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Human tissue kallikreins, uPA and PAI-1 as prognostic markers in ovarian carcinoma

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Meiner Familie

1. Introduction

1.1 Ovarian cancer, prognostic and predictive factors

Every year, almost 7,500 women are newly diagnosed with ovarian cancer in Germany and as a result up to 6,000 of these patients die of the malignant disease per year. Epithelial ovarian carcinoma (ovarian cancer) is the third most common neoplasm of the female reproductive tract, but the leading cause of death from a gynecological malignancy *(73; 187)*. Early ovarian cancer detection could potentially decrease mortality, still, the tumor commonly shows no obvious, often only unspecific, signs or symptoms such as general abdominal discomfort or pain, bowel irregularity, loss of appetite, weight gain or loss or persisting fatigue until late in its development *(175)*. Moreover, current ovarian cancer screening methods are limited as sensitivity and specificity of such methods are not high enough. Given the low prevalence of epithelial ovarian cancer (40 per 100,000 women > 50 years) *(205)*, a clinically acceptable positive predictive value of 10 % could only be acquainted with a specificity of >99 % and a sensitivity of > 67 % *(73)*. Therefore, one goal is to tailor the therapeutic regimen for each of the patients afflicted with ovarian cancer based on the tumor´s unique molecular signature by finding reliable prognostic and/or predictive factors so that the patients that are at risk can be directed to clinical trials or more effective ways of treatment.

<u>Established clinical prognostic factors in ovarian carcinoma are:</u>

- Staging of the disease at time of diagnosis
- Retroperitoneal nodal status
- Residual tumor mass after primary surgery
- Histomorphology and cellular differentiation (nuclear grading) of the tumor
- Ascitic fluid volume
- Clinical circumstances such as age, anemia, and performance status

According to the guidelines of the International Federation of Gynecology and Obstetrics, FIGO (see Appendix), staging of the disease at time of diagnosis represents the major prognostic factor in ovarian cancer *(23)*. FIGO stage I patients have a 5-year survival of 80-90 % compared to only 15-20 % for women with advanced disease stage III or IV. Unfortunately, 65 % of all patients are diagnosed in advanced FIGO stage III or IV *(193)*. Appropriate intra-operative staging is vitally important for effective post-operative therapy decision-making. According to recommendations of the German national treatment guidelines (AGO, Arbeitsgemeinschaft gynäkologischer Onkologen), a staging laparotomy should be executed by longitudinal abdominal section with bilateral adnectomy, hysterectomy, infragastric omentectomy, radical paraaortic and pelvic lymphadenectomy, and appendectomy. Furthermore, multiple cytological washings and random biopsies of the

peritoneum, the diaphragm, and of suspect regions is required *(158; 164)*.

Assessment of the nodal status, i.e. lymphogenic metastasis of the iliac, sacral, paraaortal, and inguinal area is of prognostic value. To assess the nodal status adequately, at least 10 lymph nodes have to be removed and analyzed histologically *(50)*. A systematic removal of all pelvic and paraaortal lymph nodes does not prove to be of therapeutic use, but nevertheless, a radical pelvic and paraaortal lymphadenectomy is recommended if intraabdominal tumor-free status can be achieved, as nodal status is often positive in advanced ovarian carcinoma *(120)*.

Radical surgery and platinum-based chemotherapy are cornerstones for treatment of ovarian carcinoma. As the amount of postoperative residual tumor mass has been proven to be the strongest independent prognostic factor, optimal surgical cytoreduction of the tumor mass is imperative for increasing the patient's chance of survival *(24; 85; 100; 129)*. There is no general definition of optimal debulking. Early studies considered a residual tumor size of < 2 cm as optimal *(102)*, while in a survey among gynecologists in the USA only 13 % of the gynecologists considered a tumor of 1.5 to 2.0 cm as optimal and 61 % chose a 1 cm threshold to distinguish optimal from suboptimal debulking. Regarding the fact that above a tumor diameter of 2 cm no significant differences in survival can be found, and a subset analysis of smaller diameters in residual disease showed improved patient prognosis *(100)*, the most rational approach might be to define microscopic residual tumor mass or 0 cm as optimal, < 1 cm as minimal, 1 to 2 cm as intermediate and > 2 cm as gross *(158)*.

Despite aggressive approaches to the surgical treatment of patients with ovarian carcinoma, extensive tumor mass, the presence of tumor in specific locations, and intraperitoneal tumor cell spread may prohibit optimal resection at primary surgery. The success rate of achieving optimal cytoreductive surgery varies among institutions, ranging from 40-50 % *(173)*. Considering that patients who undergo suboptimal debulking derive minimal benefit from surgical debulking, it would appear practical to identify these patients preoperatively to offer them an alternate approach to the current standard of care, for example neoadjuvant chemotherapy.

Several approaches have been made to find valid predictors of optimal debulking. Some studies have identified the following clinical, histomorphological, and radiological factors, assessable by clinical examination, CT, MR-Scan or laparoscopy, that were associated with suboptimal debulking: older age, preoperative evidence of ascites, higher preoperative CA125 levels, advanced FIGO stage, serous tumors, low performance status *(25; 237)*, and diffuse peritoneal thickening *(67; 75; 232)*.

Another prognostic factor in ovarian cancer is the histological subtype. Regarding pathological diagnosis, invasive carcinoma has to be delineated from epithelial tumors of low malignant potential (LMP, borderline ovarian carcinoma), a histological variant less aggressive than their invasive epithelial counterparts, which is found in younger women, and

often confined to the ovary at diagnosis *(64)*. Of malignant ovarian neoplasms, nearly 90 % are classified as epithelial ovarian carcinomas, i.e. arising from the germinal epithelium lining the ovary. Epithelial ovarian cancer can be further subdivided into several histological cell types: serous, mucinous, endometrioid, clear cell, transitional, and undifferentiated carcinomas. A tumor classified as clear cell or undifferentiated histology predicts a significantly worse prognosis than the rest of the histologies *(50)*.

Nuclear grading describes the grade of dedifferentiation of the tumor cells, implicating tumor aggressiveness. The prognostic value of nuclear grade assessed by the WHO system *(see Appendix)* is somewhat controversial, as this system lacks reproducibility and consistency: criteria are poorly defined, which makes microscopical diagnosis subjective, it cannot be used for all histologies and doubts have arisen as to wether the prognostic influence of grading is only valid for early stages *(45; 207)*. A newer grading system proposed by Silverberg et al in 1998 *(206)* is regarded to be an improvement. It is based on well-defined nuclear and architectural criteria and mitotic count, it can be used in all histological groups and its prognostic impact has been validated *(151)*. Due to the fact that our patient cohort was operated between 1985 and 1999, the WHO system was applied to assess the grading status.

Ovarian cancer is often associated with the presence of ascitic fluid in the peritoneal cavity. Ascites formation is caused by reduced lymphatic drainage due to obstruction of lymphatic vessels by tumor cells, angiogenesis, and hyperpermeablity of microvessels in the abdominal cavity *(10)*. As intraperitoneal tumor spread is facilitated by ascites formation, a large amount of ascites volume is a sign for intraperitoneal carcinosis, which is associated with a poor prognosis *(45; 76; 121; 122)*. Other prognostic factors are a reduced performance status (Karnofsky-Index < 70), age > 60 years, and anemia *(26; 193; 226)*.

1.2 Tumor biological markers in ovarian cancer

In ovarian cancer, reliable tumor markers are in short supply. A tumor marker is a molecule, a process, or a substance that is altered quantitatively or qualitatively in pre-cancerous or cancerous conditions, the alteration being detectable by an assay. These alterations can be produced either by the tumor itself or by the surrounding normal tissue as a response to tumor cells *(92)*. Several categories of markers can be defined *(53; 199)*: Screening or diagnostic tumor markers to detect a malignant disease in an individual, like the approved cancer screening markers human tissue kallikrein 3 (hK3/PSA) in serum for prostate cancer and hemoglobin (faecal occult blood test) for colon cancer *(32)*. Prognostic markers, which are tools for estimating the risk of disease recurrence and/or cancer-related death for an individual patient after primary surgery (plus/minus initial therapy), while predictive tumor markers will foretell how well the tumor responds to a given therapy; for example estrogen and progesterone receptor (ER, PR) for endocrine therapy in breast cancer. Monitoring markers, which are used to assess efficacy of a given therapy or to detect disease recurrence or remission during follow-up can also be helpful for localizing tumors, for targeting of cytotoxic agents, and for developing new therapeutic approaches.

1.2.1 CA125

CA125 is currently the best established tumor-associated marker available for the management of patients with ovarian cancer. Cancer antigen 125 (CA125) is a high-molecular-weight glycoprotein of unknown function, contributed on the endothelium of fallopian tubes, the endometrium, the endocervix, and also in the normal ovary. Elevation in serum concentrations is a result of vascular invasion, tissue destruction and inflammation in malignant disease *(86)*. It is expressed by more than 80 % of non-mucinous epithelial ovarian cancers. Although serum levels are elevated in most women with advanced ovarian cancer, only 50 % of patients with early-stage disease have an elevated CA125, and mucinous epithelial ovarian cancers express this antigen poorly *(80)*. Furthermore, an elevated CA125 is not specific for ovarian cancer. 95 % of healthy adult women have a CA125 serum value under 35 U/mL, but many non-gynecologic and benign gynecologic conditions, i.e. endometriosis, genital and peritoneal infections, uterus myomatosus, liver diseases, autoimmune diseases, and pregnancy are associated with elevation in this serum antigen *(192)*.

In a study by Jacobs et al, a general screening was performed in a group of 22,000 postmenopausal, asymptomatic women. The positive predictive value of CA125 was 3.1 %, which means that only 3.1 % of the patients suspected to suffer of ovarian carcinoma actually had cancer when being operated *(105)*. Therefore ovarian cancer screening is currently not recommended except for high-risk patients. Attempts are being made to improve the performance of screening by using algorithms and testing of multiple tumor

markers *(80; 209; 238)*.

The best established application of CA125 is in monitoring ovarian cancer, either with respect to therapy response or to detect disease recurrence. The rate of decline of CA125 during cytotoxic chemotherapy has been shown to be an independent prognostic factor in multiple trials by indicating treatment response *(7; 42; 190; 230; 233)*. Furthermore, CA125 even seems to be able to replace standard radiological response assessment to determine eligibility for subsequent additional chemotherapy *(86)*. However, caution is needed since levels in the individual undergo random fluctuation and can fall in response to the drainage of malignant effusions or ascites, whereas debulking surgery or a paracentesis can cause a temporary rise in CA125 levels *(222)*. With CA125, recurrent disease can be predicted two to six months before any clinical or radiological symptoms. However, as a palliative salvage chemotherapy started before the beginning of any clinical symptoms does not seem to have an effect on survival *(74)* while it is shortening treatment-free time, CA125 analysis should be reserved as an aid to the diagnosis of relapse when clinically suspected *(153)*.

Studies examining the prognostic implications of preoperative CA125 have been contradictory *(228)* and many investigators have found CA125 to be of no prognostic value *(202; 229)*. Naegele et al identified CA125 as a strong prognostic factor in a study including only patients with limited disease (stage I). Therefore CA125 might be helpful to decide whether adjuvant therapy is appropriate or not *(161)*. Despite the fact that CA125 alone seems to be useless in screening and is a poor predictor of long-term prognosis, it is useful to monitor therapy success, potentially even more so when combined with other tumor markers *(153)*.

1.2.2 Serine proteases

Behavior of tumor cells is modulated by its microenvironment, including extracellular matrix (ECM) components and stromal cells. By cleaving ECM and non-ECM components, proteases or proteolytic enzymes regulate tumor microenvironment and therefore influence tumor growth, apoptosis, angiogenesis, invasion, and metastasis *(132)*. Many studies have shown that a variety of proteolytic enzymes are overproduced either by the cancer cells themselves or by the surrounding stromal cells, and that overexpression alters clinical prognosis. Serine proteases, which account for one third of the total proteases encoded by the human genome, are proteolytic enzymes that hydrolyze peptide bonds or other ester bonds. Hydrolysis starts by the oxygen atom of the hydroxyl group of the serine residue which attacks the carbonyl carbon atom of the susceptible peptide bond. At the same time, the serine transfers a proton to the histidine residue of the catalytic triad, then to the nitrogen atom of the susceptible peptide bond which is thus cleaved and released. The other part of the substrate is now covalently bound to the serine by an ester bond. The charge that develops at this stage is partially neutralized by the third (aspartate) residue of the catalytic

triad. This process is followed by deacylation where the histidine draws a proton away from a water molecule and the `OH´ ion attacks the carbonyl carbon atom of the acyl group that was attached to the serine. The histidine then donates a proton to the oxygen atom of the serine, which will then release the acid component of the substrate *(5; 167; 181; 208)*.

The serine protease urokinase plasminogen activator system with uPA, uPA-receptor (uPAR), and plasminogen is known to be responsible for extracellular proteolysis in cancer *(196)*. During the last years, more factors have been recognized to participate in this proteolytic network, like for example the serine proteases of the human tissue kallikrein family.

1.2.3 Urokinase-type plasminogen activator system

The urokinase-type plasminogen activator system plays a key role in tumor invasion and metastasis, but also in a variety of other biological processes, such as fibrinolysis, inflammation, arteriosclerosis, and wound healing. The fibrinolytic system encompasses the pro-enzyme plasminogen, which is converted by the urokinase-type plasminogen activator uPA or tissue-type plasminogen activator, tPA, into the enzymatically active serine protease plasmin, and the urokinase receptor (uPAR, CD87) and is inhibited by the serpins PAI-1 and PAI-2. tPA is mainly involved in intravascular fibrin homeostasis *(5; 189)*.

Plasmin has broad substrate specificity and is able to degrade many ECM proteins, such as fibronectin (FN), vitronectin (VN), and fibrin. It also catalyzes activation of the zymogen forms of several metalloproteinases (MMPs). Formation of active MMPs allows further degradation of ECM-components like interstitial and type IV collagen *(27)*. Plasmin can also activate or release growth factors such as fibroblast growth factor 2, vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β), leading to enhanced tumor progression and stimulation of angiogenesis and migration *(69)*.

uPA is initially released from normal (e.g. phagocytes, keratinocytes, fibroblasts, trophoblasts) or tumor cells in its one-chain zymogen form, pro-uPA, and then converted to the enzymatically active two-chain form HMW-uPA by proteases such as plasmin, plasma kallikrein, blood coagulation factors, cathepsin B and L, and human tissue kallikrein 3 (hK3/prostate-specific antigen/PSA) *(5)*. Binding of uPA to its membrane-anchored receptor uPA-R (CD87) leads not only to enhanced and focused proteolysis, but also to activation of intracellular signal transduction via tyrosine and serine-protein kinases *(16)*.

uPA activity can be neutralized by two endogenous inhibitors, PAI-1 and PAI-2 *(4)*. These serpins have shown, independently from their interaction with uPA, to be capable to inhibit apoptosis. Additionally, PAI-1 modulates cell adhesion and migration by interacting with vitronectin *(46; 111; 127; 137)*. Interaction of PAI-1 (but not PAI-2) with the uPA/uPA-R-complex induces internalization of the ternary complex uPA-R/uPA/PAI-1 via help of transmembrane receptors of the LDL-receptor family, which results in intracellular

degradation of uPA and PAI-1, while uPA-R is recycled to the cell surface *(43)*.

Binding of pro-uPA to uPA-R provides the cell surface with a potential proteolytic activity in specific localizations that may induce interactions with the adhesive machinery of the cell and thus may initiate a signal-transduction pathway. pro-uPA is converted to active uPA, which catalyzes activation of plasminogen on cell surfaces. uPAR-bound uPA can react with VN-bound PAI-1. The formed complexes can be endocytosed and degraded by endocytosis receptors *(16)*.

In order to alter the course of malignant disease, WX-UK1, a novel small-size synthetic inhibitor directed to serine proteases such as uPA and plasmin, has been developed. WX-UK1 has shown to significantly inhibit metastasis without major side-effects and is now studied in phase I/II clinical trials *(197)*.

Clinical findings have demonstrated that elevated tumor antigen levels of uPA and/or PAI1 are conductive to tumor cell spread and metastasis and are associated with poor disease outcome in a variety of solid tumors, like in cancer of the breast, ovary, lung, prostate, stomach, pancreas, cervix, and colon *(5; 12; 69; 95; 99; 110; 118; 165; 172; 177; 178; 196; 210; 234)*. In breast cancer, uPA and PAI-1 can be used as a tool for therapy decision making and as predictors of response to therapy *(89; 90)*. Numerous international studies have shown that breast cancer patients with low levels of components of the uPA-system in tumor tissue have a significantly better survival rate than patients with high levels of either factor. Its components uPA and PAI-1 have been validated at the highest level of evidence with regard to their clinical utility *(107; 136)*.

Several studies have indicated that uPA and PAI-1 are involved in ovarian tumor progression and have a clinical impact on prognosis. Higher antigen content of uPA and/or PAI-1 in plasma, cytosol, and/or ascites fluid is associated with advanced or more aggressive disease and predicts poor patient outcome. A number of independent research groups have evidenced that uPA and PAI-1 content is increasing from benign ovarian over low malignant potential tumors (LMP) to invasive ovarian cancer *(83; 119; 122; 195; 231)*. In a clinical study conducted by Konecny et al *(119)*, that included 82 patients suffering from primary ovarian carcinoma, uPA and, to a lesser extent, PAI-1 have shown to be of prognostic relevance: uPA correlated to higher FIGO stage and protein levels of uPA in tissue samples of intra-abdominal metastasis were significantly higher than those present in primary tumors. The group with high antigen levels in primary tumor, defined by optimized cut-off values calculated by log-rank statistics, showed shortened progression-free survival and overall survival and uPA retained a statistically significant prognostic factor in multivariate analysis.

Kuhn et al *(122)* concentrated on the clinically most relevant ovarian cancer patients classified as FIGO III and demonstrated that overall survival is prolongued in patients with a lower uPA and/or PAI-1 content in primary tumor cytosols (using optimized cut-off values). In a study by van der Burg *(231)*, these findings could only be approved for PAI-1 as a trend for

shorter PFS and OS, but no significant impact could be assessed. Nevertheless, the prognostic value of PAI-1 could be confirmed in a cohort of patients with FIGO IIIc, even by multivariate analysis. This prognostic power was lost, however, over time *(125)*. Neither Kuhn nor van der Burg found any correlation of uPA or PAI-1 content with established prognostic factors such as age, FIGO stage, amount of ascitic fluid, or nodal status *(122; 231)*. In another group of patients with ovarian cancer staged FIGO I-IV, uPA did not correlate with stage or cell type, but grade 3 tumors had significantly higher uPA levels than grade 1 or 2 and uPA was higher in recurrent than in primary tumors *(83)*.

Schmalfeldt et al *(195)* demonstrated in a group of 39 patients with advanced ovarian carcinoma (FIGO IIIc/IV) that median uPA concentrations in omentum metastases were four times higher than in primary tumors, while PAI-1 content increased two-fold *(194)*. In the group of patients classified FIGO IIIc, PAI-1 turned out to be a strong, statistically independent prognostic parameter only surpassed by residual tumor mass and was more important than clinical parameters such as nodal status, ascitic fluid volume, grading, and age *(194)*.

Using immunohistochemistry, Chambers et al *(31)* pointed to the prognostic impact of PAI-1 expression in the group of FIGO III and IV patients regarding overall survival. Findings are sometimes contradictory, for example is plasma PAI-1 correlated with the presence of malignant tumor and higher stage of disease *(96)*, while high PAI-1 levels in ascites predict prolongued progression-free survival *(30)*.

1.2.4 Tissue kallikrein family

The term “kallikrein” was introduced by Werle et al in the 1930s to describe enzymes which cleave small vasoactive peptides, kinins, from high-molecular-weight precursors – kininogenes *(236)*. Kallikrein enzymes are divided into two different groups: Tissue kallikreins and plasma kallikreins. Plasma kallikrein (*KLK*B1) is located on chromosome 4, expressed exclusively in the liver, and involved in blood clotting, fibrinolysis, inflammation, and regulation of blood pressure *(9)*. The human tissue kallikrein family consists of 15 homologous serine proteases, but until the mid 1990s, it was thought that it contained only the three following members:

- Human tissue kallikrein 1/pancreatic/renal tissue kallikrein (*KLK1*/hK1),
- Human tissue kallikrein 2/glandular tissue kallikrein-1 (*KLK2*/hK2), and
- Human tissue kallikrein 3/prostate-specific antigen (*KLK3*/PSA/hK3).

In the past seven years, the collective efforts of a number of independent researchers, primarily those of the group of E.P. Diamandis, Toronto, Canada, and J. Clements, Brisbane, Australia, have contributed to the expansion of the human tissue kallikrein gene family *(82; 91; 216; 254)*. According to the official nomenclature, tissue kallikrein gene and protein

symbols are denoted as `*KLK*´ and `hK´, respectively (see Appendix: Official and alternative tissue kallikrein gene and protein names). Only pancreatic/renal tissue kallikrein (*KLK1*) enzyme has kininogenase activity; the membership of the other tissue kallikreins is defined by conserved gene and protein structures and the same gene locus.

Prostate-specific antigen (PSA, hK3) is the best characterized tissue kallikrein so far and the most valuable biomarker in clinical medicine for prostate cancer diagnosis and monitoring in high-risk populations. With the identification and characterization of all members of the tissue kallikrein gene family, accumulating reports started to indicate that in addition to the established tissue kallikreins hK1, hK2, and hK3, the novel tissue kallikreins hK4 to hK15 might also be related to hormonal malignancies such as that of the prostate, testis, breast, and ovary, as there is strong experimental and clinical evidence linking tissue kallikreins and cancer, i.e. as new serum and/or tissue biomarkers *(39; 61; 169; 246)*. The human tissue kallikrein gene locus spans a 300 kb region on chromosome 19q13.3-13.4 and is comprised of 15 tandemly arranged genes bound centromerically by the testicular acid phosphate gene (ACPT) and telomerically by Siglec-9, a member of the immunoglobulin family *(78; 250)*. Except *KLK2* and *KLK3*, all genes are transcribed from telomere to centromere.

All human *KLK* genes consist of five coding exons and four intervening introns. Sizes of the genes range from 4-10 kb with most of the differences relating to variable intron sizes, although intron phases are completely conserved *(140)*. The five coding exons are very similar both in size and organization. Coding exon 1 harbors the start codon, coding exons 2, 3, and 5 contain the histidine (H), aspartic acid (D), and serine (S) codons of the catalytic triad. Coding exon 5 harbors the stop codon. In contrast to the `classical´ *KLK* genes, most of the new members of the human tissue kallikrein family have one or two non-coding exons in the 5´ untranslated region (UTR). The 3´ UTR typically varies in length *(21)*.

hK proteins are single-chain secreted serine endopeptidases of 25-30 kDa. They are synthesized as pre-pro-enzymes containing an amino-terminal single peptide (Pre), that directs them to the endoplasmic reticulum for secretion, followed by a pro-peptide (Pro) of four to nine amino acids, that maintains them as inactive precursors (zymogen) and a catalytic domain, which comprises the mature, enzymatically active protein. hK proteins have fully conserved amino acids around the catalytic residues, as well as overall amino acid sequence identity of 40-80 % *(169)*. Most of the tissue kallikrein enzymes denote a trypsin-like action, while enzymatic activity of hK3, hK7, and hK9 are chymotrypsin-like *(39)*. **Figure 1** shows common features of the human tissue kallikrein family.

Alternatively processed mRNA transcripts are common among members of the tissue kallikrein family. In fact, all *KLK* genes possess at least two transcripts as a result of alternative splicing, promoters/transcription start sites, polyadenylation signals and combinations thereof. A total of 70 alternative *KLK* mRNA isoforms have been identified to date, exclusive the classical form. Most of alternative splicing occurs in coding regions, and

only few within the 5´ UTR, primarily involving exon skipping, exon extension/truncation and intron retention *(21)*. The degree of tissue specificity in expression and the relationship to a particular patho-physiological condition requires further investigation. Emerging data indicate that *KLK* transcriptome alteration occurs in neoplastic progression and harbors several cancer-specific mRNAs: three *KLK*4 *(66)*, three *KLK*5 *(65; 126; 267)*, one *KLK*7 *(65)* and two *KLK*8 *(149)* alternative transcripts for example are overexpressed in ovarian tumors and/or cell lines compared with normal ovaries.

Tissue kallikreins are expressed in several tissues, at both the mRNA and protein level. Using Northern blot, RT-PCR, and/or ELISA, tissue kallikreins are found with highest expression levels within a few major tissues like the salivary gland, the CNS, the prostate, and the breast and with lower levels in many others (summarized in **Figure 2)**.

Figure 1: Tissue kallikrein locus, gene, and protein characteristics *(18)*

a) KLK gene locus on chromosome 19q13.4. `Classical´ KLK genes (KLK1, KLK2, KLK3/PSA): black arrowheads, KLK4-KLK15: grey arrowheads, tissue kallikrein pseudogene (ΨKLK1): white arrowhead

b) KLK genes (4-10 kb) are organized very similarly regarding exons (grey boxes), intron phases (white boxes), codons of the catalytic triad histidine (H), aspartic acid (D), serine (S), start codon (↓) and stop codon (*)

c) hK proteins are synthesized as pre-proenzymes containing an amino-terminal signal sequence (Pre), a propeptide (Pro), and a catalytic serine-protease domain

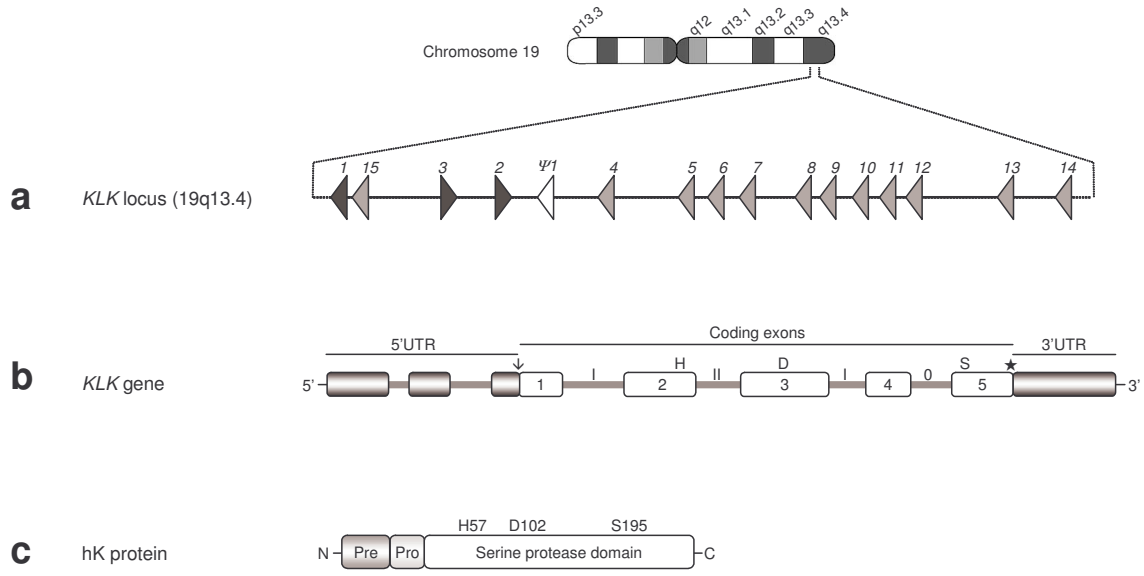


Figure 2: Tissue expression of tissue kallikreins verified by Northern Blots and RT-PCR

High expression is marked with a dark grey square, low expression with a grey square, no expression with a white square *(6; 22; 36; 38; 82; 88; 98; 134; 140; 157; 166; 182; 216; 240; 241; 244-246)*

	KLK1	KLK2	KLK3	KLK4	KLK5	KLK6	KLK7	KLK8	KLK9	KLK10	KLK11	KLK12	KLK13	KLK14	KLK15
BREAST															
CNS															
LUNG															
KIDNEY															
OVARY															
PANKREAS															
PROSTATE															
SALIVARY GLAND															
SKIN															
TESTIS															
THYROID															
TRACHEA															
UTERUS															

Tissue kallikreins are often co-expressed in the same tissues, including steroid hormone-producing or hormone-dependent tissues like the prostate, breast, testis, and ovary. The parallel expression of many tissue kallikreins in the same tissue under physiologic and/or pathologic situations suggests that they may participate in enzyme cascade reactions similar to those established for the processes of digestion, fibrinolysis, coagulation, complement activation, wound healing, angiogenesis, and apoptosis *(248)*. The diversity of expression in human tissues further suggests that they may act on different substrates in different tissues.

Tissue kallikrein gene expression is regulated by a number of stimulating and inhibiting factors that influence many signaling pathways. All tissue kallikrein genes are regulated by steroid hormones and are differentially expressed in endocrine-related malignancies or cell lines *(21; 249)*. *KLK2,* and *KLK3*, which are mainly expressed in the prostate, are the classical androgen-regulated genes *(242)*, whereas *KLK1, KLK5, KLK6* and *KLK10 (143; 144; 255; 259)* are more responsive to estrogen. Expression of all other *KLK* genes is also affected by androgenic and/or estrogenic stimulation, sometimes dependent on the tissue: *KLK4* is upregulated by androgens in prostate and breast cancer cell lines *(166; 258)* and by estrogen in endometrial cell lines *(159)*, while *KLK12* is upregulated by androgens and progestin in prostate cancer cell lines and by estrogens and progestin in breast cancer cell lines *(257)*.

All tissue kallikrein proteins are synthesized as inactive precursors or zymogens, with an inhibitory pro-peptide that sterically blocks the active site and thereby prevents substrate binding. Zymogen conversion to the active enzyme generally occurs by limited proteolysis of the pro-peptide via diverse mechanisms, intra- or extracellular and needs a trypsin-like activity except in hK4 *(113)*. In vitro, hK2 *(155)*, hK6 *(148)*, and hK13 *(212)* are autoactivated while some tissue kallikreins are activated by other hKs: pro-hK3 is activated by hK2 *(138)*, hK4 *(221)*, and hK15 *(219)*; pro-hK7 is activated by hK5 *(29)* and other serine proteases, including trypsin *(220)* and metalloproteinases *(218)*.

Activated zymogen function of tissue kallikreins is controlled by endogenous inhibitors such as α_2-macroglobulin and other serpins (serine protease inhibitors), for example α_1-antichymotrypsin, α_2-antiplasmin, antithrombin III, or PAI-1 (plasminogen activator inhibitor type–1), which form complexes with several hKs *(36; 104; 131; 148; 154; 156; 188; 214; 252)*. Other forms of inactivation, like internal cleavage and subsequent degradation, has been described for several hKs *in vitro (148; 188; 212)*. Only the three classical tissue kallikreins have been assigned a specific biological function. hK1 exerts its biological activity mainly through the release of lysylbradikinin (kallidin) from low-molecular-weight kininogen. The diverse expression pattern of hK1 has led to the suggestion that the functional role of this enzyme may be specific to different cell types and in the processing of growth factors and peptide hormones *(14; 191)*.

The physiologic function of hK2 protein has been examined only recently. hK2 has very low

kininogenase activity compared to hK1 *(34)*. Seminal plasma hK2 cleaves seminogelin I and seminogelin II, but at different cleavage sites and at a lower efficiency than i.e. PSA *(47)*. A role of hK2 in regulating growth factors, through insulin-like growth factor binding protein-3 (IGFBP-3) proteolysis, has been suggested. hK2 was also found to activate the single-chain proform of urokinase-type plasminogen activator (uPA) *(79)*. As uPA has been implicated in the promotion of cancer metastasis, hK2 may be part of this pathway in cancer. hK3 (human tissue kallikrein 3/prostate-specific antigen/PSA) is present at very high concentrations in seminal plasma, rapidly hydrolyzing both seminogelin I and seminogelin II, as well as fibronectin, causing liquefaction of the seminal plasma clot after ejaculation *(130)*. Several other potential substrates for hK3 have been identified, including IGFBP-3, tumor growth factor-β, basement membrane, parathyroid hormone-related peptide, and plasminogen *(59)*. The physiologic relevance of these findings is still not clear. hK3 is found in nipple aspirate fluid, breast cancer cyst fluid, milk of lactating women, amniotic fluid, and breast cancer tumor tissue. It is thus very likely that hK3 has extraprostatic biological functions in breast and other tissues and may also play a role during fetal development *(52)*.

Among all the other 12 novel human tissue kallikreins, only a few have been connected to physiologic processes and/or pathologic conditions. Putative functions have been proposed for several of the tissue kallikreins, based on the sites of expression and/or the activity of orthologue proteins. Isolation or cloning of human tissue kallikreins from specific tissues such as the skin (*KLK5, KLK7, KLK11*) and brain (*KLK6, KLK8, KLK11*) suggests a role at these sites *(38; 82)*. *KLK7* is linked to the desquamation of stratum corneum and it is suggested to play a role in pathological keratinization and psoriasis *(22; 70; 139; 211)*. *KLK6* is hypothesized to be important in the deposition of amyloid plaques in Alzheimer´s disease *(134)*. Potential substrates and functions have been identified only by in vitro biochemical and cell-culture systems and/or extrapolated from the known actions of their orthologues. That is why the complex action and interaction of members of the human tissue kallikrein family yet is to be fully elucidated.

With the identification and characterization of all members of the tissue kallikrein gene family, accumulation reports started to indicate that in addition to the established tissue kallikreins hK1, hK2, and hK3 the novel tissue kallikreins hK4 to hK15 might also be related to hormonal malignancies such as that of the prostate, testis, breast, and ovary. Until now, the tissue kallikrein family proved to be a rich source of cancer biomarkers *(21; 58; 59; 247)*. The mechanism by which tissue kallikreins might be involved in the pathogenesis and/or progression of cancer is not yet fully understood, but preliminary reports indicate that hKs participate early and via several steps in neoplastic progression.

Growth and survival: hKs promote or inhibit cancer cell proliferation by modulating the bioavailability and activity of latent growth factors. hK1, hK2, and hK3 degrade insulin-like growth factor binding proteins (IGFBP2, 3, 4, 5) *in vitro* and thereby liberate insulin-like

growth factor 1 (IGF1), which, by binding to its receptor (IGF1R), has a proliferate and anti-apoptotic activity *(41; 183; 185; 217)*. hK2 and hK4 interact with the urokinase plasminogen activator system by activating single-chain pro-uPA *(79; 221)*, and inactivating (only hK4) plasminogen inhibitor type-1 (PAI-1) *(154)*, leading to release and/or activation of growth factors from the extracellular matrix (ECM). hKs may also act as growth factors themselves by activating protease-activated receptor (PAR) signaling *(171)*. Conversely, hK3 can also release transforming growth factor-β (TGF-β) from its latent complex, which, bound to its receptor, might suppress tumor growth *(48)*.

Angiogenesis: hKs might promote angiogenesis by modulating its activation, facilitating endothelial-cell proliferation, migration and capillary-tube formation through direct or indirect ECM degradation. *In vitro* hKs cleave structural components of the subendothelial basement membrane (BM) and extracellular matrix (ECM) *(13; 40; 148; 235)*. Human tissue kallikreins also interact with the uPA system *(79; 154; 221)* and matrix metalloproteinases (MMPs) *(49; 152; 227)*. These systems further promote ECM degradation through plasmin, liberation or activation of proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and pro-MMPs and therefore tumor invasion and metastasis. Activation of TGF-β (by hK3) *(48)* and release of bradykinin from kininogen (by hK1) *(72)* leads to stimulation of angiogenesis. PAR signaling (see above) induces endothelial cell proliferation *(108)*. Angiogenesis may be inhibited by hK3, hK6, and hK13 by generating angiostatin-like fragments from plasminogen *(8; 93; 212)*. Angiostatin is a potent inhibitor of endothelial cell proliferation and angiogenesis *in vivo (18)*.

Invasion and metastasis: hKs may directly or indirectly regulate invasion by dissolution of ECM barriers. hKs could activate PAR signaling with a consequent stimulatory or inhibitory effect on tumor cell invasion *(94; 109)*. hK3 liberates TGF-β *(48)* from its latent complex and therefore promotes epithelial-to-mesenchymal transition (EMT), which is necessary for tumor cells to detach, invade, and metastasize. All human tissue kallikreins are differentially expressed or have altered protein content in fluids, tumor specimens, or cell lines of hormone-dependent malignancies like prostate cancer, breast cancer, testicular cancer and ovarian cancer, as summarized in Table 1, Table 2, and Table 3.

In prostate cancer, human tissue kallikrein hK3 (PSA) is used as a screening and diagnostic marker for prostate cancer and for monitoring tumor recurrence *(81)*. hK2, to a lesser degree, is also being examined as a diagnostic/prognostic marker for prostate disease *(184)*. hK2 can also activate pro-PSA, demonstrating the close relationship of these two prostatic tissue kallikreins *(220)*. At the mRNA level, *KLK2, 3, 5, 6, 10* and *13* are down-regulated in cancerous prostate tissue compared to normal adjacent tissue *(1; 87; 150; 179; 180; 261)*, whereas *KLK11, 14,* and *15* are overexpressed *(56; 215; 266)*. Higher *KLK5* and *KLK11* mRNA levels and lower tissue hK3 concentration is associated with more aggressive forms of cancer and poor patient prognosis. On the protein level, approximately 60 % of men with

prostate cancer show elevated hK11 serum levels **(Table 1)** *(213)*.

In breast cancer tissues and/or cell lines, tissue kallikrein genes *KLK3, KLK10, KLK12, KLK13,* and *KLK14* are downregulated at the mRNA level *(51; 84; 135; 244; 256; 257; 269-271)*. *KLK6* gene is down-regulated in metastatic breast cancer and upregulated in primary breast cancer *(6)*. *In silico* analysis of tissue kallikrein mRNA expression levels in normal and cancerous breast tissues and cell lines suggest that tissue kallikrein genes *KLK5, KLK6, KLK8,* and *KLK10* are down-regulated in breast cancer, partially consistent with recent findings *(268)*. hK3 expression is not only associated with prostate but also with breast diseases *(269)* and has recently been suggested to be a useful prognostic marker for breast cancer *(15)*. Although human tissue *KLK5 and KLK14* mRNA levels are reduced in breast cancer, elevated serum levels of the hK5 and hK14 proteins were observed in a subgroup of breast cancer patients *(259)*.

Table 1: Differential expression of human tissue kallikreins at the protein or mRNA level and potential clinical applicability in prostate cancer

Tissue kallikrein	Source	Clinical relevance	Reference
hK2	Serum and tissue from prostate cancer patients	Marker of diagnosis	*(44; 150)*; *(174; 188)*
hK3	Serum and tissue from prostate cancer patients	Population screening, marker of diagnosis, prognosis, monitoring	*(150; 188)*
KLK5	mRNA from matched normal and cancerous prostate tissues	Prognostic marker	*(261)*
KLK11	mRNA from matched normal and cancerous prostate tissues	Prognostic marker	*(56; 163)*
hK11	Serum from prostate cancer patients	Diagnostic marker	*(56; 162; 213)*

This discrepancy between hK5 and hK14 mRNA and serum protein levels in breast cancer has also been observed for hK3/PSA in prostate cancer. In these cases, the elevation of tissue kallikrein proteins in the serum may be due to angiogenesis and/or destruction of glandular architecture during carcinogenesis, thereby facilitating the outflow of hKs into the circulation. For some hKs, a prognostic impact in breast cancer can be observed **(Table 2)**: higher mRNA expression of *KLK5, KLK7* and *KLK14* are associated with poor prognosis *(223; 243; 263)*, while expression levels of *KLK9, KLK13,* and *KLK15* as well as hK3 protein content are indicative for a favorable disease outcome *(33; 77; 264; 265; 270; 271)*. High levels of hK3 and hK10 proteins in breast carcinomas predict a poor response to tamoxifen therapy *(77; 142)*.

Table 2: Differential expression or protein content of human tissue kallikreins in breast cancer

Tissue kallikrein	Source	Clinical relevance	Reference
hK3	Serum and tissue from breast cancer patients	Favorable prognosis, predictive response to tamoxifen	*(77; 101; 269-271)*
KLK5	mRNA from cancerous breast tissues	Unfavorable prognosis	*(263)*
hK5	Serum from breast cancer patients	Diagnostic marker	*(259)*
KLK7	mRNA from cancerous breast tissues	Unfavorable prognostic marker	*(223)*
KLK9	mRNA from breast cancer tissues	Favorable prognostic marker	*(265)*
KLK10	mRNA in breast ductal carcinoma in situ	Predictive of invasiveness	*(51; 268; 272)*
hK10	Cytosols from cancerous breast tissues	Predictive of response to tamoxifen	*(142)*
KLK13	mRNA from cancerous breast tissues	Favorable prognosis	*(33)*
KLK14	mRNA from breast cancer tissues	Unfavorable prognosis	*(243)*
hK14	Serum and tissue from breast cancer patients	Diagnostic marker	*(20)*
KLK15	mRNA from breast cancer tissues	Favorable prognostic marker	*(264)*

With respect to ovarian cancer, numerous studies using Northern-Blot, RT-PCR, or immunoassay have shown that the tissue kallikreins hK4, hK5, hK6, hK7, hK8, hK10, hK11, hK13, hK14, and hK15 are overexpressed in ovarian carcinoma tissues, serum and/or cell lines at the mRNA and/or protein levels, as summarized in **Table 3**. The up-regulation of tissue kallikrein genes *KLK5, KLK6, KLK7, KLK8, KLK10, KLK11,* and *KLK14* was further verified by *in silico* analysis of tissue kallikrein gene expression in normal and cancerous ovarian tissues and cell lines by Yousef et al *(260)*. *KLK4, KLK5/hK5, KLK6/hK6, KLK7, hK10,* and *KLK15* seem to be markers of poor prognosis, while *KLK8, KLK9,* hK11, hK13, and *KLK14* have shown to be markers of favorable prognosis.

Using ELISA, elevated serum or tissue levels of hK5, hK6, hK8, hK10, hK11, hK13, and hK14 protein were found in a proportion of ovarian cancer patients and seem to have clinical value either as biomarkers for detection or diagnosis or as predictors of prognosis *(20; 56; 57; 62; 116; 141; 146; 200; 259)*.

hK5 is elevated in 69 % of ovarian cancer patient serum samples compared to almost

undetectable serum levels of normal individuals or patients suffering from other malignancies *(259)*. hK5 can also be detected in ascitic fluid of ovarian cancer patients *(259)* and in the specimens: analysis of ovarian tumor tissue extracts has indicated that hK5 is elevated in cancerous compared to normal or benign ovarian tissue cytosols *(259)*, also compared to tumors with low malignant potential (LMP) *(55)*. hK5 overexpression in ovarian cancer patients, defined by exceeding the optimized cut-off value, predicts poor outcome and is associated with more aggressive forms of ovarian cancer *(55)*.

hK6, like hK5, is significantly elevated in malignant tumor serum samples compared to normal or benign ovarian tumor serum samples. Pre-surgical serum hK6 levels increase the diagnostic sensitivity of CA125 in patients with early stage (I/II) ovarian cancer and predict overall and progression-free survival *(57)*. Analysis of hK6 in tumor cytosols using ELISA indicates that hK6-positive tumors are more likely attached to advanced disease, serous histology, and suboptimal debulking. hK6 has a prognostic impact on overall survival and progression-free survival, especially in subgroups with a good prognosis at first sight, e.g. optimally debulked and low-grade tumors *(97)*. hK8 is not only elevated in serum of ovarian cancer patients, but also in the ascitic fluid, whereas higher levels indicate a better prognosis *(115)*.

A promising finding is the distinct value of hK10 as serum biomarker. hK10 is elevated in ovarian cancer serum samples, but not in healthy individuals *(141)*. Similar to hK6, hK10 increases sensitivity and specificity of CA125 in detecting early stage ovarian cancer *(146)*. Multiple studies have indicated that hK10 is associated with a more aggressive disease, i.e. higher FIGO stage, serous histotype, higher nuclear grade, suboptimal debulking, and low response to chemotherapy. In the subgroup with advanced disease, hK10 is predicting survival *(141; 146; 147)*.

hK11 antigen content in serum is not only elevated in prostate cancer patients but also in 70 % of ovarian cancer patients compared to healthy individuals *(56)*. hK11 concentration is evaluated in ovarian cancer tissue, being an indicator of favorable prognosis and associated with early stage, low grade, and complete or partial response to chemotherapy phenotype *(19; 54)*. Analogous to hK11, hK13 antigen analysis in tumor cytosols indicates a good prognosis for hK13-positive patients, who tend to have less advanced tumors (FIGO stage I or II) and are subject to optimal surgical debulking *(200)*. Still, serum content of hK13 in ovarian cancer patients and its clinical relevance is to be elucidated. Preliminary data indicate that hK14 is also elevated in tumor tissues of a proportion of ovarian cancer patients as well as in the serum of a subset of ovarian cancer patients *(20)*.

Table 3: Differential protein or mRNA expression of human tissue kallikreins in ovarian cancer patients

Tissue kallikrein	Source	Clinical relevance	References
KLK4	mRNA from normal and cancerous ovarian tissues	Unfavorable prognosis	*(170)*
hK4	Ovarian cancer tissue	Marker of resistance to paclitaxel therapy	*(239)*
KLK5	mRNA from cancerous ovarian tissue	Unfavorable prognostic marker	*(114; 260)*
hK5	Cytosols and serum of ovarian cancer patients	Marker of diagnosis and unfavorable prognosis	*(55; 259)*
KLK6	mRNA from ovarian cancer tissue	Unfavorable prognostic marker	*(224; 260)*
hK6	Cytosols of normal, benign and cancerous ovarian tissue Serum of ovarian cancer patients	Marker of diagnosis, unfavorable prognosis, monitoring	*(57; 62; 97)*
KLK7	mRNA from cancerous ovarian tissue	Unfavorable prognosis	*(65; 128; 225)*
hK7	Cytosols from normal and cancerous ovarian tissue	Unfavorable prognostic marker	*(225)*
KLK8	mRNA from ovarian cancer tissues	Favorable prognostic marker	*(149; 204)*
hK8	Serum and ascites from ovarian cancer patients	Marker of diagnosis, prognosis and monitoring	*(115; 204)*
KLK9	mRNA from ovarian cancer tissues	Favorable prognosis	*(253)*
hK10	Serum and tissue from ovarian cancer patients Cytosol from normal, benign and cancerous tissues	Marker of diagnosis, unfavorable prognosis and monitoring	*(141; 146; 147)*
KLK11	mRNA from normal and cancerous ovarian tissues	Unfavorable prognostic marker	*(203)*
hK11	Cytosols and serum from ovarian cancer patients	Diagnostic and favorable prognostic marker	*(19; 54; 56)*
hK13	Cytosols of ovarian cancer tissues	Favorable prognostic marker	*(200)*
KLK14	mRNA from normal, benign and ovarian cancer patients	Favorable prognostic marker	*(251)*
hK14	Serum and tissue from ovarian cancer patients	Diagnostic marker	*(20)*
KLK15	mRNA from benign and cancerous ovarian tissues	Unfavorable prognosis	*(244; 262)*

2. Aim of the study

Numerous studies have focused on a better understanding of the underlying tumor biology in ovarian cancer. Still today, no single marker or parameter was found which predicts exactly disease progression, response to therapy or surgical outcome for the individual ovarian cancer patient.

The aim of this study therefore was:

- to identify and test adequate tumor biomarkers,
- to better understand the tumor biology in ovarian cancer, and
- explore the potence of these markers to support individualized therapy decisions.

To achieve this, the following steps were taken:

1. Tissue cytosols from epithelial ovarian carcinoma patients obtained at primary surgery, from primary tumor, and omentum metastasis, were assessed by ELISA for the content of human tissue kallikrein protein, hK5, hK6, hK7, hK8, hK10, hK11, hK13 and key factors of the plasminogen-activator system, uPA and PAI-1.
2. The statistical associations between the protein levels of these tumor tissue-associated factors, in particular according to subgroups defined by FIGO stage and nuclear grading, were explored and thus interactions between the serine proteases, which are hypothesized to participate in a cascade, were further investigated.
3. The clinical utility of these proteases as novel tumor markers to predict prognosis were analyzed in this homogeneous patient collective treated according to national treatment guidelines valid at the time. The statistical relevance and clinical impact of the analyzed antigens on progression-free and overall survival was assessed as well as its potential to predict therapy success.
4. For some of the antigen levels, previously defined cutoffs to distinguish between low- and high-risk groups were tested for their clinical utility in this collective.
5. Not only in primary tumor but also in omentum metastasis, the content of these biomarkers was determined and their differences between primary tumors and omentum metastases was evaluated.
6. In addition to the new biomarkers, statistical analyses encompassing clinically established prognostic / predictive factors such as age, FIGO stage, residual tumor, histology, presence and volume of ascites and nuclear grade was performed to determine overall and progression-free survival.

3. Materials and Methods

3.1 Patients

One-hundred and forty-two patients afflicted with ovarian cancer stage FIGO I to IV (Fédération Internationale de Gynécologie et d'Obstétrique) were enrolled in a retrospective study conducted between 1985 and 1999 at the Department of Obstetrics and Gynecology of the Technical University of Munich. Standard surgical procedures were performed including partial resection of the small and large intestine, diaphragmatic peritoneum, peritonectomies and upper abdominal surgery, as well as pelvic and para-aortic lymphadenectomy if indicated *(122; 125; 194)*. In younger patients (< 35 years) with tumor stage FIGO I less radical surgery was performed in order to preserve the fertility of the patients *(194)*. The study to collect tissue from ovarian cancer patients to assess the patients proteolytic factor profile was approved by the Ethics Committee of the University Hospital Klinikum rechts der Isar of the Technical University of Munich. All patients gave written informed consent. Patients were chosen regarding availability of sufficient tumor material for the different analyses and complete clinical data. Following surgery, all patients received adjuvant platinum-containing chemotherapy treatment according to consensus recommendations at that time. None of the patients received any neoadjuvant therapy before surgery. Seventy-two patients (50.7 %) were optimally debulked without macroscopical sign of residual tumor after surgery. Median follow-up in patients still alive at time of analysis was 41 months **(Table 5a)**. In a subgroup of 54 patients, not only primary tumor but also metastasis specimens were available **(Table 5b)**.

3.2 Tissue collection and extraction

At the laboratory of the Clinical Research Unit, Department of Obstetrics and Gynecology, Klinikum rsechts der Isar, Technical University of Munich, tissue collection and tissue extraction is performed routinely *(195; 198)*. Tissue samples were collected during surgery, immediately examined by the pathologist and then the specimens stored in liquid nitrogen until extraction. Approximately 200 mg of deep-frozen ovarian tissue was pulverized for 30 seconds at maximum speed to fine powder by a micro-dismembrator (Braun-Melsungen, Melsungen, Germany) and resuspended in 1.8 ml cold extraction buffer containing 0.02 M Tris-HCl (pH 8.5), 0.125 M NaCl (Merck, Darmstadt, Germany) and 1 % Triton X-100 (Sigma-Aldrich, Munich, Germany). After gentle rotation for 15 h at 4 °C and ultracentrifugation for 45 min at 100,000 x g (4 °C), the supernatant was stored in liquid nitrogen in cryovials (1.2 ml, Nunc, Wiesbaden, Germany) until further analysis.

3.3 ELISA (Enzyme-linked immunosorbent assay)-analysis

CA125 antigen in serum analysis was performed routinely by the Department for Clinical

Chemistry, Klinikum rechts der Isar, Technical University of Munich, Germany, while CA125 antigen in cytosol was quantified by the Department of Clinical Chemistry at the Mount Sinai Hospital, Toronto, Canada, using the CA125 II assay by Roche Diagnostics GmbH, Mannheim, Germany.

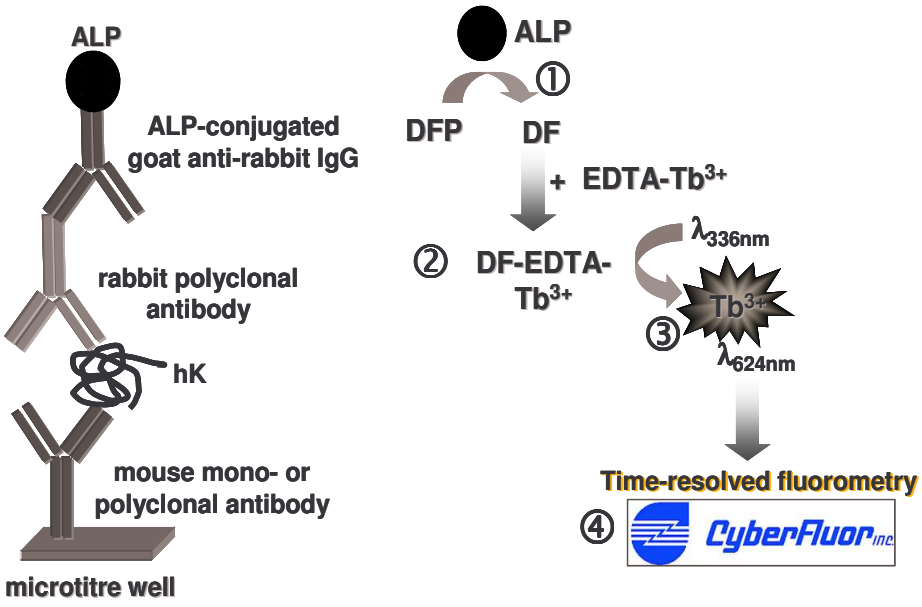
Protein content in the samples was determined together with the staff of the Clinical Research Unit of the Department of Obstetrics and Gynecology, Technical University of Munich, using the BCA Protein Assay reagent kit (Pierce, Rockford, IL, USA) according to the manufacturer's manual. This method is based on reduction of Cu^{++} by protein to Cu^{+} in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) by a reagent containing bicinchoninic acid (BCA). The absorption of the resulting purple color reaction is measured at 540 nm, the detection range is 5 to 250 µg/ml.

uPA and PAI-1 antigen was quantified by the staff of the Clinical Research Unit of the Department of Obstetrics and Gynecology, Technical University of Munich, Germany, using the commercially available Immunoassays Imubind uPA (Product #894) and PAI-1 (Product #821) ELISA kits (American Diagnostica, Stamford, CT, USA) according to the manufacturer's manual. These ELISAs (enzyme-linked immunosorbent assays) employ a murine monoclonal antibody against human uPA or PAI-1, respectively, as the capture antibody. Samples were incubated in antibody-precoated microtest wells and detected by a second, biotinylated antibody recognizing the bound uPA-/PAI-1- molecules. Addition of streptavidin horseradish peroxidase-conjugated (HRP) completes the formation of the antibody-enzyme detection complex. Addition of a perborate/3,3´,5,5´-tetramethylbenzidine (TMB) substrate, and its subsequent reaction with HRP, creates a blue-colored solution. After addition of a sulphuric acid containing stop solution, which yields a yellow color, uPA/PAI-1 levels are quantified by measuring the solution absorbances at 450 nm and comparing the values with those of a standard curve. Material (uPA, PAI-1) to generate a standard curve is included in the kit. Analysis was performed in duplicates. The detection range is 0.01-1.0 ng/ml for uPA and 0.05-10 ng/ml for PAI-1. uPA and PAI-1 antigen content was expressed as ng/mg protein.

Antigen content ofoncentration of human tissue kallikreins was quantified using highly sensitive and specific noncompetitive `sandwich-type´ immunoassays, developed at the department of Prof. Dr. E.P. Diamandis, Institute of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Canada. The design and setup of the human tissue kallikrein ELISAs is shown in **Figure 3**. Microtiter wells are incubated with a capture antibody (mouse-anti-hK). The capture antibody is generated by immunizing mice with recombinant hK protein (monoclonal for hK5, hK11, hK13, polyclonal for hK6, hK7, hK8, hK10), detection antibody

for hk by immunizing rabbits (all polyclonal). hK is bound and the complex detected by an alkaline phosphatase-conjugated goat-antibody to rabbit-IgG. Binding is detected is performed by using a DFB (diflunisal phosphate) containing developing solution. After removal of a phosphate ester of DFP by ALP (alkaline phosphatase) [1], the resulting DF forms a complex with Tb^{3+}-EDTA [2], which upon excitation at 336 nm causes Tb^{3+} fluorescence at 624 nm [3]. Fluorecence signal detection and data analysis were performed automatically by the CyberFluor 615 Immunoanalyzer. Fluorescence measurements are time-resolved, that is, taken during the first 100-200 µs after excitation, by the elimination of all short-lived fluorescence background signals [4] *(37)*.

Figure 3: Configuration of the hK-sandwich-type ELISA and signal detection. See text for details



In detail, white polystyrene microtiter plates were coated with a sheep anti-mouse IgG, Fc fragment-specific antibody (Jackson ImmunoResearch, Pennsylvania, USA) by overnight incubation of 100 µl of coating antibody solution (5 mg/L antibody diluted in 50 mM Tris-HCl buffer, pH 7.8) in each well. Then, plates were washed six times with washing buffer (9 g/L NaCl, 0.5 g/L Tween 20, 10 mM Tris-HCl, pH 7.4). Mouse anti-hK (monoclonal for hK5, hK11, hK13, polyclonal for hK6, hK7, hK8, hK10, produced in-house) was diluted 2,000-fold in a general diluent (60 g/L BSA, 0.5 g/L sodium azide, 50 mM Tris, pH 7.8) and 100 µl of this solution were applied to each well. After 2 h of incubation, plates were washed six times with washing buffer. Samples were then pipetted into each well (50 µl/well) along with 50 µl of general diluent and incubated for 1 h with shaking; then, plates were washed six times with washing buffer. Subsequently, 100 µl of polyclonal rabbit anti-hk, diluted 500-fold in buffer A

(containing the components of the general diluent plus 25 ml/L normal mouse serum, 100 ml/L normal goat serum, and 10 g/L bovine IgG), were applied to each well and incubated for 1 h; plates were then washed as described earlier. Finally, 100 µl of alkaline phosphatase-conjugated goat anti-rabbit IgG, Fc fragment-specific antibody (Jackson ImmunoResearch, Pennsylvania, USA), diluted 3,000-fold in buffer A, were added to each well and incubated for 45 min, and plates were washed as described above. Diflunisal phosphate (100 µl of a 1 mM solution) in substrate buffer (0.1 M Tris-HCl, pH 9.1, 0.1 M NaCl, and 1 mM $MgCl_2$) was added and incubated for 10 min. Developing solution (100 µl, containing 1 M Tris base, 0.4 M NaOH, 2 mM $TbCl_3$, and 3 mM EDTA) was pipetted into each well and mixed for 1 min. Fluorescence was measured with the time-resolved Cyberfluor 615 Immunoanalyzer (MDS Nordion, Kanata, Ontario, Canada).

Table 4: Range of detection for different human tissue kallikrein ELISAs

Detection range (µg/L)		Reference
hK5	0.10 - 50	*(259)*
hK6	0.50 - 200	*(63)*
hK7	0.20 - 10	*(117)*
hK8	0.20 - 20	*(116)*
hK10	0.05 - 10	*(145)*
hK11	0.10 - 50	*(54)*
hK13	0.05 - 20	*(112)*

All samples were analyzed in duplicates. To generate a standard curve, diluted recombinant hK-protein (produced in-house) was used. Upper and lower detection limits are shown in **Table 4**. No cross-reactivity of the respective hK-specific antibodies with any other members of the human tissue kallikrein family was detected. If not cited otherwise, all chemicals used for the hK-ELISA were from SigmaAldrich, Canada.

3.4 Statistical methods

Statistical analyses were performed using the SPSS software (SPSS Inc. Chicago, Illinois, USA, version 13.0). A p-value <0.05 was considered as statistically significant.

The joint distributions of proteolytic factors uPA, PAI-1, and the tissue kallikreins hK5, hK6, hK7, hK8, hK10, hK11, hK13 in the population as a whole and particularly in subgroups defined by nuclear grade (i.e. differentiation: high, moderate, and slight or none) and FIGO classification were investigated. Analyte measurements below the limits of sensitivity were coded as zero. Since the univariate distributions of all proteolytic factors depart considerably from a normal distribution **(Figure 4)**, fractional ranks were constructed for all these factors.

(For the analytes with many zero values, the lowest fractional rank is obviously somewhat greater than zero, because the rank in case of ties is defined at the midpoint).
All correlations are Spearman (rank) correlations with respect to the original analytes. Correlations $R \geq 0.5$ were regarded as "strong". Associations between FIGO status and central tendency (average rank) of proteolytic factors were studied by grouping factors in FIGO stage I/II or stage III/IV and then performing the Mann-Whitney U-test. Associations between nuclear grade (in the three classes G1, G2, and G3) and central tendency of proteolytic factor distributions were studied by performing the Kruskal-Wallis test. More detailed relationships (i.e., those not affecting central tendency) were studied by defining quartiles of all proteolytic factors and performing chi-squared tests. Missing values were excluded pairwise.

Outcome variables were progression-free survival (PFS), overall survival (OS), and residual tumor presence (RT). PFS is defined as the time interval between the date of surgery and the date of diagnosis of disease recurrence or distant metastasis. Overall survival is defined as the time interval between the date of surgery and the date of death. RT is defined as 1 if macroscopic residual tumor mass was visible, zero if completely absent. Ascites volume, age, nuclear grade, and nodal status were coded as binary variables (ascites: >500 ml vs. less; age >60 years vs. younger; nuclear grade: G3 vs. G1/G2; nodal status: 0 for N0, otherwise 1). FIGO status was coded by three binary indicators: (II/III/IV vs. I), (III/IV vs. I/II), (IV vs. I/II/III).
As mentioned above, in view of their long-tailed distributions (**Table 6**), all antigen levels (uPA, PAI-1, and the tissue kallikreins) were coded as fractional population ranks. The fractional rank difference was also coded and considered as a separate indicator. Mann-Whitney or Kruskal-Wallis tests were computed for associations between continuous and categorical variables. The impacts of the different factors on PFS and OS were expressed as hazard ratios (HR) with respect to the above coding and were estimated by Cox proportional hazards regression using forward selection and retaining factors with $p \leq 0.05$ (unless otherwise stated). Univariate and multivariate Cox models were estimated including clinical factors and fractionally ranked antigen levels, entered as continuous variables; the resulting hazard ratios and p-values thus reflect their prognostic significance over and above that of clinical factors (in particular FIGO and RT). Kaplan-Meier curves and log-rank statistics were also computed.

Impact of factors on RT was estimated by univariate / multivariate binary logistic regression and expressed in terms of the unadjusted / adjusted odds ratio (OR), respectively. For each patient, a score (estimated probability of RT) was obtained and used to construct a receiver operating characteristic (ROC; sensitivity / specificity). The AUC (area under the curve)

provides an in-sample measure of model quality and corresponds to the quality of the score, with an AUC of 0.5 being consistent with a coincidental forecast.

For uPA, PAI-1 and some of the analyzed human tissue kallikreins, positive and negative status was defined in earlier publications, using other cohorts or a smaller subset of the same patients. To test the impact of these defined antigen-statutes on PFS and OS in this cohort, log rank statistics were used. Significant findings were shown by KM-curves.

In a subgroup of patients, not only primary tumor, but also metastasis specimens were available. Differences in antigen concentrations between specimens from the same patient were tested for significance using the Wilcoxon test for combined samples. Results are shown in boxplots, where the bottom boundary represents the 25^{th} percentile, the top boundary represents the 75^{th} percentile, the bars indicate minimum and maximum values without extremes, median values are marked within the boxes by horizontal bars.
Level differentials, defined as antigen level in metastasis minus antigen level in primary tumor in units of ng/mg protein, were calculated for each proteolytic factor and for each patient. Level differentials were ranked, and a level differential score (defined as fractional rank between 0 and 1) was coded for each proteolytic factor. Level differential scores of all proteolytic factors that were significant in univariate Cox analysis, as well as the established prognostic factors residual tumor, ascites volume, and nuclear grade were included in multivariate Cox analysis of PFS and OS. The raw hazard ratios associated with level differentials represent the risk of the highest vs. lowest rank. Reported is the hazard ratio for the 75^{th} percentile compared to the 50^{th} (median). The hazard ratio of the 75^{th} compared to the 25^{th} is easily obtained as the square of this ratio.
For level differentials significant in Cox analysis, Kaplan-Meier survival curves were drawn, using the median as cut-off. Significance was assessed by log rank. The impact of level differentials on response to chemotherapy was tested by univariate logistic regression on each level differential score. Possible categories defining response to chemotherapy could be progression, no change, partial and complete remission. Only progression and no change were actually present in this cohort with measured level differentials, so binary logistic regression was used.

4. Results

4.1 Patient cohort

The present investigation is a retrospective study of a defined cohort of ovarian cancer patients with respect to the clinical impact of proteolytic factors on disease- and progression-free survival and clinical management. **Table 5a** summarizes clinical and histomorphological characteristics of the patients. **Table 5b** shows the patient subgroup for which tissue samples of primary tumors and omentum metastases were available.

Table 5a: Clinical data of ovarian cancer patients

n				142			
Median age (range)				57.5 (19-85) years			
Median observation time of patients alive (range)				41 (1-125) months			
		n	%			n	%
FIGO stage				Histological Subtype			
	I	25	17.6		serous	99	69.7
	II	10	7.0		mucinous	13	9.2
	III	78	54.9		endometrioid	6	4.2
	IV	29	20.4		clear-cell	4	2.8
Nuclear grade					undifferentiated	12	8.5
	G1/G2	55	38.8		others	8	5.6
	G3/G4	86	60.5	Nodal status			
	no data	1	0.7		negative	54	38.0
Relapsed					positive	57	40.1
	no	73	51.4		no data	31	21.8
	yes	69	48.6	Volume of ascitic fluid			
Deceased					no ascites	36	25.4
	no	78	54.9		≤ 500 ml	47	33.1
	yes	64	45.1		> 500 ml	48	33.8
Residual tumor mass					no data	11	7.7
	0 cm	72	50.7	Response to Chemotherapy			
	> 0 cm ≤ 1 cm	28	19.6		progress	17	12.0
	> 1 cm, ≤ 2 cm	13	9.2		no change	65	45.8
	> 2 cm	14	9.9		no data	60	42.3
	no data	15	10.6				

Table 5b: Ovarian cancer patient subgroup with tissue samples from primary tumors and omentum metastases

n				54			
Median age (range)				58.5 (25 – 85) years			
Median observation time of patients alive (range)				24.5 (1 – 125) months			
		n	%			n	%
FIGO stage				Histological subtype			
	III	39	72.2		serous	39	72.2
	IV	15	27.8		mucinous	3	5.6
Nuclear grade					endometrioid	3	5.6
	G1/G2	38	70.4		undifferentiated	6	11.1
	G3/G4	16	29.6		others	3	5.6
Relapsed				Nodal status			
	no	20	37.0		negative	13	24.1
	yes	34	63.0		positive	26	48.1
Deceased					no data	15	27.8
	no	17	31.5	Volume of ascitic fluid			
	yes	37	68.5		no ascites	9	16.7
Residual tumor mass					≤ 500 ml	17	31.5
	0 cm	17	31.5		> 500 ml	25	46.3
	> 0 cm ≤ 1 cm	14	26.9		no data	3	5.6
	> 1 cm, ≤ 2 cm	11	20.4	Response to Chemotherapy			
	> 2 cm	8	14.8		progress	7	13.0
	no data	4	7.4		no change	22	40.7
					no data	25	46.3

4.2 Antigen levels of the proteolytic factors in primary tumor extracts of ovarian cancer patients FIGO I-IV

Antigen content of human tissue kallikreins hK5, hK6, hK7, hK8, hK10, hK11, hK13, uPA, PAI-1, and CA125 were determined in primary tumor tissue extracts of ovarian cancer patients by ELISA. Antigen distributions are summarized in **Table 6** and **Figure 4**. All are long-tailed, as illustrated by the mean, median, and selected percentile values. **Table 6** also shows the number of patients who are negative for certain tissue kallikreins

The distribution of uPA antigen levels **(Table 6)** was positively skewed, ranging from 0.02 to 24.0 ng/mg protein (median 0.9, mean 1.7, standard deviation 2.6)**.** Similarly to uPA, distribution of PAI-1 antigen levels **(Table 6)** was positively skewed, ranging from 0 to 389.8 ng/mg protein (median 14.7, mean 32.8, standard deviation 54.7). The median level of PAI-1 is thus about 16 times that of uPA in tumor tissue extracts.

In view of these skewed distributions, it is interesting to investigate a possible association of high levels with FIGO stage: there was no significant difference between patients with early (FIGO I/II) vs. advanced (FIGO III/IV) stage of the disease with respect to central tendency of uPA, according to the Mann-Whitney test **(Table 7)**; however, a borderline significant difference in central tendency (p=0.075) was observed for PAI-1. More subtle associations between these factors and stage are discussed below.

In ovarian cancer primary tumor tissues hK5, 6, 7, 8, 10, 11, 13 protein was detected **(Table 6)**, reaching antigen levels between 155 (hK11) to 478 (hK8) ng of tissue kallikrein per mg of tissue protein extracted, except for hK13 with a low absolute level of expression (maximum of 18 ng/mg protein). In contrast to uPA and PAI-1, some of the tissue kallikreins could not be detected in a considerable number of patients, although the ELISA test formats used are of high sensitivity and specificity *(18)*. Remarkably, 40.8 (hK5), 4.9 (hK6), 21.8 (hK7), 13.4 (hK8), 14.8 (hK11), 23.2 (hK11), 62.0 (hK13) percent of the ovarian cancer patient tumor tissue specimens were not positive for the antigens examined (coded as zero). Note that the detection rate of <50 % in the case of hK13 implies that the median (but of course not the mean) is zero **(Table 6)**.

Table 6: Distribution of tissue kallikreins hK5, 6, 7, 8, 10, 11, 13, uPA, and PAI-1 in tumor tissue extracts of ovarian cancer patients FIGO I-IV

FIGO I-IV	ng of analyte per mg of protein present in tumor tissue extract (FIGO I-IV)								
	hK5	hK6	hK7	hK8	hK10	hK11	hK13	uPA	PAI-1
n of tissue samples	142	141	142	142	141	141	141	141	141
n=0 [a]	58	7	31	19	21	33	88	0	1
n > 0 [b]	84	134	111	123	120	108	53	141	140
%=0 [c]	40.8	4.9	21.8	13.4	14.8	23.2	62.0	0	0.7
% > 0 [d]	59.2	95.1	78.2	86.6	85.2	76.8	38.0	100	99.3
minimum	0	0	0	0	0	0	0	0.02	0.0
maximum	219.6	432.6	283.6	477.8	233.6	155.4	18.4	24.0	389.8
25th percentile	0.0	1.8	0.2	1.4	1.3	0.1	0.0	0.4	6.0
median	0.4	9.4	2.1	9.5	3.9	1.5	0.0	0.9	14.7
75th percentile	1.9	19.9	5.2	24.7	12.0	6.2	0.3	1.8	34.4
mean	3.1	20.5	7.8	28.9	10.7	8.3	0.6	1.7	32.8
standard deviation	18.6	44.2	26.0	58.7	22.4	21.4	2.2	2.6	54.7

[a] number of tissue extract values below detection limit;
[b] number of tissue extract values above detection limit
[c] percentage of tissue extract values below detection limit
[d] percentage of tissue extract values above detection limit

As for uPA and PAI-1, all tissue kallikrein levels had skewed distributions **(Table 6, Figure 4)**. Testing for a possible difference between patients with early (FIGO I/II) vs. advanced (FIGO III/IV) stage with respect to the tissue kallikreins, there was no significant difference in central tendency for hK6, 7, 8, 10, 11, or 13 **(Table 7)**.

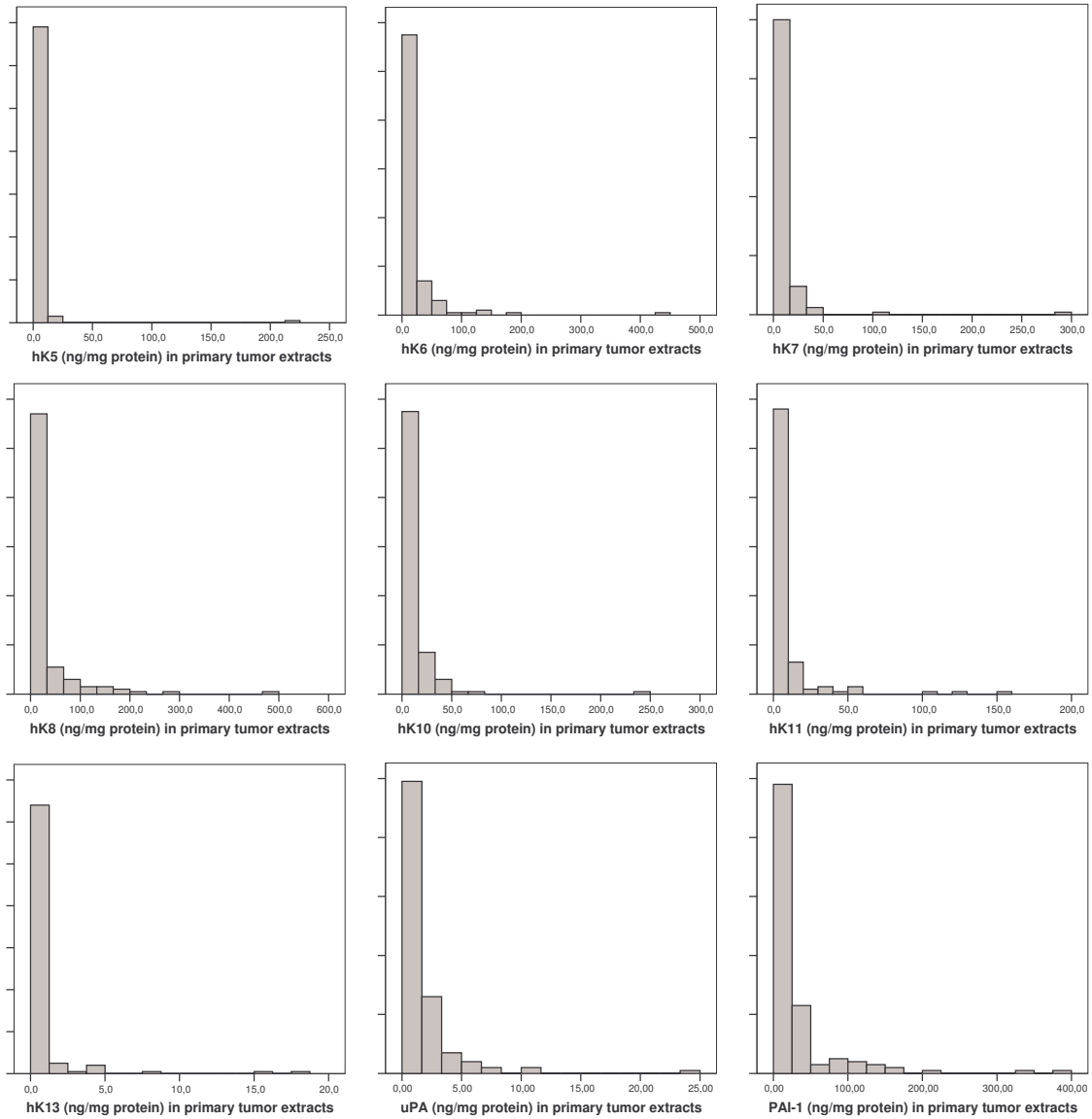
For hK5, however, we observed a statistically significant difference (p=0.007) between FIGO I/II and FIGO III/IV patients, corresponding to an elevated median level of 0.5 ng hK5 in FIGO III/IV compared to median 0 in FIGO I/II **(Table 6, 7)**. Fractionally ranked hk5 predicts FIGO stage III/IV vs. I/II with OR=7.75 (1.7-35.5). A median of zero means that in this FIGO subgroup more than half of the patients had hK5 levels below the limits of detection.

Defining quartiles of all proteolytic factors and performing chi-squared tests, we found that uPA, PAI-1, hk5, and hk8 had significant relationships to FIGO status when FIGO I/II was compared to FIGO III/IV. Qualitatively describing these relationships, the lower FIGO group is underrepresented in the highest (4th) quartile of uPA compared to the 3rd quartile (p=0.022). For PAI-1, the lower FIGO group is overrepresented in the lowest (1st) quartile of PAI-1 and underrepresented in the 3rd quartile (p=0.022). For hk5, the lower group is overrepresented in the 1st quartile and overrepresented in the 4th (p=.008). For hk8, the lower group is overrepresented in the 1st quartile and 4th quartiles and underrepresented in the 3rd.

For hk11, the lower group is overrepresented in the 3rd quartile and underrepresented in the 4th.

The differing significance of associations according Mann-Whitney and Chi-squared tests does not represent a contradiction, but rather complementary information, since the Mann-Whitney test summarized above describes central tendency, whereas comparison of quartiles as reflected in the Chi-squared test reflects more finely resolved features of the statistical distributions.

Figure 4: Histograms of the skewed distribution of the antigen levels in primary ovarian tumor extracts



4.3 Stratification of antigen levels of the proteolytic factors in primary tumor extracts of ovarian cancer patients by nuclear grading

Less than half of the 142 tumor specimens were of grade G1 (highly differentiated) or grade G2 (medium cellular differentiation) (G1: n=14, 9.9 %; G2: n= 41, 29.9 %); the majority were undifferentiated (G3: n=86, 61.0 %); nuclear grade was unknown for one patient (FIGO II). In view of the skewed distributions of tissue antigens, it is also interesting to investigate their possible association with nuclear grading **(Table 7)**. There was no significant difference of the antigens among G1 / G2 / G3 with respect to central tendency, except for hK5 (p=0.004; Kruskal-Wallis test).

Using quartiles of the proteolytic factor distributions as above to study more detailed putative relationships with nuclear grading (differentiation), we found that PAI-1 (p=0.012) and hk5 (p=0.023) had significant relationships with differentiation. Qualitatively speaking, the 4th (highest) quartile of PAI-1 was overrepresented in poorly differentiated patients, as was the 4th (highest) quartile of hk5. This association between the high quartile of hK5 and poor differentiation could account for the signficant relationship with respect to central tendency seen in the Kruskal-Wallis test reported above.

Table 7: Level of significance of differences between FIGO I/II vs. FIGO III/IV or between nuclear grade G1 / G2 / G3 regarding the antigen levels of individual proteolytic factors (uPA, PAI-1, hK5, 6, 7, 8, 10, 11, 13) expressed in primary tumor tissue specimens

Proteolytic factor analyzed	Level of significance between factor present in tumor tissue extracts of FIGO I/II or FIGO III/IV patients (Mann-Whitney test)	Level of significance between factor present in tumor tissue extracts of G1 vs. G2 vs. G3 patients (Kruskal-Wallis test)
uPA	0.206	0.340
PAI-1	0.075	0.106
hK5	0.007	0.004
hK6	0.270	0.740
hK7	0.784	0.558
hK8	0.753	0.602
hK10	0.286	0.662
hK11	0.535	0.159
hK13	0.150	0.810

4.4 Association of clinical parameters within the patient collective

Clinical and histomorphological parameters such as FIGO stage, age, amount of ascitic fluid, nuclear grading, residual tumor mass, and nodal status were correlated, applying Spearman´s Rho correlation. As listed in **Table 8**, established clinical prognostic factors as FIGO stage, residual tumor and nodal status do correlate strongly, and to a lesser extent, most of the other clinical and histomorphological parameters.

When correlating the same clinical parameters (FIGO stage, age, amount of ascitic fluid, nuclear grading, residual tumor mass mass and nodal status) with the above mentioned tumor-associated factors uPA, PAI-1, hK5, hK6, hK7, hK8, hK10, hK11 and hK13, of the analyzed proteolytic factors only PAI-1 and hK5 showed some weak correlation with clinical and histomorphological parameters such a FIGO stage, residual tumor mass, and nuclear grading **(Table 9)**.

Table 8: Correlation (R) between clinical and histomorphological factors FIGO stage, age, amount of ascitic fluid, nuclear grading, residual tumor mass and nodal status with level of significance (p) determined in tumor tissue extracts of ovarian cancer patients

age	R	0.049				
	p	0.56				
	n	142				
amount of ascitic fluid	R	0.488	0.230			
	p	< 0.001	0.008			
	n	131	131			
nuclear grading	R	0.338	0.159	0.281		
	p	< 0.001	0.059	0.001		
	n	141	141	131		
residual tumor mass	R	0.582	0.104	0.390	0.236	
	p	< 0.001	0.244	< 0.001	0.008	
	n	127	127	119	126	
nodal status	R	0.604	0.148	0.387	0.259	0.484
	p	< 0.001	0.131	< 0.001	0.006	< 0.001
	n	111	111	107	111	102
		FIGO	age	amount of ascitic fluid	nuclear grading	residual tumor mass

White: no significant correlation
Light yellow: Significant, but moderate correlation ($R < 0.5$, $p < 0.05$)
Yellow: Significant and strong correlation ($R \geq 0.5$, $p < 0.05$)

Table 9: Correlation (R) between clinical and histomorphological factors and uPA, PAI-1, and tissue kallikreins hK5, 6, 76, 8, 10, 11, 13 with level of significance (p) determined in tumor tissue extracts of ovarian cancer patients

uPA	R	0.105	0.033	0.048	0.045	0.121	0.213
	p	0.216	0.701	0.589	0.598	0.176	0.025
	n	141	141	130	140	126	111
PAI-1	R	0.191	0.113	0.096	0.177	0.058	0.210
	p	0.023	0.180	0.276	0.037	0.517	0.027
	n	141	141	130	140	126	111
hK5	R	0.194	-0.100	0.091	0.258	0.223	0.115
	p	0.021	0.239	0.303	0.002	0.012	0.228
	n	142	142	131	141	127	111
hK6	R	0.058	0.037	-0.097	-0.042	0.174	0.089
	p	0.495	0.667	0.272	0.626	0.052	0.355
	n	141	141	130	140	126	111
hK7	R	-0.023	-0.010	-0.128	-0.026	0.058	0.124
	p	0.786	0.904	0.144	0.755	0.521	0.196
	n	142	142	131	141	127	111
hK8	R	-0.022	-0.043	-0.106	-0.093	0.062	-0.015
	p	0.791	0.614	0.230	0.274	0.489	0.874
	n	142	142	131	141	127	111
hK10	R	0.053	-0.024	-0.075	-0.036	0.063	0.041
	p	0.533	0.775	0.396	0.676	0.486	0.668
	n	141	141	130	140	126	111
hK11	R	-0.108	-0.014	-0.075	-0.148	0.027	0.010
	p	0.204	0.871	0.395	0.081	0.767	0.920
	n	141	141	130	140	126	111
hK13	R	-0.095	-0.105	-0.075	-0.068	-0.162	-0.094
	p	0.263	0.214	0.394	0.427	0.070	0.331
	n	141	141	130	140	126	110
		FIGO	age	amount of ascitic fluid	nuclear grading	residual tumor mass	nodal status

White: no significant correlation
Light yellow: Significant, but moderate correlation ($R < 0.5$, $p < 0.05$)
Yellow: Significant and strong correlation ($R \geq 0.5$, $p < 0.05$)

4.5 Assessment of correlation between proteolytic factors uPA, PAI-1, and tissue kallikreins hK5, hK6, h7, hK8, hK10, hK11, hK13

Nonparametric (Spearman) correlations of protein expression of the nine proteolytic factors (uPA, PAI-1, and tissue kallikreins hK5, 6, 7, 8, 10, 11, 13) determined in extracts of ovarian cancer tissues by ELISA are summarized in **Table 10.** Associations between uPA or PAI-1 and the tissue kallikreins were weak, though in some cases significant, as were those between hK13 and the other tissue kallikreins.

In contrast, there were significant, and (with only a few exceptions) strong correlations up to about R=0.78 between all pairs within the cluster of tissue kallikreins hK5, 6, 7, 8, 10, and 11. The bivariate distributions among these factors often significantly depended on nuclear grading: generally, the tightest correlations occurred in low-grade (G1) patients, weakening with poorer differentiation. As a typical example, the combination of hK8 vs. hK11 is displayed as a scatter diagram in **Figure 5**. Here, one sees that there is a much tighter relation (less scatter) between these tissue kallikreins in patients with highly differentiated tumors grade G1 (R=0.9) compared to tumor specimens classified G2 (R=0.465) or G3 (R=0.660). Even though regarding G1 the numbers are small (n=14), Spearman correlations are very strong for four additional combinations: hK6 *vs.* hK7 (G1: 0.94, G2: 0.749, G3:0.731), hK6 *vs.* hK8 (G1: 0.94, G2: 0.85, G3: 0.689), hK6 *vs.* hK11 (G1: 0.8, G2: 0.523, G3: 0.595), hK7 *vs.* hK8 (G1: 0.87, G2: 0.738, G3: 0.796), all with R-values exceeding 0.8.

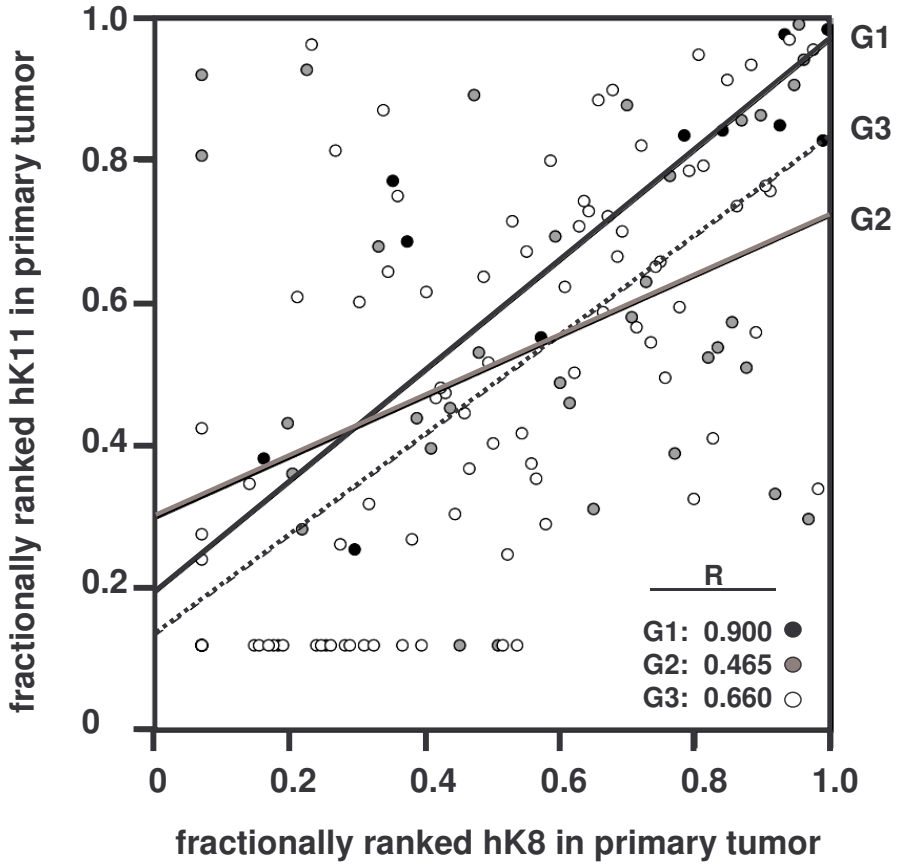
It is also worth mentioning that 9 of G1 classified tumor specimens belonged to patients staged FIGO I (n=8) or FIGO II (n=1); only 5 cases of G1 were FIGO III (n=4) or FIGO IV (n=1).

Table 10: Correlation (R) between uPA, PAI-1, and tissue kallikreins hK5, 6, 76, 8, 10, 11, 13 with level of significance (p) determined in tumor tissue extracts of ovarian cancer patients FIGO I - IV

PAI-1	R	0.05						
	p	0.86						
	n	13						
hk5	R	-0.28	-0.41					
	p	0.35	0.17					
	n	13	13					
hk6	R	0.30	-0.64	0.60				
	p	0.32	0.02	0.03				
	n	13	13	13				
hk7	R	0.32	-0.53	0.58	0.94			
	p	0.29	0.06	0.03	0.00			
	n	13	13	14	13			
hK8	R	0.35	-0.44	0.57	0.94	0.87		
	p	0.25	0.13	0.03	0.00	0.00		
	n	13	13	14	13	14		
hk10	R	0.20	-0.31	0.50	0.71	0.73	0.73	
	p	0.52	0.29	0.08	0.01	0.00	0.00	
	n	13	13	13	13	13	13	
hk11	R	0.42	-0.42	0.47	0.80	0.69	0.90	0.73
	p	0.16	0.16	0.10	0.00	0.01	0.00	0.00
	n	13	13	13	13	13	13	13
hk13	R	-0.22	-0.06	0.63	0.20	0.32	0.20	0.11	0.14
	p	0.46	0.85	0.02	0.52	0.27	0.50	0.72	0.64
	n	13	13	14	13	14	14	13	13
		uPA	PAI-1	hk5	hk6	hk7	hk8	hk10	hk11

White: no significant correlation
Light yellow: Significant, but moderate correlation ($R < 0.5$, $p < 0.05$)
Yellow: Significant and strong correlation ($R \geq 0.5$, $p < 0.05$)

Figure 5: Correlation (Spearman) for hK8 vs. hK11 of fractionally ranked antigen values determined by ELISA in extracts obtained from primary tumor tissues of ovarian cancer patients FIGO I-IV. The correlation factor R is depicted for patients with tumors G1, G2, and G3, separately.



4.6 Cox uni- and multivariate proportional hazard regression analysis of clinical, histomorphological, and proteolytic factors´ influence on overall (OS) and progression-free survival (PFS)

Cox uni- and multivariate proportional hazard regression analysis was used to assess the impact of proteolytic factors and established clinical factors on patient survival (OS, PFS) and surgical success (residual tumor mass after surgery, RT). For this, antigen levels of uPA, PAI-1, hK5, hK6, hK7, hK8, hK10, hK11, hK13, (fractionally ranked), age, nodal status, nuclear grading, ascitic volume and residual tumor mass (RT) were included.

Variables entered in the univariate and multivariate Cox proportional hazard regression analyses for PFS in all patients were FIGO I-IV, presence of RT, ascites volume, age, nuclear grade, nodal status; fractionally ranked antigen levels of uPA, PAI-1, and the difference of their fractional ranks; hK5, 6, 7, 8, 10, 11 and 13 (all coded as described under *Statistical Methods*) **(Table 11)**. Among the clinical variables, the univariate predictors were amount of RT, ascites volume, nodal status, FIGO III/IV vs. I/II, and FIGO IV vs. I/II/III. **Figure 6a** and **6b** illustrate the impact on PFS of ascites volume (>500ml vs. ≤ 500 ml) and RT (absent vs. present). hk11 was significant in G2/G3 patients (HR=0.40, 0.17–0.96, p=0.039). The fractional rank difference between PAI-1 and uPA had significant univariate impact.

In multivariate analysis of PFS in all patients **(Table 11)**, RT had a HR > 4.5. Neither FIGO status nor the other clinical variables entered the model, while of the antigen levels only hk11 was significant. To illustrate HR=0.30 for fractional ranks, consider two hypothetical patients with equal RT but hk11 levels of 6.2 ng/mg (75^{th} percentile) and 0.1 ng/mg (25^{th} percentile), respectively. Since these fractional ranks differ by 0.5, their HR would be $(0.30)^{0.5} \approx 0.55$.

In the univariate and multivariate Cox proportional hazard regression analyses for OS in all patients **(Table 12)**, variables entered were FIGO (I-IV), presence of RT, ascites volume, age, nuclear grade, nodal status, and fractionally ranked antigen levels for uPA, PAI-1, hK5, 6, 7, 8, 10, 11, and 13, coded as described under *Statistical Methods*. HR for antigens refers to fractional ranks.

For overall survival (OS), in all patients univariate predictors were RT, FIGO stage, nodal status, ascites volume, nuclear grade, as well as hk10 and hk13. The clinical factors RT (HR = 7.49) and ascites volume (HR = 1.97) -- but not FIGO -- as well as antigen hk10 (HR = 0.196) have entered the multivariate OS model.

Table 11: Univariate and multivariate Cox proportional hazard regression analyses for PFS in all patients. Although FIGO variables were strong univariate survival factors, they are not significant in the mulitvariate model.

Variable	univariate			multivariate		
	HR	95% CI	p-value	HR	95% CI	p-value
Presence of residual tumor mass	4.37	2.55-7.47	<0.001	4.53	2.59-7.95	<0.001
FIGO III/IV vs. I/II	9.25	2.25-38.1	0.002	---	---	---
FIGO IV vs. I/II/III	2.18	1.25-3.82	0.006	---	---	---
Ascites vol. > 500 ml	2.37	1.45-3.88	0.001	---	---	---
Nodal status	1.87	1.11-3.17	0.019	---	---	---
(fract. rank PAI-1) – (fract. rank uPA)	1.98	1.01-3.89	0.046	---	---	---
fract. ranked hk11	0.44	0.19-1.02	0.055	0.30	0.11-0.76	0.011

Table 12: Univariate and multivariate Cox proportional hazard regression analyses for OS in all patients. Although FIGO variables are strong univariate survival factors, they are not significant in the multivariate model.

Variable	univariate			multivariate		
	HR	95% CI	p-value	HR	95% CI	p-value
Presence of residual tumor mass	8.23	4.20-16.0	<0.001	7.49	3.63-15.45	<0.001
FIGO III/IV vs. I/II	6.35	1.96-20.6	0.002	---	---	---
FIGO IV vs. I/II/III	3.32	1.94-5.67	<0.001	---	---	---
Ascites vol. > 500 ml	3.52	2.12-5.87	<0.001	1.97	1.12-3.46	0.018
Nodal status	3.73	1.98-7.01	<0.001	---	---	---
Nuclear grade	2.07	1.19-3.62	0.010	---	---	---
fract. ranked hK10	0.41	0.17-0.99	0.048	0.196	0.072-0.54	0.002
fract. ranked hK13	0.27	0.08-0.85	0.026	---	---	---

Figure 6a: Progression-free survival (PFS) by ascites volume. Ascitic fluid of more than 500 ml is associated with a significantly shorter PFS.

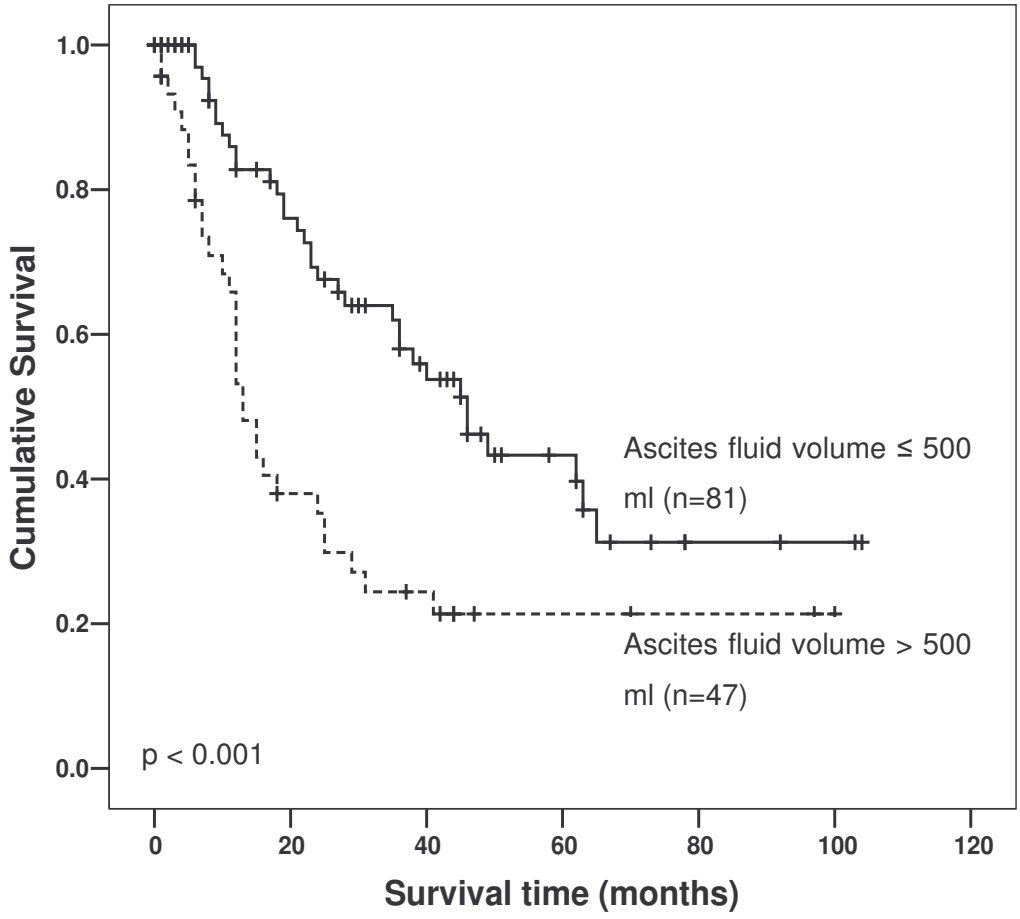
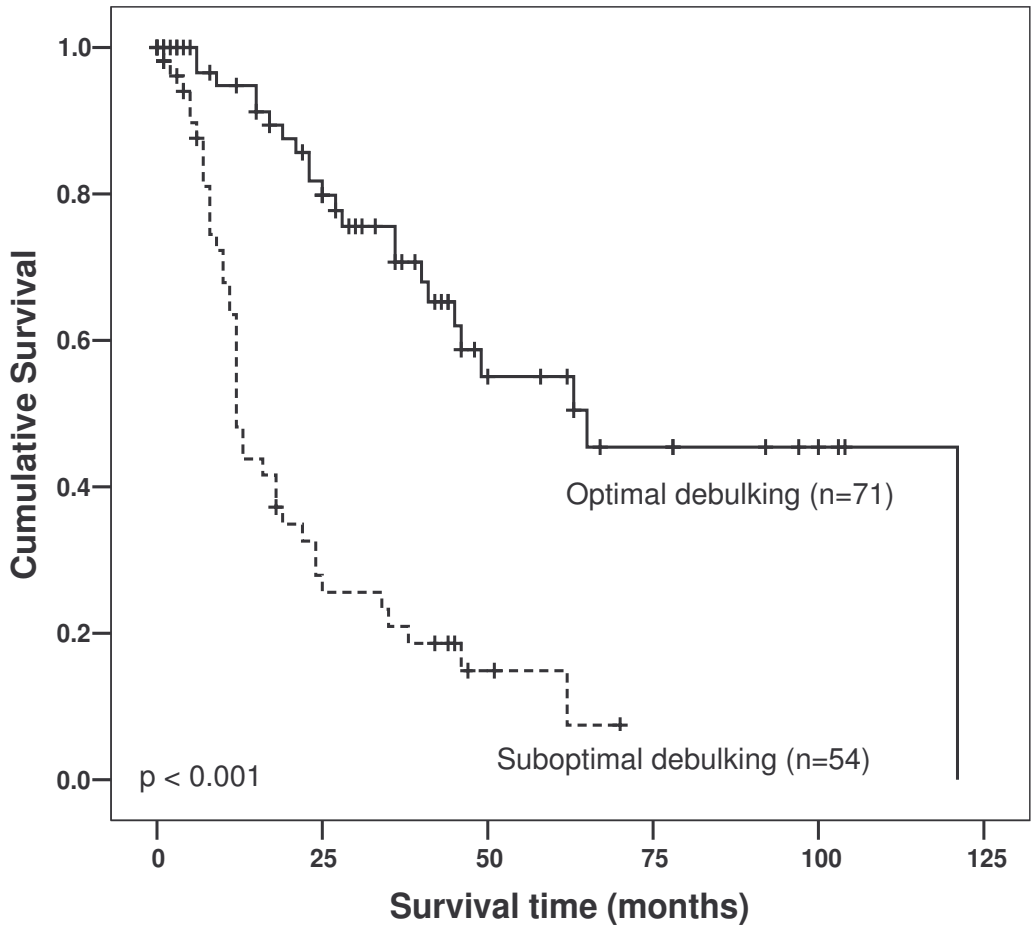


Figure 6b: PFS by residual tumor mass (macroscopically visible residual tumor presence, RT). Absence and presence of tumor mass after surgery influences the progression-free survival



Due to the strong impact of surgical success on PFS and OS, any factor influencing surgical success could affect survival -- independent of its significance in multivariate PFS and OS models. Clinical factors ascites volume, age, nuclear grade and nodal status, as well as fractionally ranked antigens of proteolytic factors were entered into univariate and multivariate logistic regression for residual tumor mass **(Table 13)**. Ascites volume, (OR = 8.92), nuclear grade (OR = 3.01), and hK5 (HR = 1.52) were statistically significant univariate factors. In multivariate models excluding clinical factors but including the proteolytic factors **(Table 14)**, higher hK5 (OR = 6.40) and lower hK13 (OR = 0.16) were associated with unfavorable surgical outcome**.**

Entering the relevant clinical and proteolytic factors (ascites volume, age, nuclear grade, fractionally ranked antigens) one obtains the multivariate scoring model for RT summarized in **Table 15.** Of the clinical factors, larger ascites volume (OR = 13.13) and poor nuclear grade (OR = 2.92) were associated with poor surgical outcome; regarding the antigens, higher hK6 (OR = 8.54) and lower hk13 (OR = 0.14) contributed to poor surgical outcome. The model provides a risk assessment for residual tumor mass based on ascites volume, nuclear grade, hK6, and hK13, which could be estimated before surgery.

Figure 7 shows the ROC (sensitivity, specificity for RT) of the probability score computed from the logistic regression model of **Table 15**; its AUC of 0.833 illustrates the in-sample modeling quality.

Table 13: Unadjusted odds ratios (OR) for factors that were significant predictors of presence of residual tumor mass (RT) according to univariate logistic regression.

Variable	OR	95% CI	p-value
Ascites volume	8.92	3.67-21.7	<0.001
Nuclear grade	3.01	1.40-6.40	0.005
hK5	1.52	1.25-16.6	0.021

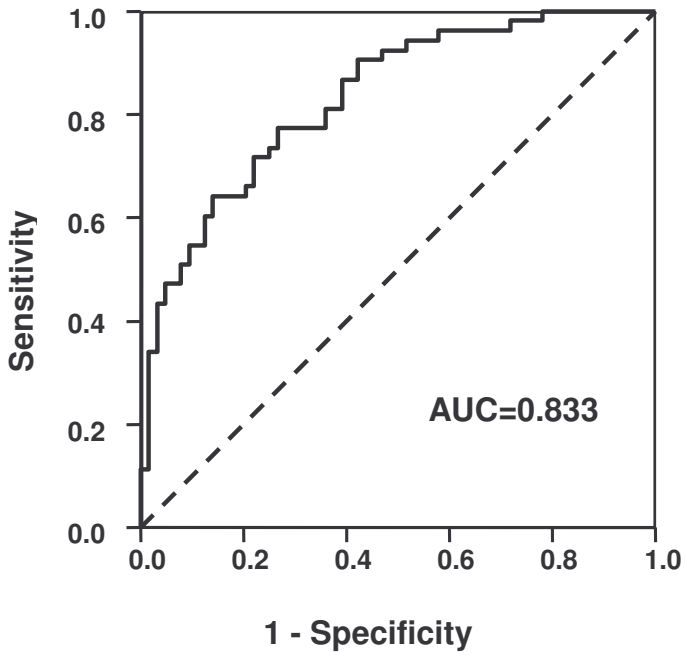
Table 14: Multivariate logistic regression model for presence of residual tumor mass (RT), excluding clinical factors but including fractionally ranked antigens (uPA, PAI-1, and the tissue kallikreins). Adjusted odds ratios (OR) of hk5 and hk11 refer to fractional ranks.

Variable	OR	95% CI	p-value
hK5	6.40	1.63-25.0	0.008
hK13	0.16	0.03-0.74	0.019

Table 15: Multivariate logistic regression model for presence of residual tumor mass (RT). All variables entering this model could be estimated prior to surgery.

Variable	OR	95% CI	p-value
Ascites volume	13.13	4.50-38.2	<0.001
Nuclear grade	2.92	1.13-7.5	0.027
hK6	8.54	1.57-46.4	0.013
hK13	0.14	0.02-0.93	0.042

Figure 7: Receiver-operator-curve (ROC) for prediciton of residual tumor (RT), based on the model of Table 15 and including ascites volume, nuclear grade, and tissue kallikreins hK6 and hK13. Area under the curve (AUC) is 0.833.



4.7 Antigen levels in primary tumors compared with omentum metastases – impact on survival

In a subgroup of patients, not only primary tumor but also metastasis specimens were available for analysis. Mean values, median, range, and standard deviation of antigen levels in primary tumor tissue and omentum metastasis are shown in **Table 16a** and **16b**.

Table 16a: Population distribution characteristics of hK5, hK6, hK7, hK8, hK10, hK11, hK13, uPA and PAI-1 in primary tumor extract of patients of which primary tumor and omentum metastasis specimens were available

	ng of analyte per mg of protein present in primary tumor tissue extract								
	hK5	hK6	hK7	hK8	hK10	hK11	hK13	uPA	PAI-1
number of tissue samples	49	48	49	49	48	48	49	53	53
minimum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
maximum	15.3	185.0	28.5	170.3	37.9	48.9	4.5	10.2	336.1
median	0.5	9.1	1.8	8.0	3.5	1.1	0.0	0.9	16.8
mean	1.4	17.3	4.6	19.3	8.7	4.3	0.4	1.7	30.8
standard deviation	18.6	44.2	26.0	58.7	22.4	21.4	2.2	2.6	54.6

Table 16b: Population distribution characteristics of the above named antigen levels in omentum metastasis tumor extracts of patients of which primary tumor and omentum metastasis specimens were available

	ng of analyte per mg of protein present in omentum metastasis tumor tissue extract								
	hK5	hK6	hK7	hK8	hK10	hK11	hK13	uPA	PAI-1
number of tissue samples	49	48	49	49	48	48	49	53	53
minimum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.9
maximum	12.4	98.8	61.0	117.0	51.6	20.5	42.6	19.8	262.1
median	1.3	13.1	1.5	6.0	5.8	1.2	0.0	3.2	30.6
mean	2.6	18.0	5.1	15.8	9.4	2.4	1.3	4.3	49.5
standard deviation	3.42	20.30	10.33	23.92	11.20	4.02	6.28	4.21	50.84

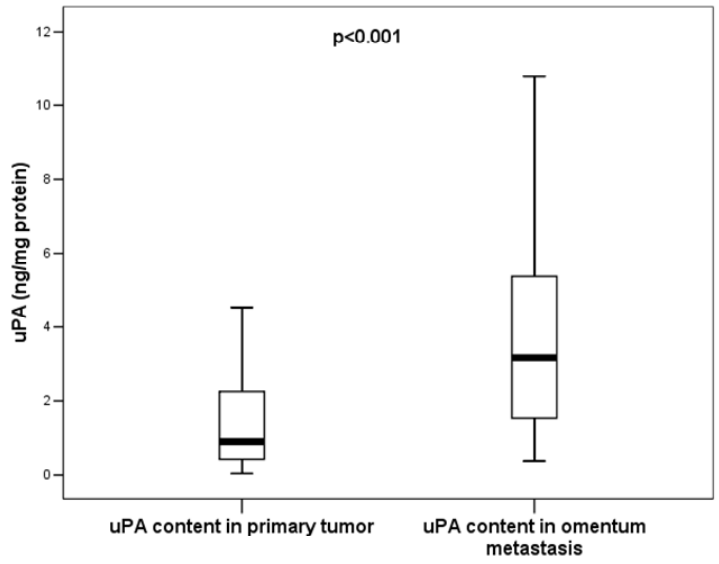
When correlating antigen levels of uPA, PAI-1, and the human kallikreins determined in primary tumor tissue extracts with the antigen content in omentum metastasis tissue extracts, some of the proteolytic factors are correlated at a statistically significant level, hK5, hK7, and hK11 being the strongest **(Table 17)**.

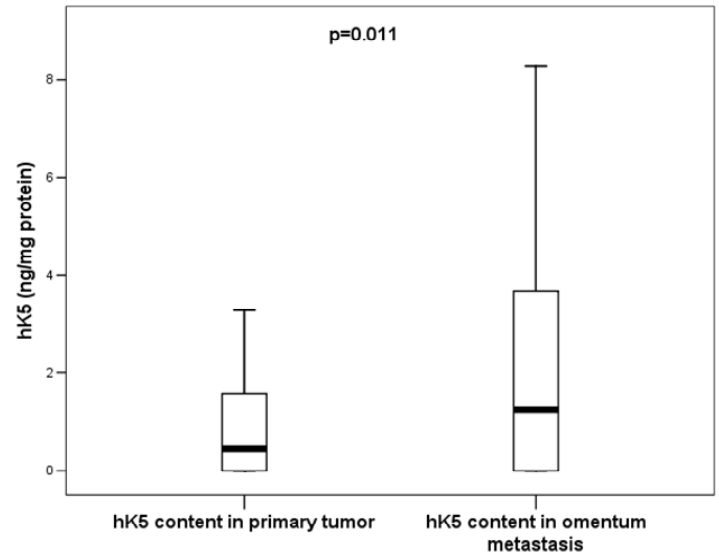
Table 17: Correlation of antigen levels determined in tissue extracts of primary tumors and omentum metastases

	uPA	PAI-1	hK5	hK6	hK7	hK8	hK10	hK11	hK13
r	0.28	-0.06	0.53	0.32	0.71	0.43	0.35	0.53	-0.59
p	0.046	0.07	<0.001	0.025	<0.001	0.002	0.014	<0.001	0.69
n	53	53	49	48	49	49	48	48	49

White: no significant correlation
Light yellow: Significant, but moderate correlation ($R < 0.5$, $p < 0.05$)
Yellow: Significant and strong correlation ($R \geq 0.5$, $p < 0.05$)

Using the Wilcoxon test, differences in antigen concentrations between specimens of the same patient were tested. Significant increases in pair-wise comparison from primary tumor to omentum metastasis could be found for uPA (158% of mean level in primary tumor), PAI-1 (86% increase), and hK5 (61%) which is shown in **Figure 8.**





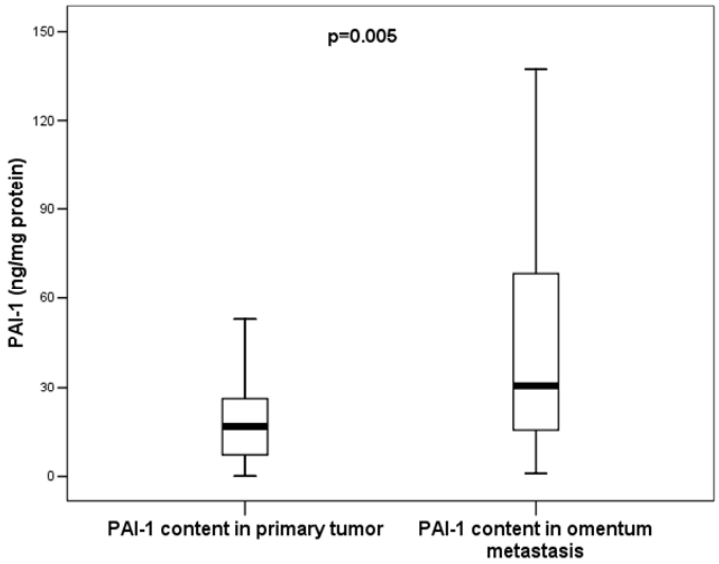


Figure 8: Boxplots of antigen concentration in primary tumor tissue extracts and corresponding omentum metastases for uPA, hK5 and PAI-1.

The content of all of the analyzed proteolytic factors was higher in omentum metastases than in the primary tumor tissues, except for hK8 and hK11, two tissue kallikreins known to be predictors of a better prognosis in ovarian cancer patients.

(Bottom boundary: 25th percentile, top boundary: 75th percentile, bars: minimum and maximum values without extremes, horizontal line: median values)

For each of the patients, with additional samples of the omentum metastasis analyzed, level differentials, defined as antigen level in metastasis minus antigen level in primary tumor in units of ng/mg protein, were calculated for each proteolytic factor. For none of the proteolytic factors analyzed, a significant impact of level differential on response to first-line chemotherapy using binary logistic regression was found.

Assessing the impact of antigen level differential on PFS, again Cox uni- and multivariate analysis was performed. In Cox univariate proportional hazard regression, larger level differentials were significantly associated with disease progression for uPA, hK6, and hK10 **(Table 18)**. Level differential scores of all proteolytic factors that were significant in univariate Cox analysis, as well as the established prognostic factors residual tumor mass, ascites volume, and tumor nuclear grade were included in multivariate Cox analysis for PFS and OS. In multivariate analysis for PFS, residual tumor mass was the strongest established prognostic factor. When proteolytic factors were included, only level differential of hK10 remained significant for PFS **(Table 18)**.

Table 18: Univariate and multivariate analysis of the impact of level differentials of the proteolytic factors and clinical factors on progression-free survival (PFS)

Variable	Univariate			Multivariate		
	p value	HR	CI	p value	HR	CI
residual tumor mass	n.s.	--	--	0.009	1.02	1.01-1.04
uPA_{diff}	0.013	1.43	1.08-1.89	n.s.	--	--
$hK6_{diff}$	0.021	1.55	0.07-2.25	n.s.	--	--
$hK10_{diff}$	0.017	1.54	0.08-2.20	0.008	1.65	1.14-2.38

In univariate analysis for overall survival (OS), only the level differential of uPA (HR 1.32; 95% CI 1.01-1.74; p=0.044) was significant. In multivariate analysis for OS, residual tumor mass and ascitic fluid volume were the strongest established prognostic factors. The level differentials of uPA remained significant for OS **(Table 19).**

Table 19: Univariate and multivariate analysis of the impact of level differentials of the proteolytic factors and clinical factors on overall survival

Variable	Univariate			Multivariate		
	p value	HR	CI	p value	HR	CI
residual tumor mass	n.s.	--	--	0.015	1.02	1.00-1.04
ascitic fluid volume	n.s.	--	--	0.018	1.68	1.09-2.57
uPA_{diff}	0.044	1.32	1.01-1.74	0.008	1.59	1.13-2.25

Level differentials that showed significance in uni- and multivariate Cox analysis were used to dichotomize the patient cohort, using the median as the cut-off. Kaplan-Meier survival curves show the impact of uPA on OS and hK10 on PFS in **Figure 9** and **Figure 10**. The distribution of level differentials is pictured in the respective insets, showing that for all antigen levels, the median cut-off is approximately 0.

Parts of the significant impact in patients with tumor stage FIGO III-IV may be attributable to poorer debulking results (and consequently an increased residual tumor mass burden) in patients with higher levels in metastasis. Therefore, the subgroup of patients with minimal residual tumor mass after primary surgery (residual tumor mass $\leq$ 1 cm) was analyzed separately: level differentials of hk5, hK6, hK7, and hK10 had a significant impact on PFS. Again, larger differentials were associated with disease progression. For OS, only the level differential of hk10 remained significant in optimally debulked patients **(Table 20)**.

Table 20: Univariate analysis for progression-free and overall survival (optimally debulked, residual tumor mass ≤ 1 cm)

Variable	PFS			OS		
	p value	HR	CI	p value	HR	CI
$hK5_{diff}$	0.006	2.18	1.25-3.79	n.s.	--	--
$hK6_{diff}$	0.015	1.85	1.13-3.05	n.s.	--	--
$hK7_{diff}$	0.016	1.95	1.13-3.36	n.s.	--	--
$hK10_{diff}$	0.015	1.79	1.12-2.86	0.04	1.74	1.02-2.96

Figure 9: Kaplan-Meier survival curves displaying the overall survival of the patient groups dichotomized by the median uPA level differential (left). Range diagram displaying the distribution of level differentials for uPA (right).

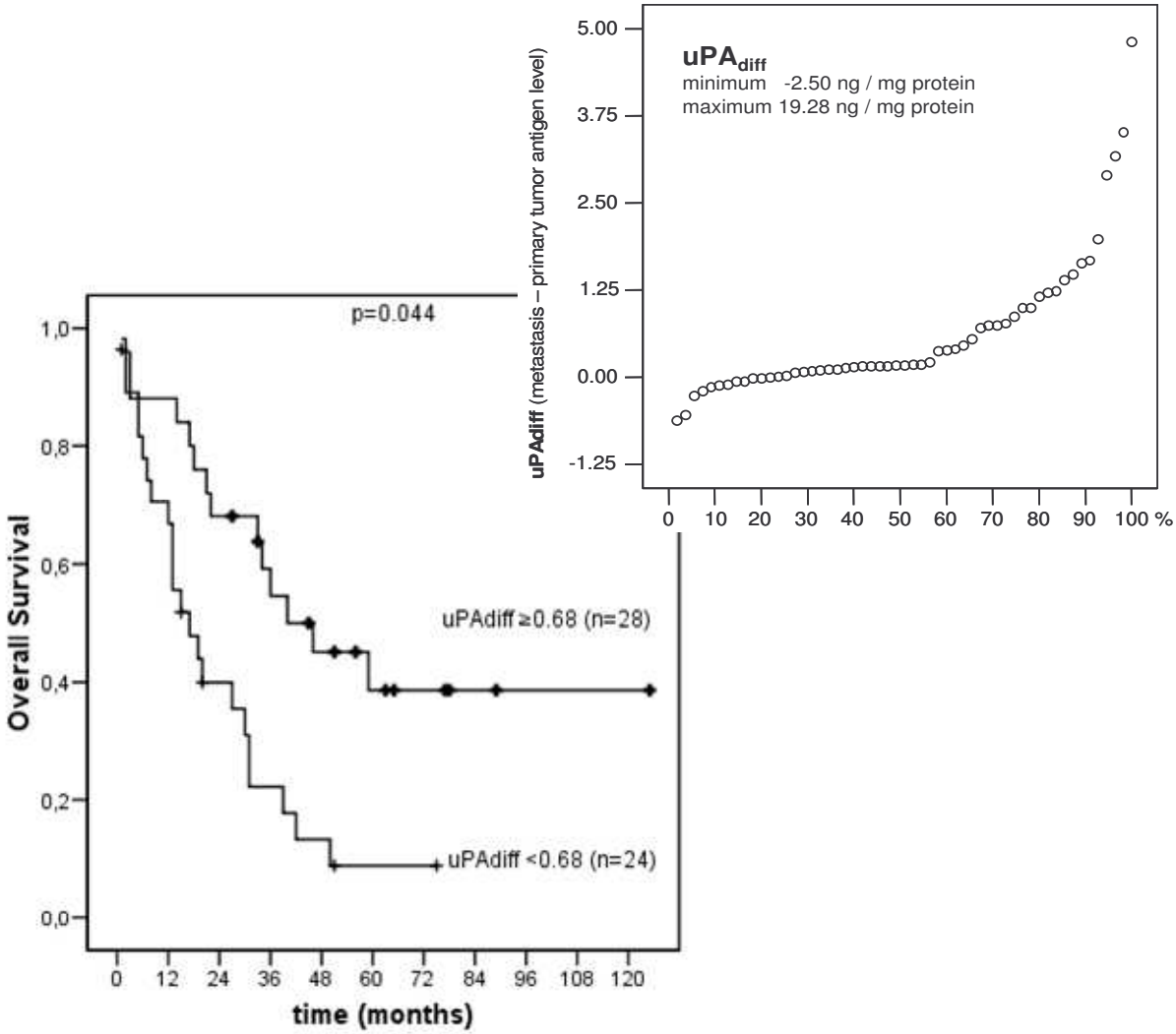
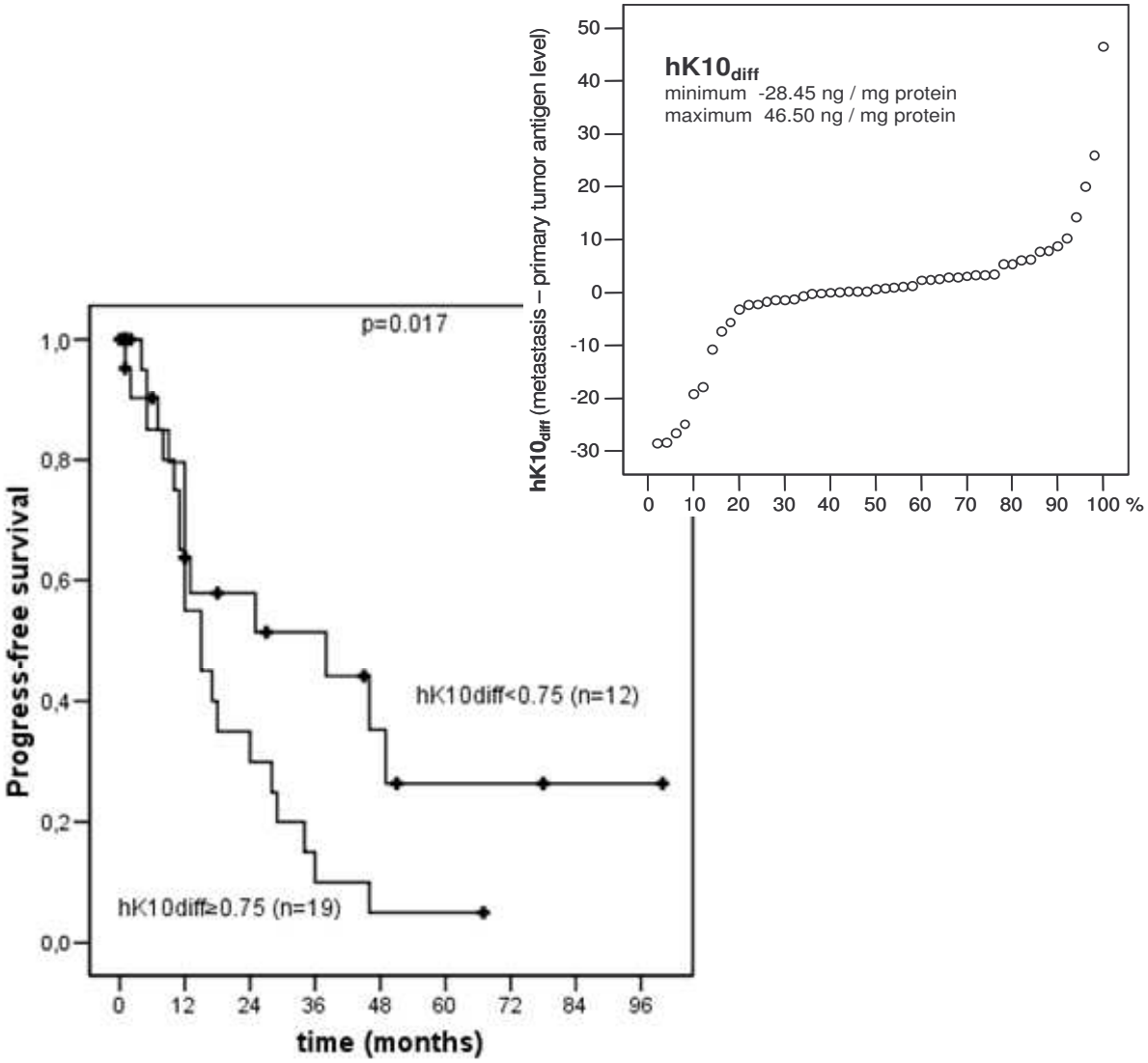


Figure 10: Kaplan-Meier curves displaying the progression-free survival of the patient groups dichotomized by the median hK10 level differential (left). Range diagram displaying the distribution of level differentials for hK10 (right).



4.8 Validation of previously described optimized cut-offs

For the analyzed antigen levels of uPA, PAI-1, hK5, hK6, hK10, hK11, hK13, optimized cut-offs were described earlier **(Table 21)**. These optimized cut-off values are used to define positive and negative tumor extract status. These cut-off antigen values have been identified in other cohorts or a smaller subset of the same patients by χ^2, CART, or log rank statistics, based on the ability to predict PFS and/or OS. For validation of these cut-offs in our cohort, log rank statistics was used. None of these optimized cut-offs had an impact on OS or PFS in the cohort or the subgroups analyzed within this work (data not shown).

Table 21: Optimized cut-offs to be validated

Patient cohort	Analyte cut-off	Reference
FIGO I-V (n=103)	uPA: 5.5 ng/mg protein PAI-1: 18.8 ng/mg protein	*(119)*
FIGO III (n=54)	uPA: 0.92 ng/mg protein PAI-1: 13.50 ng/mg protein	*(122)*
FIGO IIIC (n=86)	uPA: 2.0 ng/mg protein PAI-1: 27.5 ng/mg protein	*(125)*
FIGO I-IV (n=132)	hK5: 0.15 ng/mg protein	*(55)*
FIGO I-IV (n=180)	hK6: 35 ng/mg protein	*(97)*
FIGO I-IV (n=182)	hK10: 1.35 ng/mg	*(147)*
FIGO I-IV (n=134) FIGO I-IV (n=104)	hK11: 6.3 ng/mg protein hK11: 0.54 ng/mg protein	*(19; 54)*
FIGO I-IV n=131	hK13: 0.13 ng/mg protein	*(200)*

5. Discussion

Ovarian cancer is the principal cause of death among female genital malignancies although survival has improved during the last decades due to refined and more radical operation techniques together with combination therapy *(106; 121)*, but still more than two-third of the patients relapse and die of their disease *(125)*.

In ovarian cancer patients, uPA and its inhibitor PAI-1 have emerged as markers of poor prognosis, indicating an elevated risk of the patient to experience early disease recurrence (metastasis) and poorer survival compared to patients with low levels of these proteolytic factors in their tumor tissue *(17; 83; 125; 231)*. In addition, several of the tissue kallikreins (hK4, 5, 6, 7, 8, 10, 11, 13, 14, and 15) are overexpressed in ovarian carcinoma tissues and are often associated with patient prognosis *(18; 21; 169; 260)*. KLK4, KLK5/hK5, KLK6/hK6, KLK7, hK10, and KLK15 expression at the mRNA or protein level are markers of poor prognosis in ovarian cancer; elevated levels correlate with more aggressive forms of the disease and decreased disease-free and overall survival. KLK8, KLK9, hK11, hK13, and KLK14, however, are markers of favorable prognosis of ovarian cancer patients *(21)*.

Tissue remodeling, involving degradation of structural and extracellular matrix proteins -- thereby mediating cell migration in embryogenesis, pregnancy, wound healing, and angiogenesis -- are key physiologic functions involving different types of proteases as MMPs, cathepsins, caspases, tissue kallikreins, and members of the plasminogen activation system, e.g. urokinase-type plasminogen activator uPA and its inhibitor PAI-1 *(28; 160; 186; 196)*.

Tissue kallikreins display diverse physiological functions, from the regulation of blood pressure and electrolyte balance to tissue remodeling, prohormone processing, neural plasticity, and skin desquamation *(18; 21)*. Recent evidence suggests that tissue kallikreins may be involved in cascade reactions, and that cross-talk may exist with proteases of similar or different catalytic types, such as the plasminogen activation system *(79; 133; 221)*. Dysregulated expression of tissue kallikreins, uPA and PAI-1 is a feature of malignancies as of the ovaries *(21; 196)*.

Several reports have previously indicated an association between deregulated tissue kallikrein expression and ovarian cancer progression and the potential use of tissue kallikreins as diagnostic/prognostic biomarkers or therapy targets for this type of cancer. However, little is known about the joint distributional characteristics of tissue kallikrein levels, about their correlations with uPA and PAI-1, and their prognostic impact. The predictive power of established clinical and histomorphological markers like age, FIGO stage, nodal status, residual tumor mass after operation, histology, nuclear grading and amount of ascites fluid might be enhanced by taking into account antigen concentrations of components of the urokinase plasminogen activator system uPA and PAI-1 as well as human tissue kallikreins hK5, hK6, hK7, hK8, hK10, hK11, and hK13.

We have now determined these characteristics for the first time by measurement and direct

comparison of all these factors in one collective of 142 tumor tissue specimens of ovarian cancer patients FIGO I-IV.

5.1 Distribution and correlation

We observed that uPA and PAI-1 are present in tissues of almost all of the ovarian cancer patients analyzed, independent of tumor stage or cellular differentiation, indicating concerted expression of these two proteolytic factors. We also noted that several of the ovarian cancer patients assessed did not express detectable tissue kallikrein protein at all, although the detection limit of the assays is in the low-nanogram range. This finding is especially remarkable for hK5, hK7, hK11, and hK13 with high proportions (41, 22, 23, 62 %, respectively) of patients negative for these tissue kallikreins, indicating a non-concerted tissue kallikrein protein expression in some patients, but not in all.

An association of the central tendency (median) of the distributions of the 9 proteolytic factors with FIGO stage was seen only in the case of hK5; the median of hK5 was lower in FIGO I/II than in FIGO III/IV patients. In addition, some of the quartiles of uPA, PAI-1, and hk8 depended on FIGO status. Similarly, only hK5 showed an association of the median with poor nuclear grading (cell differentiation). The highest quartiles of both PAI-1 and hK5 were overrepresented in G3 tumors.

The moderate to strong mutual correlations found within the cluster hK5, hK6, hk7, hK8, hK10, and hK11 (particularly among the trio hK6, hk7, hK8) for the collective as a whole would support the hypothesis of concerted expression of proteolytic factors within this cluster. The bivariate distributions among these factors depended significantly on nuclear grading, weakening with poor differentiation: particularly noteworthy were the tight correlations observed in the subgroup of G1 patients, particularly for the combinations of hK6 vs. hK7, hK6 vs. hK8, hK6 vs. hK11, hK7 vs. hK8, and hK8 vs. hK11 with $R > 0.8$.

On the other hand, for uPA, PAI-1, and hK13, no considerable correlations with any of the other proteolytic factors were observed, even considering the highly-differentiated subgroup. In contrast, in the FIGO III subgroup Kuhn et al detected a correlation of both uPA and PAI-1 with nodal status only, while Konecny et al. found uPA to be associated with FIGO stage I-IV, but not PAI-1 *(119; 122)*. In any case, FIGO stage, nuclear grading, and residual tumor mass are factors reflecting tumor progression and aggressiveness *(23; 24; 85; 100; 129; 193)*, acting in concert with PAI-1 and hK5.

From a tumor biological view, one might consider the patients with highly differentiated tumors G1 to be at an early stage of cellular dedifferentiation. Keeping in mind that about two-thirds of these patients belong to early-stage FIGO I/II, we speculate that the particularly tight correlations among hk6, 7, 8, and 11 in this group of low-risk patients might be giving a hint of molecular processes with a common cause in the early phase of ovarian cancer

progression. As tumors become less differentiated, one would generally expect additional processes to be unleashed, thereby adding additional scatter to these relationships, consistent with what is observed in the collective as a whole.

5.2 Outcome prediction

Uni- and multivariate analysis assessing the impact of clinical and histomorphological markers as well as antigen levels of uPA, PAI-1, hK5, hK6, hK7, hK8, hK10, hK11 and hK13 on progression-free and overall survival showed residual tumor mass (RT) to be a decisive (unfavorable) clinical determinant of PFS and OS, in accordance with various publications including a meta-analysis *(24; 85; 100; 121; 125; 129)*.

In univariate analysis (PFS), aside from RT, ascites volume and nodal status were significant clinical parameters; the fractional rank difference of PAI-1 and uPA -- i.e., high PAI-1 compared to uPA – was significant for poor PFS; hK11 was of borderline significance; none of the other proteolytic factors was significant. In multivariate analysis (PFS), hK11 was the only statistically significant biomarker, taking RT into account. Elevated hK11 tumor antigen levels were associated with prolonged PFS.

In previous studies, the clinical impact of hk11 in ovarian cancer depended on whether protein or mRNA levels were considered: KLK11 mRNA expression levels were associated with poor survival in ovarian cancer *(203)*. However, in agreement with our findings, elevated tissue hK11 antigen levels determined by a similar ELISA were favorable for PFS *(19)*.

Kuhn et al previously found that uPA and PAI-1 are significant prognostic factors regarding OS in FIGO IIIC ovarian cancer patients. Although uPA was univariately significant, its impact was not to be seen in multivariate analysis; the impact of PAI-1 was time-varying and more pronounced within the first two years *(125)*. In the present collective, high PAI-1 compared to uPA was significantly associated with poor DFS. These results taken together suggest that PAI-1 and uPA play important but distinct roles in the complex process of tumor invasion in ovarian cancer.

In multivariate analysis, low hK10 levels, RT and ascites volume were independently associated with poor OS. It is noteworthy that although high hK13 was a good univariate predictor of improved OS, it was not significant in the multivariate model upon inclusion of RT; hence, the univariate impact may be partly attributable to its ability to predict absence of residual tumor mass. In contrast, elevated hK10 is an even stronger predictor of favorable OS in a multivariate model that includes RT than in univariate analysis of OS. These findings appear consistent with hK10 down-regulation during tumor progression in nude mice inoculated with breast or prostate cancer cell lines *(84; 135)*.

Unfavorable outcome in FIGO III/IV ovarian cancer patients was reported to be associated

with hK10 tissue levels *(147)* when exceeding a predefined cutoff; however, in this study hK10 was neither significant as a continuous variable, nor in multivariate analysis including all FIGO I-IV patients. In view of the still unresolved underlying biological complexity, a non-monotonic relationship between hK10 and tumor aggressiveness cannot be ruled out. The partly discrepant results in the literature indicate that the existing data on the diagnostic and prognostic role of human tissue kallikreins in ovarian cancer merit further investigation. It must be pointed toward the fact that the tissue kallikreins are subject to regulation by gene expression as all of the human tissue kallikrein genes present in endocrine-related tissues are under steroid hormone regulation control *(18; 21)*. In addition, while measuring tumor-associated proteolytic factors in tumor tissue extracts, one has to take into account possible post-translational modifications or proteolytic activation/degradation of the tissue kallikrein proteins expressed. In this case, certain putative molecular forms of the tissue kallikreins, including tissue kallikrein-inhibitor complexes, may escape detection by the antibodies employed in the ELISA test kits because these antibodies have been generated against recombinant tissue kallikreins. Apart from that, differences in patient selection, way of treatment, molecular marker determination methods, and use of optimized cut-offs could be relevant.

5.3 Predictors of optimal tumor debulking

Since chemotherapy (platinum compounds ± taxane) was rather homogeneously administered to our primary ovarian cancer patients, the observed impact of tumor biological factors on survival (PFS, OS) reflects a superposition of their biological role in the natural course of the disease and their influence on therapy response: the observed survival impact of a factor could be modified or masked by a predictive component with respect to therapy response. However, since ovarian cancer spreading is primarily loco-regional, tumor burden within the abdominal cavity already reflects tumor aggressiveness apart of patient survival. Hence, studying relationships between tumor-associated factors and probability of surgical success reveals key information on localized tumor aggressiveness free of confounding effects of therapy response. Moreover, since completeness of cytoreductive surgery is a key determinant of outcome *(24; 68; 100)*, pre-operative determination of factors influencing surgical success may be helpful in individualizing treatment for ovarian cancer, e.g. administration of pre-operative chemotherapy, that is suggested nowadays to be a valid alternative to radical surgery as it improves patient prognosis and quality of life and may reduce morbidity and mortality while retaining the survival benefits of primary surgery *(103; 176; 232)*.

Data on the rate of optimal debulking varies a lot in the literature, presumably due to the absence of a clear definition for “residual tumor”. Tumor mass reduction down to < 2 cm can be achieved in approximately 40-50% *(124; 173)*. Some studies even report debulking rates

of more than 70% *(35; 168)*. In this cohort of patients operated at the Department of Obstetrics and Gynecology of the Technical University of Munich between 1985 and 1999, optimal debulking (microscopic tumor residue 0 cm) was achieved in 50.7% of the patients, a tumor rest < 2 cm in 70.2%.
Radical tumor resection may cause considerable morbidity (e.g. postoperative subileus, re-laparotomy due to anastomosis defects, cardio-respiratory failure, thromboembolism, sepsis, or lymphoceles). Systematic lymph node dissection -- a recommended procedure when optimal debulking seems achievable -- by itself often causes significantly higher blood loss and consecutive transfusion rates compared to surgery without radical lymphadenectomy *(11)*. Our overall morbidity rate following advanced ovarian cancer surgery is about 40%, but radical surgery may lead to up to 80% morbidity *(71)*. High morbidity is certainly acceptable if optimal debulking with its attendant survival advantage can be achieved, but it must be viewed critically for patients in whom optimal debulking was not achievable and who thus have a short remaining life expectancy.

In order to estimate risks and benefits of surgical interventions pre-operatively, clinical or biological factors that could more accurately predict individual debulking success are urgently needed. In our study, high hK5, large ascites volume, and high nuclear grade by univariate analysis predicted poor surgical success. In multivariate analysis, favorable grade, low ascites volume, low hK6, and high hK13 predicted surgical success. The predictive impact of pre-operative ascites for surgical outcome in FIGO IIIc ovarian cancer has previously been described *(125)*. Regarding human tissue kallikreins, there is limited information regarding their impact on surgical success: for elevated hK10, a significant association with large residual tumor was reported *(147)*; for hK11, a significant correlation with response to chemotherapy, but not with surgical outcome, was observed *(19)*.

Using a logistic regression model, a score (RT-score) was obtained, based on hK6, hK13, ascites volume, and tumor grade that predicted surgical outcome with good performance (AUC=0.833). Such a score could be calculated before definitive surgery: pre-operative ascitic fluid volume is easily estimated by ultrasound, tumor biopsies can be obtained for tissue analysis. The score could support pre-operative risk stratification: in patients with considerable co-morbidity, a favorable RT-score would reinforce the decision for radical surgery whereas an unfavorable RT-score might indicate an alternative therapeutic approach, such as pre-operative chemotherapy. So far, no reliable parameters exist to predict which patients are likely to benefit from pre-operative chemotherapy. In a pilot study, Kuhn et al. demonstrated that patients with large ascites volume, which is a sign for peritoneal carcinomatosis, who received pre-operative chemotherapy followed by radical surgery had less RT at time of definitive surgery and a substantial survival advantage over

comparable patients receiving chemotherapy after surgery *(123)*. A predictive score combining tumor biological with clinical information – in our case using tissue kallikreins hK6, hK13, ascites volume, and nuclear grade – may thus help to identify suitable candidates for pre-operative chemotherapy.

5.4 Impact of antigen content in primary tumor and omentum metastasis

Cancer metastasis includes cancer cell detachment from their original localization, migration, invasion into surrounding tissue and lymphatic vessels and evasion to colonize at distant sites of the organism. Involved in this process are proteolytic enzymes like human tissue kallikreins and plasminogen activator system members uPA and PAI-1 *(5)*. Therefore, the amount of antigen in tissue of metastatic sites and how these levels differ from the antigen content in primary tumor tissue might give a hint on the role of uPA, PAI-1, hK5, hK6, hK7, hK8, hK10, hK11, and hK13 during metastasis.

Of the 54 patients, not only primary tumor tissue but also tissue of omentum metastases was accessible for analysis. By pair-wise comparison of primary tumor levels with that of the metastases, significant increases were shown for uPA, PAI-1 and hK5. The results are consistent with the hypothesis that elevated levels of uPA and PAI-1 may contribute to invasiveness and metastasis of ovarian cancer: uPA and PAI-1 concentration is lowest in benign ovarian tumor tissues but higher in extracts of low malignant potential tumors (LMP) and highest in invasive ovarian cancer tumor tissue *(83; 119; 122; 195; 231)*. Schmalfeldt et al. *(194)* reported on an up to 4-fold increase of uPA in tumor tissue extracts from omentum metastases compared to primary tumor tissues while PAI-1 content doubled *(194; 195)*. hK5 seems to have a similar role in ovarian cancer as uPA and PAI-1.

In contrast to all other measured proteases, levels of human kallikrein 8 and 11 in omentum metastases decreased compared to primary tumor tissues. This is not surprising since hK8 and hK11 were shown to be predictors of good prognosis *(19; 54; 115; 204)*.

As mentioned above, some studies have proven that serine proteases uPA and PAI-1 are elevated in omentum metastasis compared to primary tumor tissues *(83; 119; 122; 194; 195; 231)*, but it has never been interrogated whether the amount of increase, i.e. the value of the level differential, has an impact on outcome in ovarian cancer.

In Cox analysis, higher uPA, hK6, and hK10 level differentials are associated with a significant shorter progression-free survival, with hK10diff remaining significant in a multivariate setting. For overall survival, only the level differential uPA diff showed statistical significance.

For patient groups dichotomized using the median of the level differentials uPAdiff and hK10diff, which is approximately 0, log rank statistics and Kaplan-Meier survival curves indicate a significant difference in OS and PFS. It can be concluded that patients whose antigen levels of uPA, hK10, and, to a lesser extent, hK6, are higher in omentum metastases

than those in primary tumor tissues, have a worse outcome.

Extend of residual tumor mass is a strong prognostic factor, but nevertheless, patients with minimal (< 1cm) tumor rest may experience early disease recurrence *(125)*. Level differentials hk5diff, hK6diff, hK7diff, and hK10diff might therefore be useful tools to identify these individuals at risk in the subgroup of patients with minimal residual tumor volume, as they have shown to influence PFS and even OS (only hK10 diff).

In view of the fact that for some of the proteolytic factors analyzed, level differentials have a clinically significant impact on disease outcome and that their level differentials are not correlated with response to conventional chemotherapy, these factors are considered interesting targets for novel tumor biological therapeutic approaches.

5.5 Optimized cutoffs proteolytic factors

In previous investigations, cut-off values have been estimated for some of the human tissue kallikreins as well as for uPA and PAI-1 to allow assessment of the univariate clinical impact of individual proteolytic factors *(19; 57; 62; 115; 125; 141; 146; 149; 200; 253)*. Keeping in mind that multiple factors are involved, cutoffs for individual factors do not provide an adequate basis for clinical decision support, since classification of low vs. high risk groups according to a cutoff on one such factor will not necessarily be consistent with the classification according to a different factor, why it does not surprise that none of the preliminary defined optimized cutoffs had any significance in this cohort.

Apart from that, the method of defining optimal cutoffs with data of a cohort is controversial among statisticians, as reported by Altman et al *(2)*. The used method, called minimum p-value approach, holds the problem of multiple testing: as statistical tests with a nominal type I error rate (α) of 5 % are repeated serially with the same data, the whole procedure might have an up to 10-fold higher global error rate. The cited literature on the role of uPA, PAI-1, and hK11 in ovarian cancer highlights the problem, since the proposed cutoffs are too different to be all `optimal´. Altmann et al recommend to use an adjusting formula for estimating the real p-value and not to overestimate existing cut points. In the same time, they dissuade from using the minimum p-value approach at all but to pre-specify cut points guided by for example biological reasons or measurement techniques *(2)*.

5.6 Conclusions and outlook

Knowledge of the regulation of the tissue kallikrein expression, of their biological functions and their role in cancer has grown by the identification of all members of the human tissue kallikrein family and the discovery of the tissue kallikrein locus, but is still to explore.

This study demonstrates that together with clinical factors like residual tumor mass, some of the human tissue kallikreins and uPA and / or PAI-1 are statistically significant independent prognostic and / or predictive markers in ovarian cancer. Their role in disease progression is

evidently complex, as different serine proteases have a strong impact for different disease endpoints. Nevertheless, they have the potential to support clinical therapy decisions by helping to identify those patients who cannot be debulked optimally at primary surgery and who consequently could be possible candidates for alternative therapeutic approaches such as neoadjuvant chemotherapy. Level differentials, i.e. the antigen content in omentum metastasis compared to primary tumor, are not only valid markers of prognosis but also give insight in underlying pathways of metastasis.

Hence, optimal use of these factors for definition of prognostic risk groups and support of clinical decisions will require validation in bigger cohorts using multivariate analyses and weighted risk assessment taking into account not just single tissue kallikreins, but rather a panel of several tissue kallikreins plus uPA and PAI-1, as well as the clinical and histomorphological parameters.

But already it can be stated that tissue kallikreins and, to a lesser extent, the plasminogen activator system components uPA and PAI-1 are linked to tumor aggressiveness and therefore play a clinically important role in ovarian cancer. Apart of using tissue kallikreins as biomarkers for diagnosis and prognosis of cancer, as illustrated in this work, tissue kallikreins seem to represent potential targets for the development of cancer therapeutics as well, for example by using a member of the serine protease inhibitor group.

6. Abstract

In epithelial ovarian cancer, the high mortality rate is usually ascribed to late diagnosis, since epithelial ovarian tumors commonly lack early-warning symptoms. Tumor-associated biomarkers useful for diagnosis, prognosis, and/or therapy response prediction are in short supply. The serine protease urokinase-type plasminogen activator and its inhibitor PAI-1, both members of the plasminogen activation system of fibrinolysis, and the recently described members of the interfacing system of tissue kallikrein serine proteases (hK1 to hK15) are reported to be associated with malignancy and tumor progression of early and/or advanced stage ovarian cancer patients.

The protein levels of nine proteolytic factors (uPA, PAI-1, and tissue kallikreins hK5, hK6, hK7, hK8, hK10, hK11, hK13) were determined simultaneously by ELISA in detergent-released extracts of 142 ovarian cancer patient primary tissue, and, in a subgroup of 54 patients, also in corresponding metastasis tumor tissue specimens. After radical surgery, absence of macroscopically visible residual tumor was achieved in 72 patients; all patients received postoperative platinum-containing chemotherapy.

The strength of the associations between the protein expression levels of these tumor tissue-associated factors was studied, in particular according to subgroups defined by FIGO stage and nuclear grading. The distributions of uPA, PAI-1, hk5, and hk8 are associated with FIGO status (FIGO I/II vs. FIGO III/IV); hK5 expression was higher in FIGO III/IV than in FIGO I/II patient tissues. The distributions of PAI-1 and hk5 also differed significantly according to cell differentiation (nuclear grading); expression of hK5 was higher in G3 compared to G1/2 tumors. Associations between uPA or PAI-1 and the tissue kallikreins were weak, though in some cases significant, as were those between hK13 and the other tissue kallikreins. There were strong, significant pairwise correlations within the cluster of tissue kallikreins hK5, 6, 7, 8, 10, and 11, but their bivariate distributions depended on nuclear grading: the tightest correlations occurred in low-grade (G1) patients, weakening with poorer cell differentiation.

These results support the notion that several of the tandemly co-localized genes of the tissue kallikrein family on locus 19q13.4 are co-expressed in ovarian cancer patients, substantiating the possible existence of a steroid hormone-driven tissue kallikrein cascade in patients with malignant disease.

When investigating the use of clinical factors, uPA, PAI-1 or any of the human tissue kallikreins as outcome predictors, significant univariate predictors of poor progression-free survival (PFS) were RT (residual tumor > 0), FIGO stage (III/IV vs. I/II and IV vs. I/II/III), ascites volume > 500 ml, nodal status, and the difference between PAI-1 and uPA (fractionally ranked). In multivariate analysis, significant independent factors for poor PFS were RT (HR=4.53) and low hK11 fractional rank (HR=0.30). Univariate predictors of poor OS were RT, FIGO stage, nodal status, ascites volume, nuclear grade, and low hK10 and

hK13. In multivariate analysis, statistically significant independent factors for poor OS were RT (HR=7.49), ascites volume (HR=1.97), and low hK10 (HR=0.196).

A multivariate scoring model estimating RT probability has been constructed, based on ascites volume (OR=13.1), nuclear grade (OR=2.92), hK6 (OR=8.54) and hK13 (OR=0.14), with good in-sample predictive performance (area under the curve = 0.833). In view of risks and benefits of radical surgery, such a score could support pre-operative risk stratification and identify candidates for alternative therapeutic strategies. These results highlight the distinct roles of the human tissue kallikreins for different disease endpoints in ovarian cancer and their potential to support individualized therapy decisions.

For 54 patients, also corresponding omentum metastasis was available for analysis. Level differentials were defined as level in metastasis minus level in primary tumor tissue. By pairwise comparison, significant increases in metastasis level compared to primary tumor level were only found for uPA (148% of mean) and hk5 (84% of mean).

In all patients, including those with residual tumor mass, differences in uPA had significant impact on progression-free survival (PFS), as well as tissue kallikreins hK6, 7 and 10 (others not significant). Larger differentials of uPA, hK6, hK7 and hK10 were associated with disease progression. Some of the impact for these proteolytic factors in all patients may be attributable to poorer debulking results (and consequently an increased residual tumor burden) in patients with higher antigen levels in metastasis.

In patients with optimal debulking result (i.e., with no macroscopically visible residual tumor), only the level differentials of hk5, hK7, hK8, and hK11 had a significant impact on progression-free survival: again, larger differentials were associated with shorter disease-free survival.

In brief, for the first time, distribution, correlation, the impact on overall survival and the presence of changes from primary to metastatic tumor tissue have been fully explored for all proteolytic factors listed above. Using this homogeneous patient collective treated according to national guidelines valid at the time, it could be shown that certain human tissue kallikreins, uPA and PAI-1 are clinically relevant in ovarian cancer.

7. Appendix

7.1 TNM and FIGO staging system for ovarian cancer

TNM	FIGO	
TX		Primary tumor cannot be assessed
T1	I	Tumor limited to one or both ovaries
T1aN0M0	IA	Tumor limited to one ovary; capsule intact with no tumor on ovarian surface; no malignant cells in ascites or peritoneal washings
T1bN0M0	IB	Tumor limited to both ovaries; capsules intact with no tumor on ovarian surface; no malignant cells in ascites or peritoneal washings
T1cN0M0	IC	Tumor limited to one or both ovaries with capsule rupture; tumor on ovarian surface or malignant cells in ascites or peritoneal washings
T2	II	Tumor involves one or both ovaries with pelvic extensions or cells in ascites or peritoneal washings
T2aN0M0	IIA	Tumor has spread and/or attaches to the uterus and/or fallopian tubes. No malignant cells in ascites or peritoneal lavage.
T2bN0M0	IIB	Extension to other pelvic tissues; no malignant cells in ascites or peritoneal washings
T2cN0M0	IIC	Pelvic extension (2a or 2b) with malignant cells in ascites or peritoneal washings
T3 and/or N1	III	Tumor involvement with one or both ovaries and microscopic peritoneal metastases outside the pelvis and/or regional lymph nodes metastasis
T3aN0M0	IIIA	Microscopic peritoneal metastases beyond pelvis
T3bN0M0	IIIB	Macroscopic peritoneal metastasis beyond pelvis (2 cm or less) in greatest dimension
T3cN0M0 or	IIIC	Peritoneal metastasis beyond pelvis (more than 2 cm) in greatest; any TN1M0 dimension and/or regional lymph node metastasis
M1	IV	Distant metastasis (excludes peritoneal metastasis)

References *(3; 201)*

7.2 Classification of nuclear grading of ovarian tumors

GX	Grade cannot be assessed	
GB	Borderline cancer (malignant)	
G1	Well-differentiated cancer	low grade
G2	Moderately differentiated cancer	
G3	Poorly differentiated cancer	high grade
G4	Undifferentiated	

References *(50)*

7.3 Official and alternative kallikrein gene and protein names

Official gene name	Other names	Official protein name	Other names
KLK1	-	hK1	Pancreatic/renal kallikrein, hPRK
KLK2	-	hK2	Human glandular kallikrein 1, hGK-1
KLK3	-	hK3	Prostate-specific antigen, PSA
KLK4	PRSS17, KLK-L1	hK4	Prostase, KLK-L1 protein EMSP1
KLK5	KLK-L2	hK5	KLK-L2 protein, HSCTE
KLK6	PRSS9	hK6	Zyme, Protease M, Neurosin
KLK7	PRSS6	hK7	HSCCE
KLK8	PRSS19	hK8	Neuropsin, Ovasin, TADG-14
KLK9	KLK-L3	hK9	KLK-L3 protein
KLK10	PRSSL1, NES1	hK10	NES1 protein
KLK11	PRSS20	hK11	TLSP/hippostatin
KLK12	KLK-L5	hK12	KLK-L5 protein
KLK13	KLK-L4	hK13	KLK-L4 protein
KLK14	KLK-L6	hK14	KLK-L6 protein
KLK15	-	hK15	-
Taken from reference *(60)*			

7.4 Abbreviations

ALP	alkaline phosphatase	pAK	polyclonal antibody
AUC	area under the curve	PBS	phosphate-buffered saline
bp	base pair	PCR	polymerase chain reaction)
BSA	bovine serum albumin	PrCa	prostate Cancer
cDNA	complementary desoxyribonucleic acid	PRK	pancreatic/renal kallikrein
CT	computerized tomography scan	pro-uPA	enzymatic inactive proenzyme form of uPA
DFP	diflunisal phosphate	PRSS	protease, serine
DNA	desoxyribonucleic acid	PRSSL	protease, serine-like
EDTA	dthylendiamintetraessigsäure	PSA	prostate-specific antigen
ELISA	enzyme-linked immunosorbent assay	Q RT-PCR	quantitative real-time polymerase chain reaction
GIT	gastrointestinal tract	RT-PCR	reverse transcriptase polymerase chain reaction
h	hour	RNA	ribonucleic acid
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazin]ethansulfonacid	ROC	receiver-operator characteristics
hK	human kallikrein protein	rpm	rounds per minute
HSCCE	human stratum corneum chymotryptic enzyme	s	second
HSCTE	human stratum corneum tryptic enzyme	suPAR	soluble uPAR
IgG	immunoglobulin G	Tb3+	terbium ion
KLK	human kallikrein gene	TBS	tris-buffered saline
KLK-L	kallikrein-like	TLSP	trypsin-like serine protease
BrCa	breast cancer	tPA	tissue type plasminogen activator
mAK	monoclonal antibody	Tris	tris-buffered saline
min	minute	Triton	octylphenolpolyethylenglykol
MMP	matrixmetalloproteinase	TVS	transvaginal sonography
NES1	normal epithelial cell-specific 1	U	unit
OvCa	ovarian cancer	uPA	urokinase-type plasminogen activator (CD 87)
PAI-1	plasminogen-activator-inhibitor type 1	uPAR	urokinase-type plasminogen activator receptor
PAI-2	plasminogen-activator-inhibitor type 2	UTR	untranslated region

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7.6 Publication list

Parts of this work have been used for publications or posters:

Journal Publications:

G.M. Yousef, M-E. Polymeris, L. Grass, A. Soosaipillai, P-C. Chan, A. Scorilas, C. Borgono, N. Harbeck, B. Schmalfeldt, **J. Dorn**, M. Schmitt, E.P. Diamandis: *Human Kallikrein 5: A Potential Novel Serum Biomarker for Breast and Ovarian Cancer,* Cancer Res. 2003 Jul 15;63(14):3958-65

T. Kishi, L. Grass, A. Soosaipillai, A. Scorilas, N. Harbeck, B. Schmalfeldt, **J. Dorn**, M. Mysliwiec, M. Schmitt, E.P. Diamandis: *Human Kallikrein 8, a Novel Biomarker for Ovarian Carcinoma,* Cancer Res. 2003 Jul 15;63(11): 2771-2774

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J. Dorn, N. Harbeck, R. Kates, V. Magdolen, L. Grass, A. Soosaipillai, B. Schmalfeldt, E. P. Diamandis, M. Schmitt: Disease processes may be reflected by correlations among tissue kallikrein proteases but not with proteolytic factors uPA and PAI-1 in primary ovarian carcinoma, submitted in Biol. Chem, 2006

J. Dorn, M. Schmitt, R. Kates, B. Schmalfeldt, M. Kiechle, A. Scorilas, E. P. Diamandis, N. Harbeck: *Primary tumor levels of human tissue kallikreins impact surgical success and*

survival in ovarian cancer patients, submitted in J Clin Oncol 2006

Poster:

T. Kishi, L. Grass, A. Soosaipillai, A. Scorilas, C. Shimizu-Okabe, N. Harbeck, B. Schmalfeldt, **J. Dorn,** M. Schmitt, E.P. Diamandis: *Human Kallikrein 8: Identification and Clinical Impact in Advanced Ovarian Cancer* (ISFP 2002)

G.M.Yousef, L. Grass, M-E. Polymeris, A. Soosaipillai, A. Scorilas, C. Borgono, N. Harbeck, B. Schmalfeldt, **J. Dorn**, M. Schmitt, E.P. Diamandis: *Human Kallikrein 5 (hK5): A Novel Serum Biomarker for Breast and Ovarian Cancer* (AACR 2003)

T. Kishi, L. Grass, A. Soosaipillai, A. Scorilas, N. Memari, M. Schmitt, N. Harbeck, **J. Dorn,** B. Schmalfeldt, E.P. Diamandis: *Human Kallikrein 8 as a Serum Biomarker for Ovarian Cancer* (AACR 2003)

J. Dorn, N. Harbeck, R. Kates, A. Scorilas, L. Grass, A. Soosaipillai, E.P. Diamandis, M. Kiechle, B. Schmalfeldt, M. Schmitt: Analysis of the Differential Expression of Proteolytic Factors uPA, PAI-1, and seven Tissue Kallikreins between Primary Ovarian Carcinoma Tissues and Corresponding Omentum Metastases (AACR 2005 und Plasminogen Workshop 2005)

N. Harbeck, R. Kates, **J. Dorn**, A. Scorilas, L. Grass, A. Soosaipillai, E.P. Diamandis, M. Kiechle, M. Schmitt, B. Schmalfeldt: *Impact of Proteolytic Factors on Surgical Success and Survival in Ovarian Cancer* (ASCO 2005)

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