TYPE I AND TYPE III COLLAGEN METABOLITES AND PERITONEAL CELLS IN PREDICTING THE CLINICAL OUTCOME OF EPITHELIAL OVARIAN CANCER PATIENTS

MARIJA SIMOJOKI

Department of Obstetrics and Gynaecology and Department of Clinical Chemistry, University of Oulu

OU LU 2003
MARJA SIMOJOKI

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Department of Obstetrics and Gynaecology; Department of Clinical Chemistry, University of Oulu,
P.O.Box 5000, FIN-90014 University of Oulu, Finland
Oulu, Finland
2003

Abstract
Malignant tissue growth induces marked biochemical and structural changes in the extracellular matrix of the tumour and its surrounding tissues. In the present study, we evaluated the prognostic value of the serum concentration of the markers of synthesis of type I collagen (PICP, PINP) and type III collagen (PIIINP) as well as the marker of type I collagen degradation (ICTP) and compared them with the conventional indicators of prognosis (clinical stage, grade of differentiation, histological subtype, residual tumour load and the age of the patient). The prognostic value of peritoneal cytological findings at operation was an additional object in our studies.

High preoperative serum ICTP (> 5.6 µg/L) and PIIINP (> 3.2 µg/L) concentrations and a low PICP:PINP ratio (> 2) correlated with poor prognosis in ovarian carcinoma in univariate analysis and in multivariate analysis when each variable was analyzed separately with the conventional factors. However, ICTP concentration was the only prognostic variable in multivariate analysis including PIIINP, PINP, ICTP and CA125. When analyzed with conventional prognostic factors (clinical stage, grade, residual tumour, presence of ascites, histology), clinical stage and ICTP were independent indicators of prognosis. In addition, malignant cells in the peritoneal fluid aspirate at primary operation, grade and the age of the patient predicted poor prognosis in multivariate analysis.

Postoperative serum ICTP concentration 9-months after the operation was the strongest prognostic factor as compared to the preoperative ICTP and CA125 values and clinical variables.

These results indicate that serum collagen metabolites, especially ICTP, are indicators of prognosis in epithelial ovarian cancer. The present ICTP-test does not detect the degradation products of immature type I collagen, the dominating form in ovarian cancer tissue. Therefore, the excess ICTP in invasive ovarian cancer might originate through the degradation of trivalently matured collagens in non-malignant tissues surrounding the malignancy. ICTP may thus be an indicator of invasive properties of the tumor and its determination opens up new perspective to predict the clinical outcome of ovarian cancer.

Keywords: carcinoma, collagen metabolism, matrix metalloproteinases, ovarian neoplasms, peritoneal cytology, procollagen, prognosis, tumor markers
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Oulu, January, 2003

Marja Simojoki
### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CASA</td>
<td>cancer associated antigen</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinases</td>
</tr>
<tr>
<td>CD44</td>
<td>cluster of differentiation 44, adhesion molecule</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CSF</td>
<td>colony-stimulating factor</td>
</tr>
<tr>
<td>ERBB2</td>
<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 2</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Obstetrics and Gynaecology</td>
</tr>
<tr>
<td>GOG</td>
<td>Gynecologic Oncology Group</td>
</tr>
<tr>
<td>HCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>hCGβ</td>
<td>human chorionic gonadotropin beta</td>
</tr>
<tr>
<td>ICTP</td>
<td>carboxyterminal telopeptide of type I collagen</td>
</tr>
<tr>
<td>IAP</td>
<td>immunosuppressive acidic protein</td>
</tr>
<tr>
<td>KRAS</td>
<td>gene encoding Harvey virus homolog Kirsten type</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygoty</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MMPi</td>
<td>matrix metalloproteinase inhibitor</td>
</tr>
<tr>
<td>MYC</td>
<td>v-myc myelocytomatosis viral oncogene homolog (avian)</td>
</tr>
<tr>
<td>p21</td>
<td>cell cycle inhibitor</td>
</tr>
<tr>
<td>p53</td>
<td>nuclear phosphoprotein p53</td>
</tr>
<tr>
<td>PAI</td>
<td>plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PICP</td>
<td>carboxyterminal propeptide of type I procollagen</td>
</tr>
<tr>
<td>PINP</td>
<td>aminoterminal propeptide of type I procollagen</td>
</tr>
<tr>
<td>PIINP</td>
<td>aminoterminal propeptide of type III procollagen</td>
</tr>
<tr>
<td>UPAR</td>
<td>urokinase type plasminogen activator receptor</td>
</tr>
<tr>
<td>TATI</td>
<td>tumour associated trypsin inhibitor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>tumour growth factor β</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
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List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


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1 Introduction

Ovarian cancer is the second most common gynecological cancer in developed countries. Due to its silent nature and lack of specific symptoms during early development it is often at an advanced stage at the time of diagnosis. Over 70% of the cases present with metastatic growth beyond the ovary and pelvis. The recent introduction of platinum-based combination chemotherapy with paclitaxel has slightly improved the prognosis of this disease. Ovarian cancer has, however, remained the most common cause of death of the gynecological malignancies. (Kristensen & Tropé 1997, Runnebaum & Stickeler 2001.)

Tumour growth involves several alterations in the extracellular matrix of the tumour and the surrounding tissues. These include active cell migration and an increased synthesis of extracellular matrix components (Liotta 1986, Van den Hooff 1986). The prerequisite for tumour invasion is disruption of the basement membranes, followed by a degradation of the interstitial stroma. The malignant tumour cells must be able to detach from neighbouring cells as well as to adhere to the extracellular matrix components in order to enable the migration of tumour cells through the extracellular matrix (Heino 1996). Several proteolytic enzymes (e.g. the matrix metalloproteinases) induced by tumour cell-fibroblast interaction are involved in the degradation of extracellular matrix components during tumour invasion (Boyd & Balkwill 1999). The most important constituents of the extracellular matrix are fibrillar type I and type III collagens, which are responsible for the structural integrity of soft and bone tissues (Bateman et al. 1996). Malignant growth is known to induce a fibroproliferative response characterized by an increased expression of type I and type III collagens in the tissue surrounding malignant lesions. At the same time, the formation of a collagenous network also takes place inside the malignant tumour. The metabolism of these collagens is a continuously ongoing active process, in which synthesis and degradation take place concomitantly. These phenomena are reflected by the serum concentrations of different collagen metabolites which can be measured using immunoassays. The aminoterminal propeptide of type III procollagen (PIIINP) and the amino- (PINP) and carboxyterminal propeptides (PICP) of type I procollagen are markers of synthesis, while the carboxyterminal telopeptide of type I collagen (ICTP) is a marker of degradation. (Risteli et al. 1988, Melkko et al. 1990, Risteli et al. 1993, Melkko et al. 1996, Santala et al. 1998.)
Epithelial ovarian cancers are thought to form metastases via both the lymphatic system and the exfoliation of tumour cells inside the peritoneal cavity, as well as hematogenously, to a minor extent. Shedding of the malignant cells inside the peritoneal cavity can be detected with a peritoneal cytological examination. Malignant cytological findings also divide the early stage ovarian cancer into subclasses (IC and IIC) according to the FIGO surgical staging classification. (Kristensen & Tropé 1997.)

The clinical outcome of ovarian cancer depends on several clinical, biological and pathological variables including the clinical stage of the disease, histopathological grade and type, extent of residual disease and the age of the patient. Recently, many biological, tumour-related factors have been under investigation in relation to the prognosis of ovarian cancer. An understanding of the biological and other prognostic factors associated with ovarian cancer serve to improve treatment decisions, assist in the design of clinical trials, increase the knowledge of the pathogenesis of the malignancy while also providing valuable information for the patients. (Dembo et al. 1990, Makar et al. 1995, Concato 2001.)

This study was conducted to elucidate the clinical importance of markers of type I and type III collagen synthesis (PINP, PICP, PIIINP) and degradation (ICTP) and peritoneal cytological findings in predicting the clinical behaviour of epithelial ovarian cancer both before and during the different phases of therapy.
2 Review of the literature

2.1 Epidemiology and clinical features of ovarian cancer

Ovarian cancer is the fourth most common malignancy among Finnish women and the leading cause of death of the gynecological cancers. In 1997, a total of 628 new cases were diagnosed and 343 ovarian cancer related deaths documented in Finland. The age-adjusted incidence was 14.6/100 000 (Finnish Cancer Registry 2002), which corresponds to that in other industrialized countries in Northern (19/100 000/year) and Western Europe (18/100 000) and in the USA (16/100 000). Low incidence rates (3/100 000–6/100 000) are observed in Japan and Asia. The incidence has remained somewhat stable during recent years. (Runnebaum & Stickeler 2001.)

The risk of ovarian cancer increases with age and is highest in women aged 60–69 years. Approximately 90 % of all ovarian cancers are epithelial and, according to the prevailing view arise from the surface epithelium covering ovarian inclusion cysts (Kruk & Auersperg 1992). The exact etiology of epithelial ovarian cancer is unknown. There may be multiple genetic changes in activating certain oncogenes or inactivating tumor suppressor genes including \textit{ERBB2} (HER-2/neu), \textit{CMYC}, KRAS and p53 (Gallion \textit{et al.} 1995, Lynch \textit{et al.} 1998). Incessant ovulation, persistent gonadotropin or androgen stimulation and exposure of the ovaries to pelvic contaminants (e.g. talc powder) and carcinogens have been suggested to explain the development of ovarian cancer (Edmondson & Monaghan 2001, Runnebaum & Stickeler 2001). Ovulation involves repetitive minor trauma to the covering epithelium as well as repeated exposure of the epithelium to estrogen-rich follicular fluid (Fathalla 1971). It has been hypothesized that with ovulation the germinal epithelium is entrapped within the stroma and the inclusion cysts formed are thought to undergo malignant transformation. Whether or not there are precursor neoplastic lesions for epithelial ovarian cancer is unclear (Gallion \textit{et al.} 1995, Holschneider & Berek 2000). Evidence from the observations of coincidently diagnosed early ovarian carcinomas suggests that these may arise \textit{de novo} without a recognizable precursor phase, as these invasive tumors were not associated with any identifiable precursor lesions (Bell & Scully 1994, Chapman 2001). Endometrioid and clear cell carcinomas are related to endometriosis. In 1.7 % of all cases of endometriosis, atypical
changes have been recognized suggestive of a premalignant phase within the endometriosis but only a very small percentage of these become malignant (Ogawa et al. 2000, Chapman 2001).

One age-related reason for an increased risk of cancer may be an accumulation of somatic cell mutations. As an example, the loss of heterozygosity on chromosome 17 (deletion of a proportion of a chromosome containing a putative tumor suppressor gene) increases with age (Pieretti & Turker 1997).

The risk of ovarian carcinoma is increased among nulliparous women and women with low parity, late age at first birth and unexplained infertility. The lifetime risk for developing ovarian carcinoma is 1.4%. Among women with one first-degree relative with epithelial ovarian cancer it is 5 %, but among those with hereditary ovarian cancer syndrome the risk may be as high as 66 % (Antoniou et al. 2000). Hereditary, highly penetrating carcinomas account for up to 5–10% of cases and most ovarian carcinomas are therefore sporadic or have a weak hereditary background (Runnebaum & Stickeler 2001).

Combination oral contraceptives and pregnancies have a protective effect on ovarian carcinoma (Rosenberg et al. 1982, La Vecchia et al. 1984). Even one full-term pregnancy and the sometime use of combination oral contraceptives reduce the risk by 30–40% (Hankinson et al. 1992, Rosenberg et al. 1994, Ness et al. 2000, Riman et al. 2002) whereas the effect of late menarche and early menopause is weak (Whittemore et al. 1992, Hankinson et al. 1995, Riman et al. 2002). In some studies the protective effect of oral contraceptives is weaker in mucinous than nonmucinous ovarian carcinoma, suggesting different etiological mechanisms for mucinous tumors (Risch et al. 1996, Purdie et al. 2001, Riman et al. 2002). In addition to these other hypotheses explaining ovarian carcinogenesis, a hormonal theory of conditions with progesterone deficiency has been suggested (Risch 1998). The protective effect of pregnancy could also be explained by progesterone predominance during pregnancy. In accordance, the use of progestin-only contraception also reduces the risk of ovarian carcinoma (Rosenberg et al. 1994). Hysterectomy and tubal ligation reduce ovarian cancer risk by 20–30 % (Hankinson et al. 1993) as reviewed by Ylikorkala in 2001.

Current screening techniques to detect early ovarian cancer are not sufficiently sensitive or specific to be applied to the general population. However, the screening of high risk populations, i.e. patients with BRCA1 and BRCA2 mutations or two or more first degree relatives with ovarian cancer, with twice yearly transvaginal ultrasound sonography and serum CA-125 testing has been recommended, although no data of the impact of such screening on the stage of cancer detected or the quality of life are available (Einhorn et al. 2000). Prophylactic bilateral oophorectomy should be considered for those with increased genetic risk when their family is complete (Kristensen & Trope 1997).

2.2 Prognosis of ovarian cancer

Ovarian cancer has a poor prognosis due mainly to the fact that 70 % of the patients are diagnosed at advanced stages. In addition, the recurrence rate after initial treatment is as high as 60% (Ozols 1999). In Norway the age-adjusted 5-year survival increased from 39% to 43 % and the median survival from 29 months to 39 months from 1975–1979 to
1990–1994. This was probably due to the introduction of cisplatinum-based chemotherapy and more aggressive surgery. However, no major improvement in long-term survival was seen (Bjorge et al. 1998). In the 1990’s, the introduction of paclitaxel has improved the initial complete response (51% vs. 31%), progression free survival (18 months vs. 13 months) and overall survival (38 months vs. 24 months) (McGuire et al. 1996). However, there is a wide spectrum of clinical behaviours from an excellent prognosis and high likelihood of cure to those with rapid progression and poor prognosis irrespective of clinical stage of the disease, most probably reflecting different biological properties of the tumour.

2.3 Clinicopathological prognostic variables

2.3.1 Stage

The International Federation of Obstetrics and Gynaecology (FIGO) staging system is based on a careful examination of the extent of spread of the malignancy at initial laparotomy. It is the main determinant of prognosis in epithelial ovarian cancer (Högberg et al. 1993, Brun et al. 2000). The five-year survival rates vary in different stages: stage I 76%–93%, stage II 35%–79%, stage III 11%–50%, stage IV 4%–17% (Aure et al. 1971, Bjorkholm et al. 1982, Einhorn et al. 1985, Sweeney et al. 1985, Balvert-Locht et al. 1991, Venesmaa 1994a, Bjorge et al. 1998, Holschneider & Berek 2000). The large differences in the reported survival rates of the patients with the same FIGO stage suggest that inadequate or under-staging is common, although evolving treatment methods also affect survival rates (Högberg et al. 1993, McGowan 1993, Friedlander 1998). Each stage is divided into substages according to surface excrescences, tumour rupture, ascites, positive peritoneal cytology or the size of the abdominoperitoneal implants in stage III. There are differences in 5-year survival rates between the substages as follows: stage IA 71%–92%, stage IB 62%–85%, stage IC 57%–82%, stage IIA 53%–67%, stage IIB 38–56%, stage IIC 37%–51%, stage IIIA 39–60%, stage IIIB 13–30%, stage IIIC 15–17% (de Souza & Friedlander 1992, Nguyen et al. 1993, Friedlander 1998). In many studies the patients are divided into two groups: early (Stage I and II) and advanced stage (stage III and IV), because the survival in early stage disease is significantly better than that in advanced disease. The factors which have been found to correlate with poor prognosis in early stage disease are histopathological type (serous vs. non-serous), degree or grade of differentiation, the presence of dense adhesions, large volume ascites and spontaneous rupture of a cyst (Malkasian et al. 1984, Dembo et al. 1990, Vergote et al. 2001) and in advanced stage disease the amount of residual tumour, performance status, type of chemotherapy, substage, age of the patient, grade and – in some studies – histological type (Malkasian et al. 1984, Einhorn et al. 1985, Hunter et al. 1992, Venesmaa 1994a, Makar et al. 1995, Mukararah et al. 1997, Eisenkop et al. 1998, Bristow et al. 1999, Naik et al. 2000, Akahira et al. 2001, Vergote et al. 2001).
2.3.2 Histopathological grade

There is no generally accepted grading system for ovarian carcinoma mainly because the same criteria are not applicable to all histological types. The grading is subjective and is based on architectural and/or nuclear features; the more nuclear atypia and mitotic figures and solid areas the higher the grade. The reproducibility of grading is poor, because of subjective judgement, with high intra- and inter-observer variability (Hernandez et al. 1984). According to most studies the histopathological grade correlates with prognosis. Some authors suggest that tumour grade is of good prognostic value in early stage ovarian cancer, but its value decreases in patients with advanced stage disease (Sorbe et al. 1982, Einhorn et al. 1985, Friedlander & Dembo 1991). The estimated 5 year survival for all stages with grade 1 tumours is 73–82 % and with grade 2 and grade 3, 41–43% and 20–21%, respectively (Swenerton et al. 1985, Baker et al. 1994). For stage I the 5-year cumulative survival rate according to the grade of the tumour is 91% in grade 1, 71% in grade 2 and 46% in grade 3, for stage II 82%, 56% and 41% and for stage III 39%, 18% and 9%, respectively (Swenerton et al. 1985). In a more recent study, the 5-year survival rates for stage I disease of various grade were 94%, 81% and 61%, respectively (Vergote et al. 2001).

2.3.3. Histopathological type

Over 90 % of ovarian neoplasms arise from the epithelial surface of the ovary, the rest from germ cells and stromal cells. The epithelial neoplasms are classified as serous (30–70%), mucinous (5–20%), endometrioid (10–20%), clear cell (3–10%) and undifferentiated (1%) and the 5-year survival rates for these subtypes are 20–35%, 40–69%, 40–63%, 35–50% and 11–29%, respectively (Bjorkholm et al. 1982, Sorbe et al. 1982, Högberg et al. 1993). There may be differences between subtypes with regard to risk factors, biological behaviour and optimal treatment. Mucinous and endometrioid tumours are more often diagnosed at early stages, which contributes to their more favourable prognosis (Malkasian et al. 1984, Friedlander 1998). Advanced stage mucinous tumours are known to have poor prognosis, probably due to their chemoresistance (Makar et al. 1995). Clear cell carcinomas are suggested to have a more aggressive biological nature compared to the other types (Makar et al. 1995). The prognostic significance of the histological type is limited, however, when taken independent of clinical stage and histological grade (Friedlander 1998).

2.3.3 Residual tumor

Several studies show an improved survival of patients with residual tumours less than 1–2 cm in diameter after primary surgery as compared to patients with larger lesions (Hoskins et al. 1994, Makar et al. 1995, Brun et al. 2000, Akahira et al. 2001, Bristow et al. 2002). Even in stage IV disease with or without hepatic metastases, optimal cytoreduction is
associated with a more favourable survival time of 25–40 months compared with 10–18 for those to whom only explorative laparotomy was performed (Curtin et al. 1997, Liu et al. 1997, Munkarah et al. 1997, Bristow et al. 1999, Naik et al. 2000, Akahira et al. 2001). In some studies, however, patients with large volume intra-abdominal metastases before operation had a much worse survival than the others, in spite of optimal cytoreduction (Hacker et al. 1983, Hoskins et al. 1992). Optimal cytoreduction can only be achieved in 15–45% of cases with advanced disease (Curtin et al. 1997, Munkarah et al. 1997, Bonnefoi et al. 1999).

2.3.4 Age

Younger women (<30 years) have a better prognosis than older ones (5-year survival 71% vs. 47%), which is due to a higher rate of early stage (44% vs. 35%) and low grade tumours (68% vs. 37%) (Markman et al. 1993, Massi et al. 1996). The survival advantage among younger women is seen even after correction for stage and for death from other causes, reflecting different tumour character, co-morbidity, less aggressive treatment methods in older women and possibly a different immunological defence capability. A ten year increase in age induces a 1.6 times greater risk of death. The overall 5-year survival for different age categories has been as follows: 66% for 14–45 years, 39% for 45–59 years, 24% for 60–74 years and 20% for women 75 years or older (Balvert-Locht et al. 1991, Markman et al. 1993, Baker et al. 1994, Curtin et al. 1997, Eisenkop et al. 1998).

2.4 Treatment of ovarian cancer

The currently accepted treatment for ovarian cancer is primary debulking surgery followed by adjuvant chemotherapy (Ozols 2000). However, surgery alone is regarded as adequate for patients with well-differentiated carcinomas limited to the ovaries. Consequently, a critical aspect of the management is a comprehensive staging laparotomy to determine the risk of recurrence and to identify patients who are eligible for postoperative adjuvant therapy (Thigpen 1999). Staging laparotomy should include total hysterectomy with a salpingo-oophorectomy, omentectomy, pelvic and para-aortal lymph-node biopsies and cytological evaluation of ascites or peritoneal washings (Trimbos 2000). Random peritoneal biopsies or from suspected areas should be obtained throughout the peritoneal cavity. Early stage patients are categorized as low risk or high risk patients according to the findings from staging procedures. Low risk tumours are defined as those with grade 1 histopathological differentiation, intact capsule, no tumour on the external surface of the ovary, non-clear cell carcinoma, no ascites and benign cytological findings. Stage I patients with low risk tumours can be treated with fertility-sparing surgery with no adjuvant therapy (Thigpen 1999).
Patients with advanced stage tumors should be operated with maximal cytoreduction followed by adjuvant chemotherapy. Optimal cytoreduction is defined as leaving no residual tumor greater than 1–2 cm in diameter. Standard, first-line chemotherapy today includes six courses of platinum-based combination (paclitaxel or docetaxel) chemotherapy. In 1996 the Gynecologic Oncology Group (GOG) first reported more favorable results of paclitaxel/cisplatin compared to cisplatin/cyclophosphamide regimens in terms of response to therapy (73% vs. 60 %), clinical complete response rate (51% vs. 31%), percentage of patients grossly disease-free at second-look laparotomy (40 % vs. 26 %), median progression-free survival (18 months vs. 13 months), and overall survival (38 months vs. 24 months) (McGuire et al. 1996). If optimal cytoreduction cannot be achieved, the optimal sequence of surgery and chemotherapy i.e. intervals for debulking and neoadjuvant chemotherapy, is not exactly known.

2.5 Tissue tumor markers as prognostic factors in epithelial ovarian cancer

2.5.1 Oncogenes

The ERBB2 (HER-2/neu) gene codes for a growth factor receptor structurally related to epidermal growth factor receptor. Overexpression of this gene, which is commonly due to multiple copies of the normal gene (i.e. amplification) has been observed in 10–30 % of breast and ovarian carcinomas by immunohistochemical techniques and has been associated with a poor prognosis in some (Berchuck et al. 1990, Meden et al. 1994, Felip et al. 1995, reviewed by Friedlander 1998) but not all (Rubin et al. 1994, van der Zee et al. 1995) studies. Relative tumour specificity and transmembranous expression provide possibilities for targeted therapy, for example via the application of recombinant, humanized ERBB2 (anti-HER-2)–antibodies, which improve the chemosensitivity of the tumour (Gallion et al. 1995, Runnebaum & Stickeler 2001).

A point mutation in the KRAS proto-oncogene may result in the production of a protein, which is constitutively activated and stimulates growth. KRAS mutation is seen in mucinous (45%) more often than in serous (16%) ovarian cancers and has not been shown to correlate with prognosis (Gallion et al. 1995, Cuatrecasas et al. 1998, Aunoble et al. 2000).

CMYC oncogene amplification is seen in 29–37% of ovarian carcinoma, but its prognostic significance is not clear (Baker et al. 1990, Tashiro et al. 1992). Patients whose tumors have both ERBB2 and CMYC amplification have a significantly shorter survival than those without such amplification (Wang et al. 1999).

The ETS1 proto-oncogene is a transcription factor activating the expression of several proteases including collagenase 1 (MMP-1), stromelysin (MMP-3), gelatinase (MMP-9) and urokinase-type plasminogen activator. ETS1 expression in stromal cells has been found to correlate with poor prognosis in ovarian cancer in both univariate and multivariate analyses (Davidson et al. 2001).
2.5.2 Tumor suppressor genes

p53 is a nuclear phosphoprotein involved in many important cellular functions such as in the control of apoptosis. Mutations of the p53 are the most common in human malignancies and have been detected in about 50% of epithelial ovarian cancers (Levine et al. 1991). In multivariate analysis the expression of mutant p53 has been shown to correlate with both recurrence-free (Anttila et al. 1999 a) and overall survival (Klemi et al. 1995, Levesque et al. 1995, Geisler et al. 2000), especially in well-differentiated ovarian carcinomas (Levesque et al. 1995, Anttila et al. 1999 a), but some studies have not confirmed these findings (Kohler et al. 1993, Hartmann et al. 1994).

The somatic mutation of the PTEN suppressor-suppressor gene is frequent (21%) in endometrioid ovarian cancer but not in other histological types. This supports the hypothesis that epithelial ovarian cancers arise through developmentally distinct pathways (Maxwell et al. 1998, Obata et al. 1998). PTEN mutation occurs more often in low grade and low stage malignancies, suggesting that the inactivation of this gene is an early event in ovarian tumorogenesis and may thus be associated with a more favorable prognosis (Obata et al. 1998).

2.5.3 Cell cycle regulators

P21 inhibits CDK/cyclin complexes, which are involved in the transition from the G1 to the S phase. The p21 gene contains two p53 binding sites and is a major downstream effector of p53. P21 gene mutations are rare and the prognostic significance of p21 in ovarian cancer is controversial. The expression of p21 correlates with improved survival in some (Anttila et al. 1999 b, Schmider et al. 2000), but not in all studies (Baekelandt et al. 1999 b, Werness et al. 1999, Levesque et al. 2000). The independent character of p21 as a prognostic variable has not been confirmed by multivariate analysis (Anttila et al. 1999 a, Baekelandt et al. 1999 b).

P27 prevents and inhibits the activation of cyclin CDK complexes regulating cell growth negatively. Conflicting data on the prognostic role of p27 in ovarian cancer have been reported. In some studies, P27 expression has been found to correlate with longer time to progression and overall survival (Newcomb et al. 1999, Masciullo et al. 2000), while in another study only a trend toward reduced survival was noticed in patients whose tumor lacked the p27 expression completely (Baekelandt et al. 1999 a).

The deletion of CDKN2/p16 and/or MTS2/p15 genes is a poor prognosis factor in advanced ovarian cancer and a potential indicator for poor chemotherapy response (Kudoh et al. 2002).
2.5.4 DNA ploidy

DNA ploidy, measured by flow cytometry reflects cellular DNA content, whether diploid i.e. normal or aneuploid i.e. pathologic. Tumour aneuploidy has been seen to correlate with a poor prognosis of ovarian cancer both in advanced (Rodenburg et al. 1987, Friedlander et al. 1988, Kuhn et al. 1989) and early stage disease (Kallioniemi et al. 1988, Schueler et al. 1993, Gajewski et al. 1994, Kimmig et al. 2002).

2.5.5 Markers of cell proliferation

Ki67 is a protein expressed in the G1, S and G2 phases in the cycling cells but not in G0 cells. It is thus regarded as a good marker of proliferative activity (van Diest et al. 1998). Ki67 has been found to correlate with poor survival in ovarian cancer in both univariate and multivariate analyses (Anttila et al. 1998).

The activator protein AP2 is a transcription factor, which mediates cell differentiation and gene expression in response to various signals (Williamson et al. 1996). In cancer cells, AP2 has been shown to exert growth inhibition (Zeng et al. 1997) and in ovarian cancer tissues the cytoplasmic expression of AP2 indicates a good prognosis whereas nuclear AP2 expression correlated with a decrease in survival (Anttila et al. 2000).

Topoisomerase II is a nuclear enzyme catalyzing DNA replication. It is expressed during S, G1, G2 and M phases of the cell cycle. In short-term survivors (less than 3 years) topoisomerase II expression was 70% compared to 12% in long-term survivors (more than 5 years) (Gotlieb et al. 2001).

2.5.6 Growth factors

The abnormal expression of growth factors may trigger cell growth and carcinogenesis via autocrine pathways. TGFβ, PDGF and VEGF, elicit numerous cellular effects including the differentiation and proliferation of epithelial cells, the modulation of angiogenesis, the synthesis of extracellular matrix proteins and the production of matrix-degrading proteinases and their inhibitors. TGFβ enhances ovarian cancer invasiveness in advanced tumours, which is partly mediated by the induction of matrix metalloproteinase (MMP) activity (Rodriguez et al. 2001).

VEGF expression in carcinoma cells is an independent prognostic factor for poor prognosis in ovarian cancer (Paley et al. 1997, Shen et al. 2000). The expression of the PDGF α-receptor in ovarian cancer tissue has been associated with poor prognosis in univariate analysis (Henriksen et al. 1993) while the expression of epithelial macrophage colony-stimulating factor (CSF-1) in ovarian carcinoma tissue along with its receptor correlated with poor outcome and the stromal expression of CSF-1 correlated with a good outcome of the disease (Chambers et al. 1997).
2.5.7 Adhesion molecules

Ovarian cancer cells attach to peritoneal mesothelial cells using adhesion molecules on the cell membrane. Standard CD44 (CD44S), a receptor for hyaluronic acid, is the principal matrix adhesion molecule expressed by normal ovarian epithelium and ovarian carcinoma cell lines (Saegusa et al. 1999). High CD44s expression has been demonstrated to correlate with poor prognosis in ovarian cancer in one study (Kayastha et al. 1999) but with good prognosis in another (Saegusa et al. 1999). Reduced α-catenin expression predicts poor prognosis in stage I tumors in univariate and multivariate analysis (Anttila et al. 1998).

Intercellular adhesion molecule-1 (ICAM-1) protein expression levels are reduced in adenocarcinoma cells, which is associated with poor survival (Arnold et al. 2001).

2.5.8 Proteolytic enzymes

Degradation of the extracellular matrix requires activation of proteinases. The main proteinases produced by ovarian cancer cells are plasminogen activators and MMPs (Moser et al. 1994). Urokinase-type plasminogen activator (uPA) must bind to its membrane receptor (uPAR) to facilitate cell invasion and metastasis. The enzymatic activity of uPA is regulated by the plasminogen activator-inhibitors PAI1 and PAI2. High levels of uPA are correlated with good prognosis (Chambers et al. 1995). High uPA (Konecny et al. 2001) and PAI1 and/or uPA (Kuhn et al. 1999) expression in ovarian cancer tissue has been observed to correlate with poor prognosis in advanced stage ovarian cancer in some, but not all studies (van der Burg et al. 1996).

Kallikrein gene 5 (KLK-5) is a member of the serine protease family, the expression of which is associated with a poor clinical outcome in low grade ovarian tumors (Kim et al. 2001).

MMPs comprise a family of proteinases including collagenases, stromelysins, gelatinases, membrane type MMPs and others (Harris & Krane 1974). Together they are able to degrade most of the proteins of the extracellular matrix and most of them are able to degrade some type of collagen in a substrate-specific manner. They may therefore play an important role in tumor invasion and metastatic spread. Gelatinase A (MMP-2) degrades type IV collagen in basement membranes and its expression in cancer cells correlates with poor prognosis in ovarian cancer (Westerlund et al. 1999, Wu et al. 2002).

Prominent trypsin expression has been seen in 45% of ovarian cancers but not in benign or borderline tumors, suggesting that tumour-derived trypsin may be associated with invasiveness and malignant potential of the tumour (Hirahara et al. 1998).
Table 1. Results of some interesting studies on A) cancer tissue markers and B) serum markers as predictors of prognosis in ovarian cancer. In multivariate analysis the prognostic value is shown over the variables tested in each analysis, not over all the known clinicopathological prognostic variables.

<table>
<thead>
<tr>
<th>Tumor marker</th>
<th>Prognostic value demonstrated (+ yes, – no)</th>
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<tbody>
<tr>
<td></td>
<td>Univariate analysis</td>
</tr>
<tr>
<td><strong>A) Tissue</strong></td>
<td></td>
</tr>
<tr>
<td>Oncogenes</td>
<td></td>
</tr>
<tr>
<td>ERBB2</td>
<td>+ (1, 3, 4, 5)</td>
</tr>
<tr>
<td></td>
<td>– (2)</td>
</tr>
<tr>
<td>MYC</td>
<td>+(6) (with ERBB2)</td>
</tr>
<tr>
<td>Ets-1</td>
<td>+ (7)</td>
</tr>
<tr>
<td>Tumor suppressor genes</td>
<td></td>
</tr>
<tr>
<td>BRCA 1</td>
<td>+ (8, 9, 10)</td>
</tr>
<tr>
<td></td>
<td>– (11)</td>
</tr>
<tr>
<td>p53</td>
<td>+ (12, 13, 14, 16, 17)</td>
</tr>
<tr>
<td></td>
<td>– (15, early stage)</td>
</tr>
<tr>
<td>Cell cycle regulators</td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td>+ (18)</td>
</tr>
<tr>
<td></td>
<td>– (19, 20)</td>
</tr>
<tr>
<td>p27</td>
<td>+ (21, 22)</td>
</tr>
<tr>
<td></td>
<td>– (23)</td>
</tr>
<tr>
<td>DNA ploidy (aneuploidy)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ (24 stage III+IV, 25, 26, 27, 28 stage I+II, 29)</td>
</tr>
<tr>
<td>Markers of cell proliferation</td>
<td></td>
</tr>
<tr>
<td>Ki67</td>
<td>+(30)</td>
</tr>
<tr>
<td>AP2</td>
<td>+(31)</td>
</tr>
<tr>
<td>Topoisomerase II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+(32)</td>
</tr>
<tr>
<td>Growth factors</td>
<td></td>
</tr>
<tr>
<td>PDGFRα</td>
<td>+(33)</td>
</tr>
<tr>
<td>VEGF</td>
<td>+(34, 35)</td>
</tr>
<tr>
<td>CSF</td>
<td>+(36)</td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>+(37, 38)</td>
</tr>
<tr>
<td>α-catenin</td>
<td>+(39)</td>
</tr>
<tr>
<td>Proteolytic enzymes</td>
<td></td>
</tr>
<tr>
<td>UPA</td>
<td>+(40 stage III, 41)</td>
</tr>
<tr>
<td></td>
<td>– (42)</td>
</tr>
<tr>
<td>UPAI1</td>
<td>+(40, 41)</td>
</tr>
<tr>
<td></td>
<td>– (42)</td>
</tr>
<tr>
<td>KLK-5</td>
<td>+(43)</td>
</tr>
<tr>
<td>MMP2</td>
<td>+(44)</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>– (45)</td>
</tr>
<tr>
<td>Steroid hormone receptors</td>
<td></td>
</tr>
<tr>
<td>Estrogen and/or progesterone receptors</td>
<td>+ (46, 47)</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>+(49)</td>
</tr>
<tr>
<td>Bax</td>
<td>+(50)</td>
</tr>
<tr>
<td>Others</td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>+(50)</td>
</tr>
<tr>
<td></td>
<td>– (51)</td>
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Table 1. Continued.

<table>
<thead>
<tr>
<th>Tumor marker</th>
<th>Prognostic value demonstrated (+ yes, – no)</th>
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<tr>
<td></td>
<td>Univariate analysis</td>
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<tr>
<td><strong>B) Serum tumor markers</strong></td>
<td></td>
</tr>
<tr>
<td>CA125</td>
<td></td>
</tr>
<tr>
<td>Preoperative/</td>
<td>+ (52, 59)</td>
</tr>
<tr>
<td>prechemotherapy</td>
<td>– (53, 58, 60)</td>
</tr>
<tr>
<td>postoperative</td>
<td>+ (53, 54, 55, 56, 57)</td>
</tr>
<tr>
<td>TATI</td>
<td>+ (61)</td>
</tr>
<tr>
<td>CEA</td>
<td>+ (62)</td>
</tr>
<tr>
<td>CASA</td>
<td>+ (63)</td>
</tr>
<tr>
<td>Inhibin A</td>
<td>+ (64)</td>
</tr>
<tr>
<td>IAP</td>
<td>+ (65)</td>
</tr>
<tr>
<td>VEGF</td>
<td>+ (66, 67)</td>
</tr>
<tr>
<td>CSF-1</td>
<td>+ (69)</td>
</tr>
<tr>
<td>soluble FAS</td>
<td>+ (70)</td>
</tr>
<tr>
<td>interleukin 6</td>
<td>+ (71)</td>
</tr>
<tr>
<td>MAGE4</td>
<td>+ (72)</td>
</tr>
<tr>
<td>Tetranectin</td>
<td>+ (73)</td>
</tr>
<tr>
<td>HCG</td>
<td>+ (74)</td>
</tr>
<tr>
<td>Progesterone/</td>
<td>+ (75)</td>
</tr>
<tr>
<td>estrogen</td>
<td></td>
</tr>
</tbody>
</table>

2.5.9 Collagen IV

Type IV collagen is responsible for the structural integrity of the basement membrane (BM). Malignant tumours, including epithelial ovarian cancer, often exhibit discontinuity and focal absence in BM structures (Liotta 1984). In ovarian cancer, however, low type IV collagen expression in the BMs has not been found to be an indicator of adverse prognosis (Anttila et al. 1998).

2.5.10 Steroid hormone receptors

Estrogen and progesterone receptor status has been shown to be a prognostic indicator in endometrial and breast cancer, the survival prospects being poorer for patients with receptor-poor or negative tumours (Vihko et al. 1986). Their role in ovarian cancer is controversial. A more favourable clinical course of the disease has been seen in estrogen receptor negative and progesterone receptor positive ovarian cancers compared with all other combinations (5-year survival 81.3% vs. 45.3%) (Kauppila et al. 1983, Munstedt et al. 2000). Improved survival has also been seen in patients with high serum progesterone in combination with progesterone receptor expression (Lindgren et al. 2001). In other studies, however, patient survival was not related to steroid hormone receptor status (Scambia et al. 1995 a, Konecny et al. 2001). On the other hand, low estrogen and progesterone receptor concentrations predicted unresectable tumours and a shorter survival time in advanced ovarian cancer than other receptor combinations (Kauppila et al. 1983).

2.5.11 Other markers

Hyaluronan is an extracellular polysaccharide particularly abundant in connective tissues. Increased concentrations of hyaluronan in the extracellular matrix may facilitate cancer invasion by providing a less dense matrix for cancer cells, stimulating cancer cell motility and forming an immunoprotective coat for cancer cells (Yang et al. 1993). Elevated levels of stromal hyaluronan are seen in ovarian carcinoma tissue and the levels are higher in metastatic foci than in the corresponding primary tumour. In multivariate analysis, a high level of stromal hyaluronan was an independent prognostic factor for poor outcome (Anttila et al. 2000).

The Bcl-2 family of apoptosis-regulating proteins is critical in determining the apoptotic threshold of the cells. Bax and Bcl-2 expression in tumor tissue cytoplasm correlate with improved prognosis in some (Baekelandt et al. 1999 b, Baekelandt et al. 2000), but not all studies (Geisler et al. 2000) and Mcl-1 expression correlates with a poor prognosis in advanced ovarian cancer in univariate analysis (Baekelandt et al. 2000).

Cyclooxygenase-2 (Cox-2) expression is a prognostic factor for poor survival in both uni- and multivariate analyses (Denkert et al. 2002).
2.6 Serum tumor markers as prognostic factors in epithelial ovarian cancer

A tumour marker is generally defined as an antigen or protein, which is secreted by the tumour itself or the surrounding tissues in response to the tumour and can be determined in the serum samples of the patient (Canney et al. 1984). An ideal tumour marker is both specific and sensitive for the malignancy and signals recurrence before clinical symptoms occur. Ideally, tumour markers can be used in the differential diagnosis of carcinomas from benign tumours and in monitoring the treatment response and disease recurrence. Tumour markers have also been evaluated as prognostic markers of the disease. Poor sensitivity and specificity in ovarian cancer limits the clinical use of most of the tumour markers, except CA125, which is the gold standard to which new tumour marker candidates are compared.

2.6.1 CA125 as a prognostic factor

CA125 antigen is a large glycoprotein expressed on the epithelium of the fallopian tubes, the endometrium, the endocervix and the ovary as well as in mesothelial cells of the pleura, pericardium and peritoneum (Bast et al. 1981). CA125 serum concentration is elevated in 83% of all ovarian cancer patients (Bast et al. 1984). The sensitivity of CA125 correlates with the clinical stage of the disease; serum levels being elevated in 51% of the patients with stage I, 71% with stage II, 91% with stage III and 98% of patients with stage IV disease (reviewed by Tuxen et al. 1995). In stage I ovarian cancer patients, the preoperative serum CA125 concentration with a cut-off value of 65 is an independent prognostic factor over the tested variables (substage, grade, age) in multivariate analysis, the risk of dying of disease being 6.35 times higher in CA125 positive patients (Nagele et al. 1995). The preoperative serum CA125 level shows poor correlation with prognosis in patients with advanced stage disease (van der Burg et al. 1988, Makar et al. 1992) but in one study increased risk of dying was observed with increasing preoperative CA125 levels (Cooper et al. 2002). In contrast to these findings, changes in serum CA125 levels during adjuvant chemotherapy have been identified as independent indicators of prognosis in several studies (Redman et al. 1990, Tomas et al. 1990, Makar et al. 1993, Yedema et al. 1993, Gadducci et al. 1995, Buller et al. 1996). A half-life of less than 20 days correlated to longer progression-free (van der Burg et al. 1988) and overall survival (Hunter et al. 1990). In addition, the absolute serum level of CA125 at 3 months after the operation correlated with prognosis (Sevelda et al. 1989, Mogensen 1992). Patients whose serum levels do not normalize (>35U/ml) within three months of surgery have a 3.1 times higher risk of dying of the disease than those with normal (≤35) serum CA125 levels (Sevelda et al. 1989). The combined criteria of a serum CA125 level lower than 65 U/ml four weeks after the second course of the chemotherapy and at least a 50 % decrease in serum CA125 level compared to the prechemotherapy level may indicate a favourable prognosis in patients with advanced
stage disease with residual tumour after cytoreductive surgery (Makar et al. 1993). However, the prognostic value of serum CA125 regression appears to be temporary and may be limited to the first year after surgery (Peters-Engl et al. 1999).

2.6.2 Other serum tumor markers

Tumour-associated trypsin inhibitor (TATI) is most specific for mucinous ovarian tumours. TATI serum concentrations are elevated in 60–70 % of the patients with mucinous ovarian cancer (Halila et al. 1988). Elevated serum concentrations of TATI have been shown to correlate with poor prognosis in non-mucinous advanced stage ovarian cancer in a multivariate analysis (Venesmaa et al. 1994 b).

Carcinoembryonic antigen (CEA) is a glycoprotein whose serum concentration is often elevated in cancers of the gastrointestinal tract, breast, lung and in gynecological malignancies (Tuxen et al. 1999). CEA is elevated in approximately 34–37% of the patients with ovarian cancer and more often in mucinous (88%) than in non-mucinous (19%) cancers (Bast et al. 1984, Tholander et al. 1990, Stenman et al. 1995, Tuxen et al. 1995). Serial CEA determinations can be used to predict prognosis in ovarian cancer. Declining or persistently low CEA levels are associated with a better prognosis than high or rising levels (Khoo et al. 1979).

The assay for cancer-associated serum antigen (CASA) uses monoclonal antibodies, which binds to an epitope on a polymorphic epithelial mucin. In a univariate analysis, elevated serum CASA levels have been shown to correlate with a poor prognosis for ovarian cancer (Ward et al. 1993).

Elevated circulating levels of immunoreactive inhibin A is typically seen in granulosa cell tumors of the ovary and often in mucinous cystadenocarcinomas (Lappöhn et al. 1989, Kauppila and Santala 1999, Ala-Fossi et al. 2000). The tumor stroma is the major source of excess inhibin in ovarian carcinoma (Fuller et al. 1999). Elevated serum inhibin A concentrations correlated with poor survival in postmenopausal women (Frias et al. 1999).

Immunosuppressive acidic protein (IAP) is a glycoprotein being involved with host-immune responses and impaired immunologic reactivity often found with advanced malignancies. High preoperative serum IAP levels have been seen to correlate with poor prognosis in ovarian cancer in multivariate analysis (Scambia et al. 1996).

The prognostic value of the pre-treatment concentration of serum growth factor, VEGF, is controversial; it correlated to a poor prognosis of ovarian cancer in multivariate analysis in some (Tempfer et al. 1998, Chen et al. 1999), but not in all studies (Gadducci et al. 1999). In addition to neovascularization and angiogenesis, VEGF also reflects the presence of ascites and tumor progression in ovarian cancer (Gadducci et al. 1999, reviewed Brown et al. 2000).

High serum CSF-1 concentrations have been shown to predict poor prognosis in ovarian cancer (Kacinski et al. 1989, Scholl et al. 1994).
Soluble Fas is a form of the Fas protein which lacks the transmembrane domain. Fas and its ligand, FasL, trigger apoptosis. Soluble Fas binds to and neutralizes FasL, thus functionally antagonizing Fas/FasL-driven apoptosis. Increased serum soluble Fas levels have been shown to be a significant prognostic factor for shorter disease-free and overall survival in ovarian cancer in a multivariate analysis (Hefler et al. 2000).

Several cytokines (e.g. interleukins) are involved in normal ovarian function and may also play a role in tumor development. High serum interleukin-6 levels correlate with poor prognosis in ovarian cancer (Scambia et al. 1995 b). The serum concentration of soluble interleukin-2 receptors is elevated in ovarian cancer patients more than in benign disease, but the prognostic value of this observation is unclear (Gebauer et al. 1999).

The MAGE gene family (MAGE –1, –2, –3, –4) encode tumor rejection antigens recognized by cytotoxic T-lymphocytes. Expression of the MAGE gene family at both mRNA and protein levels are seen in ovarian cancer. Serum MAGE-4 positivity correlates with a poor prognosis of the disease (Kawagoe et al. 2000).

Tetranectin is a plasminogen binding protein, and high serum concentrations and low tumor tissue stromal concentrations of tetranectin are associated with improved survival in ovarian cancer (Hög dall et al. 1994).

High serum hCGβ concentration in epithelial ovarian cancer has been demonstrated to be an independent prognostic factor of poor prognosis in multivariate analysis together with the stage and grade of the tumor (Vartiainen et al. 2001).

High serum progesterone in poorly differentiated ovarian cancer, especially in combination with expression of progesterone receptors, is a sign of improved prognosis in univariate analysis (Lindgren et al. 2001). High Ki67 expression is seen in some areas of ovarian cancer tissue with high estrogen receptor density. A lower apoptotic activity is seen in tumors with high estrogen receptor expression compared to others, but the prognostic significance of estrogens or its receptors has not been precisely determined (Lindgren et al. 2001).

2.7 Fibrillar type I and III collagens

Fibrillar type I and III collagens are the main constituents of the extracellular matrix and responsible for the structural integrity of the tissues. The collagen molecules consist of three polypeptide chains (α-chains) each of which is coiled into a left-handed helix and the three chains are twisted around each other into a right-handed super-helix forming a rod-like molecule. Each polypeptide chain has repetitive Gly-X-Y-sequences, with proline and hydroxyproline residues in the X and Y position, respectively. Hydroxyproline makes up about 12 % of the mass of a fibrillar collagen molecule and can be used as a general measure of the collagen content of a tissue. Urin hydroxyproline has been used as a marker of collagen breakdown in bone diseases, however, its non-specificity for type I collagen reduces its clinical use. The hydroxyproline content in benign ovarian tumours was 4.7 times higher than in ovarian carcinoma, but it has not been evaluated as a prognostic marker in ovarian carcinoma (Kauppila et al. 1999 a, Demers et al. 2000).
Type I collagen is the most abundant collagen in the human body, comprising about 70 \% of the total collagen. It is widely distributed in bone, tendon, skin and internal organs. About 90 \% of the organic matrix of the bones consists of type I collagen. Type III collagen is the second most abundant collagen in the human organism and occurs particularly in tissues exhibiting elastic properties such as the skin, blood vessels, internal organs and placenta, often in association with type I collagen. (Prockop et al. 1979 a, Prockop et al. 1979 b, Kühn et al. 1982, Prockop & Kivirikko 1995, Risteli & Risteli 2002.)

Type I and III collagens are synthesized by fibroblasts in the endoplasmic reticulum as larger precursors molecules, procollagens, which have large propeptide extensions at both the aminoterminal and the carboxyterminal ends of the molecule (Fig. 1). After secretion into the extracellular space, the propeptides are cleaved off from procollagen en bloc by specific proteases, leading to formation of mature type collagen with short non-helical telopeptide regions at both ends of the molecule. These telopeptide regions are the primary sites for intermolecular cross-linking, which are important for stabilizing the collagen fibers. The telopeptide lysyl and hydroxyllysyl aldehydes are capable of reacting with the residues of other collagen molecules to form immature divalent cross-links. In the hydroxylysyl aldehyde pathway, which predominates in bone, tendons and most internal organs, the divalent cross-link spontaneously matures further into trivalent pyridoline or pyrrole cross-links (Eyre et al. 1984, Kauppila 1998 a, Risteli & Risteli 1999). Deoxypyridinoline is a bone-specific, cross-linking residua. Both pyridinoline and deoxypyridinoline can be measured in urine and serum using either high pressure liquid chromatography or by immunoassay for the free cross-link measurements (Demers et al. 2000). In metastatic bone tumors, urinary pyridinoline and deoxypyridinoline may be useful markers in monitoring response to treatment but no data is available so far on their use in soft tissue tumors (Paterson et al. 1991, Walls et al. 1999, Coleman 2002).

The degradation of collagens occurs both intra- and extracellularly. Intracellular pathway involves phagocytosis and the lysosomal degradation of collagens during balanced turnover conditions whereas the extracellular route is active during rapid turnover of collagens (Everts et al. 1996). MMPs, which include collagenases, stromelysins, gelatinases and membrane type MMPs, play a crucial role in the extracellular degradation of collagens (Kähäri & Saarialho-Kere 1999). The major collagen degrading enzyme in bone is the cysteine protease cathepsin K, which is expressed by osteoclasts (Delaisse et al. 2000).
Fig. 1. The structure of type I and type III collagen molecules. The arrows indicate the sites for cleavage of amino- (PINP, PIIINP) and carboxyterminal (PICP) telopeptides. Thick arrow shows the structure of trivalently cross-linked ICTP molecule. The ICTP assay detects only fragments having two phenylalanine rich regios (solid box), which are brought together with a trivalent cross-link. N, aminoterminal end of the propeptide, C, carboxyterminalend of the propeptide, H, helical domain in the aminoterminal telopeptide, T, telopeptide domains at each end of the collagen molecule (modified from Kauppila S. 1998 and Risteli J. & Risteli L. 1999).

### 2.7.1 The structure and metabolism of PIIINP

Type III collagen is a homotrimer of three α1(III) chains. The aminoterminal propeptide of type III procollagen (PIIINP) is cleaved off by a specific N-proteinase (Halila & Peltonen 1984). The PIIINP molecule is an indicator of both the synthesis and the degradation of type III collagen. Occasionally, the removal of PIIINP from the newly-synthesized type III collagen is not complete and the propeptide may remain attached to some of the molecules, known as type IIIpN-collagen. PIIINP is a degradation product of type IIIpN collagen (Fleischmajer et al. 1990). PIIINP has a molecular weight of 42 000 and contains three distinct domains: a triple-helical domain (Col 3) in the middle of the molecule, the Col 1-domain at the aminoterminal and Col 2-domain at the carboxyterminal end of the propeptide (Bruckner et al. 1978). PIIINP is cleared from the circulation by scavenger receptors in liver endothelial cells. The measurement of PIIINP in human serum by radio-immunoassays facilitates the assessment of altered metabolism of type III collagen in situations such as fibrosis and malignancy. The radioimmunoassay used in this study only measures trimeric PIIINP molecules, not the monomeric degradation products of PIIINP (Risteli et al. 1988).
Type I collagen is a heterotrimer consisting of two α1(I) chains and one α2(I) chain. In addition to the typical type I collagen, there is evidence of a type I α1-homotrimer collagen consisting of three α1(I) chains (Jimenez et al. 1977). A third variant consisting of one α1(I), one α1(III) and one α2(I) chain, referred to as oncofetal collagen, has also been identified (Pucci-Minafra et al. 1995). The carboxyterminal propeptide of type I procollagen (PICP) and the aminoterminal propeptide of type I procollagen (PINP) are enzymatically removed from the procollagen molecule by specific proteases. PICP and PINP are indicators of the synthesis of type I collagen. Cleavage of PICP is required for the initiation of fibril formation, whereas PINP may be retained in the collagen molecule resulting in type I pN-collagen. The retention of PINP is transient, unlike the presence of HHpN-collagen, and has been considered to regulate fibril diameter (Fleischmajer et al. 1985). The PINP molecule is similar to PIII NP consisting of three distinct structural domains: Col 1 is on the aminoterminal side of the molecule, while Col 2 and Col 3 are situated on the middle of the helically structured molecule (Kühn et al. 1982). The PINP molecule has a molecular mass of 35 000 and is cleared by scavenger receptors in liver endothelial cells (Melkko et al. 1994). PINP often occurs in circulation in two forms of different molecular sizes. One is identical to the trimeric authentic antigen (intact PINP) whereas the other consists of smaller forms of PINP, resembling a single domain of the proα1(I) chain of PINP and is probably a degradation product of type I procollagen or I pN-collagen. Thus, an assay of intact PINP rather than total PINP appears to be more sensitive in detecting changes in the rate of type I collagen synthesis (Melkko et al. 1996, Risteli & Risteli 1999).

PICP is a trimeric, globular protein consisting of three polypeptide chains: two proα1(I) and one proα2(I) chains. The molecular mass of PICP is 100 000 and it is cleared by mannose receptors in liver endothelial cells (Smedsrød et al. 1990).

The carboxyterminal telopeptide of type I collagen (ICTP) is an indicator of degradation of type I collagen. ICTP antigen consists of a trivalent collagen cross-link joining three polypeptide chains of which two are α1 chains of one collagen molecule while the third is derived from either an α1 or an α2 chain of the helical region of another molecule (Fig. 1) (Risteli et al. 1993). The major determinant for antigeneity must contain two phenylalanine-rich regions, which is only possible if the cross-link is trivalent in nature (Sassi et al. 2000). In osteoclasts, cathepsin K cleaves the trivalently cross-linked ICTP structure at two sites between the phenylalanine-rich region and the cross-link, thereby destroying any reactivity with ICTP antibodies (Sassi et al. 2000). Therefore degradation of type I collagen in bone is not measurable by the present ICTP-test. ICTP is cleared from circulation by the kidneys and has a molecular mass of about 12 000–20 000 (Risteli et al. 1993).
2.8 Collagen metabolism in epithelial ovarian cancer

Malignant growth affects the extracellular matrix in several ways and vice versa. During invasive growth, malignant cells must be able to detach from each other, penetrate through the basement membrane, degrade the surrounding collagenous stroma by utilizing proteolytic enzymes and adhere to the extracellular matrix component via integrin receptors to migrate through the extracellular matrix. In addition to the degradation of collagens, tumour cells may also synthesize matrix components including fibrillar collagens. (Liotta 1986, Van den Hooff 1986, Heino 1996.) The active cell migration and increased synthesis of extracellular matrix components by fibroblasts during tumour growth resemble the tissue remodelling in healing wounds (Dvorak 1986). In addition the cells surrounding the tumour may increase production of extracellular matrix components, which phenomenon is known as desmoplasia (Dvorak 1986).

2.8.1 PIIINP

In ovarian cancer tissues, irregularly organized PIIINP positive fibres have been seen close to the cancer cells as well as further away in the stroma in immunohistochemical studies. This phenomenon correlates with increased serum PIIINP concentrations (Zhu et al. 1993). In an in situ hybridisation study, data demonstrated an increased production of mRNA for PIIINP with increasing grade of the malignancy. In anaplastic tumours, some of the neoplastic cells have been found to participate in the production of type III procollagens, whereas in well differentiated tumours the fibroblasts alone are responsible for this function (Kauppila et al. 1996). The PIIINP concentration is ten to a hundred-fold higher in the peritoneal ascitic fluid than in serum, indicating active fibroproliferation in the peritoneal cavity. Concentrations in tumour veins and tumour cyst fluid are similar in benign and malignant tumours (Zhu et al. 1993). The synthesis of type III procollagens is increased in both the tumour tissue and peritoneal cavity, as measured using a radioimmunoassay (Zhu et al. 1993). Enhanced metabolism of type III collagen is also evidenced by increased serum concentrations of PIIINP, which correlates with the clinical behaviour of the disease (Risteli et al. 1988, Kauppila et al. 1989, Tomas et al. 1990 a, Risteli et al. 1992). Pathologically elevated serum concentrations are seen in 77% of the patients with advanced ovarian cancer and in 55% in early stage disease. Serum PIIINP concentrations correlate with the clinical stage of the disease, and among patients with poor prognosis an inverse correlation with survival time has been observed. In monitoring the disease, serum PIIINP concentration has been shown to be complementary to serum CA125 concentration in one-third of the patients with recurrent disease (Tomás et al. 1990 a).
2.8.2 ICTP

Irregular, destructured, disorganized ICTP-positive fibres are seen in poorly differentiated ovarian cancer tissues in immunohistochemical studies (Zhu et al. 1995, Kauppila et al. 1999a). The serum concentration of ICTP is increased in ovarian cancer patients compared to patients with benign tumours (Santala et al. 1998). In a preliminary study, an elevated serum ICTP concentration reflects a poor prognosis (Santala et al. 1995). The ICTP concentration in peritoneal fluid in ovarian cancer was about two times higher than that in serum but about the same as in malignant cyst fluid and peritoneal fluid in benign tumours (Santala et al. 1998). Gel filtration and biochemical studies of ovarian cancer tissues have shown that the majority of the ICTP is immature and divally cross-linked, whereas ICTP in benign tumours is mature and trivalently cross-linked, indicating marked structural difference between these two tumour types (Kauppila et al. 1999 a).

2.8.3 PICP and PINP

The serum concentration of PICP in patients with malignant ovarian tumour does not differ from that of patients with benign disease. Changes in the serum PICP concentration during tumour progression or regression remain within the reference interval. Only during the final progression of the disease may serum concentrations increase rapidly above the reference intervals (Zhu et al. 1994 b). In a gel filtration study of ascitic fluid in ovarian cancer, large variants of PICP representing incompletely processed procollagens have been observed (Zhu et al. 1994 a). In in situ hybridisation studies of mRNAs for polypeptide chains type I and III procollagens, strong signals were seen in stromal fibroblasts next to tumour cell islets, especially in poorly differentiated (grade 3) tumours, in which the mRNAs were also seen in the neoplastic cells themselves. In well-differentiated (grade 1) tumours the mRNA was only seen in the stromal fibroblasts close to the tumour cells (Kauppila et al. 1996).

Marked irregularities were observed in immunohistochemical studies in the deposition of PINP-positive collagen fibers in poorly differentiated ovarian carcinomas (Zhu et al. 1995). The PINP expression is about twofold in soluble serous ovarian cancer tissue extracts compared to benign serous cystadenomas (Kauppila et al. 1999 a). The PINP concentration in peritoneal fluid is about fifty times higher, and in the cyst fluid about eight times higher than in serum in ovarian cancer. The serum concentrations of PINP were significantly higher in malignant tumors compared to benign ones. The serum concentration of intact PINP is increased in approximately 10 % of patients with ovarian cancer (Santala et al. 1998).
2.8.4 Collagen metabolism in other gynecological malignancies

Elevated serum levels of PIIINP have been observed in 35% of patients with endometrial cancer, 40% with cervical and 33% with vulvar carcinoma, but no correlation with the prognosis of the disease has been shown (Tomás 1994, Tomás et al. 1990 b, Tomás et al. 1991). In a manner analogous to that of anaplastic ovarian cancer, poorly differentiated malignant mixed Müllerian tumours express type I procollagen mRNAs in both epithelial and stromal components. Intracellular type I procollagen staining was also observed in malignant cells in mixed Müllerian tumours of the uterus (Kauppila et al. 1999 b).

In malignant breast tumors, an increased expression of type I and III procollagen mRNAs has been observed in the fibroblastic cells of the stroma (Kauppila et al. 1998 b). There is a discrepancy between the serum levels of PICP and PINP in aggressive breast cancer, the levels of PINP dominating over those for PICP (Jukkola et al. 1997).

2.9 Peritoneal cytology in epithelial ovarian cancer

The first report of an intraoperative examination of peritoneal washing cytology to detect subclinical metastases was presented in 1971. Patients with normal peritoneal cytological specimens had better survival rates than patients with abnormal findings, but only one abnormal cytologic specimen was found in early stage disease (Creasman & Rutledge 1971). FIGO incorporated the results of peritoneal washing cytology into the staging classification of ovarian cancer in 1975. Peritoneal washing cytology from cul-de sac aspirates has been used with promising results in the follow-up of the disease to detect recurrent intraperitoneal disease (VillaSanta & Jovanovski 1980, Venesmaa et al. 1986, Redman et al. 1991). Both pre- and post-treatment peritoneal lavage cytology have been seen to be of prognostic value in ovarian cancer (Redman et al. 1991).

The presence of ascites at the time of laparotomy for ovarian cancer has been associated with poor prognosis (Puls et al. 1996). Malignant cells in ascites are found in 20%–100% of cases, depending on the stage of disease, the involvement of ovarian surface and ascitic volume. A finding of malignant cells in peritoneal washings or ascites during the laparotomy correlates with the stage of the disease (Yoshimura et al. 1984a,b). In the study by Zuna and coworkers, a significantly worse survival was seen in patients with malignant cytology when each stage, except stage II (3 patients), was analyzed separately in univariate analysis (Zuna & Behrens 1996).
3 Purpose of the present study

The purpose of this study was to evaluate the clinical value of the serum concentration of the collagen metabolites PIIINP, ICTP, PINP and ICTP and peritoneal cytological findings in relation to conventional clinicopathological prognostic factors and CA125 in predicting clinical outcome in epithelial ovarian cancer at different time-points of treatment (preoperatively, intraoperatively or postoperatively). The specific aims were:

1. to evaluate the prognostic value of preoperative serum PIIINP, PINP and ICTP concentrations in comparison to CA125 and conventional prognostic variables in assessing the long-term clinical outcome of epithelial ovarian cancer
2. to assess the prognostic value of the ratio of the preoperative serum concentrations of PICP and PINP and to correlate it to conventional prognostic factors and preoperative serum CA125 concentrations
3. to evaluate the prognostic value of intraoperative peritoneal cytological findings in correlation to conventional prognostic variables
4. to assess the prognostic value of serum concentrations of PIIINP, and ICTP and the PICP/PINP ratio during the treatment and follow-up of patients measured at three to six months intervals and to correlate the findings with conventional prognostic variables.
4 Patients and methods

The patients included in this study were referred to the Oulu University Hospital during the period 1989–1995 due to an adnexal mass suspicious of ovarian cancer. Preoperative blood samples were collected and the primary debulking surgery was performed at the Oulu University Hospital in 284 patients included in the study. Patients with benign or borderline disease, non-epithelial tumours or a co-existing carcinoma were excluded from the study. Seventy-eight patients met the study criteria. The patients were followed for three years or until death in studies 2 and 3, until January, 1997 in Study 5, until January, 1999 in study 1, and until January, 2000 in study 4.

Maximal cytoreductive surgery was performed using a vertical midline incision. The fluid for cytological analysis was collected during primary surgery from the lower and upper abdominal cavity. If no fluid was present at the time of laparotomy, the peritoneal cavity was lavaged with saline solution, and the fluid was then collected for analysis. During postoperative chemotherapy and follow-up, blood samples for PIIINP, PINP, PICP and ICTP analysis were collected at 3 –6 months intervals.

The demographic data and clinical characteristics of the patients from the different studies are shown in Table 2.
Table 2. The demographic data and clinical characteristics of the patients.

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<td>Follow-up time (months) Median (range)</td>
<td>35 (1–107)</td>
<td>31 (12–36)</td>
<td>31 (4–36)</td>
<td>54 (1–132)</td>
<td>35 (1–107)</td>
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4.1 Blood samples and radioimmunoassays

Blood samples collected before operation and during follow-up were centrifuged and the sera were immediately frozen and stored at −20°C until analysed. The PIIINP, PINP, PICP and ICTP concentrations were determined by radioimmunoassays using commercial kits from Orion Diagnostica, (Espoo, Finland). Unfrozen serum samples were used for PIIINP analysis. The serum CA125 concentration was analysed using the Centocor Europe (I) and Abbott AxSYM assay, (Abbott Laboratories, IL) from unfrozen serum samples and in work I with a kit from Centocor Europe.

The upper limits of the reference interval for serum PIIINP, PINP, PICP and ICTP were 4.2 µg/L, 79 µg/L, 170 µg/L, 4.6 µg/L, respectively, and 35 U/ml for CA125. The intra- and inter-assay coefficients of variations for the collagen metabolites were less than 6% at the concentrations encountered in this study.

4.2 Cytological analysis

One cytologist examined all cytological samples. The evaluation included an assessment of cellularity, cell arrangements and background appearance. The criteria for malignancy included nuclear abnormalities, enlargement and variability in nuclear size,
hyperchromasia and nucleolar abnormalities. Benign findings included hyperplastic or reactive changes in mesothelial cells. The cytological findings were interpreted without knowledge of the histology of the tumours. The Papanicolaou classification was used in the interpretations of the results.

4.3 Statistical methods

Statistical analyses were performed using SPSS version 8.0–9.0 for Windows 98 (Statistical Package for Social Science, Inc., Chicago, IL).

The Mann-Whitney U-test was used to analyze continuous variables due to the skewed distribution of the variables, while the $\chi^2$-test was used for contingency tables for discrete variables. Spearman rank correlation and linear regression analysis were used to estimate correlation between the variables.

A receiver-operator characteristic (ROC) curve analysis was carried out in study IV to determine the clinical validity of the variables studied in discriminating between good or poor prognosis over a range of cut-off points. A poor prognosis was defined as the patients’ death during the study-period. The sensitivity and specificity and positive and negative predictive values to discriminate between good or poor prognosis were calculated for the parameters analyzed in study 2.

Survival was analyzed by univariate survival analysis according to the Kaplan-Meier method using the log-rank test. A multivariate analysis of survival was performed using the Cox proportional hazard model with a backward, stepwise procedure. In the Cox regression analysis the results were expressed as relative risk (RR or Exp(B)) with 95% confidence intervals (CI). A p-value less than 0.05 was considered to be statistically significant when combined with a CI not crossing zero.
5 Results

5.1 Collagen metabolites as preoperative prognostic factors in ovarian cancer

5.1.1 PIIINP (Study I and II)

The frequency of patients with a high serum PIIINP concentration (>3.2µg/l) before operation was 57% for the total population, and was significantly higher in patients with a poor than in those with a good prognosis (74% vs. 45%, p=0.034). The median preoperative serum concentration of PIIINP was 4.0 µg among patients with a poor prognosis and 3.2 µg among those with a good prognosis (p=0.094). The median preoperative serum PIIINP concentration was significantly higher in patients with advanced stage disease (4.7 vs. 3.6µg/L), a histologically poorly differentiated tumour (5.0 vs. 3.5µg/L) or with residual disease after primary surgery (4.9 vs. 3.5µg/L), than for the respective reference group.

The sensitivity of the PIIINP-test prior to operation in identifying patients with a poor clinical outcome was 74%, while its specificity was 55%. The values for the reference marker CA125 were 87% and 27%, respectively.

In univariate analysis, a preoperative serum PIIINP concentration of 3.2 µg/L or 3.9 µg/L significantly differentiated between patients with good or poor prognosis (Fig. 2A).

A preoperative serum concentration of CA125 divided the patients into two prognostic groups (Study I) at a cut-off value 170 IU/ml, but failed to do so when using a cut-off value of 65 IU/ml (Study II).

In multivariate analysis in which the clinicopathological indicators of prognosis were evaluated together with preoperative and postoperative values of PIIINP and CA125, the preoperative PIIINP-test and 12-month CA125 test were significant prognostic variables for ovarian cancer (Table 3C). In multivariate analysis where preoperative serum concentration of PIIINP, PINP, ICTP and CA125 were evaluated, serum ICTP
concentration was the only prognostic factor of significance. When all biochemical and clinicopathological variables were analyzed together, however, only clinical stage appeared as an independent prognostic marker (Table 3B).

<table>
<thead>
<tr>
<th>Factors analyzed in multivariate analysis</th>
<th>Prognostic factors remaining in equation</th>
<th>Exp(B) (95%CI)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>A (Study I) Preoperative: PIIINP, ICTP, CA125</td>
<td>ICTP</td>
<td>1.098 (1.035–1.164)</td>
<td>0.0019</td>
</tr>
<tr>
<td>B (Study I) Preoperative: PIIINP, ICTP, CA125, stage, grade, residual tumor, ascites, histology</td>
<td>Stage ICTP</td>
<td>4.493 (1.609–12.55)</td>
<td>0.0041</td>
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<tr>
<td>C (Study II) Preoperative: PIIINP, CA125, stage, grade, residual disease 12-month: PIIINP, CA125</td>
<td>Preop. PIIINP 12-mo CA125</td>
<td>2.5960 (1.02–6.61)</td>
<td>0.0454</td>
</tr>
<tr>
<td>D (Study III) Preoperative: ICTP, CA125 stage, grade, histological subtype, residual disease, age 9-month: ICTP, CA125</td>
<td>9-mo ICTP</td>
<td>13.00 (3.64–46.45)</td>
<td>0.0001</td>
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<td>E (Study III) Stage I and II patients (same factors as in D)</td>
<td>Preop. ICTP</td>
<td>20.44 (1.81–230.77)</td>
<td>0.0147</td>
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<td>F (Study III) Stage III and IV patients (same factors as in D and E)</td>
<td>9-mo ICTP</td>
<td>8.63 (1.89–39.48)</td>
<td>0.0054</td>
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<td>G (Study IV) Preoperative: PICP:PINP, ICTP, CA125, stage, grade, residual disease</td>
<td>Stage ICTP</td>
<td>3.83 (1.31–11.19)</td>
<td>0.014</td>
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<tr>
<td>H (Study IV) Same factors as in G, except ICTP</td>
<td>Stage PICP:PINP</td>
<td>3.69 (1.31–10.37)</td>
<td>0.013</td>
</tr>
<tr>
<td>I (Study IV) Stage I and II, same factors as in H</td>
<td>PICP:PINP</td>
<td>6.48 (1.10–38.19)</td>
<td>0.04</td>
</tr>
<tr>
<td>J (Study IV) Stage III and IV, same factors as in H</td>
<td>PICP:PINP</td>
<td>2.96 (1.068–8.1958)</td>
<td>0.037</td>
</tr>
</tbody>
</table>
5.1.2 ICTP (Studies I, III, IV)

The median serum ICTP concentration before operation of the patients with good prognosis was 4.2 µg/L, which was significantly lower than that of the patients with poor prognosis (6 µg/L, p=0.006). The concentrations exceeded the upper reference limit in approximately 49% of cases. The median preoperative serum concentration of ICTP was significantly higher in patients with advanced stage disease (5.1 vs. 3.7 µg/L), anaplastic carcinoma (5.4 vs. 4.2 µg/L) and residual disease after primary surgery (6.3 vs. 4.1 µg/L) than that in the respective reference groups (studies I and III).

The best cut-off value for discriminating between good and poor prognosis patients was 5.6 µg/L (study III) – 5.7 µg/L (Study I). In a univariate analysis using these cut-off values, the preoperative serum ICTP concentration separated patients with a good from those with a poor prognosis (Fig. 2B).

In a multivariate analysis of patients with early stage disease, preoperative serum ICTP concentration was a significant prognostic factor (Table 3E). In a multivariate analysis of the entire study material or of patients with advanced stage disease, the postoperative but not preoperative serum ICTP concentration was a significant prognostic factor. (Table 3 D and F).

The sensitivity of the preoperative serum ICTP concentration for discriminating between patients with good or poor prognosis was 56 %, and the specificity 85 %.

5.1.3 PICP, PINP and the PICP:PINP ratio (Study IV)

The median preoperative ratio of circulating PICP to PINP was 2.4 in the poor prognosis group and 3.4 in the good prognosis group, p=0.014. The median value of PINP before operation was 43 µg/L in the poor prognosis group and 31 µg/L in the good prognosis group, p=0.057. On the other hand, the serum PICP concentrations did not differ between the groups. The circulating PINP concentration was significantly increased (78µg/L vs. 40 µg/L; p=0.003) and the PICP/PINP ratio was lower (2.3 vs. 3.1; p=0.031) in the patients with fast progression.

The preoperative PICP:PINP ratio did not correlate with clinical stage, histological grade, presence of residual disease or age of the patient, while it correlated with the preoperative ICTP concentration in poor prognosis patients (Spearman correlation coefficient –0.454; p=0.029)

No cut-off concentration was found for PINP and PICP to discriminate between patients with good or poor prognosis (Study I). A cut-off value of 2.0 was chosen for PICP:PINP, as it represents the lowest level of the reference range, even though the sensitivity with the cut-off value of 2.1 was slightly better according to ROC analysis. The sensitivity and specificity of the preoperative PICP:PINP -test with a cut-off value of 2.0 were 32% and 100%, respectively. All patients with a preoperative ratio of PICP:PINP less than 2 (N=9) died during follow-up while about one half of the patients with a ratio ≥2 were alive (N=22/41; p=0.003).

In a Kaplan-Meier analysis, the preoperative serum PICP:PINP ratio significantly divided the patients into good or poor prognosis using a cut-off value of 2. (Fig. 2C)
In a multivariate analysis in which the preoperative PICP:PINP ratio and the serum ICTP concentration were compared with a number of clinical parameters, the significant prognostic variables were clinical stage and serum ICTP concentration, in this order with the PICP:PINP ratio being the last variable to drop out (Table 3G). When the serum ICTP concentration was excluded from the analysis, the remaining prognostic factors were clinical stage and the PICP:PINP ratio (Table 3H). Early stage (I and II) and advanced stage patients were also analyzed separately in survival analysis. In the univariate analysis for early stage patients, the PICP:PINP ratio detected the patients with good or poor prognosis. In multivariate analysis, the PICP:PINP ratio was the only prognostic variable remaining in the equation over the factors analyzed (Table 3I). In advanced stage patients, the preoperative PICP:PINP ratio was able to discriminate between patients with good or poor prognosis until 46-months of follow-up (at two, three and four years follow-up), but failed thereafter. In multivariate analysis, the PICP:PINP ratio was the only prognostic variable remaining in the equation at four years follow-up (Table 3J).

Fig. 2. Cumulative survival curves for patients with preoperative serum concentrations of A) PIIINP ≤3.2 µg/L or >3.2 µg/L; B) ICTP ≤5.6 µg/L or >5.6 µg/L; C) PICP:PINP ratio ≥2 or <2 and D) benign or malignant cytological findings.
5.2 Peritoneal cytology as an intraoperative prognostic marker

Peritoneal cytological analysis was carried out from ascites (N=39) or peritoneal fluid (fluid less than 100 ml, N=12) in 51 patients and from peritoneal washings in 22 patients. Malignant or possibly malignant cytology (Class III–IV) was found in 71 % (52/73) of the cases and findings were benign in 29 %. The cytological finding in ascitic fluid was malignant in 88% of cases (45/51) and in the peritoneal wash in 33 % of cases (7/21). Malignant cells were present in 7/27 (26%) of patients with early stage (Stage I or II) disease, of which the findings were suggestive of malignancy (Class III) in four and malignant (Class IV and V) in three cases. In advanced stage disease (Stage III–IV) only one patient out of 46 had benign cytological findings in peritoneal washing samples and was alive at the end of the study period.

Peritoneal cytological findings (benign vs. malignant) correlated significantly with clinical stage (stage I+II vs. III+IV), presence of ascites (no vs. yes), presence of residual disease (no vs. yes) and histopathological type (serous and endometrioid vs. mucinous and others) (p< 0.05), but not with the age of the patient (≤50 vs. 50).

In a Kaplan-Meier survival analysis the peritoneal cytology (Fig. 2D), clinical stage, histopathological grade, presence of ascites, the amount of residual disease and the patient's age, all correlated significantly with prognosis. Histopathological type did not correlate with the outcome of the patients. In Cox multivariate analysis, the significant prognostic factors were peritoneal cytology, histopathological differentiation and the age of the patient, in this order (Table 3K).

5.3 Collagen metabolites as prognostic factors during the follow-up of ovarian cancer patients

5.3.1 PIIINP (Study II)

The frequency of high serum PIIINP concentration (>4.2) was higher in patients with poor prognosis than in those with good prognosis at 9 (12/23 vs. 6/33) and 12 (15/23 vs. 9/33) months after the operation. At six months, the groups did not differ from each other. Similar findings were also observed for the reference marker CA125 when using a cut-off value of 65 U/ml during follow-up of the patients. The median serum PIIINP concentration was higher in the patients with a poor prognosis during the follow-up from six months onward until the end of the follow-up. The median serum CA125 concentration was higher in the poor prognosis patients than in the others only at 9- and 12 months. At 6-months, the median serum PIIINP concentration increased in both good (from 3.2µg/L to 3.9µg/L) and poor prognosis group (from 4.0µg/L to 4.3µg/L) while that of CA125 dropped markedly in both groups (from 197 U/ml to 7 and from 433 to 7, respectively).
A marked change (>25%) in PIIINP levels did not have any association with the clinical outcome of ovarian cancer at any time. On the contrary, a change (>50%) in CA125 concentration at 9- and 12-months examinations compared to the preceding examination correlated significantly with the clinical outcome of the disease.

In univariate survival analysis, serum PIIINP concentrations at 9- and 12-months follow-up with a cut-off value of 4.2 µg/l discriminated significantly between patients with good or poor prognosis (p=0.0062).

In multivariate analysis in which conventional predictors of prognosis were analyzed together with preoperative and 12-month PIIINP and CA125 concentrations, the significant predictors of prognosis were preoperative PIIINP and 12-month CA125 concentrations (Table 3C).

The sensitivity and specificity of the serum PIIINP test at the 12-month follow-up were 67 % and 73 %, respectively. The corresponding values for CA125 were 63 % and 75 %.

### 5.3.2 ICTP (Study III)

The median serum ICTP concentration in patients with a good prognosis was significantly lower at every time point examined during the two years of follow-up compared to patients with a poor prognosis. The frequency of patients with a high serum ICTP concentration (>5.6) was higher in patients with poor prognosis than in those with good prognosis at any time point examined, eg. 81% versus 10% at 9 months. The serum ICTP concentration did not correlate with clinical stage, histological grade or presence of residual disease at any postoperative time-point.

In univariate survival analysis using a cut-off value of 5.6, the serum ICTP concentration significantly discriminated between patients with good or poor prognosis at every time point of the follow-up.

The sensitivity and specificity of the 9-month serum ICTP-test was 81% and 90 %, respectively.

In multivariate analysis comprising conventional clinicopathological prognostic factors, preoperative, and 9-month serum ICTP, as well as CA125 concentrations, the significant prognostic factor was the 9-month serum ICTP concentration (Table 2D).

### 5.3.3 PINP, PICP and PICP:PINP ratio (Study IV)

Neither the serum PICP or PINP concentrations nor the PICP/PINP ratio correlated with the prognosis of the patients at the 3, 6, 9 or 12 months follow-up postoperatively. No cut-off value for discriminating between good and poor prognosis patients was found for serum PICP or PINP concentrations or for the PICP:PINP ratio during the postoperative follow-up and they were therefore not included in the multivariate analyses.
6 Discussion

The interaction between the malignant epithelium and its surrounding non-neoplastic stroma during tumour growth and the formation of metastasis involves both the synthesis and degradation of extracellular matrix components, including type I and III collagens. The metabolites of these collagens may therefore reflect the invasive properties of the malignancy. The most important and novel finding of this study was the prognostic value of ICTP, the degradation product of type I collagen, in predicting clinical outcome in ovarian cancer, as was repeatedly observed in multivariate analyses. The products of type I and III collagen synthesis also correlated with the clinical outcome of ovarian cancer patients, but were less efficient than ICTP in this function. In accordance with earlier studies, clinical stage was also a strong and independent determinant of clinical outcome. Both ICTP-test and FIGO stage were shown to be independent prognostic indicators in multivariate analysis, with the FIGO stage being stronger than the ICTP-test.

The material in this study is homogenous with regard the type and extent of operation and adjuvant treatment, as all patients were operated on by the gynecologic oncologist in the Department of Gynecological Oncology of the Oulu University Hospital. Adjuvant treatment included cisplatin-based combination chemotherapy as a first-line treatment for all patients. They were also monitored using similar clinical, radiological and biochemical methods in the same department. The study was prospective, with patient information and serum samples gathered prior to primary surgery. Patients with ovarian cancer detected serendipitously, and had been operated on in other hospitals, were excluded from the study as were patients with co-existing carcinoma and non-epithelial cancer or borderline malignancy. The relatively small sample size in this study limited the possibilities to analyze different subgroups, e.g. each stage separately. Slightly over 50 % of the patients had advanced disease, which is less than the 70 % often reported in the literature, but undifferentiated cancers were more common (10 %) than the 1% usually reported in literature (McGowan 1993, Friedlander 1998). These differences may be explained by relatively small material in our study. Under-staging may be one reason for the large proportion of early stage disease, as the lymph node status was not completely examined in 27–34 % of cases with early stage disease.

Exfoliation of ovarian cancer cells into the peritoneal cavity is the main route for the spread of ovarian cancer and can be diagnosed through cytological examination. The formation of metastasis is a complex process involving many enzymes, cytokines and
adhesion molecules, eg. CD44, which facilitates the binding of cancer cells to the peritoneum (Cannistra et al. 1995). In this study, (study V) malignant cytology was shown to be an independent prognostic factor of poor prognosis in ovarian cancer in multivariate analysis. This confirms the previous findings in univariate analyses showing a correlation with malignant cytological findings and poor prognosis of the patient (Creasman & Rutledge 1971, Yoshimura et al. 1984 b, Zuna & Behrens 1996). The finding of malignant cells in the peritoneal washing samples is also one criterion in dividing the early stage patients into substages IC and IIC, in addition to the findings of tumour capsule rupture or tumour present on the surface of the ovary. The substage IC or IIC is an important factor affecting treatment decisions for an individual ovarian cancer patient. There are reports, however, demonstrating no significant survival differences between stages IB-IC and IIB-IIC and no effect of the tumour capsule state on survival (Sigurdsson et al. 1983, Nguyen et al. 1993). This suggests that stages IB and IC and IIB and IIC might be combined (Nguyen et al. 1993). The strong prognostic significance of malignant cytology shown here in study V, however, supports the importance of dividing early-stage patients into substages according to FIGO criteria. Despite many interesting, but so far only promising, prognostic biological factors for clinical purposes, the clinical stage of the disease, also between the substages (Einhorn et al. 1985, Swenerton et al. 1985), remains one of the most important determinants of prognosis as was seen in this series (studies I and IV), as well.

Collagen is synthesized as large procollagen molecules by fibroblasts in soft tissue and by osteoblasts in the bone. The procollagen molecule has large extra domains at both the carboxy- and amino-terminal ends of the molecule. These bulky domains are removed en bloc from procollagen by specific N- and C-proteinases when the molecule is released into the extracellular space. The concentrations of these propeptides in blood reflect the rate of synthesis of type I and III collagens. (Risteli & Risteli 2002.)

Type I collagen is the most abundant collagen in soft tissues and in the skeleton. The basic pathways of synthesis and degradation of collagens are similar in all tissues, but there are, however, some major differences in the metabolism of collagens in bone and soft tissues. This makes it possible to evaluate the sources of type I collagen metabolites between these two tissues. First, the inter-molecular cross-linking of the telopeptide region of the type I collagen molecules required for the fibril formation differs between bone and soft tissues, especially in the carboxyterminal end of the molecule. More than half of the intermolecular cross-links in the bone may remain immature, divalent or non cross-linked, because the mineralization of bone slows down the maturation of bone cross-links (Oxlund et al. 1995, Risteli & Risteli 1999). In comparison, most of the collagen found in non-malignant soft tissues is mature, trivalently cross-linked (Kauppila et al. 1999 a). Second, the degradation of type I collagen in bone and soft tissues is mediated by different proteases. In normal bone turnover the degradation of type I collagen by osteoclasts is mediated by cysteine protease cathepsin K, which cleaves the carboxy-terminal telopeptide of the α1-chain of type I collagen between two phenylalanine rich regions. The degradation products resulting from this breakdown do not react in the ICTP assay, since the epitope requires two phenylalanine rich regions (Risteli et al. 1993, Sassi et al. 2000). The extracellular degradation mediated by MMPs in soft tissues is the most common breakdown pathway and the degradation product of the breakdown of type I collagen contains two phenylalanine-rich regions which will therefore react in the ICTP assay (Sassi et al. 2000). Due to these differences in cross-linking and degradation, it is thus possible to evaluate the degradation of type I collagen
in soft tissues with the ICTP assay used. In this context it is also important to notice that
the cross-linking of type I collagen is predominantly defective in malignant ovarian tissue
compared to the non-malignant tissue (Kauppila et al. 1999 a). We may therefore assume
that the excess of ICTP measured in ovarian cancer patients is derived from the tissues
surrounding the malignancy rather than from the cancer tissue itself. Hence, we conclude
that ICTP reflects the invasive properties of the tumor. Because the high ICTP
collection was associated with a poor prognosis in this disease, we further hypothesize
that the ICTP-test reflects the aggressive potential of invasion of the tumor. The present
findings support the view that ICTP may be regarded as an indicator of invasion in this
disease.

The origin (bone or soft tissue) of the markers of synthesis of type I or III collagens,
cannot be revealed by the present assays, because the changes in the synthesis markers
are not as specific for soft tissue as is the case for ICTP. The products of the synthesis of
these molecules in bone affect the results. Circulating PINP consists of two forms with
different molecular sizes. The larger form is identical to the trimeric antigen (intact PINP)
while the smaller contains at least the globular part (Col 1) of PINP. The smaller forms
are probably produced during the synthesis of type I pN collagen with retained
propeptide. Type I pN collagen is not found on mineralized bone whereas in soft tissues it
is on the surface of newly formed collagen fibers. For this reason the intact PINP analysis
measuring the trimeric antigen more sensitively reflects the rate of bone collagen
synthesis than the other assays (PINP Col 1 or total PINP), which both also measure the
smaller forms of PINP. In this study we performed an analysis of intact PINP (Melkko et
al. 1996). It is not possible to estimate the proportion of PINP derived from bone
metabolism in this assay. However, as none of the patients in this study had bone
metastases and the study population was comparable with regards treatment and clinical
characteristics, we assume that the bone collagen metabolism of patients with good or
poor prognosis did not differ, and thus did not affect the results of this study. In breast
cancer patients with aggressive disease and no evidence of bone metastases, the results
were comparable with our findings in ovarian cancer (Jukkola et al. 1997). For PICP, the
only form of the circulating antigen so far recognized is the authentic propeptide (Risteli
& Risteli 1999).

The fact that the markers of synthesis are weak tumor markers and are not as efficient
indicators of prognosis as ICTP may be dependent on the fact that they are derived from
the metabolism of both bone and soft tissue. This was clearly seen in this study with the
low power of the PICP- and PINP-tests in discriminating between patients with good or
poor prognosis pre- and postoperatively. Interestingly, a low PICP:PINP ratio
preoperatively correlated with fast progression of the disease and poor prognosis. The
increased expression of PINP has also been seen in several other diseases such as
aggressive breast cancer or Paget’s disease of bone (Sharp et al. 1996, Jukkola et al.
1997). In addition, the phenomenon is also seen in normal physiological conditions with
active growth such as in growing children and in amniotic fluid (Tähtelä et al. 1997,
Kauppila et al. 1998 c). The reason for the much higher PINP concentration in relation to
PICP may be caused by malignancy-associated changes in the clearance of the two
metabolites or in the synthesis of type I collagen variants with different recognition of the
PINP and PICP assays. The propeptides are metabolized by liver endothelial cells through
two separate receptor systems. PICP is cleared by the mannose receptor and PINP by the
scavenger receptor. In macrophages several hormones and other modulators, i.e. vitamin
D and thyroxin regulate the expression of the mannose receptors (Stahl 1990).
Disproportion between the two metabolites may also be caused by an accumulation of ligands competing with PINP for the scavenger receptor or by altered function of the liver clearance system caused by the malignancy. The discrepancy can also be due to synthesis of several variants of PINP. At least two variants from this type I collagen have been found or suggested: \( \alpha_1 \)-homotrimeric collagen, also known as \( \alpha_1 \)-homotrimer (hotPINP) (Jimenez et al. 1977) and oncofetal collagen (Pucci-Minafra et al. 1995). The intact PINP assay used in this study detects classical, heterotrimeric PINP (hetPINP) and \( \alpha_1 \)-homotrimeric PINP (hotPINP) equally. The specificity of the PICP assay for variant forms of type I collagen is so far unknown. In malignant pleural lesions, however, discrepant PICP and PINP levels did not correlate with the presence of the variant hotPINP (Kauppila et al. 2001).

Interestingly, collagen metabolism is also linked to tumor suppressor factors. Lysyl oxidase, the enzyme catalyzing the first reaction in collagen cross-linking, resembles the tumor suppressor protein rrg (Kenyon et al. 1991). A low expression of lysyl oxidase has been shown in malignant cells in vitro and in breast cancer in vivo (Hämäläinen et al. 1995, Peyrol et al. 1997). The lack of this enzyme may be one reason for the defective cross-linking of type I and III collagens in ovarian cancer tissues.

The treatment of ovarian cancer with the drugs inhibiting matrix metalloproteinase activity is a new possible treatment option (Davies et al. 1993, Rasmussen & McCann 1997). Extracellular degradation of type I and III collagens in soft tissues is mediated by many types of enzymes, of which the MMPs are most common. They are very likely responsible for the increased degradation of type I collagens in this disease, in particular in the close vicinity of the malignancy, which will thus promote invasive growth. Special enzymes, i.e. the tissue inhibitors of matrix metalloproteinases (TIMPs), inhibit these proteinases (Stetler-Stevenson et al. 1989). In theory, drugs resembling TIMP could slow down the degradation process surrounding the malignancy and consequently slow down the invasive process. In addition, as a degradation product of MMP mediated degradation of type I collagen, ICTP could theoretically be a marker of the effects antiprotease treatment. So far, the results of clinical trials show no significant survival advantage in patients treated with matrix metalloproteinase inhibitors (MMPIs) (Coussens et al. 2002). Current trials are assessing the efficacy of MMPIs in the maintenance of remission after other modalities of therapy or in combination with cytotoxic agents (Yip et al. 1999).

Type III collagen makes up about 35% of the sum total of type I and III collagens in benign ovarian tissue (Kauppila et al. 1996). In this study, we demonstrated a correlation between high preoperative and 9-month serum PIIINP concentrations and poor prognosis in ovarian cancer (Study II). However, the changes in the serum concentrations of PIIINP during the follow-up did not correlate with prognosis. The malignancy-induced changes in serum PIIINP concentration may be masked by several other conditions with increased PIIINP synthesis. For example, gynecological surgery induces an increase in serum PIIINP concentration for at least 2-months. The background bone metabolism may also cover the malignancy-induced changes in serum PIIINP and weaken its value as a tumour marker. Indeed, in multivariate analysis, PIIINP lost its independent character as a prognostic factor (Study I). Also, when the findings of study IV are taken into account, where ICTP and clinical stage were the only significant prognostic variables in multivariate analysis, we can conclude that the ICTP-test is the strongest and most specific prognostic variable of the fibrillar type collagen metabolites in epithelial ovarian
cancer to be developed so far. This finding confirms preliminary data demonstrating that a simultaneous increase in serum CA125, PIIINP and ICTP concentrations is associated with progressive disease in individual ovarian cancer patients (Santala et al. 1995).

Knowledge of prognostic markers in ovarian cancer is at present insufficient for clinical purposes. New markers characterizing the aggressiveness and invasive potential of ovarian malignancy more precisely are interesting and clinically important. In this study we demonstrated marked alterations in the turnover of fibrillar type I and type III collagens in this disease with the measurement of the different collagen metabolites in serum samples of ovarian cancer patients. Changes in the concentrations of synthesis or degradation products of these collagens have the potential to predict clinical course of the disease. The most powerful indicator of clinical outcome of the biochemical variables studied here was the ICTP-test both pre- and postoperatively. Further comparison to new biological prognostic factors in multivariate analysis is required to further assess the usability of collagen metabolites in predicting the prognosis of ovarian cancer with relation to other markers of prognosis. ICTP provides valuable information on prognosis, with a sensitivity of 56–81 % and a specificity of 85–90%. The low degree of sensitivity, however, reduces its viability in everyday, clinical practice. According to these present results, FIGO stage remains the most powerful indicator of clinical outcome in ovarian cancer patients, while collagen metabolites, especially ICTP, provide additional information on the invasive properties of the tumor.
7 Conclusions

In previous studies, a marked alteration in the metabolism of fibrillar type I and type III collagens has been observed in ovarian cancer tissues as well as in the normal tissues surrounding the malignancy. In this study, the prognostic value of the markers of the synthesis and degradation of type I and III collagens and peritoneal cytology were studied with the following observations:

1. High preoperative serum ICTP and PIIINP concentrations and a low PICP:PINP ratio correlated with a poor prognosis in ovarian carcinoma. However, when analyzed together with ICTP concentration, the most powerful biochemical prognostic factor for poor prognosis was a high preoperative serum ICTP concentration, which had moderate sensitivity and relatively good specificity. As the present ICTP-test only detects the mature, trivalently cross-linked telopeptides of type I collagen seen in non-malignant tissues, but not defective i.e. divalent or non-cross-linked telopeptides present in ovarian carcinoma tissue, the excess ICTP in ovarian cancer patients very likely comes from tissues surrounding the malignancy and would thus reflect the aggressiveness and invasive properties of the tumor. Thus, ICTP may open up new perspectives for the assessment of the clinical course and outcome of this disease.

2. The finding of malignant cells in the peritoneal fluid aspirate during primary surgery was a strong indicator of poor prognosis in both univariate and multivariate analysis. This emphasizes the independent character of this finding with respect to the clinical outcome of the disease.

3. On the basis of the postoperative serum PIIINP concentration it was possible to discriminate between patients with poor or good prognosis in univariate analysis, but not in multivariate analysis. In addition, individual changes in PIIINP concentration did not correlate with the clinical outcome and the postoperative PICP:PINP ratio was also unable to discriminate between patients in the different prognostic groups. Here again, ICTP was superior to synthesis markers in this respect. The serum ICTP concentration at 9-months after operation was the most powerful prognostic factor, even in comparison to CA125 and clinical variables. From the 9-month follow-up
onward, CA125 analysis was quite comparable to ICTP-test as a prognostic variable. These results are in accordance with earlier findings, which suggest that the prognostic value of CA125 is limited during the early postoperative period.

Taken together, the current results suggest that the ICTP-test provides valuable information on the clinical nature and outcome of epithelial ovarian cancer both before and after operation. It is assumed to reflect the degradation of type I collagen in the stromal tissue around the malignancy, thus reflecting different phenomena than the epithelial marker CA125. Before operation and during the first six months after surgery, the ICTP-test is superior to other collagen metabolites and CA125 in predicting the clinical outcome of the disease. Together with the clinical stage of the disease, which is still the most important clinical determinant of prognosis in ovarian carcinoma, ICTP provides complementary information on the clinical prospects of the patient.
8 References


