NOVEL BIOMARKERS IN THE DIAGNOSIS OF OVARIAN CANCER

Doctoral (PhD) Dissertation
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TABLE OF CONTENTS

1 LIST OF ABBREVIATIONS 4

2 INTRODUCTION 7

3 NORMAL FEMALE PELVIC ANATOMY 8

4 OVARIAN FUNCTION 13

4.1. Physiological ovarian function 13

5 OVARIAN CANCER 15

5.1. Types of ovarian cancer 15

5.2. Staging of ovarian cancer 18

5.3. Causes and pathogenesis of ovarian cancer 22

5.4. Epithelial ovarian cancer 24

5.5. Biomarkers in ovarian cancer 26

5.6. Current treatment of ovarian cancer 28

6 AIMS OF THE STUDY 30

7 INVESTIGATING THE CLINICAL POTENTIAL FOR 14-3-3 ZETA PROTEIN TO SERVE AS A BIOMARKER FOR EPITHELIAL OVARIAN CANCER 31

7.1. Materials and methods 31

7.1.1. Patients and follow-up study design 31

7.1.2. Computed tomography (CT) scans 34

7.1.3. Laboratory methods 36

7.1.4. Enzyme-linked immunosorbent assay (ELISA) 36

7.1.5. Quantitative electrochemiluminescence assay (ECLIA) 36

7.1.6. Risk of Ovarian Malignancy Algorithm (ROMA) index.....37

7.1.7. Statistical analysis.....38

7.2. Results 39

7.2.1. Radiological assesment fololwing therapeutic procedures 39

7.2.2. Detection of CA-125, HE4 and 14-3-3 Zeta protein.....41

7.2.3. Correlation between radiological findings and serum parameters.....48

7.3. Discussion.....51

7.4. Conclusion.....55

8 REFERENCES 56

9 PUBLICATIONS OF THE AUTHOR.....61

10 ACKNOWLEDGEMENTS.....63

1 LIST OF ABBREVIATIONS

BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BSO	Bilateral salpingo-oophorectomy
CA-125	Cancer antigen 125
COX2	Cyclooxygenase-2
CP	Corpus luteum
CT	Computed tomography
ECLIA	Quantitative electrochemiluminescence assay
ELISA	Enzyme-linked immunosorbent assay
EOC	Epithelial ovarian cancer
FIGO	International Federation of Obstetrics and Gynecology
FSH	Follicle-stimulating hormone

CGIG	Clinical Global Impression-Global Improvement
GnRH	Gonadotropin releasing hormone
HE4	Human epididymis protein 4
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LD	Longest diameter
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
OEIC	Ovarian epithelial inclusion cysts
OSE	Ovarian surface epithelium/cells
PI	Predictive index
RMI	Risk of malignancy index
ROMA	Risk of ovarian malignancy algorithm

SEM	Standard error of the mean
SHBG	Sex hormone-binding globulin
TAH	Total Abdominal Hysterectomy
TNM	Tumor-Lymph nodes-Metastasis
UICC	Union for International Cancer Control
VEGF	Vascular endothelial growth factor
WAP	Whey acid protein
WHO	World Health Organization

2 INTRODUCTION

Amongst all gynecological cancers, ovarian cancer poses the most difficult in diagnosis and treatment. This type of gynecological malignancy is either diagnosed by chance (e.g. during a diagnostic laparoscopy) owing to the fact that in early stages it is almost symptomless, or when it is already in an advanced stage that gives rise to symptoms. For the above-mentioned reasons ovarian cancer has earned in the United States of America the nickname (The Silent Killer). This phenomenon is due to the fact that our current early diagnostic tools (biomarkers) are extremely limited. Its high mortality rate has made it one of the most investigated fields in gynecological oncology.

As the father of medicine Hippocrates of Kos said, "He who diagnoses best also cures best" that is the main reason that we as physicians should strive to discover novel diagnostic tools, that will help us to better serve humankind. Even though we are in the 21st-century our arsenal of weapons against in diagnosing ovarian cancer is extremely limited. As physicians whose solemn duty under oath is to serve humanity we should strive for excellence and not rest until we have obtained the tools necessary to diagnose and fight this malicious disease.

3 NORMAL FEMALE PELVIC ANATOMY

The organs that occupy the female pelvis are the bladder, the ureters, the urethra, the fallopian tubes, the ovaries, the vagina, and the rectum. With the exception of the inferior portion of the rectum and most of the vagina, all lie immediately beneath the peritoneum. The uterus, fallopian tubes and ovaries are almost completely covered with peritoneum and are suspended in peritoneal ligaments. The remainders are partially covered. These organs do not completely fill the cavity; the remaining space is occupied by the ileum and sigmoid colon. (1)

The urinary bladder is a muscular, hollow organ that lies posterior to the pubic bones and anterior to the uterus and broad ligament. It's form; size and position vary with the amount of urine it contains. A thin layer of fascia, the vesical sheath, encloses the bladder. Its blood supply comes from branches of the hypogastric artery. (1)

The ureters are slightly flattened tubes that extend from the termination of the renal pelvis to the lower outer corner of the base of the bladder, a distance of about 26-28 cm. They are partly abdominal and partly pelvic and lie entirely behind the peritoneum. Their diameter varies from 4 to 6 mm, depending on distention, and their size is uniform except for 3 slightly constricted portions. The first of these constrictions is found at the junction of the ureters with the renal pelvis and is known as the upper isthmus. The second constriction (the lower isthmus) is at the point where the ureters cross the brim of the minor pelvis. The third (intramural) constriction is at the terminal part of the ureters as they pass through the bladder wall. (1)

The female urethra is a canal 2.5-5.25 cm long. It extends downward and forward in a curve from the neck of the bladder (internal urethral orifice), which lies nearly opposite the pubic symphysis. (1)

The uterus is a pear-shaped, thick-walled, muscular organ, Situated between the base of the bladder and the rectum. Covered on each side by the 2 layers of the broad ligament, it communicates above with the fallopian tubes and below with the vagina. It is divided into 2 main portions, the larger portion or body above and the smaller cervix below, connected by a transverse constriction, the isthmus. The fallopian tubes join the uterus at the superior (lateral) angles. The cervix is divided into a supravaginal and vaginal portion by the line of attachment. Normally, the uterus forms a sharp angle with the vagina so that it's anterior surface lies on the upper surface of the bladder and the body is in a horizontal plane when the woman is standing erect. There is a bend in the area of the isthmus, at which the cervix then faces downward. This position is the normal anteflexion, although it may be placed backwards retroflexion, without angulation (military position), or to one side (lateral flexion). Anteriorly, The body of the uterus rests upon the upper and posterior surfaces of the bladder, Separated by the uterovesical pouch of the peritoneum. Posteriorly the peritoneal covering extends down as far as the uppermost portion of the vagina; therefore the entire posterior surface of the uterus is covered by peritoneum, and the convex posterior wall is separated from the rectum by the pouch of Douglas. Although the cervix of the uterus is fixed, the body is free to rise and fall with the filling and emptying of the bladder. The ligaments supporting the uterus consist of the uterosacral ligaments, the transverse ligaments of the cervix (cardinal ligaments, cardinal supports, ligamentum transversum colli, ligaments of Mackenrodt), the round ligaments and the broad ligaments. The

cervix is embedded in tissue called the parametrium. The wall of the uterus is very thick and consists of 3 layers: serous, muscular and mucous. The blood supply of the uterus is from the uterine and ovarian arteries. The arterial supply of the cervix is primarily through the cervical branches of the right and left uterine arteries, which form a rete around the cervix (coronary artery). (1)

The fallopian tubes serve to convey the ova to the uterus. They extend from the superior angles of the uterus to the region of the ovaries, running into the superior border of the broad ligament (mesosalpinx). Each tube is 7-14 cm long and may be divided into three parts: isthmus, ampulla and infundibulum. The wall of the fallopian tubes has 4 layers: serous (peritoneal), adventitial, muscular and mucous. The blood supply of the fallopian tubes is derived from the ovarian and uterine arteries. (1)

The ovaries are paired organs situated close to the wall on either side of the minor pelvis. Each measures 2.5-5 cm in length, 1.5-3 cm in diameter, weighing about 4-8 gr. The ovaries have 2 surfaces, medial and lateral; 2 borders, anterior or mesovarium and posterior or free; and 2 poles, tubal and uterine. The ovaries are suspended by means of the mesovarium, the suspensory ligament of the ovaries, and the ovarian ligaments. The mesovarium consists of 2 layers of peritoneum, continuous with both the epithelial coat of the ovaries and the posterosuperior layer of the broad ligament. The ovarian arteries provide blood supply to the ovaries. Though both arteries may originate as branches of the abdominal aorta, the left frequently originates from the left renal artery, the right less frequently. (1)

The vagina is a strong canal of muscle approximately 7.5 cm long that extends from the uterus to the vestibule of the external genitalia, where it opens to the exterior. Its long axis is almost parallel with that of the lower part of the sacrum, and it meets the cervix of the uterus at an angle of 45-90 degrees. In virgins, an incomplete fold of highly vascular tissue and mucous membrane, the hymen, partially closes the external orifice. The vaginal wall is composed of a mucosal layer and a muscular layer. The chief blood supply to the vagina is through the vaginal branch of the uterine artery. (1)

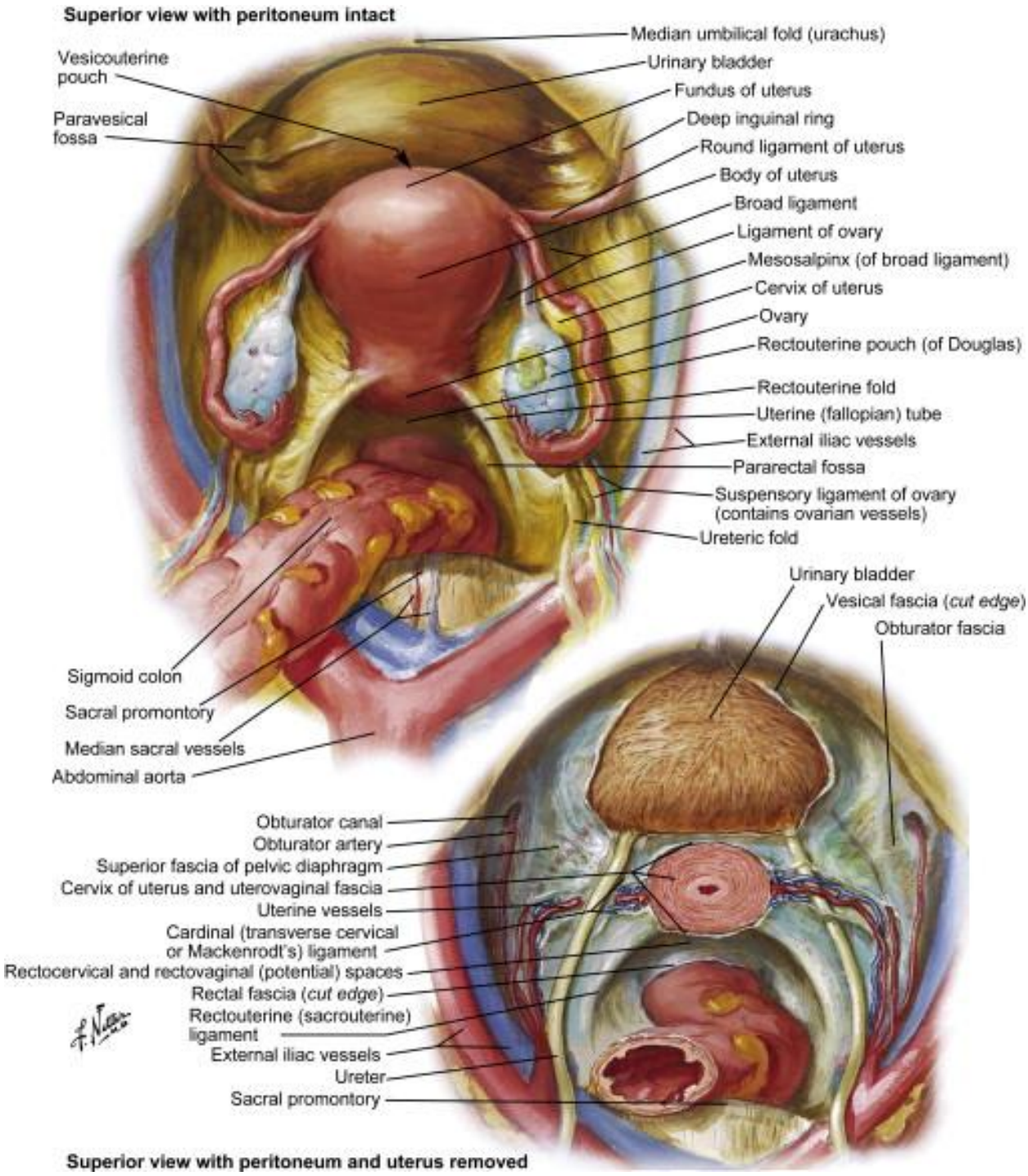

Figure 1: *Superior view of the internal female genitalia with and without the uterus.*

(Atlas of Human Anatomy 5th Edition, Frank Netter)

4 OVARIAN FUNCTION

4.1 Physiological ovarian function

Physiological ovarian function is controlled primarily by the gonadotropins LH (luteinizing hormone) and FSH (follicle-stimulating hormone) secreted by the anterior pituitary gland. This secretion is regulated by the hypothalamic hormone, gonadotropin releasing hormone (GnRH). (2)

In females, GnRH is secreted in a cyclical pattern that maintains the menstrual cycle. LH acts on the ovarian follicle to induce ovulation and maintain the corpus luteum. FSH causes development of the ovarian follicle and stimulates secretion of estradiol and progesterone. These steroidal hormones inhibit GnRH release and, subsequently, LH and FSH release via a negative feedback loop. (2)

However, estradiol in sustained high levels causes an LH surge that is associated with ovulation. The effects of leptin (a hormone emanating from fat cells) and insulin from the pancreas alter the bioavailability of estradiol and testosterone by affecting production of sex hormone-binding globulin (SHBG) from the liver. Insulin can also function directly on the ovary. (2)

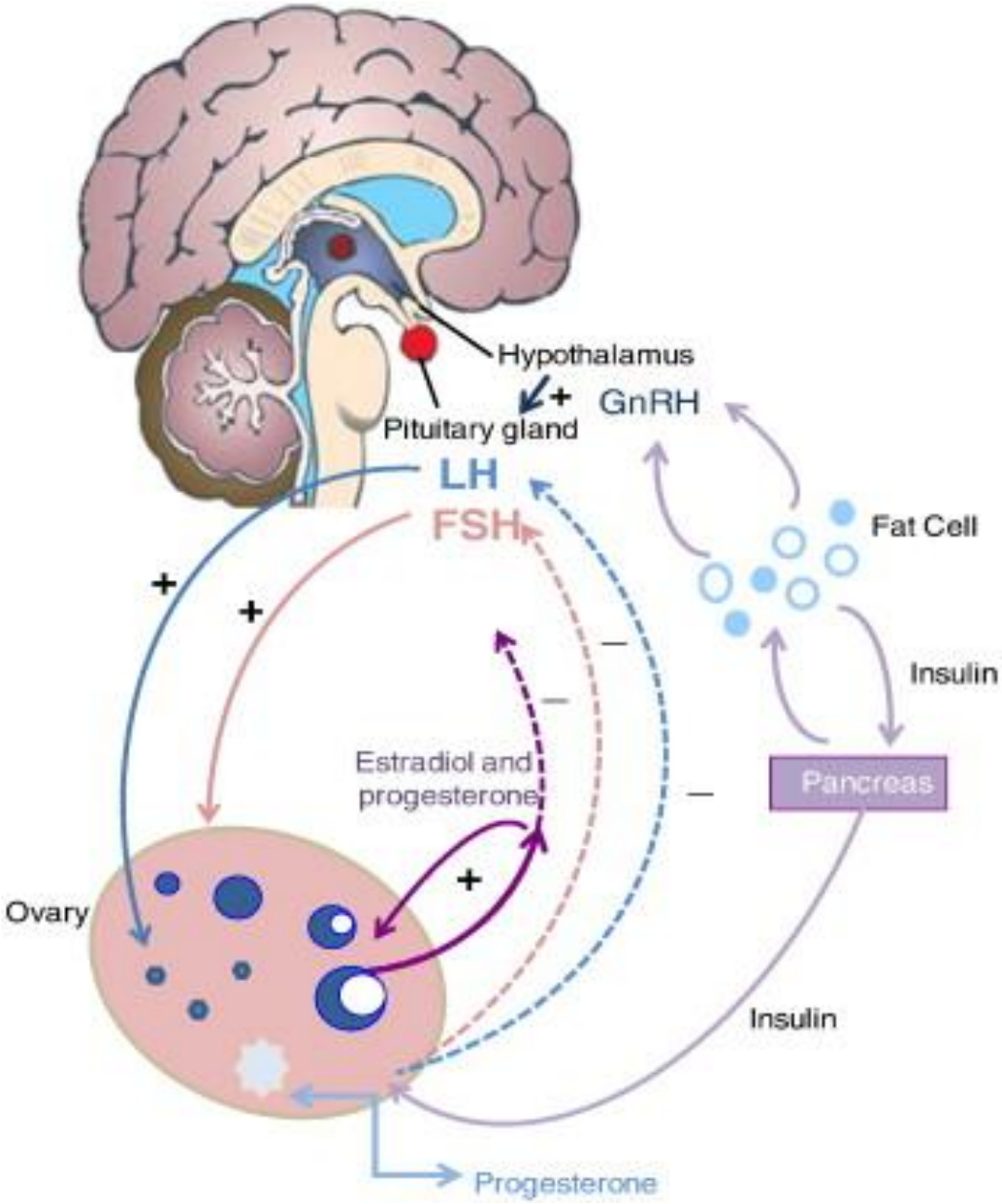

Figure 2: *Physiological ovarian function.*

(Monica L. Anderson et al Journal of Brain Research, October 2011)

5 OVARIAN CANCER

5.1 Types of ovarian cancer

The task forces of FIGO endorse the histologic typing of ovarian tumors as presented in the WHO publication no. 9, 1973, and recommend that all ovarian epithelial tumors be subdivided according to a simplified version of this. The types of tumors classified are as follows: serous, mucinous, endometrioid, clear cell (mesonephroid), un-differentiated and unclassified.

- Serous tumors
  - Benign serous cystadenomas
  - Of borderline malignancy: serous cystadenomas with proliferating activity of the epithelial cells and nuclear abnormalities, but with no infiltrative destructive growth (carcinomas of low potential malignancy)
  - Serous cystadenocarcinomas
- Mucinous tumors
  - Benign mucinous cystadenomas
  - Of borderline malignancy: mucinous cystadenomas with proliferating activity of the epithelial cells and nuclear abnormalities, but with no infiltrative destructive growth (carcinomas of low potential malignancy)
  - Mucinous cystadenocarcinomas

- Endometrioid tumors
  - Benign endometrioid cystadenomas
  - Endometrioid tumors with proliferating activity of the epithelial cells and nuclear abnormalities, but with no infiltrative destructive growth (carcinomas of low potential malignancy)
  - Endometrioid adenocarcinomas
- Clear cell tumors
  - Benign clear cell tumors
  - Clear cell tumors with proliferating activity of the epithelial cells and nuclear abnormalities, but with no infiltrative destructive growth (low potential malignancy)
  - Clear cell cystadenocarcinomas
- Brenner
  - Benign Brenner
  - Borderline malignancy
  - Malignant
  - Transitional cell
- Undifferentiated carcinomas: a malignant tumor of epithelial structure that is too poorly differentiated to be placed in any other group.

- Mixed epithelial tumors: these tumors are composed of two or more of the five major cell types of common epithelial tumors (types should be specified).

- Cases with intraperitoneal carcinoma in which the ovaries appeared to be incidentally involved and not the primary origin should be labeled as extra-ovarian peritoneal carcinoma.

5.2 Staging of ovarian cancer

The most common staging system is the FIGO system, as modified in 1988, and is based on findings made mainly through surgical exploration. Table 1 provides the current (2006) FIGO staging classification for cancer of the ovary. Also it is useful to be aware of the equivalence within the Union for International Cancer Control (UICC) TNM classification (Table 2). Figure 4 provides a graphical representation of ovarian cancer according to FIGO and TNM staging. (3)

FIGO Stage	Description
I	Growth limited to the ovaries
IA	Growth limited to one ovary; no ascites present containing malignant cells. No tumor on the external surface; capsule intact
IB	Growth limited to both ovaries; no ascites present containing malignant cells. No tumor on the external surfaces; capsules intact
IC[a]	Tumor either Stage IA or IB, but with tumor on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings
II	Growth involving one or both ovaries with pelvic extension
IIA	Extension and/or metastases to the uterus and/or tubes
IIB	Extension to other pelvic tissues
IIC[a]	Tumor either Stage IIA or IIB, but with tumor on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings
III	Tumor involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive regional lymph nodes. Superficial liver metastases equals Stage III. Tumor is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum
IIIA	Tumor grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologic proven extension to small bowel or mesentery
IIIB	Tumor of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative
IIIC	Peritoneal metastasis beyond the pelvis >2 cm in diameter and/or positive regional lymph nodes
IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV

[a] In order to evaluate the impact on prognosis of the different criteria for allotting cases to Stage IC or IIC, it would be of value to know if rupture of the capsule was spontaneous, or caused by the surgeon; and if the source of malignant cells detected was peritoneal washings, or ascites.

Table 1: *Ovarian cancer staging (FIGO 2006).*

FIGO Stage	Union for International Cancer Control (UICC)		
	T (tumor)	N (lymph nodes)	M (metastasis)
IA	T1a	N0	M0
IB	T1b	N0	M0
IC	T1c	N0	M0
IIA	T2a	N0	M0
IIB	T2b	N0	M0
IIC	T2c	N0	M0
IIIA	T3a	N0	M0
IIIB	T3b	N0	M0
IIIC	T3c	N0	M0
	Any T	N1	M0
IV	Any T	Any N	M1

Table 2: *Ovarian cancer, FIGO staging (2006) compared with TNM classification.*

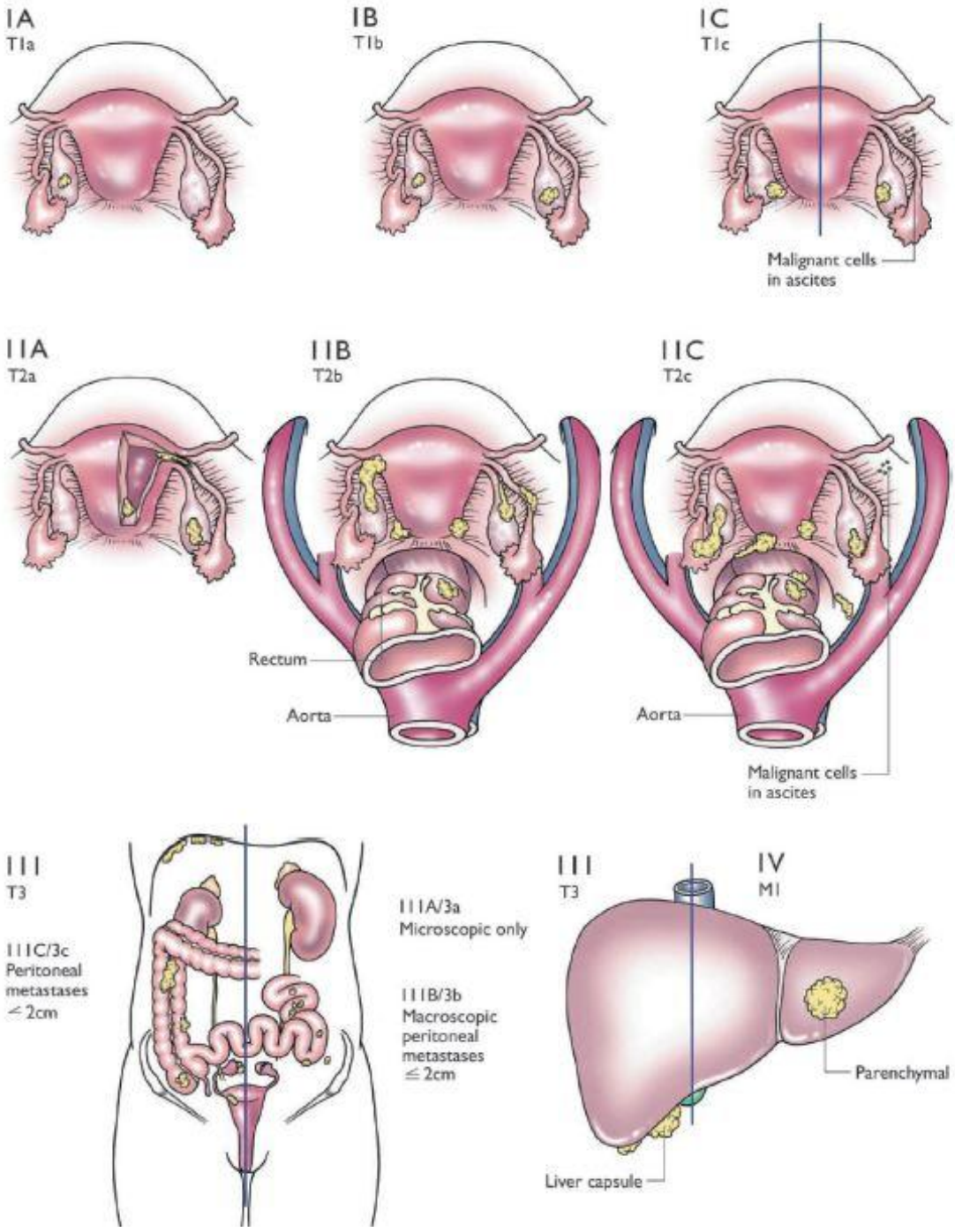

Figure 4: *Graphical representation of ovarian cancer staging depicting the primary tumor and metastases (FIGO and TNM).*

(APM HEINTZ, F ODICINO et al Journal of Gynecoology and Obstetrics, November 2006)

5.3 Causes and pathogenesis of ovarian cancer

Three inter-related theories have been proposed to explain the pathogenesis of epithelial ovarian cancer (figure 5). (4) Recently a fourth theory suggesting a possible origin of ovarian cancer to be from the fallopian tubes has been published.

First, the incessant ovulation hypothesis postulates that repetitive wounding of the ovarian surface epithelium and cell proliferation in postovulatory repair result in a stepwise accumulation of genomic abnormalities. Ovarian epithelial inclusion cysts occur as a result and might increase risk of carcinogenesis by trapping cells in an environment of aberrant autocrine or paracrine stimulation by growth factors including hormones, phospholipids, and vascular endothelial growth factor (VEGF), which activate intracellular processes such as kinase signalling. (4)

Second, the gonadotropin theory postulates that surges of pituitary gonadotropins at ovulation and persistent high concentrations after menopause stimulate surface epithelium cells, resulting in accumulation of genetic changes and carcinogenesis. (4)

The third theory suggests a role for inflammation and changes in redox potential in the setting of ovulation and surface-epithelium repair and might account for the increased risk of epithelial ovarian cancer that is associated with talc or asbestos exposure, endometriosis, pelvic inflammatory disease, and mumps. (5) Whatever the stimulus, repair of genomic damage is diminished in women with defective *BRCA1* and *BRCA2* function, leading to an increased risk of disease.

Since the inflammation-like setting in which ovulation occurs is dependent on

cyclooxygenase-2 (COX2), this theory lends support to exploration of the chemopreventive potential of COX2 inhibitors. (6)

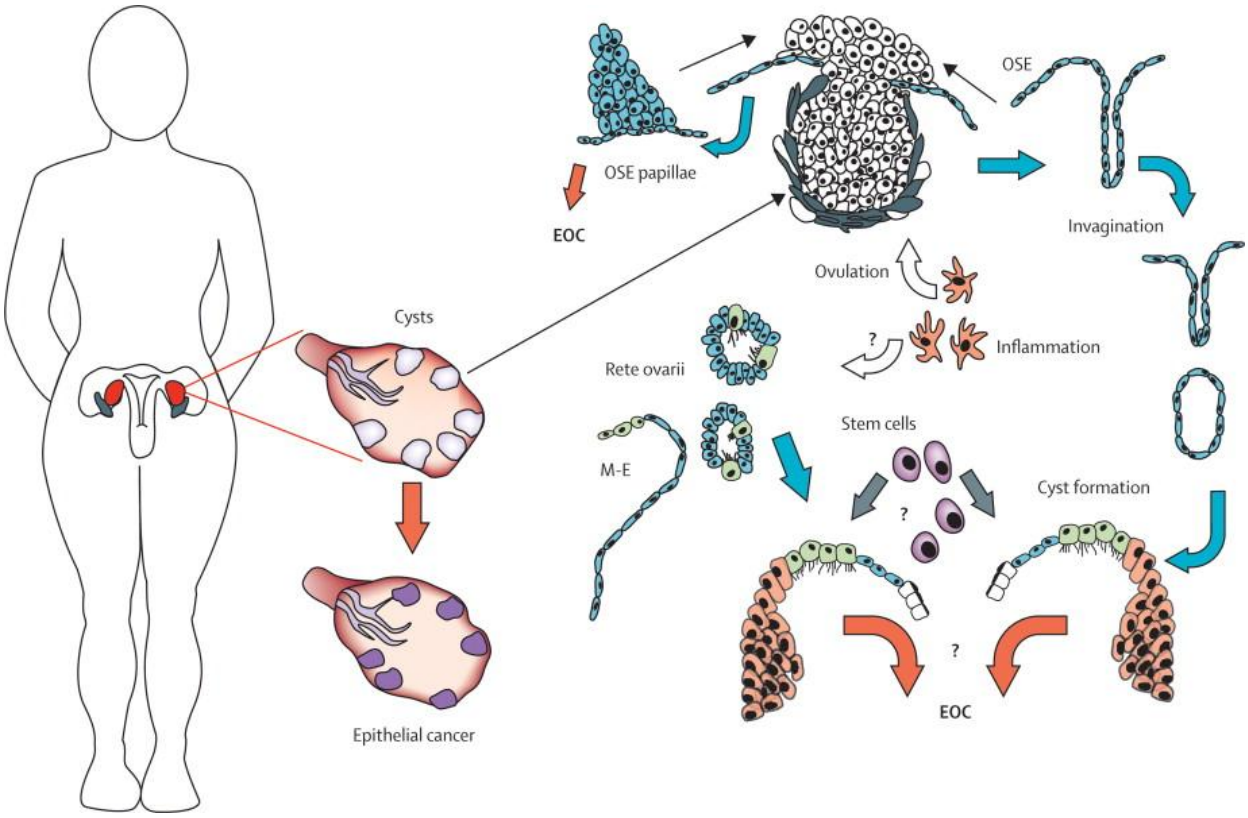

Figure 5: *Epithelial ovarian carcinogenesis.*

(Fleming JS et al Journal of Molecular Cell Endocrinology, March 2006)

During postovulatory re-epithelialisation, ovarian surface epithelial cells (OSE) (blue) divide and migrate to cover the ovulatory lesion (black arrows). Hyperplasia and transformation of the OSE to adenocarcinoma can occur as a result of many factors including surges of pituitary gonadotropins (red arrow, top left). The preovulatory surge of luteinising hormone induces increased expression of cytokines and invasion of

macrophages and monocytes (orange cells), leading to differentiation of follicle cells into luteal cells. Ovulation also stimulates formation of invaginations and inclusion cysts. Cyst cells can differentiate to take on Müllerian characteristics and proliferate under the effect of continuous hormonal and cytokine stimulation and become ciliated (yellow cells) or secretory (tan cells). Eventually, these cells accumulate genetic aberrations. Rete ovarii tubules at the hilus of the ovary, close to the mesothelium to OSE (M-E) transition, also contain ciliated and secretory cells and can dilate to form cysts. Whether cells in both cyst types can become cancerous is unknown. The role of any proposed ovarian stem or progenitor cells (purple cells) in epithelial ovarian carcinogenesis remains to be elucidated. EOC=epithelial ovarian cancer.

The fourth and newest theory proposed by Daniel J. O'Shannessy *et al* was published in the International Journal of Molecular Sciences in July of 2013. The authors state that they demonstrated that genes that encode biomarkers previously considered to be overexpressed in EOC (e.g.,CA-215 and HE4) were indeed significantly overexpressed in EOC when compared to normal ovarian tissue. Importantly however, none of these markers of EOC, with the possible exception of CA-125, showed significant upregulation when compared to fallopian tube tissue, either normal tissue or fallopian adenocarcinoma. Cluster analysis produced two very distinct clusters, one of which contained normal ovarian tissue and a second cluster that contained normal and cancerous fallopian tissue as well as EOC. Taken together, these data support the hypothesis that EOC derives from fallopian fimbriae and, further, that markers previously considered to be upregulated or overexpressed in EOC are most likely not of ovarian origin, but fallopian in derivation.

5.4 Epithelial Ovarian cancer

Epithelial ovarian cancer (EOC) is a highly malignant gynecological neoplasia with an incidence of 12/100 000 women (7), and this rate has only slightly decreased in the last 80 years. While women of any age are at risk for this malignancy, postmenopausal women have a higher incidence. For example, 90 % of women who suffer from EOC are older than 40 years of age, and the greatest number are 55 years or older. Moreover, due to the anatomic position of the ovaries, pelvic malignancies can remain obscured. In addition, a lack of symptoms until the advanced stages of tumor development results in an increased rate of metastasis at the time of diagnosis. Correspondingly, 80 % of the ovarian neoplasias diagnosed are stage III-IV according to International Federation of Obstetrics and Gynecology (FIGO) criteria. While FIGO stage I EOC has a relatively high five-year survival rate (> 90 %), survival rates markedly drop for patients with stage III-IV EOC (25-30 %) (8,9). Therefore, it is crucial to diagnose EOC as early as possible. Accordingly, the identification of serum biomarkers to detect EOC would represent an important and valuable advance for the monitoring and treatment of EOC progression.

5.5 Biomarkers in ovarian cancer

Algorithms and triage protocols designed to evaluate potential cases of ovarian cancer in their early stages are currently limited, and rely on pelvic ultrasonography and CA-125 determination (10). Moreover, the sensitivity and specificity of these approaches range from 70-80 %. (11). Regarding CA-125, its levels are elevated in less than 50 % of EOC cases, and it is undetectable in another 20 % of EOC cases. In addition, high serum levels of CA-125 are also associated with benign gynecological diseases (e.g., cysts, endometriosis, etc.) (12). CA-125 also known as mucin 16 or MUC16 is a protein that in humans is encoded by the *MUC16* gene. MUC16 is a member of the mucin family of glycoproteins. CA-125 has found application as a tumor marker or biomarker that may be elevated in the blood of some patients with specific types of cancers or other benign conditions. MUC16 is a component of the ocular surface (including the cornea and conjunctiva), the respiratory tract and the female reproductive tract epithelia. Since MUC16 is highly glycosylated it creates a hydrophilic environment that acts as a lubricating barrier against foreign particles and infectious agents on the apical membrane of epithelial cells. CA-125 is the most frequently used biomarker for ovarian cancer detection. Normal values range from 0 to 35 (U/mL).

In 2008, Moore and colleagues identified human epididymis protein 4 (HE4) as a biomarker for ovarian cancer (13). HE4 is a protein that in humans is encoded by the WAP- four disulfide core domain 2 (WFDC2*)* gene. This gene encodes a protein that is a member of the WFDC domain family. The WFDC domain contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease

inhibitor in many family members. This gene is expressed in pulmonary epithelial cells, and was also found to be expressed in some ovarian cancers.

Based on these findings, a risk of ovarian malignancy algorithm (ROMA) was developed, and is currently used to predict the presence of malignant ovarian cancer using a combination of CA-125 levels, HE4 expression, and menopausal status. In particular, the combination of HE4 and CA-125 in the ROMA has been associated with a higher sensitivity than any single biomarker (14).

The Risk of Ovarian Malignancy Algorithm (ROMA) Predictive Index (PI) to classify patients as being at a low or high risk for malignant epithelial ovarian cancer disease was calculated using the following algorithms proposed by Moore (14):

Pre-menopausal PI = −12.0 + 2:38 x LN [HE4] + 0.0626 x LN [CA125]

Post-menopausal PI = −8.09 + 1:04 x LN [HE4] + 0.732 x LN [CA125]

Predicted probability ROMA (%): exp (PI)/[1 + exp (PI)] x100.

Menopausal status was defined as absence of menstruation for more than six months and presence of menopausal symptoms.

5.6 Current treatment of ovarian cancer

The standard initial management of epithelial ovarian cancer consists of surgical staging, operative tumor debulking including total abdominal hysterectomy (TAH) and bilateral salpingo-oophorectomy (BSO), and administration of six cycles of intravenous chemotherapy with carboplatin and paclitaxel. Extensive and largely retrospective experience has shown that optimum surgical debulking to leave residual tumor deposits that are less than 1 cm in size is associated with improved patient outcomes. (15)

However, 75% of patients present with advanced (FIGO stage III or IV) disease and, although more than 80% of these women benefit from first-line therapy, tumor recurrence occurs in almost all these patients at a median of 15 months from diagnosis. Second-line treatments can improve survival and quality of life but are not curative. (15)

If epithelial ovarian cancer is suspected on the basis of physical examination and imaging, an exploratory laparotomy is usually done for histological confirmation, staging, and tumor debulking. The standard comprehensive surgical staging approach consists of a total abdominal hysterectomy and BSO along with examination of all peritoneal surfaces, an infracolic omentectomy, biopsies of pelvic and para-aortic lymph nodes and clinically uninvolved areas, and peritoneal washings. A unilateral salpingo-oophorectomy could be considered as an alternative in women with stage IA or IC ovarian cancer who desire preservation of fertility. (15)

Since grade and ovarian capsule rupture are the most powerful prognostic indicators in stage I disease, these factors are used in decisions about therapy in clinical practice and in staging sub-classification of stage II disease. (15)

Patients with early stage (stages I or II) disease are thus divided into two categories relevant to a role for adjuvant chemotherapy. For patients with well differentiated (grade 1) stage IA or B disease, adjuvant chemotherapy is not recommended since more than 90% of these women will have long-term (>10 year) disease-free survival without further treatment. Patients with stage IA or B (high grade), stage IC, and stage II cancers will have improved survival if given cisplatin or carboplatin-based therapy after surgical staging. (15)

Controversy exists in the gynecological cancer community regarding the need to treat patients with stage I moderately differentiated (grade 2) cancers with platinum-based chemotherapy. Furthermore, since 50–60% of patients with high-risk early-stage disease will not have recurrence if monitored, there is a crucial need to identify molecular markers that will allow accurate identification of patients who will benefit from chemotherapy. (15)

Results of clinical trials done over the past 30 years have defined the standard of care in the initial chemotherapeutic management of advanced disease as the combination of a platinum (carboplatin or cisplatin) and a taxane (paclitaxel or docetaxel) given intravenously; the GCIG Consensus Meeting in 2005, defined the standard of care as carboplatin (AUC5-7.5) plus paclitaxel (175 mg/m^2) every 21 days for six cycles. (15)

6 AIMS OF THE STUDY

14-3-3 zeta is an important regulatory protein, which mediates intracellular signaling pathways by interfering with approximately 100 cellular proteins, including oncogenes and protooncogenes. Recently, two independent research groups, Waldemarson et al. (16) and He et al. (17), advocated 14-3-3 zeta as a potential biomarker for EOC. In addition, Kobayashi et al. (18) recently demonstrated that 14-3-3 zeta protein is present in malignant ascites of patients with EOC, and is secreted by ascetic monocytes and macrophages. However, while the role of 14-3-3 zeta protein as an intracellular adaptor protein has been widely investigated, the function of the secreted protein is unclear.

1. The goal of the current pilot study was to assess the potential for 14-3-3 zeta protein to serve as a novel biomarker for monitoring patients with FIGO stage II-III EOC that undergo chemotherapy.

7.1 Materials and methods

7.1.1 Patients and follow-up study design

This prospective study was approved by the University of Pecs Institutional Ethical Review Board (#4076.316-251/KK15/2011), and written informed consent was obtained from all enrolled patients.

Peripheral blood samples were collected preoperatively from 13 patients admitted for six cycles of first line chemotherapy (paclitaxel/carboplatin; Hungarian OEP Chemotherapy protocol # 7167).

Chemotherapy dosage was calculated using (AUC5-7.5) for carboplatin and (175 mg/m^2) for paclitaxel, and treatments were administered in 21 d intervals at the University of Pecs Medical Center Department of Obstetrics and Gynecology/Oncology (Pecs, Hungary) in 2012.

Blood samples were collected 1-2 h before each treatment into citrate tubes. These tubes were then centrifuged (5000 rpm for 10 min), and blood plasma samples were collected and stored at −80 °C. When needed, samples were thawed at room temperature, and then were thoroughly vortexed as indicated by the manufacturer's recommendation.

In all patients, aged 51 to 73 y (mean, 60 y), only sub-optimal gynecological surgery could be performed. Computed tomography (CT) scans were performed prior to and following the completion of chemotherapy treatment. These images were used to

assess changes in both target and non-target lesions. Each diagnosis was verified according to histopathology studies of the original tumors. Histopathological grade and stage of disease (according to FIGO criteria) were available for all malignant cases, and included FIGO stage IIa (n = 2), stage IIIb (n = 2), and stage IIIc (n = 9) cases (Table 3).

Patient no.	Histology	FIGO stage	Age, y	Tumor grade*
1.	Serous	III C	69	High
2.	Serous	III C	57	High
3.	Serous	III B	51	High
4.	Serous	II A	50	High
5.	Serous	III C	67	High
6.	Serous	III C	64	High
7.	Serous	III B	73	High
8.	Adenosquamous	II B	51	High
9.	Adenosquamous	III C	69	High
10.	Serous	III C	55	High
11.	Serous	III C	71	High
12.	Serous	III C	60	High
13.	Serous	III C	53	High

*According to International Federation of Obstetrics and Gynecology (FIGO) criteria.

Table 3: *Clinicopathological features of the patients enrolled in this study that underwent six cycles of paclitaxel/carboplatin-based chemotherapy for treatment of EOC.*

7.1.2 Computed tomography (CT) scans

CT scans were performed in the Department of Radiology (University of Pecs, Hungary). Contiguous 5 mm axial slices obtained through the abdomen and pelvis. Prior to examination high-concentration iodinated contrast agent was administered intravenously (Iomeron 400, Bracco Diagnostic Imaging). Field-of-view was adjusted to body habitus (to include the whole body including the skin). Target and non-target lesions were defined based on Responsive Evaluation Criteria In Solid Tumors (RECIST) 1.1 guidelines (www.recist.com). These guidelines are a set of rules published in January 2009 by an international collaboration including the European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States of America and the National Cancer Institute of Canada Clinical Trials Group that define when cancer patients improve ("respond"), stay the same ("stable") or worsen ("progression") during treatments. Today, the majority of clinical trials evaluating cancer treatment for objective response in solid tumors are using RECIST 1.1. A lesion was measurable and defined as a target lesion if the tumor was ≥ 10 mm along its longest diameter (LD) on a CT axial image with ≤ 5 mm reconstruction intervals, or if lymph nodes were ≥ 15 mm along their short axis on CT images. Non-target lesions were considered to be: masses with a diameter < 10 mm, lymph nodes with a diameter of 10-14 mm along the short axis. Ascites, pleural or pericardial effusion, abdominal masses, or organomegaly were identified by physical exam. Furthermore, these could not be measured by reproducible imaging techniques. CT scans were performed 1-2 weeks after radical surgeries were performed, 1-2 weeks prior to chemotherapy, and 1-2 weeks after the final chemotherapy treatment.

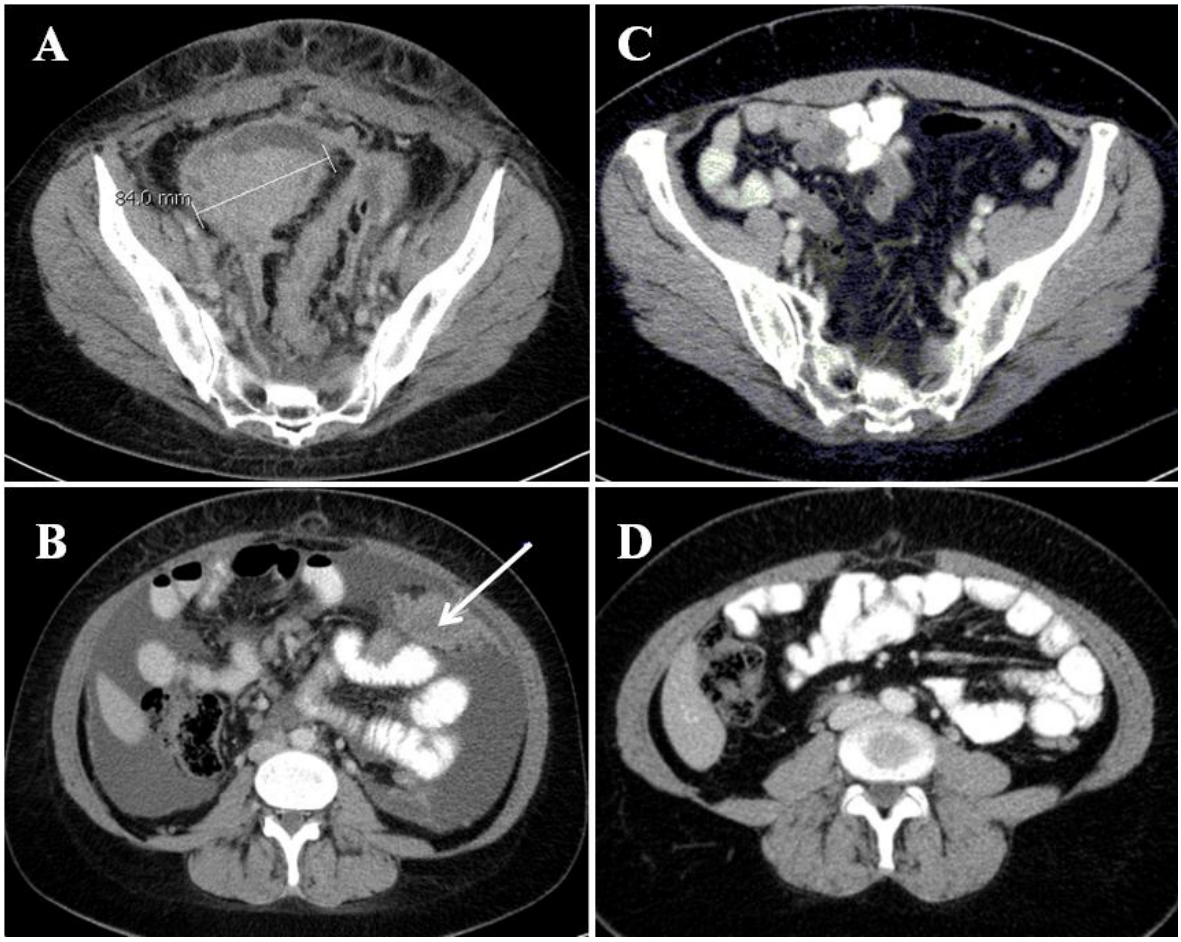

Figure 6: *Axial CT slices of patient # 3 after contrast material was intravenously administered. A target lesion with a SLD of 84 mm is localized near the minor pelvis before* ***(A)*** *and after* ***(C)*** *six cycles of first-line chemotherapy.* ***(B)*** *The arrow represents a mesenteric peritoneal carcinosis (non-target lesion) in the same patient that is level with the lower edge of the liver prior to chemotherapy. A significant amount of ascites associated with the non-target lesion is also observed* ***(B)****.* ***(D)*** *Both non-target lesions are absent after the completion of chemotherapy.*

7.1.3 Laboratory methods

7.1.4 Enzyme-linked immunosorbent assay (ELISA)

Levels of serum CA-125 (Fujirebio Diagnostics, Malvern, PA; Catalog #: 400-10, Lot. # 29191), HE4 (Fujirebio Diagnostics, Malvern, PA; Catalog #: 404-10, Lot# 28373), and 14-3-3 zeta protein (Cusabio Biotech, Wuhan, China; Catalog #: CSB-EL026293HU, Lot. #A26174460) were determined using a quantitative sandwich enzyme immunoassay according to each manufacturer's protocol. Serum concentrations were calculated using Optima 2.10 R2 built-in data calculator software.

7.1.5 Quantitative electrochemiluminescence assay (ECLIA)

Tumor marker levels were measured using a Roche electrochemiluminescent fully automated immunoassay system (ECLIA, Roche Diagnostics, http://www.roche-diagnostics.com). To determine serum levels of CA-125 (Cat. no. 11776223), and HE4 (Cat. no. 05950929), samples were processed using a Roche Cobas e411 analyzer. Master calibration, imprecision, and inaccuracy were checked using bi-level quality controls prior to the analysis of patient serum samples.

7.1.6 Risk for Ovarian Malignancy Algorithm (ROMA) index

The ROMA used serum levels of HE4 and CA-125 measured either by ELISA and ECLIA, and was calculated using an Excel spreadsheet with preset formulas to generate the predictive index (PI) value for EOC (19) as follows:

For postmenopausal women: PI = -8.09 + 1.04*LN [HE4] + 0.732*LN [CA125]

A ROMA value was then calculated as follows: ROMA value (%) = exp(PI) / [1 + exp(PI)]*100. According to the manufacturer's manual, the detection of HE4 by ECLIA and CA-125 by ELISA in post-menopausal women identified an EOC high-risk index value equal to, or higher than, 25.3 % (20).

7.1.7 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistic 20 (IBM Corporation) at the University of Pecs, Institute of Bioanalysis. The sample size (n) was 13, and comparisons were made between treatments and between methods according to the Wilcoxon signed-rank test. To evaluate trends between the number of treatments and serum levels of tumor markers, linear regression and correlation analyses were applied. To examine the relationship between tumor marker levels and CT scan results, Spearman's rank correlation coefficient was used. Mean data are reported ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$, or $p < 0.01$.

7.2 Results

7.2.1 Radiologic assessment following therapeutic procedures

CT scans were obtained one to two weeks after radical gynecological surgeries were performed. After an initial laparotomy, 10 out of 13 (76.92 %) patients were found to have residual tumor present prior to induction of paclitaxel/carboplatin-based chemotherapy. After six consecutive cycles of treatment within 21 d intervals, CT scans were repeated. At this point, residual tumor with a LD value >1 cm was only detected in 4 out of 13 (30.76 %) patients (Table 4). Based on the detection of 26 non-target lesions in pre-chemotherapy CT scans, and only 3 non-target lesions in post-chemotherapy scans, the efficacy of chemotherapy for EOC treatment is demonstrated (Figure 6).

Patient no.	SLD of target lesions 10 (+/- 4) d before chemotherapy (mm)	SLD of target lesions 10 (+/- 4) d after the last cycle of chemotherapy (mm)	Non-target lesion changes*
1	22	0	-1
2	56	0	-1
3	84	0	-1
4	0	0	0
5	137	0	-4
6	0	0	0
7	0	0	0
8	441	202	-1
9	256	18	-3
10	147	0	-3
11	125	0	-1
12	46	18	-1
13	39	14	-3

SLD: sum of length diameter; according to RECIST 1.1 guidelines.

*Negative values represent a decrease in lesion size.

Table 4: *Evaluation of tumor size before and after paclitaxel/carboplatin chemotherapy using CT scans.*

7.2.2 Detection of CA-125, HE4 and 14-3-3 Zeta protein

Levels of CA-125, HE4, and 14-3-3 zeta protein were monitored throughout the treatment period by ELISA and ECLIA.

On the first day of chemotherapy, the mean concentration of CA-125 was 147.87 ± 55.98 U/ml and 648.26 ± 186.52 U/ml, respectively. After completing the sixth cycle of chemotherapy, CA-125 levels were lower, with the mean concentrations detected being 58.54 ± 30.89 U/ml and 119.70 ± 22.75 U/ml, respectively. Similarly, mean serum levels of HE4 detected on the first day of chemotherapy by ELISA were 455.32 ± 106.39 pM, and decreased to 120.52 ± 23.76 pM upon completion of chemotherapy.

Using the ECLIA method, serum levels of HE4 were 1383.49 ± 577.23 pM on the first day of chemotherapy, and decreased to 70.12 ± 26.44 pM upon completion of chemotherapy. ROMA index values were subsequently calculated, and decreased from 58.17 ± 10.05 % to 28.95 ± 7.67 %, and from 69.62 ± 9.91 % to 30.78 ± 7.91 % for ELISA and ECLIA, respectively.

According to Wilcoxon statistical analyses, the differences in the values determined at the start of treatment versus upon completion of treatment were significant ($p < 0.05$), thus further demonstrating the effectiveness of chemotherapy for EOC (Figure 6/A-F, Table 5).

For 14-3-3 zeta protein levels detected in patients prior to chemotherapy by ELISA, the mean concentration was 1.93 ± 0.57 ng/ml. In contrast, the mean concentration of 14-3-3 zeta protein for healthy postmenopausal women (mean age, 58 y), was 0.39 ±

0.11 ng/ml. Subsequently, at the start of chemotherapy, the mean serum level of 14-3-3 zeta protein in EOC patients was 2.38 ± 1.44 pg/ml, and 2.17 ± 1.71 pg/ml after the final treatment. Neither the difference in levels detected for EOC patients and healthy postmenopausal patients, either at the beginning or end of chemotherapy, was found to be significant (Figure 6/G).

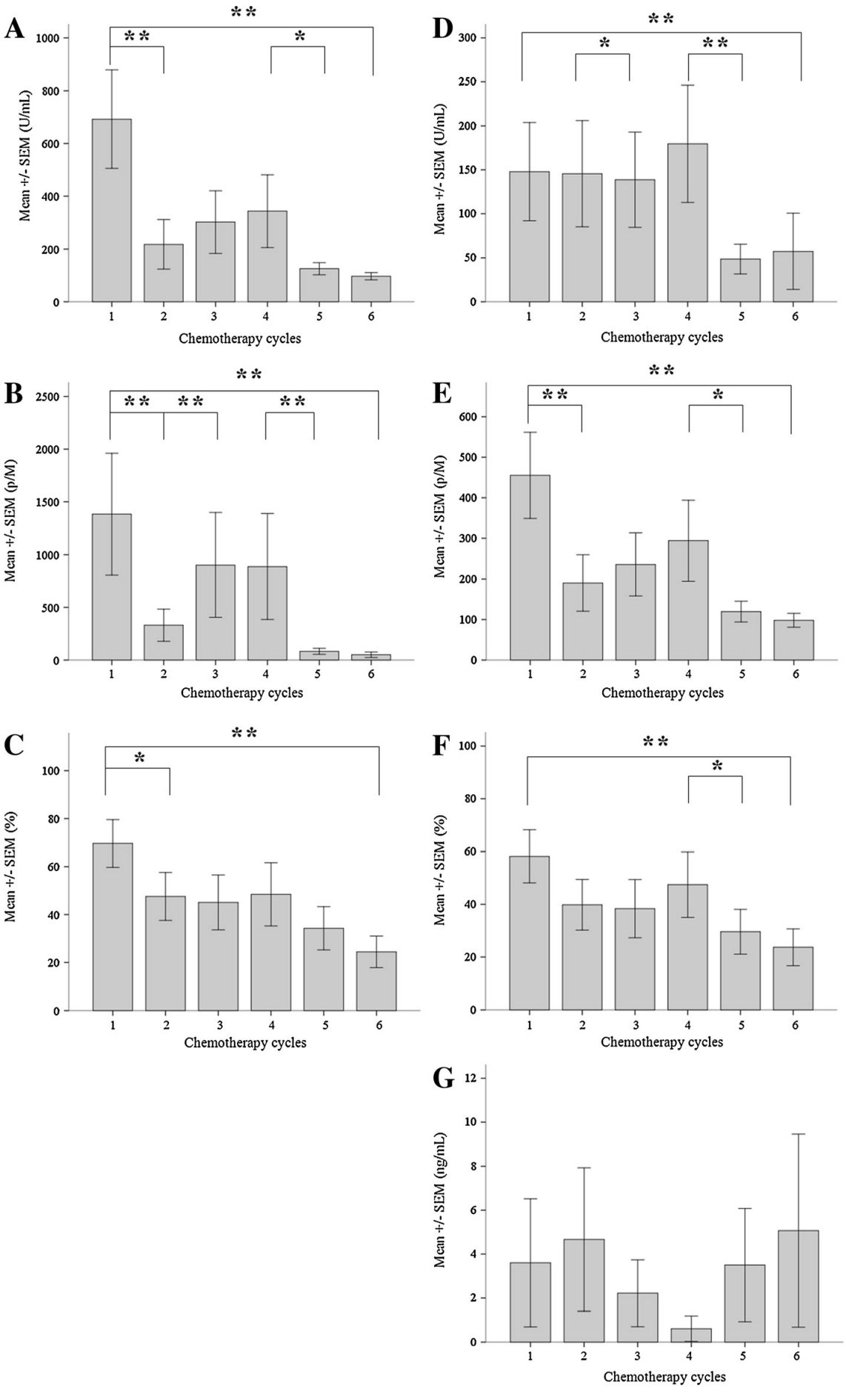
A
Mean +/- SEM (U/mL)
Chemotherapy cycles
B
Mean +/- SEM (p/M)
Chemotherapy cycles
C
Mean +/- SEM (%)
Chemotherapy cycles
D
Mean +/- SEM (U/mL)
Chemotherapy cycles
E
Mean +/- SEM (p/M)
Chemotherapy cycles
F
Mean +/- SEM (%)
Chemotherapy cycles
G
Mean +/- SEM (ng/mL)
Chemotherapy cycles

Figure 6: *Mean levels of serum biomarkers (CA-125, HE4 and 14-3-3 zeta protein) measured with ELISA and ECLIA methods, and the ROMA index values. Average values of CA-125 (U/mL) +/− SEM determined by ECLIA* ***(A)*** *and by ELISA* ***(D)*** *are shown according to the chemotherapy cycles (consistently on each x-axis from 1 to 6). Mean concentrations of HE4 (p/M), measured by ECLIA* ***(B)*** *and by ELISA* ***(E)*** *are also shown. Furthermore average values determined by ELISA* ***(G)*** *of 14-3-3 zeta protein (ng/ml) are depicted. Based on the above-mentioned data, we calculated the postmenopausal ROMA index values (%) for ECLIA* ***(C)*** *and for ELISA* ***(F)*** *techniques. We present the mean levels of 14-3-3 zeta protein (ng/mL) for each of the treatment days. Wilcoxon signed-rank test analysis was performed for each diagram, and statistical significance represented with an asterisk (*) was set at $p < 0.05$, and (**) when $p < 0.01$.*

Patient no.	Chemo Cyle no.	ELISA			ECLIA			ELISA
		CA-125 (U/mL)	HE4 (p/M)	ROMA %	CA-125 (U/mL)	HE4 (p/M)	ROMA %	14-3-3 Z (ng/mL)
1	1	23.38	95.87	26.19	105.70	79.63	46.90	38.28
	2	18.09	65.94	16.60	76.82	30.69	20.57	37.95
	3	7.78	64.47	9.49	73.55	15.77	11.20	18.78
	4	NA	NA	NA	NA	NA	NA	NA
	5	10.71	67.85	12.26	77.76	8.58	6.5	32.01
	6	7.90	62.81	9.35	75.21	9	6.60	39.82
2	1	26.93	334.49	58.99	353.50	176.10	83	0
	2	24.28	102.01	28	116.10	106.20	56	0
	3	19.31	66.42	17.37	79.16	29.24	20.10	0
	4	10.16	87.74	14.97	88.92	18.22	14.30	0
	5	3.33	81.38	6.7	88.28	15.06	12	0
	6	0.08	85.94	0.49	87.33	13.11	10.50	0
3	1	1.45	43.73	2	83.1	52.97	32.6	0
	2	0	49.24	0	67.49	28.04	17.70	0
	3	3.47	52.41	4.48	69.24	23.49	15.4	0
	4	NA	NA	NA	NA	NA	NA	NA
	5	0	44.09	0	52.38	17.25	9.7	0
	6	9.12	52.37	8.68	62.76	24.63	15.10	0
4	1	0	48.51	0	55.06	6.78	4	1.34
	2	0	55.9	0	58.43	6.91	4.30	1.42
	3	3.63	47.48	4.18	54.72	6.71	4	0.39
	4	2.55	45.86	3.15	53.12	6.08	3.50	0.32
	5	4.35	51.67	5.16	58.04	6.59	4.10	0.26
	6	NA	NA	NA	NA	NA	NA	NA
5	1	9.23	101.40	15.93	133.4	14.60	15.20	0
	2	12.40	93.07	17.77	107.40	8.69	8.20	0

	3	2.78	85.49	6.21	103.30	7.08	6.50	0
	4	14.24	69.76	15.03	75.26	6.66	4.90	0
	5	9.10	75.68	12.20	80.91	7.95	6.20	0
	6	9.73	74.47	12.55	80.21	7.89	6.10	0
6	1	153.04	904	93.53	1500	701.5	98.30	0
	2	445.65	904	96.94	1218	644.20	97.90	0
	3	368.55	714.78	95.57	843.10	394.10	95.50	0
	4	249.51	541.89	92.41	540.10	243.90	90.30	0
	5	95.43	259.90	73.68	227.30	84.13	65.40	0
	6	39.94	167.57	48.43	142	46.16	38.30	0
7	1	77.86	118.41	51.49	111.90	68.66	44.10	4.36
	2	17.01	122.23	26.50	124.90	22.64	22.64	15.02
	3	9.25	120.03	18.50	127	18.20	17.80	8.28
	4	8	107.76	15.47	111.10	16.34	15	6.38
	5	1.07	53.14	1.98	132.50	15.39	15.80	13.13
	6	9.03	202.84	27.82	186.80	26.99	30.30	5.74
8	1	475.23	259.74	90.07	248.50	4810	99.2	0.04
	2	500	90.83	75.92	80.07	1265	92.7	0
	3	189.18	67.99	53.34	70.60	642.10	85.20	0
	4	500	904	97.18	1500	5000	99.8	0
	5	172.63	57.11	47.22	57.21	254.10	65.30	0
	6	402.03	62.74	64.64	57.66	256.20	65.60	0
9	1	21.16	904	77.35	1500	185.40	93.70	2.46
	2	29.53	170.69	43.41	176.10	61.61	49.50	1.61
	3	4.97	97.08	10.38	103.70	21.29	18.10	1.32
	4	8.13	96.13	14.09	102.70	15.37	13.50	0
	5	5.90	105.57	12.56	108.90	13.93	12.80	0.13
	6	8.57	84.25	12.96	87.24	15.70	12.40	0.06
10	1	500	904	97.18	1500	5000	99.8	0
	2	189.91	62.04	51.08	60.85	243.10	65.30	0

	3	500	441.44	94.22	514.20	4789	99.5	0
	4	500	149.23	84.07	155.70	833.50	93.10	0
	5	84	127.48	54.97	121.70	197.90	71.60	0
	6	29.43	90.79	28.37	92.78	54.27	35	0
11	1	7.85	904	62.12	1500	117.70	90.20	0
	2	9.58	201.95	28.59	200.90	36.28	38.40	0
	3	0.93	131.04	4.42	125.90	18.88	18.30	0
	4	3.75	105.53	9.34	104.40	13.74	12.30	0
	5	2.65	107.64	7.54	107.80	13.41	12.30	0
	6	NA	NA	NA	NA	NA	NA	NA
12	1	126.02	397.04	84.2	404.30	1772	98.30	0.38
	2	500	365.72	93.07	323.20	1517	97.70	0
	3	192.79	273.67	83.2	258.90	761.40	94.70	0.09
	4	178.79	223.37	79.09	192.20	360.10	86.80	0
	5	121.86	168.58	68.17	169	265.70	81.30	0
	6	NA	NA	NA	NA	NA	NA	NA
13	1	500	904	97.18	1500	5000	99.8	0
	2	NA	NA	NA	NA	NA	NA	NA
	3	500	904	97.18	1500	5000	99.8	0
	4	500	904	97.18	853.90	3241	99.50	0
	5	120.65	355.03	82.16	349.30	171.90	82.5	0
	6	NA	NA	NA	NA	NA	NA	NA

Table 5: *Levels of CA-125, HE4, and 14-3-3 Z protein determined by ELISA and ECLIA methods.*

7.2.3 Correlation between radiological findings and serum parameters

Neither ELISA nor ECLIA measurements of CA-125 and HE4 serum biomarkers provided significant linear regression correlations. However, the ROMA index values that were calculated based on these values did provide a strong significant regression correlation ($r = 0.840$, $p = 0.036$ and $r = 0.920$, $p = 0.009$, respectively) (Figure 7/C and F). Moreover, with a p-value margin of 0.01, a significant linear correlation was found for all ECLIA measurements.

Linear regression analysis of 14-3-3 zeta protein levels at each treatment day, showed no significant correlation between the mean serum values and the chemotherapy cycles ($r = 0.073$; $p = 0.089$). (Figure 7/G)

Spearman's correlation analysis further identified a significant correlation between CA-125 serum levels determined by ELISA and the largest tumor diameter measured by CT scans obtained following chemotherapy ($p = 0.011$). Levels of HE4 detected by ECLIA were also found to significantly correlate with tumor diameter ($p = 0.04$), while levels of 14-3-3 zeta protein did not significantly correlate with any of the examined parameters.

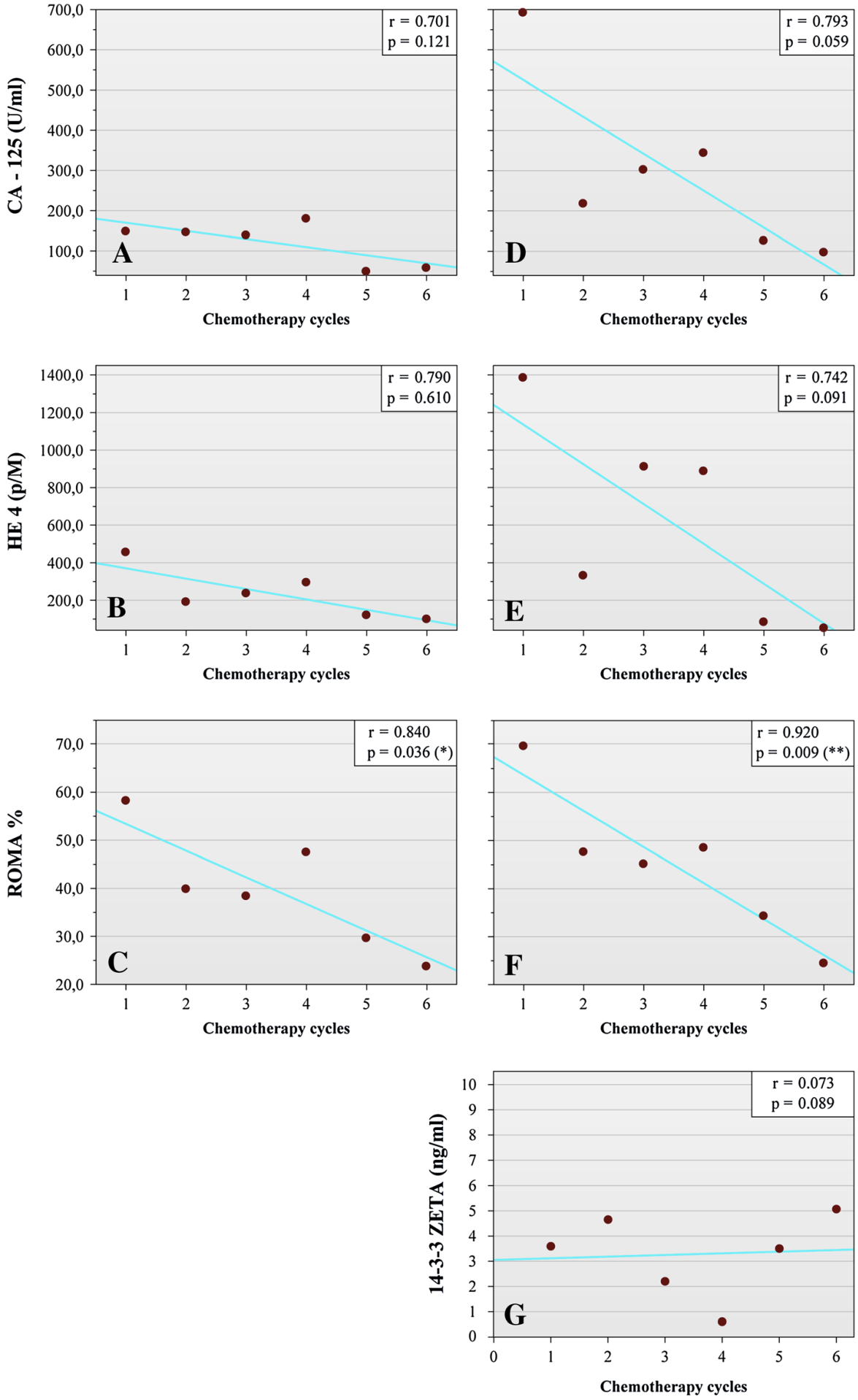
CA - 125 (U/ml)
700,0
600,0
500,0
400,0
300,0
200,0
100,0
r = 0.701
p = 0.121
A
1 2 3 4 5 6
Chemotherapy cycles
r = 0.793
p = 0.059
D
1 2 3 4 5 6
Chemotherapy cycles
HE 4 (p/M)
1400,0
1200,0
1000,0
800,0
600,0
400,0
200,0
r = 0.790
p = 0.610
B
1 2 3 4 5 6
Chemotherapy cycles
r = 0.742
p = 0.091
E
1 2 3 4 5 6
Chemotherapy cycles
ROMA %
70,0
60,0
50,0
40,0
30,0
20,0
r = 0.840
p = 0.036 (*)
C
1 2 3 4 5 6
Chemotherapy cycles
r = 0.920
p = 0.009 (**)
F
1 2 3 4 5 6
Chemotherapy cycles
14-3-3 ZETA (ng/ml)
10
9
8
7
6
5
4
3
2
1
0
r = 0.073
p = 0.089
G
0 1 2 3 4 5 6
Chemotherapy cycles

Figure 7: *Changes in CA-125, HE4, and 14-3-3 zeta protein serum levels, and ROMA index values during the six cycles of paclitaxel/carboplatin-based chemotherapy that were performed. Mean concentrations of CA-125 determined by ELISA* ***(A)*** *and by ECLIA* ***(D)*** *are shown for each of the treatment days. Mean levels of HE4 determined by ELISA* ***(B)*** *and ECLIA* ***(E)*** *are also shown for each of the treatment days. Mean concentrations of 14-3-3 zeta protein were determined by ELISA* ***(G)*** *are represented at each chemotherapy day. Postmenopausal ROMA index values were calculated based on ELISA* ***(C)*** *and ECLIA* ***(F)*** *data. Linear regression analysis was performed for each diagram (see r value), and statistical significance was set at* $p < 0.05$.

7.3 Discussion

Several studies have demonstrated the limitations associated with depending on any single tumor marker for the detection of EOC. Initially, CA-125 was widely used. However, other malignant and benign diseases also express CA-125, thereby limiting its reliability as a tumor marker. In particular, CA-125 has a high false-positive rate among women with benign gynecological conditions such as endometriosis (20), and a low sensitivity in identifying patients with early-stage ovarian cancer (21).

Accordingly, when EOC is diagnosed, 80 % of cases are in an advanced stage of disease (e.g., FIGO III-IV) (22). To improve the specificity and sensitivity of an ovarian cancer diagnosis, additional tumor markers have been investigated. One novel tumor marker is HE4, which contains two whey acid protein (WAP) domains and eight cysteine residues that constitute a four-disulphide bond core (23). HE4 localizes to human chromosome 20q12-13.1 and its expression significantly increases during malignant transformation. However, HE4 is expressed in normal tissues as well, and therefore, is not tumor specific. Correspondingly, it has been hypothesized that the function of HE4 is related to both spermiotelcosis (a protease inhibitor involved in sperm maturation) and natural immunity, although the details of HE4 function remain to be clarified (24).

As a tumor marker for the early detection of ovarian cancer, Moore et al. reported a sensitivity of 72.9 % and a specificity of 95 % for HE4 (13). Moreover, when both HE4 and CA-125 were detected, the sensitivity increased to 76.4 %. Therefore, the

detection of more than one biomarker resulted in a 33.1 % increase in the sensitivity of CA-125, and a 3.5 % increase in HE4 sensitivity (13).

In the present study, ROMA values provided a PI based on the postmenopausal status of a patient, and the presence and levels of biomarkers CA-125 and HE4. As such, this PI relies on an accurate determination of serum levels of HE4 and CA-125.

Moreover, in a recent study, the ROMA was found to be more effective in predicting ovarian cancer than the widely used risk of malignancy index (RMI), which employs ultrasound findings, CA-125 concentrations, and menopausal status (25).

Furthermore, when the specificity was set to 75 %, the RMI had a sensitivity of 84.6 %. For the same specificity, the sensitivity of the ROMA was significantly higher (94.3 %). Although biomarker concentrations can be assayed by various methods (e.g., ELISA, chemiluminescent microparticle immunoassay), a recent study conducted by Ruggeri et al. demonstrated that chemiluminescent immunoassays are more adequate and more reproducible than commercially available ELISA kits that are characterized by interassay imprecision percentages (CV %) ranging from 6.8-10.3 %, compared to < 4 % for ECLIA (26). The results of the present study are consistent with these findings, and they further support the use of the ECLIA method for routine determinations of CA-125 and HE4 levels. Furthermore, the deviation in accuracy for ELISA versus ECLIA can be attributed to the fully automated format of ECLIA, while ELISAs are manual assays that also require testing samples in duplicate.

14-3-3 zeta protein plays an important role in several different biological mechanisms. For example, it has been reported to be an adaptor protein for intracellular signaling since it contains tandem repeats of phosphoserine motifs that have the capacity to bind upstream and downstream signaling molecules (27-30). 14-3-3 zeta protein also facilitates cell migration by forming a ternary complex with integrin alpha-4 and paxillin (31).

However, 14-3-3 zeta also has potential roles in cancerogenesis, based on its ability to bind NF-kappa B, beta-catenin, and Bcl-2, and to augment cancer cell proliferation (31). Furthermore, 14-3-3 zeta protein has been shown to block activation of p38 mitogen-activated protein kinase (MAPK), thereby mediating an anti-apoptotic mechanism (32). Numerous investigations have also suggested that 14-3-3 zeta protein is a key molecule in the malignant pathological processes of several malignancies, including oral, esophageal, lung, and breast cancers, as well as B cell lymphoma.

Recently, He et al. reported that 14-3-3 zeta protein represents a candidate biomarker and a metastasis-promoting factor in ovarian cancer based on a serum proteomic analysis of a nude mouse xenograft model containing SKOV-3 cells and a mass spectrometry [liquid chromatography-tandem mass spectrometry (LC-MS/MS)] analysis to identify metastasis-related serum proteins (17). Significantly higher expression of 14-3-3 zeta was detected in EOC patients than in patients with benign gynecological diseases. Furthermore, compared to CA-125, serum levels of 14-3-3 zeta protein were significantly upregulated when microscopic peritoneal metastasis was present, or when both ovaries were involved. Accordingly, the authors suggested

that 14-3-3 zeta protein may be a useful tool in differentiating FIGO stage Ib and Ic ovarian cancers from stage Ia ovarian cancers in the clinic (17).

However, the results of the present study are not consistent with these findings. For example, significant differences in the serum levels of 14-3-3 zeta protein were not detected in healthy menopausal women versus patients with advanced stage EOC. Furthermore, significant changes in serum levels of 14-3-3 zeta protein was not detected during the six consecutive cycles of chemotherapy treatment that were administered (Figure 7/G), although CT scans and CA-125 and HE4 levels unambiguously indicated the efficacy of the treatment.

A possible explanation for these results is the insufficient number of patients enrolled in the current study. Thus, future studies should include a larger cohort in order to identify statistically significant changes. It is also possible that serum proteins may undergo degradation, even when stored at -80 °C. In particular, it may be that 14-3-3 zeta is an unstable protein that needs to be assayed shortly after collection.

Furthermore, an intriguing possibility is that 14-3-3 zeta may bind proteins activated by chemotherapeutic agents, or present as a result of chemotherapy, thereby obscuring detection of 14-3-3 zeta protein in serum. In the future a large-scale clinical investigation is necessary to evaluate the efficacy of 14-3-3 zeta protein, and to determine the sensitivity and the specificity of this biomarker comparing it to CA-125 and to HE4.

7.4 Conclusion

In conclusion, determination of CA-125 and HE4 serum levels for the ROMA represents a useful tool for the prediction of chemotherapy efficacy for EOC patients. However, based on our current findings, levels of 14-3-3 zeta protein were not found to reliably correlate with the clinical behavior of EOC, and therefore we question if it would be a useful biomarker for this disease.

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9 PUBLICATION OF THE AUTHOR

Original Research Articles upon which the Dissertation is based on:

Hatzipetros I, Gocze P, Koszegi T, Jaray A, Szereday L, Polgar B, Farkas B

Investigating the clinical potential for 14-3-3 zeta protein to serve as a biomarker for epithelial ovarian cancer.

JOURNAL OF OVARIAN RESEARCH 2013 Nov. 15;6(1):79 PMID: 24238270

IF: 2.429

Other Original Research Articles:

Hatzipetros I, Gocze PM, Cziraky K, Kovacs K, Kalman E, Farkas B.

Assessment of cells in the ascitic fluid of women with ovarian hyperstimulation syndrome: the clinical implications for subsequent ovarian malignancy.

REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY 2013 Sep 12;11(1):91. doi: 10.1186/1477-7827-11-91. PMID**:** 24028152 **IF: 2.144**

Letters to the Editor:

Hatzipetros I, Gocze PM, Farkas B.

Oral Contraceptive Pills as Primary Prevention for Ovarian Cancer: A Systematic Review and Meta-analysis.

OBSTETRICS AND GYNECOLOGY 2013 Nov;122(5) p. 1114. doi:

10.1097/01.AOG.0000435076.57585.21. PMID: 24150022 **IF: 4.798**

Hatzipetros I, Drozgyik I, Bodis J.

What is new in gynecologic oncology? Thought-provoking articles from the past year.

OBSTETRICS AND GYNECOLOGY 2013 Aug;122(2 Pt 1):393-4. doi:

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