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Assessment of kallikrein-related peptidases 4, 5, 7 and 12 as prognostic biomarkers in advanced high-grade serous ovarian cancer and triple-negative breast cancer

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For my family

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1 Introduction

1.1 Diagnosis, histopathology, treatment, and prognosis of ovarian cancer

Ovarian cancer is one of the most common malignancies and is the eighth leading cause of cancer-related death worldwide among women, followed by breast, lung, colorectum, cervix uteri, stomach, liver, and pancreas cancers (Bray et al., 2018). In 2018, 295,414 females were diagnosed with ovarian carcinoma and 184,799 patients died from this disease throughout the world (Bray et al., 2018). Globally, the highest age-standardized incidence rates of ovarian cancer were present in Europe (9.9 per 100,000), where it ranked as the fifth most commonly diagnosed carcinoma among women, followed by cancers of breast, colorectum, lung, and uterus (Ferlay et al., 2015).

Known factors leading an increased risk of developing ovarian cancer are familial genetic syndromes, pelvic inflammatory disease (borderline tumors), endometriosis, smoking, obesity, postmenopausal hormone therapy (particularly for more than five years), as well as older age at menopause and younger age at menarche. In contrast, long-term oral contraceptive use, pregnancy, and breastfeeding have been viewed as protective factors for this disease (Doubeni et al., 2016; Webb and Jordan, 2017; Lisio et al., 2019). There is evidence that ovarian cancer is most frequently diagnosed among women aged 55-64 (median age 63) and the highest death rate is among women aged 65-74 (median age 70) (Noone et al., 2018). Additionally, during the clinical practice, several biological and clinical variables have been identified as prognostic predictors for ovarian cancer patients, such as age, FIGO (the Féderation Internationale de Gynécologie et d'Obstétrique) stage, histological subtype, nuclear grade, residual tumor mass after debulking surgery, ascites fluid volume, and performance status (Berman, 2003).

According to the guidelines of FIGO and the TNM classification system (T: the primary tumor; N: regional lymph nodes; M: distant metastasis) (see **Appendix 9.1**), staging is determined at the time of initial diagnosis and serves as the major prognostic factor in ovarian cancer (Mutch and Prat, 2014). Patients afflicted with early-stage (FIGO I) ovarian cancer have a 5-year survival rate of up to 92.3%, which considerably drops to 29.2% for advanced stage (FIGO III/IV) patients (Noone et al., 2018). Unfortunately, only 14.9% of ovarian cancer patients are diagnosed at FIGO I, while the majority of

patients (up to 59%) present at advanced stages (FIGO III or IV), resulting in the overall poor prognosis and high mortality rate (Noone et al., 2018). Thus, early detection is the key to improve the rate of survival of ovarian cancer.

Nevertheless, early diagnosis of ovarian cancer is difficult, mainly due to the fact that the early stage of this disease is generally asymptomatic or presents nonspecific and/or low sensitive symptoms, such as abdominal pain/bloating, pelvic pain, constipation, fatigue, or urinary symptoms (Paulsen et al., 2005). To date, transvaginal ultrasonography and use of the blood assay for CA125 are major screening approaches for ovarian cancer in the clinical practice. However, both methods present limited sensitivity and specificity for early detection of this disease. For example, these two screening strategies failed to diminish the mortality risk in a U.S. clinical trial (Buys et al., 2011). Moreover, pelvic examination is also recommended as a screening strategy for early detection of ovarian cancer. Similarly, up to now, no study presented that pelvic exam could provide a benefit for ovarian cancer morbidity or mortality (Guirguis-Blake et al., 2017). Therefore, it is not surprising that the mortality of this gynecologic malignancy decreased only slightly over the past forty years (Kurman, 2013). Based on this, effective screening strategies for the early diagnosis of ovarian cancer are in demand.

After diagnosis, the following treatment options are recommended for the ovarian cancer patients. The present cutting-edge treatment modalities comprise surgery, platinum-/taxane-based chemotherapy, sometimes plus radiotherapy, depending upon tumor stage and the extent of surgical debulking (Jelovac and Armstrong, 2011; Sundar et al., 2015). Comprehensive staging laparotomy followed by platinum-containing combined chemotherapy is potentially curative for women with early-stage ovarian cancer, *i.e*. the disease is confined to ovaries (FIGO I) or pelvis (FIGO II). Numerous retrospective studies and meta-analyses have indicated that the amount of post-operative residual tumor mass is the strongest independent prognostic factor (Burges and Schmalfeldt, 2011; Dorn et al., 2015). Furthermore, the survival of ovarian cancer patient has been proven to benefit from increasing surgical debulking rates (Aletti et al., 2006; Chi et al., 2006; Winter et al., 2007; Chi et al., 2009; Du Bois et al., 2009; Elattar et al., 2011). Additionally, it has been shown that late-stage ovarian cancer patients with a small volume of residual tumor mass after primary surgery, followed by systemic

chemotherapy, have a better outcome than those with a larger volume of residual tumor mass after initial surgery (Pölcher et al., 2014). Thus, for the subset of ovarian cancer patients with advanced stages, surgical cytoreduction aims to reach maximal resection of all visible tumors (Aebi and Castiglione, 2009; Burges and Schmalfeldt, 2011; Pölcher et al., 2014). Based on this, for more than 30 years, the maximum debulking surgery following platinum-/taxane-based chemotherapy has been acknowledged as the standard therapeutic management of advanced ovarian cancer (Kim et al., 2012). Meantime, many antitumor agents, involving angiogenesis inhibitors and molecular-targeted agents, or combined treatment schemes have been investigated in clinical trials. However, both therapeutic options have not obviously improved the overall survival rate of this lethal gynecologic malignancy (Davis et al., 2014).

Ovarian cancer is a heterogeneous disease encompassing diverse subtypes, with widely differing morphology, molecular-genetic features, and clinical behavior (**Table 1**). Morphologically, ovarian cancer is broadly categorized into epithelial ovarian cancer (EOC) and non-epithelial ovarian cancer (NEOC) (Kim et al., 2018). Only a small fraction (2%) of ovarian cancer cases are NEOC, including two types: germ-cell tumors and sex cord-stromal tumors (Koulouris and Penson, 2009; Boussios et al., 2016; Ray-Coquard et al., 2018). The majority (98%) of ovarian cancer are EOC (Bast et al., 2009; Jayson et al., 2014), containing four well-defined histological subtypes: serous (75%), endometrioid (10%), clear cell (10%) and mucinous carcinomas (3%) (Prat, 2014; Sundar et al., 2015). Based on histopathology and molecular genetic changes, EOC, which comprises heterogeneity of clinicopathologic features and behavior, is subdivided into type I and type II (Kurman et al., 2014). The type I tumor category, accounting for 25% of EOC, comprises subtypes of low-grade serous, low-grade endometrioid, clear-cell, mucinous, and transitional cell (Brenner) carcinomas (Jayson et al., 2014; Au et al., 2015). These neoplasms mostly derive from pre-malignant or borderline lesions and are characterized by slow tumor growth (Shih and Kurman, 2004; Jones and Drapkin, 2013; Kurman and Shih, 2016). Moreover, the type I ovarian tumors are genetically stable, harbouring somatic mutations of genes encoding protein kinases, such as KRAS, BRAF, CTNNB1, and PIK3CA (Shih and Kurman, 2004; Jones and Drapkin, 2013; Kurman and Shih, 2016), but not TP53 or BRCA (Bashashati et al., 2013). In contrast, the type II ovarian tumors are depicted as high-grade, more

aggressive, and genetically unstable. They are predominantly diagnosed at an advanced stage (FIGO stage III or IV: 60–80%), resulting in poor outcome (Seidman et al., 2004; Kurman and Shih, 2010; Lengyel, 2010; Peres et al., 2019). The type II ovarian cancer comprises high-grade serous, high-grade endometrioid, undifferentiated carcinomas, as well as malignant mixed-mesodermal tumors (Kurman and Shih, 2008; Lim and Oliva, 2013). High-grade serous ovarian cancer (HGSOC) is the prototype of type II ovarian neoplasms with a high-frequency TP53 mutations (>80% of cases) (Cho and Shih, 2009), representing about 75% of all EOCs (Karst and Drapkin, 2010; Jayson et al., 2014) and accounting for a large majority (70–80%) of deaths from all ovarian cancer (Bowtell et al., 2015; Kurman and Shih, 2016). Upon that, the present study focused on this most common and lethal subtype of ovarian cancer.

Table 1. Histopathological subtypes of ovarian cancer

Ovarian cancer	Subtype	
NEOC (2%)	germ-cell tumors sex cord-stromal tumors	
EOC (98%)	Type I (25%)	low-grade serous tumors low-grade endometrioid tumors clear-cell tumors mucinous tumors transitional cell (Brenner) tumors
	Type II (75%)	high-grade serous tumors high-grade endometrioid tumors undifferentiated carcinomas tumors malignant mixed-mesodermal tumors

NEOC: non-epithelial ovarian cancer; EOC: epithelial ovarian cancer.

Due to the heterogeneity of distinct subtypes, the lack of obvious symptoms in early stage, the inefficient primary debulking surgery and the rapid development of chemo-resistance, the majority of patients are diagnosed at advanced stages and have a high mortality rate (Jemal et al., 2011; Pölcher et al., 2014). The present treatment for patients afflicted with ovarian cancer is based on traditional cancer factors, such as tumor stage and reductive surgery. Moreover, most of the tumor biomarkers are not cancer and/or tumor-type specific for ovarian cancer detection, diagnosis, and

prognosis. Therefore, new specific tumor biomarkers for different subgroups of ovarian cancer to predict the course of disease and therapy response are urgently needed.

1.2 Diagnosis, histopathology, treatment, and prognosis of breast cancer

Globally, breast cancer is the most frequently diagnosed carcinoma and the leading cause of cancer-related death among females (Bray et al., 2018). In 2018, approximately 2.1 million new cases were diagnosed with breast cancer worldwide and 626,679 patients died from this disease (Bray et al., 2018). Numerous environmental and lifestyle factors have been recognized to increase the risk of breast cancer. In the clinical practice, more than 80% breast cancer cases are diagnosed among women aged 50 or older (Kamińska et al., 2015) and accordingly age is documented as one of the most important risk factors (Justo et al., 2013; Sun et al., 2017). Other known risk factors include gender, family history, hormone therapy, physical inactivity, alcohol, smoking, overweight and obesity (Kolak et al., 2017). Moreover, reproductive factors also increase the possibility of breast cancer, such as delayed childbearing, early menarche, and late menopause (Jerônimo et al., 2017). Additionally, various gene mutations and abnormal gene amplifications have been determined to facilitate the initiation and progression of breast cancer, *e.g.* BRCA 1/2, c-Myc, and the Ras gene family (Jerônimo et al., 2017).

The mortality rate of breast cancer has been reduced in the past 30 years due to earlier detection and improved treatment options (Berry et al., 2005). The screening methodologies commonly used for breast cancer detection include breast self-examination, mammography, ultrasonography, magnetic resonance imaging, and blood testing of tumor markers (Kolak et al., 2017; Akram et al., 2017). Mammography, which has been proven to decrease breast cancer mortality, is regarded as the mainstay of breast cancer imaging strategies, showing high sensitivity (77-95%) and excellent specificity (94-97%) (Humphrey et al., 2002; Berry et al., 2005). Furthermore, the addition of digital breast tomosynthesis was found to again remarkably increase the cancer detection rate and to diminish the rate of false-positive cases (Friedewald et al., 2014).

Besides the screening strategies, the continuous improvement of breast cancer therapies is another key factor to reduce the mortality rate. In breast cancer management, surgery

is the foremost treatment for patients without distant metastasis, including lumpectomy (breast-conserving surgery), mastectomy as well as reconstructive surgery. Moreover, surgery reveals the histologic grade and tumor stage, which both play crucial roles in the prediction of patient prognosis (Akram et al., 2017; Martei et al., 2018). Additionally, systemic therapy is also indispensable, including chemotherapy (neoadjuvant/adjuvant), endocrine therapy, and HER2-directed therapy (Harbeck and Gnant, 2017). Neoadjuvant chemotherapy is frequently applied to increase possibilities of breast conservation for females with the following indications: larger tumor size (> 5cm), tumor fixed to the chest wall, locally advanced disease, and inflammatory breast cancer (McDonald et al., 2016). Furthermore, postoperative radiation therapy has been verified to be beneficial by randomized trials, showing an improvement in local-regional control and reduction in the recurrence and mortality rates in this tumor entity (Correa et al., 2010; Darby et al., 2011; McCormick et al., 2015).

However, these managements are not always efficacious owing to the fact that breast cancer is a very heterogeneous disease comprising numerous subtypes, which are distinct in biology, molecular features, clinical behaviors, treatment sensitivity and prognosis (Perou et al., 2000; Geyer et al., 2009). Breast cancer is clinically categorized according to the TNM-staging system (see **Appendix 9.2**), which is based on the primary tumor size (T), regional lymph node status (N), and distant metastasis (M) (Hortobagyi et al., 2017) (www. cancerstaging.org). Histologically, breast cancer can be classified into ductal carcinoma and lobular carcinoma, which are the two major subtypes and make up 80% of malignant breast tumors (Akram et al., 2017). The very rare subtypes (20%) include medullary carcinoma, mucinous carcinoma, tubular carcinoma, inflammatory breast cancer and Paget's disease of the breast (Akram et al., 2017).

Several pathological markers have been extensively studied to characterize molecular subtypes of breast cancer, including estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and the tumor proliferation factor Ki67, which are clinically used to guide therapeutic decisions and predict patient outcome. ER and PR belong to the nuclear receptor superfamily and the abnormal expression of both receptors has been implicated in breast cancer tumorigenesis and development (Lanari and Molinolo, 2002). Overexpression of HER2 has been observed

in approximately 15-30% of breast cancer cases and associated with an increased risk of relapse and shortened survival (Moasser, 2007; Ross et al., 2009; Krishnamurti and Silverman, 2014).

Additionally, it has been recognized that expression patterns of tumor-related genes are associated with breast cancer progression through modulation of the cell cycle, invasion, proliferation, and angiogenesis (Perou et al., 2000; Van't Veer et al., 2002). Thus, the molecular classification of breast cancer, which is more efficacious than the traditional TNM classification system, was established in daily clinical practice for planning patient-tailored therapy treatment (Sørlie et al., 2001; Merino et al., 2017). Based on the above-named immunochemistry markers, breast cancer is categorized into four subtypes (**Table 2**), including luminal A, luminal B, HER2-enriched and triple-negative breast cancer (TNBC) (Senkus et al., 2015; Merino et al., 2017).

Table 2. Molecular subtypes of breast cancer and recommended therapies

Subtype		**Pathological markers**	**Recommended therapies**
Luminal A		ER positive HER2 negative Ki67 low PR high (> 20%) low-risk molecular signature (if available)	endocrine therapy alone in the majority of patients; chemotherapy for cases with high-risk factors (high tumor burden; grade 3)
Luminal B	**HER2-negative**	ER positive HER2 negative Ki67 high or PR low (≤ 20%) high-risk molecular signature (if available)	endocrine therapy plus chemotherapy for the majority of patients
	HER2-positive	ER positive HER2 positive any Ki67 any PR	endocrine therapy plus chemotherapy plus HER2 target therapy for all patients
HER2-enriched		HER2-positive ER and PR absent	chemotherapy plus HER2 target therapy
Triple-negative		ER and PR absent HER2 negative	chemotherapy

ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor 2 receptor. Adapted from Senkus et al. 2015 and Merino et al. 2017.

Luminal A accounts for almost 50% of all breast cancers and is normally of low histological grade, having the most favorable prognosis with 5-year survival rates of 80% (Sørlie et al., 2001; Trop et al., 2014). The incidence of luminal B is around 15%, with 5-year survival rates of 40% (Sørlie et al., 2001; Trop et al., 2014). According to the current treatment guidelines for all luminal subtypes with hormone receptor-positive, the standard treatment is 5-years adjuvant endocrine therapy as monotherapy or after chemotherapy, including selective estrogen receptor modulators (e.g. tamoxifen) and aromatase inhibitors (e.g. anastrozole) (Harbeck and Gnant, 2017). The HER2-enriched subtype, 15-30% of all breast tumors (Wang et al., 2008), is usually of high or intermediate histological grade and has a relative unfavorable outcome (Youk et al., 2012). Before introduction of HER2-targeting therapy, 5-year survival rates of this subtype were around 31% (Sørlie et al., 2001). Anti-HER2 treatment, such as the monoclonal antibody trastuzumab (Herceptin®) and the tyrosine kinase inhibitor lapatinib (Tykerb®), decreases the risk of relapse as well as tumor-induced death and improves 5-year survival rates up to 90% (Brown-Glaberman et al., 2014). Moreover, the combination of anti-HER2 treatment and chemotherapy is recommended as the first-line treatment for breast cancer patients with HER2-overexpression (Romond et al., 2005; Harbeck and Gnant, 2017).

TNBC accounts for up to 17% of primary breast cancers and is more commonly found in females younger than 50 years old (Foulkes et al., 2010). Histologically, the common features of TNBC comprise high nuclear grade, increased mitotic activity, central necrosis, enhanced tumor proliferation rate, as well as conspicuous lymphocytic infiltrate and fibrosis (Livasy et al., 2006; Elsawaf and Sinn, 2011). Clinically, TNBC shows a more aggressive phenotype with large tumor size, axillary infiltration and frequent visceral metastases (Choi et al., 2011; Park et al., 2012). Patients with TNBC have an elevated risk of recurrence during the first 1-3 years after diagnosis and an increased 5-year survival rate, compared to patients with other molecular subtypes (Dent et al., 2007; Pogoda et al., 2013). Last but not least, only about 10% of TNBC patients are diagnosed at an early stage (grade I, T1N0) (Badve et al., 2011), which also contributes to the poor prognosis (Kaplan et al., 2009).

Furthermore, unlike ER/PR positive or HER2-overexpressing breast carcinomas, TNBC is characterized by a negative profile of ER, PR, and HER2, suggesting that the

available anti-targeted therapy has very limited or no impact on this entity. As shown in **Table 2**, besides surgery and radiotherapy, anthracycline-taxane-based chemotherapy is the only option for TNBC patients (Foulkes et al., 2010). Therefore, patients with TNBC have the worst outcome among all breast cancer subtypes (Foulkes et al., 2010). Thus, the identification of new biomarkers for the improvement of the therapeutic management of patients with TNBC is in demand.

1.3 Kallikrein-related peptidases (KLKs)

The original definition of kallikreins included two serine proteinases, plasma kallikrein (KLKB1) and tissue kallikrein (KLK1), responsible for proteolytically releasing kinin peptides from kininogens (Werle et al., 1969). KLKB1 differs from KLK1 in that it spans 15 exons and 14 introns and is located on chromosome 4q34-35 (Beaubien et al., 1991; Bhoola et al., 1992; Sainz et al., 2007). Furthermore, KLKB1 is generated by the liver and is then secreted into the blood circulation system, where it is involved in activating clotting, fibrinolysis, and inflammation (Lawrence et al., 2010). As the founding member of the kallikrein-related peptidase family, KLK1 was discovered from pancreatic extracts already in the 1930s and was found to have kininogenase activity (Kraut et al., 1930; Werle et al., 1969). Subsequently, 14 orthologic kallikrein genes (KLK2-15) were identified, encoding serine proteases with conserved structures. However, unlike KLK1, KLK2-15 lack the enzyme activity to release kinin peptides from kininogens (Lundwall et al., 2006). Therefore, they were formally named kallikrein-related peptidases.

The KLK gene family, located on the long arm of human chromosome 19q13.4 (**Figure 1A**), is the largest contiguous cluster of serine proteases in the human genome (Prassas et al., 2015). This gene family is composed of 15 members, which are tightly arrayed in a tandem cluster without intervention of non-KLK gene. All 15 members share distinct sequence similarity (**Figure 2**): KLK1-3 share 61-79% sequence similarity to each other, while for KLK4-15 the similarity is 38-57% (Goettig et al., 2010). Based on that, KLK1-3 were named the classical KLKs, while KLK4-15 were the new KLKs. Furthermore, all KLK genes share many common genomic features (**Figure 1B**): all contain 5' and 3'- untranslated region (UTR), five coding exons and four conserved introns (intron phases: I, II, I, 0) (Dong et al., 2014). Most KLK genes have one or two additional non-coding exons in the 5'UTR. Except for KLK2 and KLK3, the direction

of transcription of all KLK genes is from telomere to centromere (Paliouras and Diamandis, 2006). The coding exon 1 harbors the start codon and 5'UTR, while the termination codon and 3'UTR are in the coding exon 5. The codons for the three amino acids of the serine-type catalytic triad, histidine (His), aspartic (Asp), and serine (Ser), are highly conserved and located in coding exons 2, 3 and 5, respectively (Clements et al., 2001; Lawrence et al., 2010). In contrast to the coding exons, the untranslated regions of KLKs are more variable in length (ranging from 4.3 to 10.5 kb) and sequences (Kryza et al., 2016). To date, approximately 80 alternative mRNA splicing variants, which encode structurally and functionally unique proteins, have been described for KLKs (Lai et al., 2016).

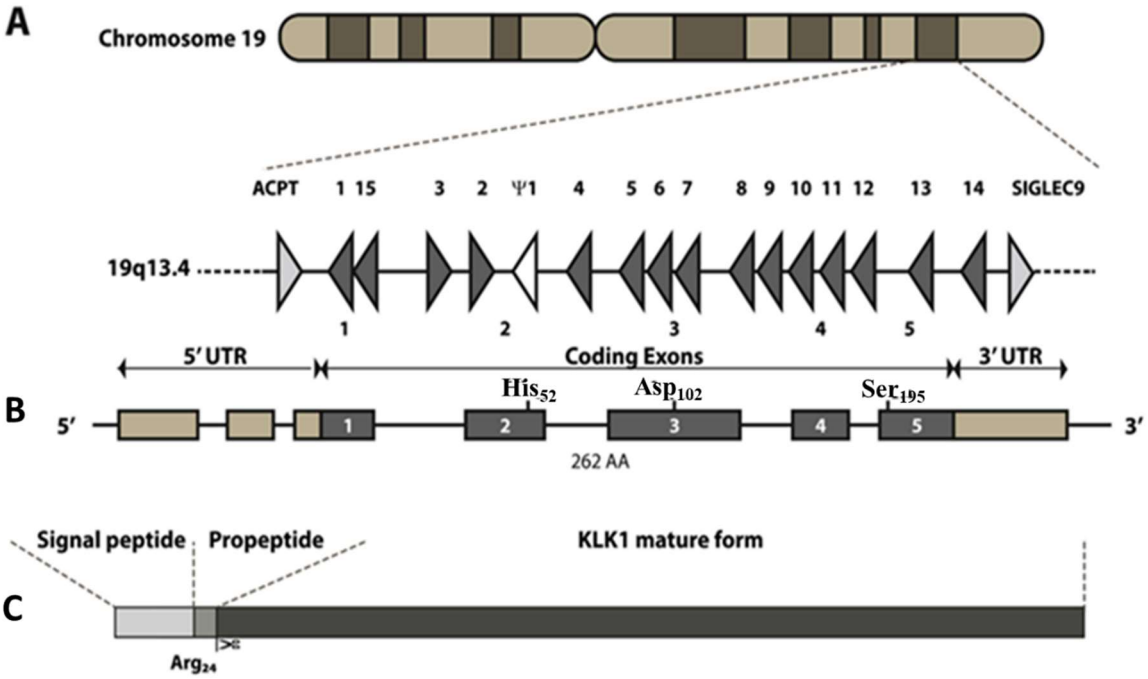

Figure 1. Gene, mRNA, and protein characteristics of KLKs

A. The KLK gene family is located on chromosome 19q13.4 and flanked by the genes encoding testicular acid phosphatase (ACPT) and sialic acid-binding Ig-like lectin 9 (SIGLEC9). It encompasses 15 members and representing the largest contiguous cluster of serine proteases in the human genome. **B**. Generally, KLKs contain 5 coding exons (dark gray boxes), where exon 2 carries catalytic His52, exon 3 carries Asp102, and exon 5 carries Ser195, as exemplified here by KLK1 mRNA. **C**. KLKs are firstly synthesized as pre-pro-enzymes comprising a signal peptide (pre-), a pro-peptide (pro-) and a serine protease domain responsible for the catalytic activity. The figure is taken and adapted from Kalinska et al. 2016.

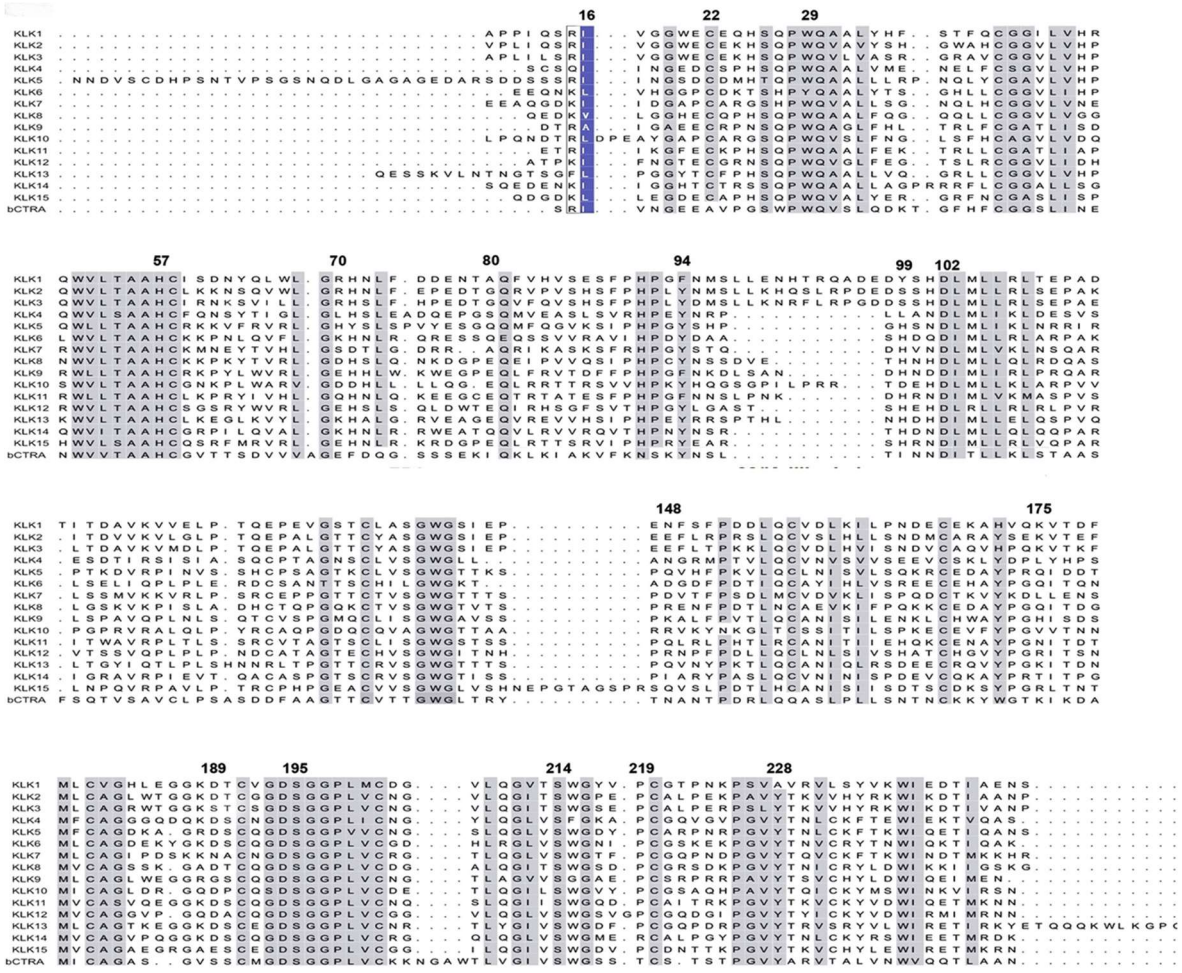

Figure 2. Sequence alignment of human KLKs with bovine chymotrypsin (bCTRA)

All 15 members share a great similarity in sequence. The classical KLKs (KLK1-3) share 61-79% sequence similarity to each other, while the similarity for the new KLKs (KLK4-15) is 38-57%. Highly conserved residues are shown with a grey background. Residue 16 position when the propeptide is cleaved off is shown with a blue background. The figure is taken and adapted from Goettig et al., 2010.

Human KLK gene expression is modulated by numerous factors, e.g. steroid hormones, which influence various signaling pathways (Shaw and Diamandis, 2008). For example, KLK4 is sensitive to androgen in prostate (Nelson et al., 1999) and breast cancer cell lines (Yousef et al., 1999) and to estrogen in endometrial cell lines (Myers and Clements, 2001). KLK12 is responsive to androgen and progestin in prostate cancer cell lines and to estrogen as well as progestin in breast cancer cell lines (Yousef et al., 2000). Furthermore, it is highlighted that single nucleotide polymorphisms (SNPs) are

involved in modulation of KLKs expression and the proteolytic activity of KLK proteases (Lai et al., 2007). Moreover, epigenetic-related mechanisms, like histone modification, DNA methylation, and miRNA-mediated control of mRNA levels, have been demonstrated to affect the transcriptional and post-transcriptional regulation of KLKs (Pasic et al., 2012; Samaan et al., 2014; Pasic et al., 2015).

Based on the structural organization and proteolytic mechanism, human KLKs belong to the chymotrypsin-like S1 family of serine peptidases and are secreted as trypsin- or chymotrypsin-like serine endopeptidases of around 25-30 kDa (Lawrence et al., 2010). KLKs are firstly synthesized as single-chain pre-pro-enzymes comprising three domains (**Figure 1C**): (i) the N-terminal signal peptide (pre-domain) with 16-33 amino acids, directing pre-pro-enzymes into the endoplasmic reticulum for secretion; (ii) the pro-peptide (pro-domain) with 3-37 amino acids, rendering them as inactive precursors; (iii) the KLK core domain (mature form) with 227-252 amino acids, containing the invariant catalytic triad (His, Asp, Ser), responsible for the catalytic activity (Avgeris and Scorilas, 2016). After activation, the majority of KLKs (KLK1-2, KLK4-6, KLK8, and KLK10-15) exhibit a trypsin-like activity, while KLK3, KLK7, and KLK9 possess a chymotrypsin-like activity with regard to substrate specificity (Lawrence et al., 2010).

The activity of KLKs is modulated by multiple mechanisms and factors, comprising zymogen activation cascades, endogenous KLK inhibitors and micro-environmental pH (Goettig et al., 2010). Activation of pro-KLKs is a crucial process with regard to modulating active KLK levels in human tissues (Yousef and Diamandis, 2002). Guided by the single peptide, the newly synthesized inactive pre-pro-KLKs are secreted into the extracellular space after, in most cases, being glycosylated in the endoplasmic reticulum and Golgi apparatus (Guo et al., 2014). For activation and conversion to the mature forms, the propeptides of pro-KLKs have to be proteolytically removed (Kryza et al., 2016). This step is achieved by a trypsin-like cleavage after an arginine or a lysine residue for all KLKs, with the exception of KLK4 which is activated after a glutamine residue (Kalinska et al., 2016; Kryza et al., 2016). The pro-KLK-activating proteinases comprise plasmin, plasma kallikrein, urokinase-type plasminogen activator (uPA), factor Xa, thrombin, and matrix metalloproteinases (MMPs) (Yoon et al., 2008, 2013). Furthermore, Yoon et al. (2007) have identified the interaction profiles of KLKs, which may lead to proteolytic cascades as seen in the skin for KLK5, 7, 14 and others (Prassas

et al., 2015). In addition, KLK2, KLK6, and KLK13 have been reported to be autocatalytically activated (Yoon et al., 2007).

The human tissue proteome data, based on an integrated omics approach, shows that KLKs are expressed with distinct expression profiles in diverse human tissues (Uhlén et al., 2015; Loessner et al., 2018). For example. KLK1 is highly expressed in kidney and pancreas (Lawrence et al., 2010), whereas KLK2 and KLK3 are two of the most abundant proteins in the prostate (Stanbrough et al., 2006). Nevertheless, none of KLKs is completely tissue-specific and different KLKs are usually observed to be expressed in the same tissues with distinct expression levels (Lawrence et al., 2010). Intriguingly, many KLKs are co-expressed in the same tissues, e.g. KLK2-4 show parallel expression pattern in the prostate, suggesting that these KLKs may be involved in enzymatic cascade reactions (Lawrence et al., 2010).

KLKs are widely expressed in a variety of human tissues and are secreted into majority of physiological fluids (Kalinska et al., 2016), indicating that KLKs broadly participate in physiologic and pathological processes. Indeed, multiple studies have revealed the well-characterized roles of KLKs in various mechanistic pathways, such as the roles of KLK5 and KLK7 in desquamation of the skin (Yamasaki et al., 2006; Lundwall and Brattsand, 2008), of KLK3 in semen liquefaction (Pampalakis and Sotiropoulou, 2007), of KLK4 in the formation of tooth enamel (Bartlett, 2013) and of KLK6 in neuroinflammation (Ashby et al., 2010).

1.4 Kallikrein-related peptidases in carcinoma

In tumor research, the KLK family became more well-known, after KLK3 was shown to be remarkably associated with prostate carcinoma (Catalona et al., 1994). KLK3, also known as prostate-specific antigen (PSA), is a powerful biomarker for diagnosis and motility in prostate cancer. Therefore, expression of other KLK family members was extensively studied to determine their possible functions in neoplastic diseases. Indeed, nearly all KLKs have been found to be dysregulated in various cancer types, like lung, prostate, breast, ovarian and gastric cancer (Clements et al., 2004; Avgeris et al., 2012; Dorn et al., 2014; Scorilas and Mavridis, 2014). Moreover, KLKs have been shown to be involved in cancer progression (Sotiropoulou et al., 2009; Fuhrman-Luck et al., 2014; Yu et al., 2014). Previous investigations have shown that KLKs, as secreted

peptidases, could modulate various molecular signaling pathways in the tumor microenvironment, cleaving growth factors, cell surface receptors, and/or extracellular matrix (ECM) proteins. Thus, KLKs can positively or negatively influence tumor growth, metastasis, invasion, and chemoresistance (Lawrence et al., 2010; Kryza et al., 2016).

1.4.1 Roles of KLKs in tumor growth

As mentioned above, KLKs have been shown to regulate tumor growth through modulating diverse signaling cascade pathways. The protease-activated receptor (PAR) family (PAR1-4) has been demonstrated to accelerate multiple intracellular signaling networks, thus contributing to a number of physiological and pathological processes (Gieseler et al., 2013). Previous studies found that several KLKs could initiate trans-membrane signal transduction by activating PARs via proteolytic cleavage of the extracellular N-terminus, thus revealing a tethered ligand initiating cell signaling (Yu et al., 2014). For example, KLK14 could promote proliferation of colon cancer cells by initiating PAR2 and the downstream mitogen-activated protein kinase (MAPK) pathway (Gratio et al., 2011; Chung et al., 2012). In prostate cancer cells, KLK2 is able to activate PAR1 by modulating the extracellular signal-regulated kinase signaling, while KLK4 modulates both PAR1 and PAR2 signaling pathways and facilitates tumor growth (Mize et al., 2008; Ramsay et al., 2008). In addition, KLK6 was shown to stimulate cell proliferation and to reduce apoptosis in non-small lung cancer, through modulating the transactivation mechanism of PAR2-dependent epidermal growth factor receptor (EGFR) (Michel et al., 2014).

Besides, accumulating evidence suggests a proliferative effect of KLKs in regulation of the hormone-associated signaling pathway. In prostate cancer, KLK2 (Shang et al., 2014) and KLK3 (Niu et al., 2008) were reported to induce androgen receptor (AR) activation to facilitate cell proliferation. Also, enhanced expression of KLK4 (Lai et al., 2014) and KLK14 (Lose et al., 2012) could modulate AR activation, via cleaving sex hormone-binding globulin, to promote prostate cancer growth.

Moreover, KLKs may affect tumorigenesis by modulating activity and bioavailability of growth factors, which are essential for tumor progression. KLK1-5, KLK11, and KLK14 were reported to directly degrade insulin-like growth factor binding proteins

(IGFBPs), thus accelerating the release of insulin-like growth factor (IGF) and increasing the availability of IGF and the interaction with IGFBPs (Michael et al., 2006; Samani et al., 2007; Sano et al., 2007). This process reduces the anti-apoptotic effects of IGFs on tumor cells and the tumor suppression activity of IGFBPs, thereby resulting in tumor proliferation. Additionally, bioavailability of growth factors is modulated by KLKs through cleavage of the extracellular matrix (ECM) proteins, which are involved in sequestration of growth factors, such as fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor-beta (TGF-ß) (Lawrence et al., 2010). Furthermore, some members of the KLK family, e.g. KLK4 and KLK5, can indirectly activate the hepatocyte growth factor/scatter factor (HGF/SF) pathway, a growth factor-associated signaling pathway, effecting cancer progression (Mukai et al., 2008, 2015).

Nevertheless, it has been reported that several KLKs have a tumor suppressive effect in carcinomas, *e.g.* elevated expression of KLK4 decreases proliferation in the prostate cancer cells (Veveris-Lowe et al., 2005). Besides, *in vivo* and *in vitro* studies have demonstrated that KLK10 inhibits cell proliferation in different cancer types, such as prostate (White et al., 2012), ovarian (White et al., 2010) and tongue cancer (Zheng et al., 2012).

1.4.2 Roles of KLKs in tumor migration and invasion

KLKs have been reported to be implicated in cell migration and invasion in malignancies, including lung (Chou et al., 2011), breast (Pampalakis et al., 2009), colon (Kim et al., 2011), pancreatic (Ramani et al., 2008), ovarian (Dong et al., 2013) and prostate (Mo et al., 2010) cancer. This might be due to the fact that KLKs mediate degradation of ECM barriers, which is essential for the dissemination of cancer cells (Kryza et al., 2016). Indeed, increasing evidence suggests that KLKs are able to degrade the major components of ECM, including fibronectin, vitronectin, collagens, and laminin (Lawrence et al., 2010). For example, elevated KLK13 expression in lung cancer induces laminin degradation and N-cadherin expression, thus facilitating tumor cell motility and metastasis (Chou et al., 2011). Besides, in the colon cancer cell line Caco2, KLK6 was shown to contribute to KRAS-dependent invasion and migration through laminin and the basement membrane-like substrate Matrigel (Henkhaus et al., 2008).

Moreover, KLKs are involved in cell migration and invasion also via the proteolytic processing of cell adhesion and cell-cell cohesion proteins, such as E-cadherin, corneodesmosin, desmocollin, and desmoglein 1/2 (Lawrence et al., 2010). In prostate cancer cells, KLK3 and KLK4 have been demonstrated to decrease expression of the tumor suppressor E-cadherin, thus promoting tumor cell aggressiveness and invasiveness (Veveris-Lowe et al., 2005; Canel et al., 2013). Additionally, KLKs have been shown to regulate the process of epithelial-to-mesenchymal transition (EMT), where epithelial cells transform into mesenchymal-like cells, stimulating cell motility (Lawrence et al., 2007). KLK7 has been observed to promote the EMT-like phenotype in prostate tumor cells, as evidenced by the upregulation of the mesenchymal marker vimentin (Mo et al., 2010). Last but not least, KLKs may indirectly influence the migratory and invasive potential of cancer cells by modulating other molecular signaling pathways, such as PARs (Gao et al., 2010), MMPs (Ashby et al., 2010) and uPA-uPAR system (Beaufort et al., 2006). Inhibition of KLK14 by small interfering RNA (siRNA) in ovarian cancer cells was shown to suppress cell growth and invasion through down-regulating MMP2 expression and up-regulating the expression of caspase 9 and cleaved caspase 3 (Zhang et al., 2012).

On the contrary, several KLKs have been shown to inhibit migration and invasion of tumor cells. Unlike in lung cancer, overexpression of KLK13 in oral squamous cell carcinoma cells was suggested as a tumor suppressor, reducing cell invasive potential and inducing the expression of adhesion molecules (Ishige et al., 2014). Also, overexpression of KLK8 was found to suppress cell proliferation and invasion in the lung adenocarcinoma cell line CL1-5, which might due to cleavage of fibronectin by KLK8, which can inhibit tumor cell motility by retarding actin polymerization (Sher et al., 2006).

1.4.3 Roles of KLKs in tumor chemo-resistance

A growing number of evidence indicates that KLKs might influence the efficacy of chemotherapies. In the ovarian cancer cell lines OVCA432 and SKOV-3, KLK4 overexpression was associated with paclitaxel resistance by inducing the formation of multicellular aggregates (MCA), which could stimulate cell survival and chemoresistance (Dong et al., 2013). This effect was inhibited by KLK4-blocking antibodies or the selective active site KLK4 sunflower trypsin inhibitor (SFTI-FCQR)

(Dong et al., 2013). Furthermore, KLK7 has been reported to be associated with resistance to paclitaxel and tumor metastasis via enhancing the MCA formation and α5/β1integrin–dependent cell adhesion in ovarian cancer cells (Dong et al., 2010). Moreover, combined overexpression of KLK4-7 increased the insensitivity to paclitaxel in ovarian cancer cells by reducing the expression of α5β1/αvβ3 integrins (Loessner et al., 2012). Accordingly, this effect was shown to be abrogated via inhibition of KLK4-7 or MAPK in a spheroid-based animal model (Loessner et al., 2013). Besides, modulation of transforming growth factor-β (TGF-β) in cycling rates and/or tumor microenvironments was found to be responsible for tumor heterogeneity and ultimately affect chemoresistance in squamous cell cancer stem cells (Oshimori et al., 2015). Furthermore, inhibition of TGF-β can promote chemotherapy action against TNBC (Bhola et al., 2013). This may explain that the overexpression of KLK4-7 in ovarian cancer cells promotes resistance to paclitaxel and invasion in a xenograft model by stimulating the expression of TGF-β (Shahinian et al., 2014).

Conversely, some studies have suggested that overexpression of KLKs is potentially modulated by chemotherapeutic agents in tumor cells. For example, in prostate cancer cells, KLK5 mRNA expression levels were found to be up-regulated after treatment with etoposide, doxorubicin, and carboplatin, while KLK11 mRNA expression was increased following chemotherapy with mitoxantrone (Thomadaki et al., 2009). In BT-20 breast cancer cells, KLK4 and KLK5 mRNA expression levels were shown to be decreased after treatment with epirubicin, docetaxel, and methotrexate, while KLK14 mRNA expression was increased in epirubicin- and reduced in methotrexate-treated cells (Papachristopoulou et al., 2013).

1.4.4 Roles of KLKs in tumor angiogenesis

Angiogenesis, the growth of new blood vessels in tissues, is considered as an important hallmark of neoplasm, contributing to tumor growth, invasion and distant metastasis (Avgeris et al., 2012). KLKs promote key regulatory mechanisms in this process directly via diminishing ECM proteins or indirectly via activating uPA- and MMPs-related cascades in the tumor microenvironment (Avgeris et al., 2012). Overexpression of KLK1 in endothelial progenitor cells was found to improve the secretion of vascular endothelial growth factor (VEGF) and to enhance neo-angiogenesis (Kryza et al., 2016). Furthermore, exogenous recombinant KLK1 was shown to induce invasive and

proangiogenic activities of proangiogenic cells by kinin receptors B2 (B2R)- and MMP2- mediated mechanisms (Spinetti et al., 2011), further suggesting an important role of KLK1 in angiogenesis. Moreover, KLK3 has been observed to stimulate angiogenesis through activating the proangiogenic factor TGFβ2 (Dallas et al., 2005). Besides, KLK12 was reported to enhance cancer cell proliferation and pro-angiogenic activity by indirectly modulating several growth factors, such as the bone morphogenic protein (BMP) 2, TGFβ and VEGF (Guillon-Munos et al., 2011).

Nevertheless, some members of the KLK family, including KLK3 (Mattsson et al., 2009), KLK5 (Michael et al., 2005), KLK6 (Bayés et al., 2004), and KLK13 (Sotiropoulou et al., 2003), have been demonstrated to inhibit angiogenesis. These four KLKs were found to exert an anti-angiogenic effect through releasing angiostatin-like fragments from plasminogen, which potentially inhibit endothelial cell proliferation and angiogenesis (Avgeris et al., 2012; Kryza et al., 2016).

1.5 Kallikrein-related peptidases as biomarkers in ovarian and breast cancer

Regarding the impact on tumorigenicity, it is speculated that KLKs may be utilized as biomarkers for diagnosis and prognosis in carcinomas. KLKs have been reported to be aberrantly expressed in nearly all human solid tumors, especially in hormone-related cancers, including prostate, testicular, ovarian, and breast cancer (Tan et al., 2006; Avgeris et al., 2012; Schmitt et al., 2013; Lai et al., 2014). Expression and clinical relevance of KLKs in ovarian and breast cancer are summarized in **Table 3** and **Table 4**.

Table 3. Expression and clinical relevance of KLKs in ovarian cancer

KLK	Expression/ Source	Clinical relevance		References
		Favorable	Unfavorable	
4	↑ mRNA and protein in tissue		PFS; OS	Davidson et al., 2005; Obiezu et al., 2001
5	↑ mRNA in tissue ↑ protein in serum and ascites fluid	PFS; OS	PFS; OS	Diamandis et al., 2003; Dorn et al., 2011; Dorn et al., 2016 Kim et al., 2001; Oikonomopoulou et al., 2008
6	↑ mRNA and protein in tissue ↑ protein in serum		PFS; OS	Ahmed et al., 2016 Diamandis et al., 2003; Shan et al., 2007
7	↑ mRNA in tissue	PFS; OS	PFS; OS	Dorn et al., 2014; Dorn et al., 2015; Kyriakopoulou et al., 2003; Shan et al., 2006; Psyrri et al., 2008
8	↑ mRNA in tissue ↑ protein in serum	PFS; OS	PFS	Magklara et al., 2001; Kishi et al., 2003; Kountourakis et al., 2009
9	ND	PFS; OS		Yousef et al., 2001
10	↑ protein in tissue ↑ protein in serum	PFS; OS	PFS; OS	Dorn et al., 2007; Luo et al., 2001; Luo et al., 2003; Zheng et al., 2007; Oikonomopoulou et al., 2008
11	↑ protein in tissue ↑ protein in serum	PFS; OS	PFS; OS	Borgoño et al., 2003; Geng et al., 2017 Shigemasa et al., 2004; Zheng et al., 2007; Oikonomopoulou et al., 2008
12	ND			
13	↑ protein in tissue	PFS; OS	PFS	Dorn et al., 2007; Scorilas et al., 2004; Zheng et al., 2007; White et al., 2009
14	↑ mRNA in tissue ↑ protein in serum ↓ protein in tissue	PFS; OS		Dettmar et al., 2018; Yousef et al., 2003
15	↑ mRNA in tissue	OS	PFS; OS	Geng et al., 2017; Yousef et al., 2003

↑ increase; ↓ decrease; ND: not determined; OS: overall survival; PFS: progression-free survival.

Table 4. Expression and clinical relevance of KLKs in breast cancer

KLK	Expression/ Source	Clinical relevance		Reference
		Favorable	Unfavorable	
3	↑ in breast cell lines ↓ Protein in tissue	OS; DFS		Black and Diamandis, 2000; Yousef et al., 1999; Yu et al., 1998
4	↑ mRNA and protein in tissue		DFS	Mangé et al., 2008; Yang et al., 2017
5	↓ mRNA in tissue ↑ mRNA in tissue ↑ protein in serum		OS; DFS	Li et al., 2009; Yousef et al., 2002; Yousef et al., 2003; Yousef et al., 2004
6	↓ mRNA in tissue	ND	ND	Yousef et al., 2004
7	↓ mRNA in tissue	DFS	OS; DFS	Holzscheiter et al., 2006; Li et al., 2009; Talieri et al., 2004
8	↓ mRNA in tissue		DFS	Michaelidou et al., 2015; Yousef et al., 2004
9	ND	OS; DFS		Yousef et al., 2003
10	↓ mRNA in tissue ↑ mRNA in tissue ↑ protein in serum ↑ DNA methylation	OS	OS; DFS	Dhar et al., 2001; Ewan et al., 2007; Kioulafa et al., 2009; Luo et al., 2002; Yousef et al., 2004; Wang et al., 2016
11	↓ mRNA in tissue	ND	ND	Yousef et al., 2004
12	↓ mRNA in tissue ↑ in breast cancer cell lines	DFS; OS		Papachristopoulou et al., 2018; Talieri et al., 2012; Yousef et al., 2000
13	↓ mRNA in tissue	OS; DFS		Chang et al., 2002; Yousef et al., 2000
14	↓ mRNA in tissue ↑ mRNA in tissue ↑ protein in serum		OS; DFS	Borgoño et al., 2003; Papachristopoulou et al., 2011; Yousef et al., 2001; Yousef et al., 2002;
15	ND	OS; DFS		Yousef et al., 2002

↑ increase; ↓ decrease; ND: not determined; OS: overall survival; DFS: disease-free survival.

In ovarian cancer (**Table 3**), many members of the KLK family have been shown to be up-regulated. KLK4-8, KLK14, and KLK15 mRNA expression levels are elevated in ovarian cancerous cohorts. Enhanced protein levels of KLK4-8, KLK10-11, KLK13, and KLK15 have also been observed in ovarian cancer tissues, compared to healthy and benign tissues. In breast cancer (**Table 4**), mRNA expression of KLK5-8 and KLK10-13 was reported to be decreased, compared with normal breast tissues. Additionally, KLK4 was found to be overexpressed both at the mRNA and protein levels in breast malignant tissues (Mangé et al., 2008; Yang et al., 2017). These findings further suggest that KLKs might serve as biomarkers for screening and/or diagnosis in ovarian and breast cancer. However, contradictory results have been published for several KLKs. In ovarian cancer, KLK14 was up-regulated in tumor tissues (Dettmar et al., 2018), while it was downregulated in the serum of ovarian cancer patients (Yousef et al., 2003). Similarly, in breast cancer, KLK5 (Li et al., 2009), KLK10 (Dhar et al., 2001), and KLK14 (Yousef et al., 2001) were decreased in tumor tissues, whereas they were enhanced in the serum of breast cancer patients (Yousef et al., 2003; Ewan et al., 2007; Borgoño et al., 2003). These discrepancies may be due to the fact that glandular destruction and angiogenesis potentially promote the release of KLK protein into serum during tumorigenesis.

Numerous previous studies have determined the value of KLKs as prognostic and/or predictive biomarkers in ovarian and breast cancer. In ovarian cancer (**Table 3**), KLK4-7 and KLK10 overexpression levels are associated with unfavorable outcome, while elevated expression of KLK8-9, KLK11, and KLK13-14 represent favorable predictive biomarkers. In breast cancer (**Table 4**), increased expression of KLK4-5, KLK8, KLK10, and KLK14 indicate an unfavorable prognosis, whereas up-regulation of KLK3, KLK9, KLK12-13, and KLK15 imply a favorable prognosis. Interestingly, however, there are controversial observations for several KLKs with regard to prognosis in ovarian and breast cancer. Dorn et al. (2016) suggested that overexpression of KLK5 by stromal cells, not tumor cells, was correlated with prolonged progression-free survival (PFS) and overall survival (OS) in ovarian cancer, in contrast to the other studies indicated that elevated KLK5 expression was associated with shortened PFS and OS (Kim et al., 2001; Diamandis et al., 2003; Oikonomopoulou et al., 2008; Dorn et al., 2011). Also, in most cases, elevated KLK7 (Kyriakopoulou et al., 2003; Shan et

al., 2006; Psyrri et al., 2008; Dorn et al., 2015) and KLK10 (Luo et al., 2001; Zheng et al., 2007) levels were described as unfavorable markers in ovarian cancer, while two publications showed that ovarian cancer patients with positive expression of KLK7 (Dorn et al., 2014) and KLK10 (Dorn et al., 2007) exhibited a favorable outcome. Similar observations have also been revealed for KLK8, KLK11, KLK13, and KLK15 in ovarian cancer (**Table 3**) and KLK7 as well as KLK10 in breast cancer (**Table 4**). These conflicting findings can possibly be explained, on one hand, by different methods which were applied for detection and analysis in these studies, such as RT-PCR, ELISA and immunohistochemistry; on the other hand, in most of these studies rather heterogeneous patient cohorts were investigated, including distinct clinical stages, low/high grade tumors, and various subtypes of ovarian and breast cancer. The diverse expression patterns of KLKs in low versus high grade tumors and distinct subtypes may certainly result in erroneous conclusions. Therefore, the present study only enrolled patients with advanced high-grade serous ovarian cancer (HGSOC, FIGO stage III/IV) and triple-negative breast cancer (TNBC) to investigate the impact of KLKs on prognosis.

1.6 Tumor biological roles of KLK4, KLK5, KLK7, and KLK12

1.6.1 Kallikrein-related peptidase 4

KLK4，also known as prostase/KLK-L1, is located immediately downstream of KLK2 and upstream of KLK5 on chromosome 19q13.3–19q13.4 (Stephenson et al., 1999; Yousef et al., 1999). Two major alternative KLK4 transcripts have been reported, including the full-length KLK4-254 transcript and the exon 1-deleted KLK4-205 transcript, both proteolytically encoding active serine proteases (Dong et al., 2005; Kurlender et al., 2005). Besides, several KLK4 transcripts with splice variations between exons 2 and 5 were observed, exhibiting a frame-shift in the coding region and producing truncated proteins, which do not have the essential serine and/or aspartic acid residues of the catalytic triad (Obiezu and Diamandis, 2000; Dong et al., 2001; Korkmaz et al., 2001; Myers and Clements, 2001). The full-length KLK4 transcript encodes a 254-amino acid pre-pro-serine protease, comprising an N-terminal signal peptide with 26 amino acids, followed the pro-peptide with 4 amino acids and the active protease domain with 224 amino acids (Nelson et al., 1999). The exon 1-deleted transcript (KLK4-205) encodes an N-terminally truncated 205-amino acid protein with

the absence of the signal peptide and the pro-peptide (Dong et al., 2005). The lack of exon 1 suggests that KLK4-205 has an intracellular localization and function, distinct from full-length KLK4 transcript and other KLKs, which are characterized by major extracellular functions (Korkmaz et al., 2001). Thus, KLK4, which is predominantly localized in the nucleus, is considered as a unique member of KLK family (Dong et al., 2005).

KLK4, a trypsin-like serine protease, shows an arginine/lysine-specific protease activity and is involved in physiological and pathological processes. It is well-known that KLK4 facilitates degradation of enamel matrix proteins during tooth maturation, exhibiting a key role in enamel mineralization (Hu et al., 2007; Lu et al., 2008; Opal et al., 2015). Furthermore, KLK4 is supposed to be an oncogene in various cancer types, including but not limited to oral, breast, ovarian, prostate, and colon carcinomas. Elevated KLK4 mRNA and protein expression levels were correlated with a higher probability of invasion and metastasis in oral squamous cell carcinomas, suggesting that KLK4 could serve as a potential therapeutic target (Papagerakis et al., 2015). Obiezu et al. (2001) observed that positive KLK4 mRNA expression is an independent unfavorable prognostic biomarker in patients with ovarian cancer. Consistently, Dong and co-workers (Dong et al., 2001, 2013) have observed the association of KLK4 mRNA and antigen overexpression with advanced stage and paclitaxel resistance in serous ovarian cancer. Furthermore, KLK4 was found to be up-regulated in prostate carcinoma and to be modulated by steroid hormones in prostate and breast cancer cell lines (Nelson et al., 1999; Yousef et al., 1999). In breast cancerous tissues, KLK4 mRNA levels were shown to be higher compared to normal and benign breast tissues (Papachristopoulou et al., 2009). Moreover, overexpression levels of KLK4 mRNA and protein showed a strong correlation with high grade and poor disease-free survival in this tumor entity (Mangé et al., 2008; Yang et al., 2017).

1.6.2 Kallikrein-related peptidase 5

KLK5/KLK-L2 was initially described as human stratum corneum tryptic enzyme (HSCTE), owing to its high expression in skin and potential effects on stratum corneum turnover and desquamation in the epidermis (Brattsand and Egelrud, 1999). Further studies indicated that KLK5 is telomeric to KLK4 and upstream of KLK6 mapping to chromosome 19q13.3-q13.4 (Yousef et al., 2000), encoding a 25 kDa trypsin-like serine

protease (Brattsand and Egelrud, 1999; Yousef and Diamandis, 1999). In addition, KLK5 was found to structurally resemble KLK4 with approximately 54% amino acid sequence identity and present a high degree of homology with other KLKs (Kim et al., 2001). KLK5 is produced as an inactive pre-pro-enzyme, comprising a 29-amino acid signal peptide, followed by a 37-amino acid activating pro-peptide and a 237-amino acid catalytic domain (Yousef and Diamandis, 1999). This inactive precursor can be activated by matriptase and KLK14 as well as by self-activation (Zhu et al., 2017). Moreover, KLK5 is supposed to be implicated in KLK activation cascades, thus activating numerous protease zymogens, including other pro-KLKs (De Veer et al., 2016).

KLK5 was originally found to be expressed in testis, skin, brain, and breast (Brattsand and Egelrud, 1999; Yousef and Diamandis, 1999), and was subsequently found also in the prostate (Michael et al., 2006) and kidney (Kriegel et al., 2012). KLK5 is predominantly responsible for skin desquamation by directly cleaving corneodesmosomal cadherins (Ekholm et al., 2000; Caubet et al., 2004). It also stimulates inflammation via induction of PAR2 signaling (Oikonomopoulou et al., 2006; Yamasaki et al., 2006; Stefansson et al., 2008) and is involved in pro-filaggrin processing (Sakabe et al., 2013). Furthermore, dysregulation of KLK5 might contribute to pathophysiological processes, due to its function of cleaving substrates or activating protease zymogens. For example, KLK5 has been reported to be involved in the pathogenesis of several skin diseases, such as Netherton syndrome (Yamasaki et al., 2006; Furio et al., 2015) and atopic dermatitis (Komatsu et al., 2007; Kubo et al., 2012). Moreover, accumulating reports have suggested potential functions of KLK5 in human cancers, including oral (Jiang et al., 2011), breast (Yousef et al., 2002; Yang et al., 2015), urinary bladder (Shinoda et al., 2007), ovarian (Kim et al., 2001; Dorn et al., 2016), prostate (Yousef et al., 2002; Korbakis et al., 2009), and colorectal (Wu et al., 2016) malignancies. In ovarian cancer, KLK5 overexpression was shown to be positively associated with tumor grade and risks for relapse and death (Kim et al., 2001; Diamandis et al., 2003; Dorn et al., 2011). However, Dorn et al. (2016) have quantified KLK5 protein levels in 95 ovarian cancer patients, showing that the overexpression of KLK5 by stromal cells, but not by tumor cells, was significantly associated with prolonged DFS and OS. Similarly, in breast cancer, Papachristopoulou et al. (2013) has

revealed that KLK5 might serve as a tumor-suppressor. In contrast, most publications have demonstrated that overexpression of KLK5 is associated with estrogen receptor status, larger tumors, positive nodes, distal metastasis, and shortened DFS as well as OS (Yousef et al., 2002; Talieri et al., 2011; Yang et al., 2015).

1.6.3 Kallikrein-related peptidase 7

KLK7, also known as PRSS6 or the human stratum corneum chymotryptic enzyme (HSCCE), is located at the chromosomal locus 19q13.3–q13.4 between KLK6 (centromere) and KLK8 (telomere) (Hansson et al., 1994; Yousef et al., 2000). The genomic structure of KLK7 comprises six exons, of which the first exon is non-coding, and five intervening introns highly conserved with other KLKs, encoding 253 amino acids pre-pro-enzyme (Yousef et al., 2000). Unlike most members of the KLK family, KLK7 is a chymotrypsin-like serine protease due to the absence of aspartate at position 189 (Yousef et al., 2000; Debela et al., 2008). KLK7 expression has been identified to be up-regulated by estrogens and glucocorticoids in the breast cancer cell line BT-474 (Yousef et al., 2000).

Prior studies have reported that KLK7 is implicated in various pathophysiological processes in human organs, such as skin, lung, breast, prostate, and ovary (Shaw and Diamandis, 2007; Avgeris et al., 2010). KLK5, KLK7, and KLK14 are considered as major functional proteases in normal skin, where they exhibit crucial roles in the process of skin desquamation (Caubet et al., 2004). Furthermore, elevated expression of KLK5 and KLK7 have also been found in skin diseases like skin barrier disorders (Fischer and Meyer-Hoffert, 2013; Kalinska et al., 2016). Moreover, the tumor biological role of KLK7 has been evaluated individually or in panels in different types of cancer. Overexpression of KLK7 was observed in squamous (Zhao et al., 2011), cervical (Li et al., 2014; Raju et al., 2016), ovarian (Shan et al., 2006; Dong et al., 2010), pancreatic (Du JP et al., 2018) and colorectal (Walker et al., 2014) carcinomas, whereas down-regulation of KLK7 was shown in breast (Li et al., 2009; Ejaz et al., 2017), prostate (Xuan et al., 2008) and lung carcinomas (Planque et al., 2005). In ovarian cancer, elevated KLK7 expression was shown to be associated with chemoresistance and poor prognosis (Kyriakopoulou et al., 2003; Shan et al., 2006; Psyrri et al., 2008; Dong et al., 2010). However, Dorn et al. (2014) revealed that patients with high KLK7 levels have a lower risk of relapse and cancer-related death than those with low KLK7

levels. Similarly, in breast cancer, most studies have indicated that KLK7 overexpression was connected with advanced stage, distant metastasis, and shortened survival (Talieri et al., 2004; Prezas et al., 2006; Li et al., 2009). However, Holzscheiter et al. (2006) determined KLK7 mRNA expression in tumor tissue specimens from 155 breast cancer patients and observed a favorable prognostic value of KLK7 mRNA expression in breast cancer.

1.6.4 Kallikrein-related peptidase 12

KLK12, originally known as KLK-L5, contains five coding exons and one 5' untranslated exon (Yousef et al., 2000). KLK12 is structurally very similar to other KLKs, with the transcribed direction of being 21.3 kb telomeric to KLK13 and 1.5kb centromeric to KLK11 (Yousef et al., 2000). To date, four splice forms of KLK12 have been identified to produce secreted proteins, including the classical form and three alternative splice variants named KLK12sv1, KLK12sv2, and KLK12sv3 (Yousef et al., 2000; Kurlender et al., 2005). KLK12 is synthesized as a pre-pro-enzyme, consisting of a 17-residue signal peptide, followed by a 21-residue cleavage site for activation and a 227-residue mature enzyme with a predicted molecular weight of 24.5 kDa (Memari et al., 2007). The secreted inactive pro-enzyme gains enzymatic activity via auto-activation (Memari et al., 2007). Active KLK12 displays trypsin-like activity and thus cleaves peptide bonds after arginine and lysine (Memari et al., 2007). Furthermore, the activity of KLK12 was found to be quickly lost due to self-digestion or to be rapidly inhibited by interaction with Zn^{2+} as well as by covalent complex formation with α2-antiplasmin (Memari et al., 2007). Besides, KLK12 has been identified to be up-reglated by estrogen, androgen and progestin in LNCaP prostate cells and breast cancer cell line BT-474 (Yousef et al., 2000).

KLK12 is widely expressed in human tissues, and particularly abundant in the human respiratory tract (Shaw and Diamandis, 2007; Hamilton and Whittaker, 2013). However, its physiological roles in the respiratory tract as well as other tissues are still ill-defined. Recent studies pointed to a potential effect of KLK12 on cell proliferation, angiogenesis, invasion, and migration, thereby influencing cancer progression (Kryza et al., 2014; Li and He, 2016; Kryza et al., 2018). In gastric cancer, KLK12 mRNA and protein expression levels were positively associated with lymph node status, histological type and TNM stages (Zhao et al., 2012). There, patients with elevated KLK12 expression

levels have a worse 5-year survival rate in this tumor entity, compared to those with low KLK12 expression. Furthermore, Planque et al. (2008) have revealed that KLK12 protein expression is down-regulated in non-small cell lung cancer (NSCLC). Here, KLK12 protein expression was positively correlated with tumor stage, smoking status, gender, and risk of NSCLC. In breast cancer, KLK12 mRNA levels were found to be decreased in tumor tissues and were increased by steroid hormones in breast cancer cell lines (Yousef et al., 2000). With regard to the splice variants of KLK12 in breast cancer, two studies have reported that KLK12sv3 expression was correlated with early stage, lower grade, and smaller tumor size (Talieri et al., 2012; Papachristopoulou et al., 2018). Moreover, breast cancer patients with elevated mRNA expression of KLK12sv3 presented longer DFS and OS than those with lower KLK12sv3 mRNA levels, suggesting the potential prognostic value of KLK12 in this tumor entity.

In summary, the evidence mentioned above highlights the tumor biological roles of KLK4, 5, 7, and 12 in various types of cancer, especially in ovarian and breast carcinomas. Of note, these four KLK genes may exert different effects on tumor progression depending on the tumor type or even subtype. Thus, the current project focuses on analyzing the mRNA expression and clinical relevance of KLK4, 5, 7, and 12 in the well-defined homogenous cohorts of patients with advanced high-grade serous ovarian cancer and triple-negative breast cancer.

2 Aims of the study

Numerous studies have demonstrated that KLK genes are dysregulated in various types of tumors and are implicated in cancer progression. Previous studies have focused on the diagnostic and prognostic values of KLKs in ovarian and breast malignancies in terms of refining clinical management for the individual cancer patient. However, advanced high-grade serous ovarian cancer and triple-negative breast cancer should receive more attention due to their aggressive behaviors and/or the lack of effective therapeutic targets. To date, KLK4, KLK5, KLK7, and KLK12 have been reported to play decisive roles in processes of ovarian and breast cancer. Nevertheless, these previously reported results were sometimes paradoxical. Therefore, the current study aimed at characterizing the mRNA expression patterns of KLK4, KLK5, KLK7 and KLK12 in homogenous patient cohorts with advanced high-grade ovarian cancer and triple-negative breast cancer, respectively.

To achieve this, the following analyses were performed:

1. Quantitative PCR assays to evaluate mRNA expression levels of KLK4, 5, 7 and 12 in HGSOC and TNBC tissues.
2. Correlation of KLK5 and KLK7 mRNA expression levels with their corresponding protein levels (available data from previous studies), respectively, in HGSOC.
3. Correlation between continuous variables of KLKs in HGSOC and TNBC.
4. Assessment of the statistical associations of mRNA expression levels of KLK4, 5, 7 and 12 with clinicopathological parameters in patients with HGSOC and TNBC.
5. Assessment of the statistical associations of mRNA expression levels of KLK4, 5, 7 and 12 with clinical outcome in patients with HGSOC and TNBC.

3 Patients, Materials and methods

3.1 Tissue collection and extraction

3.1.1 Cohort 1: patients afflicted with advanced high-grade serous ovarian cancer

A total of 139 tumor tissue specimens from patients afflicted with HGSOC, FIGO stage III/IV, were enrolled at the Department of Obstetrics and Gynecology, Klinikum Rechts der Isar, Technical University of Munich (TUM), Germany, between years 1990 and 2013. All patients were treated with standard stage-related primary radical debulking surgery. Following surgery, all patients received systemic adjuvant treatment according to the consensus recommendations at that time, including platinum-based adjuvant chemotherapy. None of the cases received any neoadjuvant therapy previous to surgery. The clinical data of HGSOC patients are documented in **Table 5**.

This study to assess the KLK expression in the collected ovarian cancer tissues was approved by the local Ethics Committee (Faculty of Medicine, TUM, Germany; project 491/17 S). Written informed consent to utilize tissue specimens for research purposes was obtained from all patients.

Table 5. Clinical characteristics of advanced high-grade serous ovarian cancer patients in cohort 1

Clinical variables	n	%
All patients	139	
Median age	64	
(range)	(33-88) years	
Median observation time of patients alive	29	
(range)	(2-279) months	
Age		
≤ 60 years	58	41.7
> 60 years	81	58.3
Residual tumor mass		
0 mm	70	50.4
> 0 mm	67	48.2
no data	2	1.4
Ascitic fluid volume		
≤ 500 ml	78	56.1
> 500 ml	54	38.9
no data	7	5.0
FIGO stage		
III	108	77.7
IV	31	22.3
Disease recurrence		
yes	76	54.7
no	33	23.7
no data	30	21.6
Alive recurrence		
yes	78	56.1
no	47	33.8
no data	14	10.1

3.1.2 Cohort 2: patients afflicted with triple-negative breast cancer

The tumor tissue samples of 125 primary TNBC in this retrospective study were collected conducted between the year 1988 and 2012 at the Department of Obstetrics and Gynecology, Klinikum Rechts der Isar, TUM, Germany. All patients received standard surgical procedures, including breast conservation surgery and mastectomy. Following surgery, patients accepted adjuvant therapy based on consensus recommendations at that time, including anthracycline- or cyclophosphamide-based chemotherapy, endocrine therapy, and radiotherapy. The histomorphologic parameters and clinical data were summarized in **Table 6**.

Tumor samples were routinely assessed for expression of HER2 and steroid hormone receptors (ER and PR) at the Department of Pathology before storage. Tumor specimens were classified as TNBC following the rules previous described (Yang et al., 2017): negative statues of ER and PR and lack or low levels of HER2 protein expression (immunohistochemically determined score 0 or 1+, or 2+ with negative fluorescence in situ hybridization [FISH] test for testing HER2 amplification).

Table 6. Clinical and pathological data of triple-negative breast cancer patients in cohort 2

Clinical parameters	n	%
All patients	125	
Median age	55	
(range)	(30-96) years	
Median observation time of patients alive	79	
(range)	(4-286) months	
Age		
≤ 60 years	68	54.4
> 60 years	57	45.6
Menopausal status		
pre-menopausal	41	32.8
peri-menopausal	3	2.4
post-menopausal	81	64.8
Histological subtype		
invasive ductal	113	90.4
medullary	2	1.6
lobular	1	0.8
other	9	7.2
Tumor size		
pT1	34	27.2
pT2	75	60.0
pT3	7	5.6
pT4	9	7.2
Nodal status		
pN0	71	56.8
pN1	40	32.0
pN2	10	8.0
pN3	4	3.2
Metastasis		
yes	32	25.6
no	93	74.4
Histological grade		
II	12	9.6
III	112	89.6
IV	1	0.8
Disease recurrence		
yes	54	43.2
no	67	53.6
unknown	4	3.2
Adjuvant treatment		
chemotherapy	92	73.6
endocrine therapy	20	16.0
radiotherapy	94	75.2
unknown	1	0.8

3.2 Cell culture

OV-MZ-6, a human epithelial ovarian cancer cell line, was cultured in Dulbecco's Modified Eagle Medium (DMEM), which is supplemented with 10% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.2% arginine/asparagine. The culture medium was replaced twice a week and cells were passaged after they reached a confluence of 80%. OV-MZ-6 cells grow adherently in a 75 cm^2 culture flask (5% CO^2 (v/v), 95% humidity, 37 °C). The reagents and materials were summarized in **Table 7**.

Table 7. Reagents and materials used in the cell culture

Cell culture flask (25 cm^2, 75 cm^2)	Greiner Bio-one GmbH, Frickenhausen, Germany
Cell culture microscope	CK30, Olympus, Tokyo, Japan
Centrifuge	Rotina 48R, Andreas Hettich, Tuttlingen, Germany
CO^2 incubator	Heraeus Function Line Serie 7000
Cryogenic vials	Thermo Fisher Scientific Inc., Rochester, NY, USA
DMEM (Dulbecco's modified eagle's Medium)	#61965-026, Gibco, Invitrogen, Paisley, United Kingdom
DMSO (dimethyl sulfoxide)	#317275, Merck Chemicals, Darmstadt, Germany
EDTA (ethylenediaminetetraacetic acid), 1% (w/v)	#L2113, Biochrom GmbH, Berlin, Germany
FBS (fetal bovine serum)	#10270-106, Invitrogen, Carlsbad, USA
Hemocytometer	0.1 mm, Neubauer improved chamber, Laboroptik
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	#15630-080, Gibco, Invitrogen, Darmstadt, Germany
Laminar flow cabinet (Hera Safe)	M1199, Heraeus, Hanau, Germany
L-arginine	#A8094, Sigma, Munich, Germany
L-asparagine	#A7094, Sigma, Munich, Germany
PBS (phosphate-buffered saline)	#10010-015**,** Gibco, Invitrogen, Carlsbad, CA, USA
Serological pipette	Greiner Bio-one GmbH, Frickenhausen, Germany

After thawing and properly passaging, adherent cells were used in the experiments. OV-MZ-6 cells were detached using ethylenediaminetetraacetic acid/phosphate-buffered saline (EDTA/PBS; 1% w/v) and washed with phosphate-buffered saline (PBS). The

cell suspension was centrifuged at 1,300 rpm for 3 min and the supernatant was discarded. The steps of resuspension and centrifugation repeated again. Then, the OV-MZ-6 cells were detached from the culture flask and $1x10^6$ cells were rapidly resuspended in the freezing medium (FBS containing 10% dimethylsulfoxide [DMSO]) on ice. The cell suspension was transferred into cryogenic storage vials. Finally, the cells were frozen at -80 °C in cryogen storage vials for 24 h and transferred into liquid nitrogen at -196°C for storage.

3.3 Quantitative polymerase chain reaction (qPCR)

3.3.1 RNA isolation

Human ovarian cancer OV-MZ-6 cells were employed, which had been stably transfected with pRcRSV-derived expression plasmids encoding the complete coding region of target KLKs (OV-KLK4, OV-KLK5, OV-KLK7, and OV-KLK12, respectively). The automated extraction and purification of total DNA and RNA from cell lines and tumor tissues was performed by using the QIAcube sample preparation device (Qiagen) and the All Prep DNA/RNA Universal kit (Qiagen).

Tumor tissue specimens from both HGSOC and TNBC cases were immediately stored in liquid nitrogen after inspection by the pathologist until RNA extraction. Deep-frozen tumor tissues were obtained from the Tumor Bank of the Medical Faculty of TUM. The frozen tumor tissues were pulverized on ice and the still-frozen powder (10-20 µg) was immediately homogenized in 600 µl of RLT plus buffer (Qiagen) supplemented with 6 µl 2-β-mercaptoethanol. Then, tumor lysates were centrifuged at full speed (3 min, RT) in QIAshredder spin columns (Qiagen). The supernatants were inserted into the QIAcube machine and the manufacturer's recommendations were exactly followed.

Concentration and purity of extracted RNA were quantified employing the NanoDrop 2000c spectrophotometer and the NanoDrop 2000/2000c software (Thermo Fisher Scientific, Wilmington, USA). The RNA quality was assessed by determination of absorbance ratios at 260/280 nm and 260/230 nm. All RNA samples were stored at -80°C until further use.

3.3.2 Reverse transcription

According to the manufacturer's recommendations, reverse transcription of the isolated

mRNA was performed using the AMV First Strand cDNA Synthesis Kit (Invitrogen, Darmstadt, Germany). First, the annealing of primers was conducted in a PCR reaction tube as follows:

Components	Volume
Isolated RNA	1000 ng (cell lines) 500 ng (tumor tissues)
Random hexamer primers (50 ng/µl)	1 µl
dNTP (10 mM)	2 µl
DEPC-treated H_2O	x µl
Total	12 µl

The PCR reaction tubes were incubated in a thermal cycler (65 °C, 5 min) and then transferred on ice. Next, the following master reaction mix was added at 8 µl per well to the RNA/primers mixture:

Components	Volume
cDNA synthesis buffer (5x)	4 µl
DTT (0.1 M)	1 µl
RNase out (40 U/µl)	1 µl
Cloned AMV RT (15 U/µl)	0.9 µl
DEPC-treated H_2O	1.1 µl

Then, the reaction tubes were transferred to a thermal cycler (Qiagen) with the following PCR program:

Steps	Temperature	Time
1	25 °C	10 min
2	50 °C	50 min
3	85 °C	5 min

The resulting cDNA from frozen tumor tissues was diluted with RNase-free water resulting in a final cDNA concentration of 5 ng/µl, while the cDNA from cell lines was diluted to 10 ng/µl prepared for dilution series and calibrators. All cDNA samples were stored at -20°C until further use.

3.3.3 qPCR analysis using Universal ProbeLibray probes

Universal ProbeLibray probes (Roche) were employed for the quantitative polymerase

chain reaction (qPCR) method. These probes are short hydrolysis probes (8-9 nucleotides), which are attached by a reporter with fluorescein (FAM) at the 5' end and by a dark quencher dye at the 3' end. The fluorogenic probes were able to target specific genes and the reporter dye was cleaved by the DNA polymerase, emitting its characteristic fluoresce. Hypoxanthine-Guanine Phosphoribosyl transferase 1 (HPRT1) was used as the reference gene, suitable for the assessment of biomarkers in ovarian and breast cancer (de Kok et al., 2005).

For KLK5 and HPRT1, assays have been established in-house using the ProbeFinder software (https://lifescience.roche.com/products/universal-probe-library) and the Universal ProbeLibrary (Roche, Penzberg, Germany). The gene-specific primers were shown in **Table 8**.

Table 8. Gene-specific primers of KLK5 and HPRT1

		KLK5	HPRT1
Transcript variants		NM_001077491.1 NM_001077492.1 NM_012427.4	NM_000194
Primers (position)	**Forward**	5'-AAGGCCCAACCAGCTCTACT-3' (476-495, 408-427, 611-630)	5'-TGACCTTGATTTATTTTGCATACC-3' (218-241)
	Reverse	5'-CCGAGACGGACTCTGAAAAC-3' (555-574, 487-506, 690-709)	5'-CGAGCAAGACGTTCAGTCCT-3' (300-319)
Probe (amplicon size)		5'-FAM-GCAGGAAG-3'-dark quencher (99 bp)	5'-FAM-GCTGAGGA-3'-dark quencher (102 bp)

The assay detects all the major transcript variants of KLK5 and HPRT1, respectively, encoding full-length proteins.

Regarding to KLK4, KLK7 and KLK12, assays were optimized to apply the Biosystems TaqMan gene expression assays from ThermoFisher, which consist of a pair

of unlabeled PCR primers and a TaqMan probe with a FAM dye label at the 5 'end and a minor groove binder (MGB) as well as a non-fluorescent quencher (NFQ) at the 3' end. The specific assays were summarized in **Table 9**.

Table 9. Gene-specific assays of KLK4, KLK7, and KLK12

KLK	Transcript variants	Assay ID	amplicon size
KLK4	NM_004917.4 NM_001302961.1	Hs05041835_s1	120 bp
KLK7	NM_005046.3 NM_139277.2 NM_001207053.1 NM_001243126.1	Hs00192503_m1	70 bp
KLK12	NM_019598.2 NM_145894.1 NM_145895.1	Hs00377603_m1	108 bp

The assays detect all the major transcript variants of KLK4, KLK7, and KLK12, respectively, encoding full-length proteins.

The experimental reaction mixture was prepared as shown in **Tables 10 and 11**. All qPCR reactions were conducted in triplicates (input: 15 ng/well for clinical samples and 30 ng/well for cell lines). cDNA from OV-MZ-6 cells overexpressing the target KLKs was used as the calibrator and positive control. Negative controls consisted of no-template control (water as substrate), genomic DNA (OV-MZ-6 cells), and no-RT control (untranscribed RNA from cell line as substrate).

Table 10. qPCR reaction mixture for KLK5 and HPRT1

Components	Volume
2x Brilliant III qPCR master mix with low ROX	10 µl
UPL Probe (10 µM)	0.4 µl
Primer forward (20 µM)	0.4 µl
Primer reverse (20 µM)	0.4 µl
H_2O	5.8 µl
Total	17 µl

Table 11. qPCR reaction mixture for KLK4, KLK7, and KLK12

Components	Volume
2x Brilliant III qPCR master mix with low ROX	10 µl
2x TaqMan® Gene Expression Master Mix	1 µl
H_2O	6 µl
Total	17 µl

The reaction mixture was distributed to a 96-well qPCR reaction plate (Biozym, Hamburg, Germany) and 3 µl of cDNA was added to each reaction well. Then, the plate was centrifuged at 3,000 rpm for 3 min and transferred to the Agilent Mx3005P instrument (Agilent, Darmstadt, Germany) for qPCR analysis. The steps of qPCR cycling program were shown in **Table 12**.

Table 12. qPCR cycling program

Segment	Cycles	Parameter	Temperature	Time
1	1	Polymerase Activation	95 °C	3 min
2	40	Denature	95 °C	15 sec
		Anneal/Extend	60 °C	1 min

3.3.4 Standard dilution series

Due to sample heterogeneity and variation in extraction/conversion efficiency, standard dilution series were utilized to estimate the efficiency of KLK assays. Efficiency validation was carried out using a two-fold dilution series with cDNA from positive control cell lines (OV-MZ-6 cells overexpressing the target KLKs). Five dilution steps (cDNA0-cDNA4, range from 30 ng to 1.875 ng) were performed to establish the standard dilution curves of the KLKs and HPRT. The 2-fold dilution series for each gene was analyzed by qPCR in three independent experiments. The cycle threshold (Ct) value for each dilution step (y-axis) was plotted against the logarithm of the

corresponding cDNA input (log input 1.477-0.273; x-axis). The slope of the standard dilution series was calculated based on a linear regression analysis and further used to determine the efficiency (E) by the following calculation formula: $E = 10^{(-1/slope)}$. The acceptable range of efficiency (E) is 95-100% for qPCR assay validation, with the E value between 1.6 and 2. A ΔE between KLK and HPRT1 was estimated for calculation of ΔE-related error margins.

3.3.5 qPCR evaluation method

Relative quantitation analyses for mRNA expression of target KLKs were performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method (Pfaffl, 2012): ΔCt = Ct (KLK) - Ct (HPRT1); ΔΔCt = ΔCt (tumor sample) – ΔCt (calibrator).

All measurements are subject to error or uncertainty. Thus, the random errors in observable quantities are measured by estimating the error propagation. Relative error propagation (EP) was calculated for each step by the following formula (STDEV: standard deviation):

$$\mathrm{EP}\ (\Delta\mathrm{Ct}) = \sqrt{\frac{(STDEV_{KLK})^2 + (STDEV_{HPRT1})^2}{2}}$$

$$\mathrm{EP}\ (\Delta\Delta\mathrm{Ct}) = \sqrt{\frac{(EP_{\Delta\mathrm{Ct}_{tumor\ sample}})^2 + (EP_{\Delta\mathrm{Ct}_{calibrator}})^2}{2}}$$

Absolute error propagation was calculated by the following formula (ln: natural logarithm): $\mathrm{EP\ (absolute\ error)} = \ln 2 \times EP(\Delta\Delta\mathrm{Ct}) \times 2^{-\Delta\Delta\mathrm{Ct}}$.

Accounting for the detection limitations and the variations of sample qualities, the following quality criteria were applied in the present project to exclude unassertive results (Ahmed et al., 2016): (1) the Ct value for HPRT was > 35; (2) the 2exp-ΔΔCt error progression% was > 30% even after repetition; (3) the % STDEV of the 2exp-ΔΔCt for 2 valid runs was > 47.1%. Based on this, sample numbers in statistical analyses do not always add up to 139 for ovarian cancer and to 125 for breast cancer.

3.4 Antigen expression levels of KLK5 and KLK7 in advanced high-grade serous ovarian cancer patients

The KLK5 (Dorn et al., 2016) and KLK7 (Dorn et al., 2014) antigen levels of 46 of the 139 cases of the present patient cohort 1 have been determined by an immunofluorometric assay (ELISA) in previous studies. The ELISA determinations and the qPCR analyses of the present project were performed with independent tissue samples of the same patient.

3.5 Statistical analyses

The association of KLK mRNA expression levels with clinical parameters of patients was analyzed using the Chi-square test. Associations of tumor biological factors and clinical survival were calculated by univariate and multivariate proportional hazard regression analyses and expressed as hazard ratio (HR) as well as its 95% confidence interval (95% CI). Survival curves were plotted according to Kaplan-Meier survival analysis, using the log-rank test to compare group differences of survival functions. The database from The Cancer Genome Atlas (TCGA) was applied to validate *in silico* the prognostic power of KLKs in HGSOC (not available for TNBC).

The Mann-Whitney U test and Spearman rank correlation (r_s) were utilized to assess the correlations between continuous variables of KLKs. The Mann–Whitney U test and Spearman rank correlation were also applied to analyze the relationship of KLK5 and KLK7 mRNA expression levels with their corresponding protein expression levels, respectively. Box plots were drawn to indicate differences.

All calculations were performed employing the SPSS statistical analysis software (version 20.0; SPSS Inc., Chicago, IL, USA). P-values ≤ 0.05 were considered statistically significant.

4 Results

4.1 KLK mRNA expression determined by qPCR in advanced high-grade serous ovarian cancer (cohort 1)

4.1.1 Establishment of quantitative PCR assays for KLKs

In an ideal qPCR assay, the amplification efficiency is supposed to be 100%, corresponding to an E value of 2. Actually, the qPCR efficiency has a wide dynamic range from 95% to 100%, with corresponding E values from 1.6 to 2 (Ruijter et al., 2013). Thus, in the current study, the standard dilution series method was applied for evaluating the efficiencies of the target KLKs and the housekeeping gene HPRT1 during qPCR assays (Bustin and Nolan, 2013). Based on this, 2-fold dilution series of cDNA from cell lines OVMZ6-KLK4, OVMZ6-KLK5, OVMZ6-KLK7, and OVMZ6-KLK12, which individually overexpressed the respective KLK, were generated to calculate the efficiencies for each assay, with five dilution steps (cDNA0-cDNA4: range from 30 ng to 1.875 ng). As shown in **Figure 3**, the Ct value for each dilution step (y-axis) was plotted against the logarithm of the corresponding cDNA input (log input 1.477-0.273; x-axis).

Three independent experiments were performed for each dilution series. As exemplified by the KLK4 dilution curves in **Figure 3A**, the slopes of the lines for KLK4 (1^{st} -3.23; 2^{nd} -3.42; 3^{rd} -3.67) are parallel with those of the reference gene HPRT (1^{st} -3.36; 2^{nd} -3.24; 3^{rd} -3.40). Furthermore, the E values of KLK4 (E_{KLK4}: 1^{st} 2.0; 2^{nd} 1.96; 3^{rd} 1.89; **Table 13**) are also comparable with those of HPRT (E_{HPRT}: 1^{st} 1.98; 2^{nd} 2.04; 3^{rd} 1.97; **Table 13**). Similarly, the E values of KLK5, KLK7, and KLK12 are comparable to those of HPRT (**Table 13**). Therefore, efficiency correction was not required for normalization and the relative KLK mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method in the succeeding analyses (see chapter 3.3.5 for details).

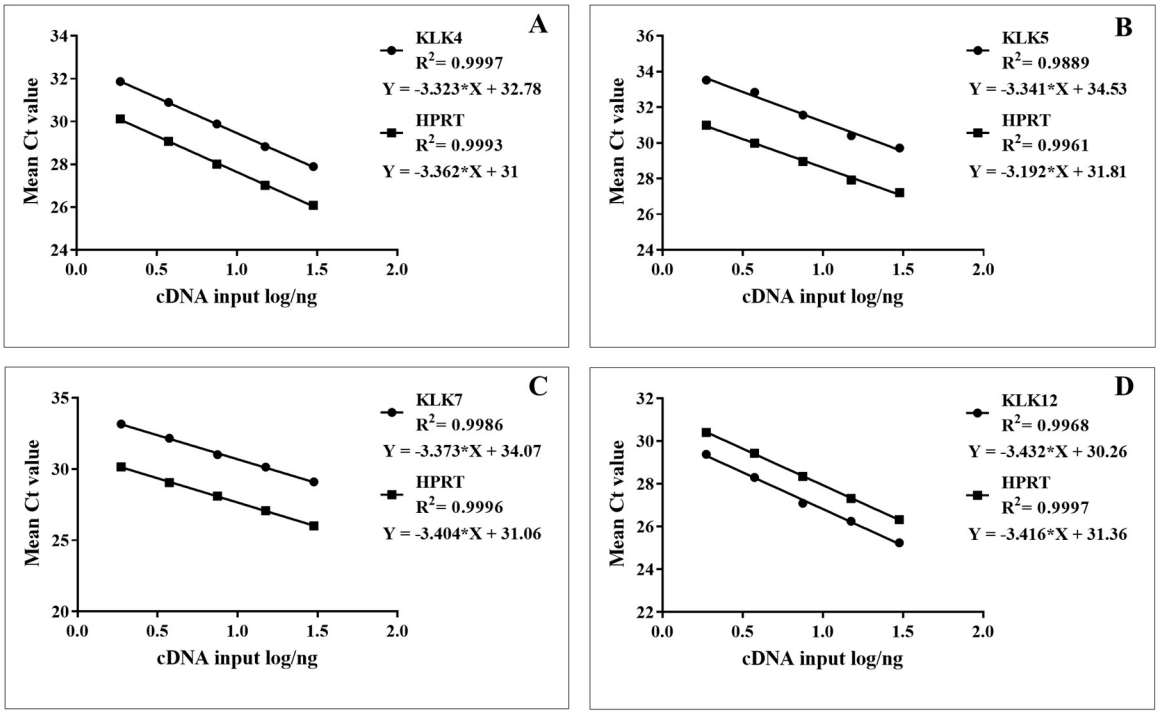

Figure 3. Exemplary dilution series of cDNAs for detection of KLKs and HPRT by qPCR

HPRT was used as the reference gene. Total RNA was isolated from OV-MZ-6 cell lines overexpressing KLK4, 5, 7, and 12, respectively, and reverse transcribed. Standard dilution series curves were plotted by 2-fold serial dilutions starting with 30 ng cDNA input (dots from left to right: cDNA4, cDNA3, cDNA2, cDNA1, cDNA0). Slopes: (**A**) KLK4 -3.32, HPRT -3.36; (**B**) KLK5 -3.34, HPRT -3.19; (**C**) KLK7 -3.37, HPRT -3.40; (**D**) KLK12 -3.43, HPRT -3.41.

Table 13. Efficiency values of KLKs in three independent dilution series are comparable to those of HPRT

Dilution series	Efficiency values			
	E_{KLK4}/E_{HPRT}	E_{KLK5}/E_{HPRT}	E_{KLK7}/E_{HPRT}	E_{KLK12}/E_{HPRT}
1st run	2.0/1.98	1.99/2.05	1.98/1.97	1.99/1.97
2nd run	1.96/2.04	1.95/2.02	2.04/1.99	2.01/2.0
3rd run	1.89/1.97	1.93/1.99	2.0/1.97	1.96/1.96

To validate the qPCR assay, the relative KLK mRNA expression levels of cDNA1-4 were calculated with normalization against HPRT (Ct_{KLK}-Ct_{HPRT}) and cDNA0 (Ct_{cDNA}-Ct_{cDNA0}), as demonstrated in **Figure 4**. In the three independent repetitions, the standard deviations (SDs) of the relative KLK mRNA expression in cDNA1-4 were all lower

than 20%.

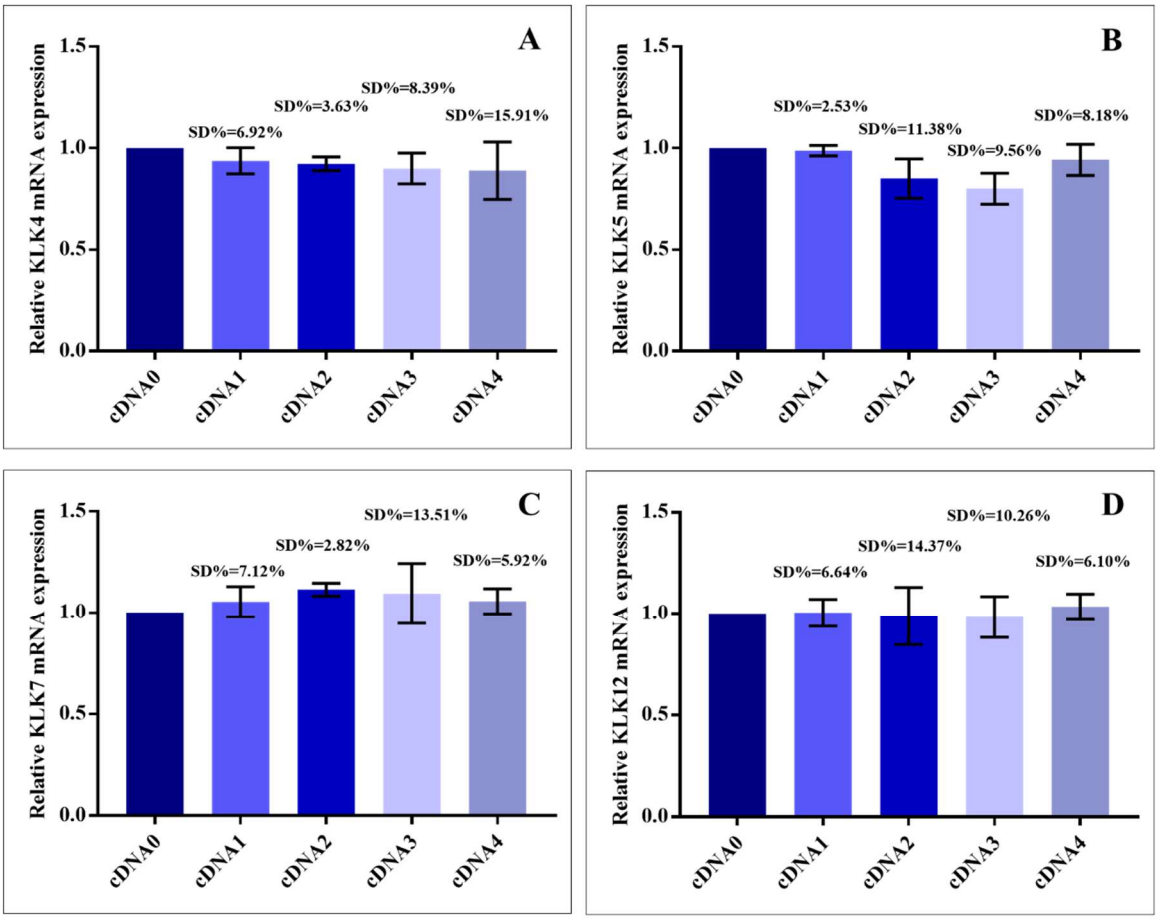

Figure 4. Mean values and standard deviations of relative KLK mRNA expression in three independent qPCR dilution experiments

Relative mRNA expression of KLK4 (**A**), KLK5 (**B**), KLK7 (**C**), and KLK12 (**D**) were calculated in cDNA1-4 with normalization against HPRT and cDNA0. Mean values of relative KLK mRNA expression were in close proximity and all SDs were lower than 20% in cDNA1-4.

Next, three cDNA samples from ovarian cancer tissue were randomly collected for a pretest. For each clinical sample, triplicates were analyzed regarding the relative KLK mRNA expression in three independent assays. For KLK4, 5 and 7, all Ct values of HPRT and the $2^{-\Delta\Delta Ct}$ error progression % met the inclusion criteria (see chapter 3.3.5 qPCR evaluation method). Furthermore, all SDs of the relative KLKs mRNA expression levels in the three repetitions were less than 47.1% (**Figure 5**). Thus, based on our criteria, none of the three samples would have been excluded for subsequent

analyses, which also validated the stability of the established qPCR assays for KLK4, 5, and 7. However, KLK12 mRNA expression was not detected in the three samples, even after repetitions. We further analyzed 32 ovarian cancer specimens and observed that KLK12 mRNA was not detected in these ovarian cancer tissues as well. Thus, in this ovarian cancer subgroup, expression of KLK12 mRNA seems to be very low, even absent. Therefore, subsequent quantification of KLK12 was not performed in the HGSOC cohort.

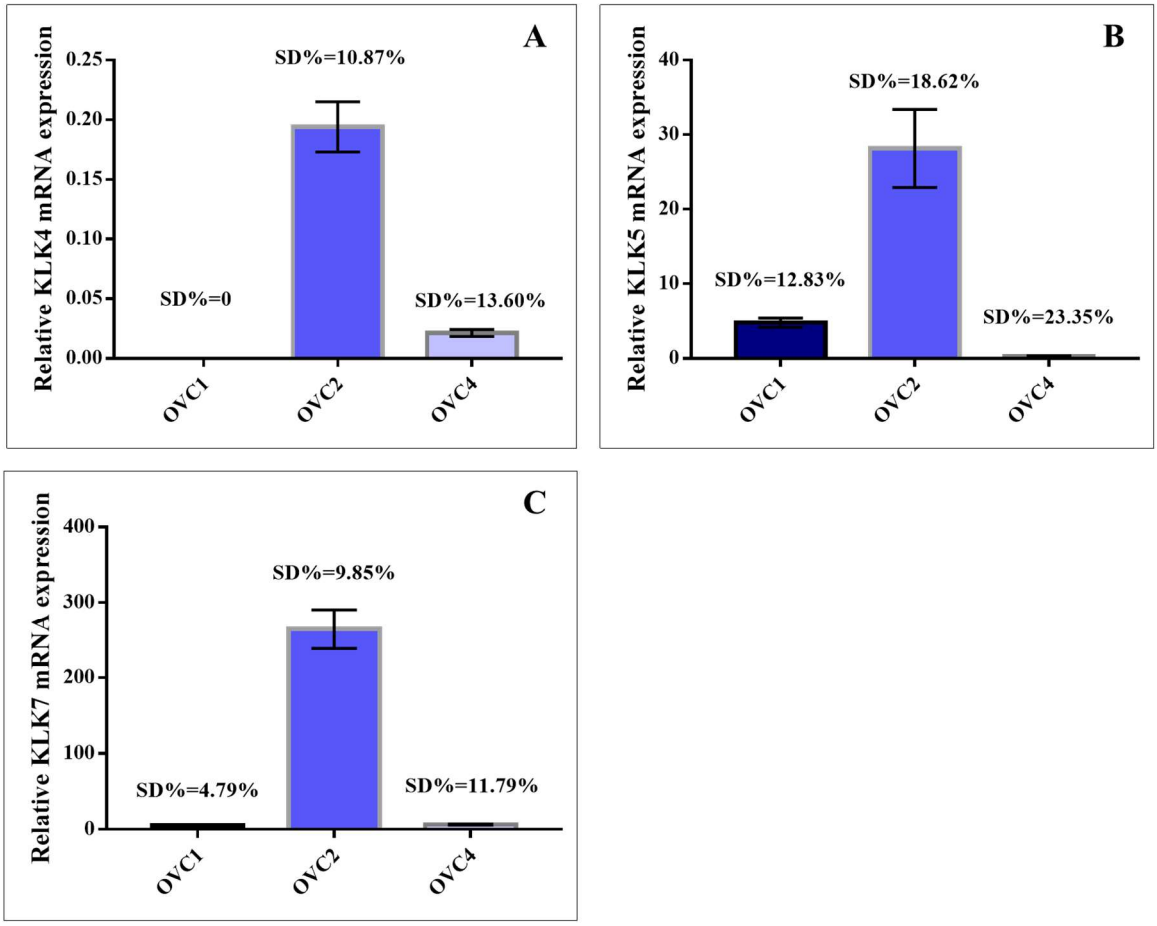

Figure 5. Mean values and standard deviations of relative KLK mRNA expression in three ovarian cancer specimens

In three separated qPCR experiments, standard deviations of relative mRNA expression of KLK4 (**A**), KLK5 (**B**), and KLK7 (**C**) were all less than 47.1% in three ovarian cancer specimens. KLK12 mRNA expression was not detected in the three ovarian cancer samples during the three repetitions. cDNA from OV-MZ-6 cell lines overexpressing KLK4, 5, 7, and 12, respectively, were used as calibrators. OVC: tumor tissues of ovarian cancer patients.

4.1.2 Determination of KLK mRNA expression by qPCR in advanced high-grade serous ovarian cancer

The KLKs mRNA levels were determined in the well-defined homogeneous cohort including 139 patients with HGSOC, FIGO stage III/IV (for details see chapter 3.1; Gong et al., 2019a, 2019b). Relative KLK mRNA expression was calculated with normalization against HPRT and the calibrators. The relative KLK4 mRNA expression levels were in the range of 0 to 0.44 (median, 0.019). Thus, rather low KLK4 mRNA expression was observed in the majority of cases (**Figure 6A**). Based on the expression pattern, the median value (50^{th} percentile) was defined as the cut-off point for KLK4 to categorize its mRNA expression levels into a low- and a high-expression group.

Relative KLK5 mRNA expression levels ranged from 0 to 644.31 (median, 16.87), while relative KLK7 mRNA expression levels were in the range of 0 to 953.22 (median, 79.25). Most ovarian cancer specimens displayed robust KLK5 as well as KLK7 expression (**Figure 6B**, **Figure 6C**, respectively). Based on the expression patterns, KLK5 and KLK7 mRNA expression levels were both dichotomized by the cut-off point at the 67^{th} percentile into a low-expressing group (tertiles 1+2) versus a high-expressing group (tertile 3).

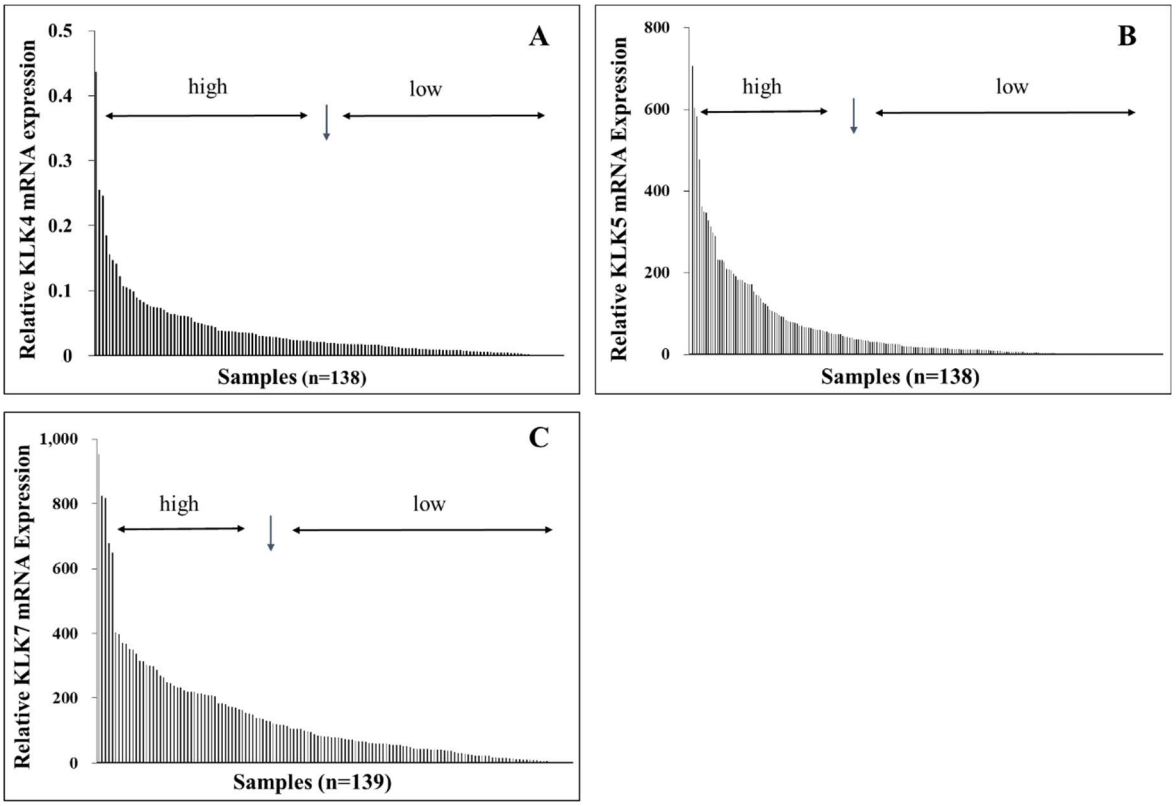

Figure 6. Relative KLK mRNA expression in tumor tissues of patients afflicted with advanced high-grade serous ovarian cancer

The cumulative histograms represent relative KLK mRNA expression levels. The majority of samples presented very low KLK4 mRNA expression, while most cases displayed robust mRNA expression levels of KLK5 and KLK7. For further analysis, we classified KLK4 (**A**) mRNA levels into a low- versus a high-expression group by the median (50^{th} percentile), while both KLK5 (**B**) and KLK7 (**C**) mRNA levels were categorized into a low-expressing group (tertiles 1+2) versus a high-expressing group (tertile 3) by 67^{th} percentile.

Including available protein expression data of KLK5 and KLK7, previously determined by ELISA method from a partially-overlapping cohort (Dorn et al., 2014, 2016), the relationship of their mRNA expression with corresponding protein expression was evaluated (n=46).

Spearman rank correlation analysis showed that KLK5 protein expression levels are significantly associated with its mRNA expression levels (r_s=0.689, $p<0.001$). This finding was also investigated by box plot analysis (**Figure 7A**), where higher KLK5 protein levels are present in the group with elevated KLK5 mRNA expression and vice

versa (Mann-Whitney test, $p<0.001$). Similarly, a strong positive correlation between KLK7 mRNA and protein levels was observed using Spearman rank correlation analysis ($r_s=0.663$, $p<0.001$), which was further validated by box plot analysis (Mann-Whitney test, $p=0.007$). As demonstrated in **Figure 7B**, high KLK7 protein levels are significantly associated with increased KLK7 mRNA expression.

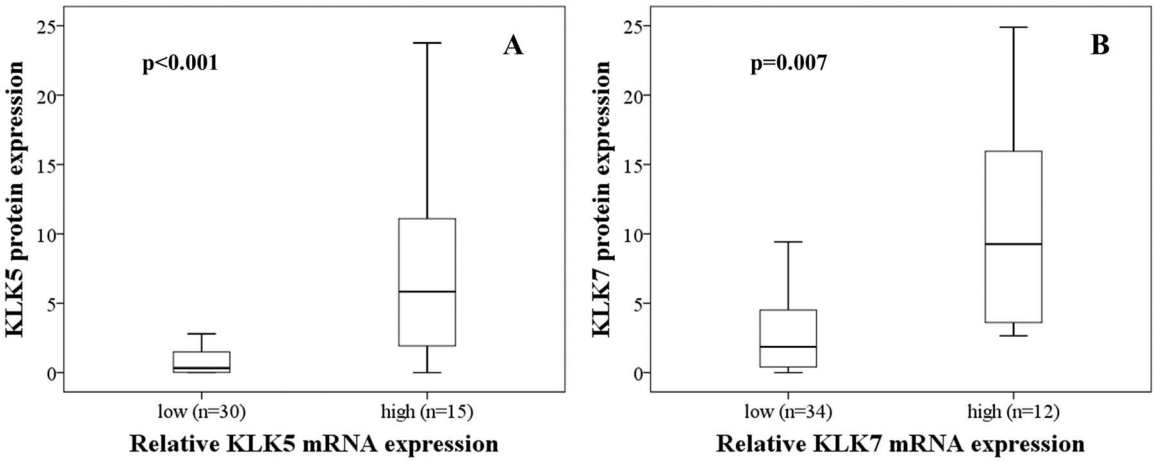

Figure 7. Correlation of KLK5 and KLK7 mRNA levels with their protein levels in tumor tissues of advanced high-grade serous ovarian cancer

(**A**) Higher KLK5 protein levels are present in the KLK5 mRNA high expression group and *vice versa* (Mann-Whitney test, $p<0.001$). (**B**) High KLK7 protein levels are significantly associated with increased KLK7 mRNA expression (Mann-Whitney test, $p=0.007$). Relative KLK mRNA expression was determined by qPCR and protein levels were measured by ELISA.

Moreover, Spearman correlation analysis was also performed among these KLKs. KLK5 mRNA levels are significantly correlated with KLK7 mRNA levels ($r_s=0.568$, $p<0.001$); also, a similar association was observed between KLK5 and KLK7 protein expression levels ($r_s=0.805$, $p<0.001$). This relationship was further evident in box plot analysis, where KLK7 mRNA levels are significantly higher in the KLK5 mRNA high group (Mann-Whitney test, $p<0.001$; **Figure 8A**), and, similarly, KLK7 antigen levels are elevated in the KLK5 antigen high group (Mann-Whitney test, $p<0.001$; **Figure 8B**). These results strongly suggested the coordinate expression of KLK5 with KLK7 in HGSOC. There is no obvious correlation among other KLKs in HGSOC ($r_s<0.2$).

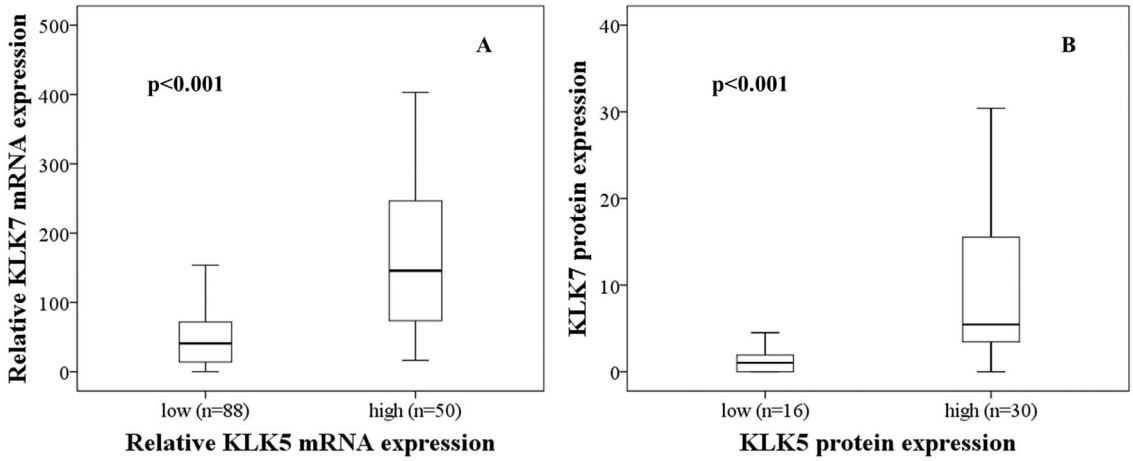

Figure 8. Correlation between KLK5 and KLK7 mRNA expression in tumor tissues of advanced high-grade serous ovarian cancer

(**A**) Higher KLK7 mRNA levels were observed in the KLK5 mRNA high group (Mann-Whitney test, $p<0.001$). (**B**) Higher KLK7 antigen levels were observed in the KLK5 antigen high group (Mann-Whitney test, $p<0.001$. Relative KLK mRNA expression was determined by qPCR and protein levels were measured by ELISA.

4.1.3 Association of KLK mRNA expression with clinical parameters in advanced high-grade serous ovarian cancer

The relationship of dichotomized KLK mRNA expression (low/high) with established clinical parameters, including age, residual tumor mass, and pre-operative ascites fluid volume, are summarized in **Table 14**. KLK4 mRNA levels correlated with age and pre-operative ascites fluid volume ($p=0.006$, $p=0.042$, respectively). A significant association was also observed between KLK5 mRNA expression and residual tumor mass ($p=0.041$). As to KLK7, its mRNA levels did not display any association with the above mentioned clinical variables.

Table 14. Association between KLK mRNA expression and clinical parameters in patients with advanced high-grade serous ovarian cancer

Clinical parameters	KLK4[a] low/high	KLK5[a] low/high	KLK7[a] low/high
Age	**p=0.006**	p=0.476	p=0.564
≤ 60 years	37/21	35/23	40/18
> 60 years	32/48	53/27	56/25
Residual tumor mass	p=0.303	**p=0.041**	p=0.234
0 mm	38/32	51/19	51/19
> 0 mm	30/36	37/29	44/23
Ascites fluid volume	**p=0.042**	p=0.087	p=0.261
≤ 500 ml	45/33	54/23	54/24
> 500 ml	21/32	30/24	41/13

[a] Chi-square test (cut-off point: KLK4 = 50th percentile, KLK5 = 67th percentile, KLK7 = 67th percentile).
Bold values indicate statistical significance (p≤0.05).

4.1.4 Association of KLK mRNA expression and established clinical parameters with progression-free survival and overall survival in advanced high-grade serous ovarian cancer

The prognostic values of KLK mRNA expression and clinical parameters for the patient outcome (PFS/OS) were estimated by univariate and multivariate Cox regression analyses. In univariate Cox regression analysis (**Table 15, Table 16**), residual tumor mass and ascites fluid volume are univariate predictors both for PFS and OS. Patients with residual tumor mass (>0 mm) have a significantly higher risk of disease relapse and cancer-related death, compared to the tumor-free cases. A larger ascites fluid volume (>500 ml) predicts worse PFS and OS.

In HGSOC, KLK4 mRNA expression (**Table 15**) represents an unfavorable predictive factor for OS (HR: 2.28, 95%CI: 1.38-3.76, p=0.001), indicating an approximately two-fold increased probability of cancer-related death in the KLK4 high-expressing group. However, no significant association was observed between KLK4 mRNA levels and PFS. Regarding KLK5 (**Table 16**), elevated mRNA levels are significantly associated

with shortened PFS (HR: 1.60, 95% CI: 1.01-2.55, p=0.047), but not with OS. Elevated KLK7 mRNA levels (**Table 16**) present an unfavorable prognostic value for PFS (HR: 1.75, 95% CI: 1.07-2.84, p=0.025) and show a trend towards significance in case of OS (HR: 1.66, 95% CI: 0.99-2.79, p=0.055).

Table 15. Univariate Cox regression analysis of KLK4 mRNA expression and clinical parameters with patient survival in advanced high-grade serous ovarian cancer

Clinical parameters	PFS			OS		
	No[a]	HR (95% CI)[b]	p	No[a]	HR (95% CI)[b]	p
Age			0.627			0.348
≤ 60 years	43	1		50	1	
> 60 years	65	1.12 (0.70-1.79)		76	1.27 (0.77-2.08)	
Residual tumor mass			**<0.001**			**<0.001**
0 mm	59	1		64	1	
> 0 mm	49	2.53 (1.60-4.02)		60	3.76 (2.18-6.48)	
Ascites fluid volume			**0.018**			**0.011**
≤ 500 ml	63	1		72	1	
> 500 ml	39	1.78 (1.10-2.87)		47	1.93 (1.16-3.18)	
KLK4 mRNA[c]			0.121			**0.001**
low	55	1		62	1	
high	52	1.44 (0.91-2.78)		63	2.28 (1.38-3.76)	

[a] Number of patients;
[b] HR: hazard ratio (CI: confidence interval) of univariate Cox regression analysis;
[c] Dichotomized into low and high levels by the 50th percentile;
Bold values indicate statistical significance (p≤0.05).

Table 16. Univariate Cox regression analysis of KLK5 and KLK7 mRNA expression and clinical parameters with patient survival in advanced high-grade serous ovarian cancer

Clinical parameters	PFS			OS		
	No[a]	HR (95% CI)[b]	p	No[a]	HR (95% CI)[b]	p
Age			0.762			0.414
≤ 60 years	43	1		49	1	
> 60 years	62	1.08 (0.67-1.72)		70	1.24 (0.74-2.06)	
Residual tumor mass			**<0.001**			**<0.001**
0 mm	58	1		60	1	
> 0 mm	47	2.41 (1.53-3.90)		57	3.80 (2.17-6.65)	
Ascites fluid volume			**0.019**			**0.005**
≤ 500 ml	61	1		66	1	
> 500 ml	38	1.79 (1.10-2.90)		46	2.10 (1.25-3.54)	
KLK5 mRNA[c]			**0.047**			0.269
low	62	1		73	1	
high	42	1.60 (1.01-2.55)		45	1.33 (0.80-2.20)	
KLK7 mRNA[d]			**0.025**			*0.055*
low	73	1		82	1	
high	32	1.75 (1.07-2.84)		37	1.66 (0.99-2.79)	

[a] Number of patients;
[b] HR: hazard ratio (CI: confidence interval) of univariate Cox regression analysis;
[c] Dichotomized into low and high levels by the 67th percentile;
[d] Dichotomized into low and high levels by the 67th percentile;
Bold values indicate statistical significance ($p \leq 0.05$); values in italics indicate a trend towards significance.

Furthermore, the impact of KLKs on clinical outcome was validated by the respective Kaplan-Meier survival analysis. The patients in the KLK4 mRNA high group (**Figure 9**) have a worse OS, compared to the low subgroup. KLK5 overexpression (**Figure 10**) as well as KLK7 overexpression (**Figure 11**) were significantly correlated with shortened PFS.

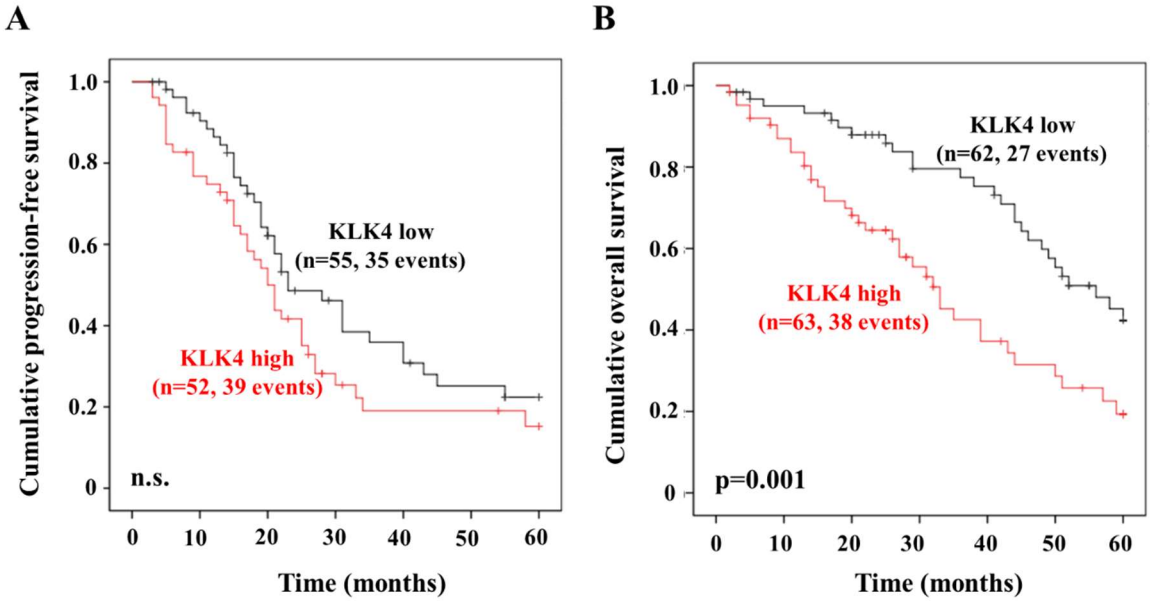

Figure 9. Kaplan–Meier survival analysis concerning KLK4 mRNA expression in patients with advanced high-grade serous ovarian cancer

Patients with elevated KLK4 mRNA levels display a significantly worse OS (p=0.001, **B**) but not PFS (**A**), compared with those with low KLK4 mRNA levels.

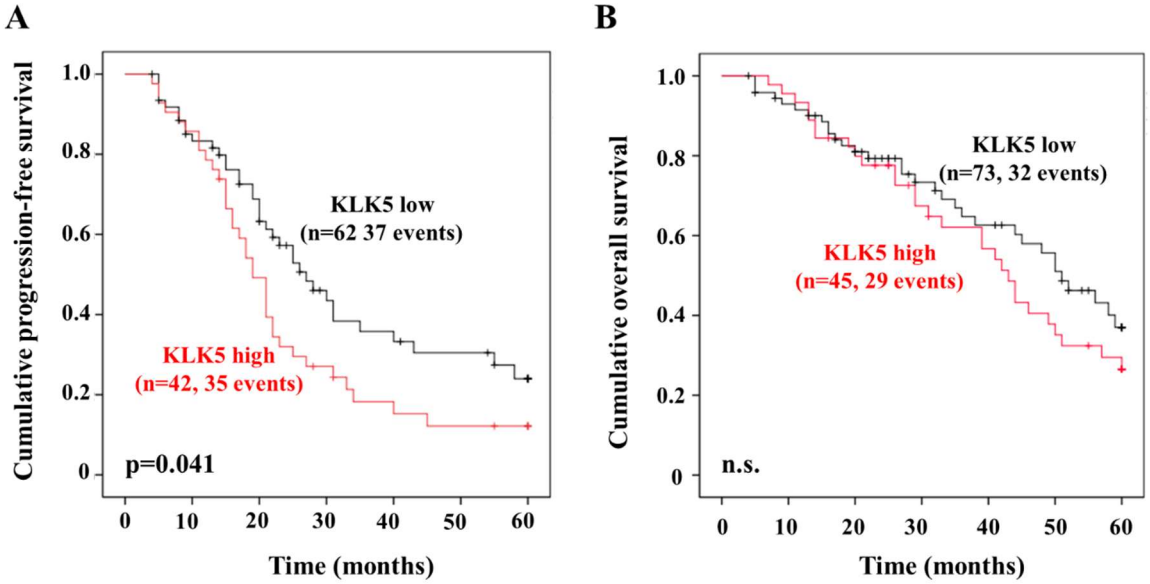

Figure 10. Kaplan–Meier survival analysis concerning KLK5 mRNA expression in patients with advanced high-grade serous ovarian cancer

Patients with elevated KLK5 mRNA levels display a significantly shortened PFS (p=0.041, **A**) but not OS (**B**), compared with those with low KLK5 mRNA levels.

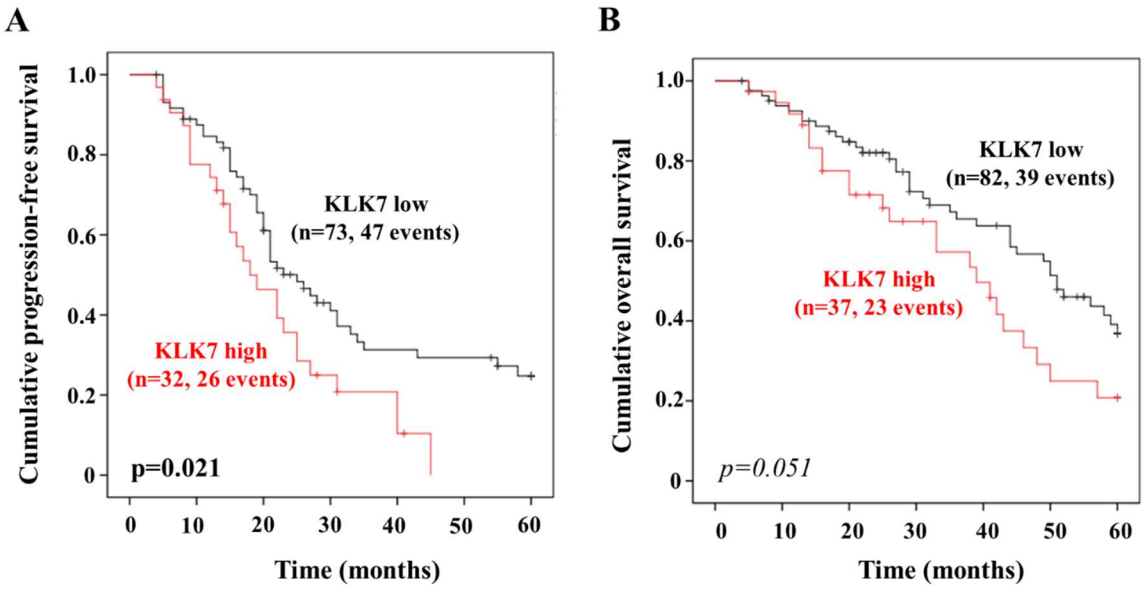

Figure 11. Kaplan–Meier survival analysis concerning KLK7 mRNA expression in patients with advanced high-grade serous ovarian cancer

Patients with elevated KLK7 mRNA levels display a significantly worse PFS (p = 0.021, **A**) and show a trend towards significance in case of OS (**B**), compared with those with low KLK7 mRNA levels.

Multivariate Cox regression analysis was performed to assess the independent prognostic value of the clinical parameters and KLK mRNA expression in HGSOC. First, a base model was established, consisting of the established clinical parameters age, residual tumor mass, and ascites fluid volume. Here, residual tumor mass is the only independent predictive marker for the outcome, while ascites fluid volume loses its prognostic value. Next, the KLKs were individually added to the base model, summarized in **Table 17-19**. When subjected to multivariate Cox regression analysis, KLK4 mRNA expression retains the unfavorable predictive power for OS (HR: 2.31, 95% CI: 1.27-4.20, p=0.006; **Table 17**). KLK5 mRNA expression may have a trend towards significance for PFS (HR: 1.53, 95% CI: 0.93-2.51, p=0.095; **Table 18**). In case of KLK7 (**Table 19**), mRNA expression represents an unfavorable predictor for PFS (HR: 2.19, 95% CI: 1.23-3.89, p=0.007) and OS (HR: 1.94, 95% CI: 1.06-3.55, p=0.032). To sum up, KLK4 and KLK7 mRNA expression were demonstrated to be independent biomarkers for patient outcome in HGSOC.

Table 17. Multivariate Cox regression analysis of KLK4 mRNA expression and clinical parameters with patient survival in advanced high-grade serous ovarian cancer

Clinical parameters	PFS			OS		
	No[a]	HR (95% CI)[b]	p	No[a]	HR (95% CI)[b]	p
Age			0.733			0.470
≤ 60 years	41	1		47	1	
> 60 years	60	0.92 (0.56-1.51)		69	1.22 (0.72-2.07)	
Residual tumor mass			**0.002**			**<0.001**
0 mm	58	1		63	1	
> 0 mm	43	2.36 (1.38-4.05)		53	3.58 (1.90-6.74)	
Ascites fluid volume			0.474			0.911
≤ 500 ml	63	1		71	1	
> 500 ml	38	1.22 (0.71-2.10)		45	1.03 (0.58-1.86)	
KLK4 mRNA[c]			0.284			**0.006**
low	52	1		58	1	
high	49	1.32(0.79-2.20)		58	2.31 (1.27-4.20)	

The biological marker KLK4 mRNA was added to the base model of clinical parameters, which included age, residual tumor mass, and ascites fluid volume. Significant p-values ($p \leq 0.05$) are indicated in bold.

[a] Number of patients;

[b] HR: hazard ratio (CI: confidence interval) of multivariable Cox regression analysis;

[c] Dichotomized into low and high levels by 50th percentile.

Table 18. Multivariate Cox regression analysis of KLK5 mRNA expression and clinical parameters with patient survival in advanced high-grade serous ovarian cancer

Clinical parameters	PFS			OS		
	No[a]	HR (95% CI)[b]	p	No[a]	HR (95% CI)[b]	p
Age			0.592			0.660
≤ 60 years	41	1		46	1	
> 60 years	57	0.87 (0.53-1.44)		63	1.13 (0.65-1.96)	
Residual tumor mass			**0.005**			**<0.001**
0 mm	57	1		59	1	
> 0 mm	41	2.20 (1.17-3.81)		50	3.29 (1.69-6.41)	
Ascites fluid volume			0.363			0.605
≤ 500 ml	60	1		64	1	
> 500 ml	38	1.33 (0.76-2.31)		45	1.18 (0.64-1.91)	
KLK5 mRNA[c]			0.095			0.718
low	59	1		69	1	
high	39	1.53 (0.93-2.51)		40	1.11 (0.64-1.91)	

The biological marker KLK4 mRNA was added to the base model of clinical parameters, which included age, residual tumor mass, and ascites fluid volume. Significant p-values ($p \leq 0.05$) are indicated in bold.

[a] Number of patients;

[b] HR: hazard ratio (CI: confidence interval) of multivariable Cox regression analysis;

[c] Dichotomized into low (tertiles 1+2) and high (tertile 3) levels by 67th percentile.

Table 19. Multivariate Cox regression analysis of KLK7 mRNA expression and clinical parameters with patient survival in advanced high-grade serous ovarian cancer

Clinical parameters	PFS			OS		
	No[a]	HR (95% CI)[b]	p	No[a]	HR (95% CI)[b]	p
Age			0.633			0.609
≤ 60 years	41	1		46	1	
> 60 years	58	0.89 (0.54-1.46)		64	1.15 (0.67-1.99)	
Residual tumor mass			**0.003**			**<0.001**
0 mm	57	1		59	1	
> 0 mm	42	2.26 (1.32-3.89)		51	3.42 (1.77-6.62)	
Ascites fluid volume			0.363			0.696
≤ 500 ml	61	1		65	1	
> 500 ml	38	1.29 (0.75-2.23)		45	1.13 (0.61-2.09)	
KLK7 mRNA[c]			**0.007**			**0.032**
low	72	1		80	1	
high	27	2.19 (1.23-3.89)		30	1.94 (1.06-3.55)	

The biological marker KLK4 mRNA was added to the base model of clinical parameters, which included age, residual tumor mass, and ascites fluid volume. Significant p-values ($p \leq 0.05$) are indicated in bold.

[a] Number of patients;

[b] HR: hazard ratio (CI: confidence interval) of multivariable Cox regression analysis;

[c] Dichotomized into low (tertiles 1+2) and high (tertile 3) levels by 67th percentile.

4.1.5 Validation of the association of KLK mRNA expression with patients survival in advanced high-grade serous ovarian cancer by *in silico* analysis using publicly available data

To further estimate the association of KLK mRNA expression with patient outcome in HGSOC, the online biomarker assessment tool, Kaplan-Meier Plotter, was used to analyze the publicly available Affymetrix-based mRNA data of ovarian cancer patients from The Cancer Genome Atlas (Gyorffy et al., 2012). For this, patients with clinical characteristics of advanced stage (FIGO III+IV), high-grade (grade 3) and serous ovarian cancer, receiving platinum-based chemotherapy, with a follow-up of 5 years

and dichotomized by the same cut-off point used in the present study, were selected to evaluate and plot the Kaplan–Meier survival curves. For KLK4 (Affymetrix probe ID: 1555737_a_at), 249 cases for PFS and 252 cases for OS, respectively, were enrolled for Kaplan–Meier survival analysis, showing that elevated KLK4 mRNA expression is significantly linked with poor OS (p=0.047), but also with PFS (p=0.032) (**Figure 12**). In case of KLK5, 377 (PFS) and 398 (OS) patients could be enrolled to evaluate the predictive power of KLK5 (Affymetrix probe ID: 222242_s_at). Here, KLK5 represents an unfavorable biomarker for PFS (p=0.027), but not for OS (**Figure 13**). In contrast to our finding, KLK7 mRNA (Affymetrix probe ID: 239381_at) did not contribute to the prediction of OS or PFS in the selected cohort consisting of 249 patients for PFS and 252 patients for OS (**Figure 14**).

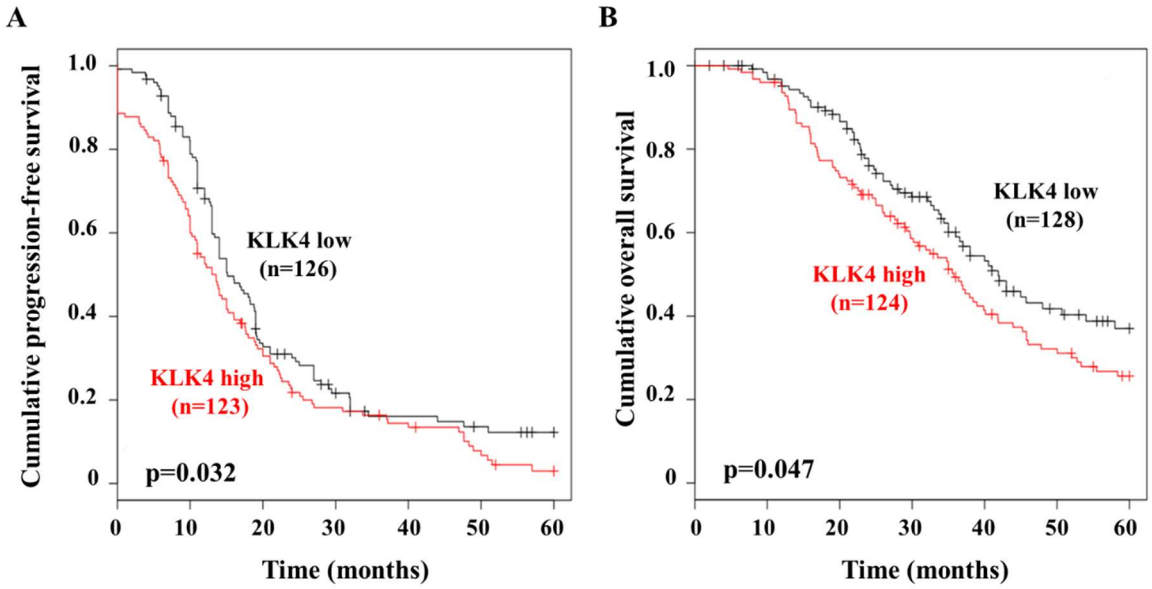

Figure 12. Association of KLK4 mRNA expression with patient outcome in the publicly available Affymetrix microarray data set

In this *in silico* analysis, 249 patients for PFS and 252 patients for OS were enrolled for Kaplan-Meier analysis. The Kaplan-Meier survival curves indicate that elevated KLK4 mRNA expression (probe ID: 1555737_a_at) is significantly linked with poor PFS (p=0.032, **A**), but also for OS (p=0.047, **B**).

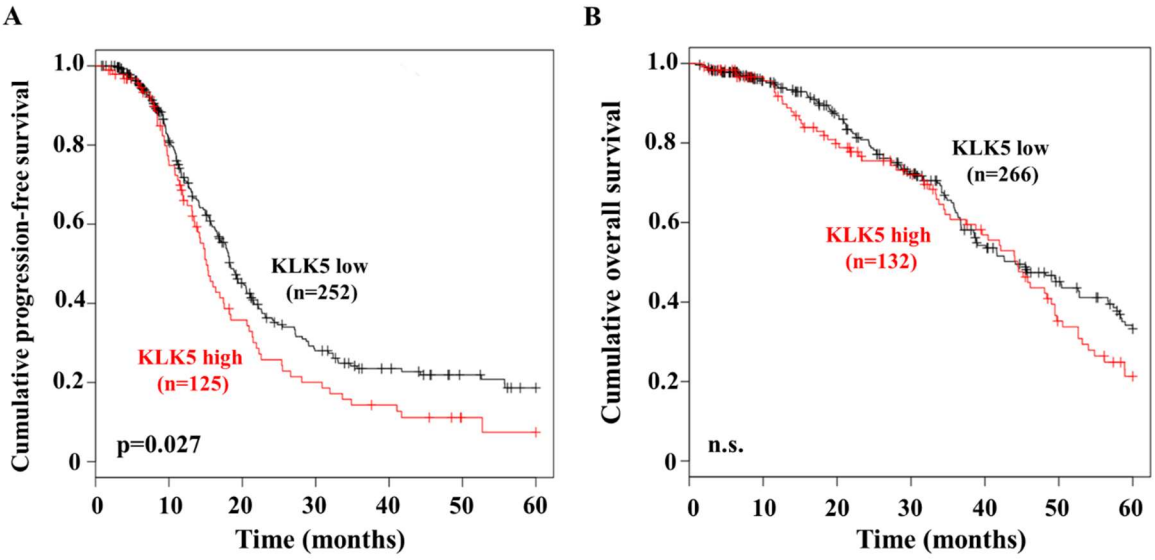

Figure 13. Association of KLK5 mRNA expression with patient outcome in the publicly available Affymetrix microarray data set

377 patients (PFS) and 398 patients (OS) were selected to calculate the predictive power of KLK5 (probe ID: 222242_s_at). The Kaplan-Meier survival curves indicated that KLK5 represents an unfavorable biomarker for PFS (p=0.027, **A**), but not for OS (**B**).

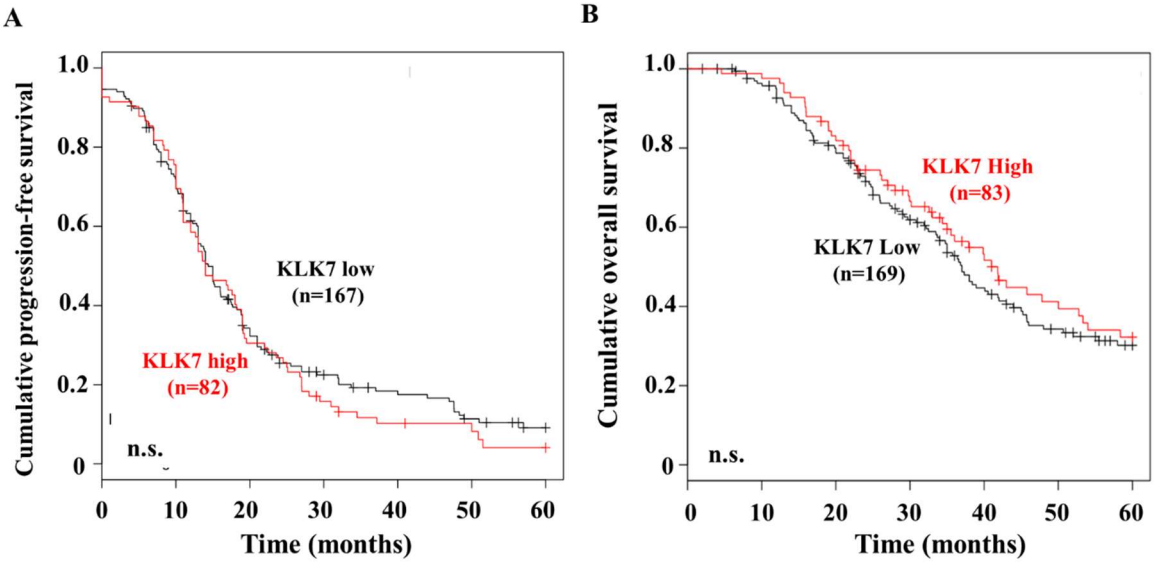

Figure 14. Association of KLK7 mRNA expression with patient outcome in the publicly available Affymetrix microarray data set

As to KLK7 (probe ID: 239381_at), 249 patients were analyzed for PFS (**A**) and 252 patients for OS (**B**). The Kaplan-Meier survival curves indicated that KLK7 mRNA did not contribute to the prediction of OS or PFS.

4.2 Assessment of KLK mRNA expression by qPCR in triple-negative breast cancer (cohort 2)

4.2.1 KLK mRNA expression in tumor tissues of triple-negative breast cancer

The mRNA levels of KLK4, KLK5, KLK7, and KLK12 were also quantified in primary tumor tissues from a well-defined breast cancer cohort, including 125 patients afflicted with TNBC, applying the newly established qPCR assay. The relative KLK4 mRNA expression levels ranged from 0 to 8.19 (median, 0.08); regarding KLK5, the relative mRNA expression was in the range of 0 to 1778.77 (median, 8.08); relative KLK7 mRNA levels ranged from 0 to 302.33 (median, 4.44). In most of the TNBC specimens, KLK4 mRNA displayed a low expression, while robust KLK5 and KLK7 mRNA expression levels were observed. Based on the expression patterns, KLK4 mRNA expression levels (**Figure 15A**) were categorized into a low-expressing group (tertiles 1+2) versus a high-expressing group (tertile 3) by 67th percentile, which was also the cut-off point used for KLK5 (**Figure 15B**) and KLK7 (**Figure 15C**).

In case of KLK12, the relative mRNA expression levels ranged from 0 to 0.38 (median, 0.00), whereby more than half of the cases (62/114, 54%) displayed a negative expression (**Figure 15D**). Based on this, KLK12 mRNA expression was categorized into a negative-expressing group (54%) versus a positive-expressing group (46%).

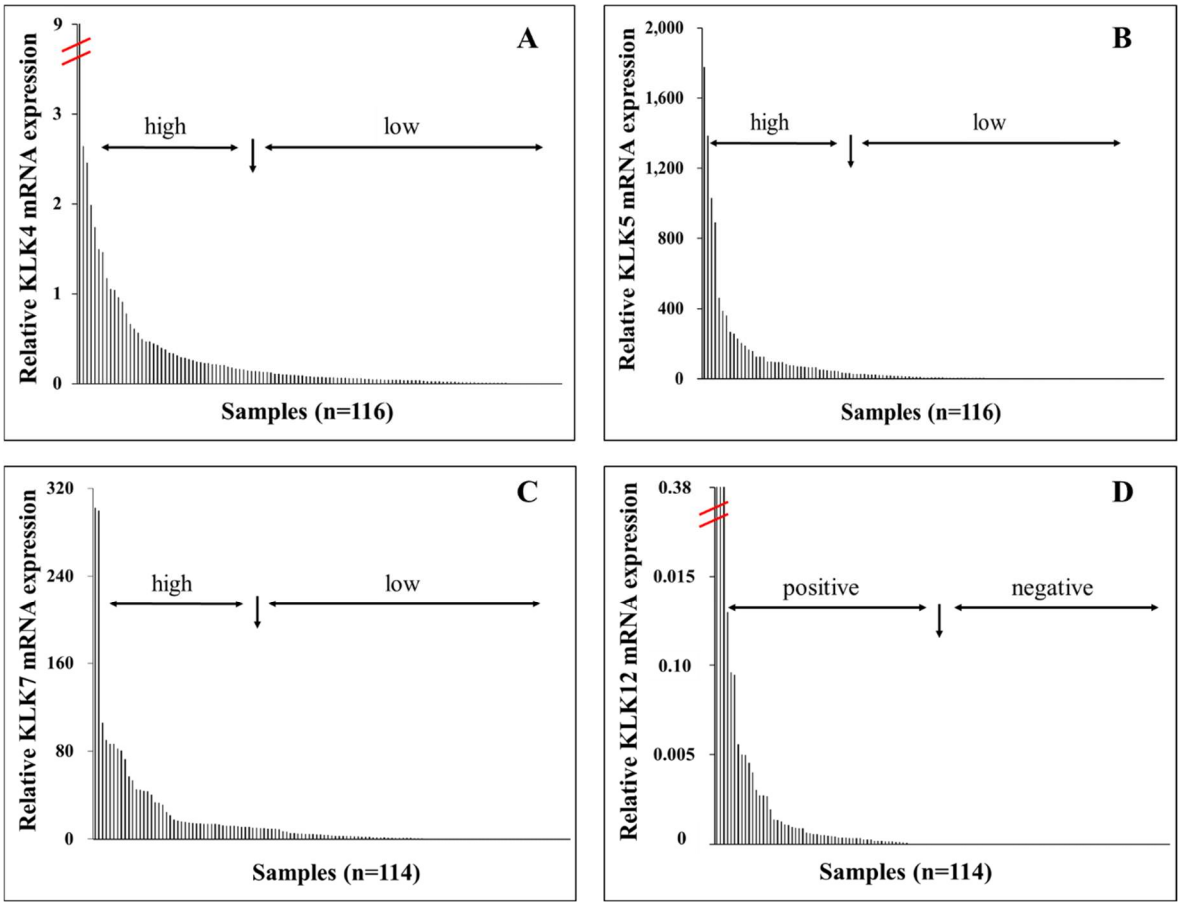

Figure 15. Relative KLK mRNA expression in tumor tissues of patients afflicted with triple-negative breast cancer

Most of the cases in the TNBC cohort display low mRNA expression of KLK4 and KLK12, while KLK5 and KLK7 show robust mRNA expression patterns. Based on this, the relative mRNA expression levels of KLK4 (**A**), KLK5 (**B**) and KLK7 (**C**) were categorized into a low-expressing group (tertiles 1+2) versus a high-expressing group (tertile 3) by 67th percentile, whereas relative KLK12 mRNA expression (**D**) was classified into a negative-expressing group (54%) versus a positive-expressing group (46%).

Spearman correlation analysis was also performed among these KLKs. A strong correlation was observed between KLK5 mRNA expression and KLK7 mRNA expression (r_s=0.735, p<0.001). This relationship was also evident in box plot analysis, where KLK7 mRNA levels are significantly higher in the KLK5 elevated group (Mann-Whitney test, p<0.001; **Figure 16**), strongly suggesting the coordinate expression of KLK5 with KLK7 in TNBC. There is no obvious correlation among other KLKs in

TNBC (r_s<0.2).

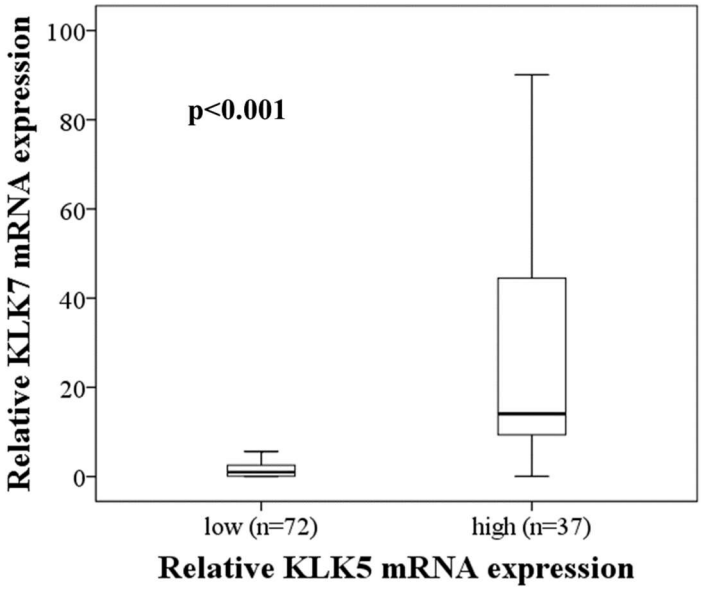

Figure 16. Correlation between KLK5 and KLK7 mRNA expression in tumor tissues of triple-negative breast cancer

KLK7 mRNA levels are significantly higher in the KLK5 elevated group (Mann-Whitney test, p<0.001), compared to the KLK5 low group. Relative KLK mRNA expression was determined by qPCR.

4.2.2 Association of KLK mRNA expression with clinicopathological parameters in triple-negative breast cancer

In the TNBC cohort, the association between KLK mRNA expression levels and the established clinicopathological parameters (age, lymph node status, tumor size, and histological grade) was estimated applying the Pearson Chi-square (χ^2) test. As depicted in **Table 20**, histological grade is significantly associated with KLK4 mRNA expression levels (p=0.004). No other relationship between KLK mRNA expression and the mentioned clinicopathological parameters was observed in this tumor entity.

Table 20. Association between KLK mRNA expression and clinicopathological parameters in patients with triple-negative breast cancer

Clinicopathological parameters	KLK4[a] low/high	KLK5[b] low/high	KLK7[c] low/high	KLK12[d] negative/ positive
Age	p=0.320	p=0.681	p=0.353	p=0.492
≤ 60 years	45/19	42/22	43/18	35/26
> 60 years	32/20	36/16	33/20	27/26
Lymph node status	p=0.127	p=0.189	p=0.595	p=0.215
N0	47/18	47/18	40/22	37/25
N1/N2/N3	30/21	31/20	38/16	25/27
Tumor size	p=0.969	p=0.453	p=0.936	p=0.731
≤20 mm	20/10	22/8	20/10	17/13
>20 mm	57/28	56/29	56/27	44/39
Histological grade	**p=0.004**	p=0.279	*p=0.052*	p=0.747
Grade II	3/8	9/2	11/1	6/6
Grade III	74/31	69/36	65/37	56/46

[a] Chi-square test (dichotomized into low and high groups by the 67th percentile);
[b] Chi-square test (dichotomized into low and high groups by the 67th percentile);
[c] Chi-square test (dichotomized into low and high groups by the 67th percentile);
[d] Chi-square test (dichotomized into negative and positive group).
Bold values indicate statistical significance (p≤0.05), values in italics indicate a trend towards significance.

4.2.3 Assessment of the prognostic impact of KLK mRNA expression and clinicopathological parameters on disease-free survival and overall survival in triple-negative breast cancer

The impact of KLKs and the clinicopathological parameters on patient outcome (DFS and OS) was investigated applying univariate and multivariate Cox regression analyses in the TNBC cohort.

As shown in **Table 21**, among the clinical parameters, age is a univariate prognostic factor for the patient outcome (DFS and OS). The patients in the advanced age subgroup (>60 years) have an elevated risk of cancer-related death (HR: 2.92, 95% CI: 1.61-5.30, p<0.001) as well as disease recurrence (HR: 2.35, 95% CI: 1.36-4.07, p=0.002). Lymph

node status (N0 vs. N+) is a univariate predictor for OS (HR: 1.78, 95% CI: 1.01-3.13, p=0.046), while it only approaches statistical significance for DFS (HR: 1.63, 95% CI: 0.95-2.78, p=0.075). The KLK4 mRNA represents an unfavorable predictor for DFS (HR: 1.83, 95% CI: 1.03-3.26, p=0.040) and OS (HR: 2.07, 95% CI: 1.13-3.80, p=0.019), revealing an approximately two-fold increased probability of disease progression and cancer-related death in the high KLK4 mRNA expression subgroup. Patients with positive KLK12 mRNA expression experience an increased risk of disease relapse (HR: 2.15, 95% CI: 1.20-3.84, p=0.010) and cancer-related death (HR: 2.00, 95% CI: 1.09-3.67, p=0.025), compared to the cases with negative KLK12 expression. Neither KLK5 nor KLK7 mRNA levels contribute to the patient outcome in the TNBC cohort.

Table 21. Univariate Cox regression analysis of KLK mRNA expression and clinicopathological parameters for the prediction of clinical outcome in triple-negative breast cancer

Clinicopathological parameters	DFS			OS		
	No[a]	HR (95% CI)[b]	p	No[a]	HR (95% CI)[b]	p
Age			**0.002**			**< 0.001**
≤ 60 years	66	1		66	1	
> 60 years	56	2.35 (1.36-4.07)		57	2.92(1.61-5.30)	
Lymph node status			*0.075*			**0.046**
N0	68	1		69	1	
N1/N2/N3	53	1.63 (0.95-2.78)		54	1.78 (1.01-3.13)	
Tumor size			0.130			0.118
≤20 mm	32	1		32	1	
>20 mm	88	1.70 (0.86-3.39)		90	1.83 (0.86-3.92)	
Histological grade			0.762			0.956
Grade II	12	1		12	1	
Grade III	109	1.15 (0.46-2.90)		111	1.03 (0.41-2.59)	
KLK4 mRNA[c]			**0.040**			**0.019**
low	74	1		75	1	
high	38	1.83 (1.03-3.26)		39	2.07 (1.13-3.80)	
KLK5 mRNA[d]			0.945			0.651
low	75	1		77	1	
high	37	1.02 (0.56-1.87)		37	0.86 (0.45-1.66)	
KLK7 mRNA[e]			0.544			0.847
low	74	1		76	1	
high	37	1.21 (0.66-2.21)		37	1.07 (0.56-2.02)	
KLK12 mRNA[f]			**0.010**			**0.025**
negative	59	1		61	1	
positive	52	2.15 (1.20-3.84)		52	2.00 (1.09-3.67)	

[a] Number of patients;

[b] HR: hazard ratio (CI: confidence interval) of univariate Cox regression analysis;

[c] Dichotomized into low and high groups by the 67th percentile;

[d] Dichotomized into low and high groups by the 67th percentile;

[e] Dichotomized into low and high groups by the 67th percentile;

[f] Dichotomized into negative and positive groups;

The bold value indicates statistical significance ($p \leq 0.05$). Italics indicate trends towards significance ($p \leq 0.09$).

The impact of KLKs on patient outcome was further validated by the respective Kaplan-Meier survival analyses. As shown in **Figure 17**, overexpression of KLK4 mRNA is significantly correlated with shortened DFS (p=0.037) and OS (p=0.016) in TNBC patients. In line with the finding in univariate Cox analysis, KLK5 and KLK7 mRNA expression levels are not related to the prognosis (**Figure 18**). Patients with KLK12 positive expression exhibit a significantly worse DFS (p=0.008) and OS (p=0.022), compared to the negative expression cases (**Figure 19**).

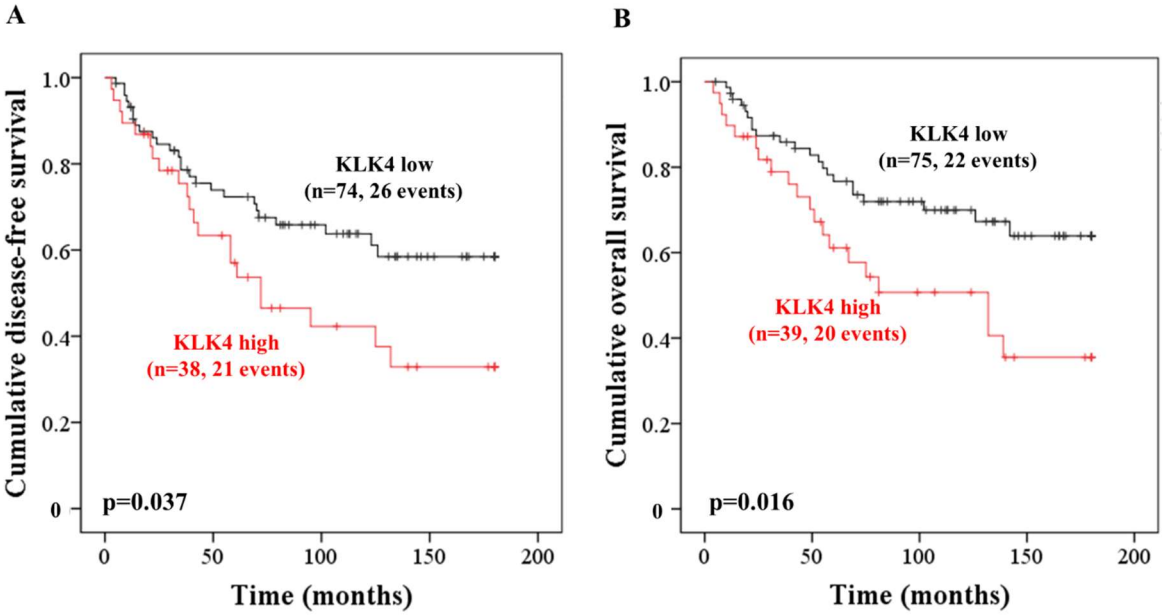

Figure 17. Association of KLK4 mRNA expression with clinical outcome, analyzed by Kaplan–Meier survival analysis in tumor tissues of triple-negative breast cancer

Patients with elevated KLK4 mRNA levels display a significantly shortened DFS (p=0.037; **A**) and OS (p=0.016; **B**), compared to those with low KLK4 mRNA levels.

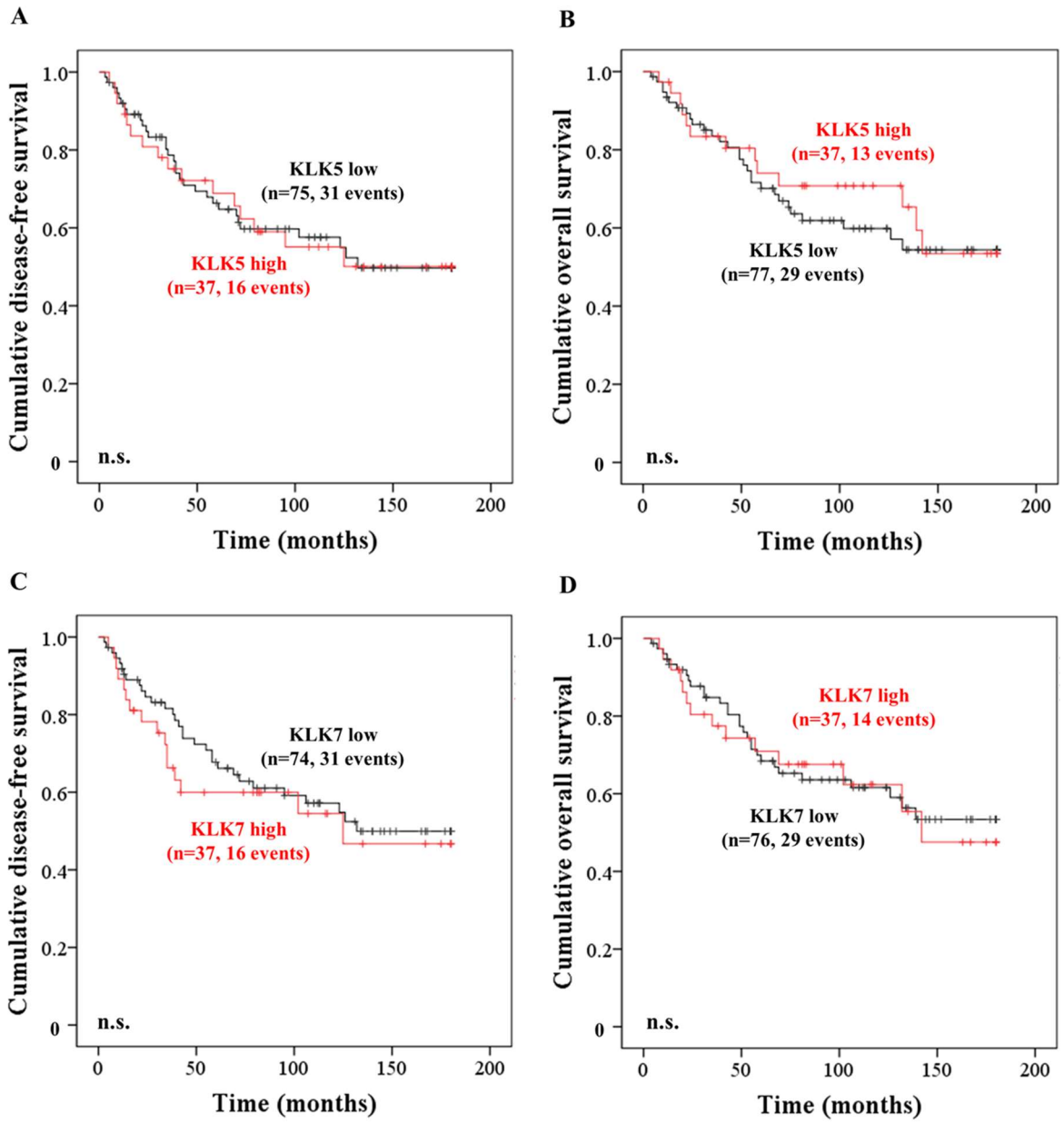

Figure 18. Association of KLK5 and KLK7 mRNA expression with clinical outcome analyzed by Kaplan–Meier survival analysis in tumor tissues of triple-negative breast cancer

Neither KLK5 nor KLK7 mRNA levels contribute to patient outcome in this TNBC cohort.

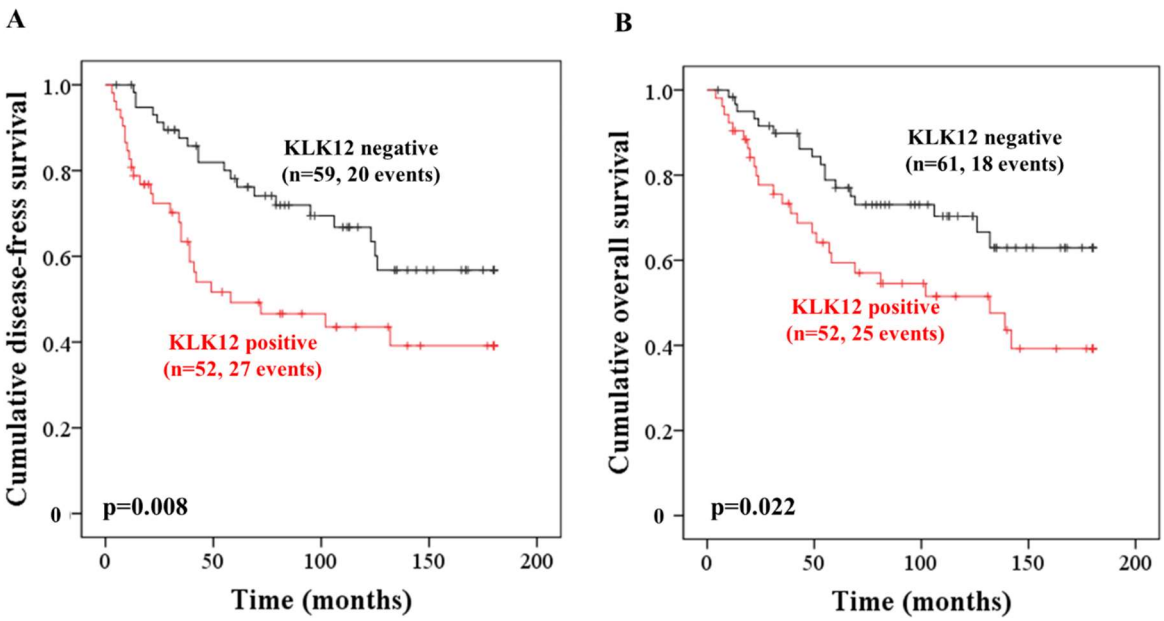

Figure 19. Association of KLK12 mRNA expression with clinical outcome, analyzed by Kaplan–Meier survival analysis in tumor tissues of triple-negative breast cancer

Patients with KLK12 mRNA positive expression show a significantly worse DFS (p=0.008; **A**) and OS (p=0.022; **B**), compared those with KLK12 mRNA negative expression.

Additionally, the independences of KLK4 (**Table 22**) and KLK12 (**Table 23**) in the prediction of prognosis were further investigated by the multivariate Cox regression analyses. Similar to the analyses in HGSOC, a base model was firstly established comprising age, lymph node status, tumor size, and histological grade. Upon addition to the base model (**Table 22**), KLK4 mRNA expression does not prove to be statistically significant, however, presents a trend towards significance for OS (HR: 1.83, 95% CI: 0.96-3.49, p=0.067). As to KLK12 (**Table 23**), after adjustment for the base model, its mRNA expression remains an unfavorable prognostic biomarker for DFS (HR: 2.16, 95% CI: 1.19-3.93, p=0.011), however, shows a trend towards significance for OS (HR: 1.82, 95% CI: 0.97-3.41, p=0.060). In summary, KLK12 was demonstrated to be an independent predictive marker for DFS in TNBC.

Table 22. Multivariate Cox regression analysis of KLK4 mRNA expression and clinicopathological parameters for the prediction of clinical outcome in triple-negative breast cancer

Clinicopathological parameters	DFS			OS		
	No[a]	HR (95% CI)[b]	p	No[a]	HR (95% CI)[b]	p
Age			**0.001**			**< 0.001**
≤ 60 years	61	1		61	1	
> 60 years	50	2.64 (1.45-4.82)		52	3.50 (1.80-6.82)	
Lymph node status			0.198			0.115
N0	62	1		63	1	
N1/N2/N3	49	1.47 (0.82-2.63)		50	1.66 (0.88-3.10)	
Tumor size			0.275			0.284
≤20 mm	30	1		30	1	
>20 mm	81	1.51 (0.72-3.15)		83	1.57 (0.69-3.59)	
Histological grade			0.732			0.911
Grade II	11	1		11	1	
Grade III	100	1.20 (0.42-3.39)		102	1.06 (0.37-3.03)	
KLK4 mRNA[c]			0.114			*0.067*
low	74	1		75	1	
high	37	1.64 (0.89-3.04)		38	1.83 (0.96-3.49)	

The hazard ratios of tumor markers were adjusted for the base model, including age, lymph node status, tumor size, and histological grade.

[a] Number of patients;

[b] HR: hazard ratio (CI: confidence interval) of univariate Cox regression analysis;

[c] Dichotomized into low and high group by the 67th percentile;

Bold values indicate statistical significance ($p \leq 0.05$), values in italics indicate a trend towards significance.

Table 23. Multivariate Cox regression analysis of KLK12 mRNA expression and clinicopathological parameters for the prediction of clinical outcome in triple-negative breast cancer

Clinicopathological parameters	DFS			OS		
	No[a]	HR (95% CI)[b]	p	No[a]	HR (95% CI)[b]	p
Age			**0.001**			**< 0.001**
≤ 60 years	59	1		59	1	
> 60 years	51	2.84 (1.54-5.24)		53	3.54 (1.81-6.90)	
Lymph node status			*0.052*			**0.037**
N0	60	1		61	1	
N1/N2/N3	50	1.80 (0.99-3.24)		51	1.96 (1.04-3.67)	
Tumor size			0.246			0.220
≤20 mm	30	1		30	1	
>20 mm	80	1.54 (0.74-3.21)		82	1.67 (0.74-3.79)	
Histological grade			0.757			0.947
Grade II	12	1		12	1	
Grade III	98	1.16 (0.45-2.98)		100	1.03 (0.40-2.68)	
KLK12 mRNA[c]			**0.011**			*0.060*
negative	58	1		60	1	
positive	52	2.16 (1.19-3.93)		52	1.82 (0.97-3.41)	

The hazard ratios of tumor markers were adjusted for the base model, including age, lymph node status, tumor size, and histological grade.

[a] Number of patients;

[b] HR: hazard ratio (CI: confidence interval) of univariate Cox regression analysis;

[c] Dichotomized into negative and positive group;

Bold values indicate statistical significance ($p \leq 0.05$), values in italics indicate a trend towards significance.

5 Discussion

A growing body of evidence has implied the potential predictive values of KLKs in a variety of carcinomas, including prostate (Samaan et al., 2014), gastric (Jiao et al., 2013), skin (Avgeris and Scorilas, 2016), colorectal (Alexopoulou et al., 2013), ovarian (Loessner et al., 2018) and breast (Avgeris et al., 2012) cancer. For instance, elevated KLK4 mRNA levels are associated with favorable outcome in laryngeal squamous cell carcinoma (Foteinou et al., 2014), while KLK4 overexpression represented an unfavorable predictor in oral (Papagerakis et al., 2015), prostate (Avgeris et al., 2011) and breast cancer (Yang et al., 2017). Both ovarian and breast cancer are heterogeneous diseases, of which the subtypes are distinct concerning morphology, biology, behaviors, and response to therapy (Jelovac and Armstrong, 2011; Cedolini et al., 2014). Conflicting results were often observed in previous studies, which may be ascribed to the heterogeneous patient cohorts analyzed, comprising different clinical stages and histological types. Thus, specific tumor biomarkers for different subgroups of ovarian and breast cancer to predict the course of disease and/or therapy response are in demand. To further investigate the clinical relevance of tumor-related KLKs, in the current study, we aimed at determining the mRNA expression levels of KLK4, KLK5, KLK7 and KLK12 and correlating their expression levels with clinical outcome in well-defined homogeneous cohorts, including 139 patients with advanced high-grade serous ovarian cancer and 125 patients with triple-negative breast cancer.

5.1 Assessment of KLKs as potential prognostic biomarkers in advanced high-grade serous ovarian cancer

To date, several studies have reported that the expression levels of KLK4 (Dong et al., 2001), KLK5 (Dorn et al., 2011) and KLK7 (Dong et al., 2003) are up-regulated in ovarian carcinomas compared to benign and/or low malignant potential (LMP) tumors, suggesting potential diagnostic values of these KLKs in ovarian cancer. Moreover, either at mRNA levels or at protein levels, KLK4 (Dong et al., 2001; Obiezu et al., 2001), KLK5 (Kim et al., 2001; Diamandis et al., 2003) and KLK7 (Kyriakopoulou et al., 2003; Shan et al., 2006) were observed to be more frequently expressed in advanced stages and were associated with higher nuclear grade of ovarian malignancies. In the present study, we observed that the majority of tumor tissues displayed robust mRNA expression patterns of KLK5 and KLK7, while low KLK4 mRNA expression was

observed. These observations were consistent with data from The Cancer Genome Atlas (TCGA) (Loessner et al., 2018), which also reports low KLK4 mRNA expression levels and high expression levels of KLK5 and KLK7 mRNA in ovarian cancer.

The antigen levels of KLK5 (Dorn et al., 2016) and KLK7 (Dorn et al., 2014), respectively, have also been quantified in a partially overlapping ovarian cancer cohort. In the present study, we demonstrate that KLK5 mRNA expression is significantly and positively associated with its protein expression (r_s=0.689) in HGSOC, which has been further validated by box plot analysis. A similar association between KLK7 mRNA and protein expression was observed in our study (r_s=0.663) as well.

Prior studies have reported that KLK4 expression is highly expressed in the effusion fluid of serous epithelial ovarian cancer patients (Davidson et al., 2005; Dong et al., 2013). In addition, Dong and co-workers (2013) observed that higher KLK4 levels are present in the ascites of serous epithelial ovarian cancer cells compared to primary tumor cells, also indicating its involvement in the ascites microenvironment of ovarian cancer. In accordance with this, in the present study, KLK4 mRNA levels were observed to be significantly correlated with the amount of pre-operative ascites fluid volume in our cohort. A remarkably increasing proportion of patients displayed higher KLK4 mRNA expression in the subgroup with larger ascites fluid volume (>500 ml; 60%, 32/53), compared to those with ascites fluid volume ≤500 ml (42%, 33/78). However, contrary results were reported by Shih et al. (2007), showing that KLK4 expression was generally low in effusion fluid of ovarian cancer patients, compared to benign effusions. This discrepancy may be attributed to the fact that the present study assessed the mRNA levels of KLK4 by qPCR, whereas Shih and co-workers (2007) determined secreted KLK4 protein expression by ELISA, implicating that KLK4 could be expressed in ovarian tumor cells but not be secreted to extracellular environment. All KLKs have been described to be highly conserved concerning exon number as well as exon/intro phases and show a high similarity of structure (Clements et al., 2001; Paliouras et al., 2007). Interestingly, Korkmaz et al. (2001) found that KLK4 in prostate cancer, due to an alternative transcription initiation site, was the only member of the KLK family that lacks the characteristic first exon for coding signal peptide, which resulted in the retention of the physiologically related major form of KLK4 in the cell. This behavior of KLK4 exhibited a distinct perinuclear localization, which was dissimilar to the other

KLKs having major extracellular functions.

Obiezu and co-workers (2001) have previously quantified KLK4 mRNA expression levels in a cohort containing 147 malignant ovarian tissues, showing that KLK4 mRNA overexpression was associated with shortened PFS and OS. There, elevated KLK4 expression also represented an unfavorable predictor for prognosis in subset of ovarian cancer patients with lower grade (grade 1 and 2). Moreover, Dong et al. (2001) found that elevated KLK4 protein levels were connected with more aggressive histological subtypes and/or advanced stage of ovarian tumors, suggesting an association between KLK4 expression and the proliferative status of ovarian cancer. Furthermore, KLK4 has been demonstrated as a predictive marker for paclitaxel resistance in patients with ovarian malignancies (Xi et al., 2004; Dong et al., 2013). These observations indicated that KLK4 may serve as a tumor biomarker for monitoring the progression and/or prognosis, but also as a therapeutic target in ovarian cancer. Consistent with this, in the present study, KLK4 mRNA expression was found to be an independent unfavorable predictor for poor OS, but not for PFS. In the *in silico* analysis (Gyorffy et al., 2012), high KLK4 mRNA levels were confirmed to be remarkably associated with shortened PFS and OS in this subtype of ovarian cancer. The lack of significant predictive power for PFS might be due to the relative low numbers of included samples in our cohort. KLK4 expression may thus turn out to represent a prognostic biomarker for PFS, if we enrolled more ovarian cancer cases.

There are several possible mechanisms to explain the prognostic functions of KLK4 in ovarian cancer. Dong et al. (2001) have observed that KLK4 protein levels were increased by estrogen in the ovarian cancer cell line OVCAR-3. Consistent with this, several prior studies also reported that KLK4 mRNA expression was up-regulated by steroid hormones like androgen, estrogen, and progesterone in prostate cancer cells (Nelson et al., 1999; Xi et al., 2004) and by androgen as well as progestin in breast cancer cells (Yousef et al., 1999). Although the potential function of the steroid hormones in ovarian cancer is a matter of discussion, several studies have demonstrated their association with tumor progression of ovarian cancer (Høgdall et al., 2007; Jönsson et al., 2015; Feng et al., 2016). Thus, it may be hypothesized that KLK4 may perform through hormone-related regulatory mechanisms to promote tumor cell spreading in ovarian tumor. Moreover, several studies indicated that KLK4 promoted

tumor progression and metastasis by activating enzymatic cascades. In the PC-3 prostate cancer cell line, the function of KLK4-accelerating tumor cell migration was shown to be related to the loss of E-cadherin and an epithelial-mesenchymal transition-like effect (Veveris-Lowe et al., 2005). Additionally, KLK4 could also modulate the expression of secreted growth factors such as HGF/SF (Mukai et al., 2008, 2015), IGF (Matsumura et al., 2005) and TGF-β (Shahinian et al., 2014), which are involved in tumor progression, thus affecting tumor cell proliferation, invasion, angiogenesis, and metastasis.

In case of KLK5, accumulating studies have demonstrated that it also represents a predictive biomarker in ovarian cancer. Kim et al. (2001) have investigated KLK5 mRNA levels in the cohort containing 142 epithelial ovarian cancer cases, showing that elevated KLK5 mRNA expression was significantly associated with an increased risk of disease recurrence and cancer-related death. Moreover, Diamandis and co-workers (2003) have reported that KLK5 overexpression was correlated with unfavorable prognosis in ovarian cancer. Furthermore, KLK5 antigen levels have also been measured in serum of ovarian cancer patients, describing that KLK5 protein expression was higher in serum of ovarian cancer patients, compared to those of benign controls and LMP tumors (Bandiera et al., 2009; Dorn et al., 2011). A significant correlation was observed between high KLK5 protein levels and advanced disease stage in ovarian cancer (Bandiera et al., 2009). Moreover, elevated KLK5 protein expression was found to be associated with poor outcome of ovarian cancer patients, suggesting an unfavorable prognostic value of KLK5 in this tumor entity (Oikonomopoulou et al., 2008; Dorn et al., 2011). Taken all together, KLK5 overexpression may represent an unfavorable prognostic biomarker and correlate with more aggressive phenotypes of ovarian cancer. However, contrary results were reported by Dorn and co-corkers (2016) assessing KLK5 protein levels in this tumor entity, showing that the overexpression of KLK5 by stromal cells, but not by tumor cells, was significantly correlated with prolonged PFS and OS. This finding suggested a tumor-suppressive function of KLK5 in ovarian cancer, indicating that KLK5 may perform divergent functions in different cell types.

In the current study, residual tumor mass was the only clinical variable correlated with KLK5 mRNA expression in HGSOC. Compared to the tumor-free subgroup (27%,

19/70), an enhanced proportion of high KLK5 mRNA levels was observed in the subgroup with residual tumor (44%, 29/66), pointing to a tumor-supporting role of KLK5. Furthermore, elevated KLK5 mRNA levels were significantly associated with shortened PFS in univariate analysis, however, only showed a trend towards significance when subjected to multivariable analysis. In line with our finding, in *in silico* analyses (Gyorffy et al., 2012), a significant association of higher KLK5 mRNA levels with worse PFS, but not OS, was observed in this subtype of ovarian cancer. These observations are in agreement with prior studies, which also showed an association of high KLK5 mRNA levels with poor PFS, rather than OS, in ovarian cancer (Zheng et al., 2007; Dorn et al., 2011, 2011). Nevertheless, in two separated studies by Kim et al. (2001) and Diamandis et al. (2003), respectively, KLK5 overexpression was found to be associated with both worse PFS and OS of ovarian cancer patients. Whether these discrepancies are attributed to the heterogeneous patient cohorts encompassing low/high grade, early/advanced stage and different histological subtypes of ovarian cancer cannot be presently answered.

The proposed molecular mechanism may explain the tumor-supporting role of KLK5 in neoplasm. Homology studies from Yousef and Diamandis (1999) suggested that KLK5 was up-regulated by estrogen and progestin in breast cancer cell line BT-474. In Matrigel-based assays, siRNA-mediated KLK5 inhibition was shown to decrease the invasion of bladder cancer cells (Shinoda et al., 2007). Under physiological conditions, KLK5 is involved in skin desquamation by degrading the cell-cell and cell-matrix adhesion molecules. In fact, inhibition of KLK5 enforced cell-cell adhesion in oral squamous cell carcinoma, further increasing the metastatic speed by promoting loss of junctional integrity (Jiang et al., 2011). Therefore, the tumor-supporting role of KLK5 in ovarian cancer may be attributed to the promotion of tumor cell shedding and the cleavage of ECM proteins during metastasis. Indeed, KLK5 was found to efficiently degrade various ECM proteins, including collagen I, II, III and IV, laminin and fibronectin (Michael et al., 2005). Furthermore, KLK5 targets a variety of substrates such as TGF-β and PARs, which upon (in-)activation by KLK5 modulate important tumor-associated signaling pathways (Oikonomopoulou et al., 2006; Paliouras and Diamandis, 2006). Moreover, KLK5 could also play a crucial role in the extracellular proteolytic network in tumor cell microenvironment through activating the zymogen

forms of other tumor-associated proteases, such as pro-uPA and pro-KLK11 (Beaufort et al., 2010a, 2010b).

Regarding KLK7, it has been well described to be implicated in the desquamation process of skin *in vivo* as well (Lundström and Egelrud, 1991). Accumulating evidence further showed that KLK7 is up-regulated in ovarian (Dorn et al., 2006; Shaw and Diamandis, 2007; Psyrri et al., 2008), pancreatic (Avgeris et al., 2010), colon (Talieri et al., 2009) and cervix malignancies (Termini et al., 2010), whereas it is down-regulated in kidney (Gabril et al., 2010), breast (Holzscheiter et al., 2006; Ejaz et al., 2017) and prostate (Xuan et al., 2008) cancer..

Additionally, KLK7 has been well described for its prognostic value in ovarian cancer. Psyrri et al. (2008) observed that elevated KLK7 protein expression was associated with inferior DFS and OS in ovarian cancer patients. A similar trend was reported by Kyriakopoulou et al. (2003) evaluating 125 ovarian tumor specimens, illustrating that KLK7 overexpression is an independent unfavorable predictor for DFS and OS in the subgroup of ovarian cancer patients with lower grades (grade 1+2). Furthermore, Shan and co-workers (2006) reported that elevated KLK7 antigen levels were more frequently observed in patients with advanced stage and higher-grade (G3), and were correlated with shortened PFS of ovarian cancer patients. Additionally, KLK7 has been shown to represent a predictive biomarker for paclitaxel chemoresistance in patients with serous epithelial ovarian carcinoma (Dong et al., 2010). All these evidence suggest a tumor-promoting role of KLK7 in ovarian malignancies. In line with previous studies, in the present study, elevated KLK7 mRNA expression was observed to be significantly associated with high risk of tumor progression and turned out to represent an independent unfavorable predictive biomarker in HGSOC.

Nevertheless, a contrary result was reported by Dorn et al. (2014) analyzing KLK7 antigen concentrations in tumor tissue extracts from 98 ovarian cancer patients by ELISA, showing that higher KLK7 protein expression was associated with prolonged PFS and OS, revealing that KLK7 may represent a favorable clinical determinant of prognosis in this tumor entity. The hypothesized explanation for this discrepancy might be attributed to the cohort analyzed by Dorn et al. (2014). Patients were of different subtypes of ovarian malignancies (serous, endometrioid, undifferentiated, mucinous, and clear cell types) and several cases were of earlier grades (FIGO I/II, 20%), whereas

the homogenous patient cohort in our study enrolled cases afflicted with HGSOC only. The pathophysiological function of KLK7 in ovarian tumors still needs to be further investigated.

KLK7 has been proposed to mediate several signaling pathways to affect tumorigenicity. In epithelial ovarian carcinoma (EOC), KLK7 was shown to stimulate peritoneal dissemination and reinvasion through increasing multicellular aggregates (MCA) and α_5/β_1 integrin-dependent cell adhesion (Dong et al., 2010). Indeed, integrin signaling has been reported to be involved in MCA/spheroids formation (Casey et al., 2001) and disaggregation (Burleson et al., 2004), which could promote EOC cell invasion and metastasis (Auersperg et al., 2001; Bast et al., 2009). Furthermore, elevated α_5 integrin levels were associated with unfavorable outcome of EOC patients (Goldberg et al., 2001). Moreover, KLK7 has been demonstrated to cleave ECM proteins (Ramani and Haun, 2008) and/or junction/adhesion molecules including corneodesmosomes (Caubet et al., 2004), desmoglein-1 and desmocollin-1 (Ramani et al., 2008), which could lead to the detachment of tumor cells from the primary tumor and dissemination (McGary et al., 2002; Ganguly et al., 2013). These evidence indicate that KLK7 may facilitate tumor cell migration and invasion processes. Last but not least, Ramani et al. (2011) found that KLK7 was associated with the activation of matrix metalloproteinase-9 (MMP-9) in tumors, influencing angiogenesis, tumor cell migration, invasion, and metastasis, further suggesting that KLK7 could be implicated in tumor development and progression.

KLK12 mRNA expression was not detected in 35 samples of our cohort of HGSOC patients, which is in line with the data from TCGA (Loessner et al., 2018).

Accumulating evidence has also demonstrated that the combined expression of KLK4-7 is part of enzymatic cascades to accelerate progression and metastasis in ovarian cancer (Dong et al., 2014). Prezas et al. (2006) showed that simultaneous overexpression of KLK4-7 led to a remarkable increase of cell invasion *in vitro* and tumor burden in xenograft models of ovarian cancer. Moreover, the concomitant expression of KLK4-7 was observed to be associated with reduced cell adhesion and insensitivity to paclitaxel in ovarian cancer cells (Loessner et al., 2012). Thus, it is tempting to speculate that co-expression of KLK4-7 might be regulated through similar modulatory mechanisms in ovarian cancer. In fact, KLKs have been indicated to form

activation cascades by activating zymogen forms of other proteases, including other members of the KLK family (Yoon et al., 2007). Firstly, as noted in the previous sections, KLK4, KLK5, and KLK7 are regulated by steroid hormones in ovarian carcinomas. Secondly, KLK4-7 have a pronounced effect on the secreted proteome in OV-MZ-6 ovarian cancer cells, suggesting the capability of activating signaling cascades in tumor microenvironment of ovarian cancer, such as TGFβ and epithelial-mesenchymal transition (EMT) pathways (Shahinian et al., 2014). Moreover, the data from Mukai et al. (2008) suggested that KLK4 and KLK5 represented novel factors to mediate the activation of pro-HGF/SF induced by hepatocyte growth factor activator (HGFA) within tumor tissues, indicating vital functions of KLK4 and KLK5 in the processes of tissue morphogenesis, regeneration, and tumor progression. Last but not least, a recent study revealed that KLK4-7 could modulate various cancer-related genes and proteins in ovarian cancer (Wang et al., 2018), including MSN, KRT7, KRT19, and JUNB, which are strongly associated with tumorigenesis, further supporting the important role of KLK4-7 in progression of this tumor entity.

5.2 Clinical impact of KLKs mRNA expression on patients with triple-negative breast cancer

As stated above, many members of the KLK gene family, including the four analyzed KLKs, were implicated in hormone-dependent tumors. As outlined in the previous chapter (5.1), we have evaluated the clinical impact of these KLKs on patient outcome in HGSOC, which may be modulated by steroid hormones-related regulatory mechanisms. Similarly, in breast cancer, KLKs could also play crucial roles in tumor progression and metastasis. However, to date, no research has correlated mRNA expression of these KLKs with patient outcome in TNBC, lacking the expression of ER and PR, and with no or low HER2 protein expression.

In the present project, most of the TNBC specimens displayed low mRNA expression levels of KLK4 and KLK12, while robust KLK5 and KLK7 mRNA expression was observed, showing a very similar trend to the expression patterns observed in the HGSOC cohort. We observed that mRNA expression of these KLKs was not detectable in some tumor tissues of TNBC patients. This was especially notable for KLK12 with a high proportion of patients displaying negative expression (62/114, 54%).

Among the 15 members of KLK gene family, only expression of KLK4 was found to be upregulated, both at mRNA and protein levels, in breast malignancies, compared to normal breast tissues (Davidson et al., 2007; Papachristopoulou et al., 2009; Schmitt et al., 2013). Furthermore, elevated KLK4 mRNA levels were associated with advanced stage and higher tumor grade in a qPCR-based study including 16 benign and 45 cancerous breast tissues (Papachristopoulou et al., 2009), suggesting that KLK4 overexpression was associated with a more aggressive behavior of breast cancer cells. Additionally, KLK4 mRNA levels were significantly and negatively correlated with PR staining in this tumor entity, which was considered as a favorable indicator of hormone-related breast cancer, indicating the unfavorable prognostic value of KLK4 in breast cancer (Papachristopoulou et al., 2009). Consistent with previous studies, in our study, a remarkably high proportion of patients exhibited elevated KLK4 mRNA levels in the subgroup with higher grade breast cancer (grade III; 70%, 74/105), compared to the cases with lower grade (grade II; 27%, 3/11), indicating that KLK4 may serve as a tumor-promoting biomarker in TNBC. Furthermore, in the present study, elevated KLK4 mRNA levels were significantly associated with shortened DFS and OS. A similar trend was previously reported by Yang et al. (2017) evaluating the protein data of KLK4 in 188 TNBC patients, illustrating that KLK4 overexpression in tumor-associated stromal cells was associated with poor DFS, also suggesting that KLK4 represented an unfavorable predictor in this tumor entity.

To date, the potential molecular mechanisms of KLK4 promoting tumorigenicity have not been well defined. KLK4 has been described to be involved in the enzymatic cascade pathway to regulate the tumor microenvironment via PAR1/2 signaling or the uPA-plasminogen axis. KLK4 could initiate cell signaling through PARs *in vitro*, facilitating tumor cell proliferation, migration, invasion, and metastasis (Ramsay et al., 2008). KLK4 could also accelerate tumor growth and development by activating uPA (Takayama et al., 2001), which was validated as an unfavorable indicator in breast cancer (Duffy et al., 2014; Dovnik and Takac, 2017). Additionally, KLK4 have been suggested to activate KLK3 by cleaving its precursor (pro-KLK3) (Takayama et al., 2001), whereby active KLK3 could promote tumor invasion and metastasis by cleaving IGF-binding protein 3 (IGFBP3) (Cohen et al., 1994) and parathyroid-hormone-related protein (PTHRP) (Cramer et al., 1996; Iwamura et al., 1996).

Furthermore, a recent study indicated that KLK4 silencing suppressed tumor cell proliferation and enhanced apoptosis in oral squamous cell carcinoma cells through inhibition of the activation of Wnt/b-catenin signaling pathway (Cui et al., 2017). Moreover, KLK4 has been reported to activate MMP1 (Fuhrman-Luck et al., 2016), enhancing the prostate cancer cell growth, migration, invasion and metastasis *in vitro* (Pulukuri and Rao, 2008). KLK4 was also validated to be a tumor-promoting factor by liberating N-terminal fragments of thrombospondin-1 (TSP1) and directly cleaving TSP1 in prostate cancer (Fuhrman-Luck et al., 2016). Additionally, KLK4 has been found to cleave the extracellular domain of murine ephrin-B2 in the research of 3D protein models applying an *in silico* approach (Lisle et al., 2015). Ephrin-B2 is the single physiologically-relevant ligand of EphB4, which is a member of type 1 transmembrane receptor tyrosine kinases and normally contribute to tumor suppression in many epithelial cancers, including breast cancer (Noren et al., 2006; Rutkowski et al., 2012; Barneh et al., 2013; Hu et al., 2014). Lisle and co-workers (2015) found that KLK4 may facilitate tumor invasiveness and angiogenesis by modulating EphB4-ephrinB2 interactions via selectively cleaving murine ephrin-B2. All in all, KLK4 may represent a potential multifunctional modulator for tumorigenesis in TNBC.

Regarding KLK5, it has been reported to exhibit the prognostic power in various carcinoma types. Shinoda et al. (2007) have reported that KLK5 levels were frequently upregulated in invasive bladder tumors, compared to superficial tumors, indicating the potential role of KLK5 to stimulate cell invasion in bladder carcinoma. In colorectal cancer, KLK5 overexpression was clearly associated with an advanced tumor stage, suggesting that KLK5 may represent a biomarker predicting tumor recurrence and metastasis in this tumor entity (Wu et al., 2016). Similarly, in a plethora of studies, KLK5 mRNA has been underlined the crucial effects on the progression of breast cancer and represented a predictor for the diagnosis and prognosis of breast cancer patients. In a RT-qPCR based study containing 102 breast cancer cases, KLK5 mRNA levels were found to be highly expressed in benign tumor specimens, compared to cancerous breast tumor specimens (Avgeris et al., 2011). Moreover, Yousef et al. (2002) have described that KLK5 overexpression was associated with reduced DFS and OS in 179 patients with different stages and grades of breast malignancies, indicating an unfavorable predictive value of KLK5 in this tumor entity. Additionally, Yang and co-

workers (2015) also reported a similar trend determining KLK5 protein levels in 180 TNBC patients, illustrating that elevated KLK5 protein levels in tumor stromal cells were significantly associated with distal metastasis and shortened OS. Besides, KLK5 expression was observed to be significantly increased in BT-20 breast cancer cells treated with docetaxel and methotrexate, suggesting that KLK5 may represent a potential indicator for predicting chemotherapy response (Papachristopoulou et al., 2013). However, in view of our data, no significant correlation was observed between KLK5 mRNA expression and clinical outcome in the TNBC cohort.

Likewise, in the current study, KLK7 also did not present any significant association with DFS or OS of TNBC patients. Nevertheless, it should be noted that KLK7 has been previously identified as a predictor in various malignancies, including breast cancer. In pancreatic cancer cells, inhibition of KLK7 was found to efficiently reduce pancreatic tumor cells proliferation, migration, and invasion, suggesting that KLK7 may be a therapeutic target in this tumor entity (Du JP et al., 2018). In intracranial tumor cells, Prezas et al. (2006) have quantified KLK7 levels in a cohort containing 73 tumor tissue specimens, showing that KLK7 overexpression was associated with the increased invasion potential and worse OS. Additionally, higher KLK7 mRNA expression has been demonstrated to represent an unfavorable prognostic biomarker in colorectal cancer (Inoue et al., 2010). In breast cancer, KLK7 expression levels were found to be highly expressed in normal and benign tissues and under-expressed in cancerous tissues (Li et al., 2009; Ejaz et al., 2017). Talieri et al. (2004) have quantified KLK7 mRNA levels in 92 breast cancer tissues, showing that KLK7 overexpression was significantly associated with shortened DFS and OS, revealing an unfavorable prognostic effect in this tumor entity. However, Holzscheiter and co-workers (2006) have reported a contrary result in a population-based cohort containing 176 primary breast cancer patients, describing that KLK7 overexpression was a significant predictor of prolonged DFS.

The potential explanation for these discrepancies for KLK5 and KLK7 may be due to the fact that the current study assessed KLK5 and KLK7 mRNA levels in the well-defined cohort of TNBC patients, whereas the cohorts previously analyzed encompassed heterogeneous subtypes of breast cancer, including the ER and PR positive as well as negative cases. Secondly, it is hypothesized that the lack of

significant association may also be due to the rather low patient numbers. Further study is required to investigate their prognostic values in ovarian cancer.

KLKs have been reported to be involved in the progression of malignancies by produced cancer-specific transcript forms (Xu and Lee, 2003; Kurlender et al., 2005; Tan et al., 2006). KLK12 was found to generate three alternative splice variants (KLK12sv1/2/3) (Yousef et al., 2000) and the classical form (Kurlender et al., 2005), which exhibited various physiological functions and displayed a key role in the progression of malignancies (Landry et al., 2003). In breast cancer cells, Yousef et al. (2000) observed that KLK12 mRNA expression was downregulated by steroid hormones. In the current study, we quantified all KLK12 transcripts in TNBC. Here, positive KLK12 mRNA expression was significantly associated with shortened DFS and OS in the univariate cox regression analysis and represented an independent unfavorable predictor for DFS in TNBC patients. Similar associations have also been observed in other tumor types. In gastric carcinoma, KLK12 overexpression was found to be significantly associated with more aggressive behaviors and patients with elevated KLK12 expression presented a significantly worse 5-year survival rate compared to those with low KLK12 levels (Zhao et al., 2012). KLK12 mRNA overexpression has also been shown to represent an unfavorable predictive biomarker for prognosis in pulmonary carcinoid (Swarts et al., 2013). These findings are in accordance with previous functional studies, which observed pro-tumorigenic role of KLK12 protease. Knockdown of KLK12 diminished proliferation and migration of gastric cancer cells by arresting cells in the G0/G1 phase (Zhao et al., 2012; Li et al., 2016). KLK12 could also promote cell migration by stimulating the proteolysis of the human extracellular matrix proteins fibronectin and tenascin, which are implicated in the regulation of endothelial cell adhesion and migration (Kryza et al., 2018). Furthermore, KLK12 has been described for its proangiogenic effect, thereby displaying a crucial role in the process of carcinoma. KLK12 was found to indirectly modulate the bioavailability and/or activity of various growth factors, such as VEGF165, BMP2, TGF-β1, and FGF-2, via hydrolyzing matricellular proteins (Guillon-Munos et al., 2011), which are implicated in angiogenesis and tumorigenesis (Dallas et al., 2005). Additionally, KLK12 could also modulate the availability of platelet-derived growth factor B (PDGF-B) via cleaving its C-terminal retention motif, stimulating tumor growth and

angiogenesis (Kryza et al., 2014). Last but not least, KLK12 is secreted as an inactive pro-enzyme, which autoactivates to acquire enzymatic activity. Moreover, KLK12 has been demonstrated to activate pro-KLK11 *in vitro*, suggesting that KLK12 might participate in the proteolytic cascades to facilitate specific physiologic processes (Borgoño and Diamandis, 2004; Memari et al., 2007).

However, two research groups, which measured expression of distinct KLK12 transcripts in breast cancer, have described contradictory observations of the prognostic value of KLK12 in this tumor entity. Talieri and co-workers (2012) found that KLK12sv3 overexpression was significantly associated with lower grade, earlier stages, and positive estrogen and progesterone receptor status. Patients with high KLK12sv3 expression displayed longer DFS in breast cancer. Therefore, KLK12sv3 may be considered as a favorable indicator for prognosis in breast cancer but possibly not for TNBC owing to that this transcript is poorly or not expressed in ER and PR negative breast tumors (Talieri et al., 2012). Similarly, Papachristopoulou et al. (2018) also determined the potential functional role of distinct KLK12 splice variants in 122 tissue specimens from patients with the surgical removal of cancerous or benign breast tumors. There, both KLK12sv1/2 and KLK12sv3 overexpression predicted long-term DFS and OS for breast cancer patients. Thus, it seems likely that the overall expression of KLK12 examined in our study mainly equates to the KLK12sv1/2 transcripts encoding this protease. Convergences between the prognostic effects of transcripts encoding either a KLK protease or an alternative transcript coding for a truncated form have also been reported for KLK8 in lung carcinoma (Planque et al., 2008), suggesting that the same KLK gene may probably generate two products with opposite functions in the process of neoplasm. Moreover, KLK12sv3 expression was found to be higher in benign breast tumors compared to cancerous breast tissues, and elevated KLK12sv3 levels were significantly associated with a more aggressive behavior in breast cancer cells (Papachristopoulou et al., 2018). Hence, all these observations indicate that KLK12 exhibits a pro-tumorigenic role in breast carcinoma, whereas KLK12vs3, encoding a truncated protein lacking a functional catalytic triad, may play a tumor-suppressive function. Further studies would be necessary to examine this hypothesis.

5.3 Coordinate expression of KLK5 and KLK7 in advanced high-grade serous ovarian cancer and triple-negative breast cancer

As mentioned above, in the present study, not only the parallel expression of KLK5 and KLK7 was observed in HGSOC and TNBC, but irrefutable evidence was observed for coordinated modulation of both KLKs concerning expression on the mRNA level (HGSOC: r_s=0.568, p<0.001; TNBC: r_s=0.735, p<0.001) and protein level (HGSOC: r_s=0.805, p<0.001). This indicates that KLK5 and KLK7 may be implicated in synergistic or independent mechanisms contributing to tumor-related processes. Other KLKs, including KLK4, KLK6, KLK8, KLK9, KLK10, KLK11, KLK13, KLK14, and KLK15, exhibit weak associations with either KLK5 or KLK7 in the same ovarian cancer cohort (with r_s<0.3; N. Ahmed, L. Dettmar, and X. Geng, pers. comm.). Likewise, prior studies from our lab determined co-expression of several other KLK pairs, such as KLK6 versus KLK8 (Ahmed et al., 2016), KLK9 versus KLK15 (Geng et al., 2017), and KLK10 versus KLK11 (Geng et al., 2017, 2018). The combination of KLK6 with KLK8 was found to aid in determining patients with prolonged survival (Ahmed et al., 2016), whereas the concomitant expression of KLK10 and KLK11 could identify patient outcome with better accuracy (Geng et al., 2018). Besides, the consistent expression of KLKs has also been identified in several other malignancies. Martins et al. (2011) reported a parallel under-expression of KLK6, 7, 8 and 13, highlighting a potential role of these KLKs in the transition of epithelial into mesenchymal in primary melanoma. Furthermore, the parallel overexpression of KLK7 and KLK14 was observed in colon carcinoma, indicating their potential cascade-like implication in this tumor entity (Devetzi et al., 2013). All these findings point to an involvement of certain KLKs in shared cascades/pathways influencing the progression of malignancies.

Concordant expression of KLK5 and KLK7 has been described in several human organs and tissues. It was initially observed in skin tissues, suggesting there may be a relationship between these two proteases (Brattsand and Egelrud, 1999; Ekholm et al., 2000). Furthermore, KLK5, KLK7, and KLK14 were found in catalytically active form, resembling a proteolytic cascade, in the stratum corneum (Brattsand et al., 2005). Moreover, in Netherton syndrome, both KLK5 and KLK7 play important roles in inflammation, while simultaneous deficiency of both KLKs could rescue the epidermal

barrier and the postnatal lethality in a mouse model (Kasparek et al., 2017). Indeed, KLKs have been proposed to activate themselves and each other, thus initiating an activity amplification cascade in pathophysiological processes (Debela et al., 2008). For example, KLK4 could process the zymogens of KLK3 and KLK11 into their active counterparts (Takayama et al., 2001; Yoon et al., 2007). (pro-)KLK5 has been found to be auto-activated and then activate several pro-KLKs, including KLK2, KLK3, KLK6, KLK7, KLK12, and KLK14 (Brattsand et al., 2005; Michael et al., 2006; Blaber et al., 2007; Yoon et al., 2007).

Furthermore, cumulative evidence have implied cooperative interaction between KLK5 and KLK7 in various neoplasms. In oral squamous cell carcinoma, concomitant inhibition of KLK5 and KLK7 was observed, which was associated with poor prognosis in this tumor entity (Leusink et al., 2015). In ovarian cancer, Dong and co-workers (2003) have reported that KLK5 and KLK7 were expressed abundantly in ovarian cancer, especially in advanced-stage serous tumors. They also found that KLK7 could reduce the surrounding matrix as the cancer progresses and verified the interaction between them *in vitro* in an activation cascade (Dong et al., 2003). Interestingly, the combined expression of KLK4, 5, 6 and 7 has been shown the potential to promote an increase of TGFβ-1 signaling and decrease of integrin and MAPK independent interactions, thereby diminishing cell adhesion and inducing cell invasion as well as resistance to paclitaxel in OV-MZ-6 ovarian cancer cells (Loessner et al., 2012; Shahinian et al., 2014). Similarly, in breast cancer, Talieri et al. (2011) observed parallel overexpression of KLK5 and KLK7 in a Greek population-based cohort containing 80 breast tissues, while Li et al. (2009) found parallel under-expression of KLK5 and KLK7 in an Asian population-based cohort. The coordinated up- or down-regulation of KLK5 and KLK7 in breast cancer is potentially regulated by the same pathways, such as steroid hormones, thus contributing to carcinogenesis and tumor development (Yousef et al., 1999, 2000). Consistent with this, we showed that, both in HGSOC and in TNBC cohorts, KLK5 and KLK7 were coordinately expressed and both of them represented unfavorable predictive biomarkers in HGSOC, further supporting the hypothesis that KLK5 and KLK7 may be involved in an activation cascade and exhibit similar roles in the progression of malignancies.

6 Summary

Kallikrein-related peptidases have been determined to be involved in both physiological and pathological processes, like skin desquamation, inflammation, and especially in tumor-relevant processes. High-grade serous ovarian cancer commonly presents with nonspecific or low sensitive symptoms and triple-negative breast cancer lacks specific molecular targets, both resulting in high mortality rates and limitation of therapy options. Several KLKs represent biomarkers for diagnosis and prognosis in ovarian and breast cancer. However, paradoxical observations of the effects of some KLKs on prognosis were often delineated in these tumor entities, which may be due to the rather heterogeneous patient cohorts previously analyzed. Therefore, the present project aimed at lighting up the promising roles of tumor-relevant KLK expression as prognostic biomarkers in more homogenous cohorts of patients with advanced high-grade serous ovarian cancer and triple-negative breast cancer.

In HGSOC, KLK4, 5, 7, and 12 mRNA expression levels were analyzed by quantitative PCR. Together with available protein data overlapping with the mRNA cohort, KLK5 mRNA expression was found to be significantly and positively associated with the protein expression (r_s=0.689, p<0.001). Similarly, KLK7 mRNA expression was also correlated with its protein levels (r_s=0.663, p<0.001). These suggest that there is no major post-transcriptional regulation for the expression of KLK5 and KLK7. Therefore, the mRNA and protein data are comparable. Moreover, pronounced associations were observed between KLK5 and KLK7 both at the mRNA levels (r_s=0.568, p<0.001) and the antigen levels (r_s=0.805, p<0.001), strongly supporting that KLK5 and KLK7 are concomitantly expressed in this tumor entity. In univariate Cox regression analysis, patients with elevated KLK4 mRNA expression levels displayed shortened OS (HR=2.28, p=0.001), but did not show any significant difference in PFS. Higher KLK5 mRNA expression was associated with worse PFS (HR=1.60, p=0.047), but not correlated with OS. Patients with elevated KLK7 mRNA levels exhibited an unfavorable prognosis of PFS (HR=1.75, p=0.025) and showed a trend towards significance in case of OS (HR=1.66, p=0.055). These results were further confirmed by *in silico* analysis based on the publicly available mRNA expression profiles from The Cancer Genome Atlas. In multivariate analysis, KLK4 turned out to be an independent unfavorable predictive biomarker of OS (HR=2.31, p=0.006), while KLK7

represented an independent unfavorable predictor of both PFS (HR=2.19, p=0.007) and OS (HR=1.94, p=0.032). KLK5 did not prove to be statistically significant, however, showed a trend towards significance for PFS (HR=1.53, p=0.095).

In addition, we also quantified KLKs mRNA expression in a selected cohort containing 125 TNBC patients. Tumor specimens of TNBC subgroup were characterized by a negative status for ER and PR, and by lack or low levels of HER2 protein expression. Patients with TNBC have the worst outcome among all breast cancer subtypes, due to the fact that the available anti-targeted therapy has very limited or no impact on this entity. In addition to the findings in HGSOC, a remarkable correlation between KLK5 and KLK7 mRNA expression was also observed in TNBC (r_s=0.735, p<0.001), indicating that the parallel and coordinated expression also exists in this tumor entity. Univariate Cox regression analysis showed that elevated KLK4 mRNA levels were significantly associated with shortened DFS (HR=1.83, p=0.040) and OS (HR=2.07, p=0.019), while KLK12 overexpression was also remarkably correlated with poor DFS (HR=2.15, p=0.10) and OS (HR=2.00, p=0.025). However, both KLK5 and KLK7 mRNA expression did not show any predictive value in this subgroup of breast cancer. In multivariate analysis, KLK4 mRNA expression did not prove to be statistically significant, however, presented a tendency towards significance concerning prediction of OS (HR=1.83, p=0.067). Upon addition to the base model, KLK12 mRNA expression remained an unfavorable prognostic biomarker for DFS (HR=2.16, p=0.011), however, showed only a trend towards significance for OS (HR=1.82, p=0.060).

In conclusion, KLK4, KLK5, KLK7, and KLK12 were investigated in HGSOC and TNBC, whether they can serve as prognostic biomarkers and may represent attractive targets for tumor therapy, showing the potential to assist in making decisions on systemic therapy for patients with poor prognosis in these tumor entities. Further studies aimed at determining the potential cancer-related pathways of which KLKs are involved in are warranted. This would guide the improvement of cancer intervention strategies and help patients suffering from these malignancies.

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8 List of publications

Thesis-related publications:

1. Gong W, Liu Y, Seidl C, Dreyer T, Drecoll E, Kotzsch M, Bronger H, Dorn J, Magdolen V. 2019. Characterization of kallikrein-related peptidase 4 (KLK4) mRNA expression in tumor tissue of advanced high-grade serous ovarian cancer patients. PloS one. 14(2): e0212968.

2. Gong W, Liu Y, Seidl C, Diamandis, E. P., Kiechle, M., Drecoll, E., Kotzsch M., Magdolen V. & Dorn, J. 2019. Quantitative assessment and clinical relevance of kallikrein-related peptidase 5 mRNA expression in advanced high-grade serous ovarian cancer. BMC cancer. 19(1): 696.3.

3. Gong W, Liu Y, Preis S, Geng X, Petit-Courty A, Kiechle M, Muckenhuber A, Dreyer T, Dorn J, Courty Y, Magdolen V. 2020. Prognostic value of kallikrein-related peptidase 12 (KLK12) mRNA expression in triple-negative breast cancer patients. Mol Med. 26(1):19.

Oral presentations at international meetings:

1. Gong W, Liu Y, Seidl C, Kiechle M, Dorn J, Magdolen V. Establishment of qPCR assays for quantification of kallikrein-related peptidase 4 and 5 mRNA expression in tumor tissue of ovarian cancer patients. 7th International Symposium on Kallikreins and kallikrein-related peptidases. 2017, Tours, France. Oral presentation.

2. Gong W, Liu Y, Seidl C, Kiechle M, Dorn J, Magdolen V. Establishment of qPCR assays for quantification of kallikrein-related peptidase 4 (KLK4) and 5 (KLK5) mRNA expression in tumor tissue of ovarian cancer patients. 7th International Symposium on Kallikreins and kallikrein-related peptidases. 2017, Tours, France. Poster presentation.

3. Gong W, Liu Y, Seidl C, Kiechle M, Dorn J, Magdolen V. Clinical relevance of kallikrein-related peptidase 4 (KLK4) mRNA expression levels in tumor tissue of high-grade advanced (FIGO III/IV) serous ovarian cancer patients. 35th Winter School On Proteases And Inhibitors. 2018, Tiers, Italy. Oral presentation.

4. Gong W, Tobias Dreyer, Seidl C, Dorn J, Magdolen V. Characterization of kallikrein-related peptidase 12 (KLK12) mRNA expression in tumor tissue of advanced high-grade serous ovarian cancer patients and triple-negative breast cancer patients. Minisymposium, From the frying pan into the fire: proteases and receptors in disease. 2019, Munich, Germany. Oral presentation.

5. Gong W, Liu Y, Seidl C, Kiechle M, Dorn J, Magdolen V. Clinical relevance of kallikrein-related peptidase 4 (KLK4) mRNA expression in tumor tissue of advanced high-grade serous ovarian cancer patients. DAAD program: Study Visits and Study Seminars for Groups of Foreign Students to Germany. 2019, Munich, Germany. Oral presentation.

6. Gong W, Liu Y, Seidl C, Kiechle M, Dorn J, Magdolen V. Quantitative assessment of KLK5 and KLK7 mRNA expression in advanced high-grade serous ovarian cancer. Minisymposium, From the frying pan into the fire: proteases and receptors in disease. 2019, Tours, France. Oral presentation.

7. Gong W, Liu Y, Seidl C, Kiechle M, Dorn J, Magdolen V. Clinical relevance of kallikrein-related peptidase 5 and 7 mRNA expression levels in tumor tissue of advanced high-grade serous ovarian cancer patients (FIGO III/IV). 8th International Symposium on Kallikreins and kallikrein-related peptidases. 2019, Prague, Czech Republic. Oral presentation.

9 Appendix

9.1 Ovarian cancer FIGO and TNM stage systems

9.1.1 Ovarian cancer FIGO stage system

Stage	Description
I	Tumor limited to ovaries or fallopian tube(s).
IA	Tumor limited to one ovary (capsule intact) or fallopian tube; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings.
IB	Tumor limited to both ovaries (capsules intact) or fallopian tubes; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings.
IC	Tumor limited to one or both ovaries or fallopian tubes, with any of the following:
IC1	Surgical spill intraoperatively.
IC2	Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface.
IC3	Malignant cells in the ascites or peritoneal washings.
II	Tumor involves one or both ovaries with pelvic extension (below the pelvic brim) or primary peritoneal cancer
IIA	Extension and/or implant on uterus and/or fallopian tubes and/or ovaries
IIB	Extension to other pelvic intraperitoneal tissues
III	Tumor involves one or both ovaries, or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside of the pelvis and/or metastasis to the retroperitoneal lymph nodes.
IIIA	Metastasis to the retroperitoneal lymph nodes with or without microscopic peritoneal involvement beyond the pelvis.
IIIA1	Positive retroperitoneal lymph nodes only (cytologically or histologically proven).
IIIA1(i)	Metastasis ≤10 mm in greatest dimension,
IIIA1(ii)	Metastasis >10 mm in greatest dimension.
IIIA2	Microscopic extra-pelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes.
IIIB	Macroscopic peritoneal metastasis beyond the pelvic brim ≤2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes.
IIIC	Macroscopic peritoneal metastases beyond the pelvic brim >2 cm in greatest dimension, with or without metastases to the retroperitoneal nodes.
IV	Distant metastasis excluding peritoneal metastases
IVA	Pleural effusion with positive cytology
IVB	Metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity).

Adapted from Mutch and Prat, 2014.

9.1.2 Ovarian cancer TNM stage system

Stage	T	N	M
IA	T1a	N0	M0
IB	T1b	N0	M0
IC	T1c	N0	M0
IIA	T2a	N0	M0
IIB	Tab	N0	M0
IIIA	T3a	N0	M0
	T3a	N1	M0
IIIB	T3b	N0	M0
	T3b	N1	M0
IIIC	T3c	N0	M0
	T3c	N1	M0
IV	Any T	Any N	M1

Adapted from Mutch and Prat, 2014.

9.1.3 Ovarian cancer grading system

Grade	Description
GX:	Grade cannot be evaluated (undetermined grade)
G1	Well-differentiated (low grade)
G2	Moderately differentiated (intermediate grade)
G3	Poorly differentiated (high grade)
G4	Undifferentiated (high grade)

Adapted from Edge and Compton, 2010 (Edge and Compton, 2010).

9.2 Breast cancer TNM-staging system according to American Joint Committee on Cancer (AJCC)

9.2.1 T Classifications (Primary tumor)

Category	Definition
TX	Primary tumor cannot be assessed.
T0	No evidence of primary tumor.
Tis (DCIS)	Ductal carcinoma in situ
Tis (Paget)	Paget disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS) in the underlying breast parenchyma. Carcinomas in the breast parenchyma associated with Paget disease are categorized based on the size and characteristics of the parenchyma disease, although the presence of Paget disease should still be noted.
T1	Tumor ≤20 mm in greatest dimension.
T1 mi	Tumor ≤1 mm in greatest dimension.
T1a	Tumor >1 but ≤5 mm in greatest dimension (round any measurement >1.0-1.9 mm to 2 mm).
T1b	Tumor >5 mm but ≤10 mm in greatest dimension.
T1c	Tumor >10 mm but ≤20 mm in greatest dimension.
T2	Tumor >20 mm but ≤50 mm in greatest dimension.
T3	Tumor >50 mm in greatest dimension.
T4	Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or macroscopic nodules); not including invasion of the dermis alone.
T4a	Extension to the chest wall; not including only invasion or adherence to pectoralis muscle
T4b	Ulceration and/or ipsilateral macroscopic satellite nodules and/or edema (including peau d'orange) of the skin that does not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b are present.
T4d	Inflammatory carcinoma.

Adapted from Hortobagyi et al. 2017.

9.2.2 N Classification (Lymph node status)

Clinical definition (cN)

Category	Definition
cNX	Regional lymph nodes cannot be assessed.
cN0	No regional lymph node metastasis (by imaging or clinical examination).
cN1	Metastasis to movable ipsilateral Level I, II, axillary lymph node(s).
cN1 mi	Micrometastases (approximately 200 cells, >0.2 mm but ≤2.0 mm).
cN2	Metastases in ipsilateral level I II axillary lymph nodes that are clinically fixed or matted; *or* in ipsilateral internal mammary nodes in the absence of axillary lymph node metastases.
cN2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures.
cN2b	Metastases only in ipsilateral internal mammary nodes in the absence of axillary lymph node metastases.
cN3	Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s) with or without level I, II axillary lymph node involvement; *or* in ipsilateral internal mammary lymph node(s) with level I, II axillary lymph node metastases; *or* metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement.
cN3a	Metastases in ipsilateral infraclavicular lymph node(s).
cN3b	Metastases in ipsilateral internal mammary lymph node(s) and axillary lymph node(s).
cN3c	Metastases in ipsilateral supraclavicular lymph node(s).

Adapted from Hortobagyi et al. 2017.

Pathological definition (pN)

Category	Definition
pNX	Regional lymph nodes cannot be assessed.
pN0	No regional lymph node metastasis identified or ITCs only.
pN0(i+)	ITCs only (malignant cell clusters ≤0.2 mm) in regional lymph node(s).
pN0(mol+)	Positive molecular findings by reverse transcriptase-polymerase chain reaction (RT-PCR); no ITCs detected.
pN1	Micrometastases; or metastases in 1-3 axillary lymph nodes; and/or clinically negative internal mammary nodes with micrometastases or macrometastases by sentinel lymph node biopsy.
pN1 mi	Micrometastases (approximately 200 cells, >0.2 mm but ≤2.0 mm).
pN1a	Metastases in 1-3 axillary lymph nodes, at least one metastasis >2.0 mm.
pN1b	Metastases in ipsilateral internal mammary sentinel nodes, excluding ITCs.
pN1c	pN1a and pN1b combined.
pN2	Metastases in 4-9 axillary lymph nodes; or positive ipsilateral internal mammary lymph nodes by imaging in the absence of axillary lymph node metastases.
pN2a	Metastases in 4-9 axillary lymph nodes (at least one tumor deposit >2.0 mm).
pN2b	Metastases in clinically detected internal mammary lymph nodes with or without microscopic confirmation; with pathologically negative axillary nodes.
pN3	Metastases in 10 or more axillary lymph nodes; *or* in infraclavicular (Level III axillary) lymph nodes; *or* positive ipsilateral internal mammary lymph nodes by imaging in the presence of one or more positive Level I, II axillary lymph nodes; *or* in more than 3 axillary lymph nodes and micrometastases or micrometastases by sentinel lymph node biopsy in clinically negative ipsilateral internal mammary lymph nodes; *or* in ipsilateral supraclavicular lymph nodes;
pN3a	Metastases in 10 or more axillary lymph nodes (at least one tumor deposit >2.0 mm); *or* metastases to the infraclavicular (Level III axillary) lymph nodes.
pN3b	pN1a or pN2a in the presence of cN2b (positive internal mammary nodes by imaging); *or* pN2a in the presence of pN1b.
pN3c	Metastases in ipsilateral supraclavicular lymph nodes.

ITCs: Isolated tumor cell clusters. Adapted from Hortobagyi et al. 2017.

9.2.3 M classification (Distant metastasis)

Category	Definition
M0	No clinical or radiographic evidence of distant metastases.
cM0(i+)	No clinical or radiographic evidence of distant metastases in the presence of tumor cells or deposits <0.2 mm detected microscopic or by molecular techniques in circulating blood, bone marrow, or other non-regional nodal tissue in a patient without symptoms or signs of metastases
cM1	Distant metastases detected by classic clinical and radiographic means.
pM1	Any histologically proven metastases in distant organs; or if in non-regional nodes, metastases >0.2 mm.

Adapted from Hortobagyi et al. 2017.

9.2.4 AJCC stage group

Stage	T	N	M
0	Tis	N0	M0
IA	T1	N0	M0
IB	T0	N1 mi	M0
	T1	N1 mi	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

Adapted from Hortobagyi et al. 2017.

10 Abbreviations

ACPT	acid phosphatase
AJCC	American Joint Committee on Cancer
AR	androgen receptor
Asp	aspartic
BMP	bone morphogenic protein
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
Ct	cycle threshold
DFS	disease-free survival
DMEM	dulbecco's modified eagle's Medium
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleoside triphosphate
E	efficiency
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assays
EMSP1	enamel matrix serine proteinase 1
EMT	epithelial-mesenchymal transition
EOC	epithelial ovarian cancer
EP	error propagation
ER	estrogen receptor
FBS	fetal bovine serum
FGF	fibroblast growth factor
FIGO	Féderation Internationale de Gynécologie et d'Obstétrique
FISH	fluorescence in situ hybridization
GFP	green fluorescent protein
HER2	the human epidermal growth factor receptor 2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGFA	hepatocyte growth factor activator
HGF/SF	hepatocyte growth factor/scatter factor
HGSOC	high-grade serous ovarian cancer
His	histidine
HPRT	hypoxanthine-guaninephosphoribosyltransferase
HR	hazard ratio
HSCCE	human stratum corneum chymotryptic enzyme
IGF	insulin-like growth factor
IGFBPs	insulin-like growth factor binding proteins

kDa	kilo Dalton
KLK	kallikrein-related peptidase
KLK1	tissue kallikrein
KLKB1	plasma kallikrein
L1CAM	cell adhesion molecule L1
LMP	low malignant potential
M	distant metastasis
MAPK	mitogen-activated protein kinases
MCA	multicellular aggregates
MMP	matrix metalloproteinase
N	regional lymph nodes
NEOC	non-epithelial ovarian cancer
NSCLC	non-small cell lung cancer
OS	overall survival
PARs	protease-activated receptors
PBS	phosphate-buffered saline
PDGF-B	platelet-derived growth factor B
PFS	progression-free survival
PR	progesterone receptor
PSA	prostate-specific antigen
PTHRP	parathyroid-hormone-related protein
qPCR	quantity polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	real-time polymerase chain reaction
SDs	standard deviations
Ser	serine
SFTI-FCQR	sunflower trypsin inhibitor
SIGLEC9	sialic acid-binding Ig-like lectin 9
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
STDEV	standard deviation
T	the primary tumor
TCGA	The Cancer Genome Atlas
TGF-ß	transforming growth factor-beta
TNBC	triple-negative breast cancer
TSP1	thrombospondin-1
TUM	Technical University of Munich
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
UTR	untranslated region

VEGF	vascular endothelial growth factor
WHO	the World Health Organization

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