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Prognostic Role of Cell Adhesion Factors and Angiogenesis in Epithelial Ovarian Cancer

Doctoral dissertation

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Ovarian cancer is the most lethal of the gynecological malignancies with the highest incidence rates in the developed countries. Unfortunately, the incidence rates have been increasing in many Western countries. Although the past decades have brought some advancements in the treatment and thus some improvement in the survival, the prognosis of ovarian cancer patients still remains bleak. Therefore, additional tools to achieve a more precise assessment of prognosis are required to identify those patients that could gain benefit from more aggressive treatment.

The aim of this retrospective study was to evaluate the prognostic significance of clinicopathological and biological factors related to cell adhesion and angiogenesis in epithelial ovarian carcinoma. The material of the study consisted of 310 women diagnosed and treated for epithelial ovarian malignancy in Kuopio University Hospital and Jyväskylä Central Hospital, Finland, between 1976 and 1992, with a follow-up until January 2004. The expression patterns for versican, E-cadherin, β- and γ-catenins, iNOS and CD34 were determined by means of immunohistochemistry, and quantification of angiogenesis was performed by the Chalkley method. The associations of these biological markers were investigated with relation to the previously assessed hyaluronan, CD44 and α-catenin expression as well as to the clinicopathological features and the survival of patients.

Versican expression in ovarian cancer stroma was frequent and thus different from the normal ovarian stroma, and furthermore cancer cell-associated versican expression was noted in tumour epithelium but not detected in epithelial cells of normal ovary. The levels of E-cadherin and β-catenin on cancer cell membrane were reduced in poorly differentiated carcinomas. In addition, nuclear expression of β-catenin was seen especially in endometrioid and γ-catenin in serous ovarian cancers, whereas high iNOS expression was typical for the mucinous histological subtype and a low Chalkley count was associated with serous and clear cell histological subtypes.

Increasing stromal versican expression predicted poorer disease-related survival in the univariate analysis during the first five years, but not any longer after ten years of follow-up. The recurrence-free survival at ten years was significantly better when the tumour epithelium was versican positive. The previously defined independent prognostic value of stromal hyaluronan expression was confirmed. Nuclear expressions of β-catenin and γ-catenin were significant prognosticators of better outcome in the univariate analysis of endometrioid tumours, as were also preserved membranous expressions of E-cadherin and β-catenin in the whole study cohort, but none of these factors possessed independent prognostic value for epithelial ovarian cancer outcome. High iNOS expression was associated with a better disease-related survival in the univariate analysis, whereas it did not retain its statistical significance in the multivariate analysis. A high Chalkley count was a significant and independent predictor of poor survival.

In conclusion, besides confirming the independent prognostic significance of conventional clinicopathological factors such as primary residual tumour and histological subtype, these results suggest that the determination of angiogenesis by the Chalkley count can provide additional prognostic value in epithelial ovarian cancer.

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Kirsi Suhonen
ABBREVIATIONS

APC  adenomatous polyposis coli, tumour suppressor gene
β-TrCP  beta-transducin repeat containing proteins
BRAF  v-raf murine sarcoma viral oncogene homolog B1
BRCA1, BRCA2  breast cancer 1 and 2 genes
CA-125  cancer antigen 125
CD31  cluster of differentiation 31
CD34  cluster of differentiation 34, glycoprotein on immature hematopoietic and endothelial cells
CD44  cluster of differentiation 44, adhesion molecule
CI  confidence interval
CTNNB1  constitutively expressed gene for β-catenin
DRS  disease-related survival
E-cadherin  epithelial calcium dependent adhesion molecule
eNOS, NOS3  endothelial nitric oxide synthase
FIGO  International Federation of Gynecology and Obstetrics
GAG  glycosaminoglycan
GSK-3β  glycogen synthase kinase 3 beta
HAS1, HAS2, HAS3  hyaluronan synthase 1, 2 and 3
HIF-1α, HIF-1β  hypoxia inducible factor-1α and -1β
hMLH1  mutL, e.coli, homolog 1, gene
hMSH2  mutS, e.coli, homolog 2, gene
HNF-1beta  hepatocyte nuclear factor-1beta
IHC  immunohistochemistry
iNOS, NOS2  inducible nitric oxide synthase
KRAS  v-ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog
LEF  lymphoid enhancer-binding factor
MI  microsatellite instability
mRNA  messenger ribonucleic acid
nNOS, NOS1  neuronal nitric oxide synthase
NO  nitric oxide
p53  nuclear phosphoprotein p53
PBS  phosphate buffered saline
PDGF  platelet-derived growth factor
PIGF  placenta growth factor
PTEN  phosphatase and tensin homolog gene
RFS  recurrence-free survival
RR  relative risk
TCF  T cell transcription factor
TGFbetaR2  transforming growth factor beta receptor 2
TSP-1  thrombospondin-1
VEGF  vascular endothelial growth factor
WHO  World Health Organization
WNT  wingless type protein family
LIST OF ORIGINAL PUBLICATIONS

This summary is based on the following original publications referred to in the text by their Roman numerals I-IV.


This summary includes also unpublished data.
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1. INTRODUCTION

Ovarian cancer is the fifth most common cancer in women and the leading cause of mortality from the gynecologic cancers in Finland, resulting in approximately 300 deaths annually (1). Worldwide, approximately 125 000 women died of ovarian cancer in 2002 (2). The incidence rates are highest in developed countries, especially in northern Europe (2). A slight increase in incidence of ovarian cancer has occurred during the past decades in Finland, with an incidence of 10.2 per 100 000 women and a total of 486 new diagnoses made in 2004, excluding borderline tumours of the ovary (1).

The majority of ovarian cancers are diagnosed only when there is distant spread of the disease (3), leading to a bleak prognosis for the patients. The relative 5-year survival rate in Finland is 49% (4). Some improvement in the overall survival of the patients with ovarian cancer has been achieved during recent decades (5) due to the advancement of surgical treatment and platinum-based chemotherapy regimens (6). However, the prognosis of patients with apparently similar conventional prognostic factors is variable and difficult to predict. An improved understanding of ovarian cancer biology would make it easier to predict the disease outcome and help to select those patients who would benefit from different treatments. In addition, it could provide new targets for therapeutic interventions.

In the present study, the expression and the prognostic value of several factors related to cell adhesion (versican, E-cadherin, β- and γ-catenins) and angiogenesis (iNOS, CD34) were evaluated in epithelial ovarian cancer.
2. REVIEW OF THE LITERATURE

2.1. Epidemiology of epithelial ovarian cancer
Ovarian cancer is the sixth most common cancer and the seventh cause of death from cancer in women worldwide, accounting for approximately 125,000 deaths annually on a global basis. The highest incidence rates are observed in northern and western Europe as well as in northern America (2). The general view in Europe is that there is a slowly increasing trend in ovarian cancer incidence, particularly in older women. In terms of age-standardised mortality, there seems to be a declining trend (7). In Finland, the age-adjusted incidence rate of ovarian cancer was 10.2 per 100,000 person-years in 2004. During the same year, 486 new ovarian cancer cases and 302 deaths due to ovarian cancer were reported, i.e. the age-adjusted ovarian cancer mortality was 5.3 per 100,000 person-years (1).

2.2. Etiology, risk factors and progression of epithelial ovarian cancer
The origin of epithelial ovarian cancer is believed to be malignant transformation of ovarian surface epithelium, which undergoes repetitive rupture and repair at the time of ovulation (8, 9). There are several, not mutually exclusive, hypotheses attempting to explain the development of ovarian cancer lesions; these include the incessant ovulation hypothesis (10), the gonadotropin hypothesis (11), the hormonal hypothesis (12), and the inflammation hypothesis (13). The incessant ovulation hypothesis proposes that continuous damage of the ovarian surface epithelium, followed by proliferation of surface epithelial cells after ovulation may increase the probability of mutations and thus lead to an increased risk of developing epithelial ovarian cancer (10, 14). The gonadotropin hypothesis postulates that exposure to high levels of gonadotropins may be the trigger for malignant transformation, probably by enhancing cell growth and inhibiting apoptosis either directly or indirectly through estrogenic stimulation of ovarian surface epithelium (11, 14, 15). Furthermore, the hormonal hypothesis claims that excess androgen stimulation leads to increased epithelial ovarian cancer risk, which in turn may be decreased by progesterone stimulation (12, 14). Finally, the inflammation hypothesis starts from the assumption that ovarian tumourigenesis may be
enhanced in response to genetic damage caused by the inflammatory factors, such as those deriving from environmental factors, endometriosis, genital tract infections, or the ovulatory process itself (13, 14).

A strong family history of ovarian or breast cancer constitutes the most important risk factor for ovarian cancer and this can be traced to an inherited mutation in one of two genes, BRCA1 and BRCA2, which account for approximately 10% of all ovarian cancers (16). In addition, increased ovarian cancer risk is associated also with hereditary nonpolyposis colorectal cancer (HNPCC, also known as Lynch II) syndrome with inherited mutations in DNA mismatch repair genes, primarily hMSH2 and hMLH1 (16). In addition to genetic factors, aging is a clear risk factor for ovarian cancer, since the incidence increases with age (17). In support of the incessant ovulation hypothesis, factors reducing the number of lifetime ovulations have been associated with a reduced risk of epithelial ovarian cancer. These include the number of pregnancies, oral contraceptive use, breastfeeding (17, 18), and possibly late age at menarche as well as an early age at menopause (19). In addition, tubal ligation and hysterectomy have been associated with reduction of ovarian cancer risk (17, 18, 20). Infertility itself may be a significant risk for ovarian cancer development (21, 22), but, at present, there is no convincing data of an increased risk associated with infertility treatment (23). Postmenopausal hormone replacement therapy has been suggested to associate with increased ovarian cancer risk, although the data on association between combined hormone replacement therapy and ovarian cancer are not entirely consistent (24-27). Furthermore, in particular long-duration use of unopposed estrogen has been associated with ovarian cancer risk in recent prospective studies (25, 28).

Epithelial ovarian cancers appear to arise from ovarian surface epithelial cells via one of at least two pathways: Type I tumours by slow development of precursor lesions, from an inclusion cyst to a benign adenoma or cystadenoma of low malignant potential through to metastatic adenocarcinoma, and type II tumours arising spontaneously and aggressively from the surface epithelium or inclusion cysts without any precursor lesions (29-31). The different histological types of epithelial ovarian cancer are associated with different molecular genetic alterations and pathways of development (Figure 1) (29-31). Low- and high-grade serous carcinomas most probably arise via
different pathways, the former progressing along an adenoma-borderline tumour-carcinoma sequence and being characterised by KRAS or BRAF mutations, and the latter appearing to arise de novo from morphologically normal or dysplastic epithelium within inclusion cysts or on the surface of the ovary involving mutations of p53 and BRCA1 and/or BRCA2 dysfunction (29-31). High-grade endometrioid ovarian carcinomas involve molecular genetic alterations similar to high-grade serous carcinomas and are probably closely related, whereas low-grade endometrioid carcinomas display mutations in CTNNB1 (the gene encoding β-catenin) and PTEN as well as microsatellite instability (MI), and probably originate from ovarian endometriosis or from endometrioid borderline tumours (31). Mucinous carcinomas exhibit mutations in KRAS and seem to arise via an adenoma-borderline tumour-carcinoma sequence (29-31). Furthermore, clear cell carcinomas probably have their origin in ovarian endometriosis and possess mutations of TGFβR2, overexpression of HNF-1β, abnormalities of BRCA1 and/or BRCA2, and microsatellite instability. The molecular changes present in transitional-cell carcinomas of the ovary remain largely unknown (29, 31), and malignant mixed mesodermal tumours as well as undifferentiated carcinoma have been designated as type II tumours (29).
Figure 1. Model of epithelial ovarian cancer development and molecular alterations associated with the different histological subtypes. Modified from Christie and Oehler 2006 (31).

The spread of epithelial ovarian cancer occurs mainly via three mechanisms: direct extension into contiguous pelvic structures, dissemination of free cancer cells shed from the ovary into the peritoneal cavity and their distribution by normally circulating peritoneal fluid, and spread by the lymphatic system (32-34). In contrast, hematologic spread of ovarian cancer is not a common mode of ovarian cancer extension (32, 33). The lymphatics of the ovary drain into the external iliac, common iliac, hypogastric, lateral sacral, para-aortic nodes, and occasionally, to the inguinal nodes (3). As a consequence of these ways of dissemination, a common site for metastases is the peritoneum, including the omentum and pelvic and abdominal viscera, with frequent diaphragmatic and liver-surface as well as pulmonary and pleural involvement (3, 32, 35). The ovarian cancer is staged according to the International Federation of Gynecology and Obstetrics (FIGO) staging system (36) based on the width of the
ovarian cancer spread (Table 1) determined by surgical, cytological, and histopathological findings in laparotomy, and possibly modified by clinical and radiological findings (3).

**Table 1.** Staging of ovarian cancer according to the International Federation of Gynecology and Obstetrics (1988; Ref. (36)).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Ovarian cancer with growth limited to the ovaries</td>
</tr>
<tr>
<td>Ia</td>
<td>growth limited to one ovary; no ascites present containing malignant cells. No tumour on the external surface; capsule intact</td>
</tr>
<tr>
<td>Ib</td>
<td>growth limited to both ovaries; no ascites present containing malignant cells. No tumour on the external surfaces; capsules intact</td>
</tr>
<tr>
<td>Ic</td>
<td>tumour either Stage Ia or Ib, but with tumour on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings</td>
</tr>
<tr>
<td>II</td>
<td>Ovarian cancer with growth involving one or both ovaries with pelvic extension</td>
</tr>
<tr>
<td>IIA</td>
<td>extension and/or metastases to the uterus and/or tubes</td>
</tr>
<tr>
<td>IIB</td>
<td>extension to other pelvic tissues</td>
</tr>
<tr>
<td>IIC</td>
<td>tumour either stage IIA or stage IIB, but with tumour on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings</td>
</tr>
<tr>
<td>III</td>
<td>Ovarian cancer with tumour involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals Stage III. Tumour is limited to the true pelvis but with histologically proven malignant extension to small bowel or omentum</td>
</tr>
<tr>
<td>IIIA</td>
<td>tumour grossly limited to the true pelvis with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces</td>
</tr>
<tr>
<td>IIIB</td>
<td>tumour involving one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative</td>
</tr>
<tr>
<td>IIIC</td>
<td>abdominal implants greater than 2 cm in diameter and/or positive retroperitoneal or inguinal nodes</td>
</tr>
<tr>
<td>IV</td>
<td>Ovarian cancer with growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV</td>
</tr>
</tbody>
</table>
2.3. Diagnosis and management of epithelial ovarian cancer

Although a significant proportion of patients with ovarian cancer apparently are symptomatic even months before diagnosis, the symptoms are unspecific e.g. abdominal pain, abdominal swelling, bloating, gastrointestinal disturbances, urinary symptoms, fatigue and malaise (37-39). The lack of clear pathognomonic symptoms contributes to the difficulty of making a clinical diagnosis of ovarian cancer and to the resulting diagnostic delay (37, 38), and thus the majority of the ovarian cancers are diagnosed at an advanced stage (stage III or IV) (3). Currently, there is no effective screening protocol with an acceptable level of sensitivity or specificity available for the general population (40). A suspected diagnosis of ovarian cancer is confirmed after a complete physical pelvic and rectovaginal examination including a transvaginal ultrasound examination. The additional preoperative assessment includes family history, a chest x-ray, and ultrasound examination and CT scan of the abdomen and pelvis. Tumour markers studied should include CA-125 and also carcinoembryonic antigen (CEA), alpha-feto-protein, β-chorionic gonadotropin (βHCG) and lactate dehydrogenase (LDH) in the sense of differential diagnostics. The histological diagnosis is usually confirmed at the time of surgery with frozen section analysis (41).

The management for the patient who has completed childbearing is a surgical procedure: comprehensive staging of ovarian cancer and aggressive cytoreduction of advanced disease (34). Comprehensive surgical staging is the most important factor in determining the appropriate adjuvant management (41). The operation should include total hysterectomy with bilateral salpingo-oophorectomy, infracolic omentectomy, para-aortic and pelvic lymphadenectomy, careful examination, palpation and random and focused biopsies of diaphragm and peritoneum, as well as cytologic evaluation of ascites or washings (41). The primary aim of surgery is to achieve maximal cytoreduction with no gross residual disease after primary surgery. After the primary operation, only those patients with stage Ia or Ib, grade 1 cancers, except for those with clear cell histology, can be followed without the need for adjuvant chemotherapy (34, 41). All other patients need to be considered for adjuvant chemotherapy, this most commonly consisting of a combination of a taxane and platinum therapy (34, 41).

However, despite this treatment up to 75% of advanced-disease patients eventually
suffer a recurrence and succumb to the disease (42, 43). At the present, there is no established treatment for recurrent ovarian cancer, and the major influencing criterion is platinum sensitivity, i.e. if the recurrence occurs within (platinum-resistant) or more than 6 months (platinum-sensitive) after completion of primary platinum-based chemotherapy. Active agents in the treatment of recurrent ovarian cancer include docetaxel, topotecan, liposomal doxorubicin, etoposide, gemcitabine, paclitaxel, carboplatin, cisplatin, and vinorelbine, all of which have been shown to have similar response rates, ranging from 10-20% in platinum-resistant and 20-35% in platinum-sensitive patients (34). Patients experiencing a recurrence more than 6 months after primary therapy can be rechallenged with platinum-based chemotherapy, usually in combination with paclitaxel, whereas other agents are considered for second recurrence or platinum insensitivity (34). In addition, expectations of toxicity and impact on patient's life quality contribute to the choice of second- or third-line chemotherapy. Surgical reassessment by nature of a "second look" is rarely indicated (34, 41).

2.4. Clinicopathological prognostic factors in epithelial ovarian cancer

The most consistent prognostic factors observed in different studies are stage and post-operative residual disease (44-46). In addition, although less often, also age at diagnosis, histological grade and histological subtype have independently predicted survival in some studies (44-46). Potential prognostic importance has been suggested also for several molecular markers, but none have been conclusively shown to be of independent prognostic significance and require clarification.

2.4.1. Age

The median age at diagnosis of ovarian cancer has been reported as being 63 years in USA during the period 2000-2003 (47). The incidence of ovarian cancer increases with advancing age, and higher proportions of the disease are seen in patients aged 50-69 years, with only 11% of patients being diagnosed younger than 40 years (3). The age of the patient has been shown to be an independent predictor of survival in several studies (5, 45, 48-52). The poor survival in the elderly could be due to high probability of being diagnosed at an advanced stage (3, 53), or perhaps less aggressive treatment is used in
the elderly than for younger women (48, 54-56), possibly at least partly because of co-morbidity (57, 58).

2.4.2. Stage
Survival rates between the FIGO stages differ from each other, with overall survival rates at 5 years of 89.3-78.2% for stage Ia-c, 79.2-68.2% for stage IIa-c, 49.2-28.9% for stage IIIa-c, and only 13.4% for stage IV according to the FIGO annual report (3). The FIGO stage is found to predict prognosis more consistently than many of the other factors (44-46) and represents the basic criterion for selecting patients for different treatment strategies emphasising the need for accurate surgical staging (41, 59). Understaging of the disease has been observed in up to 30% of the patients (60-62). Systemic aortic and pelvic lymphadenectomy has been shown to detect a higher proportion of patients with metastatic lymph nodes as compared with lymph node sampling (63), and the thoroughness of the staging has been proposed as being a determinant of survival of early-stage ovarian cancer patients (64-66). Under these circumstances, it is unfortunate that a significant number of patients with an apparent early-stage ovarian cancer are still not staged according to the recommended surgical protocol (64, 67).

2.4.3. Primary surgery
The amount of residual disease after primary cytoreductive surgery is one of the key prognostic factors in ovarian cancer. Since this was described by Griffiths and colleagues in 1970's (68), a large number of studies have shown the survival benefit of primary optimal cytoreduction (44-46, 52, 69-71). The definition of "optimally resected disease" is not consistent, but a residual tumour of less than 1 cm in maximal diameter constitutes optimal cytoreduction according to The Gynecological Oncology Group (72). However, patients in whom tumours are primarily debulked to no gross residual disease derive the greatest survival benefit (3, 71, 73, 74) and it has been recommended that optimal debulking surgery should be defined as no visibly residual tumour load (74, 75).
2.4.4. Histological subtype

Epithelial ovarian tumours, which constitute the majority (90%) of malignant ovarian tumours, are further classified as serous, mucinous, endometrioid, clear cell, transitional cell, squamous cell, mixed epithelial, undifferentiated and unclassified histological subtypes according to the World Health Organization (WHO) (Table 2) (76). The most frequent subtype is a serous neoplasm, followed by endometrioid, mucinous, clear cell, undifferentiated, and mixed epithelial subtypes (Table 2). Serous carcinoma is predominantly found in advanced stages of the disease, peaking at stage III, whereas clear cell, endometrioid and mucinous carcinomas tend to remain more frequently confined to the ovary or pelvis (stages I-II). Among the six most common histological subtypes, the overall survival rate at five years is poorest for the serous (37%) and undifferentiated (37%) histological subtypes, while mucinous tumours are associated with the most favourable prognosis (63%) especially at early stages (88%) (3). In addition, there are conflicting data on the behaviour of clear cell carcinoma of the ovary. In some studies, the prognosis appears to be similar to that of other ovarian carcinomas (77, 78), whereas in other studies, clear cell subtype in comparison to serous and other non-clear-cell epithelial ovarian carcinomas, has been suggested to exhibit a poor prognosis at advanced stages (79-81) with insensitivity to platinum-based chemotherapy (80-83). However, the significance of histological subtype as an independent predictor of prognosis has remained controversial in epithelial ovarian cancer (44-46, 50, 70, 73, 84).

<table>
<thead>
<tr>
<th>Histological subtype</th>
<th>Frequency</th>
<th>Overall survival rate at 5 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous adenocarcinoma</td>
<td>30-70%</td>
<td>37%</td>
</tr>
<tr>
<td>Mucinous adenocarcinoma</td>
<td>5-20%</td>
<td>63%</td>
</tr>
<tr>
<td>Endometrioid adenocarcinoma</td>
<td>10-20%</td>
<td>60%</td>
</tr>
<tr>
<td>Clear cell adenocarcinoma</td>
<td>3-10%</td>
<td>59%</td>
</tr>
<tr>
<td>Transitional cell carcinoma (TCC)/</td>
<td>rare</td>
<td>35% for TCC</td>
</tr>
<tr>
<td>Malignant Brenner tumour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>rare</td>
<td>28%</td>
</tr>
<tr>
<td>Mixed epithelial</td>
<td>0.5-4%</td>
<td>57%</td>
</tr>
<tr>
<td>Undifferentiated carcinoma</td>
<td>4-7%</td>
<td>6-37%</td>
</tr>
<tr>
<td>Unclassified adenocarcinoma</td>
<td>rare</td>
<td>not yet known</td>
</tr>
</tbody>
</table>
2.4.5. Histological grade

There is no universally accepted grading system for epithelial ovarian carcinoma. The most widely utilised grading systems are those of the FIGO (87) and the WHO (76, 88), based mainly on architectural structures of the tumours, although not included in the ovarian cancer staging system of either FIGO (36) or WHO (76). In Finland, a three-class grading system based on architecture and nuclear atypia of the tumour has been recommended by the Finnish division of the International Academy of Pathology (89). The overall survival rate at five years has been reported to be 49-86% for well-differentiated (grade 1) tumours, 26-78% for moderately differentiated (grade 2) tumours, and 27-66% for poorly differentiated (grade 3) tumours (3). Histological grading appears to have prognostic value in epithelial ovarian cancer, particularly for early-stage disease (44, 65, 90, 91). However, its independent contribution has not been firmly established (44-46, 73), although grading can have important implications for therapeutic decisions, in particular in FIGO stage I (41). Assessment of grading's contribution to prognosis is hampered by interobserver variability between pathologists, lack of standardisation of grading schemes, and differences in the treatment protocols (45, 46, 92-94).

2.4.6. Other prognostic factors

2.4.6.1. CA-125

CA-125 is a mucin (95) with a widespread distribution in human tissues and present to varying degrees in the serum of patients with a variety of tumours. The CA-125 concentration is most consistently elevated in epithelial ovarian cancer, but CA-125 levels are elevated also in multiple gynecological and non-gynecological benign diseases as well as in many other cancers e.g. cancers of the lung, breast, endometrium, cervix, fallobian tube and gastrointestinal tract (96). Approximately 50% of the patients with stage I ovarian cancer and 90% of those with the disease disseminated outside the ovary have elevated concentrations of CA-125 in their sera (97), and the frequency of positivity is greater in patients with nonmucinous tumours (97, 98). In clinical practice, measurement of CA-125 may aid in differentiation between benign and malignant
pelvic masses in postmenopausal women (96), whereas CA-125 is not suitable for ovarian cancer screening as a single marker due to its limited sensitivity and specificity (40). CA-125 has been shown to be useful in measuring the response to initial chemotherapy since an indication for cessation or continuation of treatment can be based on trends in CA-125 levels. In addition, it has been claimed that CA-125 can provide a short lead-time for the detection of relapsed ovarian cancer before clinical progression of disease, though the clinical value of this is less clear (73, 96). Both absolute levels before therapy (99-103) and half-life (104-106) of CA-125 have been shown in several publications to be independent prognostic indicators.

2.4.6.2. Ploidy
Aneuploidy is found in about 45-50% of stage I (107, 108) and in about 75% of stage III-IV (108, 109) ovarian carcinomas. In contrast to the subjectivity of histological grading and typing of ovarian cancer, ploidy determination offers the advantage of being an objective and reproducible measurement and therefore has attracted great attention in prognostic studies, although some with rather small materials (44). Tumour aneuploidy has been clearly demonstrated to be an independent adverse prognostic factor in both early- and advanced-stage epithelial ovarian cancer in several multivariate studies with more than 100 patients included (107, 110-115), although also differing results exist (116-122). Additionally, tumour ploidy has been suggested to be of assistance in selecting patients with early ovarian cancer for adjuvant treatment after surgery (111).

2.4.6.3. p53
p53 is a tumour suppressor gene, which is located on chromosome 17 (123) and plays a role in both cell proliferation and apoptosis (124). p53 is inactivated in about half of human cancers through mutations in the gene, and disruption of the p53 tumour suppressor pathway is thought to contribute to the malignant phenotype. A mutation of the p53 gene is the most common genetic alteration in ovarian cancer, with mutations being present in approximately 50% of advanced-stage and in 10-20% of early-stage ovarian carcinomas. In addition, mutations of the p53 gene have been observed also in
borderline ovarian tumours, although not so frequently as in malignant tumours (123, 124). Especially, mutations of the p53 gene have been found to be common in high-grade as opposed to low-grade ovarian serous carcinomas, and are thought to provide evidence supporting the dualistic tumour progression model for ovarian carcinoma development (29, 30). Due to the longer half-life of the mutant p53 protein, it accumulates in the nucleus and this permits the immunohistochemical detection of the mutant protein (125), although not entirely extensively (126, 127). The p53 gene is one of the most widely studied genes in relation to the prognosis of ovarian cancer. p53 expression has been reported to have independent prognostic value in some multivariate studies (128-137) also in one prospective study setting (138), but not in others (139-150) probably partly due to the marked methodological divergencies between the studies.

2.5. Prognostic value of biological factors in epithelial ovarian cancer

2.5.1. Extracellular matrix and cell adhesion related factors

The extracellular matrix is a highly organised molecular network comprising a variety of collagen superfamily and non-collagenous molecules such as glycoproteins, proteoglycans, and hyaluronan (85). This delicate composition is constantly interacting with the adjacent parenchymal cells, modulating the functional activity of these cells as well as undertaking the continuous remodelling of the extracellular matrix structure and composition during many physiological and pathological processes including normal development, inflammation, wound healing and tumour development (85, 151).

Proteoglycans are molecules characterised by the presence of long, unbranched, high molecular weight side chains, called glycosaminoglycans (GAGs) that are covalently attached to a core protein and include chondroitin, dermatan, heparin, and keratan sulphate. Proteoglycans include commonly more than one type of GAG chains and can be classified as heparin sulphate proteoglycans, lecicans with side chains consisting mainly of chondroitin sulphate, and small leucine-rich proteoglycans with predominantly chondroitin/dermatan sulphate or keratan sulphate side chains. These so-called lecicans, also known as hyalectans or large aggregating proteoglycans, include
aggrecan, versican, neurocan and brevican (85, 152). Proteoglycans can interact with other extracellular matrix molecules and regulate a spectrum of cellular functions including cell adhesion, signalling, migration, proliferation and differentiation (152). Several cytokines, including transforming growth factor β, platelet derived growth factors and epidermal growth factor, seem to co-operatively regulate proteoglycan levels (153).

Cell adhesion to the adjacent structures is essential for the formation as well as the maintenance of cellular and tissue integrity. Cell adhesion regulates many important cellular processes including motility, growth, differentiation, and survival (154). Cells adhere either directly to other cells or to the extracellular matrix. The types of cell-cell adhesion in epithelial cell sheets consist of tight-, adherens- and gap-junctions. The different junctions are built up of a transmembrane protein connected to a number of intracellular proteins, which in turn connect to the cytoskeleton thus stabilising the complex (155). The most common type of cell-cell adhesion, adherens junctions, are made up of the cadherin-catenin complex (154).

Cell adhesion receptors can be divided into five groups i.e. 1) the integrin family mediating both cell-cell and cell-extracellular matrix adhesion, 2) the cadherin family, 3) the selectin family and 4) the immunoglobulin family mediating cell-cell adhesion, and 5) other transmembrane proteoglycans, such as CD44, mediating cell-extracellular matrix adhesion (156). Seamless co-ordination between these molecules is essential for tissue integrity and morphogenesis (157). In addition to their structural role, cell adhesion molecules function also by modulating intracellular signalling pathways in response to extracellular conditions and in that way they regulate gene expression, cell adhesion, migration, proliferation, death and differentiation status (156). Decreased adhesion and aberrant adhesion-mediated signalling are typical of malignant transformation, contributing to enhanced migration, proliferation, invasion and metastasis of tumour cells (154).

2.5.1.1. Hyaluronan and CD44

Hyaluronan is a unique glycosaminoglycan that forms a major component of the extracellular matrix. It is composed of repeats of disaccharides of glucuronic acid and
N-acetylglucosamine. The hyaluronan chain extrudes through the plasma membrane onto the cell surface or into the extracellular matrix after its synthesis at the inner surface of the plasma membrane by one of three hyaluronan synthases (HAS1, -2, or -3) (158, 159). Hyaluronan has a remarkable ability to retain water, leading to an important role in tissue homeostasis and biomechanical integrity. Hyaluronan also forms a template for interactions with proteoglycans (Figure 2) and other extracellular macromolecules such as versican, aggrecan and other hyaladherins, that is important in the assembly of extracellular and pericellular matrices. This modulation of extracellular space by hyaluronan contributes to the genesis of a favourable environment for tumour cell division and migration (160). In addition, hyaluronan can influence cell behaviour by interacting directly with the cell surface either by binding to cell surface receptors, such as CD44 and receptor for hyaluronic acid mediated motility (RHAMM), or by sustained attachment to hyaluronan synthase. This interaction leads to signal transduction and cytoskeletal rearrangements that regulate cell growth, survival and motility (161). Furthermore, hyaluronan may promote tumour progression also by enhancement of angiogenesis (162-164).

![Figure 2](image.png)

**Figure 2.** The structural demonstration of E-cadherin-catenin complex and binding of hyaluronan to CD44 and proteoglycans (versican) in peri- and extracellular matrix assembly. Modified from Wijnhoven et al. 2000 (165) and Toole 2004 (161).
Increased hyaluronan expression has been correlated with increased invasiveness of cancer cells in vitro (166), and high levels of stromal hyaluronan have been associated with poor survival in several cancers, for example ovarian (167), breast (168), prostate (169, 170), and non-small cell lung (171) adenocarcinomas. Additionally, cancer cell-associated hyaluronan accumulation has been associated with poor outcome in the patients with breast (168) and colorectal (172) carcinomas, and also elevated levels of the enzymes that cleave hyaluronan, namely hyaluronidases (usually HYAL1), have been found in some malignant tumours (164, 173) and might promote tumour progression through the stimulative effects of hyaluronan breakdown products on angiogenesis (163, 174).

CD44 is a transmembrane protein that is encoded by a single gene located on human chromosome 11 and which exists as a standard isoform (CD44s) as well as several CD44 variant isoforms produced through alternative splicing (175). In addition to alternative splicing, CD44 function can be modulated also by post-translational modifications such as phosphorylation and glycosylation (176). CD44 binds hyaluronan (177) (Figure 2), and interactions between CD44 and hyaluronan have been suggested to affect cell adhesion (178), migration (178, 179), growth (180) and peritoneal implantation of ovarian cancer cells (181, 182). Expression of CD44 variants is associated with clinically aggressive behaviour in some human tumours (183, 184). Studies investigating CD44 expression and survival in ovarian cancer have reported contradictory results. Some studies have demonstrated that high CD44 expression in primary tumours is associated with poor (185-188) or improved (189-192) outcome, while others have found no association between CD44 and survival (193-199).

2.5.1.2. Versican
Versican is a member of the family of large aggregating proteoglycans also known as hyalectans or lecicans. It is composed of a core protein with chondroitin sulfate glycosaminoglycans attached to the core (151). Versican is encoded by a single gene localised on chromosome 5q12-14 in the human genome (200), and four versican isoforms resulting from alternative splicing processes have been identified: the full-length isoform V0 and smaller isoforms V1, V2, V3 with differences in the central
portion of the core proteins. In versican V0, two chondroitin carrying segments, GAG-α and GAG-β, are present, whereas the smaller V1 and V2 isoforms lack the GAG-α or the GAG-β domain, respectively (201, 202), and the GAG carrying modules are both deleted from the V3 isoform (203). All versican splice forms include globular domains at the amino terminus (G1) and carboxyl terminus (G3). The G1 domain binds hyaluronan with a high affinity (204), and the G3 domain consists of a set of lectin-, epidermal growth factor- and complement binding protein-like subdomains (205, 206).

In normal tissues, versican is found in connective tissues, most smooth muscle tissues, veins and arteries, cartilage, neural tissue, glandular epithelia, and skin (207). Versican interacts with its binding partners through its N- and C-terminal globular regions as well as its central GAG-binding region. It can bind to extracellular matrix components such as hyaluronan, type I collagen, tenascin-R, fibulin-1 and -2, fibrillin-1, fibronectin and chemokines. It also binds to the cell surface proteins CD44, P- and L-selectin, integrin β1, epidermal growth factor receptor, and P-selectin glycoprotein ligand-1 (208). Versican is one of the main components of the extracellular matrix where it participates in forming a loose and hydrated matrix (Figure 2). Since it undergoes direct or indirect interactions with cells and molecules, versican is able to regulate cell adhesion and survival, cell proliferation, cell migration, and extracellular matrix assembly (209).

Versican has been found in many malignancies, including breast (210, 211), endometrial (212), prostate (213) and colon (214) carcinoma, being localised mainly in the peritumoural stromal tissue but also cancer cell-associated expression has been reported in melanoma (215) as well as in prostate (216), endometrial (212), pharyngeal squamous cell (217) and non-small cell lung (218) cancers. Versican has been suggested to cause decreased cell-cell and cell-matrix adhesion, thus facilitating local cancer cell invasion and the formation of metastases (209). Elevated levels of versican have been associated with poor outcome in many cancers including breast (211, 219), endometrial (212), prostate (213) and oral squamous cell (220) carcinomas. However, the distribution and prognostic value of versican has not yet been elucidated in epithelial ovarian cancer.
2.5.1.3. E-cadherin-catenin complex

In general, the E-cadherin-catenin complex is important for maintaining tissue architecture, and this complex can limit cell movement and proliferation. The major epithelial cell cadherin, E-cadherin, binds via its cytoplasmic domain to β- or γ-catenin (plakoglobin), which are linked to the actin cytoskeleton via α-catenin (154). E-cadherin can bind also p120-catenin, which contributes to stabilisation of cadherin-catenin complex (221) (Figure 2). These interactions are critical for the establishment of stable and functional adherens junctions. The disruption of normal cell-cell adhesion by the downregulation of the cadherin or catenin expression may lead to enhanced cell migration and proliferation as well as invasion and metastasis of tumour cells (154). Indeed, the loss of E-cadherin expression has been associated with the transition from adenoma to invasive pancreatic carcinoma and the acquisition of a metastatic capability (222), furthermore experiments where cadherin expression has been restored have confirmed E-cadherin as an invasion suppressor (223). In addition, altered expression and localisation of the catenins can play an important role in tumourigenesis (224, 225).

In addition to providing a link between cells, the cadherin-catenin complex can influence various signalling pathways (154). Accordingly, β-catenin plays a dual role in the cells: in addition to its structural role in the complex, β-catenin can act as a transcription cofactor in the nucleus by interacting with the LEF/TCF (lymphoid enhancer factor/T-cell factor) DNA binding proteins. β-catenin-mediated transcription is activated by the Wnt pathway, the activation of which results in the inhibition of β-catenin degradation, its nuclear accumulation and transcriptional activation of LEF/TCF target genes, such as Cyclin D1 and Myc (Figure 3). Translocation of β-catenin into the nucleus might be required to induce the expression of genes that promote cell proliferation and invasion (154, 226).
Figure 3. Representation of the central role of β-catenin in Wnt signalling. β-catenin can exist in a cadherin-bound form, taking part in the regulation of adhesion, or it can be sequestered in a complex with axin, APC, and GSK-3β, enabling its degradation by β-TrCP. Activation of Wnt pathway or other abnormalities in this degradation pathway results in the entry of β-catenin to the nucleus, where it can bind to transcription factors (LEF/TCF) and stimulate transcription of target genes. Modified from Wijnhoven et al. 2000 (165) and Nelson and Nusse 2004 (226).

In accordance with the mesodermal origin of ovarian surface epithelium and its less firmly determined differentiation compared to many other epithelia (227), the E-cadherin expression, an epithelial characteristic, is rarely detected in normal ovarian surface epithelium (228, 229). However, E-cadherin expression has been found to increase in metaplastic ovarian surface epithelium, benign and neoplastic ovarian tumours (228-230) as the cells become increasingly committed to epithelial phenotypes. During the progression of ovarian cancer, the tumour cells once again lose their differentiation when they undergo an epithelial-mesenchymal transition (231), and accordingly, a decrease in E-cadherin expression is observed in poorly differentiated ovarian cancers (228, 228, 232, 233), most probably because of silencing the E-cadherin gene via methylation of its promoter (233) or by transcriptional repressors such as Snail and Slug (234), whereas somatic mutations of E-cadherin gene have been reported to be
rare in ovarian cancer (235). Consequently, in ovarian cancer E-cadherin has been suggested to contribute to neoplastic progression in the earliest stages but to act as a late stage tumour suppressor (236, 237).

In contrast to E-cadherin, the expression of catenins can be observed in normal ovarian surface epithelium (228, 238), possibly in association with cadherins other than E-cadherin (238), whereas the expression is found to be reduced in ovarian cancer (228). Furthermore, the reduced expression of α-, β- or γ-catenins has been found in ovarian cancers with adverse clinicopathologic features (239, 240). The prognostic significance of E-cadherin-catenin complex is still unclear, but aberrant expression of E-cadherin has been shown to associate with poor survival in many malignancies (241-243). Additionally, reduced expressions of α-, β- and γ-catenins have been reported to predict unfavourable prognosis in many carcinomas (241, 244-247). Previous studies on E-cadherin-catenin complex in ovarian cancer are quite limited and have left the prognostic role of this complex unclear (186, 232, 248-254).

2.5.1.4. Other factors related to cell adhesion

Integrins are cell surface glycoprotein receptors that mediate cell adhesion to extracellular matrix, and also cell-cell binding to other adhesion molecules. They are composed of a heterodimer of two noncovalently associated transmembrane α- and β-subunits, that can combine to give at least 24 integrin dimers, and the particular combinations of the α- and β-chains define the specific repertoire of ligands (156). The cytoplasmic tails of the α- and β-chains interact with cytoskeletal proteins and activate signal transduction pathways to regulate cell proliferation, apoptosis, gene expression, differentiation, and cell migration (255). Cells can gain more potential to invade and metastasise as a result of the altered expression, function, and activation of integrins, and several integrins have been also indirectly linked to tumour development via their role in regulating angiogenesis (256). The relationships between expression of some integrins and clinical stage, tumour progression, and prognosis have been reported e.g. in cases of colon and breast cancers (156). In addition to the proposed role in mediating the adhesion of ovarian carcinoma cells to mesothelial cells (182), an association of integrin expression with survival has been claimed also for ovarian cancer (257, 258).
Selectins are a small family of calcium-dependent transmembrane glycoproteins including E-, P-, and L-selectins, that mediate heterotypic cell-cell adhesion. E- and L-selectins may contribute to tumour growth by increasing angiogenesis or by activation of selectin-dependent signal transduction pathways, which can regulate cancer cell proliferation, migration, and survival (156). E- and P-selectins have been reported to be absent on the ovarian tumour cells in vitro (259), whereas an increased serum concentration of E-selectin in ovarian cancer has been reported (260). The significance of this observation, as well as the prognostic significance of selectins in ovarian cancer, remains unknown.

The immunoglobulin superfamily consists of adhesion molecules that mediate cell-cell adhesion and contain extracellular immunoglobulin domains. These molecules mediate interactions of endothelium with leukocytes and cancer cells, and several members of this family have been linked to cancer progression. For example, Ep-CAM, transmembrane glycoprotein expressed on the surface of most human epithelial cells, may negatively regulate cadherin-mediated adhesion and has been associated with poor prognosis in breast, colorectal, prostate (156) and ovarian cancers (261). Other members of the immunoglobulin superfamily, such as intercellular adhesion molecule 1 (ICAM-1) (262) and extracellular matrix metalloproteinase inducer (EMMPRIN) (263), have been linked to survival of ovarian cancer patients as well.

2.5.2. Angiogenesis-related factors

Angiogenesis, the growth of new capillary blood vessels is essential for tumour growth and metastasis, since neovascularisation of a tumour is necessary if the tumour is to expand beyond 2 mm³ (264). Normally the tissue microenvironment maintains a delicate balance between pro- and anti-angiogenic growth factors (265). However, depending on environmental factors such as hypoxia, the balance can be shifted in favour of angiogenesis by upregulating the levels of proangiogenic factors or by reducing those of angiogenesis inhibitors, such as thrombospondin-1 (TSP-1), angiostatin and endostatin. The major angiogenic factors include VEGF, fibroblast growth factor (FGF), interleukin-8 and angiopoietins (266). In addition, several other factors have been related to the regulation of angiogenesis. For example, hyaluronan
(163) and versican (267) have been claimed to modify angiogenesis.

2.5.2.1. Inducible nitric oxide synthase

Nitric oxide (NO) is a multifunctional gaseous compound and a short-lived highly reactive molecule that regulates many physiological and pathophysiological processes, such as vascular functions, including angiogenesis (268). NO has been shown to have both promoting and inhibiting effects on tumour progression and metastasis. These effects seem to be context-dependent and can be influenced by the concentration and duration of NO exposure and cellular sensitivity to NO (269).

NO is produced from L-arginine in the presence of cofactors by three different isoforms of NO synthases (NOS): neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2) and endothelial NOS (eNOS, NOS3), each encoded by distinct genes sharing a similar genomic structure. iNOS gene is located on chromosome 17, and like other isoforms, iNOS is composed of a amino-terminal oxygenase domain containing binding sites for haem, tetrahydrobiopterine (BH4) and L-arginine, linked by a calmodulin-recognition site to a carboxy-terminal reductase domain containing binding sites for flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN) and nicotinamide adenine dinucleotide phosphate (NADPH) (270). nNOS and eNOS are expressed constitutively and their activity is dependent on the level of calcium, whereas calcium-independent iNOS is not present in resting cells but its expression in many cells such as tumour cells, tumour-associated stromal fibroblasts and immune cells can be induced by inflammatory cytokines, endotoxin, hypoxia and oxidative stress to produce higher levels of NO than either nNOS or eNOS (269).

Expression of iNOS has been detected in many human tumours, such as breast (271), colorectal (272), prostate (273) and gynecological (274, 275), including also ovarian (276-279) tumours. In many tumours, the expression level of iNOS is increased compared to the corresponding normal tissues though there are some exceptions (280, 281). iNOS has been reported to have prognostic value, although again contradictory results have been reported with different cancers (272, 273, 275, 282). In this respect, the prognostic significance of iNOS has remained controversial in ovarian carcinoma (278, 279).
2.5.2.2. Microvessel counting and the Chalkley method in the assessment of tumour vascularisation

Tumour angiogenesis and its prognostic importance in different carcinomas have been commonly assessed by the tumour microvessel density evaluation technique introduced by Weidner et al. (283), based on counting the number of stained microvessels on areas of most intense tumour vascularisation. For this purpose to mark the endothelium, antibodies to CD34 (284-290), CD31 (291-293), von Willebrand factor (Factor VIII) (294-297) and Ulex (298) have been used in prognostic ovarian cancer studies. CD34 is a cell surface protein that is selectively expressed by human hematopoietic progenitor cells and vascular endothelial cells (299, 300), and it has been shown to identify tumour vessels also in the solid cancers of the ovary (301) in a more consistent way than CD31 or Factor VIII (302). In addition, recently the Chalkley count was introduced, based on the Chalkley eyepiece graticule and the number of grid points that hit stained microvessels, representing a relative area estimate rather than a true vessel count (303). In an international consensus report this assay with CD34 immunostaining was proposed to represent a standard method for angiogenesis quantification in solid tumour sections (304). However, no previous studies on the Chalkley method and its prognostic significance exist with respect to ovarian cancer. Ovarian cancer studies using the microvessel density counting for angiogenesis determination have resulted in conflicting claims about the prognostic significance of angiogenesis (284, 286, 288-294, 297, 298, 305-310). The studies published with multivariate survival analyses are summarised in Table 3.

Angiogenesis as evaluated by the Chalkley method and its prognostic significance have been studied previously in other carcinomas, especially in breast cancer in which the association of poor outcome of patients with increasing angiogenesis has been shown (311-314), although a lack of associations has also been reported (315, 316). In other tumours, the method has been shown to have prognostic significance (317, 318), or to lack significance (319, 320), or its prognostic significance remains equivocal (311, 321-324) probably at least partly because of differences in the methodology.
Table 3. Angiogenesis and prognosis in ovarian cancer; studies with multivariate survival analyses.

<table>
<thead>
<tr>
<th>Author (ref.)</th>
<th>N</th>
<th>Material</th>
<th>Antibody</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hollingsworth et al. 1995 (305)</td>
<td>43</td>
<td>FIGO III-IV</td>
<td>CD34</td>
<td>low average microvessel count → improved disease-free survival</td>
</tr>
<tr>
<td>Obermaier et al. 1999 (284)</td>
<td>63</td>
<td>FIGO I-III</td>
<td>CD34</td>
<td>ns</td>
</tr>
<tr>
<td>Ogawa et al. 2002 (286)</td>
<td>105</td>
<td></td>
<td>CD34</td>
<td>high microvessel density → better progression-free survival</td>
</tr>
<tr>
<td>Gadducci et al. 2006 (307)</td>
<td>101</td>
<td>FIGO III-IV</td>
<td>CD34</td>
<td>high intratumoural microvessel density → better progression-free and overall survival</td>
</tr>
<tr>
<td>Chan et al. 2004 (308)</td>
<td>46</td>
<td>FIGO III-IV</td>
<td>CD34</td>
<td>lower microvessel density → worse survival</td>
</tr>
<tr>
<td>Løsch et al. 2004 (288)</td>
<td>80</td>
<td></td>
<td>CD34</td>
<td>ns</td>
</tr>
<tr>
<td>Raspolini et al. 2005 (306)</td>
<td>60</td>
<td>FIGO IIIc, serous, grade 3</td>
<td>CD34</td>
<td>high microvessel density → poor survival (relapse and death)</td>
</tr>
<tr>
<td>Solomon et al. 2006 (289)</td>
<td>118</td>
<td>FIGO III-IV, serous, grade 2-3</td>
<td>CD34</td>
<td>low microvessel density → improved survival</td>
</tr>
<tr>
<td>Ino et al. 2006 (290)</td>
<td>67</td>
<td></td>
<td>CD34</td>
<td>ns</td>
</tr>
<tr>
<td>Gasparini et al. 1996 (291)</td>
<td>60</td>
<td>FIGO III-IV</td>
<td>CD31</td>
<td>ns</td>
</tr>
<tr>
<td>Nishida et al. 2004 (292)</td>
<td>80</td>
<td></td>
<td>CD31</td>
<td>ns</td>
</tr>
<tr>
<td>Goodheart et al. 2002 (293)</td>
<td>77</td>
<td>FIGO I</td>
<td>CD31</td>
<td>ns</td>
</tr>
<tr>
<td>Goodheart et al. 2005 (309)</td>
<td>77</td>
<td></td>
<td>CD31</td>
<td>high microvessel count → poor disease-specific and recurrence-free survival in FIGO Ic substage</td>
</tr>
<tr>
<td>Alvarez et al. 1999 (294)</td>
<td>85</td>
<td></td>
<td>vWF (FVIII)</td>
<td>ns</td>
</tr>
<tr>
<td>Abulafia et al. 1997 (310)</td>
<td>42</td>
<td></td>
<td>vWF (FVIII)</td>
<td>ns (in primary tumours; significant in omental metastases)</td>
</tr>
<tr>
<td>Shen et al. 2000 (297)</td>
<td>64</td>
<td></td>
<td>vWF (FVIII)</td>
<td>ns</td>
</tr>
<tr>
<td>van Diest et al. 1995 (298)</td>
<td>49</td>
<td>FIGO III-IV</td>
<td>Ulex</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns=no significance
* in multivariate analysis
vWF=von Willebrand factor
FVIII=Factor VIII
2.5.2.3. Other factors related to angiogenesis

2.5.2.3.1. Vascular endothelial growth factor
A number of growth factor receptor pathways promote tumour angiogenesis. One of the major pathways involved in this process is the vascular endothelial growth factor (VEGF) family of proteins and receptors. The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PIGF), which have specific binding affinities towards the VEGF receptors (VEGFR)-1, VEGFR-2 and VEGFR-3 (325). VEGF-A, commonly referred to as VEGF, is a 45-kDa homodimeric glycoprotein that undergoes alternative splicing to different isoforms with a diverse range of angiogenic activities, whereas VEGF-C and VEGF-D play key roles in the process of lymphangiogenesis but their contribution to tumour angiogenesis is unclear (325).

Activation of the VEGF/VEGF-receptor axis triggers multiple signalling networks that result in endothelial cell survival, mitogenesis, migration, differentiation, and mobilisation of endothelial progenitor cells from the bone marrow to distant sites of neovascularisation. In addition, VEGF mediates vessel permeability, leading to deposition of proteins in the interstitium and this facilitates angiogenesis (325). The production of VEGF is regulated by the local oxygen concentration, i.e. hypoxia stimulates VEGF production mainly through a stimulative effect of hypoxia inducible factor (HIF) on VEGF gene transcription. However, the oxygen concentration is not the only regulator of VEGF synthesis (326).

Overexpression of VEGF in tumour tissues has been associated with tumour progression and poor prognosis in a variety of solid tumours including colorectal (327-329), breast (330-332) and prostate (333) carcinomas. The contradictory results from ovarian cancer studies are based on small study materials, with some reports demonstrating an association of VEGF overexpression and poor outcome (292, 297, 306, 309, 334-337) while others report a lack of any association (295, 296).

2.5.2.3.2. Placenta growth factor
Placenta growth factor (PIGF) belongs to the VEGF family and is expressed in placenta,
heart, lung, thyroid gland and skeletal muscle. Four isoforms, PlGF-1-4, differing in size and binding properties are produced through alternative splicing of the human PlGF gene (338). Loss of PlGF has been shown to impair angiogenesis during ischaemia, inflammation, wound healing and cancer (339). However, the role of PlGF in pathologic angiogenesis has proved to be controversial. While PlGF has been claimed to enhance tumour growth, endothelial cell survival and angiogenesis (339, 340), it has also been reported to inhibit tumour angiogenesis and growth (341, 342).

PlGF has been shown to be upregulated in breast, colorectal and gastric cancers compared to the corresponding non-tumourous tissues, and PlGF expression is correlated with aggressive features in the tumours and a poor prognosis in these cancers (343-345). The data of the role of PlGF in ovarian cancer are scanty and indicate that PlGF expression appears to be absent in ovarian tissues (346).

2.5.2.3.3. Hypoxia inducible factor-1α

Hypoxia inducible factor (HIF)-1 is a transcription factor composed of HIF-1α and HIF-1β subunits. While the HIF-1β subunit is expressed constitutively and its activity is controlled in an oxygen independent manner, the HIF-1α is a unique, O2-regulated subunit that primarily determines HIF-1 activity (347, 348). HIF-1 is additionally overexpressed by hypoxia independent pathways, such as those caused by mutations in oncogenes or tumour-suppressor genes (349). The HIF-1α subunit is induced by cellular hypoxia and maintained at low levels in most cells under normal oxygen tension (347, 348). Nuclear accumulation of HIF-1α under hypoxic conditions can transactivate more than 60 target genes involved in many aspects of cancer biology including cell survival, glucose metabolism, cell adhesion and angiogenesis to increase O2 availability or to allow metabolic adaptation to O2 deprivation (349). These genes include those encoding for erythropoietin, iNOS, VEGF, and many enzymes involved in glucose, iron, and nucleotide metabolism (349).

Overexpression of HIF-1α has been shown to occur in many tumour types compared with the respective normal tissues, including ovarian cancer (350-352). Most cancers overexpressing HIF-1α are associated with increased mortality. Adverse effects on patient survival have been found in breast (353-356) and gynecological (357-360)
cancers, whereas HIF-1α does not seem to play such an important prognostic role for instance in colorectal cancer (361, 362). The independent prognostic significance of HIF-1α remains unconfirmed also in ovarian cancer (363-365). The effect of HIF-1α expression in individual cancers seems to be dependent on the specific cancer type as well as the presence or absence of genetic alterations that affect the balance between either pro- or anti-apoptotic effects.

2.5.2.3.4. Thrombospondin-1
The thrombospondin (TSP) family consists of five extracellular glycoproteins, TSP-1-5, of which TSP-1 has functions in platelet aggregation, inflammatory response, cellular adhesion and regulation of angiogenesis (366). The anti-angiogenic activity of TSP-1 has been shown to be mediated by inhibition of endothelial cell migration and induction of endothelial cell apoptosis as well as by its inhibitory effect on VEGF via the antagonism of VEGF-mediated survival, inhibition of VEGF mobilisation from extracellular matrix, as well as directly binding to VEGF to promote its cellular internalisation (367, 368). The regulation of TSP-1 is complex and modulated by growth factors, tumour suppressor genes and oncogenes. The tumour suppressor gene p53 has been shown to upregulate the TSP-1 gene expression at the transcriptional level, with the loss of wild-type p53 expression leading to decreased TSP-1 expression (369), whereas activation of oncogenes such as jun, src, and myc contributes to down-regulation of TSP-1 gene expression (366, 367).

TSP-1 expression has been shown to associate with better survival in colon (370) and invasive cervical (371) carcinomas, whereas no relationship to patient outcome seems to occur in endometrial (372), prostate (373, 374) and breast (375, 376) cancer. The association of TSP-1 with patient outcome has not been clarified in ovarian cancer. TSP-1 gene expression has been associated with the aggressive phenotype and poor survival (377), but conversely more intense immunohistochemical expression of TSP-1 has been shown to associate with better survival in advanced stage ovarian cancer (378) or to have no prognostic significance in early stage disease (309). Data from the literature indicate that during tumour progression and prolonged exposure to a TSP-1 rich environment, tumour cells may develop resistance to its anti-angiogenic effects and
increase the secretion of angiogenic factors such as VEGF to counterbalance the inhibitory effects of TSP-1 (367, 379). In addition, different fragments of TSP-1 may have varying degrees of angiogenesis-modulating properties and some may also possess proangiogenic effects (380), which may explain some of the conflicting results from the prognostic studies.

2.5.2.3.5. Platelet-derived growth factor

The platelet-derived growth factors (PDGFs) comprise a family of polypeptides, PDGF-A, PDGF-B, PDGF-C, and PDGF-D, that form homo- or heterodimers. They exert their cellular effects through two tyrosine kinase receptors, PDGFR-α and PDGFR-β, resulting in cell migration, proliferation and survival (381). In addition to its important role in embryonic development, PDGF overactivity has been related to many types of pathological processes, including cancer development. PDGF ligand-receptor system may function in autocrine stimulation of tumour growth, enhance tumour angiogenesis through pericyte recruitment and affect drug delivery by taking part in interstitial fluid pressure regulation (381).

The expression of PDGF or its receptors has been associated with the metastatic potential of different cancer cells (382-385), and positivity for PDGF-AA has claimed to have a negative impact on the prognosis of advanced stage breast cancer patients (386). Expression of different PDGF dimers has been reported also in ovarian cancer (387-391). In addition, although PDGF receptors were initially suggested to be absent (388, 392), there is now a growing body of evidence that these PDGF receptors exist in ovarian cancer cells (389, 390, 393) and there they may have an autocrine effect on cell proliferation (390). Furthermore, positivity for PDGFR-α has been associated with a reduced survival in epithelial ovarian cancer (387).
3. AIMS OF THE STUDY

The aim of the present study was to evaluate the expression and prognostic value of novel molecular markers as well as their association with several conventional clinicopathological factors in epithelial ovarian cancer. In addition, the purpose was to increase current knowledge of the fundamentals of epithelial ovarian cancer biology since this underlies the disease progression. The specific aims of the present study were:

1) To study the expression and prognostic value of versican as well as its association with hyaluronan expression in epithelial ovarian cancer.

2) To investigate the expression pattern and prognostic significance of E-cadherin-catenin complex in epithelial ovarian cancer.

3) To assess the expression and prognostic relevance of iNOS in epithelial ovarian cancer

4) To evaluate the Chalkley method in the assessment of angiogenesis as well as its prognostic value in epithelial ovarian cancer.
4. MATERIAL AND METHODS

4.1. Study material

The present study material was selected from a consecutive series of 445 women diagnosed and treated for ovarian malignancy in Kuopio University Hospital and Jyväskylä Central Hospital, Finland, between 1976 and 1992, and followed-up until January 2004 with the protocols valid at that time. The relevant clinical data were collected from patients' files retrospectively. Patients with non-epithelial type cancers (n=36), patients who were not operated on (n=35) or who were given any treatment before operation (n=33) and usually represent patients with an extremely poor prognosis, were excluded as were also cases with insufficient tumour material (n=36-42 in studies I-III). In addition, the study cohort in study IV was smaller compared to the other studies and consisted of 175 randomly selected patients. Thus, altogether 310 patients (175 to 305 patients in separate studies) were left for analyses (Table 5).

Tumour staging was re-evaluated from the patients' operative and histopathological files and was based on the standards of the International Federation of Gynecology and Obstetrics (FIGO) (36). Histological subtype and grade were re-evaluated according to the WHO classification (394) previously (128, 395). Death certificates were collected from the patients' files or obtained from the Finnish Cancer Registry. Patients who died because of any post-operative complications (deaths within one month after the operation) were not included in survival analyses. Tissue samples were fixed in 10% formalin and embedded in paraffin. Each 5 µm-thick sample was stained with haematoxylin and eosin.

4.2. Histochemistry of hyaluronan (I)

The biotinylated complex of hyaluronan-binding region of aggrecan and link protein (bHABC) was prepared from bovine articular cartilage and tested for purity (396, 397). The whole staining procedure of hyaluronan has been described in detail previously (167, 396).
4.3. Immunohistochemical stainings (I-IV)

The immunohistochemical stainings were performed according to the following immunoperoxidase protocol, with modifications in antigen retrieval and primary antibodies used (Table 4). Five-µm-thick paraffin-embedded tumour sections were deparaffinised and rehydrated using xylene and graded alcohols. To achieve better antigen retrieval, the sections were heated in a microwave oven in a citrate buffer (Tris-HCL for α-catenin (395)). Endogenous peroxidase activity was blocked by 5% hydrogen peroxide treatment for 5 min, followed by washings with water for 2×5 min and with PBS for 2×5 min. Nonspecific binding was blocked with 1.5% normal horse serum in PBS at room temperature. The samples were incubated overnight at 4°C with the primary antibody, and then for 35 min (30 min for iNOS and 45 min for E-cadherin) with the biotinylated secondary antibody, preceded and followed by washings twice for 5 min with PBS. Then, the slides were incubated (for 45-50 min) in preformed avidin-biotinylated peroxidase complex (ABC Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA) and washed twice for 5 min with PBS. The colour was developed with diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO, USA). The slides were counterstained with Mayer’s haematoxylin, washed, dehydrated, cleared and mounted with DePex (BDH, Poole, UK). An ovarian cancer section processed without the primary antibody served as a negative control in each staining series. Skin samples showing intense staining for versican were used as positive controls for versican staining in each batch. A known E-cadherin positive ovarian cancer sample, a β-catenin positive thyroid cancer sample, a γ-catenin positive lung cancer sample, and an iNOS positive colorectal sample were used as external positive controls in E-cadherin-catenin complex and iNOS stainings. For iNOS staining, mononuclear inflammatory cells that had been stained in the cancerous stroma served as internal positive controls. For CD34, hemangioma served as an external positive control.
Table 4. Summary of the antibodies used in the study.

<table>
<thead>
<tr>
<th>Marker (study number)</th>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versican (I)</td>
<td>anti-versican, clone 2B1, monoclonal</td>
<td>Seikagaku Corporation, Japan</td>
<td>1:1000</td>
<td>microwave + citrate buffer (pH 6.0)</td>
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<tr>
<td>E-cadherin (II)</td>
<td>anti-E-cadherin, clone HECD-1, monoclonal</td>
<td>Zymed Laboratories Inc., South San Francisco, CA, USA</td>
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<td>microwave + citrate buffer (pH 8.0)</td>
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<tr>
<td>α-catelin (Ref. (395))</td>
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<td>microwave + Tris-HCL (pH 9.7)</td>
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<td>anti-beta-catelin, clone 14, monoclonal</td>
<td>Transduction Laboratories, Lexington, KY, USA</td>
<td>1:1000</td>
<td>microwave + citrate buffer (pH 6.0)</td>
</tr>
<tr>
<td>γ-catelin (II)</td>
<td>anti-gamma-catelin, clone 15, monoclonal</td>
<td>Transduction Laboratories, Lexington, KY, USA</td>
<td>1:100</td>
<td>microwave + citrate buffer (pH 6.0)</td>
</tr>
<tr>
<td>CD44 (II) (Ref. (189))</td>
<td>anti-CD44, clone 2C5, monoclonal</td>
<td>R&amp;D Systems, Abingdon, UK</td>
<td>1:1200</td>
<td>microwave + citrate buffer (pH 6.0)</td>
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<tr>
<td>iNOS (III)</td>
<td>anti-iNOS monoclonal</td>
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<td>1:200</td>
<td>microwave + citrate buffer (pH 9.7)</td>
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<td>CD34 (IV)</td>
<td>anti-CD34, Anti-HPCA-1, clone My10, monoclonal</td>
<td>Becton Dickinson Immunocytometry Systems, San Jose, CA, USA</td>
<td>1:200</td>
<td>microwave + citrate buffer (pH 6.0)</td>
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</tbody>
</table>

4.4. Evaluation of the stainings

All stainings were evaluated using a light microscope. The samples were analyzed by two or three observers, who were unaware of the clinical data of the patients. In the case of disagreement between the observers, the slides were reviewed and a consensus was reached.

4.4.1. Versican (I)

All specimens were analysed by three observers (K.V., S.S., V.-M.K.) Versican expression was observed in stromal area and also in cancer cells. The intensity of the total peri- and intratumoural stromal versican in the whole section was evaluated and graded into three categories: weak=1, moderate=2 and strong=3, using the intense staining seen in dermis of skin samples as an external control for strong intensity. The percentage of stroma with the strong versican intensity of the total peri- and
intratumoural stromal area was evaluated and categorised into two groups: low (<15%), or high (≥15%), according to the median percentage value. The percentage of versican positive tumour cells of all cancer cells in the section was also estimated, but for statistical analyses, the tumours were grouped into two categories: versican negative or positive. A tumour was considered positive if any cancer cell-associated versican signal was observed.

4.4.2. Hyaluronan (I)
The hyaluronan staining was evaluated previously (167). Hyaluronan intensity in stroma was categorised as: weak=1, moderate=2, strong=3 using the strongest staining of hyaluronan in the peri- and intratumoural stroma as an internal positive control. The grading of the strong stromal hyaluronan staining percentage of total peri- and intratumoural stromal area was done using the 33rd and 66th percentiles in a frequency distribution: low (<35%), moderate (35-75%), high (＞75%) (167).

4.4.3. E-cadherin-catenin complex (II)
Immunostainings of E-cadherin, β- and γ-catenins were analysed by three observers (K.V., S.S., K.R.). The intensity of the staining of cancer cells was categorised into two groups: weak or strong. Strong intensity was equal to the intensity of the cancer cell membrane seen in the external positive controls, and weak staining corresponded to the intensities between strong and negative. The staining pattern was considered continuous when membranes around cancer cells showed an uninterrupted signal, whereas uncontinuous staining included fragmentary membranous staining and also cytoplasmic staining of cancer cells. The percentage area of tumour cells showing strong continuous staining was analysed from the total tumour cell area and further categorised into two groups according to the median percentage of the expression (5% for E-cadherin, β- and γ-catenins): reduced expression, when ≤5% of the tumour cells expressed strong continuous membranous staining; and preserved expression, when >5% of the tumour cells expressed strong continuous membranous staining. The presence of nuclear staining was also analysed and directly graded into two categories: positive or negative. Nuclear staining was considered positive if >5% of the tumour cell nuclei were positive.
The α-catenin staining was evaluated previously (395). The staining was located on the cell membranes and classified into two groups: normal, when all tumour cells expressed α-catenin homogenously, and reduced, when less than 100% of the tumour cells expressed α-catenin. The reduced α-catenin expression was further categorised into two groups: subnormal (30-99%), or exceedingly low (<30%) (395).

4.4.4. CD44 (II)
The CD44 staining was evaluated previously (167, 189). The expression of CD44 was observed on the cell membranes, scored as a fraction of positive cancer cells in the whole tumour cell area, and further categorised into two groups according to the median percentage of the expression: low, when <10% of the tumour cells expressed CD44, and high, when >10% of the tumour cells expressed CD44 (167, 189).

4.4.5. iNOS (III)
The samples were analysed by three observers (M.A., S.M., V.-M.K.). The expression of iNOS was analysed as a percentage of positive cancer cells in the whole tumour cell area and divided into two categories according to the 66th percentile in the frequency distribution of the expression: low, when ≤70% and high, when >70% of the tumour cells expressed iNOS. In addition, the intensity of iNOS was categorised into three groups; 0 (negative), 1 (weak to moderate) or 2 (strong).

4.4.6. CD34 (IV)
Angiogenesis in ovarian cancer samples was evaluated by two observers (K.S. and M.A.), using the Chalkley method in the assessment of tumour vascularisation. The CD34-stained sections were scanned at low magnification (x12.5 ocular, x4 and x10 objective) for the most vascularised areas within the tumour section, and three areas with the highest vascularity (hot spots) were chosen subjectively. A 25-point Chalkley eyepiece graticule (Graticules, Pyser-SGI Limited, United Kingdom) was applied to each hot spot at higher magnification (x12.5 ocular and x20 objective, corresponding to an area of 0.322 mm²), and oriented to permit the maximum number of points to hit on or within the immunohistochemically stained microvessels. The Chalkley count was
expressed as the mean value of the three counts for each tumour and further classified into two groups according to the median value of Chalkley count: low <8, or high ≥8.

4.5. Statistical analyses
Statistical analyses were carried out using the SPSS for Windows computer programme package (SPSS Inc., Chicago, IL, USA). The relationships between continuous variables were tested using Spearman correlation coefficients and Wilcoxon tests. \( \chi^2 \) test was used to evaluate the interrelationships between categorised IHC variables and their associations with the clinicopathological factors. The coefficient of variation (CV) was used to analyse the reproducibility data from the Chalkley method, and interobserver agreement for the intensity of the iNOS staining was evaluated by kappa statistics. Univariate survival analyses were performed with the Kaplan-Meier method (398), using the log-rank test to analyse the differences between survival curves. Cox’s proportional hazards model in a forward stepwise manner with the log-likelihood ratio significance test was used in the multivariate analysis to examine the independent prognostic value of the variables (399). Disease-related survival (DRS) was defined as the time interval between the date of surgery and the date of death due to ovarian cancer. Recurrence-free survival (RFS) was defined by the time interval between the date of surgery and the date of recurrence. Probability values less than 0.05 were considered as significant in the analyses.

4.6. Ethics
The research was approved by the National Authority for Medicolegal Affairs (N:o 2953/32/300/03) and by the research ethical committee of Kuopio University and Kuopio University Hospital (N:o 4/2003).
5. RESULTS

5.1 Patient characteristics (I-IV)

The clinicopathological characteristics and the treatment of the patients are summarised in Table 5. The median follow-up time of all the patients included in the study (n=310) was 28 months (range 0.3 to 334 months), and for surviving patients (n=79) it was 139 months (range 12 to 334 months). The mean age of the patients at the time of diagnosis was 60 years with a median age of 62 years (range 18 to 85 years). Of the 310 study patients, 264 (85%) were treated by chemotherapy, which was based on platinum in 164 cases (53% of the study population and 62% of the patients treated by chemotherapy). A secondary operation was performed on 121 (39%) of the patients. During the first ten years of follow-up, 76 patients (24%) with an initial complete response for treatment were known to have suffered a recurrence, 92 patients (30%) were without recurrence, the tumour was present or progressing in 129 patients (42%), and data about recurrence were missing from 13 (4%) patients. Sixty seven (85%) of the patients alive after ten years were recurrence-free. At the end of the 10-year follow-up, 199 patients (64%) had died of ovarian cancer and 32 patients (10%) were dead because of other causes. The 10-year disease-related survival rate of the patients was 32%, and the median survival of the patients was 33 months (95% CI 26-41 months). The 10-year recurrence-free survival rate was 51%. 
Table 5. Clinicopathological data of the 310 patients included in the separate studies (I-IV).

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<th>III</th>
<th>IV</th>
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<td>305</td>
<td>301</td>
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<td>28 (1-334)</td>
<td>29 (0.3-334)</td>
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<td>44</td>
<td>6</td>
</tr>
<tr>
<td>No data</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Tumour recurrence at 10 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No recurrence</td>
<td>89</td>
<td>91</td>
<td>91</td>
<td>43</td>
</tr>
<tr>
<td>Recurrence</td>
<td>74</td>
<td>75</td>
<td>73</td>
<td>44</td>
</tr>
<tr>
<td>Tumour present/progressing</td>
<td>123</td>
<td>126</td>
<td>124</td>
<td>82</td>
</tr>
<tr>
<td>Cause of death at 10 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>191</td>
<td>195</td>
<td>191</td>
<td>120</td>
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<td>Other cause</td>
<td>31</td>
<td>32</td>
<td>31</td>
<td>17</td>
</tr>
<tr>
<td>Alive</td>
<td>77</td>
<td>78</td>
<td>79</td>
<td>38</td>
</tr>
</tbody>
</table>

*includes 1 malignant Brenner tumour in studies I-III, 20 mixed epithelial tumours in studies I-
III and 9 in study IV, and 30, 32, 32, 15 unclassified epithelial tumours in studies I-IV, respectively.

#CR=complete response, PR=partial response, SD=stable disease, PD=progressing disease

*includes patients without response to primary treatment and with residual tumour

Table 6. Clinicopathological characteristics of the patients treated with platinum-based chemotherapy (N=164) and of those treated with non-platinum based or no chemotherapy (N=143).

<table>
<thead>
<tr>
<th></th>
<th>Platinum-based chemotherapy</th>
<th>Non-platinum-based/ none chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FIGO stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>27 (17)</td>
<td>58 (40)</td>
</tr>
<tr>
<td>II</td>
<td>23 (14)</td>
<td>24 (17)</td>
</tr>
<tr>
<td>III</td>
<td>92 (56)</td>
<td>50 (35)</td>
</tr>
<tr>
<td>IV</td>
<td>22 (13)</td>
<td>11 (8)</td>
</tr>
<tr>
<td><strong>Histological grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>19 (12)</td>
<td>27 (19)</td>
</tr>
<tr>
<td>2</td>
<td>60 (36)</td>
<td>42 (29)</td>
</tr>
<tr>
<td>3</td>
<td>85 (52)</td>
<td>74 (52)</td>
</tr>
<tr>
<td><strong>Histological subtype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>65 (39)</td>
<td>43 (30)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>19 (12)</td>
<td>14 (10)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>41 (25)</td>
<td>40 (28)</td>
</tr>
<tr>
<td>Clear cell</td>
<td>15 (9)</td>
<td>17 (12)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>24 (15)</td>
<td>29 (20)</td>
</tr>
</tbody>
</table>

5.2. Expressions of biological factors

5.2.1. Versican (I)

Epithelial cells of normal ovaries (n=6) were invariably versican negative, and versican staining in the normal ovarian stroma was generally of weak intensity. In ovarian cancer, cancer cell-associated versican signal was observed in 50.5% (n=151) of the samples, although the percentage of positive cells remained low (<5% in 77.3% of the samples). Versican positivity in cancer cells was localised mainly in the cytoplasm or on the plasma membrane (n=138) but in a few cases (n=13) there was also nuclear localisation. In ovarian cancer stroma, the high intensity of versican staining was relatively frequent, and 133 (44.5%) and 166 (55.5%) tumours fell into the low and high (Figure 4A) expression level categories, respectively. No significant difference was found in cancer cell-associated (z=-1.5, p=0.13; Wilcoxon test) or strong stromal (z=-1.1, p=0.28) versican expression between primary tumours and metastases.
5.2.2. E-cadherin-catenin complex (II)

The expression of E-cadherin and β- and γ-catenins was located mainly on the cancer cell membrane either continuously or uncontinuously (Figure 4B). The mean percentage of strong continuous membranous expression in primary tumours was 9%, 7% and 7%, and in metastases 10%, 5% and 6% for E-cadherin, β- and γ-catenins, respectively. Strong continuous membranous staining was limited to 5% or fewer cancer cells in the majority of cancer samples (n=204 (72%) for E-cadherin, n=228 (77%) for β-catenin, and n=218 (74%) for γ-catenin).

In addition to membranous staining, nuclear staining was seen in a few primary tumour samples (n=23 (8%) for β-catenin (Figure 4C), and n=52 (18%) for γ-catenin). Sixteen of 23 primary samples (70%) expressing nuclear β-catenin were of endometrioid histological subtype. In metastases, nuclear positivity for β-catenin was observed in 1 (2%) endometrioid tumour sample and for γ-catenin in 2 (5%) samples. There were no significant differences in E-cadherin (z=-0.3, p=0.80), β-catenin (z=-0.4, p=0.68) and γ-catenin (z=-1.9, p=0.054) expression patterns between primary tumours and metastases.

5.2.3. iNOS (III)

The expression of iNOS was detected in the cancer cell cytoplasm as granular deposits (Figure 4D), and also in mononuclear inflammatory cells in cancerous stroma. The mean percentage of iNOS positive cells was 50% in primary tumours (n=301), and 62% in metastases (n=43). Nonetheless, twelve (4%) of the primary tumours and 2 (0.6%) of the metastases were completely iNOS-negative. The intensity of the expression was strong in 37% of the primary tumours. Interobserver agreement (observers M.A, S.M.) for the intensity of the iNOS staining was moderate (Kappa value 0.6, p<0.0005). iNOS expression of the primary tumours did not differ significantly from that of the matched metastatic lesions (z=-0.61, p=0.54).

5.2.4. CD34 as evaluated by the Chalkley method (IV)

The median Chalkley count was 7.67 for primary tumours and 8.17 for metastases
(range 4.00-19.00 and 6.00-15.00, respectively). Of the 175 primary samples, ninety one (52%) had a Chalkley count <8 (low expression group, Figure 4E), and eighty four (48%) ≥8 (high expression group, Figure 4F). There was no significant difference in the Chalkley count between the primary tumours and the metastases (z=–1.9; p=0.057). The coefficient of variation was 12% for intraobserver and 22% for interobserver variability.

5.3. Interrelationships between biological factors

Stromal versican expression was correlated with stromal hyaluronan expression, and an unsubstantial correlation was found between cancer cell-associated versican and CD44 (Table 7).

Membranous expressions of E-cadherin and β- and γ-catenins were correlated with each other, and the expression of γ-catenin on cell surface was related to α-catenin expression (Table 7). A very weak correlation of membranous β-catenin staining was observed with cancer cell-associated versican expression and inversely with strong stromal versican expression. Membranous expressions of β- and γ-catenins were both inversely weakly correlated with strong stromal hyaluronan expression (Table 7). Nuclear γ-catenin positivity was associated with positivity for hyaluronan in cancer cells, whereas nuclear β-catenin positivity was associated with high CD44 expression (Table 7), with this association being particularly clear in endometrioid tumours (χ², p<0.0005).

iNOS expression correlated weakly with CD44 expression as well as membranous E-cadherin expression (Table 7). CD34 expression, as reflected in the Chalkley count, was weakly related to cancer cell-associated versican expression (Table 7).
Figure 4. A) A serous ovarian carcinoma representing a high level of strong versican expression in stroma (asterisk). Scale bar=250µm. B) Preserved expression of E-cadherin on the cancer cell membrane (arrows) of a serous ovarian cancer. Scale bar=250µm. C) An endometrioid ovarian cancer with nuclear β-catenin expression (arrow). Scale bar=100µm. D) Expression of iNOS as granular deposits in the cancer cell cytoplasm of a serous ovarian carcinoma with a high level of iNOS expression. Also the strong intensity of iNOS expression is seen (arrow). Scale bar=100µm. E) Low CD34 expression in terms of the Chalkley count seen in a serous ovarian carcinoma. Tumour vessel is marked by the arrow. Scale bar=250µm. F) High expression of CD34 is shown in an endometrioid ovarian cancer. The arrows mark tumour vessels. Scale bar=250µm. Stromal area is indicated by the asterisk in each figure.
Table 7. Relation of biological factors to each other in primary tumours (Spearman correlation coefficient and \( \chi^2 \)-test).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Versican (cells)</th>
<th>Versican (stroma)</th>
<th>Hyaluronan (cells)</th>
<th>Hyaluronan (stroma)</th>
<th>E-cadherin (membrane)</th>
<th>( \beta )-catenin (membrane)</th>
<th>( \beta )-catenin (nuclear)</th>
<th>( \gamma )-catenin (membrane)</th>
<th>( \gamma )-catenin (nuclear)</th>
<th>( \alpha )-catenin</th>
<th>CD44</th>
<th>iNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versican (cells)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>r=0.14</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>r=0.14</td>
<td>ns</td>
</tr>
<tr>
<td>Versican (stroma)</td>
<td>ns</td>
<td>ns</td>
<td>r=0.44</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>r=0.13</td>
<td>ns</td>
</tr>
<tr>
<td>E-cadherin (membrane)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>r=0.39</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>( \chi^2 )=5.6</td>
<td>p=0.018*</td>
</tr>
<tr>
<td>( \beta )-catenin (membrane)</td>
<td>r=0.14</td>
<td>r=0.13</td>
<td>ns</td>
<td>ns</td>
<td>r=0.34</td>
<td>( \chi^2 )=7.4</td>
<td>p=0.006*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>r=0.23</td>
<td>ns</td>
</tr>
<tr>
<td>( \beta )-catenin (nuclear)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>( \chi^2 )=7.4</td>
<td>p=0.006*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>( \chi^2 )=8.0</td>
<td>p=0.004</td>
</tr>
<tr>
<td>( \gamma )-catenin (membrane)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>r=0.34</td>
<td>( \chi^2 )=10.6</td>
<td>p=0.001*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>r=0.12</td>
<td>ns</td>
</tr>
<tr>
<td>( \gamma )-catenin (nuclear)</td>
<td>( \chi^2 )=4.5</td>
<td>ns</td>
<td>( \chi^2 )=5.6</td>
<td>ns</td>
<td>p=0.018*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>r=0.21</td>
<td>ns</td>
</tr>
<tr>
<td>iNOS</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
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<td>CD34</td>
<td>r=0.16</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
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<td>ns</td>
<td>ns</td>
<td>r=0.16</td>
<td>ns</td>
</tr>
</tbody>
</table>

*inverse association
5.4. Association of biological factors with the clinicopathological variables

5.4.1. Versican (I)
A high proportion of strong stromal versican was associated with serous histological subtype, advanced FIGO stage and large (>2 cm) primary residual tumour. Versican positivity in tumour cells was correlated with clear cell histological subtype, early FIGO stage and the absence of primary residual tumour (Table 8).

5.4.2. E-cadherin-catenin complex (II)
Reduced E-cadherin and β-catenin expression on tumour cell membrane was associated with serous and endometrioid histological subtypes, poor differentiation and cancer recurrence. In addition, reduced cell surface expression of β-catenin was associated with advanced FIGO stage and large (>2cm) primary residual tumour. Nuclear positivity for β-catenin was related to endometrioid histological subtype, good-to-moderate differentiation and early FIGO stage of the tumour (Table 8).

Reduced membranous expression of γ-catenin was correlated with serous and endometrioid histological subtypes and large (>2cm) primary residual tumour. Nuclear γ-catenin expression was associated with serous histological subtype, poor differentiation of the tumour and a better response to chemotherapy (Table 8).

5.4.3. iNOS (III)
High iNOS expression was significantly correlated with mucinous histological subtype, whereas low iNOS expression was associated with large (>2cm) primary residual tumour and cancer recurrence (Table 8).

5.4.4. CD34 as evaluated by the Chalkley method (IV)
A low Chalkley count was related to serous and clear cell histological subtypes but not to any of the other studied clinicopathological factors (Table 8).
Table 8. Relation of biological factors to clinicopathological variables ($\chi^2$-test).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Histological subtype</th>
<th>Histological grade</th>
<th>FIGO stage</th>
<th>Residual tumour</th>
<th>Chemotherapy response</th>
<th>Recurrence at 10 years</th>
<th>End state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versican (cells)</td>
<td>$p&lt;0.0005$ ns</td>
<td>$p=0.015$</td>
<td>$p=0.006$ ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Versican (stroma)</td>
<td>$p=0.019$ ns</td>
<td>$p&lt;0.0005$</td>
<td>$p=0.002$ ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>E-cadherin (membrane)</td>
<td>$p&lt;0.0005$ $p=0.005$</td>
<td>$p=0.009$</td>
<td>$p=0.002$ ns</td>
<td>ns</td>
<td>ns</td>
<td>$p=0.014$ ns</td>
<td>ns</td>
</tr>
<tr>
<td>$\beta$-catenin (membrane)</td>
<td>$p&lt;0.0005$ $p=0.009$</td>
<td>$p=0.002$</td>
<td>$p=0.008$ ns</td>
<td>ns</td>
<td>ns</td>
<td>$p=0.049$ ns</td>
<td>ns</td>
</tr>
<tr>
<td>$\beta$-catenin (nuclear)</td>
<td>$p&lt;0.0005$ $p=0.025$</td>
<td>$p=0.027$</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>$\gamma$-catenin (membrane)</td>
<td>$p&lt;0.0005$ ns</td>
<td>ns</td>
<td>$p=0.009$ ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>$\gamma$-catenin (nuclear)</td>
<td>$p=0.002$ $p=0.009$</td>
<td>ns</td>
<td>$p=0.028$ ns</td>
<td>ns</td>
<td>ns</td>
<td>$p=0.038$ ns</td>
<td>ns</td>
</tr>
<tr>
<td>iNOS</td>
<td>$p=0.009$ ns</td>
<td>ns</td>
<td>$p=0.007$ ns</td>
<td>ns</td>
<td>ns</td>
<td>$p=0.038$ ns</td>
<td>ns</td>
</tr>
<tr>
<td>CD34</td>
<td>$p&lt;0.0005$ ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

5.5. Prognostic factors of the study patients

5.5.1. Clinicopathological factors (I-IV)

The prognostic value of clinicopathological parameters and biological factors was evaluated in relation to disease-related survival as well as to recurrence-free survival. In univariate survival analysis of the whole study cohort, the significant factors predicting poor disease-related and recurrence-free survival were advanced FIGO stage, poor differentiation and serous histological subtype of the tumour, and the presence as well as a larger size ($>2$cm) of the primary residual tumour (Table 9).

When only patients treated with platinum-based chemotherapy were included in the univariate analysis, the significant factors predicting poor disease-related survival were advanced FIGO stage, poor differentiation and the presence and larger size ($>2$cm) of primary residual tumour. The significant predictors of poor recurrence-free survival were
advanced FIGO stage, serous histological subtype as well as the presence and a larger size (>2cm) of the primary residual tumour (Table 10).

Table 9. Summary of the prognostic value of clinicopathological factors in univariate survival analyses of the whole patient group. p-values are from log-rank analyses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>DRS p-value</th>
<th>N</th>
<th>RFS p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological grade</td>
<td>300</td>
<td>p&lt;0.0005</td>
<td>163</td>
<td>p=0.034</td>
</tr>
<tr>
<td>FIGO stage</td>
<td>300</td>
<td>p&lt;0.0005</td>
<td>163</td>
<td>p&lt;0.0005</td>
</tr>
<tr>
<td>Histological subtype</td>
<td>300</td>
<td>p=0.039</td>
<td>163</td>
<td>p=0.007</td>
</tr>
<tr>
<td>Primary residual tumour</td>
<td>276</td>
<td>p&lt;0.0005</td>
<td>155</td>
<td>p&lt;0.0005</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>300</td>
<td>p=0.005</td>
<td>163</td>
<td>ns</td>
</tr>
<tr>
<td>Adjuvant chemotherapy</td>
<td>297</td>
<td>p=0.024</td>
<td>162</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 10. Summary of the prognostic value of clinicopathological factors in univariate survival analyses of the patients treated with platinum-based chemotherapy. p-values are from log-rank analyses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>DRS p-value</th>
<th>N</th>
<th>RFS p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological grade</td>
<td>164</td>
<td>p=0.020</td>
<td>79</td>
<td>ns</td>
</tr>
<tr>
<td>FIGO stage</td>
<td>164</td>
<td>p&lt;0.0005</td>
<td>79</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Histological subtype</td>
<td>164</td>
<td>ns</td>
<td>79</td>
<td>p=0.036</td>
</tr>
<tr>
<td>Primary residual tumour</td>
<td>151</td>
<td>p&lt;0.0005</td>
<td>76</td>
<td>p=0.0001</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>164</td>
<td>ns</td>
<td>79</td>
<td>ns</td>
</tr>
</tbody>
</table>

5.5.2. Biological factors and survival

5.5.2.1. Versican (I)

Increasing (≥15%) strong stromal versican was a predictor of worse disease-related survival in the univariate analysis during the first five years of the follow-up (p=0.032), but lost its significance when the follow-up was prolonged up to ten years. Instead, the recurrence-free survival at ten years was significantly better in the univariate analysis when the tumour epithelium was versican positive, compared to negative epithelium (Table 11). Versican
expression had no prognostic value in the subgroup of the patients treated with platinum-based chemotherapy (Table 12). In multivariate analysis of the study I material, neither stromal nor cancer cell-associated versican expression exhibited any prognostic significance in DRS or in RFS at five or ten years.

5.5.2.2. E-cadherin-catenin complex (II)
In univariate analysis of the whole study material, preserved β-catenin expression on cell surface predicted better 10-year disease-related and recurrence-free survival. In addition, favourable recurrence-free survival in univariate analysis was indicated by preserved E-cadherin and marginally by preserved γ-catenin expression on tumour cell membrane (Table 11). Nuclear β- and γ-catenin positivities (n=14 and n=10, respectively) were significant predictors of better 10-year disease-related survival in univariate analysis in the subgroups of 76 (β-catenin) and 77 (γ-catenin) endometrioid ovarian cancers (p=0.008 and p=0.012, respectively) but were not significantly associated with recurrence-free survival of the patients. None of the E-cadherin-catenin complex components retained their statistical significance in predicting DRS or RFS in the multivariate analyses performed in study II.

In univariate analysis of the patients treated with platinum-based chemotherapy, better 10-year recurrence-free survival was predicted significantly by preserved β-catenin expression and marginally by preserved γ-catenin expression on the cell surface (Table 12). In addition, nuclear β- or γ-catenin positivities were not significant prognostic factors in the subgroup of endometrioid ovarian cancers.

5.5.2.3. iNOS (III)
High iNOS expression was associated with better disease-related survival in univariate analysis of the whole study material (Table 11) but possessed no prognostic value in the subgroup of the patients treated with platinum-based chemotherapy (Table 12) nor did it retain its statistical significance in predicting prognosis in multivariate analysis of the study III.
5.5.2.4. **CD34 as evaluated by the Chalkley method (IV)**

The Chalkley count was not significantly related to disease-related or recurrence-free survival in the univariate analysis of the entire study cohort (Table 11) or in the subgroup of the patients treated with platinum-based chemotherapy (Table 12). Instead, the high Chalkley count predicted poor disease-related survival in FIGO stage III-IV tumours (p=0.007). In multivariate analysis of the study IV, the high Chalkley count was an independent predictor of poor DRS in the entire study group (n=156; p=0.044, RR=1.50, 95% CI 1.01-2.21) but was not a significant predictor of RFS.

**Table 11.** Summary of 10-year prognostic significance of the tested biological factors in univariate survival analyses of the whole study material. p-values are from log-rank analyses.

<table>
<thead>
<tr>
<th>Factor</th>
<th>N</th>
<th>DRS</th>
<th>N</th>
<th>RFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versican (cells)</td>
<td>289</td>
<td>ns</td>
<td>158</td>
<td>p=0.027</td>
</tr>
<tr>
<td>Versican (stroma)</td>
<td>289</td>
<td>ns</td>
<td>158</td>
<td>ns</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>273</td>
<td>ns</td>
<td>149</td>
<td>p=0.038</td>
</tr>
<tr>
<td>β-catenin (membrane)</td>
<td>286</td>
<td>p=0.035</td>
<td>154</td>
<td>p=0.033</td>
</tr>
<tr>
<td>β-catenin (nuclear)</td>
<td>286</td>
<td>ns</td>
<td>154</td>
<td>ns</td>
</tr>
<tr>
<td>γ-catenin (membrane)</td>
<td>283</td>
<td>ns</td>
<td>150</td>
<td>p=0.053</td>
</tr>
<tr>
<td>γ-catenin (nuclear)</td>
<td>283</td>
<td>ns</td>
<td>150</td>
<td>ns</td>
</tr>
<tr>
<td>iNOS</td>
<td>291</td>
<td>p=0.009</td>
<td>159</td>
<td>ns</td>
</tr>
<tr>
<td>CD34</td>
<td>174</td>
<td>ns</td>
<td>87</td>
<td>ns</td>
</tr>
</tbody>
</table>

**Table 12.** Summary of 10-year prognostic significance of the tested biological factors in univariate survival analyses of the patients treated with platinum-based chemotherapy. p-values are from log-rank analyses.

<table>
<thead>
<tr>
<th>Factor</th>
<th>N</th>
<th>DRS</th>
<th>N</th>
<th>RFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versican (cells)</td>
<td>159</td>
<td>ns</td>
<td>76</td>
<td>ns</td>
</tr>
<tr>
<td>Versican (stroma)</td>
<td>159</td>
<td>ns</td>
<td>76</td>
<td>ns</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>152</td>
<td>ns</td>
<td>74</td>
<td>ns</td>
</tr>
<tr>
<td>β-catenin (membrane)</td>
<td>159</td>
<td>ns</td>
<td>79</td>
<td>p=0.043</td>
</tr>
<tr>
<td>β-catenin (nuclear)</td>
<td>159</td>
<td>ns</td>
<td>79</td>
<td>ns</td>
</tr>
<tr>
<td>γ-catenin (membrane)</td>
<td>158</td>
<td>ns</td>
<td>76</td>
<td>p=0.051</td>
</tr>
<tr>
<td>γ-catenin (nuclear)</td>
<td>158</td>
<td>ns</td>
<td>76</td>
<td>ns</td>
</tr>
<tr>
<td>iNOS</td>
<td>158</td>
<td>ns</td>
<td>78</td>
<td>ns</td>
</tr>
<tr>
<td>CD34</td>
<td>155</td>
<td>ns</td>
<td>77</td>
<td>ns</td>
</tr>
</tbody>
</table>
5.5.3. Conclusive multivariate analyses of the whole study material

The clinicopathological factors predicting independently disease-related survival in separate studies I-IV were FIGO stage, histological grade, and primary residual tumour. In addition, first line chemotherapy predicted disease-related survival in study I. The independent prognosticators of recurrence-free survival were primary residual tumour and histological subtype in each study. CD34 expression as evaluated with the Chalkley count was found to be a new significant and independent prognostic factor for DRS in the whole study IV material. All these variables were included in the multivariate analysis to test the independent prognostic value of each factor. In this way, the independent prognostic factors in the whole study cohort were primary residual tumour and CD34 expression for DRS, and histological subtype as well as primary residual tumour for RFS (Table 13).

Similarly, analysing the subgroup of patients treated with platinum-based chemotherapy in the multivariate analysis resulted in identification of the independent prognostic significance of primary residual tumour and CD34 expression for DRS, whereas only primary residual tumour predicted RFS (Table 14).

When hyaluronan, which has been previously shown to be an independent prognostic factor (167), was included in the multivariate analysis of the whole study group, the significant factors predicting poor DRS (n=156) were the presence of primary residual tumour (p<0.0005, RR=4.69, 95% CI 2.79-7.90) and a high level of strong stromal hyaluronan expression (p=0.024, RR=1.59, 95% CI 1.06-2.38), and poor RFS (n=82) was predicted by serous histological subtype (p=0.034, RR=2.01, 95% CI 1.05-3.82), the presence of primary residual tumour (p=0.001, RR=3.36, 95% CI 1.68-6.71), and a high level of strong stromal hyaluronan expression (p=0.029, RR=2.05, 95% CI 1.08-3.90). In the subgroup of patients treated with platinum-based chemotherapy, independent poor prognostic factors were advanced FIGO stage (p=0.034, RR=2.01, 95% CI 1.06-3.83), the presence of primary residual tumour (p=0.001, RR=3.53, 95% CI 1.71-7.26) and high level of strong stromal hyaluronan expression (p=0.005, RR=1.81, 95% CI 1.19-2.76) for DRS (n=142), and the presence of primary residual tumour (p<0.0005, RR=4.21, 95% CI 1.99-8.88) as well as high level of strong stromal hyaluronan expression (p=0.011, RR=2.42,
95% CI 1.23-4.75) for RFS (n=74).

Table 13. Independent predictors of 10-year disease-related (n=156) and recurrence-free (n=82) survival in the Cox’s multivariate analysis of the whole study patient group.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Category</th>
<th>RR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Disease-related survival</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary residual tumour</td>
<td>Negative vs. positive</td>
<td>0.20</td>
<td>0.12-0.33</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>CD34 expression</td>
<td>Low vs. high</td>
<td>0.67</td>
<td>0.45-0.99</td>
<td>0.043</td>
</tr>
<tr>
<td><strong>Recurrence-free survival</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological subtype</td>
<td>Serous vs. others</td>
<td>2.14</td>
<td>1.12-4.06</td>
<td>0.021</td>
</tr>
<tr>
<td>Primary residual tumour</td>
<td>Negative vs. positive</td>
<td>0.30</td>
<td>0.15-0.60</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Reference category
RR=relative risk
CI=confidence interval

Table 14. Independent predictors of 10-year disease-related (n=142) and recurrence-free (n=74) survival in the Cox’s multivariate analysis of the patients treated with platinum-based chemotherapy.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Category</th>
<th>RR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Disease-related survival</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary residual tumour</td>
<td>Negative vs. positive</td>
<td>0.16</td>
<td>0.09-0.30</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>CD34 expression</td>
<td>Low vs. high</td>
<td>0.66</td>
<td>0.44-0.99</td>
<td>0.045</td>
</tr>
<tr>
<td><strong>Recurrence-free survival</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary residual tumour</td>
<td>Negative vs. positive</td>
<td>0.24</td>
<td>0.11-0.50</td>
<td>&lt;0.0005</td>
</tr>
</tbody>
</table>

*Reference category
RR=relative risk
CI=confidence interval
6. DISCUSSION

6.1. Evaluation of the study material
The current study cohort consists of ovarian cancer patients diagnosed and treated in Kuopio University Hospital area during the years 1976-1992. The majority of disease recurrences and the decline in the survival rates occur during the first few years after the diagnosis (42, 400), and the vast majority of prognostic ovarian cancer studies report 5-year survival rates. In the present study, the follow-up of the patients was continued until January 2004, which provides unique survival data up to ten years. The 10-year disease-related survival of the patients in the present study was 32%, which is considerably higher than in the few publications which have reported long-term follow-up values (401-403). These three papers, however, only included advanced-stage patients and differ in this respect from the present study, but the values of the present study are comparable to the 10-year survival reported in Finland from the same time period (51). The median follow-up time of the patients included in the study (n=310) was 28 months, reflecting the bleak course of the disease. On the contrary, the median follow-up time for surviving patients (n=79) was rather long, 139 months. The mean age of the patients was 60 years, which is in line with that reported in the literature (3, 5, 51, 404). The distribution of the patients according to the histological subtype is different from some reports (3, 45) with a somewhat smaller proportion of serous type carcinomas in the present study, but nonetheless falling into the range reported by others (405, 406). Furthermore, the distribution of the patients according to the FIGO stage (52, 404, 406) and histological grade (406, 407) is comparable to previous reports so there does not seem to be any notable selection bias in the present study material.

Post-operative treatment contained chemotherapy in 85% of the study patients, and was based on platinum in 62% of these patients. The retrospective nature and the relatively long entry period for the study from 1976 to 1992 may generate heterogeneity of the patients, particularly with respect to chemotherapy and staging methods. Consequently, first line chemotherapy in the present study was based on platinum more frequently in patients
diagnosed within the last ten years of the study compared to the patients from the former study period ($\chi^2$ test), which is in line with the changes in treatment strategies occurring over that time (408, 409). However, there were no differences with respect to other clinicopathological variables between these two patient groups. Chemotherapy based on platinum regimens was received more often by patients with advanced-stage disease and those with larger (>2 cm) residual disease after primary surgery ($\chi^2$ test), which is not surprising given that these were the characteristics also of those patients included in one of the major studies establishing the advantages of platinum in front-line chemotherapy for ovarian cancer (410) which certainly affected subsequent treatment decisions.

The surgical procedure for ovarian cancer has also undergone substantial changes (408) since the implication of primary residual tumour size as an important prognostic factor (68), reports of lymph node metastasis in ovarian cancer (62, 411) and the introduction of an operative staging system (412). Therefore, some understaging may exist in the present study material. Furthermore, some of the patients had to be excluded from the study because there was insufficient tumour material available for immunohistochemical analyses, but no statistical differences in clinicopathological variables existed between the original cohort of 445 patients and the present study cohort (test of goodness for fit). To conclude, there does not seem to be any major selection bias in the present study, but the results must be interpreted with some caution bearing in mind the different treatment modalities from those of today.

6.2. Evaluation of the study methods

The histological diagnosis was confirmed earlier by re-evaluation of histological subtype and grade by the same experienced pathologist unaware of the clinical data (128, 395), thus reducing bias from interobserver variability (413-416). There are some clear problems associated with immunohistochemistry, since methodological variability, such as differences in fixation, processing and storage of the tumour tissues as well as different antibodies and cut-offs used, which can all affect the comparability of the results from different studies (417-419). All slides in this study were evaluated by one to three observers
to reach a consensus. In all the staining series, negative controls remained negative and positive controls stained appropriately. The continuous variables obtained from immunohistochemical analyses were dichotomised into two categories using the median value as the cut-off for versican, E-cadherin, β- and γ-catenins as well as for CD34 expression, and the 66th percentile as the cut-off for iNOS, as the median or some other centile have been demonstrated to be usable without introducing bias (420).

The evaluation of angiogenesis was performed with the Chalkley assay, which is recommended to be used for angiogenesis quantification in solid tumours by international consensus report (304). In general, this method is considered to be a simple and acceptable procedure for practical evaluation of intratumoural vascularity and it has been reported to be objective, rapid and have acceptable reproducibility (312, 319, 321, 421). However, it is noteworthy that both the Chalkley assay and microvessel counting method suffer from some methodological problems, for example concerning the objective nature of selection of the densely vascularised areas, "vascular hot-spots" for microvessel quantitation. Nevertheless, choosing the same hot-spot areas may not necessarily improve the reproducibility (421), and since it represents a relative vessel area estimate rather than a true vessel count, one of the advantages of the Chalkley method is that this eliminates one of the highly observer-dependent steps in microvessel counting method: the decision whether two immunostained and adjacent structures are the reflection of one single or two separate blood vessels (304). Accordingly, the Chalkley method has been shown to have less observer variation than estimation of microvessel density in breast cancer (421), and high levels of agreement between two observers have been reported in non-small cell lung cancer (321, 322) and soft tissue sarcoma (319). Furthermore, it seems to be clearly superior to the microvessel counting method in the evaluation of breast cancer prognosis (422). However, the prognostic impact of the two methods seems to vary with the type of carcinoma (311), and therefore more studies using both methods are needed.

6.3. Clinicopathological prognostic factors in epithelial ovarian cancer

Previously well-defined prognostic value of residual tumour (44-46) was confirmed also in
the current study. Age at diagnosis appeared to be significant in the univariate analysis but lost its significance when analysed with the other factors, indicating that those other variables, e.g. treatment modalities, may affect the prognosis more than simply the age of the patient. Although age has been shown to predict survival in many studies (3, 45, 50, 51, 145, 407, 423, 424), also a lack of prognostic significance has been shown (425-427). The diversity of the factors included in survival analyses may at least partly explain the differences in the results, and the prognostic significance of age may to some extent reflect the less aggressive treatment that elderly patients receive (54-56).

Recurrence-free survival was predicted by primary residual tumour and histological subtype. The histological subtype has been indicated as being an independent prognosticator in some studies (45, 50, 84, 425, 426, 428, 429), whereas it has lacked significance in others (70, 407, 430). However, inter- and intraobserver variability probably affects the results obtained for prognostic significance of histological classification (413-416, 431-434). In addition, clear deficiencies in the reproducibility of tumour grading by different pathologists have been reported (413-416) and this may lead to differences between studies about the prognostic significance of histological grade (92-94). A universal grading system in analogy to that used for evaluating breast carcinomas has been suggested (435) and shown to provide independent prognostic information (435-438).

Since the 70's, numerous studies have shown that residual tumour size has an impact on patients' survival (44-46, 52, 69-71). The possibility that improved survival of patients with more extensive cytoreduction may merely reflect the biological features of the tumour has been under debate. However, since no prospective randomised trials to investigate the efficacy of initial surgical debulking have been made and would be ethically unjustifiable, the present data support the effort to achieve maximal cytoreduction with the target of no macroscopic residual tumour. Indeed, all attempts to debulk the ovarian cancer patient to a level of no gross residual disease were recommended also by a consensus meeting of European Society of Gynaecological Oncology (ESGO) (73). The results of the present study support this conclusion by highlighting the survival advantage for the patients with no primary residual tumour, which is in line with other studies (3, 71, 73, 74) including a
prospective study with the goal of removal of all visible disease and improvement of survival for those patients in whom this was achieved (429). Although the treatment with platinum-based chemotherapy regimen has been indicated to be more powerful predictor of survival than the extension of primary cytoreductive surgery (51, 439), nowadays when virtually all the patients receive platinum-based chemotherapy, it is more crucial to define the prognostic significance of the primary cytoreductive surgery. In the current study, the presence of primary residual tumour was an independent predictor of survival in the whole study group as well as in the patient group treated with platinum-based chemotherapy which is in line with the results from a large meta-analysis of 6885 patients (69). Furthermore, in pursuit of better surgical outcomes, centralisation of ovarian cancer treatment and subspeciality training of the surgeons have been shown to result in the highest rates of optimal cytoreduction and comprehensive staging (404, 440) and may further improve the survival rates of ovarian cancer patients (440, 441).

6.4. Extracellular matrix and cell adhesion molecules in epithelial ovarian cancer

6.4.1. Versican

Previously, stromal hyaluronan has been shown to have independent prognostic value in epithelial ovarian cancer (167), and versican has been shown to bind hyaluronan (204). In the present study, increasing strong stromal versican staining was a predictor of worse disease-related survival in univariate analysis during the first five years of the follow-up, but had no independent prognostic impact when analysed in conjunction with the clinicopathological factors. This is in line with the findings of disease-free survival of breast cancer (211) and adenocarcinomas of the lung (218). In addition, versican lost also its univariate significance when the follow-up was prolonged to ten years.

In general, versican expression appears to be elevated in the cancer samples as compared to normal ovaries, and stromal versican expression increases with advancing stage of ovarian cancer. These results suggest that versican may have a potential role in the development and progression of ovarian cancer, although it may not be a good predictor of
prognosis. A high level of stromal versican was correlated with a high stromal hyaluronan level, which points to a parallel influence of versican and hyaluronan on the cancer progression. However, the prognostic value in epithelial ovarian cancer seems to be better for hyaluronan (167), and the prognostic influence of versican may be more relevant in other cancer types (212, 213, 219, 220).

6.4.2. E-cadherin-catenin complex

Membranous expression of β-catenin was found to have a weak inverse correlation to strong stromal hyaluronan, consistent with the observation that cells overexpressing HAS2 show a marked decrease in intensity of staining at the intercellular boundaries and a diffuse, cytoplasmic distribution of β-catenin (442). In addition, hyaluronan may induce a β-catenin shift from the cell-cell adhesion state leading to nuclear translocation in ovarian cancer cells (443). The present study revealed a significant correlation between nuclear β-catenin expression and high CD44 expression, which is in line with the implication that the expression of CD44 is regulated by β-catenin/Tcf-4 through enhanced transcription of CD44 (444). As stated earlier, dysregulation of β-catenin leading to its nuclear accumulation is a common feature of endometrioid type ovarian cancer (253, 445, 446), and accordingly, also the association between nuclear β-catenin expression and CD44 was focused especially on endometrioid ovarian carcinomas. Although also versican gene expression has been shown to be up-regulated via the β-catenin-Tcf complex formation in smooth muscle cells (447), no association was observed between nuclear β-catenin and versican expression. However, gene expression may be regulated by a number of different factors and the role of β-catenin in the regulation of versican expression in ovarian cancer remains to be clarified.

Previous prognostic studies on E-cadherin-catenin complex in ovarian cancer have resulted in uncertainty about the prognostic significance of the complex (186, 232, 248-254). In the present study, preserved membranous expression of E-cadherin indicated better 10-year recurrence-free survival in the univariate analysis, a result in agreement with some
previous studies (186, 248, 249), but not with one study (232). Preserved β-catenin expression on cell surface indicated better 10-year disease-related and recurrence-free survival in univariate analysis, which is in line with one previous study (250) but contradicted by others where no relationship was found between membranous β-catenin expression and survival (232, 252, 253). Nuclear positivity of β-catenin predicted better disease-related survival for patients with endometrioid ovarian cancers, and an association between accumulated nuclear β-catenin expression and a favourable prognosis has been reported in ovarian cancer also previously (252, 253). However, in serous ovarian cancer, the opposite trend has been reported (254), possibly reflecting the differences in the molecular pathways underlying the tumourigenesis of different histological subtypes (154). Accordingly, different cadherin-catenin expression patterns are associated with distinct histologic subtypes (446). Indeed, nuclear β-catenin accumulation has been found to be characteristic for endometrioid tumours, and mutations of the gene encoding β-catenin, CTNNB1, have been reported in 16-54% of endometrioid ovarian carcinomas (252, 445, 448-451), leading to nuclear accumulation of β-catenin. Tumours associated with such mutations and nuclear β-catenin positivity have been found to be more frequently low-grade and stage, and accordingly, to have a favourable prognosis (252, 253, 445) in line with the present findings. The results suggest that modulation of the Wnt signalling pathway may be one mechanism involved in the tumourigenesis of endometrioid ovarian carcinomas.

In addition, nuclear γ-catenin positivity was found to be a significant prognosticator of better 10-year disease-related survival in univariate analysis in the subgroup of endometrioid ovarian cancers. Previously, nuclear γ-catenin positivity has been observed immunohistochemically in endometrial (452), renal cell (453) and non-small cell lung (454) carcinomas. Although mutations of the γ-catenin gene have been suggested to be rare in patients with ovarian cancer (445, 455), the subregion of chromosome 17 that includes γ-catenin gene is known to be particularly subjected to genetic alterations in sporadic ovarian tumours (456), and nuclear γ-catenin positivity has been reported recently also in other
ovarian carcinoma studies (186, 446). Furthermore, γ-catenin can activate the Wnt signalling cascade directly without any interaction of β-catenin, since it possesses multiple functions as a transcriptional activator and a cell adhesion molecule like β-catenin (457), thus lending support to its possible role in regulating cell functions. While the prognostic significance of nuclear γ-catenin expression appears to be lacking in other cancers (453, 454) and remains to be confirmed in ovarian cancer, it is concluded that none of the E-cadherin-catenin complex components could overcome the prognostic significance of traditional clinocopathological factors in multivariate analysis of the present study.

6.5. Angiogenesis in epithelial ovarian cancer

6.5.1. iNOS

Hyaluronan has been suggested to induce iNOS expression by activation of the transcriptional regulator nuclear factor kappaB (458). In the current study, there was no association between the expressions of iNOS and hyaluronan. However, the stimulative effect of hyaluronan on iNOS mRNA has been shown to be dependent on the hyaluronan size (458-460), which may restrict the evaluation of the relationship with the method used in the present study which did not differentiate between different size hyaluronan molecules. On the other hand, a weak correlation was found between iNOS and CD44 expression in cancer cells, which might reflect the regulation of iNOS expression through CD44-hyaluronan interactions (459, 460).

Although iNOS has been indicated to be important for angiogenesis promotion also in ovarian cancer in vitro (461), the high concentration of NO that is produced by iNOS may inhibit proliferation of endothelial cells and vascular smooth-muscle cells (462). Unlike in gall-bladder (463), gastric (464), colorectal (465) and endometrial (466) carcinomas, in the present study there was no correlation between iNOS expression and angiogenesis as determined here by the Chalkley count after CD34 staining. However, Özel et al. (279) have previously reported similar findings of a missing association between iNOS and
microvessel density in ovarian cancer. In addition to methodological disparities, the biological differences regarding these cancers are also likely to explain the conflicting results between the studies.

Previously, iNOS expression has been reported to be an independent marker for poor survival in FIGO stage III, poorly differentiated serous ovarian carcinoma (278). On the contrary, in another ovarian cancer study with 100 patients, the median survival time of patients with low iNOS expression was shorter than that of those with high iNOS expression tumours, though the difference failed to reach statistical significance (279). In the present study with 301 samples available for iNOS analysis, high iNOS expression was found to associate with better disease-related survival in univariate analysis. This is in contrast with the results obtained in some studies with different malignancies (273, 282), but consistent with others (272, 467), though also many studies with a lack of prognostic significance have been reported (275, 468, 469). Interestingly, nitric oxide has been shown to have a dual role in tumour progression and metastasis, being able to both promote and inhibit these processes, depending on the NO-sensitivity of the tumour cells. In turn, this is thought to be determined by the expression level, duration and timing of NO delivery, the microenvironment, the genetic background and the cell type (269). With relation to ovarian cancer, both exogenously applied NO and endogenously synthesised NO have been shown to inhibit tumour growth, probably mainly by induction of tumour cell apoptosis (470-472), supporting the present finding of a favourable survival influence of iNOS in ovarian cancer. Indeed, different malignancies may exhibit divergent sensitivities to NO, which in addition to methodological differences might explain the conflicts between the different studies. However, iNOS expression did not retain its statistical significance in predicting prognosis in the multivariate analysis of this representative material, suggesting that the associations are too weak to resist the confounding factors that come from different evaluation methods and clinicopathological features of the cancer materials used by different groups. Moreover, considering the transient nature of iNOS expression (473) and the single time point representation of that in a sample of heterogeneous material, only a large material, such as in the present study with sufficient number of samples in the same phase of expression,
may provide relevant information on the association between iNOS expression and survival. Indeed, the importance of clinicopathological factors remains superior to iNOS expression in prediction of the prognosis of ovarian cancer.

6.5.2. CD34
Interestingly, both the development of a vascular supply and stromal support are essential for tumour growth. Indeed, the factors important for stromal structure have been associated also with the regulation of angiogenesis. For example, degradation products of hyaluronan have been shown to induce an angiogenic response (163). However, in the present study hyaluronan expression was not related to angiogenesis as estimated by the Chalkley count. It is possible that different subtypes of hyaluronan synthases may synthesise hyaluronans with different biological functions, which are not distinguishable by the staining technique used. Additionally, it is the degradation products of hyaluronan that have been found to stimulate angiogenesis, whereas high-molecular-weight hyaluronan has been claimed to inhibit angiogenesis (163), also complicating the evaluation of this interrelationship since the current method can not differentiate between these species. Furthermore, the contribution of hyaluronidase to tumour progression through the production of hyaluronan degradation products and their angiogenic activity may not be as significant in epithelial ovarian cancer (474) as has been suggested for prostate (173) and bladder (475) tumours. Indeed, in spite of the missing association between the hyaluronan expression and angiogenesis in this study, hyaluronan metabolism may play an important regulatory role in the control of normal and pathological neovascularisation in cell-type specific way.

In the present study, CD34 expression was weakly associated with cancer cell-associated versican expression. In line with this concept, versican has been claimed to enhance angiogenesis by stimulating endothelial cell adhesion, proliferation, and migration, probably through upregulation of and an interaction with fibronectin and VEGF (267). However, a more detailed role of versican in the regulation of angiogenesis remains to be elucidated.

The prognostic significance of angiogenesis as evaluated by microvessel density has
remained controversial in ovarian cancer multivariate studies, as some studies have shown that increased angiogenesis predicts either poor (289, 305, 306, 309) or improved survival (286, 307, 308), whereas a lack of association between angiogenesis and outcome of the patients has been reported in several studies (284, 288, 290-294, 297, 298, 310). In the present study, high CD34 expression assessed by the Chalkley evaluation method was shown to be an independent predictor of poor disease-related survival in the whole study cohort as well as in the subgroup of patients treated with platinum-based chemotherapy. However, these results are not directly comparable to those reported earlier because of the different methodology, although the modified Chalkley method has been used by Hollingsworth et al. and that group found no prognostic value in their considerably smaller study group (305). However, the Chalkley method has been claimed to be of benefit in evaluating other carcinomas. Especially in breast carcinoma, the association of poor outcome with increasing angiogenesis has been demonstrated (311-314), with only a few studies failing to detect this link (315, 316). In other types of malignancies, the method has been shown to have prognostic significance (317, 318), lack significance (319, 320) or its prognostic significance has remained controversial (311, 321-324). In addition to the lack of standardised techniques and the impact of that factor on the divergencies, the appearance of the vascularity seen in studies is attributable to complex processes which are still largely unclear. Therefore, part of the explanation as to why estimates of tumour angiogenesis do not consistently indicate poor or favourable prognosis in numerous studies could be that the biology of angiogenesis is different in different tissues. Indeed, the degree of angiogenesis seems to vary in different carcinomas, as appears to be the case between ovarian and breast carcinoma with a lower degree of angiogenesis in the former cancer type (476).

Chemotherapy resistance still remains as a great obstacle to success in the treatment of ovarian cancer. Angiogenesis has been linked to chemotherapy response with the hypothesis of improving delivery of chemotherapeutic agents with increasing angiogenesis. However, studies have failed to confirm this hypothesis i.e. on one hand increased angiogenesis has been reported to associate with improved chemotherapy response in advanced-stage ovarian cancer patients (287), but on the other hand an inverse association
between vascularity and response to platinum-based chemotherapy has been claimed to exist (291, 302). In the current study, no association was found between CD34 expression and response to chemotherapy. Despite increased vascularity, the delivery capacity may be insufficient due to structural and functional abnormalities of the tumour blood vessels, leading to chaotic blood flow and making certain regions inaccessible to drugs (477). Furthermore, malignant cells including ovarian cancer cells, may also participate in vascular channel formation independent of endothelial cells (478, 479), and these kinds of structures may remain unlabelled in endothelial marker stainings (480) despite their possible involvement in drug delivery. Accordingly, evaluation of drug delivery capacity by assessing only vessel quantities is questionable, and it remains to be determined in the future whether some angiogenesis marker could help in clinical practice to pinpoint those patients likely to benefit from anticancer drug therapies.

6.6. Future directions

Tumourigenesis is a multistep process with an accumulation of multiple genetic alterations (481). Therefore, it is unlikely that an alteration in a single gene has predictive value by itself. This is supported by the fact that many studies have not found any association between p53 expression and survival in multivariate analysis (139-150, 482). Instead, it is probable that the combination of many genetic alterations has greater importance. Expression of thousands of genes can be assayed with the use of DNA microarray analysis (483), and this holds the potential to clarify the genetic origins of ovarian cancer. In view of the fact that chemotherapy resistance severely impedes efficient treatment of ovarian cancer patients, understanding the biological mechanisms underlying this process could facilitate improvement of the treatment and outcome of ovarian cancer by directing the treatment choices or by leading to therapies targeted toward particular molecular subsets (484). Interestingly, gene expression profiling has provided preliminary data about potentially important molecular markers for assessing the chemotherapy response (484-489) and prognosis (487, 489, 490) of ovarian cancer patients, thus providing candidate targets for prospective trials, hopefully translating into clinical practice and better patient outcome in
the future.

While the knowledge of ovarian cancer biology undoubtedly will increase rapidly and carries with it a chance for more effective treatment modalities, endeavours for such are constantly ongoing. In order to achieve a more favourable treatment outcome, more effective combinations of established chemotherapeutic agents as well as entirely new cytotoxic agents are being evaluated (491), as are also strategies such as consolidation and maintenance therapy, though these regimens have not yet achieved any significant improvement in survival, whereas intraperitoneal drug delivery has resulted in notable promises of survival extension (491, 492). In addition, molecular-targeted therapies in ovarian cancer are under investigation (491). Taking into account the prognostic value of angiogenesis in the present study, albeit requiring confirmation in prospective studies before it is of clinical value, an intriguing subject in this field surrounds the potential of antiangiogenic treatment. The promising antitumour activity for bevacizumab, i.e. an antibody to VEGF, has already been reported (493) and will hopefully be further corroborated in the ongoing clinical trials (493). Additionally, other agents with antiangiogenic effects, such as enzastaurin (491) are being studied and are of interest since it is feasible that the combination of multiple antiangiogenesis agents, inhibiting different steps in the angiogenic cascade may have a synergistic effect (494). Even though the biological mechanisms underlying angiogenesis still remain largely unknown, the developments of microarrays hold the potential to identify the angiogenesis-related genes (494) and enable tailored antiangiogenic therapy.
7. SUMMARY AND CONCLUSIONS

The present retrospective study was performed in a representative series of ovarian cancer patients to analyse the distribution and prognostic value of factors related to cell adhesion and angiogenesis in epithelial ovarian cancer. The independent prognostic significance of primary residual tumour and histological subtype was confirmed, as was also that of the previously evaluated stromal hyaluronan expression. In addition to these confirmatory data, the main findings and conclusions can be stated as follows:

1. The strong stromal versican expression was significantly related to other clinicopathological factors of poor survival, whereas versican positivity in tumour cells was correlated with more favourable factors, such as the absence of primary residual tumour and early FIGO stage. Versican expression was a significant prognostic factor in the univariate but not in the multivariate analysis.

2. The expression of E-cadherin and β-catenin on cancer cell membrane was significantly reduced in poorly differentiated and serous or endometrioid histological subtypes, and expression of β-catenin was reduced also in advanced FIGO stage tumours. Nuclear positivity of β-catenin was related to early FIGO stage of the disease and to endometrioid histological subtype, and nuclear positivity of γ-catenin was observed especially in poorly differentiated and serous tumours. Preserved expressions of E-cadherin and β-catenin, and marginally also that of γ-catenin on tumour cell membrane, as well as nuclear expression of β-catenin and γ-catenin in endometrioid ovarian carcinomas were associated with better outcome of the patients in univariate analysis, but were not significant predictors of survival in the multivariate analysis.

3. The expression of iNOS was high in the mucinous histological subtype of epithelial ovarian cancer. A high iNOS expression was a significant favourable prognostic factor in
the univariate analysis but possessed no independent prognostic value in the multivariate analysis.

4. Angiogenesis, as determined by the Chalkley method after CD34 staining, was low in serous and clear cell histological subtypes but was not related to FIGO stage or histological grade of the tumour, or the presence of primary residual tumour. The high Chalkley count appeared to be a significant prognostic factor of poor disease-related survival in the multivariate analysis.

In conclusion, clinicopathological prognostic factors such as primary residual tumour and histological subtype remain the most important prognosticators of disease progression. In addition, angiogenesis evaluated here by the Chalkley count, seems to be important in the progression of epithelial ovarian cancer.
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