre-treatment CA-125 and ordinal variables such as FIGO stage, p53 mutation pattern, residual tumor (RT) and type of cytoreductive surgery. FIGO stage was ordinally categorized as stage I (1), II (2), III (3) and IV (4), with RT size as 0 cm (1), < 0.5 cm (2), 0.5-1 cm (3) and > 1 cm (4). Similarly, type of surgery was divided into primary cytoreduction (1) and interval cytoreduction (2). To assess the correlation between p53 mutation pattern and FIGO stage, Fisher's exact test was implemented.

Survival analysis was performed with Kaplan-Meier and Log rank significance test to determine whether the size of the RT, type of surgery, chemotherapy regimen and FIGO stage impact the survival of HGSOC patients. Chemotherapy regimen consisted of the following categories: no chemotherapy (1), adjuvant (2) and neoadjuvant (3) treatment protocol. Additionally, univariate Cox proportional hazard ratios (HRs) were calculated to analyse whether pre-treatment serum CA-125 concentration affects survival. The impact of p53 expression on survival was not analysed, as the vast majority of patients had an aberrant expression of this protein (Table 1). Three patients' time of death was unknown, and two patients' survival time after diagnosis was unavailable, thus they were omitted from the survival analysis. The excluded patients were all diagnosed at stage III. A p value of ≤ 0.05 was considered statistically significant. All statistical

analyses were performed with XLSTAT (Microsoft Excel Statistical Software, version 2022.2.1.1311) and MedCalc (version 20.109).

4.Results

4.1.Clinicopathological characteristics

28 patients diagnosed with HGSOC at the CHCR between July 2016 and December 2018 were included in this study (Table 2). Following the guidelines from the International Federation of Gynaecologists and Obstetricians (FIGO), the majority of HGSOCs were diagnosed at an advanced stage III (78.57%). To be more specific, the IIIC stage contained the highest number of cases (67.86%). Abnormal p53 expression was found in 96.43% of tissue samples, with it being strongly and diffusely overexpressed (in more than 80% cells) in 67.86%. Furthermore, high pre-treatment CA-125 levels were found in 27 preoperative blood serum samples (96.43 %). RT is defined as the residual macroscopic tumor in the abdomen after primary or interval cytoreduction and was found to be 0 in the majority of patients (67.86 %). One patient succumbed to the disease, before she could proceed with chemotherapy, while the rest followed an adjuvant–paclitaxel + carboplatin (TC) (53.57 %) or a neoadjuvant TC treatment protocol (42.86%). Biological therapy was an optional step.

Table 2 Clinicopathological characteristics of high-grade serous ovarian cancer patients.

CHARACTERISTICS	**N (%)**
NUMBER OF PATIENTS	28
AGE AT DIAGNOSIS	
Mean $\pm$ **SD**	60.54 $\pm$ 13.95
Range	36-91
MENSTRUAL STATUS	
Premenopausal	8 (28.57)
Postmenopausal	20 (71.43)
FIGO STAGE	
IA	2 (7.14)
IB	0 (0)
IC1	0 (0)

IC2	0 (0)
IC3	0 (0)
IIA	1 (3.57)
IIB	2 (7.14)
IIIA1(I)	1 (3.57)
IIIA1(II)	0 (0)
IIIA2	0 (0)
IIIB	1 (3.57)
IIIC	19 (67.86)
IVA	0 (0)
IVB	2 (7.14)
SERUM CA-125 (U/mL)	
Mean	1505.71 ± 2084.49
Median (IQR)	832.90 (265.50-1867.93)
Range	15.00-10000.00
p53 EXPRESSION	
Strong and diffuse positive	19 (67.86)
Diffuse negative (null-type)	7 (25.00)
Focal positive (wild-type)	1 (3.57)
Combination	1 (3.57)
TYPE OF SURGERY	
Primary cytoreduction	15 (53.57)
Interval cytoreduction	13 (46.43)
RESIDUAL TUMOR (RT)	
= 0	19 (67.86)
< 0.5 cm	3 (10.71)
0.5-1 cm	0 (0)
> 1 cm	6 (21.43)
CHEMOTHERAPY REGIMEN	
No chemotherapy	1 (3.57)
Adjuvant TC protocol (6 cycles)	15 (53.57)
Neo-adjuvant TC (3 cycles) + adjuvant TC (3 cycles)	12 (42.86)
BIOLOGICAL THERAPY	
No biological therapy	13 (46.43)
Bevacizumab	11 (39.29)

Olaparip	1 (3.57)
Other	3 (10.71)
SURVIVAL	
Alive and healthy	9 (32.14)
Alive with disease	5 (17.86)
Died of disease	12 (42.86)
No evidence	2 (7.14)

4.2.Correlation of CA-125 and p53 with clinicopathological characteristics

Pre-treatment CA-125 levels on one hand had no significant correlation with FIGO stage, p53 expression level, RT, or type of cytoreductive surgery on the other, according to rank correlation analyses. This was deduced from all the p-values obtained through Spearman's and Kendall's tau-b correlations, which were > 0.05. Furthermore, both r_s and τ had values close to 0, which confirmed that there was no significant correlation between the mentioned variables (Table 3).

Similarly, there was no significant correlation between the mentioned variables (all p-values > 0.05) when Kruskal-Wallis ANOVA non-parametric correlation analysis was performed (Table 4). Mutation pattern of p53 was also not significantly associated with FIGO stage of HGSOCs (p = 0.960).

Table 3 Spearman's rank and Kendall's tau-b rank correlation analyses between clinicopathological parameters of HGSOC. $p \leq 0.05$ was considered statistically significant.

	Spearman's rank correlation		**Kendall's tau-b rank correlation**	
Variable	**r_S (ρ)**	**p**	**τ**	**p**
CA-125 (U/mL) FIGO STAGE	0.138	0.485	0.109	0.434
CA-125 (U/mL) p53	0.177	0.368	0.143	0.297

CA-125 (U/mL) RT	0.057	0.774	0.056	0.696
CA-125 (U/mL) Type of surgery	0.004	0.982	0.004	1.000

Table 4 Kruskal-Wallis ANOVA non-parametric correlation analysis between clinicopathological parameters of HGSOC. $p \leq 0.05$ was considered statistically significant.

Variable	df	h	p
CA-125 (U/mL) FIGO stage	3	5.710	0.127
CA-125 (U/mL) p53	3	2.201	0.532
CA-125 (U/mL) RT	2	0.234	0.889

4.3.Survival analysis

Median follow-up time was 41 months (range = 1.5-67 months, IQR = 31.5-53.5 months). A total of 9 patients (39.10%) died from HGSOC (events, 1), while 14 (60.90%) were still alive at the 67-month follow-up (censored, 0). 25% of patients have died 31 months after diagnosis, while neither the median nor the 75th percentile of survival were reached (assigned the value 'not reached, n.r.') (Figure 3, Table 5, Table 6). Cummulative survival (survival probability) is defined as the total proportion of patients surviving in a given length of time after diagnosis, while the survival rate determines the proportion of patients surviving a given time interval after diagnosis (Table 6).

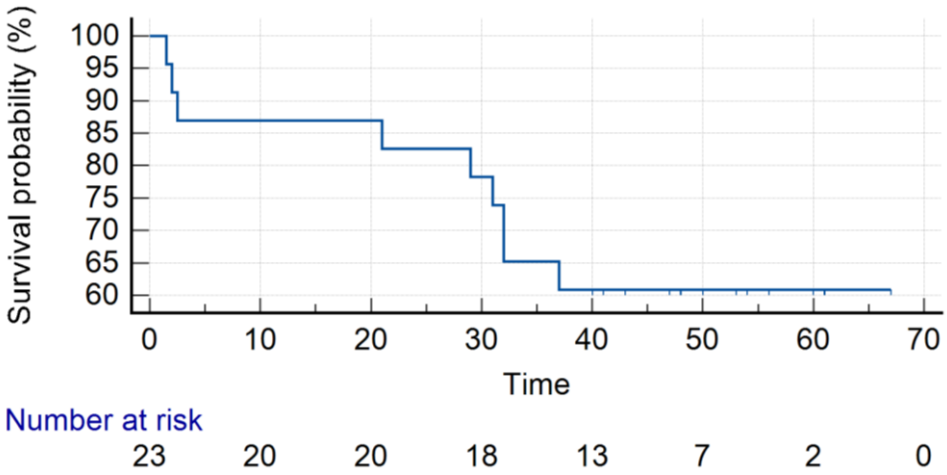

Figure 3 Kaplan-Meier survival function of 23 patients diagnosed with HGSOC.

Table 5 Summary of the Kaplan-Meier survival function of 23 patients diagnosed with HGSOC. [a]Patients who died of HGSOC. [b]Patients who remained alive at the 67-month follow-up. *not reached.

			Survival time/months		
Total sample size	**Number of events (1)[a]**	**Number censored (0)[b]**	**25th percentile**	**50th percentile (median)**	**75th percentile**
23	9	14	31.000	n.r.*	n.r.*

Table 6 Survival table of 23 patients diagnosed with HGSOC.

Survival time/months	**Survival rate**	**Cumulative propotion surviving (survival probability)**
1,5	0.957	0.957
2	0.954	0.913
2,5	0.952	0.870
21	0.950	0.826
29	0.948	0.783
31	0.944	0.739
32	0.882	0.652
37	0.934	0.609
40	1	0.609
41	1	0.609
43	1	0.609
47	1	0.609
48	1	0.609
50	1	0.609
53	1	0.609
54	1	0.609
56	1	0.609
60	1	0.609
61	1	0.609
67	1	0.609

At the 67-month follow-up, both patients diagnosed at stage I were still alive, while one patient diagnosed with stage II died of the condition (33.33%). Median survival, together with the 25th and 75th percentile of survival weren't reached for stage I patients, whereas 25% of stage II patients have died 32 months after diagnosis. There were 7 death events (43.75%) among patients diagnosed at stage III and 1 among those at stage IV (50%). 25% of stage III patients have died 29 months after diagnosis and one stage IV patient has died 2.5 months after diagnosis (Figure 4, Table 7). However, there was no significant difference in survival time between patients diagnosed at stages I, II, III and IV. In other words, FIGO stage didn't significantly affect the survival of HGSOC patients (Table 8). Similarly, FIGO stage wasn't classified as a significant prognostic predictor of survival, according HR values and non-significant 95% confidence intervals (CI) (Table 9). Significant 95% CI should not contain 1, since a HR value of 1 means no association of a predictor variable with increased/decreased risk of event.

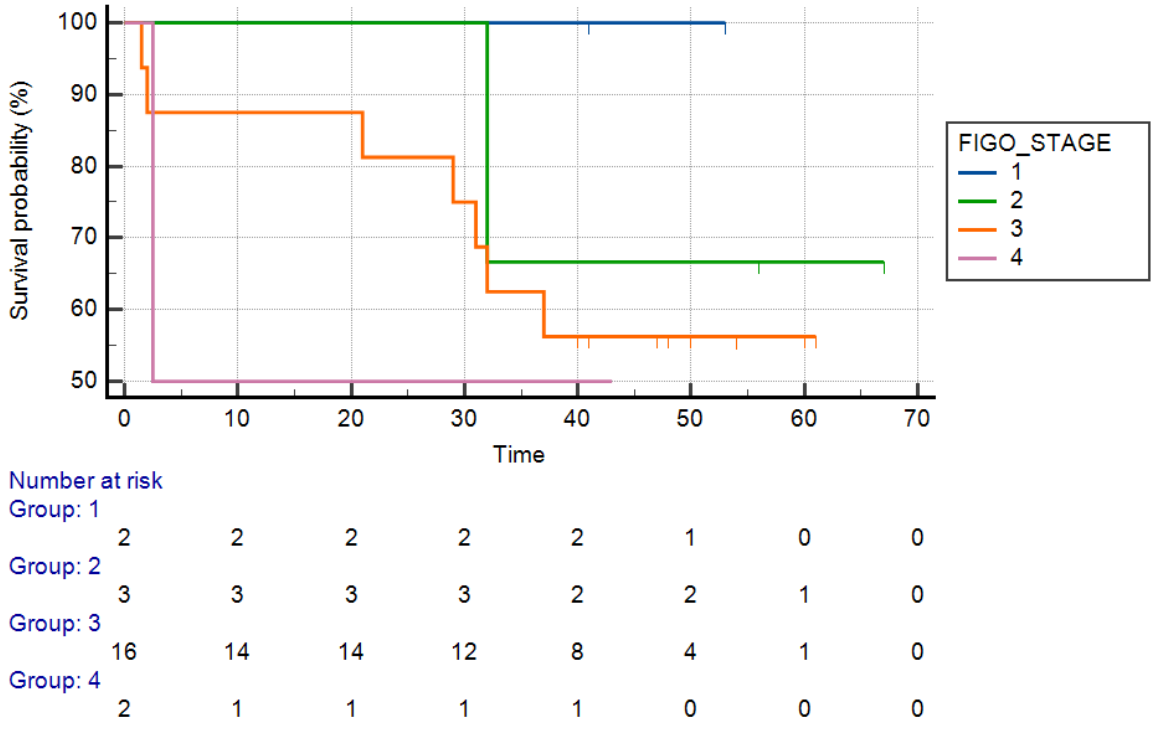

Figure 4 Kaplan-Meier survival function of 2 patients diagnosed with stage I, 3 with stage II, 16 with stage III and 2 with stage IV HGSOC.

Table 7 Summary of the Kaplan-Meier survival function of 23 patients diagnosed with differently staged HGSOC. [a]Patients who died of HGSOC. [b]Patients who remained alive at the 67-month follow-up. *not reached.

				Survival time/months		
FIGO stage	**Total sample size**	**Number of events (1)[a]**	**Number censored (0)[b]**	**25th percentile**	**50th percentile (median)**	**75th percentile**
I	2	0	2	n.r.*	n.r.*	n.r.*
II	3	1	2	32.000	n.r.*	n.r.*
III	16	7	9	29.000	n.r.*	n.r.*
IV	2	1	1	n.r.*	2.500	n.r.*

Table 8 Log-rank test parameters that show no significant difference in survival time between patients diagnosed at stages I, II and III. **χ2 (observed value) < χ2 (critical value); not statistically significant. ***p > 0.05; not statistically significant.

Test	**X^2 (observed value)****	**X^2 (critical value, df=3)**	**p*****
Log-rank	1.473	7.815	0.688

Table 9 HR and 95% CI show that FIGO stage is not a significant prognostic predictor of survival among HGSOC patients.

FIGO stage	**HR**	**95% CI HR**
II vs III	0.631	0.099 to 4.031
II vs IV	0.449	0.022 to 9.088
III vs II	1.584	0.248 to 10.116
III vs IV	0.712	0.052 to 9.794
IV vs II	2.226	0.110 to 45.040
IV vs III	1.405	0.102 to 19.341

Five patients with an RT size of 0 cm died (33.33 %) during the 67-month follow-up period, while one (33.33%) and three (60.00%) patients with RT sizes of less than 0.5 cm and higher than 1 cm died, respectively. 25% of patients with an RT size of 0 cm have died 32 months after diagnosis, while the median and 75th percentile of survival weren't reached. Similarly, 25% of patients with an RT size of less than 0.5 cm have died 37 months after diagnosis. 25% of those with an RT size of higher than 1 cm have died 2 months after diagnosis, whereas 50% of them succumbed to the disease 2.5 months after diagnosis (Figure 5, Table 10). However, there was no significant difference in survival time between patients with different RT sizes. In other words, RT size didn't significantly affect the survival of

HGSOC patients (Table 11). Furthermore, RT size wasn't classified as a significant prognostic predictor of survival, according HR values and non-significant 95% confidence intervals (CI) (Table 12).

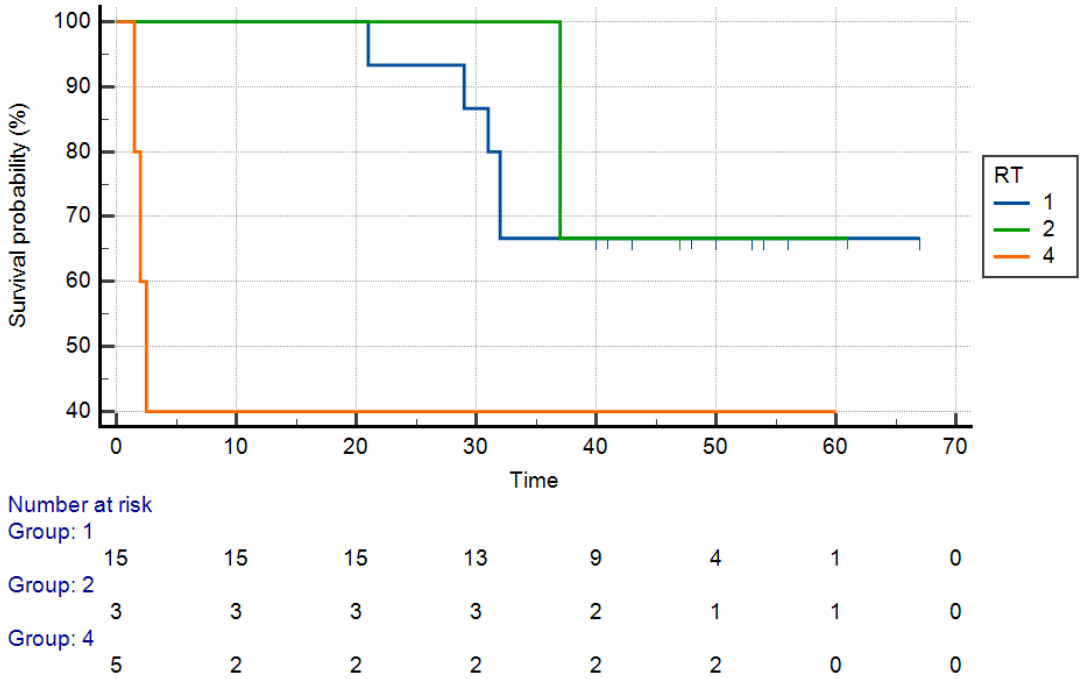

Figure 5 Kaplan-Meier survival function of 15 patients with an RT size of 0 cm, 3 with less than 0.5 cm and 5 with higher than 1 cm.

Table 10 Summary of the Kaplan-Meier survival function of 23 patients diagnosed with differently sized RT. [a]Patients who died of HGSOC. [b]Patients who remained alive at the 67-month follow-up. *not reached.

				Survival time/months		
RT	**Total sample size**	**Number of events (1)[a]**	**Number censored (0)[b]**	**25th percentile**	**50th percentile (median)**	**75th percentile**
1	15	5	10	32.000	n.r.*	n.r.*
2	3	1	2	37.000	n.r.*	n.r.*
4	5	3	2	2.000	2.500	n.r.*

Table 11 Log-rank test parameters that show no significant difference in survival time between patients with differently sized RT. **χ2 (observed value) < χ2 (critical value); not statistically significant. ***p > 0.05; not statistically significant.

Test	**X^2 (observed value)****	**X^2 (critical value, df=2)**	**p*****
Log-rank	3.049	5.991	0.218

Table 12 HR and 95% CI show that RT size is not a significant prognostic predictor of survival among HGSOC patients.

RT	HR	95% CI HR
1 vs 2	1.134	0.185 to 6.937
1 vs 4	0.323	0.047 to 2.231
2 vs 1	0.882	0.144 to 5.393
2 vs 4	0.285	0.026 to 3.166
4 vs 1	3.098	0.448 to 21.411
4 vs 2	3.514	0.316 to 39.086

Two (14.29%) and seven patients (77.78%) who have undergone primary and interval cytoreduction, respectively, have died of HGSOC. Median survival wasn't reached for patients in the primary cytoreduction group and neither were the 25 th and 75th percentile. However, 25%, 50% and 75% of patients who have undergone interval cytoreduction have died 21, 31 and 37 months after diagnosis, respectively (Figure 6, Table 13). Furthermore, there was significant difference in survival time between patients with different surgical procedure. In other words, type of cytoreductive surgery significantly affected the survival of HGSOC patients (Table 14). Similarly, type of surgery was classified as a significant prognostic predictor of survival, according HR values and significant 95% confidence intervals (CI). There was a 0.100 times lower risk of death due to HGSOC when a patient underwent primary cytoreduction. Analogously, there was a 9.985 higher risk of death when a patient underwent interval cytoreduction (Table 15).

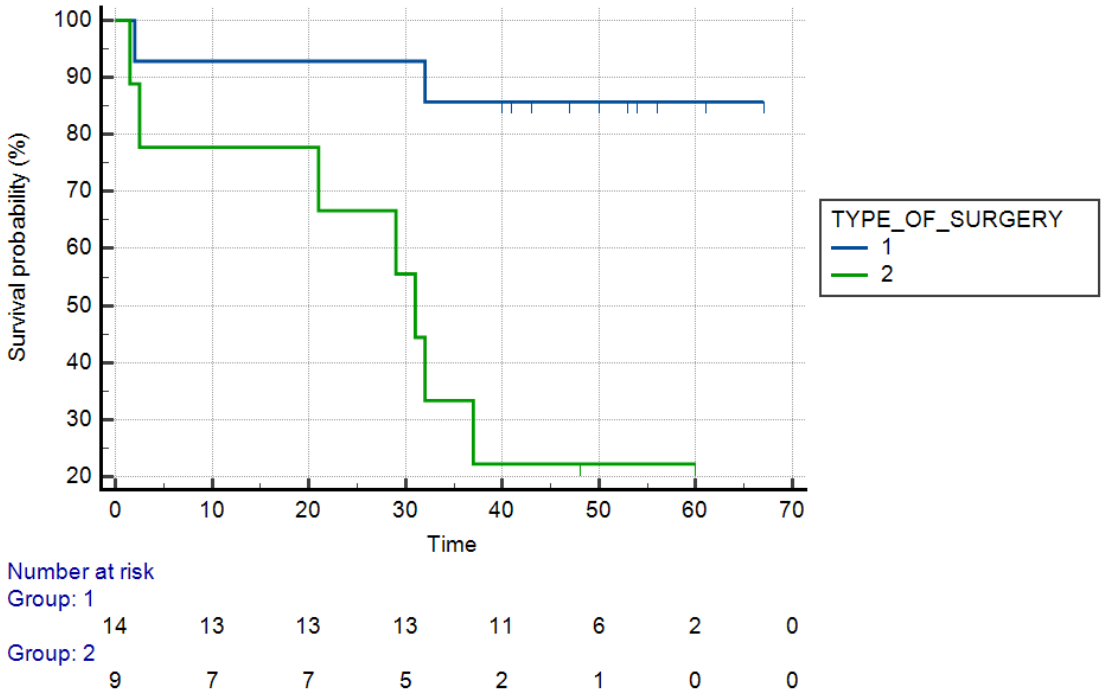

Figure 6 Kaplan-Meier survival function of 14 and 9 patients who underwent primary and interval cytoreduction, respectively.

Table 13 Summary of the Kaplan-Meier survival function of 23 patients who underwent different types of cytoreductive surgery. [a]Patients who died of HGSOC. [b]Patients who remained alive at the 67-month follow-up. *not reached.

				Survival time/months		
Type of surgery	**Total sample size**	**Number of events (1)[a]**	**Number censored (0)[b]**	**25th percentile**	**50th percentile (median)**	**75th percentile**
1	14	2	12	n.r.*	n.r.*	n.r.*
2	9	7	2	21.000	31.000	37.000

Table 14 Log-rank test parameters that show significant difference in survival time between patients who underwent different types of cytoreductive surgery. **χ2 (observed value) > χ2 (critical value); statistically significant. ***p < 0.05; statistically significant.

Test	**X^2 (observed value)****	**X^2 (critical value, df=1)**	**p*****
Log-rank	9.779	3.841	0.002

Table 15 HR and 95% CI show that type of cytoreductive surgery is a significant prognostic predictor of survival among HGSOC patients.

Type of surgery	**HR**	**95% CI HR**
1 vs 2	0.100	0.024 to 0.424
2 vs 1	9.985	2.360 to 42.235

One patient has died due to HGSOC before starting her chemotherapy treatment. Two (14.29%) and six patients (75 %) who received adjuvant (6 cycles of taxane + carboplatin) and neoadjuvant in combination with adjuvant chemotherapy (3 cycles of neoadjuvant + 3 cycles of adjuvant taxane + carboplatin) have died, respectively. Median survival wasn't reached for patients who have received only adjuvant chemotherapy and neither were the 25 th and 75th percentile. However, 25%, 50% and 75% of patients treated with a combination of neoadjuvant and adjuvant have died 21, 31 and 37 months after diagnosis, respectively (Figure 7, Table 16). Furthermore, there was significant difference in survival time between patients undergoing a different chemotherapy regimen. In other words, type of type of chemotherapy regimen significantly affected the survival of HGSOC patients (Table 17). Similarly, type of chemotherapy regimen was classified as a significant prognostic predictor of survival, according HR values and significant 95% confidence intervals (CI). There was a 0.144 times lower risk of death due to HGSOC when a patient received only adjuvant chemotherapy compared to when they received a combination of neoadjuvant and adjuvant chemotherapy. Analogously, there was a 6.928 higher risk of death when a patient received a combination of neoadjuvant and adjuvant chemotherapy (Table 18).

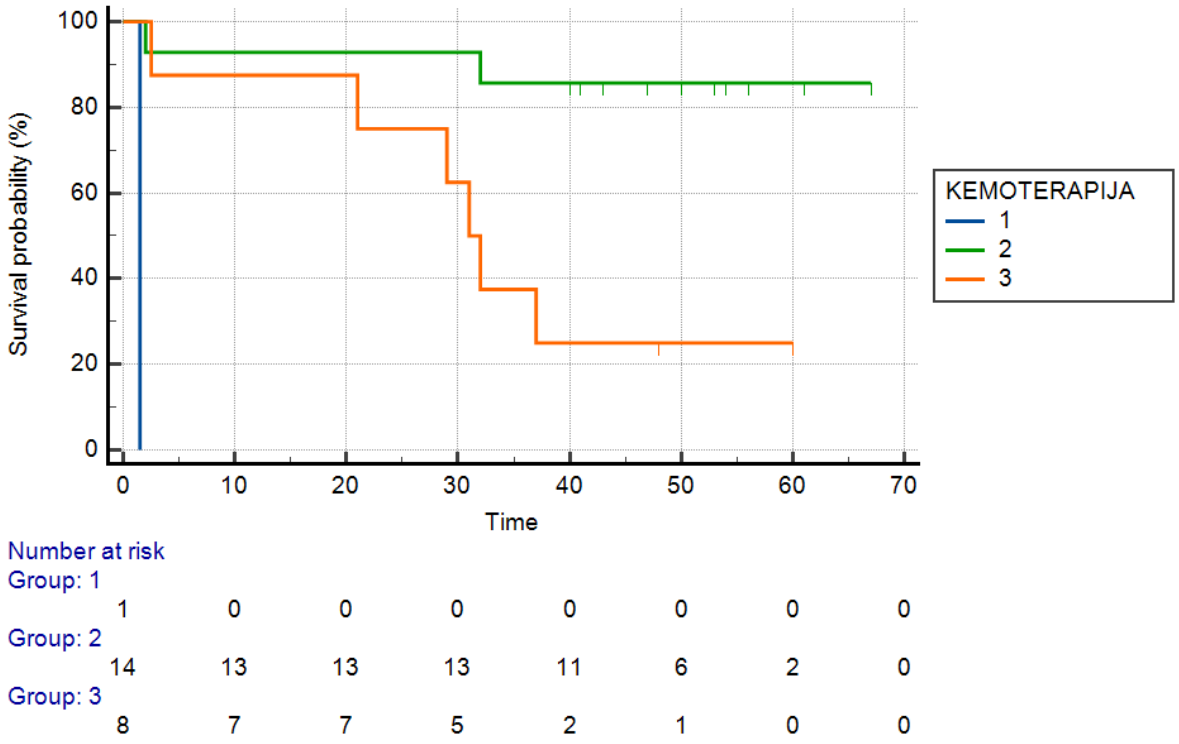

Figure 7 Kaplan-Meier survival function of 14 and 8 patients who received adjuvant and a combination of neoadjuvant together with adjuvant chemotherapy, respectively. 1 patient died before receiving any chemotherapy.

Table 16 Summary of the Kaplan-Meier survival function of 23 patients who received different chemotherapy regimens. [a]Patients who died of HGSOC. [b]Patients who remained alive at the 67-month follow-up. *not reached.

				Survival time/months		
Chemotherapy regimen	**Total sample size**	**Number of events (1)[a]**	**Number censored (0)[b]**	**25th percentile**	**50th percentile (median)**	**75th percentile**
1	1	1	0	n.r.*	1.500	n.r.*
2	14	2	12	n.r.*	n.r.*	n.r.*
3	8	6	2	21.000	31.000	37.000

Table 17 Log-rank test parameters that show significant difference in survival time between patients who received different chemotherapy regimens. **χ^2 (observed value) > χ^2 (critical value); statistically significant. ***$p < 0.05$; statistically significant.

Test	**X^2 (observed value)****	**X^2 (critical value, df=2)**	**p*****
Log-rank	29.245	5.991	<0.0001

Table 18 HR and 95% CI show that type of chemotherapy regimen is a significant prognostic predictor of survival among HGSOC patients.

Chemotherapy regimen	HR	95% CI HR
2 vs 3	0.144	0.035 to 0.601
3 vs 2	6.928	1.664 to 28.838

On the contrary, pre-treatment serum CA-125 level was not a significant prognostic factor of patient survival, indicated by a p-value higher than 0.05 and a 95% CI OH that contains 1 (Tables 19 and 20). A cummulative function of hazard for this predictor variable is shown in Figure 8.

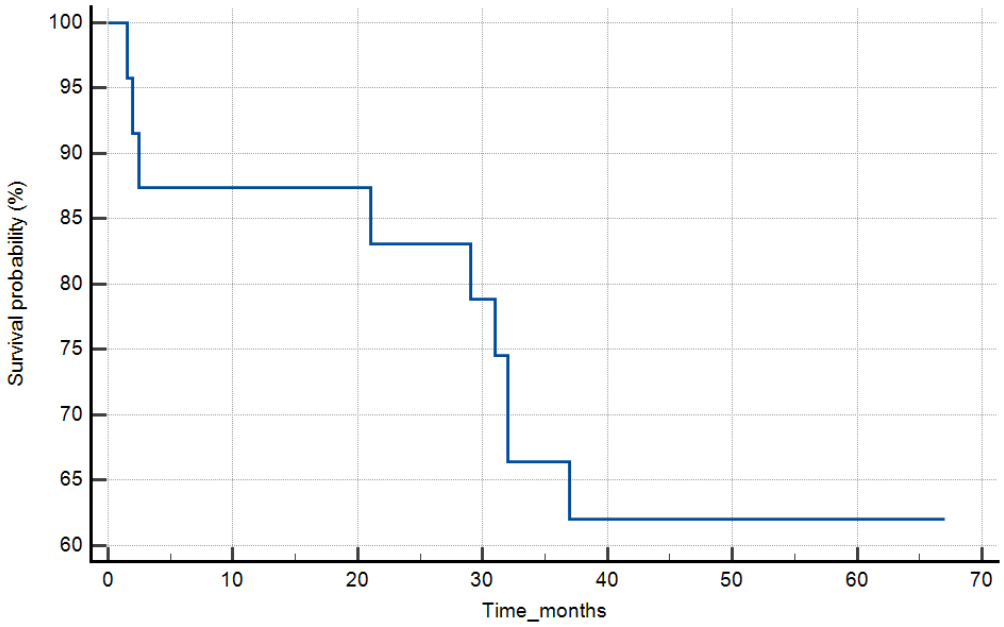

Figure 8 Cummulative function of hazard for the predictor variable 'serum CA-125 level'.

Table 19 Parameters of Cox proportional hazards model that show the insignificance of the predicting model.

Predictor variable	df	χ^2	p	Null model -2 log likelihood	Full model -2 log likelihood
CA-125	1	0.380	0,538	59.952	52.572

Table 20 Parameters of Cox proportional hazards model that show the insignificance of the variable 'serum CA-125' in predicting survival of HGSOC patients.

Predictor variable	β	SE OH	OH	95% CI OH	p
CA-125	-0.0001	0,0002	0.9999	0.9995 to 1.0003	0.577

5.Discussion

This retrospective cohort study included 28 patients diagnosed with HGSOC between July 2016 and December 2018 at the CHCR. In general, the CHCR receives around 30 patients harboring HGSOC per year, which all undergo pre-treatment serum CA-125 evaluation. Pre-treatment serum CA-125 is obtained before a patient undergoes any kind of cytoreductive surgery, chemotherapy and/or biological treatment. However, p53 expression is not immunohistochemically determined at the Department of Pathology and Cytology unless diagnosis by observing the histopathological architecture and cytological features is inconclusive. Aberrant p53 expression is a hallmark of cancer in general, as it allows for rapid development and survival of cancer cells[23,24]. Several studies have explored the correlation of pre-treatment serum CA-125 levels with patient prognosis, but the observations were rather contradictory[12,13,14,15]. Based on these findings, I have decided to evaluate pre-opreative serum CA-125 levels and p53 mutation pattern and status (aberrant/normal) in HGSOC patients admitted at the CHCR. The correlation of pre-treatment serum CA-125 and p53 mutation pattern with clinicopathological variables as well as their impact on survival were examined. The findings of my thesis will contribute to a better understanding of the relationship between clinicopathological factors and HGSOC patient survival.

The obtained data suggest that pre-treatment serum CA-125 levels don't significantly correlate with p53 mutation pattern (strong and diffuse positive, diffuse negative, focal positive/wild-type and combination of diffuse positive and negative expression). It is important to emphasize that the majority of patients (67.86 %) had a strong and diffuse positive p53 expression. Accordingly, the pattern of p53 mutation was not formerly correlated to patient overall and progression-free survival[14]. Targeting different types of p53 mutations would be interesting to explore in HGSOC in studies to come[29].

In general, all of the patients except for one in this study had a mutant *TP53* gene, which drives HGSOC pathogenesis from an early stage according to other studies[14,30]. This one patient had a focal positive stain (wild-type) of p53, which could be contributed to antigen degradation and weak staining during the IHC process rather than to the fact that this patient doesn't actually have a *TP53* mutation. To be more specific, HGSOC ubiquitously harbor *TP53* mutations[19,20,21]. As a result, HGSOC and its rapid advancement are caused by an abnormal p53 expression, which is why it hasn't been deemed a major predictor of survival in this histological subtype, regardless of FIGO stage. My hypothesis that abnormal p53 expression occurs early in HGSOC pathogenesis and is essentially one of the disease's driving mutations has been proven.

A study by Osman *et al.*[31]demonstrated no significant correlation between pre-treatment CA-125 serum levels with FIGO stage, which was also the case in this thesis. Interestingly, FIGO stage didn't have a significant impact on survival, which contraindicates current research. To be more specific, HGSOC is usually diagnosed at an advanced stage (III-IV), which is why it is associated with poor survival outcomes[9,32,33]. In this retrospective cohort study, the vast majority of patients were diagnosed in stage III, while the sample size was small. A multi-center retrospective study might be done in Croatia to enhance sample size and ensure that additional stages are represented in a larger number, allowing for a more complete examination of the impact of FIGO stage on survival.

Furthermore, pre-treatment serum CA-125 levels had no significant correlation with neither RT size nor the type of cytoreductive surgery. This means that pre-treatment serum CA-125 levels are not a significant predictor of RT size. RT size had no significant effect on survival, in contrast to claims in other research papers[9,34,35,36]. However, even with optimal cytoreduction and no indication of the disease following therapy, relapses are common in women identified at an advanced stage[37]. It is possible that the women in my thesis that were diagnosed at stages III and IV, and

in which optimal cytoreduction was achieved, relapsed. Unfortunately, data on recurrent disease was unavailable from the CHCR database. In future studies, progression-free survival should be explored and correlated to RT size, FIGO stage and p53 expression. Also, RT should be correlated to the type of cytoreductive surgery together with response to chemotherapy.

Type of cytoreductive surgery, on the other hand, significantly impacted the survival of patients. In my thesis, primary cytoreduction was associated with longer survival after diagnosis. However, there is no evidence that primary cytoreduction is actually superior to interval cytoreduction[9,35,36]. Therefore, my results could stem from the fact that the majority of patients undergoing interval cytoreduction were diagnosed at an advanced stage. Survival time was known for nine patients who underwent interval cytoreduction, out of which seven (six at stage III, one at stage IV) succumbed to the disease. As described in the last paragraph, advanced-stage patients are more prone to cancer recurrence[37].

The impact of a certain chemotherapy regimen is also a significant prognostic factor of survival. This seems logical since it is directly correlated to the type of cytoreductive surgery. In other words, patients who undergo primary cytoreductive surgery can only receive adjuvant chemotherapy, whereas neoadjuvant chemotherapy is administered before interval cytoreductive surgery. Interestingly, there is research demonstrating a correlation between platinum-resistant recurrent HGSOC and the neoadjuvant chemotherapy + interval cytoreduction approach[38]. This should be investigated in future studies to balance the benefits and drawbacks of those different treatment regimens.

Pre-treatment serum CA-125 level wasn't a significant survival predictor in my thesis, which is also claimed in a research paper by Osman *et al*.[31]. However, it would be very useful to examine pre-treatment serum CA-125 as a predictor of progression-free survival, as it was attempted before[39]. Additionally, CA-125 levels are also measured to monitor response to

therapy, as well as to check for cancer recurrence. Therefore, I propose that post-and intra-treatment serum CA-125 levels of patients at the CHCR be correlated to the onset of recurrence, FIGO stage, overall and progression-free survival. The future study should cover a broader cohort of patients, preferably through partnership with other clinical medical centers in Croatia, such as those in Split and Zagreb. This would ensure sample heterogeneity. On a final note, just as it is necessary to research prognostic HGSOC biomarkers, it is also of paramount importance to investigate diagnostic markers that would encounter the disease in its early stage.

6.Conclusion

The findings of my study indicate that pre-treatment serum CA-125 levels were abnormal in 96.43 % HGSOC patients, while p53 expression was aberrant in all of them. Despite the fact that p53 mutation pattern had no association with FIGO stage and pre-treatment serum CA-125 levels, I confirmed that its generally aberrant expression was the culprit of HGSOC. There was no link between pre-treatment serum CA-125 levels and FIGO stage, RT size, or the type of cytoreductive surgery. The only clinicopathological factors that had an impact on overall survival of HGSOC patients were the type of cytoreductive surgery and chemotherapy regimen. Interestingly, pre-treatment serum CA-125 level is not a significant outcome predictor, despite the fact that it is still assessed for the purpose of detecting ovarian cancer. It would be very useful if future multi-centered studies correlated post- and intra-treatment serum CA-125 level to the time of recurrence, FIGO stage, overall and progression-free survival. Similarly, pre-treatment CA-125 should be evaluated as a predictive variable for progression-free survival. The results obtained in this study will contribute to a better understanding of the relationship between clinicopathological factors and HGSOC patient survival. Additionally, they will serve as a foundation of future study design and focus, as discussed in the previous paragraph. Overall, I am confident that outcome predictions will one day become supplementary to screening tests developed to detect HGSOC at an early stage.

7.References

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