Function and clinical relevance of kallikrein-related peptidases and other serine proteases in gynecological cancers

Julia Dorn 1*, Nathalie Beaufort 2*, Manfred Schmitt 1, Eleftherios P. Diamandis 3, Peter Goettig 4, and Viktor Magdolen 1

1Klinische Forschergruppe der Frauenklinik der Technischen Universität München, Klinikum rechts der Isar, Munich, Germany, 2Institute for Stroke and Dementia Research (ISD), Klinikum der Universität München, Munich, Germany, 3Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Canada, and 4Structural Biology Group, University of Salzburg, Salzburg, Austria

Abstract
Gynecological cancers, including malignant tumors of the ovaries, the endometrium and the cervix, account for approximately 10% of tumor-associated deaths in women of the Western world. For screening, diagnosis, prognosis, and therapy response prediction, the group of enzymes known as serine (Ser-)proteases show great promise as biomarkers. In the present review, following a summary of the clinical facts regarding malignant tumors of the ovaries, the endometrium and the cervix, and characterization of the most important Ser-proteases, we thoroughly review the current state of knowledge relating to the use of proteases as biomarkers of the most frequent gynecological cancers. Within the Ser-protease group, the kallikrein-related peptidase (KLK) family, which encompasses a subgroup of 15 members, holds particular promise, with some acting via a tumor-promoting mechanism and others behaving as protective factors. Further, the urokinase-type plasminogen activator (uPA) and its inhibitor PAI-1 (plasminogen activator inhibitor-1) seem to play an unfavorable role in gynecological tumors, while down-regulation of high-temperature requirement proteins A 1, 2 and 3 (HtrA1,2,3) is associated with malignant disease and cancer progression. Expression/activity levels of other Ser-proteases, including the type II transmembrane Ser-proteases (TTSPs) matriptase, hepsin (TMPRSS1), and the hepsin-related protease (TMPRSS3), as well as the glycosyl phosphatidylinositol (GPI)-anchored Ser-proteases prosasin and testisin, may be of clinical relevance in gynecological cancers. In conclusion, proteases are a rich source of biomarkers of gynecological cancer, though the enzymes’ exact roles and functions merit further investigation.

Abbreviations: CA125: cancer antigen 125; ECM: extracellular matrix; EGFR: epidermal growth factor receptor; ELISA: enzyme-linked immunosorbent assay; EMT: epithelial to mesenchymal transition; FIGO: International Federation of Gynecology and Obstetrics; GPI: glycosyl-phosphatidylinositol; HGF: hepatocyte growth factor; HAI: HGF activator inhibitor; HE4: human epididymis protein 4; HtrA: high temperature requirement protein A; IHC: immunohistochemistry; IGF: insulin-like growth factor; KLK: kallikrein-related peptidase; MMP: matrix metalloprotease; PAI: plasminogen activator inhibitor; PAR: protease-activated receptor; PCR: polymerase chain reaction; PDB: protein database; PDGF: platelet-derived growth factor; PSA: prostate specific antigen; ROMA: Risk of Malignancy Algorithm; Serprotease: serine protease; SFTI: sunflower trypsin inhibitor; TGF: transforming growth factor; tPA: tissue-type plasminogen activator; TTSP: type II transmembrane Ser-protease; uPA: urokinase-type plasminogen activator; uPAR: uPA receptor; VEGF: vascular endothelial growth factor

Keywords
Cervical cancer, endometrial cancer, GPI-anchored serine proteases, high temperature requirement protein A1, ovarian cancer, transmembrane serine proteases, urokinase-type plasminogen activator

Proteases and cancer
The identification of biomarkers in diverse tumor types, including gynecological cancer, has attracted significant attention in the last decade. Screening biomarkers are employed to test the general population for early onset of the disease; diagnostic markers can distinguish healthy or benign tissues from malignant ones; prognostic markers predict the course of the cancer, including risk of disease...
recurrence; predictive markers identify those cancer patients who will benefit from or fail to respond to certain (chemo-) therapies.

Among the most extensively investigated protein candidate biomarkers are numerous proteases belonging to all major protease family subtypes; indeed, deregulated proteolysis is actually a hallmark of cancer\textsuperscript{2,3}. Not only are proteases differentially expressed in cancer; they are also thought to directly contribute to all major carcinogenic steps: malignant transformation; primary tumor growth, including tumor-associated neovascularization; metastasis formation; tumor-dependent inflammatory response. However, some proteases also display potent tumor suppressive functions\textsuperscript{2}.

**Human serine proteases**

Nearly 700 active proteases are known in humans, and these are divided into the major classes of metallo, serine, cysteine, threonine and aspartic proteases. The 176 known serine (Ser-) proteases fall into 13 clans; however, roughly 130 proteases belong to the family S1 from clan PA\textsuperscript{2}. The PA clan catalytic domains are often combined with other domains including mediators of specific protein interactions, membrane anchors or even protease inhibitors (MEROPS, http://merops.sanger.ac.uk/index.shtml). Trypsin- and chymotrypsin-like Ser-proteases exhibit a catalytic domain that consists of two \(\beta\)-barrel subdomains with a catalytic triad, comprising the nucleophile Ser195, His57 and Asp102 (according to the temperature requirement protein A (HtrA) proteases 1, 2 and 3 have been identified, including various growth factors belonging to the transforming growth factor (TGF) \(\beta\) superfamily and the aggrecans\textsuperscript{14,15}. Besides its role as activator of hepsin and prosatina, matriptase can degrade laminin in the extracellular matrix (ECM), resulting in higher motility of prostate cancer cells\textsuperscript{16}. Moreover, matriptase can contribute to signaling in prostate cancer via the platelet-derived growth factor (PDGF) receptor-\(\beta\) by activating its ligand, PDGF-D\textsuperscript{17}. Similarly, its substrate hepsin is thought to activate hepatocyte growth factor (HGF), an important factor in prostate and ovarian cancers\textsuperscript{18}. Furthermore, hepsin activates pro-uPA and promotes tumor progression, as shown in experimental tumor mouse models\textsuperscript{19}, whereas prosatina activates matriptase\textsuperscript{20}. Testisin may promote carcinogenesis in the cervix by degrading the tumor-suppressor protein maspin\textsuperscript{21}.

**Gynecological cancers**

This review will focus on current knowledge regarding the function and clinical relevance of Ser-proteases in gynecological cancers; in particular, on the more common cancers of the corpus uteri (endometrium), cervix uteri and the ovaries. In the Western world, the incidence of gynecological malignomas is highest for endometrial cancer, followed by cervical cancer and ovarian cancer, while mortality is inverted (http://globocan.iarc.fr/). Staging of all gynecological cancers is performed according to guidelines offered by the International Federation of Gynecology and Obstetrics (FIGO, http://www.figo.org, see Table 1).

**Ovarian cancer**

The term “ovarian cancer” encompasses tumors originating from the epithelial surface of the ovary, accounting for more than 80% of all solid ovarian tumors. Others like sex cord-stromal tumors, germ cell tumors and metastases of other

<table>
<thead>
<tr>
<th>FIGO Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO I</td>
<td>Tumor is limited to one or both ovaries</td>
</tr>
<tr>
<td>FIGO II</td>
<td>Tumor shows pelvic extensions or implants</td>
</tr>
<tr>
<td>FIGO III</td>
<td>Tumor is present with peritoneal metastases outside the pelvis or involves lymph nodes</td>
</tr>
<tr>
<td>FIGO IV</td>
<td>Distant metastases</td>
</tr>
</tbody>
</table>

**Endometrial cancer**

<table>
<thead>
<tr>
<th>FIGO Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO I</td>
<td>Tumor is confined to the uterus</td>
</tr>
<tr>
<td>FIGO II</td>
<td>Tumor invades cervical stroma, but not beyond the uterus</td>
</tr>
<tr>
<td>FIGO III</td>
<td>Tumor invades the serosa (uterus surface) or adjacent organs (ovaries, vagina), or lymph node involvement</td>
</tr>
<tr>
<td>FIGO IV</td>
<td>Tumor infiltrates other organs like bladder or bowels or distant metastases</td>
</tr>
</tbody>
</table>

**Cervical cancer**

<table>
<thead>
<tr>
<th>FIGO Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO I</td>
<td>Tumor is confined to the cervix area</td>
</tr>
<tr>
<td>FIGO II</td>
<td>Tumor has spread beyond the cervix but is confined to the pelvic area</td>
</tr>
<tr>
<td>FIGO III</td>
<td>Tumor has spread to the pelvic wall or lower part of the vagina</td>
</tr>
<tr>
<td>FIGO IV</td>
<td>Tumor has spread to other organs in the pelvis or distant metastases</td>
</tr>
</tbody>
</table>
tumors, which are less common. The poor prognosis of ovarian cancer patients is mainly the result of late diagnoses and high rates of disease recurrence. Until now, no sufficiently specific and sensitive screening method for the above-mentioned low-incidence ovarian cancers has been established: screening programs like cancer antigen (CA) 125 serum analyses and transvaginal ultrasound examinations have been demonstrated to support early ovarian cancer detection, but their influence on ovarian cancer mortality is not yet clear.

Diagnosis is usually performed via biopsy during standard radical surgery therapy; therefore, some patients will have laparotomy, though only suffering from a benign ovarian tumor. In case of an adnexal mass of unknown nature, CA125 laparotomy, though only suffering from a benign ovarian radical surgery therapy; therefore, some patients will have not yet clear.

have been demonstrated to support early ovarian cancer serum analyses and transvaginal ultrasound examinations above-mentioned low-incidence ovarian cancers has been sufficiently specific and sensitive screening method for the nosises and high rates of disease recurrence. Until now, no ovarian cancer patients is mainly the result of late diag-

DOI: 10.3109/10408363.2013.865701

Serine proteases in gynecological cancers

ROMA, these tests are only useful for preventing the false Anyway, with many false-positive cases for OVA1 and

staging and debulking surgery analogue to ovarian cancer

ing transfer of the patient to a gynecological oncologist for surgery. Since approximately 20% of ovarian cancer patients are CA125-negative, they would be treated like patients presenting with a benign ovarian tissue mass. Anyway, with many false-positive cases for OVA1 and ROMA, these tests are only useful for preventing the false classification of a malignant mass as benign. Currently, therapy is highly standardized – in one part, because there are no noteworthy alternative therapy regimens, and in another, because there are no valid prognostic factors to, e.g. predict the course of the disease or the incidence of disease recurrence. Furthermore, there are no valid predictive factors to aid in foreseeing the success of administered therapies. In follow-up care, routine CA125 and HE4 serum-level analyses are not recommended, as earlier diagnosis of recurrence without symptoms may lead to shorter therapy-free time but not to prolonged survival.

Ovarian carcinomas are categorized according to their histological type as either molecular type I or II. The most common histopathology is serous cystadenocarcinoma, followed by endometrioid and mucuscinous carcinoma. Molecular type I ovarian cancers are less common and are characterized by better patient outcomes, despite resistance to chemother-

apy. Type I tumors are of low-grade serous-papillary, endometrioid, and borderline histotypes and contain mutations in KRAS and/or BRAF, CTNNB1, PTEN and PIK3CA and, in low-grade mucinous tumors, TP53. Molecular type II ovarian cancers are more frequent and are associated with poor patient outcomes. Tumors of this group are believed to originate from the fallopian tubes and the peritoneum, and they include high-grade serous carcinomas, undifferentiated carcinomas and carcinosarcomas. Type II tumors are characterized by TP53 mutations and loss of heterozygosity on chromosomes 7q and 9p.

Major prognostic factors for ovarian cancer are FIGO stage (Table 1) and residual tumor mass after primary surgery, followed by histopathology, tumor grade and presence of ascites. Standard treatment comprises cytoreductive therapy completed by an often extensive primary surgery, followed by platinum-containing adjuvant chemotherapy. In advanced stages of cancer, bevacizumab (Avastin), a vascular endothelial growth factor (VEGF)-directed antibody, is administered concomitant with chemotherapy and subsequently as a monotherapy for 15 months in total.

Endometrial cancer

Endometrial cancer originates from the inner glandular layer of the uterus. Other cancers of the uterus include the rarer sarcomas or gestational trophoblastic forms of the disease. Advanced age, a history of endometrial hyperplasia, as well as increased levels of estrogen play an important role in the development of endometrial cancer, as estrogen stimulates accumulation of the glandular inner lining of the uterus. Consequently, estrogen replacement therapy (without the use of progesterone) and tamoxifen, a selective estrogen receptor modulator used for breast cancer treatment, lead to higher incidences of endometrial cancer. Furthermore, metabolic syndrome (including obesity, diabetes and dislipidemia), polycystic ovarian syndrome, early menarche and late meno-

pause are risk factors. Patients with Lynch syndrome (hereditary non-polyposis colorectal cancer), an autosomal dominant genetic condition with a DNA mismatch repair defect, have a high risk of colon cancer, but are also at an increased risk for development of endometrial and other malignant tumors.

Most endometrial cancer patients present with vaginal discharge or abnormal bleeding. These early symptoms can lead to diagnosis in this less advanced stage, improving prognosis. Endometrial cancer can be placed into two pathogenetic groups. Type I tumors usually occur in pre- and perimenopausal women with a history of unopposed estrogen exposure (namely, without concomitant progesterone therapy) and/or endometrial hyperplasia. Type I endometrial cancer patients usually receive a good prognosis. This histological subtype is a low-grade (meaning it is highly differentiated) endometrioid that is often characterized by a positive progesterone receptor status, which can be used for anti-hormonal therapy. Type II endometrial cancer is less common and occurs in elderly, post-menopausal women without a history of estrogen exposure. This histological subtype is a high-grade (poorly differentiated) endometrioid, uterine papillary serous carcinoma, or clear cell carcinoma, and is associated with a poorer prognosis.

The therapy of choice for endometrial cancer is a hysterectomy with bilateral salpingo-oophorectomy. In patients with increased risk factors (i.e. type II pathogenic group), therapy is complemented by pelvic and para-aortic lymphadenectomy and/or by adjuvant radiotherapy. Preoperative serum CA125, a well-established biomarker for ovarian cancer, is an indicator of advanced disease, though it is not necessary in routine clinical evaluation. In the most aggressive forms of carcinosarcoma of the endometrium, a staging and debulking surgery analogue to ovarian cancer treatment is indicated. Radiotherapy alone, endocrine therapy with progesterone, or chemotherapy (doxorubicin/cisplatin) are usually administered in advanced stages or in patients not fit for surgery owing to poor health.

Alternative therapeutic approaches including trastuzumab (an antibody directed to the extracellular part of the human epidermal growth factor receptor-2), temsiroliimus
(a mammalian target of rapamycin-inhibitor), bevacizumab (a VEGF-inhibitor used in ovarian cancer), or thalidomide (an angiogenesis inhibitor) did not show any impact on survival.\[^{26-28}\]

**Cervical cancer**

Due to screening through regular gynecological examination combined with a Pap smear (the microscopical examination of cervical cells after staining according to Papanicolaou) and successful therapy of pre-malignant lesions (cervical dysplasia) and early cancerous stages, cervical cancer has become a rare disease in the industrialized world. Furthermore, vaccination against human papilloma virus is now recommended for all girls before the first sexual contact, as infection and subsequent inefficient immune response to eliminate the virus is known to be an essential pre-requisite for cervical cancer development. In case of an irregular examination or Pap smear, diagnosis can be performed with colposcopy and biopsy, possibly followed by conization, a small operational procedure often sufficient for the therapy of premalignant dysplasia or very early cervical cancer. Possible symptoms of advanced cervical cancer are bleeding on contact, vaginal discharge, a putrid smell, hematuria, hydroureteronephrosis, swollen lymph nodes and cachexia.

Stage at diagnosis (Table 1) is one of the most important prognostic factors. Early invasive cervical cancer falls within the domain of the surgeon, who may perform a total radical hysterectomy, including parametrias, pelvic lymph node resection and a vaginal cuff (Wertheim-Meigs). Newer operative approaches, like total mesometrial resection and sentinel lymph node biopsy, are currently under investigation in clinical trial.\[^{29}\]. In more advanced disease stages, a primary combined radio-chemotherapy is indicated. Cancer biomarkers, at present, play a minor role in cervical cancer: squamous cell carcinoma for squamous cell carcinomas, and carcinoembryonic antigen and CA125 for adenocarcinomas, can be used for therapy monitoring, but so far there is no proven clinical or diagnostic benefit.

**Serine proteases in gynecological cancers**

**Kallikrein-related peptidases**

The kallikrein-related peptidases (KLKs), previously referred to as tissue kallikreins\[^{30}\], constitute a family of 15 secreted Ser-proteases (Figure 1). Along with uPA (see below), they are among the most promising protease-type, gynecological cancer biomarkers.\[^{31,32}\]. Notably, current knowledge regarding KLKs has recently been reviewed in a book series\[^{33,34}\].

KLK genes are located within a single locus on chromosome 19q13.4 and represent the largest protease gene cluster within the human genome.
aberrations have been reported in this locus and are suspected to be relevant in the context of ovarian cancer. Moreover, a number of KLK splice variants have been described, some of which are tumor-associated. Depending on the KLK considered, KLK expression is either tissue-specific or, in contrast, pleiotropic. Many KLK genes are under the control of steroid hormones, but are also regulated epigenetically by DNA (de)methylation and histone (de)acetylation processes. Additionally, some KLK-targeting microRNAs have been described and, in some cases, have been found to be altered in a malignant context.

KLKs are secreted as inactivezymogens, which require the proteolytic removal of a short pro-peptide for activation (Figure 1). As an example, the structure of mature KLK7 displaying chymotryptic-like activity is depicted in Figure 2(A). Proposed KLK activators include some KLKs themselves, as well as the tumor-relevant enzymes plasmin, matriptase (see below), and matrix metalloprotease (MMP).

Figure 2(A). Proposed KLK activators include some KLKs displaying chymotryptic-like activity is depicted in (Figure 1). As an example, the structure of mature KLK7.

KLKs target a large set of cancer-related substrates: they activate protease zymogens (i.e. pro-uPA, pro-MMP-2, pro-MMP-9), degrade growth factors or growth factor-associated proteins (e.g. TGF-β, insulin-like growth factor (IGF)-binding proteins), process cell/ECM- and/or cell/cell-adhesive proteins (e.g. laminin, fibronectin, vitronectin, cadherins, uPA receptor), and trigger activation of signaling receptors (i.e. PARs). Accordingly, the participation of KLKs in a variety of tumor-relevant cellular processes has been well documented. KLKs have indeed been reported to affect cell survival, proliferation, differentiation (i.e. epithelial to mesenchymal transition - EMT), adherence/detachment and migration, and chemoresistance. Importantly, KLKs' contribution to gynecological cancerogenesis has also been confirmed in vivo. Indeed, in a murine xenograft model, the concomitant overexpression of KLK4–7 in ovarian cancer cells was found to promote early-phase tumor growth, as well as late-stage metastatic spread.

KLKs in ovarian cancer

Of all malignancies, the role of the KLK family has most extensively been investigated in ovarian cancer. Compared to the normal ovary, 12 (i.e. 3–11, 13–15) of the 15 KLKs have been reported to be upregulated at the mRNA and/or protein expression level. One possible mechanism moderating this may result from epigenetical modulation common to the members of the KLKs, as stated earlier. For example, for KLK10, down-regulation by hypermethylation has been reported in ovarian cancer cell lines. In addition, dysregulated microRNAs may lead to unbalanced KLK10 expression and cell proliferation. In the previous few decades, numerous studies have aimed at shedding more light on the impact of KLKs in gynecological cancers. As detailed below, KLKs tend to be upregulated in ovarian cancer, although some exceptions have been documented. It is worth stressing here that some reports appear to conflict, an observation that may be accounted for by the different patient cohorts and methodological approaches used.

KLKs in effusions of ovarian cancer patients

Seven KLKs (KLK5–8, 10, 11, 14) are present in the serum of ovarian cancer patients at levels higher than those found in healthy individuals, patients with benign ovarian tumors, or other malignancies. In addition, KLK5, 7, 8, 10, 11, and 14 are known to be released into ascitic fluid, and KLK5–8, 10, 11, 13 and 14 into pleural effusions of ovarian cancer patients.

KLKs in effusions as diagnostic and prognostic markers

Elevated protein levels of KLK5, 6, 10 and 11 in serum are markers of poor prognosis for the ovarian cancer patient, while higher KLK8 levels in serum indicate a favorable prognosis. Some KLKs seem to enforce the diagnostic and prognostic utility of the established ovarian cancer biomarker CA125: KLK6 and KLK13 serum concentrations improve the diagnostic sensitivity of CA125 in early-stage ovarian cancer, while KLK6 is associated with advanced stages and a more aggressive tumor type. Koh et al. have observed higher CA125, KLK6 and KLK10 serum levels in late-stage FIGOIII/IV cancer patients and upregulation of CA125 and KLK6, but not KLK10, in those patients who live less than 36 months. KLK8 levels in serum and ascites of ovarian cancer patients correlate with CA125, though an impact on the diagnostic or prognostic power of CA125 has not yet been shown.

KLKs in effusions as predictive markers

Oikonomopoulou et al. determined serum protein levels of KLK5–8, 10, and 11, CA125, B7-H4 (a member of the B7 family of immune costimulatory proteins), regenerating protein IV, and spodinin-2 at baseline and after the first chemotherapy cycle to predict patients' responses to carboplatin and/or taxol-based (CBDCA/CAF; taxol/CBDCA) chemotherapy. The authors reported that a panel of these serum markers, among others that included KLK5, KLK7 and CA125, predicted chemotherapy response. All biomarkers examined, except KLK7 and regenerating protein IV, were also powerful predictors of time to progression among the chemotherapy responders. Besides KLK5 and KLK7, the clinical utility of KLKs released into the blood of ovarian cancer patients to predict response to or failure of chemotherapy was shown for three other KLKs: higher KLK6, 8 and 10 serum levels indicated poor response to platinum-based chemotherapy, while higher levels of KLK8 were found particularly in the group with no or low CA125 expression.

KLKs in ovarian cancer tissue

Diagnostic impact

As mentioned earlier, most KLKs have been reported to be overexpressed in ovarian cancer compared to normal ovarian tissue and benign ovarian tumors. In some analyses, this difference is also observed for tumors of low malignant potential or in comparisons between late and early stages of the disease or low- and high-grade tumors. Namely, KLK4–8,
Figure 2. Serine proteases with small synthetic inhibitors bound to the active sites. The proteases of Figures A–F are consistently depicted as colored ribbons, whereas the small inhibitors are always shown as ball-and-stick models with C-atoms (white), N-atoms (blue), O-atoms (red), and S-atoms (yellow).

A: The chymotryptic KLK7 (salmon) in standard orientation with the covalent chloromethyl ketone inhibitor suc-AAPF-cmk occupying the non-prime side, in particular with a Phe side chain in the S1 pocket (Protein Database [PDB] code 2QXH), Pro in S2, and two Ala in S3 and S4. Important catalytic residues of the active site are shown as stick models. For example: the I16-D194 salt bridge, N189, and A190 in the S1 pocket, and D102 and H57 of the catalytic triad. S195 is hardly visible. The latter two residues are covalently linked to the chloromethyl ketone.

B: uPA (green) with the inhibitor N-(1-adamantyl)-N0-(4-guanidinobenzyl)-urea (AGB) and the guanidinobenzyl side chain interacting with residues D189 and S190 of the S1 subsite and the adamantyl moiety occupying the S1' and S2' region (PDB 1EJN). Notably, the catalytic triad (D102, H57 and S195) adopts a non-functional conformation, while the activating salt bridge I16-D194 is formed.

C: The HtrA1 catalytic domain in the active form (orange) and the borate inhibitor DPMFKL-boro-V (boron displayed as pink ball) overlayed with the inactive form (yellow) with a broken catalytic triad (PDBs 3NZI and 3NUM). The triad consists of residues D250, H220 and S328. The S1 subsite of HtrA1 is shaped to accommodate aliphatic side chains, such as the P1-Val. Further inhibitor side chains are only well defined in the S2 (Leu), S4 (Phe) and S5 (Met) pockets.

D: Matriptase (magenta) in complex with the non-covalent inhibitor CJ-672, which exhibits a benzamidine-like side chain bound to the S1 pocket residues D189 and S190 (PDB 2GV7). Otherwise, the non-peptidic small molecule occupies the S1' to S3 subsites in an unconventional manner. D102, H57 and S190 of the catalytic triad are labeled, as well as the activating salt bridge V16-D194.

E: Hepsin (light turquoise) is another tryptic serine protease that is shown in complex with Ac-KQLR-cmk, which is covalently bound to the catalytic triad residues H203 and S353, except D257 (PDB 1Z8G). The Arg side chain of the inhibitor makes polar interactions with residues D347 and hydrophobic interactions with A348, while Leu, Gln and Lys occupy the S2, S3 and S4 subsites. The activating salt bridge is formed by I163 and D352.

F: Prostasin (blue), a prostatic serine protease that is activated by matriptase, bound to the D-FFR-cmk inhibitor (PDB 3E0N). The catalytic triad consists of D102, H57 and S195, the activating salt bridge is formed by I16 and D194, while the S1 residues D189 and A190 interact with the Arg side-chain of the inhibitor. Both S2 and S4 subsites are occupied by l-Phe and o-Phe, respectively.
10, 11 and 13–15 are overexpressed at the mRNA level, and KLK5–8, 10, 11 and 14 are overexpressed at the protein level.

**Prognostic impact**

Most KLK family members are associated with poor prognosis of ovarian cancer patients, but some are linked to a favorable course of the otherwise fatal cancer disease: KLK4–7, 10, 11, 13 and 15 mRNA overexpression is associated with late-stage, high-grade disease and shorter disease-free and overall survival. The contrary is true for elevated KLK8, 9, 11 and 14 mRNA. According to this, KLK5–7 and 10 proteins have been reported as predictors of poor patient outcomes and KLK8 for good. For some KLKs, findings are different for mRNA expression compared to protein content: KLK11 and KLK13 protein content predict favorable disease outcome, while the opposite is true for KLK11 and KLK13 mRNA expression.

This finding can be explained by the different methodologies used to assess KLK concentration at the mRNA level versus the protein level (see Table 2). One might hypothesize that the prognostic impact of KLK mRNA is different from that observed at the (active?) protein level as a result of the presumption that KLK levels can be modified during translation or after secretion within a KLK-cascade. Apart from this, findings from these different studies were acquired from different cohorts; thus, differences in patient characteristics could also explain these contrasting results. Numerous studies dedicated to evaluating the prognostic capabilities of KLKs have been conducted. Examples of these are described in detail below. KLK4 mRNA expression in ovarian tumor tissue, as well as KLK5 protein levels in tissue, serum or ascites, have been shown to be associated with shorter disease-free and overall survival.

KLK6-positive tumors (analyzed by enzyme-linked immunosorbent assay/ELISA) are more likely to be associated with advanced FIGO stage, serous histology and suboptimal debulking, with impacts on overall and progression-free survival. KLK6 cell-associated overexpression of KLK6 as assessed by immunohistochemistry is associated with shorter overall and progression-free survival. Similarly, high KLK7 and KLK10 protein levels in ovarian cancer tissue extracts are associated with advanced stage, suboptimal debulking and poor prognosis,

and have been found to be even more accurate when used in a serum-based multiparametric biomarker panel including B7-H4 and spondin.

When evaluating the impact of KLK-level differentials between primary tumor and omentum metastases on prognosis, Dorn et al. found KLK5–7 and 10 differentials to be associated with suboptimal debulking and disease progression.

Some KLKs seem to be protective biomarkers: KLK8 protein is elevated in tissue, serum and ascites of ovarian cancer patients, with higher levels indicating a better prognosis. Analogously, KLK9 gene expression in tumor cytosols has been found to be a favorable factor. As already mentioned, contradictory results have been found for KLK11 and KLK13: some studies have reported KLK11 and KLK13 tumor tissue protein levels to be correlated with early stage and favorable outcome for overall survival, while Shigemasa et al. and White et al. observed an association between higher KLK11 and KLK13 mRNA expression quantified by polymerase chain reaction (PCR) and higher-grade tumors and poorer prognosis. As discussed previously, the contrast between these observations may reflect a difference between the mRNA level and the protein level for KLK11 and KLK13. Another protective protease seems to be KLK14: in contrast to KLK15 mRNA, KLK14 mRNA in ovarian tumor tissue is associated with longer overall and progression-free survival. The prognostic impact of the KLK family could be used for decisions regarding therapy: for example, by implementing the OVSCORE, an algorithm that consists of KLK6 and KLK13 tumor tissue levels as well as tumor grading and ascites volume capable of predicting the size of residual tumor mass left in the patient after primary debulking surgery. The OVSCORE could help to identify patients who could be spared the burden of surgery.

**Predictive impact**

Adjuvant therapy in ovarian cancer is highly standardized, as there is no marker to predict the effect of chemotherapy. KLKs could help to identify non-responders: in patients with recurrent disease, KLK4 protein tumor expression (by immunohistochemistry) was found to be elevated in the group of patients progressing under taxane treatment but not in the group of responders.

In line with these findings, KLK7 protein levels and KLK6 combined with KLK8, KLK13, FIGO stage, and residual tumor mass after surgery have been shown to discriminate between responder and non-responder ovarian cancer patients.

Altogether, KLKs are promising predictive factors in ovarian cancer. However, due to the lack of sufficiently effective alternative therapy models in ovarian cancer, the clinical use of this knowledge for now is limited and should be subjected to further investigation.

**KLKs in endometrial cancer**

The impact of the KLK family members has best been evaluated for ovarian cancer. Nevertheless, some KLKs seem to play a significant role in other gynecological cancers as well, including endometrial cancer. KLK4 as assessed by immunohistochemistry (IHC) has been shown to be over-expressed in endometrial cancer patients compared to those with normal and hyperplastic endometrium, and is upregulated by steroid hormones (estrogen and progesterone) in endometrial cancer cell lines.

In the more aggressive type II form of endometrial cancer, KLK6 gene expression (quantitative PCR) has been shown to be significantly elevated compared to normal, benign and type I carcinoma; cell culture supernatants (ELISA) of type I endometrial and ovarian serous papillary carcinoma are highly positive for KLK6 protein compared to endometrioid and cervical carcinomas. Also, plasma and serum KLK6 and KLK10 protein levels are equal in healthy female individuals, in patients with benign diseases of the uterus and in patients with endometrioid carcinoma, but are significantly elevated in serous-papillary tumors. KLK8 is overexpressed in endometrial cancer tissue compared to normal tissue and associated with lower stage and grade,
Table 2: Analysis of Ser-protease expression in gynecological cancers: cohort size, sample type and analytical method. Apparent discrepancies in the literature may reflect differing study characteristics, including the size and treatment of the tested cohort, the type of sample to be analyzed (e.g. cancer subtype, primary tumor versus derived fluids), as well as the detection method (e.g. targeting mRNA or protein, applied to the whole tissue or limited to cancer cells, highly sensitive and/or robustly quantifiable).

<table>
<thead>
<tr>
<th>Type and number of samples analyzed</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK4</td>
<td>PCR, BIC, ELISA</td>
</tr>
<tr>
<td>KLK5</td>
<td>PCR, BIC, ELISA</td>
</tr>
<tr>
<td>KLK6</td>
<td>PCR, BIC, ELISA</td>
</tr>
<tr>
<td>KLK7</td>
<td>PCR, BIC, ELISA</td>
</tr>
<tr>
<td>KLK8</td>
<td>PCR, BIC, ELISA</td>
</tr>
<tr>
<td>KLK9</td>
<td>PCR, BIC, ELISA</td>
</tr>
<tr>
<td>KLK10</td>
<td>PCR, BIC, ELISA</td>
</tr>
<tr>
<td>KLK11</td>
<td>PCR, BIC, ELISA</td>
</tr>
<tr>
<td>KLK12</td>
<td>PCR, BIC, ELISA</td>
</tr>
<tr>
<td>KLK13</td>
<td>PCR, BIC, ELISA</td>
</tr>
<tr>
<td>KLK14</td>
<td>PCR, BIC, ELISA</td>
</tr>
<tr>
<td>KLK15</td>
<td>PCR, BIC, ELISA</td>
</tr>
</tbody>
</table>

Critical Reviews in Clinical Laboratory Sciences Downloaded from informahealthcare.com by University of Toronto on 01/23/15 For personal use only.
**Critical Reviews in Clinical Laboratory Sciences Downloaded from informahealthcare.com by University of Toronto on 01/23/15**

For personal use only.

DOI: 10.3109/10408363.2013.865701

Serine proteases in gynecological cancers

KLK2,3, 6-8,10,11,13

Normal ovarian tissue (n = 10), benign ovarian tumors (n = 10), ovarian cancer (n = 20).

ELISA 82

KLK5–8,10,11,13

Normal ovarian tissue (n = 15), ovarian cancer (n = 19).

GEP, IHC, PCR 67

KLK4–8,10,11,13,14

Ovarian cancer tissue (n = 142). ELISA 79

KLK5–8,10,11,13

Serum from ovarian cancer patients (n = 98). ELISA 64

KLK4–8,10,11,13,14

Ovarian cancer tissue (primary tumor and omentum metastasis, n = 54). ELISA 91

**Endometrial cancer**

KLK4

Cell lines (n = 6).

PCR, WB 96

KLK6

Endometrial biopsies: normal endometrial cells (n = 3), endometrioid carcinoma (n = 13), USPC (n = 13).

IHC 97

Tumor cultures: endometrioid carcinoma (n = 3), USPC (n = 3), ovarian serous papillary carcinoma (n = 5), cervical cancers (n = 3).

ELISA 98

Control sera (n = 22), sera from patients with benign diseases of the endometrium (n = 20), endometrioid carcinoma (n = 20), USPC (n = 17).

ELISA 99

KLK8

Normal endometrial tissues (n = 37), endometrioid carcinoma (n = 44).

PCR, IHC 100

KLK10

Endometrial biopsies: normal endometrial cells (n = 6), USPC (n = 11).

PCR 101

Control sera (n = 22), sera from patients with benign diseases of the endometrium (n = 20), endometrioid carcinoma (n = 21), USPC (n = 12).

ELISA 98

**Cervical cancer**

KLK7

Normal cervical tissue (n = 13), cervical adenocarcinoma (n = 70).

IHC 102

Normal cervical keratinocyte cultures (n = 8), cervical cancer cell lines (n = 18).

PCR 103

Primary squamous cervical tumor (n = 5), primary adenocarcinoma (n = 5).

IHC 104

Cervical tissue: cervicitis (n = 35), low-grade cervical intraepithelial neoplasia (n = 31), high-grade cervical intraepithelial neoplasia (n = 51), squamous cervical carcinomas SCC (n = 197), cervical adenocarcinomas (n = 53).

IHC 105

KLK8

Normal cervical keratinocyte cultures (n = 8), cervical cancer cell lines (n = 19).

PCR, IHC 106

**Urokinase**

**Ovarian cancer**

Normal tissue (n = 4), carcinomas (n = 9).

IHC 240

Ascites derived from malignant tumors (n = 36).

ELISA 127

Malignant tissue (n = 70).

ELISA 112

Early (n = 7) or advanced (n = 39, including lymph node and omentum metastasis tumors.

ELISA 113

Normal or benign tissue (n = 154), malignant tissue (n = 90).

ELISA 115

Malignant tissue (n = 16).

ELISA 122

Primary malignant tumor (n = 86).

ELISA 128

Plasma from healthy control (n = 36), patient with benign tumors or inflammatory disease (n = 16), cancer patients (n = 25).

ELISA 116

Primary ovarian carcinomas (n = 77), ovarian metastases (n = 14).

ELISA 129

Sera from cancer patients (n = 64).

ELISA 123

Malignant tissue (n = 70).

ELISA 117

Low malignant potential tumors (n = 9), primary carcinomas (n = 82), recurrent carcinomas (n = 12).

ELISA 118

Primary carcinomas (n = 35), abdominal metastasis (n = 10).

ISH, NB 114

Cystadenomas (n = 19), low malignant potential tumors (n = 18), advanced cancers (n = 41).

ELISA 126

Ascites derived from benign (n = 70), borderline (n = 12) or malignant tumors (n = 25).

ELISA 242

Normal tissue (n = 5), benign tumors (n = 20), borderline tumors (n = 15), invasive carcinomas (n = 16).

IHC 119

Benign (n = 7) and borderline malignant tumors (n = 5), malignant tumors (n = 51).

ELISA 130

Sera from cancer patients (n = 17).

ELISA 130

(continued )
Table 2. Continued

<table>
<thead>
<tr>
<th>Type and number of samples analyzed</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal tissue (n = 20), primary tumors (n = 100), matched metastatic lesions (n = 30). Primary tumors (n = 120), matched metastatic lesions (n = 40). Normal tissue (n = 20), benign tumors (n = 20), cancers (n = 60); Blood from control individuals (n = 49) or patients with benign (n = 49) or malignant tumors (n = 49).</td>
<td>IHC</td>
<td>120</td>
</tr>
<tr>
<td><strong>Endometrial cancer</strong></td>
<td>Malignant tumors (n = 100). Primary adenocarcinomas (n = 69). Endometrial cancer patients (n = 274). Normal tissue, hyperplasia, cancers. Epithelial cancers (n = 91). Normal tissue (n = 16), carcinomas (n = 34). Malignant tissue (n = 64), tumor-free marginal tissue (n = 56).</td>
<td>IHC</td>
</tr>
<tr>
<td>Carcinomas grade 1 (n = 10), 2 (n = 9), 3 (n = 20) Carcinomas (n = 114). Normal tissue (n = 5), squamous intra-epithelial lesions (n = 36), carcinomas (n = 42). Normal tissue, squamous intra-epithelial lesions, invasive carcinomas. Normal tissue (n = 10), tumors with (n = 28) or without (n = 34) lymph node involvement.</td>
<td>IHC</td>
<td>142</td>
</tr>
<tr>
<td><strong>HtrAs</strong></td>
<td>Normal tissue (n = 2), primary tumors (n = 16; including early and late stage). Endometrioid tumors (n = 8), serous tumors (n = 51), mixed histology (n = 1). Normal tissue (n = 19), benign tumors (n = 20), borderline tumors (n = 7), cancers (n = 44), Krukenberg tumors (n = 8). Normal tissue (n = 11), granulosa cell tumors (n = 16). Normal tissue (n = 9), granulosa cell tumors (n = 19), mucinous cystadenocarcinomas (n = 6), serous cystadenocarcinomas (n = 8).</td>
<td>NB and/or PCR (HtrA1) TMA (HtrA1)</td>
</tr>
<tr>
<td><strong>Ovarian cancer</strong></td>
<td>PCR and/or IB (HtrA1–3)</td>
<td>156</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>PCR (HtrA3) PCr and/or IB (HtrA1–3)</td>
<td>14 157</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>Normal tissue (n = 6), cancers (n = 33) Normal tissue (n = 36), cancers (n = 88). Endometrioid or mucinous tumors (n = 171), non-endometrioid tumors (n = 13).</td>
<td>PCR, WB and/r IHC (HtrA1, 3) PCR, IB (HtrA1–3) TMA (HtrA1)</td>
</tr>
<tr>
<td><strong>TTSPs</strong></td>
<td>Fibrotheomas (n = 2), granulosa cell tumor (n = 1), carcinomas (n = 9). Serous tumors (n = 18), mucinous tumors (n = 8), endometrioid tumors (n = 17), clear cell tumors (n = 9), mixed/unclassified (n = 2). Normal tissue (n = 8), serous tumors (n = 39), mucinous tumors (n = 19), endometrioid tumors (n = 17), clear cell tumors (n = 14). Brenner tumors (n = 6), fibromas (n = 5), granulosa cell tumors (n = 8), dygerminomas (n = 6), clear cell carcinomas (n = 12), yolk sac tumors (n = 6), endometrioid adenocarcinomas (n = 10), mucinous adenocarcinomas (n = 23), serous adenocarcinomas (n = 84). Low-grade serous tumors (n = 12), high-grade serous tumors (n = 200), clear cell tumors (n = 132), endometrioid tumors (n = 132), mucinous tumors (n = 31).</td>
<td>IB IHC, ISH IHC, PCR IHC TMA</td>
</tr>
<tr>
<td><strong>Endometrioid cancer</strong></td>
<td>Normal tissue (n = 20), endometrial hyperplasia (n = 11), adenocarcinomas (n = 65).</td>
<td>IHC</td>
</tr>
<tr>
<td><strong>Cervical cancer</strong></td>
<td>Normal tissue (n = 4), carcinomas (n = 11). Normal tissue (n = 10), low-grade squamous intraepithelial lesions (n = 19), high-grade squamous intraepithelial lesions (n = 2).</td>
<td>IHC, PCR</td>
</tr>
<tr>
<td><strong>Hepsin</strong></td>
<td><strong>Ovarian cancer</strong></td>
<td>Primary cancers ($n = 14$), omental metastases ($n = 17$). Normal ovaries ($n = 10$), tumors of low malignant potential ($n = 12$), serous carcinomas ($n = 19$), mucinous carcinomas ($n = 7$), clear cell carcinomas ($n = 3$), endometrioid carcinomas ($n = 3$).</td>
</tr>
<tr>
<td><strong>Endometrial cancer</strong></td>
<td>Normal endometria ($n = 34$), hyperplasic endometria ($n = 11$), adenocarcinomas ($n = 128$).</td>
<td>IHC 184</td>
</tr>
<tr>
<td><strong>TMPRSS3</strong></td>
<td><strong>Ovarian cancer</strong></td>
<td>Normal ovaries ($n = 10$), benign tumors ($n = 4$), low malignant potential tumors ($n = 10$), serous carcinomas ($n = 29$), mucinous carcinomas ($n = 12$), clear cell carcinomas ($n = 6$), endometrioid carcinomas ($n = 8$). Normal ovaries ($n = 7$), adenomas ($n = 6$), low malignant potential tumors ($n = 3$), adenocarcinomas ($n = 41$).</td>
</tr>
<tr>
<td><strong>GPI-anchored Ser-proteases</strong></td>
<td><strong>Prostasin</strong></td>
<td><strong>Ovarian cancer</strong></td>
</tr>
<tr>
<td><strong>Testisin</strong></td>
<td><strong>Ovarian cancer</strong></td>
<td>Normal tissue ($n = 7$), adenomas ($n = 4$), low malignant potential tumors ($n = 3$), carcinomas ($n = 27$).</td>
</tr>
<tr>
<td><strong>Cervical cancer</strong></td>
<td>Primary cancers ($n = 14$), omental metastases ($n = 17$).</td>
<td>GEP 183</td>
</tr>
</tbody>
</table>

ELISA: enzyme-linked immunosorbent assay; GEP: gene expression profiling; IB: immunoblot; IHC: immunohistochemistry; ISH: in situ hybridization; LIA: luminiscence immunoassay; MI: multiplex immunoassay; NB: Northern blot; PCR: polymerase chain reaction; TMA: tissue microarray; USPC: uterine serous papillary carcinoma; WB: Western blot.
suggesting that KLK8 expression is an early event in endometrial carcinogenesis.\textsuperscript{99}

**KLKs in cervical cancer**

For the assessment of cervical cancer, KLK7 appears to be a promising biomarker: while normal cervical tissue is negative for KLK7, it is highly expressed in tumor tissue.\textsuperscript{100} An inhibitor of KLK7, antileukoprotease, is significantly lower in cervical adenocarcinomas compared to normal endocervical glands, though no impact on survival has been found.\textsuperscript{101} Furthermore, KLK7 levels increase with the development from cervical intraepithelial neoplasia to invasive cervical cancer, suggesting a possible additional role for screening of cervical precursor lesions.\textsuperscript{102} Additionally, normal cervical tissue is negative for KLK8, while cancer cell lines and primary tumor cultures show KLK8 expression by PCR and IHC.\textsuperscript{103}

**Plasminergic proteases**

Plasminogen, as well as its urokinase- and tissue-type plasminogen activators (uPA and tPA, respectively), are secreted trypsin-like proteases that have long been recognized as significant pro-tumorigenic factors.\textsuperscript{104} Plasminogen is synthesized in the liver, then circulates and penetrates tissues, whereas uPA and tPA are ubiquitously expressed, including within the epithelia. Plasminogen and pro-uPA are activated upon proteolytic conversion of single-chain precursors into mature enzymes composed of two disulfide bond-linked chains.\textsuperscript{104,105} Interestingly, pro-uPA activators include a number of tumor-relevant proteases such as plasmin itself, trypsin KLKs, matriptase, hepsin and prosemin.\textsuperscript{42,106,107} Although tPA can also be processed into a two-chain form, single-chain tPA is already proteolytically active, particularly upon binding to fibrin.

Remarkably, both pro- and mature uPA associate with a high-affinity, GPI-anchored membrane receptor (uPAR/CD87; Figure 1).\textsuperscript{108} Despite its secretion, it might therefore be considered a pseudo membrane protease as opposed to a truly membrane-associated enzyme (i.e. transmembrane and GPI-anchored proteases, see above). uPA/uPAR interaction focuses and catalyzes the activity of uPA, particularly the conversion of plasminogen into plasmin, at the cell surface (for the structure of the protease domain of mature uPA, see Figure 2B). Moreover, upon binding to its receptor, uPA promotes integrin-(in)dependent intracellular signaling that affects cell proliferation, migration and survival.\textsuperscript{109} Notably, these later functions are independent from its proteolytic activity. Whereas uPA and tPA target a rather limited number of substrates, the active counterpart of plasminogen (i.e. plasmin) is a wide-spectrum protease. Its substrates include protease precursors such as some pro-KLKs and pro-MMPs, ECM, and basement membrane proteins, cytokines, and growth factors, as well as membrane receptors.\textsuperscript{104,105,108} Accordingly, the plasmin(ogen) system contributes vitally to the modulation of cell adherence, migration, differentiation and survival.\textsuperscript{104,105,108}

Within the last three decades, plasminogen system components have been extensively investigated in the tumor tissues and bodily fluids of afflicted patients. Significantly, this has resulted in the identification and validation of uPA and its inhibitor PAI-1 as biomarkers of tumor progression and patient outcome in breast cancer.\textsuperscript{110} Indeed, high tumor-associated uPA/PAI-1 levels predict that node-negative patients will benefit from adjuvant systemic chemotherapy. Accordingly, uPA/PAI-1 measurement has been recommended for use in clinical practice by various national Clinical Oncology guidelines.\textsuperscript{110} In addition to breast cancer, uPA and/or PAI-1 expression has also been assessed and has been found to be upregulated in the course of other malignancies, including gynecological ones. The next paragraphs will therefore focus on the expression and clinical relevance of uPA in ovarian, endometrial and cervical cancers. While some data exist concerning tPA and plasmin, these were excluded as a result of uPA’s status as a far more promising cancer biomarker. It is worth mentioning here that uPAR has also received considerable attention as a contributor to and marker of carcinogenesis; however, a survey of this is clearly beyond the scope of this review.

uPA tissue expression has been well-documented to: (i) gradually increase from benign to transformed, then malignant tissue, (ii) correlate with dedifferentiation, tumor grade and malignant potential, and (iii) be elevated in metastatic, as compared to primary tumors.\textsuperscript{111–121} Importantly, high uPA, either alone or in combination with elevated PAI-1, has also been proposed to be predictive for poor outcome when considering either disease-free, progression-free or overall survival.\textsuperscript{111,112,116,117,119–122} However, other studies have failed to reproduce these findings.\textsuperscript{115,123}

In conjunction with this, uPA is known to be present in ascitic fluids from patients with ovarian tumors, where high uPA levels are predictive of poorer histological subtypes. In contrast, the uPAR/uPA ratio has been found to be associated with FIGO stage, residual disease, and disease-free and overall survival.\textsuperscript{124–127} uPA is also elevated in the serum of ovarian cancer patients\textsuperscript{128}, though its evaluation has led to conflicting conclusions. While Abendstein et al. found that uPA protein levels were not predictive of response to treatment and overall survival,\textsuperscript{129} two recent studies based on protein and mRNA measurements, respectively, demonstrated its clinical utility in terms of determining clinicopathological parameters and/or patient prognosis\textsuperscript{111,130}.

In endometrial cancers, uPA has been found to be upregulated, both at the mRNA and protein level, as compared to hyperplastic, marginal tumor-free, and normal tissue.\textsuperscript{131–133} Moreover, high uPA levels have been reported to predict unfavorable tumor histology, advanced disease stages, high-grade classification, nodal involvement, lower estrogen and progesterone levels, recurrence, and shorter overall survival.\textsuperscript{132,134–136} Still, a recent pilot study did not confirm uPA utility for long-term survival prediction.\textsuperscript{137}

In cervical cancer, uPA has also been found to be elevated based on mRNA, protein, and/or activity measurements.\textsuperscript{138–141} It has been proposed to predict early invasive growth, advanced disease stages, lymph node involvement, and shorter progression-free and overall survival.\textsuperscript{139,140} However, other groups have failed to prove or confirm an association between high uPA and tumor grade, stage, or nodal status, or with disease recurrence or recurrence-free survival.\textsuperscript{141,142}
HtrAs are highly conserved trimeric Ser-proteases, which are found in organisms ranging from prokaryotes to mammals. Notably, and contrasting with the majority of Ser-proteases, HtrAs are currently considered potent tumor-suppressive enzymes. Four HtrAs – of which HtrA1 and 2 are the best characterized – are expressed in humans. In addition to their catalytic domain, these ubiquitous proteases display protein/protein interaction domains, including an IGF-binding, a Kazal (HtrA1, 3, 4), and a PDZ domain that is not observed in any other human protease (Figure 1). Interestingly, whereas most Ser-proteases require proteolytic processing for activation, activation of HtrAs involves substrate-induced active site conformational changes, as recently established for HtrA1 (Figure 2C).

HtrA1 (PRSS11), 3 and 4, which are closely related, are predominantly secreted proteases (Figure 1). Little is known regarding the regulation of their expression, which may be affected by single nucleotide polymorphisms, loss of heterozygosity, DNA methylation and histone modifications. Remarkably, HtrA1 expression is down-regulated by cell transformation and chemotherapy in vitro. HtrA1 tumor-relevant, extracellular substrates identified so far include ECM proteins (fibronectin, vitronectin), as well as growth factors or growth factor-associated proteins such as TGF-β, FGF8 and IGF-binding proteins. Additionally, HtrA1 is present intracellularly. On the one hand, it has been found to associate with microtubules, to process the tumor-suppressor tuberous sclerosis complex-2 protein, and to disrupt the cytosolic X-linked inhibitor of apoptosis. On the other hand, it has also been proposed to interact with and disrupt TGF-β in the endoplasmic reticulum, resulting in the impairment of TGF-β signaling. Accordingly, a direct contribution of HtrA1 down-regulation to malignancy is strongly supported by ex vivo and in vivo experiments. Indeed, in cell based assays, HtrA1 has been found to affect the survival/apoptosis balance, particularly in response to chemotherapy, as well as the anchorage-dependent cell growth and the invasiveness of cancer cells, including that of ovarian and endometrial cells. HtrA1 is also known to display critical vascular functions that might be relevant to tumor-associated neoangiogenesis. Notably, HtrA1 down-regulation has been reported to promote increased peritoneal dissemination of ovarian cancer cells and to enhance endometrial cell metastasis to the lungs in xenograft mouse models. In contrast, protease overexpression has been shown to be protective.

In contrast to other HtrAs, HtrA2 – also known as Omi, is an intracellular enzyme that locates to the mitochondrial inter-membrane space and is involved in the maintenance of mitochondrial homeostasis, presumably due to chaperone-like functions. Upon cellular stress, HtrA2 is released into the cytosol and contributes to programed cell death via interaction with and degradation of apoptosis modulators, including the X-linked inhibitor of apoptosis and Wilms tumor 1. It also promotes caspase-independent apoptosis via unknown mechanisms.

Shridhar et al. first noticed down-regulation of HtrA1 expression when subtracting cDNA libraries obtained from two advanced and two early ovarian malignant tumors from libraries obtained from normal epithelial cell brushings. The same group subsequently confirmed down-regulation, or even a complete loss of, HtrA1 mRNA in a small number of primary ovarian cancers. These observations were then extended to a larger cohort, including some secondary Krukenberg-type tumors. Indeed, a significant reduction in HtrA1 mRNA and protein levels was found in malignant as compared to benign tumors. In the same cohort, HtrA3 was decreased in all tumor types as compared to normal tissue. This was also the case with malignant compared to benign tumors, where the protein was found to be below the detection limit in 30% of the malignant tissue samples examined. HtrA2 mRNA was slightly decreased in all tumor types. Reduced HtrA3 expression in ovarian cancers at the mRNA and/or protein level was then confirmed in independent cohorts. However, in the latter report, Singh et al. found that HtrA1 and 2 were not significantly affected. HtrA1 may also have the potential to predict response to chemotherapy since, in a cisplatin-treated ovarian cancer patient cohort, low tumor-associated antigenic HtrA1 was found to correlate with a poor response rate.

Likewise, in endometrial cancers, mRNA and/or protein analysis has shown that HtrA1, 2 and 3 are down-regulated when compared to normal tissue and is negatively correlated with TGF-β protein levels. A similar pattern is also expected in cervical cancers, since analysis of DNA methylation in malignant versus normal cervix tissue has indicated that HtrA3 promoter methylation may serve as a potential marker.

Type II transmembrane serine proteases

Type II transmembrane serine proteases (TTSPs) are inserted into the plasma membrane via an aminoterminal, integral transmembrane domain (Figure 1). These proteases all display: (i) a short aminoterminal cytoplasmic domain that potentially connects TTSPs with the cytoskeleton and intracellular signaling proteins; (ii) a transmembrane domain; (iii) a central region of variable length and function; (iv) a carboxyterminal protease domain, including a protease-sensitive pro-peptide (Figure 1). Seventeen TTSPs are expressed in humans and have received growing interest over the last decade for their major contribution to homeostasis and disease, including gynecological malignancies.

Matriptase

Matriptase-1, also known as MT-SP1, is ubiquitously expressed by epithelial cells and is upregulated by androgens. In addition to typical TTSP domains, it displays a SEA domain, two CUB domains, and four LDL receptor class A domains (Figure 1). Upon proteolytic activation, which can be self-achieved or involve another protease (e.g. prostatasin, as discussed below), its catalytic domain (Figure 2D) remains membrane-associated via disulfide bonds. Matriptase can also be shed proteolytically. The active trypsin-like protease targets a number of tumor-relevant substrates. Indeed, it degrades ECM proteins (fibronectin, laminin, type IV collagen) and IGF-binding proteins, and activates uPA, prostatasin...
Hepsin and hepsin-related proteases

Hepsin, also known as TMPRSS1, is a trypsin-like enzyme and has been initially identified in the liver (Figure 2E). It is also expressed at lower levels in the kidney, thyroid, pancreas and testis. In addition to its Ser-protease, transmembrane and cytosolic domains, it displays a macrophage scavenger receptor-like domain of unknown function (Figure 1). Hepsin is thought to auto-activate and, in turn, to activate pro-uPA and prostatin (see above and below), as well as HGF and macrophage-stimulating protein, while processing the extracellular domain of the EGFR18,106,175,176. Whereas hepsin has been unequivocally proposed as a tumorigenic factor, it remains unclear if it affects primary tumor growth or metastatic spread – a distinction that might be dependent on the tumor type, on protease expression levels, or on its intracellular versus membranous localization177. Indeed, antibody-based neutralization of hepsin does not affect growth, but rather alters migration/invasion of ovarian cancer cells in vivo178. In line with this, in an in vivo prostate cancer model, hepsin did not impact cell growth but rather disturbed basement membranes organization179. On the other hand, hepsin overexpression has been found to affect cell cycle/apoptosis, and to decrease anchorage-(in)dependent growth of ovarian and endometrial cancer cells in vitro and in vivo177,180,181.

In addition, hepsin mRNA has been found to be ectopically expressed in tumors, whereas mRNA levels are very low in the normal ovary182. However, hepsin mRNA levels have also been reported to be lower in metastatic versus primary ovarian tumors183. Hepsin is also upregulated in endometrial cancer versus in the normal and hyperplasic endometrium, and high hepsin levels have been found to be associated with advanced disease stage and high-grade classification, as well as with depth of endometrial invasion, lymph node involvement and metastasis184.

Another tumor-relevant TTSP is the hepsin-related protease TMPRSS3, also known as TADG-12 (Figure 1). It is a sodium channel-activating protease that has been investigated to some extent in the context of deafness185. While TMPRSS3 expression is low to undetectable in the normal ovary, it is overexpressed in ovarian carcinomas186. Moreover, a TMPRSS3 splice variant, thought to result in a lack of membrane anchorage, was found to be elevated in ovarian cancers with low or high malignant potential and to be increased in early versus advanced stages187, probably due to aberrant DNA hypomethylation188.

Glycosyl-phosphatidylinositol (GPI)-anchored serine proteases

Glycosyl-phosphatidylinositol (GPI) anchors are glycolipidic groups that are post-translationally attached to the carboxy-terminal region of extracellular proteins carrying a cleavable amino-terminal signal sequence, a step that occurs in the endoplasmic reticulum. GPI moieties ultimately bridge these proteins, including some proteases, to the exterior leaflet of the plasma membrane (Figure 1)106. They are enriched in cholesterol-rich membrane domains, including lipid rafts and caveolae, and can be hydrolyzed by phospholipases, resulting in protein release into the extracellular space.

Prostatin

Prostatin, also known as PRSS8, is an epithelial trypsin-like protease initially identified in seminal fluid189. It has been well-documented to play a pivotal role in activation of epithelial sodium channels and is thought to contribute to terminal epithelial differentiation190. Interestingly, prostatin...
(which can be activated by plasmin and hepsin; see above) takes part in matriptase activation, and is reciprocally activated in vitro and in vivo by matriptase20,191 (Figure 2F). Thus, prostasin indirectly impacts the various targets of tumor-associated matriptase, including PAR-2. However, loss of prostasin expression seems to be associated with EMT163,192.

Although several attempts to investigate ovarian cancer-related prostasin have been reported, no consistent pattern has emerged so far. Indeed, Mok et al. were the first to observe upregulation of the prostasin gene in a cDNA microarray by comparing malignant with control ovarian cell lines193. Furthermore, Mok and colleagues found antigenic prostasin to be upregulated in ovarian cancer versus normal tissues193. Moreover, when exploring its clinical relevance in a larger patient cohort, prostasin was also found to be elevated in sera derived from afflicted patients compared to control sera193. However, a second research group could not demonstrate upregulation of prostasin mRNA in ovarian cancer tissues194. Along with this finding, an immunohistochemistry-based study concluded that there is a decreased number of prostasin-positive cells in malignant compared with normal ovary tissues, particularly when considering adenocarcinomas195. Hence, frequent prostasin expression was found to correlate with prolonged overall survival195. Still, elevation of prostasin in serum of ovarian cancer patients was recently confirmed in a large cohort196. In fact, among 259 candidate markers investigated, prostasin was found to be among the most informative in discriminating between benign and malignant ovarian tumors. Thus, a combination of the nine best markers identified in this study was found to perform better than the OVA1 panel, with a specificity of 88.9% for a 90% threshold196.

**Testisin**

Testisin, also known as PRSS21, was initially identified as a prostasin-related, testis-specific protein, whose expression is lost in testicular cancers197. Although it appears to be clinically relevant in a malignant context, little is known about its substrates and tumorigenic functions. In an ovarian cancer cell culture model, its down-regulation potentiates apoptosis and decreases colony formation; whereas in a xenograft mouse model, testisin overexpression induces larger primary ovarian tumors198. Hence, in cervical cancer cells examined in cell culture, testisin has been shown to interact with and inactivate the protease inhibitor maspin, a well-known tumor suppressor, thereby promoting cell growth, invasiveness and chemoresistance21. Moreover, it is also suspected to contribute to vessel morphogenesis and/or angiogenesis199.

Testisin is expressed at low levels or below the level of detection in the normal ovary and is ectopically expressed in malignant ovarian tumors199. Moreover, testisin mRNA levels are higher in carcinomas than in adenomas, and in advanced- versus early-stage carcinomas199. Testisin is also suspected to be upregulated in cervical cancers21. Conversely, using microarray-based screening, it has been found to be expressed at lower levels in metastatic versus primary ovarian tumors183.

**Other serine proteases and serine protease inhibitors**

1. **Other Ser-proteases**

In addition to the enzymes mentioned above, other Ser-proteases have also been considered for their involvement in and clinical relevance to gynecological malignancies. First, an association between cancer and thrombosis has been well established. While cancer patients are at a higher risk for thrombosis, thrombosis is also suspected to increase cancer occurrence and/or metastasis200. In addition, various coagulation Ser-proteases, including thrombin itself and several pro-/anti-coagulant factors, have been proposed to be differentially expressed in and to promote the course of gynecological malignancies201,202. Second to this, circulating inflammatory cells including macrophages, neutrophils, mast cells and lymphocytes, are recruited to and can modulate the tumor microenvironment203,204. These cells locally release Ser-proteases such as the mast cells tryptase and chymase, as well as lymphocytic granzyme B, and these in turn impact both malignant cells and the surrounding stroma. Accordingly, their contribution to tumor growth and metastasis, as well as their clinical relevance to gynecological cancers, has been investigated205–209.

Another remarkable example is provided by prosemin, a secreted epithelial protease also known as tryptase epsilon/PRSS22210. This trypsin-like enzyme is encoded by chromosome 16, a region where the genes for prostasin and testisin are also located. Prosemin, which activates pro-uPA, is overexpressed in an array of cancer cell lines in culture, including ovarian and endometrial107,210. Moreover, it has been found to be expressed at the apex of ovarian cancers, and has been detected in patient ascites as well210. These initial observations will require further investigation. Additional secreted or membrane-anchored Ser-proteases were excluded from the present review due to the fact that, although they are differentially expressed or activated in tumor tissue and thus contribute to tumorigenesis, they so far have not been investigated in gynecological cancer patient specimens.

**Protease inhibitors**

Besides tissue-specific expression, the activity of proteases is tightly regulated by proteinaceous inhibitors, of which nearly twice as many as proteases are present in humans. Often the well-balanced equilibrium of the protease-inhibitor interaction typically present under healthy conditions is seriously disturbed in cancer. As observed for proteases, the expression levels of certain Ser-protease inhibitors are altered in gynecological cancers. The tumor-associated trypsin inhibitor of the Kazal-type (SPINK1, clan IA, family I1) may serve as a valuable additional marker in cases of mucinous carcinomas and in combination with CA125211. A general increase in uPA and the plasminogen activator inhibitors PAI-1 and PAI-2, serpins that belong to clan ID and family I4, has been observed in invasive cervical carcinoma tissues140. Elevated PAI-1 protein levels, compared with healthy controls, were also detected in ovarian cancer tissues. Even higher PAI-1 levels were observed in metastases localized to the omentum and the lymph nodes. However, while some studies report an
association between increased PAI-1 expression levels and poor prognosis in ovarian cancer patients, other studies have found no significant association between PAI-1 and clinical outcome (reviewed in Schmitt et al.110). In endometrial cancer, elevated PAI-1 protein levels have been reported to be associated with poor patient prognosis110. Note that the paradoxical pro-tumoral functions of PAI are attributed to protease-independent properties that affect, for example, cell proliferation, adherence and/or survival212. Another member of the serpin family, maspin, which is predominantly localized inside tumor cells, is a marker of good prognosis for patients with gynecological cancers213. Recently, the relatively unspecific protease inhibitor α2-macroglobulin (clan IL, family I39) has been identified as a potential biomarker of breast and ovarian cancer214–216. The Kunitz-type inhibitor HAI-1 appears to act as a tumor suppressor by controlling the activity of matriptase and, concomitantly, progression of ovarian cancer168. The physiologically important matriptase/HAI-1 complex is depicted in Figure 3A. Moreover, the proteolytic activation cascade of matriptase, hepsin and prostasin is most likely regulated by HAI-1 expression, which is a favorable prognostic indicator in cervical cancer172. Similarly, HAI-1 and HAI-2 (LI02-005) appear to regulate HGF-induced invasion of human breast cancer cells217.

Aprotinin (BPTI, clan IB, family I2) has been investigated as an anti-tumor agent; however, it exhibits specific interactions with the protease only from the S2 to the S2’ subsites13,218 (Figure 3B). Nevertheless, engineering of the BPTI scaffold offers variants which display significantly improved specificities for protease targets219. Recently, several variants with the sunflower trypsin inhibitor (SFTI, clan IF, family I2) scaffold were engineered to target KLK4, KLK7 and matriptase220–222. Natural SFTI binds subsites S4 to S3’ of serine proteases as seen in the matriptase complex223. The low molecular weight and high bio-stability of this cyclic 14-residue peptide suggests a wide potential for pharmaceutical applications, since SFTI engineering with non-natural amino acids has been well established224,225. Application of SFTI-FCQR, which is specific for KLK4, showed reversion of KLK4-mediated paclitaxel resistance in ovarian cancer cells11. Serpins target proteases in a specific manner by binding from S4 to S3’. Based on phage-display data, engineered serpins α1-antichymotrypsin and α1-antitrypsin have been employed for the development of recombinant agents as inhibitors specific to human KLK2 and KLK14226,227. Since serpins such as α1-antitrypsin or the myxomavirus-derived serpin are promising therapeutic agents for various diseases, future applications as anti-cancer drugs can thus be expected as well228,229.

Figure 3. Matriptase and prostasin complexes with polypeptide inhibitors. Proteases and inhibitors are depicted as ribbons, with important residues shown as stick models. A: Matriptase (magenta) in complex with the physiological Kunitz-type inhibitor HAI-1 (white), which exhibits as major interaction a P1-Arg residue binding to D189 in the S1 subsite (PDB 4ISO). The scissile bond P1–P1’ close to the catalytic triad (D102, H57 and S195) remains intact. HAI-1 appears to be a rather specific inhibitor, with distinct interactions from the S3’ to the S4 subsite. For example, P3'-Phe and P2'-Ser in the S3’ and S2’ subsites, respectively. P2-Cys in the hydrophobic S2 pocket, bordered by H57 and F99, forms a disulfide bridge with another Cys of the inhibitor, while the P3-Arg occupies the S4 subsite, shaped by W215 and Q175. B: Prostasin (blue) in complex with BPTI (bovine aprotinin, white), another Kunitz-type inhibitor that binds with P1-Lys to the S1 pocket to D189 (3GYM). The catalytic triad residues D102, H57 and S195 are depicted and labeled. No specific interactions beyond P2’ and P2 are observed. Highly specific interactions comprise P2'-Arg, P1'-Ala, P1-Arg and P2-Cys in the subsites from S2’ to S2.
Conclusion
The identification of novel and effective biomarkers is crucial for the delineation of gynecological cancer patient subgroups and optimization of treatment in terms of both high effectiveness and low toxicity. In this context, a number of Ser- proteases have been highlighted as potential diagnostic and/or prognostic markers. Large scale, multicenter studies are now required to validate the utility of the most promising of these before they can be translated into routine clinical practice. Moreover, the use of multiplex mRNA- or protein-based arrays suitable for overall expression profiling will certainly contribute to further identification of valuable markers and marker panels. Further, one has to keep in mind that, besides their expression level, the enzymatic activity of proteases, which is tightly regulated by activators and/or inhibitors, is a major determinant of their function. Thus, the development of selective, activity-based probes represents a promising research field. Noteworthy pilot studies using activity-based probes aimed at detecting active KLKs in ovarian cancer patient ascites have recently been reported. Similarly, degradomics, which aims to identify the array of substrates targeted by a given protease, is another challenging field that will allow further understanding of the function of proteolytic enzymes. This approach has made significant progress within the last decade due to the fast development of mass spectrometry techniques. Finally, proteases in general and Ser-proteases in particular have received significant attention concerning their use as therapeutic interventions in oncology. An array of anti-tumoral protease inhibitors has been developed and a number of these have proved effective in animal models. Significantly, a synthetic uPA inhibitor has already been evaluated in some phase II clinical trials targeting patients with pancreatic or breast cancer. Moreover, protease-activated drugs that limit the impact of the highly toxic, anti-tumoral substances to the protease-rich environment of malignant cells also represent a promising focus for future research.

Declaration of interest
The authors report no declarations of interest.

References


Serine proteases in gynecological cancers


