



PRECLINICAL STUDY

Clinical relevance of the deregulated kallikrein-related peptidase 8 mRNA expression in breast cancer: a novel independent indicator of disease-free survival

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Abstract Examining for new BC biomarkers has proven that kallikrein-related peptidase (KLK) family members represent promising serum and/or tissue molecular tools for early diagnosis, effective prognosis, and treatment monitoring of patients. The aim of this study was to investigate, the previously unexplored, prognostic significance of *KLK8* in BC. *KLK8* mRNA expression was quantitatively analyzed in 150 cancerous and 100 corresponding normal breast tissue specimens via a SYBR Green-based Real-Time PCR methodology. Expression data and patients' clinicopathological parameters were used for extensive biostatistical analyses, including internal validation. *KLK8* mRNA expression was significantly downregulated in the cancerous tissue part relative to the non-cancerous counterpart ($P < 0.001$), in the majority of the paired breast tissue samples. *KLK8* expression was associated with advanced TNM stage ($P = 0.019$) and positive nodal status involvement ($P = 0.044$). Triple negative (TNBC) and HER2 overexpressing tumors exhibited higher *KLK8* expression levels ($P < 0.001$), compared to Luminal A and B molecular subtypes. Kaplan–Meier survival curve analysis revealed that BC patients with high *KLK8* expression had significantly shorter disease-free survival (DFS)

intervals ($P < 0.001$) compared to those belonging in the *KLK8*-low expression group. Cox univariate analysis confirmed the association between *KLK8* expression, analyzed as a continuous variable, and poor patients' outcome (Hazard ratio [HR] = 3.28, $P < 0.001$). Most importantly, multivariate analysis showed that *KLK8* expression is a strong and independent predictor of adverse DFS in BC ([HR] = 2.74; $P = 0.002$). Our results show that *KLK8* mRNA expression is associated with aggressive tumor characteristics and it can serve as a novel independent biomarker of unfavorable prognosis for BC patients.

Keywords *KLK8* · Kallikreins · Biological tumor marker · Prognostic biomarker

Abbreviations

AUC	Area under the curve
BC	Breast cancer
CI	Confidence interval
Ct	Threshold cycle
DFS	Disease-free survival
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
<i>HPRT1</i>	Hypoxanthine phosphoribosyltransferase 1
IHC	Immunohistochemistry
KLK	Kallikrein-related peptidase
MMPs	Matrix metalloproteinases
PR	Progesterone receptor
ROC	Receiver operating characteristic
RQ units	Relative quantification units
RT-qPCR	Quantitative real-time PCR
TNBC	Triple negative breast cancer
TNM	Tumor-node-metastasis

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Introduction

One of the major hurdles in improving breast cancer (BC) patients' management is the remarkable molecular and biological heterogeneous nature of the disease, which in turn results in distinct clinical presentations and behaviors of the tumors [1]. Although the routinely used clinicopathological factors of prognosis in BC, such as histological stage and grade, steroid hormonal receptors' (estrogen (ER) and progesterone (PR) receptors), and human epidermal growth factor receptor 2 (HER2) status, are highly useful [2], it is now apparent that these parameters have a limited capacity to predict the intrinsic complexity of BC and to tailor individual treatment [1]. Therefore, contemporary research efforts are focused towards the identification of novel and more reliable BC-specific biomarkers which can be found among the biomolecules that play key roles in fundamental processes underlying cancer establishment and progression, including members of the family of kallikrein-related peptidases (KLKs).

Kallikrein-related peptidases (KLKs) are fifteen closely related secreted serine proteases, which are expressed in a broad spectrum of human tissues and are involved in a plethora of physiological activities [3–5]. Many studies provide evidence that the deregulated KLK expression and/or function is a common event in human malignancies [6, 7] and notably, BC development and KLKs are closely related as well. In particular, KLK3, KLK6, and KLK10 display tumor suppressive characteristics, whereas KLK1 and KLK11 exhibit tumor promoting effects during BC progression [6, 8–12].

The *KLK8* gene, which maps to the human kallikrein multigene cluster, encodes for serine protease with trypsin-like activity [13]. Several experimental data propose distinct mechanisms, by which KLK8 participates in tumor progression. Rajapakse et al. showed that KLK8 can efficiently degrade a number of ECM proteins such as collagen type IV and fibronectin, and thereby facilitates pericellular proteolysis and cancer cell invasion. The same study also revealed that KLK8 is able to activate single-chain tissue-type plasminogen activator (tPA), which in turn generates plasmin from plasminogen. Plasmin also plays an important role in ECM remodeling, either directly through its own activity or indirectly via the activation of MMPs [13]. Contrariwise, Sher et al. demonstrated that overexpression of KLK8 suppresses the invasiveness of lung adenocarcinoma cells in vitro and inhibits tumor growth and invasion in vivo. This finding was attributed to the degradation of fibronectin by KLK8, which interferes with the fibronectin-integrin signaling pathway and reduces tumor cell motility by preventing actin polymerization [14].

KLKs are being actively investigated for their clinical value as molecular tumor markers [15, 16] and *KLK8* has

been proposed as a promising prognostic biomarker for ovarian [17–19] and lung cancer [14, 20]. Although previous studies have revealed that *KLK8*, like most of the *KLK* genes, is downregulated in BC compared with non-cancerous breast tissues [21, 22], no data have been reported concerning its possible prognostic importance in BC.

Therefore, the aim of this study was the comprehensive mRNA expression analysis of *KLK8* in breast tumors and adjacent non-cancerous breast tissues and the investigation of its previously unexplored, clinical usefulness and prognostic significance as a novel molecular tissue biomarker for BC.

Materials and methods

Collection of breast tissue samples and clinical data

A total of 150 breast tumor specimens and 100 matched non-malignant breast tissue sections were collected during routine therapeutic surgery of patients with newly diagnosed BC, at the “Saint Savvas” Anticancer Hospital of Athens, between 2010 and 2011. Immediately after resection, each malignant and corresponding normal breast tissue sample was histopathologically characterized and stored at -80°C until use. This study was designed according to new guidelines for reporting new tumor biomarkers [23] and it was approved by the institutional review board of “Saint Savvas” Anticancer Hospital. Research procedures of this study complied with the ethical standards of the World Medical Association's Declaration of Helsinki. All participants provided written informed consent prior to breast tissue harvesting. None of the patients had received pre-operative (neoadjuvant) treatment.

Patient characteristics and tumor clinical and histopathological features were also provided for statistical analyses. Tumors were staged according to tumor-node-metastasis (TNM) classification and graded based on the Bloom–Scarff–Richardson grading system (Supplementary Table S1).

Adjuvant systemic treatment was administered to BC patients according to the guidelines of “Saint Savvas” Anticancer Hospital and the respective consensus recommendations at the time. In particular, 28 (18.7 %) patients were treated with endocrine therapy alone; 43 (28.7 %) patients were given chemotherapy alone; 48 (32.0 %) patients had a combination of chemotherapy and endocrine therapy, and 19 (12.7 %) patients underwent adjuvant treatment with HER2-targeted therapy in addition to chemotherapy. Different treatments are shown in Supplementary Table 1.

BC-specific disease-free survival (DFS) information were available for 124 patients. DFS was defined as the time interval between the surgical resection of the tumor

Table 1 Descriptive statistics of continuous variables of the study in breast cancer patients

Variable	No. of patients	Mean \pm SE ^a	Range	Percentiles		
				25th	50th (median)	75th
<i>KLK8</i> (RQ units)						
Cancerous tissues ^b	150	9.8 \pm 2.0	0.009–171.8	0.8	2.5	6.4
Non-cancerous tissues ^b	100	11.5 \pm 1.4	0.01–89.8	3.2	7.7	12.9
Patients' age (years)	149	58.7 \pm 1.1	31.0–89.0	48.0	59.0	71.0
Tumor size (cm)	139	2.8 \pm 0.1	0.8–10.0	1.8	2.5	3.1
Ki67 labeling index	135	15.3 \pm 1.2	0.0–60.0	4.0	11.0	25.0
ER expression ^c	147	1.2 \pm 0.1	0.0–3.0	0.0	1.0	2.2
PR expression ^c	146	0.8 \pm 0.1	0.0–3.0	0.0	0.1	1.6
DFS (months)	124	35.9 \pm 0.9	3.8–47.2	33.4	39.5	43.1

^a Standard error^b Relative quantification units = $2^{-\Delta\Delta CT}$ ^c Immunohistochemical score (Hscore)

and the date of the first documented event of either local or regional recurrence, second cancer, or death from BC, and was used as an endpoint in the statistical survival analyses [24]. Table 1 summarizes clinicopathological information of the samples and patients' characteristics.

Immunohistochemical (IHC) assessment of hormone receptors' expression, HER2 status, and Ki67 labeling index

The expression of ER, PR, HER2, and Ki-67 labeling index in BC tissue samples was determined by IHC staining in the pathology laboratory of "Saint Savvas" Anticancer Hospital. The antibodies used were for ER (Dako 1D5), PR (Dako PgR636), HER2 (Dako polyclonal rabbit anti-human c-erbB2), and Ki67 (Dako MIB1).

ER and PR were scored semiquantitatively on the basis of both the staining intensity (*i*) and the corresponding percentage of positive-stained cells (*Pi*), using the equation $Hscore = \sum (Pi \times i/100)$. The cutoff for positivity of Hscore was 0.35 for ER and 0.25 for PR. HER2 expression was initially analyzed by IHC and the intensity of membrane protein staining scores were defined as following: 0, no staining; 1+, incomplete, weak membrane staining in >10 % of tumor cells; 2+, weak complete membrane staining in >10 % of tumor cells; and 3+, intense complete membrane staining in >10 % of tumor cells. HER2 expression status was considered negative if immunostaining was scored as 0 or +1, and positive if immunostaining was scored as +3. For an equivocal HER2 IHC (2+) test result, HER2 expression status was considered positive if fluorescent in situ hybridization (FISH) assay revealed a HER2: chromosome-17 amplification ratio of >2.2 [25]. For the nuclear antigen Ki67, the percentage of

positively stained nuclei was calculated. The cutoff value for Ki67 labeling index was considered as 14 % of positive cancer nuclei, in order to distinguish tumors with low (<14 %) and high (≥ 14 %) proliferative fraction [26].

Furthermore, according to the St. Gallen expert consensus, breast tumors can be classified into distinct molecular subtypes based on IHC staining results for estrogen and progesterone receptor status, IHC or in situ hybridization tests for the detection of overexpression and/or amplification HER2 and Ki67 labeling index. Therefore, the above-mentioned assessments were used to categorize breast tumors into: luminal A (ER+ and/or PR+, Ki67 low and HER2-), luminal B (ER+ and/or PR+, Ki67 high and/or HER2+), HER2-overexpressing (ER-, PR- and HER2+), and triple negative (ER-, PR-, and HER2-) subtypes [1, 26].

Total RNA extraction, RNA quality evaluation, and cDNA synthesis

Approximately 50–100 mg of each deep-frozen breast sample, were pulverized to a fine powder and homogenized by the addition of 1 mL of TRI reagent[®] (Molecular Research Center). Following the manufacturer's instructions, total RNA was extracted, diluted in RNA Storage Solution (Applied Biosystems), and stored in -80 °C. For all samples, total RNA purity and concentration were determined spectrophotometrically and RNA integrity was visually confirmed using agarose gel electrophoresis. Single-stranded cDNA synthesis was performed from 2 μ g of total RNA, using M-MuLV reverse transcriptase (Invitrogen), recombinant RNase inhibitor (Invitrogen), and oligo (dT)₁₈ primer, in a final reaction volume of 20 μ L.

Quantitative real-time PCR (RT-qPCR)

The quantification of *KLK8* mRNA expression levels was performed by RT-qPCR, using the SYBR Green chemistry on a 7500 fast real-time PCR system (Applied Biosystems). Gene-specific primer pairs were designed based on the published cDNA sequences of the reference gene *HPRT1* (NM_000194.2), and the *KLK8* type 1 mRNA transcript variant, which encodes the canonical 260-amino acid KLK8 protein (NM_007196.3). The primer sequences used in this study were as follows: *KLK8* forward 5'-GGAGCCTGGG CAGGACAC-3' and reverse 5'-AAGGACACCGCCACAG AGTAGTT-3' (PCR amplicon of 129 bp); *HPRT1* forward 5'-TGGA AAGGGTGT TTTATTCCTCAT-3' and reverse 5'-ATGTAATCCAGCAGGTCAGCAA-3' (PCR amplicon of 151 bp). We selected *HPRT1* as a housekeeping gene for data normalization as it was previously identified as the most suitable single endogenous control gene for expression studies in various solid tumors, including BC [27].

The RT-qPCR reaction mixture consisted of 10 ng of template cDNA, 5.0 μ L Kapa SYBR fast qPCR Master Mix (Kapa Biosystems), 0.2 μ L of 50 \times Rox low passive reference dye (Kapa Biosystems), 1.0 μ L of gene-specific primers (final concentration *KLK8*: 350 nM; *HPRT1*: 300 nM), adjusted to a final volume of 10.0 μ L with DEPC-treated water. All genes were amplified in technical triplicates for each sample, using the following thermal cycling conditions: an initial step at 95 $^{\circ}$ C for 3 min, followed by 40 cycles of 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 min. After the completion of RT-qPCR cycles, melting curves of amplified products were generated in order to verify the reaction specificity and the absence of primer dimers and/or contamination. In addition, randomly selected RT-qPCR products were electrophoresed on 3.0 % (w/v) agarose gel and visualized, under UV light, after ethidium bromide staining.

The relative quantification of *KLK8* mRNA expression was performed using the comparative threshold cycle ($\Delta\Delta$ Ct) method. Relative quantification units (RQ units) of *KLK8* in each sample were calculated based on the equation: RQ units = $2^{-\Delta\Delta$ Ct}. The Δ Ct is the difference between the Ct value of the target gene and the endogenous control [Δ Ct = Ct (*KLK8*) – Ct (*HPRT1*)] and $\Delta\Delta$ Ct is the difference between the average Δ Ct value of an experimental sample and the average Δ Ct of the corresponding calibrator [$\Delta\Delta$ Ct = Δ Ct (sample) – Δ Ct (calibrator)] [28]. In the current study, the human BC cell line BT-474 served as a calibrator.

The main requirement for the application of the comparative Ct method is that the amplification efficiencies of the target and reference genes are approximately equal and close to 100 %. Therefore, validation experiments were carried out using a tenfold dilution series of calibrator cDNA, covering several orders of magnitude, for the

generation of standard curves by plotting Ct values vs log of input cDNA. The RT-qPCR efficiency (E) was calculated from the slope of the standard curves according to the formula: $E = (10^{(-1/\text{slope})} - 1) \times 100$.

Statistical analyses

The *KLK8* expression data and patients' clinicopathological characteristics were subjected to extensive biostatistical analyses using the SPSS software program (SPSS Inc., version 17.0). The nonparametric Wilcoxon signed-ranks test was applied to analyze the differences of *KLK8* mRNA expression in the pairs of matched non-cancerous and tumor counterparts. We also used the Oncomine database [29] to determine whether *KLK8* mRNA expression is deregulated in clinical BC samples compared to non-cancerous breast tissues, based on microarray gene expression profiling studies employing established patient datasets. Moreover, the correlations between continuous variables of the study were examined with Spearman correlation coefficient (r_s). The Mann–Whitney *U*, Jonckheere–Terpstra, and Kruskal–Wallis tests were employed to scrutinize the differences of *KLK8* expression between distinct groups of BC samples. The capability of *KLK8* levels to distinguish triple negative breast tumors (TNBC) from other molecular BC subtypes was evaluated via the construction of Receiver operating characteristic (ROC) curve, by plotting sensitivity versus (1-specificity) values. The area under the curve (AUC) was analyzed using the Hanley and McNeil method.

We also applied the X-tile algorithm [30] in order to dichotomize BC patients into *KLK8*-low and *KLK8*-high expression populations. X-tile plots allowed the determination of an optimal cutoff value for *KLK8* expression data based on DFS, while correcting for the use of minimum *P* value statistics. Corrected *P* values for the cutoff points were calculated using the Miller–Siegmund *P* value correction and Monte Carlo simulations (e.g., Cross-validation) based on 1000 random populations. The software also splits the entire population cohort into random training and validation subsets, and subsequently finds the appropriate cutoff in the training set and then applies this cut-point in the validation cohort, allowing in this way the internal validation of the results. The cutoff point calculated by the above-mentioned approaches was defined as 4.68 RQ units of *KLK8* expression (69th percentile). *KLK8* mRNA expression was also analyzed as a continuous variable in order to minimize the loss of information due to categorization. Survival analysis was done by constructing Kaplan–Meier DFS curves and significance was evaluated using the log-rank test. Both univariate and multivariate Cox proportional hazard regression models were developed in order to determine the prognostic value, with respect to

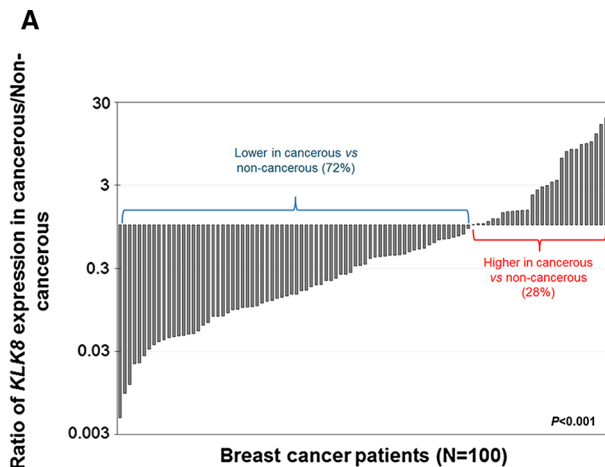


Fig. 1 The mRNA expression of *KLK8* is frequently decreased in human BC tissues. **a** Bar graph representing the relative expression levels of *KLK8* in 100-paired BC specimens and matched adjacent non-cancerous tissues. Data are presented as the ratio of *KLK8*

DFS, of *KLK8* expression and other clinical parameters. The multivariate regression model was adjusted for established clinicopathological and demographic variables including TNM stage, lymph node status, histological grade, BC molecular subtype, and patients' age. Moreover, we developed a separate multivariate model which included *KLK8* expression and the different adjuvant treatment modalities used (chemotherapy and endocrine therapy).

We next performed a complementary statistical approach, in order to analyze the prognostic value of *KLK8* mRNA expression in subgroups of patients, stratified according to the type of systemic adjuvant treatment received. Thereafter, Kaplan–Meier survival analysis was done for each patient subgroup (i.e., adjuvant chemotherapy-treated, no chemotherapy-treated, adjuvant endocrine-treated, and no endocrine-treated). A *P* value of less than 0.05 was considered as an indication of statistical significance.

Results

Quality control of the RT-qPCR assay for the quantification of *KLK8* mRNA expression

Gene-specific amplification was confirmed by the presence of a single peak with the appropriate melting point temperature (T_m) for each amplicon (*KLK8* $T_m = 86.3$ °C; *HPRT1* $T_m = 81.5$ °C) (Supplementary Fig. 1A and B) and by the appearance of a single band, of the expected amplicon size, in the agarose gel electrophoresis analyses (Supplementary Fig. 1C).

The RT-qPCR amplification efficiencies for the reference and target gene were calculated from the slopes of the corresponding standard curves deriving from validation

B

Database	Tissues analyzed	Sample size	Fold-change	P-value
The Cancer Genome Atlas	Invasive Ductal Breast Carcinoma	389	-8.597	1.47E-16
	Invasive Breast Carcinoma	76	-4.912	4.06E-9
	Invasive Lobular Breast Carcinoma	36	-5.893	3.13E-7
Curtis Breast	Mucinous Breast Carcinoma	46	-1.118	6.13E-7
	Invasive Lobular Breast Carcinoma	148	-1.103	2.05E-6
	Invasive Ductal and Invasive Lobular Breast Carcinoma	90	-1.104	5.51E-6
Karnoub Breast	Invasive Ductal Breast Carcinoma	1556	-1.064	1.14E-4
Karnoub Breast	Invasive Ductal Breast Carcinoma	7	-1.617	2.37E-4
Turashvili Breast	Invasive Ductal Breast Carcinoma	5	-2.869	0.005
Richardson Breast 2	Ductal Breast Carcinoma	40	-1.730	0.008
Ma Breast 4	Ductal Breast Carcinoma in Situ	11	-1.156	0.025

expression in cancerous tissue part versus non-cancerous counterpart. The *P* value was calculated using “Wilcoxon signed-ranks test”. **b** Studies from Oncomine database showing significant downregulation of *KLK8* levels in breast carcinomas vs normal analyses

experiments. The slopes of *HPRT1* (-3.344 ; $R^2 = 0.999$) and *KLK8* (-3.402 0; $R^2 = 0.996$) standard curves were similar (Supplementary Fig. 1D), and the calculated PCR amplification efficiencies were 99.0 and 96.7 % correspondingly, thereby allowing relative quantification by the application of the $2^{-\Delta\Delta Ct}$ formula.

Downregulation of *KLK8* mRNA expression in cancerous compared to matched histologically normal breast tissues

The expression of *KLK8* was investigated in 100 paired specimens of cancerous and matched histologically normal breast tissues located adjacent to the carcinoma. This analysis revealed that *KLK8* mRNA expression levels were significantly downregulated in BC tissue sections compared to the non-cancerous component in 72 % of the cases examined, whereas only 28 % of the paired samples exhibited higher *KLK8* expression in the cancerous part than their corresponding non-tumor breast tissues ($P < 0.001$; Wilcoxon signed-ranks test; Fig. 1a).

Using the oncomine database, we observed that *KLK8* expression levels were substantially downregulated in the majority of BCs of different subtypes, relative to non-malignant breast tissues (Fig. 1b).

KLK8 mRNA expression is associated with advanced TNM stage, lymph node positive status, and triple negative breast tumors (TNBC)

In the next step of our study, we investigated the associations between *KLK8* mRNA expression levels in BC tissues with the clinicopathological parameters of the patients examined. Our data showed that *KLK8* expression is

Table 2 Associations of *KLK8* mRNA expression with clinicopathological data of BC patients

Variable	No. of patients	Mean ± SE ^a	Median	<i>P</i> value
TNM stage ^b				
0 or I	34	6.8 ± 3.3	2.16	0.019^d
II	85	10.8 ± 3.1	2.31	
III or IV	25	11.6 ± 3.4	5.18	
Molecular subtype				
Luminal A	47	5.9 ± 2.4	1.24	0.002^e
Luminal B	42	4.9 ± 1.6	1.80	
HER2 group	14	23.6 ± 13.1	5.74	
TNBC	32	16.7 ± 5.8	3.85	
Molecular subtype				
Luminal A and B (luminal subtypes)	89	5.5 ± 1.5	1.59	<0.001^f
TNBC and HER2 (non-luminal subtypes)	46	18.8 ± 5.6	4.47	
Lymph node status				
N0 or N1	123	9.6 ± 2.3	2.32	0.044^f
N2 or N3	18	11.1 ± 3.5	5.52	
Histological grade ^c				
Grade I or II	96	8.2 ± 2.3	1.94	0.253 ^f
Grade III	50	13.5 ± 3.9	2.77	
HER2 status				
Negative	104	8.3 ± 1.9	2.43	0.209 ^f
Positive	41	14.4 ± 5.3	2.82	
Ki67 labeling index				
Negative	77	8.8 ± 2.9	2.55	0.650 ^f
Positive	58	8.7 ± 2.3	2.52	
Age (years)				
<60	80	6.9 ± 1.4	2.55	
≥60	69	13.1 ± 4.0	2.33	0.790 ^f

^a Standard error^b TNM staging system^c Bloom–Scarff–Richardson grading system^d Calculated using the “Jonckheere–Terpstra test”^e Calculated by the “Kruskal–Wallis test”^f Calculated by the “Mann–Whitney *U* test”

Bold value indicates statistical significance

associated with several clinicopathological features, indicative of unfavorable prognosis in BC patients (Table 2). In particular, *KLK8* mRNA expression levels had an increasing trend with the progression of the tumor TNM stage ($P = 0.019$) (Fig. 2a). The median *KLK8* expression in the group of patients with clinical stage III or IV (5.18 RQ units) was remarkably higher compared to TNM stage II (2.31 RQ units), and stage I or 0 (2.16 RQ units) tumors. Furthermore, a significant positive relationship between *KLK8* expression with positive nodal status was also observed ($P = 0.044$).

KLK8 mRNA expression differed remarkably ($P = 0.002$; Kruskal–Wallis Test) between BC molecular subtypes (Fig. 2b). Moreover, *KLK8* mRNA expression was found to be significantly increased ($P = 0.009$) in the group of patients with TNBC (median 3.85 RQ units) tumors compared to those

with other BC molecular subtypes (median 1.64 RQ units). ROC curve analysis revealed the ability of *KLK8* mRNA expression to distinguish patients with TNBC from patients harboring breast tumors of other molecular subtypes ([AUC] = 0.653, [95 % CI] = 0.546–0.760, $P = 0.009$) (Fig. 2d). High *KLK8* levels were also found in TNBC and HER2 overexpressing groups (collectively non-luminal subtypes) compared to luminal A and B (luminal subtypes) ones ($P < 0.001$) (Fig. 2c). The median *KLK8* expression in the group of patients with non-luminal BC subtypes (4.47 RQ units) was substantially higher compared to luminal (1.59 RQ units) carcinomas.

On the contrary, no significant association was found between *KLK8* expression and tumor grade, HER2 status, Ki67 proliferative index, or patients' age.

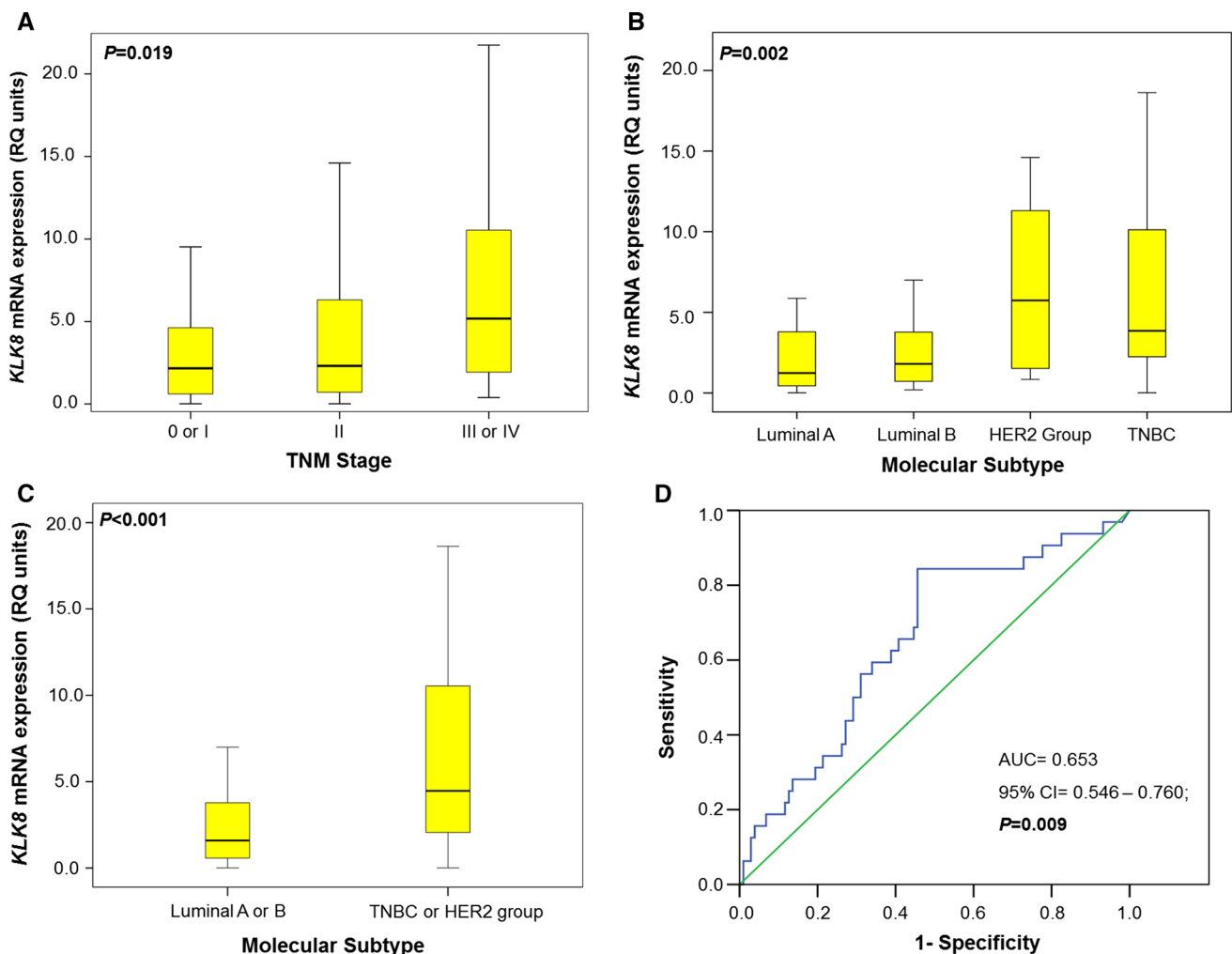


Fig. 2 Association of *KLK8* mRNA expression with clinicopathological features of breast tumors. Boxplots representing *KLK8* expression levels in early vs advanced TNM stage tumors (a), in distinct BC molecular subtypes (b) and in luminal vs non-luminal

disease (c). ROC curve analysis revealed the ability of *KLK8* mRNA expression to distinguish patients with TNBC from patients harboring breast tumors of other molecular subtypes (d)

Using the Spearman correlation coefficient analysis we also observed a weak negative correlation between *KLK8* mRNA levels and ER ($r_s = -0.281$; $P = 0.001$) and PR ($r_s = -0.273$; $P = 0.001$) expression, in breast tumors. No correlations were observed between *KLK8* expression and other continuous variables such as tumor size, patients' age, and Ki67 labeling index.

Internal validation of the prognostic significance of *KLK8* based on an optimal cutoff value

The X-tile algorithm generated an optimal cutoff value equal to the 69th percentile (4.68 RQ units) of *KLK8* expression that was able to effectively dichotomize our patient cohort based on DFS analysis, while correcting the chance of type I errors (Miller–Siegmund $P < 0.001$). The

significance of the selected cutoff point was reinforced by Monte Carlo simulations based on 1000 random populations (Cross-validation $P = 0.0010$, Monte Carlo corrected $P < 0.001$). Moreover, the patients' cohort was randomly divided into training and validation subsets (2:1 patient population ratio), in order to perform internal validation. The above-mentioned selected cutoff was identified as optimal in the training cohort ($P = 0.0012$), and after its application in the validation cohort it also reached high statistical significance ($P = 0.014$).

KLK8 expression is associated with poor DFS of BC patients

Next, we aimed to investigate the possible prognostic value of *KLK8* expression status in the entire BC patients' cohort.

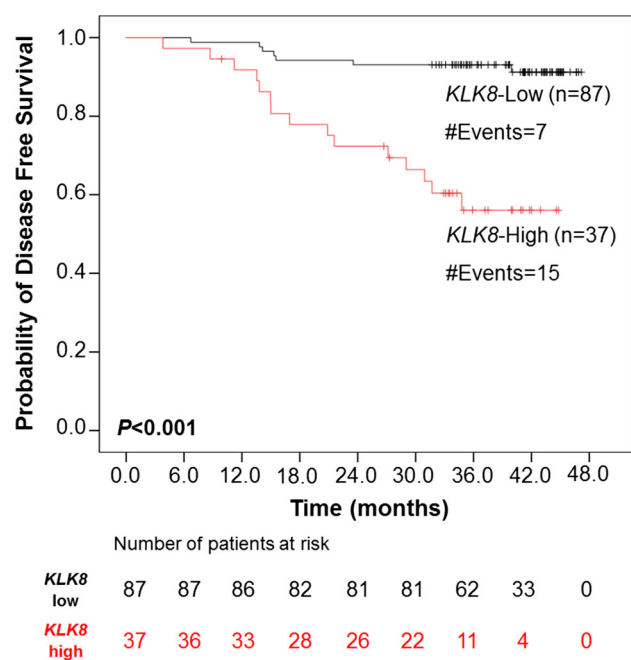


Fig. 3 Kaplan–Meier disease-free survival curve for the whole cohort of 124 BC patients. Patients were categorized as *KLK8*-high or *KLK8*-low expression groups according to the optimal cutoff value (69th percentile). *P* value was calculated via the log-rank algorithm

For this purpose, we initially performed Kaplan–Meier DFS curve analysis with log-rank test for determining statistical significance. Notably, patients with BC belonging to the *KLK8*-high expression group had significantly ($P < 0.001$) shorter DFS intervals compared to patients in the *KLK8*-low expression group (Fig. 3). The 3-year cumulative probability of BC-specific DFS for patients belonging to the *KLK8*-high group was 0.561 whereas the corresponding probability for patients in the *KLK8*-low group was 0.931.

Using univariate Cox regression analysis, we further confirmed the unfavorable prognostic value of *KLK8* expression, with respect to DFS, in BC patients. Firstly, analysis was performed using *KLK8* mRNA expression as a continuous variable (log-transformed values of RQ units). As shown in Table 3, the risk of relapse was significantly associated with *KLK8* mRNA expression (hazard ratio [HR] = 3.28, 95 % confidence interval [95 % CI] = 1.92–5.61, $P < 0.001$). Moreover, BC patients belonging in the *KLK8*-high expression group had a significantly higher risk of relapse ([HR] = 6.55, [95 % CI] = 2.66–16.15, $P < 0.001$) over time, compared to patients in the *KLK8*-low expression group.

Additional clinicopathological variables, including clinical TNM stage, BC molecular subtype, tumor size, and lymph node status were also strongly associated with poor DFS of BC patients (All HR >1.0, and *P* values <0.05; Table 3).

KLK8 expression is an independent predictor of unfavorable prognosis in BC patients

The independence of *KLK8* expression in predicting unfavorable outcome in BC patients was also evaluated using Cox multivariate analysis. In particular, after adjustment for important clinicopathological factors including TNM stage, nodal status, BC molecular subtype, histological grade, and patients' age, *KLK8* mRNA expression, as a continuous variable, was found to be a strong independent predictor of adverse prognosis for BC patients with a hazard ratio of 2.69 ([95 % CI] = 1.42–5.12, $P = 0.003$). Moreover, we repeated the analysis described above using *KLK8* expression as a binary variable. Again, high-*KLK8* expression retained its independent unfavorable prognostic nature with a hazard ratio 4.54 ([95 % CI] = 1.64–12.57, $P = 0.004$) (Table 4).

Moreover, a separate multivariate model which included *KLK8* mRNA expression and the different adjuvant treatment modalities (chemotherapy; endocrine therapy) was developed. Interestingly, after correcting for adjuvant treatment, *KLK8* mRNA expression retained its independence as an indicator of unfavorable outcome for BC patients ([HR] = 2.00, [95 % CI] = 1.08–3.73, $P = 0.029$). The same conclusions were drawn when *KLK8* mRNA expression was used as a dichotomous variable ([HR] = 3.31, [95 % CI] = 1.22–8.98, $P = 0.019$).

KLK8 mRNA expression is associated with unfavorable DFS in subgroups of BC patients, stratified according to the type of adjuvant systemic therapy received

In order to evaluate the prognostic relevance of *KLK8* mRNA expression regarding DFS in subgroups of patients based on the adjuvant systemic therapy received, a stratified Kaplan–Meier survival curve analysis was performed. In both, adjuvant chemotherapy-treated and endocrine-treated subgroups, patients with high-*KLK8* mRNA expression presented a significantly shorter DFS (chemotherapy-treated $P = 0.003$; endocrine-treated $P = 0.001$), compared to those categorized as *KLK8*-low. In patients who had not received adjuvant chemotherapy, high-*KLK8* was also associated with shorter DFS intervals reaching statistical significance ($P = 0.016$). However, in the subgroup of patients who did not receive adjuvant endocrine treatment, no impact of *KLK8* expression status on DFS was observed ($P = 0.204$) (Fig. 4).

Discussion

In the present study we quantitatively analyzed *KLK8* mRNA expression in breast tumors and matched histologically normal tissue sections, in order to investigate the

Table 3 Cox univariate regression analysis of *KLK8* expression and clinicopathological variables for the prediction of disease-free survival (DFS)

Variable	No. of patients	Univariate analysis		
		HR ^a	95 % CI ^b	<i>P</i> value
<i>KLK8</i> expression				
Log ₁₀ (<i>KLK8</i> RQ units)	124	3.28	1.92–5.61	<0.001
Negative	87	1.00		
Positive	37	6.55	2.66–16.15	<0.001
TNM stage				
0/I/II	102	1.00		
III/IV	19	4.99	2.04–12.26	<0.001
Lymph node status				
N0/N1	104	1.00		
N2/N3	15	3.85	1.47–10.02	0.006
Molecular subtype				
Luminal A or B	81	1.00		
TNBC or HER2 group	38	3.74	1.59–8.76	0.002
Grade				
I/II	81	1.00		
III	42	0.76	0.29–1.95	0.571
HER2 status				
Negative	89	1.00		
Positive	34	1.87	0.79–4.37	0.150
Ki67 labeling index				
Negative	70	1.00		
Positive	46	0.67	0.26–1.74	0.412
CEA				
Negative	90	1.00		
Positive	8	1.73	0.39–7.61	0.469
Tumor size (ordinal)	119	1.41	1.13–1.76	0.003
Age (ordinal)	124	1.02	0.98–1.05	0.228
Adjuvant chemotherapy				
No	24	1.00		
Yes	93	1.35	0.38–4.65	0.639
Adjuvant endocrine therapy				
No	47	1.00		
Yes	70	0.16	0.05–0.48	0.001

^a Hazard ratio, estimated from Cox proportional hazard regression model

^b Confidence interval of the estimated HR

Bold value indicates statistical significance

possible associations with clinicopathological features of the patients, and to further assess its prognostic performance in BC with respect to prediction of disease progression. To the best of our knowledge, this is the first study examining the clinical relevance of *KLK8* mRNA expression, in breast carcinomas.

Our data demonstrate that *KLK8* expression levels are significantly ($P < 0.001$) downregulated in BC tissue parts compared to adjacent histologically normal counterparts in the majority (72 %) of paired breast specimens examined. We next performed in silico expression analysis using information from the Oncomine expression profiling

database, which revealed that *KLK8* mRNA expression is markedly decreased in breast carcinomas than in normal breast tissues, corroborating our findings. These observations are in agreement with previous studies showing that most of the *KLK* family members, including *KLK8*, exhibit reduced mRNA and/or protein expression levels in breast tumors compared to non-malignant breast tissues [21, 22, 31]. In particular, Yousef et al., using a small set of BC and normal tissues showed that *KLK8* mRNA levels were lower in tumors than in non-cancerous breast tissues [22]. A recent study using massively parallel signature sequencing (MPSS) and isolated epithelial cells from BC and normal

Table 4 Cox multivariate analysis of *KLK8* expression regarding disease-free survival (DFS)

Variable	No. of patients	Multivariate analysis		
		HR ^a	95 % CI ^b	<i>P</i> value
<i>KLK8</i> expression ^c				
Log ₁₀ (<i>KLK8</i> RQ units)	115	2.69	1.42–5.12	0.003
TNM stage				
0/I/II	97	1.00		
III/IV	18	7.78	1.61–37.54	0.011
Lymph node status				
N0/N1	100	1.00		
N2/N3	15	0.68	0.13–3.48	0.642
Molecular subtype				
Luminal A or B	78	1.00		
TNBC or HER2 group	37	3.66	1.25–10.69	0.018
Grade				
I/II	75	1.00		
III	40	0.41	0.13–1.24	0.114
Age (ordinal)	115	1.03	0.99–1.08	0.168
<i>KLK8</i> expression ^c				
Negative	82	1.00		
Positive	33	4.54	1.64–12.57	0.004
TNM stage				
0/I/II	97	1.00		
III/IV	18	6.99	1.55–31.61	0.012
Lymph node status				
N0/N1	100	1.00		
N2/N3	15	0.54	0.11–2.64	0.446
Molecular subtype				
Luminal A or B	78	1.00		
TNBC or HER2 group	37	4.45	1.58–12.54	0.005
Grade				
I/II	75	1.00		
III	40	0.38	0.13–1.18	0.096
Age (ordinal)	115	1.04	1.00–1.09	0.046
<i>KLK8</i> expression ^d				
Log ₁₀ (<i>KLK8</i> RQ units)	117	2.00	1.08–3.73	0.029
<i>KLK8</i> expression ^d				
Negative	85	1.00		
Positive	32	3.31	1.22–8.98	0.019

^a Hazard ratio, estimated from Cox proportional hazard regression model

^b Confidence interval of the estimated HR

^c Multivariate models were adjusted for TNM stage, histological grade, nodal status, BC molecular subtype, and patients' age

^d Multivariate models were adjusted for adjuvant treatment modalities (chemotherapy and endocrine therapy)

Bold value indicates statistical significance

counterparts revealed that *KLK8*, along with *KLK5*, *KLK7*, *KLK10*, is among the set of genes which are significantly downregulated in the tumor epithelial transcriptome [32]. Several reports show that molecular mechanisms, such as epigenetic modifications and specifically DNA

hypermethylation, may contribute to the downregulation of *KLK* gene expression in BC [33]. One characteristic example is the tumor-specific *KLK10* exon 3 hypermethylation in BC cell lines and tissues [34, 35]. Therefore, we may speculate that changes in DNA methylation could

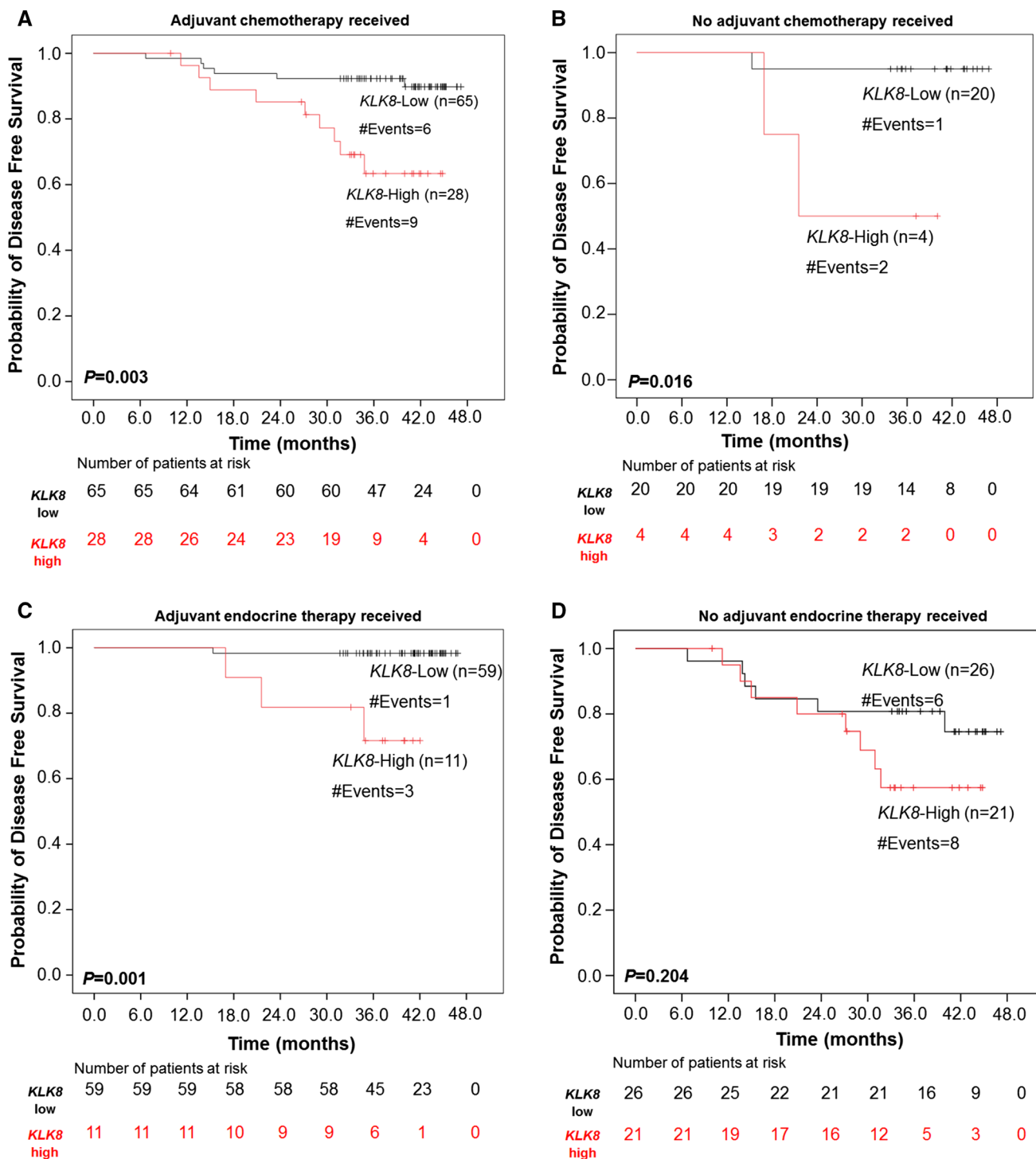


Fig. 4 Kaplan–Meier disease-free survival analyses for BC patient subgroups. Adjuvant chemotherapy-treated (a), no adjuvant chemotherapy-treated (b), adjuvant endocrine-treated (c), and no adjuvant endocrine-treated (d). P values were calculated via the log-rank algorithm

partially account for the deregulation of *KLK8* expression in breast tumors.

According to our data, *KLK8* mRNA expression was found to be associated with important indicators of poor prognosis in BC and with more aggressive forms of the

disease. In more details, *KLK8* mRNA levels were increased in a statistically significant degree ($P = 0.019$) in the group of patients with advanced TNM stage (III/IV) compared to those with TNM stage II or I breast tumors. In addition, *KLK8* expression was found to be associated with

positive nodal status of the patients ($P = 0.044$). We also observed a weak negative correlation between *KLK8* and ER ($r_s = -0.281$; $P = 0.001$) and PR ($r_s = -0.273$; $P = 0.001$) levels of expression.

Another important finding of our present research was the remarkable higher expression levels of *KLK8* in TNBC, compared to other molecular subtypes of disease. The differential diagnostic potential of this observation was also demonstrated by ROC curve analysis (AUC = 0.653; $P = 0.009$). Interestingly, mRNA expression of *KLK8* was significantly ($P < 0.001$) higher in non-luminal breast carcinomas (TNBC and HER2 overexpressing tumors) than luminal subtypes (luminal A and B) of BC. Generally, non-luminal BC molecular subtypes, and particularly TNBC, are associated with worse overall and disease-free survival rates compared to luminal disease [36–38]. Therefore, it is tentative to say that *KLK8* expression may be used in conjunction with established molecular markers in order to refine BC molecular classification and to improve treatment selection for individual patients. Supporting this hypothesis, the basal-like group of tumors, which typically lack or show low levels of hormone receptors and HER2 expression [38], exhibit high expression of a unique cluster of genes that are usually found in the basal epithelial cell layer. These include among others keratins 5, 6, and 17 and four *KLKs*, including *KLK8* [39]. Additionally, Glynn et al., using microdissected BC epithelium from high nitric oxide synthase ER-negative tumors also showed that *KLK8* was among the basal-like signature genes [40]. Furthermore, given on the one hand the recognition that gene expression signatures have the potential to identify molecular changes that can be used to predict recurrence of disease as well as response to specific therapies and on the other, the rapid rise in interest in developing multigene prognostic and predictive tools, such as real-time PCR-based assays (e.g., Oncotype DX and PAM50) and microarray-based multigene tests (e.g., MammaPrint) [41], one might consider the inclusion of *KLK8* into multifactorial biomarker panels and/or other multigene assays that may contribute in the multidimensional approach which is required for optimal management of BC patients. Interestingly, data from a “four-kallikrein panel” are very encouraging for prostate cancer patients’ management and several multicomponent *KLK*-based panels have been described for other cancers as well (e.g., ovarian and lung cancer) [15].

According to Kaplan–Meier survival curve analysis, after internal validation, patients belonging to the *KLK8*-high expression group had significantly ($P < 0.001$) shorter DFS intervals, compared to patients in the *KLK8*-low expression group. Cox univariate regression analysis confirmed the significantly poorer DFS of patients with

high-*KLK8* expression, analyzed as a continuous variable ([HR] = 3.28, $P < 0.001$). Although these results seem to be contradictory with the observed downregulation of *KLK8* mRNA expression in BC tissues compared to histologically normal counterparts, this phenomenon is, in fact, common and it was previously reported for other *KLK* family members in BC studies. For instance *KLK5*, *KLK7*, and *KLK14* are all reported to be downregulated in the BC tissues relative to non-cancerous ones. However, the expression of these *KLKs* has been associated with poor prognosis of BC patients [42–44]. The question that remains is how these *KLKs* are associated with BC progression, since their mRNA levels appear to be downregulated in BC tissues. One possible explanation is that *KLK* mRNA expression levels might not correlate with protein expression levels in BC tissues, due to a regulation on the translational level [22]. Another possible explanation comes from a microarray study by Tripathi et al., which showed that global gene expression abnormalities occur in normal epithelium of BC patients [45]. Interestingly, Schummer et al., revealed that a number of genes, usually associated with cancer pathways, are expressed at lower levels in BC compared to normal breast tissue [46]. Furthermore, growing evidence suggests, that primary tumor growth induces molecular changes in adjacent tissues that are believed to support cancer progression [47].

Multivariate Cox regression model adjusted for important clinicopathological parameters such as TNM stage, nodal status, BC molecular subtype, patients’ age, and histological grade, identified *KLK8* expression ([HR] = 2.69, $P = 0.003$) as a strong and independent indicator of unfavorable outcome with regard to DFS, for BC patients. Most importantly, *KLK8* mRNA expression retained its independence as an indicator of poor DFS for BC patients ([HR] = 2.00, $P = 0.029$), irrespective of the post-operative therapeutic modalities used. It should be noted here that *KLK8* protein expression was previously identified as a predictor of adverse progression-free survival in patients with advanced ovarian cancer [18], while *KLK8* type 4 splice variant is an independent indicator of poor OS in lung cancer patients [20].

Despite performing internal validation, a more rigorous verification of our observations through external validation on heterogeneous, larger, and multicentric datasets, is among our future goals in order to strengthen the significance of our findings and to confirm *KLK8* significance as a BC biomarker. Another interesting future perspective would be the assessment of *KLK8* protein expression in bodily fluids, such as nipple aspirate fluid or serum from BC patients, and its clinical evaluation as a soluble biomarker for non-invasive diagnosis, prognosis, and prediction or monitoring of chemotherapy response in BC.

Conclusions

Our study is the first to show that high-*KLK8* mRNA levels are significantly associated with advanced TNM stage, non-luminal (TNBC and HER2 overexpressing) molecular subtypes of BC, and with poor clinical outcome, in terms of DFS, of BC patients. Most importantly we show that *KLK8* expression is an independent indicator of unfavorable outcome for BC patients.

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Competing interests The authors declare that they have no competing interests.

Ethical standards All research procedures that took place during the completion of our study comply with the ethical standards of the 1975 Declaration of Helsinki, as revised in 2008, and were approved by the institutional review board of ‘Saint Savvas’ Anticancer Hospital. Additionally, written informed consent was obtained from all BC patients participating in the study.

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