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Review

Kallikrein-related peptidases (KLKs): a gene family of novel cancer biomarkers

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Abstract

Early diagnosis of cancer and early detection of relapse following surgery are critical for the effective treatment of the disease and for a positive clinical outcome. Identification of novel diagnostic, prognostic and predictive biomarkers will contribute utmost to clinical decision-making. The human tissue kallikrein and kallikrein-related peptidases (KLKs), encoded by the largest contiguous cluster of protease genes in the human genome, are secreted serine proteases with diverse expression patterns and physiological roles. The aberrant expression of *KLK*s in various malignancies as well as their involvement in many cancer-related processes, such as cell growth regulation, angiogenesis, invasion, and metastasis, has prompted scientists to investigate their potential as cancer biomarkers. Expression of distinct KLKs is associated with clinicopathological parameters of cancer patients. Moreover, several KLKs possess significant favorable or unfavorable prognostic value in various malignancies, with prostate-specific antigen (PSA) being the most widely used biomarker in clinical practice, today. KLKs are also considered as very promising biomarkers for cancer personalized medicine, especially for prediction and monitoring of patients' response to chemotherapy, therefore opening up new horizons towards effective patient monitoring post-treatment. This review describes the current status of KLKs as tumor biomarkers.

Keywords: cancer prognosis; personalized medicine; tumor biomarkers.

Introduction

Tissue kallikrein and kallikrein-related peptidases (KLKs) comprise a family of 15 homologous, single-chain, secreted trypsin- or chymotrypsin-like serine proteases of approximately 25–30 kDa. The *KLK* gene family locus spans ~300 kb, is located on the chromosomal region 19q13.3–q13.4, at a distance of 7.5 Mb from the telomeres of the long arm of chromosome 19, and consists of 15 *KLK* genes. The genes *KLK1*, *KLK15*, *KLK3*, *KLK2*, *KLK1*, and *KLK4–KLK14* are tightly clustered in a tandem array, and therefore represent the largest contiguous cluster of protease genes of any catalytic class within the entire human genome (1). The *KLK* genes, ranging from 4.4 up to 10.5 kb in length, are transcribed in the direction from telomere to centromere, except for *KLK2* and *KLK3* (2). Furthermore, each KLK gene is translated as a preproenzyme, which contains a signal peptide of 16 to 30 amino acids at its N-terminus, followed by a pro-peptide of four to nine amino acid residues, and a catalytic domain, which remains in the mature, enzymatically active, protein. Proenzyme and mature enzyme forms result from the sequential cleavage of the signal sequence on entry into the secretory pathway and of the pro-peptide on activation, respectively (3). The proteolytic activity of KLK proteins is regulated in several ways, including zymogen activation, complex formation with endogenous plasma and tissue inhibitors, and/or inactivation through self-fragmentation (4).

The *KLK* locus is flanked by a subfamily of genes which are transcribed into small nucleolar RNAs C/D box 88 (*SNORD88A*, *SNORD88B*, and *SNORD88C*) on the centromeric end, and by the *CTU1* gene, encoding the cytosolic thiouridylase subunit 1 homolog, on the telomeric end. In addition, to co-localization on chromosome 19q13.4, the human *KLK* genes share many common features, such as exon/intron organization (coding sequence spanning 5 exons, conservation in the respective coding exon lengths, conserved intronic phases) (5), high similarity of the respective protein sequences, and, as in all serine proteases, the three conserved catalytic residues, namely His, Asp, and Ser (6). In fact, orthologs of the human *KLK* genes have been identified in many organisms, including mammals, birds, reptiles, and amphibians, therefore allowing the scientific community to trace the emergence of KLKs to 330 million years ago. It is noteworthy that *KLK3* is unique to higher order primates (7). Evolutionary relationships of the human, chimpanzee, rat and mouse kallikreins have recently been reviewed (6, 8).

Physiological roles of KLKs

KLKs are primarily expressed by secretory epithelial cells within the glandular epithelia of many organs, including

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the skin, breast, prostate, colon, pancreas and brain. After being secreted, KLKs enter bodily fluids, such as sweat, milk, saliva, seminal plasma, cerebrospinal fluid, or pericellular spaces (9). Not surprisingly, these serine proteases are implicated in a vast range of normal physiological processes, varying from the regulation of blood pressure and electrolyte balance to extracellular-matrix remodeling, prohormone processing, neural plasticity, and skin desquamation, acting independently or as part of one or more proteolytic cascades (10). KLKs participate also in signal transduction pathways by cleaving and thus activating cell-surface receptors and/or other proteases (11). Nonetheless, the complete spectrum of the physiological roles and in vivo targets of KLKs has not been fully elucidated yet.

Recently, degradomics tools, including biological and chemical combinatorial peptide-based specificity-profiling technologies, such as phage display and fluorogenic substrate libraries, respectively, have been used to determine the preferred consensus sequence, substrate specificity, and candidate physiological targets of the majority of KLKs. For instance, Sharma et al. showed by substrate phage display that KLK6 may have a potential dual substrate specificity, acting as both a trypsin and chymotrypsin-like enzyme (12), while biochemical characterization of KLK2, based on the same technique, uncovered its strict cleavage consensus sequence and three putative protein substrates (13). In another study, cathelicidin, urokinase-type plasminogen activator (uPA), laminin, and transmembrane protease serine 3 were determined as novel plausible substrates for KLK4, KLK5, KLK13, and KLK14, with the use of fluorogenic substrate scanning-synthetic combinatorial libraries (14).

Emerging data indicate that KLKs are able to induce proliferation of endothelial cells through activation of protease-activated receptors (PARs). PARs are members of the G-protein-coupled receptor superfamily that are activated by partial proteolytic cleavage of their extracellular domains (15, 16). KLKs, such as KLK2 and KLK3, have been shown to cleave insulin-like growth factor (IGF)-binding proteins (IGFBPs), resulting in increased availability of IGFs that bind and activate their corresponding receptors and that, in turn, can modulate cell survival, mitogenesis, and differentiation. KLK3 can also activate latent TGFβ by cleaving TGFβ-binding proteins, leading to cell proliferation (10).

The large number of KLKs along with their coordinated regulation and tissue co-expression patterns led to the hypothesis that KLKs could participate in proteolytic cascades, involved in semen liquefaction, skin desquamation, neurodegeneration, and tumor-promoting or -inhibiting effects (17). KLK5 has been proposed as the key initiator molecule of the postulated prostate cascade, as it is able to activate itself as well as pro-KLK2, -KLK3, -KLK6, -KLK7, -KLK11, -KLK12, and -KLK14 (18). For example, it was demonstrated by in vitro proteolysis that KLKs secreted in prostatic fluid can participate in an interaction network leading to activation of pro-KLK3, the inactive zymogen form of KLK3. KLK3 is the physiological enzyme responsible for the resolution of the seminal clot by digestion of SgI and SgII. Hence, most members of the KLK family participate in semen liquefaction (19). Desquamation of the stratum corneum is a serine protease-dependent process, too. Two members of the human KLK family, KLK5 and KLK7, are implicated in skin desquamation by digesting corneodesmosomes. Moreover, the epidermal localization and specificity of additional KLKs raises the possibility that multiple KLKs participate in desquamation. This process involves cleavage of desmoglein 1 by KLKs and their regulation by SPINK5 (serine protease inhibitor Kazal-type 5) (20).

Historical overview

The first member of this protease subclass was identified by Kraut, Frey and Werle in the 1930s, as a proteolytic enzyme, abundantly expressed in the pancreas – "kallikreas" in Greek – and, hence, was named tissue kallikrein (KLK1). Forty years later, the search for male-specific antigens in semen that could have an application in forensics, prompted the discovery of prostate-specific antigen (PSA; later renamed KLK3). To date, KLK3 is the most well-characterized kallikrein-related peptidase and the most valuable biomarker in clinical practice for prostate cancer diagnosis and monitoring of high-risk populations (21). In 1989, the genes encoding the tissue kallikrein (*KLK1*) and PSA (*KLK3*), along with another novel gene encoding human glandular kallikrein-1 (hGK-1; later renamed KLK2) – a new player on the scene – were mapped on 19q13.4, cloned and characterized (22, 23).

During the late 1990s, 12 novel genes encoding kallikrein-related peptidases were discovered and assigned to the kallikrein gene family, based on their localization to 19q13.4 as well as sequence and structure similarities to the first three kallikrein genes (24, 25). In more detail, sequencing data from the Human Genome Project revealed that some already discovered genes mapped to the same loci as the three classical kallikreins (5, 26), namely normal epithelial cell-specific 1 (*NES1*; know designated as *KLK10*) (27), zyme/protease M/neurosin (*PRSS9*; *KLK6*) (28–30), neuropsin/tumor-associated differentially expressed gene-14 (*TADG14*; *KLK8*) (31, 32), hippostasin/trypsin-like serine protease (*TLSP*; *KLK11*) (33, 34), the human stratum corneum tryptic enzyme (*SCTE*; *KLK5*) (35) and chymotryptic enzyme (*HSCCE*/*PRSS6*; *KLK7*) (36). Studies of the whole genetic locus eventually resulted in the cloning of seven additional, formerly unknown serine proteases (37), increasing the human tissue *KLK* genes to 15, plus an additional pseudogene, namely KLKP1 (38). These genes were shown to be expressed at varying levels in a diverse range of tissues, although they exhibited quite distinct patterns of expression (39). According to the nomenclature suggested recently for the human tissue kallikreins, each gene is referred to as *KLK* followed by the appropriate number and the protein as KLK followed by the appropriate number (40).

Genomics of the *KLK* locus

Chromosomal and genomic aberrations, including copy number and structural rearrangements, are a hallmark of many

cancers, since they are capable of significantly modulating gene function. Somatically acquired chromosomal alterations constitute a major mechanism for gene activation, especially in hematological malignancies, where specific and recurrent chromosomal translocations give usually birth to oncogenic fusion transcripts. Accumulating evidence suggests that common epithelial tumors, such as prostate cancer, may also harbor chromosomal translocations, which clearly affect gene expression (41). A recent study unraveled the impact of cytogenetic and genomic aberrations of the *KLK* locus in ovarian cancer. Bayani et al. showed in more detail, for the first time, that gain of *KLK* gene copy number as well as unbalanced translocations of the *KLK* locus may account for increased protein expression of KLKs 5, 6, 7, 8, 9, 10 and 11 (42), hence contributing to ovarian carcinoma progression and aggressiveness (42, 43); however, this is not always the case. For instance, even though increased *KLK6* copy number is not likely to directly regulate the observed KLK6 overexpression per se, it is one contributing factor (43).

Except for genomic instability and copy-number heterogeneity of *KLK*s, single nucleotide polymorphisms (SNPs) of the *KLK* genes possess clinical value as putative genomic biomarkers. Interestingly, two recent studies have shown that certain SNPs of the *KLK2* gene can significantly improve the prediction of biochemical recurrence after initial prostate cancer treatment, especially when used in combinatorial models that also include clinicopathological data. These studies introduce the role of *KLK* SNPs in stratifying prostate cancer patients according to their eligibility for adjuvant therapy (44). Another very interesting example consists of a functional SNP residing within one of the androgen response elements of *KLK3*; this SNP has been associated with increased serum PSA levels, as it can increase the ability of the androgen receptor to bind to the aforementioned response element (45).

Alternative splicing of *KLK*s

All genes of the *KLK* genomic locus are subjected to alternative splicing, thus producing two or more mRNA variants, which in many cases encode functionally distinct protein isoforms. Exon skipping and exon extension constitute the most common alternative splicing events of the *KLK* genes, followed by exon truncation and intron retention (46). Among *KLKs*, *KLK3* constitutes the most prominent example of alternative splicing, since it can produce more than 10 distinct mRNA variants (46, 47).

It has been postulated that mRNA splicing variants may play a major role in the etiology of many diseases including cancer, since protein isoforms that arise by translation of alternatively spliced transcripts often contain additional functional domains or miss some of the structural motifs of the classical isoform, and therefore acquire new properties or lack some of them, respectively. From a clinical aspect, *KLK* mRNA variants are particularly important in oncology, as they provide selective drug targets or may serve as a marker set for cancer diagnosis and/or prognosis (48). For instance, *KLK3* splicing variant 5 expression has been shown to possess significant discriminatory value, distinguishing very efficiently prostate cancer patients from benign prostate hyperplasia (BPH) cases (47).

Furthermore, it is likely that a number of *KLK* splicing variants do not produce a protein, or even that some KLK isoforms produced are not functional. In fact, *KLK* mRNA variants that contain a faulty reading frame ending at a premature translation termination codon are mostly identified by a conserved RNA surveillance mechanism and subsequently subjected to degradation through a post-transcriptional process called non-sense mediated mRNA decay (NMD). In general, NMD is elicited by premature termination codons residing 5′ to a boundary of ~50 nt upstream of the last exon/exon junction (49). However, non-functional KLK isoforms could act to sequester partner molecules, such as inhibitors, thus enhancing the accessibility of active enzyme isoforms to their substrates (50). Undoubtedly, the potential clinical significance of transcript heterogeneity detected in normal and/or pathophysiological conditions dictates the further investigation of alternative splicing of *KLK*s (51). Some examples highlighting this issue are presented throughout this review.

Regulation of *KLK* gene expression

Hormonal regulation of KLK gene transcription

According to the extensive expression profiling of KLKs in cancer tissues and blood serum of cancer patients accomplished in the last 10 years, KLKs are dysregulated in a wide range of solid tumor malignancies (9). Accumulating evidence indicates that KLKs are coordinately up- or downregulated at both the transcriptional and protein levels in several neoplastic diseases, particularly adenocarcinomas derived from steroid-hormone-regulated tissues, as compared with their normal, benign and/or pre-malignant tissue counterparts, thus implying common regulatory pathways (11). A synergistic hormonal regulation of *KLK* gene transcription has been well documented as the underlying cause for this dysregulation of KLK gene expression, either through a single or through a few locus-control regions. For instance, KLK10, KLK11, KLK13, and KLK14 were shown to be coordinately regulated by dihydrotestosterone (DHT) and norgestrel in several breast cancer cell lines (52). Interestingly, none of these genes contain characterized hormone-response elements, therefore indicating an indirect function of steroid hormones as trans-acting transcriptional regulators of KLK expression (52, 53). Recently, it has even been proposed that coordinated expression of KLKs represents the transcriptional activation of a unique expression ‘cassette’, utilizing a common hormone-dependent mechanism (52).

In fact, at least 14 functional hormone response elements have been identified in the KLK locus. Although many of the factors that coordinate this complex expression profile are unknown, in a subset of tissues it is clear that KLK expression is strictly regulated by steroid hormones including androgens, estrogens, progestins, mineralocorticoids, and glucocorticoids (54). The most striking example is the hormonal control of the expression of two classical KLK family members, KLK2 and

KLK3, in prostate (55–58) and breast cancer cell lines, where their expression is upregulated in response to androgens and progestins (59). This hormonal control is directly attributed to the hormone response elements present in the promoter regions of these two genes (23). A more extensive study of the transcriptional regulation of KLK genes would contribute to the better understanding of the physiological functions of kallikreins and their effectiveness as cancer biomarkers.

KLKs and epigenetics

Regulation of *KLK* mRNA expression can alternatively occur through epigenetic factors, in particular DNA methylation. For instance, *KLK10* mRNA downregulation has been associated with the hypermethylation of CpG islands in several cancers, such as breast cancer (60), gastric cancer (61), lung cancer (62), head and neck squamous cell carcinoma (63), and acute lymphoblastic leukemia (64). A similar regulatory mechanism has been well documented for the *KLK6* gene, the inactivation of which is associated with hypermethylation of specific CpG dinucleotides located in the *KLK6* proximal promoter, while its overexpression is linked to complete demethylation (64). The protective role of KLK6 against breast tumor progression, exerted through inhibition of epithelial-mesenchymal transition (EMT), is waived by *KLK6* gene silencing. In fact, expression of KLK6 in MDA-MB-231 breast adenocarcinoma cells at normal levels results in significant reduction of vimentin, an established marker of EMT, and concurrent upregulation of calreticulin and epithelial markers cytokeratin 8 and 19 (65). In accordance with these results, the epigenetic drug decitabine (5-aza-2′-deoxycytidine), a cytidine analog hypomethylating DNA by inhibiting DNA methyltransferase, reactivates the expression of downregulated *KLK* genes in prostate, breast, and ovarian cancer cell lines (66, 67). Hence, it has become evident that epigenetic regulation provides a new mechanism for the pharmacological modulation of *KLK* levels in human cancers with potential therapeutic implications.

KLKs and microRNAs

A post-transcriptional control mechanism by microRNAs (miRNAs) has also been suggested to explain notable discrepancies between *KLK* mRNA and protein levels (68). miRNAs function by annealing to mRNA targets with partial – rather than perfect – complementarity, thus negatively regulating target protein expression (69). The high sequence similarity of KLK mRNAs, evident also in the 3′-UTRs (untranslated regions) of these transcripts, supports the notion that a single miRNA can target more than one KLK, thus controlling simultaneously the protein expression levels of multiple KLKs (68). Using different miRNA target prediction algorithms, Chow et al. provided evidence that 96 miRNAs are predicted to target one or more KLKs. KLK10 is the most frequently targeted KLK (19 miRNAs), followed by KLK5 and KLK13, whereas KLK1, KLK3, KLK8, and KLK12 are not strongly predicted to interact with known miRNAs. It is worth mentioning that KLK2, KLK4, KLK5, and KLK10 are predicted to have multiple miRNA-targeting sites on their 3′-UTRs (70). Undoubtedly, further experimental validation is needed to confirm miRNA-KLK interaction predictions.

Dysregulation of KLK-targeting miRNAs in cancer

Accumulating evidence indicates that many KLK-targeting miRNAs are dysregulated in malignancies (68). For instance, three miRNAs that are increased in ovarian cancer, let-7f, miR-224, and mR-516a, were validated as negative regulators of KLK10 expression. Indeed, increased transcription of each one of these miRNAs in a cell line model resulted in a dose-dependent decrease in KLK10 protein levels and, subsequently, had a negative effect on cell proliferation (71). In addition, to KLK10, let-7f targets KLK6, as experimentally validated in a breast cancer cell line (70). Interestingly, dysregulation of KLK expression in renal cell carcinoma (RCC) has been attributed to alterations in the levels of several miRNAs; in particular, increased miR-224, a validated negative regulator of KLK1 protein expression in human embryonic kidney (HEK293) cells, is likely to account for KLK1 downregulation in clear-cell RCC subtype (72). Moreover, repression of Klk5 by miR-382 contributes to the development of renal inner medullary interstitial fibrosis in a mouse model (73), therefore suggesting that faulty regulation of KLKs by miRNAs can, in some cases, constitute the underlying cause of a disease.

Besides direct regulation of protein levels through mRNA targeting, miRNAs can regulate the mRNA and/or protein levels of *KLK*s by indirect mechanisms. The most prominent example is the indirect suppression of KLK3 expression by miR-99 family members in prostate cancer cells. Loss of miR-99 family members (miR-99a, -99b, or -100) has been shown to affect AR-driven gene expression, particularly the expression of the *KLK3* gene, at both the mRNA and the protein level. Moreover, the lower levels of these miRNAs in C4-2 prostate cancer cells, in comparison with the parental cell line (LNCaP), are directly associated with derepression of the chromatin remodeling factors SMARCA5 and SMARCD1 as well as the cellular growth regulatory kinase mTOR. The derepression of these three genes contributes to the elevated expression of KLK3 protein along with increased proliferation in this more advanced prostate cancer cell line (C4–2), as demonstrated by Sun et al. (74). In contrast to these findings, another study showed that high levels of miR-100 are associated with biochemical recurrence of localized prostate cancer in patients treated with radical prostatectomy (75), associated with an increase in serum KLK3 levels. Obviously, the full significance of the interactions between KLKs and miRNAs has yet to be elucidated.

KLKs and cancer

Despite the fact that many KLKs are overexpressed in malignant tumors, this overexpression does not always reflect an

increase in their proteolytic activity. The underlying cause for this putative discrepancy is the existence of various KLK forms that are present in the extracellular milieu of tissues together with the active enzymes, including inactive pro-KLKs, KLKs sequestered in inhibitor complexes, KLKs inactivated by internal cleavage and/or inactive KLK isoforms. For example, monitoring of pro-KLK6 conversion to its active enzyme species in biological fluids demonstrated that only up to 5% of immunoreactive KLK6 detected in clinical samples possesses proteolytic activity (76). However, most (80%–90%) of KLK3 within the interstitial fluid of prostatic tumors is not primarily in a complex with inhibitors but enzymatically active, suggesting that KLK3 expression might more directly correlate with KLK3 activity in tumors (77).

Malignancies of the male reproductive system

Prostate cancer

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in males. Incidence rates vary dramatically worldwide, with the highest rates recorded primarily in the developed countries of Oceania, Europe, and North America, mostly due to wide population screening, including measurement of serum PSA concentration. This testing detects clinically important prostate cancer as well as other slow-growing prostate tumors that might otherwise escape diagnosis. However, the prostate cancer mortality rate reaches its peak in males of African descent in the Caribbean region, partly implying differences in genetic susceptibility (78).

The current clinical goal is the early detection of the disease and effective patient monitoring post-treatment. Along with digital rectal examination, the PSA test has become part of the routine medical checkup in many countries during the last decades (79). It is common knowledge that increased preoperative serum PSA levels constitute an independent unfavorable prognostic biomarker in prostate cancer, positively correlating with advanced-stage disease and predicting poor clinical outcome. However, despite its high popularity as a screening biomarker, the usefulness of the PSA test is still under examination. The major drawback of the use of this test is its relatively low specificity, especially in screening programs when high sensitivity is needed (80). In addition, to serum total PSA (tPSA), recent clinical trials uncovered the predictive potential of serum free PSA (fPSA) in terms of the aggressiveness of the disease and patients' outcome. Increased fPSA is strongly associated with high Gleason score, advanced stages, capsular penetration, and positive surgical margins of patients (81).

In order to improve the clinical accuracy of PSA, researchers have focused their efforts on discovering a multiparametric panel of prostate cancer biomarkers. Because of their differential expression patterns, several members of the KLK family (*KLK2*, *KLK4*, *KLK5*, *KLK11*, *KLK14*, and *KLK15*) have been considered as promising diagnostic and/or prognostic biomarkers of prostate cancer (82, 83). In particular, KLK2 has been shown to add important information with regard to the early detection and staging of prostate cancer. Preliminary analysis demonstrated that KLK2 could discriminate between pT2 and pT3 tumors, and predict Gleason grade 4/5 cancer volume better than tPSA or fPSA (84). Moreover, increased specificity was obtained using the ratio of KLK2 to fPSA and/or the ratio of tPSA to fPSA when combined in a logistic regression model for discrimination between prostate cancer cases from benign prostatic hypertrophy in individuals with "gray zone" PSA (85–87). Similarly, artificial neural networks combining tPSA, fPSA/tPSA, KLK2, KLK2/fPSA and KLK2/(fPSA/tPSA) as input factors have been suggested as promising tools for improved diagnosis, staging, and prognosis (PSA recurrence, long-term survival) of prostate cancer (88).

KLK4 and *KLK15* mRNA expression analyses revealed upregulation of both genes in prostate cancer tissue biopsies, compared to BPH tissue biopsies. In fact, expression of *KLK4* mRNA and alternative *KLK15* transcripts may serve as independent biomarkers for the discrimination between malignant and benign prostatic lesions (83, 89). Moreover, KLK4 mRNA is linked to advanced stages of the disease and strongly correlates with patient pre-operative serum tPSA levels (89). Similarly, prostate needle biopsies from cancer patients displayed significantly lower *KLK5* mRNA and higher *KLK11* mRNA levels than those from BPH patients, suggesting their application as independent biomarkers for the differential diagnosis and prognosis of prostate cancer (90–92). Furthermore, a reduction of *KLK3* mRNA levels was observed in fine needle biopsies from prostate tumors of lower grade of differentiation. Interestingly, *KLK3* mRNA expression in needle biopsy material did not appear to correlate with PSA circulating levels in prostate cancer patients (93).

Testicular cancer

The most frequent and important neoplasms of testis are germ cell tumors. Testicular cancer mainly affects young males, and its incidence is steadily increasing in affluent societies. In several regions, including North America and Northern Europe, testicular cancer has become the most common cancer in men aged from 15 to 44 years. In spite of the rising incidence of testicular cancer, there has not been much interest in further investigation for new biomarkers of the disease, mostly due to the fact that 5-year survival rates approach 95%, at least in countries with an excellent clinical oncology infrastructure (94).

Expression profiles of *KLK* genes in testicular cancer have not been fully characterized yet. According to preliminary results, KLK5, KLK10, KLK11, KLK13, and KLK14 seem to be downregulated in malignant testicular tumors (95). *KLK5* mRNA expression is associated with lower-stage tumors, suggesting a favorable prognostic value of *KLK5* mRNA in testicular cancer (96). Other KLKs, such as KLK2 and KLK4, have been detected in testes (9). Notably, some alternatively spliced variants of KLKs present in testis are tissue-specific. Consequently, it is plausible that one or more of these splice variants have potential as biomarkers for testicular cancer (95).

Renal cell carcinoma (RCC)

Cancer of the kidney amounts to 2% of the total human cancer burden, with approximately 190,000 new cases diagnosed each year (78). Early diagnosis and prognosis of renal cell carcinoma is very challenging, because the majority of early-stage tumors are asymptomatic and may be detected only by imaging. Once detected, renal tumors can be completely removed surgically; however, hematogeneous metastasis is frequent and may occur already at an early stage of the disease (24). The expression of KLK6, KLK7 and KLK10, as detected by immunohistochemistry, correlates with tumor size and the histologic type of RCC, while patients suffering from advanced-stage RCC present elevated KLK1, KLK7, and KLK11 levels. Moreover, KLK6 expression has been shown to predict shorter disease-free survival (DFS) (97).

Gynecological malignancies

Ovarian cancer

Ovarian cancer, particularly epithelial malignancy of the ovary, usually escapes detection at an early stage and, therefore, its prognosis is poor at the time of diagnosis. The vast majority of *KLK* family members (*KLK2-KLK11*, *KLK13-KLK15*), are aberrantly expressed in ovarian carcinoma tissues and cell lines. Moreover, the levels of KLKs secreted in serum or detected in ascites fluids of ovarian cancer patients differ significantly from the healthy controls' serum KLK levels (11). In particular, *KLK4* and *KLK5* mRNAs have been shown to be overexpressed in ovarian cancer, and both of them indicate poor prognosis (98). Interestingly, *KLK4* expression may also serve as a marker for predicting resistance to paclitaxel-based therapy (99). Similarly to *KLK4* and *KLK5*, *KLK10* and *KLK15* may function as indicators of an unfavorable prognosis for ovarian cancer, whereas *KLK8*, *KLK9*, and *KLK14* may have some utility as favorable prognostic markers of the disease (96). In particular, elevated mRNA levels of *KLK8*, *KLK9* and *KLK14* have been associated with lower disease stage, lower tumor grade, optimized residual tumor volume, improved DFS and OS. *KLK14* expression is also inversely correlated with carbohydrate antigen 125 (CA125) serum concentrations of ovarian cancer patients, which further supports the suggested favorable prognostic value of *KLK14*.

Expression of the *KLK6* gene, the most studied member of the family to date in ovarian cancer, has also been shown to strongly predict an unfavorable outcome for these patients. In fact, higher levels of KLK6 in ovarian cancerous tissues is associated with more aggressive phenotypes of the disease, and correlates significantly with shorter DFS and overall survival (OS) (100). High KLK6 serum concentrations also predict a strong unfavorable outcome for ovarian cancer patients (101). In addition, to its prognostic value, serum KLK6 may also contribute to diagnosis of ovarian cancer. A very recent study examining the diagnostic value of KLK6 and KLK10 vs. CA125 in ovarian cancer concluded that serum KLK6 may improve the sensitivity of CA125 testing and that serum KLK10 has the highest specificity among the three biomarkers (102).

Similar clinical utility was revealed by the analysis of KLK5 and KLK10 levels in ovarian tissue extracts and patient serum, as well as from KLK7 immunohistochemistry. Elevated levels of these KLKs were found to be associated with rapid progression of the disease and low survival probabilities for ovarian cancer patients (103). Furthermore, alternative splice variants of *KLK5* and *KLK7*, namely the short KLK5 and long KLK7 transcripts, may be useful as tumor markers for epithelial-derived serous carcinoma (104). With regard to the *KLK11* and *KLK13* genes, increased mRNA in ovarian cancer tissue specimens is indicative of aggressive disease. On the contrary, KLK11 and KLK13 protein accumulation in ovarian cancer predicts better DFS and OS probabilities (21).

Uterine papillary serous carcinoma

Uterine papillary serous carcinoma (UPSC), also known as uterine serous carcinoma (USC) and uterine serous adenocarcinoma, is an uncommon form of endometrial cancer, mostly affecting post-menopausal women. Prognosis of UPSC is affected by age, stage, and histology as well as treatment (105). The *KLK6* gene is differentially expressed in UPSC, in comparison with other subtypes of endometrial carcinoma and/or healthy tissue (96). In addition, both KLK6 and KLK10 are upregulated in UPSC patient sera (82). Similarly, *KLK8* gene expression has been shown to be higher in endometrial carcinoma tissues, both at the mRNA level and protein level (106).

Cervical cancer

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in females worldwide, with a high prevalence in developing countries (78), where the Papanicolaou (Pap smear) test is not so widely used due to financial and cultural limitations. Therefore, alternative approaches to Pap screening are still required, particularly in developing countries.

Expression profiling of the *KLK* genes suggests several KLKs as biomarkers of cervical cancer. *KLK7* and *KLK8* expression, both at the mRNA and protein levels, was upregulated in primary squamous cervical cancer cells and in established cervical tumor cell lines (107, 108). A very recent study added that differences in the KLK7 protein concentration could potentially be used as a biomarker for the characterization of different stages of cervical cancer (109). It is worth mentioning that the increased KLK7 levels in cervical adenocarcinoma tissues were accompanied by decreased production of a KLK7 inhibitor, known as secretory leukocyte proteinase inhibitor (SLPI) (110). This finding may imply the existence of a regulatory mechanism in cervical carcinogenesis affecting the proteolytic activity of KLK7, the potential clinical importance of which remains to be fully elucidated.

Gastrointestinal malignancies

Colorectal cancer

Early diagnosis of colorectal cancer (CRC) and early detection of relapse following surgery are critical for the effective treatment and/or positive clinical outcome. Nonetheless, neither circulating carcinoembryonic antigen (CEA) nor any of the other biomarkers that have been proposed in the past, such as CA19-9 (carbohydrate antigen 19), are sensitive enough (9). Consequently, the identification of novel, reliable prognostic and predictive biomarkers which will contribute to clinical decision-making, remains of the utmost importance. Proteases, including KLK family members, are considered to be associated with CRC progression because of their ability to degrade extracellular matrix (ECM) proteins, thereby facilitating tumor invasion and metastasis (11).

Several *KLK* genes have been shown to be overexpressed in colorectal cancer, and most of them also display prognostic significance in this malignancy (111). For instance, low *KLK10* and/or *KLK7* mRNA expression is significantly associated with longer DFS and OS, suggesting that both genes may be used as markers of unfavorable prognosis for CRC (112, 113). KLK5, KLK6, KLK7, KLK13, and KLK14 protein levels in cytosolic extracts from CRC tissues were significantly associated with patients' OS, but only three among them (KLK5, KLK7, and KLK14) added to the established prognostic value of staging and grading. Another KLK family member, KLK4, was shown to be aberrantly expressed in colonic tumors. Notably, KLK4 is able to induce PAR1 signaling in HT-29 colorectal adenocarcinoma cells, and hence to promote ERK1/2 activation, which indirectly confers a more aggressive phenotype to colorectal adenocarcinoma cells (114). According to our unpublished results, *KLK4* mRNA is more abundant in advanced-stage and/or poorly differentiated intestinal tumors, and is also associated with tumor size. In accordance with these findings, high *KLK4* mRNA expression in CRC predicts an increased risk of relapse, independently of the tumor size and the nodal status of CRC patients (our unpublished data).

Gastric cancer

Gastric cancer is the fifth most common cancer across European countries. Early-stage gastric cancer is often asymptomatic or causes only non-specific symptoms. When symptoms occur, this malignancy has often reached an advanced stage – one of the main reasons for its poor prognosis. As with colorectal cancer, surgical resection of the tumor at early stages represents the cornerstone of any curative strategy for gastric cancer. Overall 5-year relative survival rates of gastric cancer patients are approximately 20% (78). Undoubtedly, the overall survival and quality of patients' life bearing gastric tumors have been improved thanks to continuous developments of new chemotherapeutic drugs and various multidisciplinary approaches.

KLK13 mRNA expression in gastric cancer predicts favorable prognostic outcome of patients, as it is significantly related to prolonged DFS and OS (115). Elevated *KLK6* and *KLK10* transcription levels constitute two unfavorable prognostic indicators in gastric cancer (116). In more detail, high *KLK6* expression has been associated with positive lymph nodal status and a poorer survival rate of gastric cancer patients (117). Moreover, its reduction in gastric cancer cells led to a decrease in their invasiveness, whereas its exogenous overexpression in cells decreased the activity of the E-cadherin promoter and hence augmented their metastatic potential, since E-cadherin is a key molecule in EMT. Additionally, it has been postulated that *KLK6* represents a novel therapeutic target in gastric cancer that can be exploited using gene-silencing procedures (117). Regarding *KLK10*, its expression was found to be higher in malignant tumors and cell lines compared to normal tissues (96). Huang et al. concluded that loss or reduction of *KLK10* mRNA expression is associated with differentiation level during gastric cancer progression, implying that *KLK10* inactivation via CpG island hypermethylation might contribute to the malignant progression of gastric tumors (61).

Other malignancies

Breast cancer

Breast cancer is by far the most commonly diagnosed malignancy and the leading cause of cancer death in European females. Five year survival rates drop dramatically from 97% for localized tumors, to 79% for regionally spread tumors and to 23% for metastatic tumors (96). Significant prognostic value for breast cancer has been reported for a broad spectrum of KLKs; among them, *KLK4*, *KLK5*, *KLK7*, *KLK10*, *KLK12*, and *KLK14* constitute unfavorable prognostic indicators for breast cancer patients, whereas *KLK3*, *KLK9*, *KLK13* and *KLK15* predict good clinical outcome in breast cancer (101).

mRNA expression analysis of *KLK5* using quantitative real-time PCR has shown that upregulation of *KLK5* transcription predicts poor prognosis in breast cancer patients, even in those suffering from early-stage disease (118, 119). Moreover, mRNA expression analysis revealed *KLK5* upregulation in breast cancer tissue specimens, compared to benign breast lesions. Therefore, *KLK5* mRNA may serve as an independent biomarker for the discrimination between malignant and benign tumors of the mammary gland (120). Furthermore, *KLK14* mRNA overexpression was found to be an independent prognostic indicator of decreased DFS and OS (121). *KLK14* and *KLK4* mRNA positivity is associated with high tumor grade and size (122, 123). In accordance with the mRNA data, strong immunohistochemical KLK14 staining of the malignant breast tumors is linked to aggressive phenotypes of the disease (124).

KLK10 has been extensively studied in breast cancer, as it was originally designated as a putative tumor suppressor gene, with loss of expression in breast cancer (11). *KLK10* gene expression is dramatically decreased in breast cancer cell lines, compared to normal mammary epithelial cells, mostly – although not exclusively – due to hypermethylation

of the *KLK10* exon 3 (60, 67) and/or gene promoter (125), as aforementioned. *KLK10* mRNA analysis by in situ hybridization on tissue sections from normal breast, typical and atypical hyperplasia, and in infiltrating ductal carcinoma, has shown that although all normal specimens and the majority of hyperplastic breast samples presented *KLK10* mRNA expression, more than half of the ductal carcinoma and almost all infiltrating ductal carcinomas were *KLK10*-negative (126, 127). In fact, KLK10 expression is associated with breast cancer progression (126), and represents an independent predictive marker for tamoxifen therapy response (128). A very recent study provided evidence that *KLK10* exon 3 methylation is also an important prognosticator in early breast cancer patients (129).

Lung cancer

Lung cancer constitutes a huge burden on public health, its most important feature being its high mortality rate. In spite of the therapeutic progress, little gain has been achieved in overall lung cancer survival over the past 30 years, with approximately 15% 5-year survival rates for all stages combined. Therefore, conventional treatment remains unsatisfactory, in terms of decreasing global lung cancer burden (130). Currently, there are several available lung cancer biomarkers that have potential application in risk assessment, early detection, treatment selection, prognosis, and monitoring of recurrence. Nonetheless, because of their low sensitivity and specificity, these biomarkers are not recommended for routine clinical use (131).

Accumulating data suggest that *KLKs* may be useful as diagnostic and/or prognostic biomarkers of lung cancer. KLK8 expression was shown to confer a favorable clinical outcome in non-small cell lung carcinoma (NSCLC) by suppressing tumor cell invasiveness (132). In addition, Nathalie et al. demonstrated that high KLK6 concentration constitutes an independent unfavorable prognostic factor in NSCLC (133). Compared to adjacent non-malignant lung tissues, *KLK5* and *KLK10* are overexpressed in a subtype of NSCLC, namely the squamous cell lung carcinoma (134, 135), whereas *KLK7* is downregulated in lung adenocarcinoma, another subtype of NSCLC (134). Moreover, KLK5, KLK7, KLK8, KLK10, and KLK12 concentrations in sera of NSCLC patients were lower than in sera from normal individuals, in contrast to KLK11, KLK13, and KLK14, the levels of which were higher in sera of NSCLC patients. In particular, KLK11 and KLK12 appear to be related to disease stage (131). Overexpression of the *KLK13* mRNA and/or protein in lung adenocarcinoma is associated with positive nodal status and lower OS probabilities of patients, while KLK14 protein expression correlated with tumor size (136).

Head and neck squamous cell carcinoma

Head and neck carcinomas constitute the sixth most commonly diagnosed cancer worldwide, and the overwhelming majority of them are characterized as squamous cell carcinomas. In fact, head and neck squamous cell carcinoma (HNSCC) includes a large variety of tumors arising from different sites of the head and neck region, including oral cavity and larynx. The high mortality from HNSCC is attributed to regional and distant metastasis, and the 5-year survival rate hardly reaches 50%, thus highlighting the need for discovery of novel biomarkers of disease aggressiveness and therapeutic targets (137).

Four members of the KLK family (KLK5, KLK7, KLK8, and KLK10) are abundantly expressed in HNSCC, as demonstrated by immunohistochemical analysis (138). A very recent study suggested that KLK5 might promote metastatic dissemination of HNSCC by promoting loss of junctional integrity through cleavage of desmoglein-1 (139). Furthermore, KLK4 and KLK7 immunohistochemistry seems to have diagnostic and prognostic potential in this disease. Intense KLK4 and KLK7 staining was more usual in moderately and/or poorly differentiated neoplasms, and the respective patients had significantly shorter OS (140).

KLK11 seems to constitute a novel and independent biomarker in laryngeal squamous cell carcinoma, for diagnostic and prognostic purposes. *KLK11* mRNA expression, as assessed by quantitative real-time PCR, was significantly lower in laryngeal cancerous specimens of primary or recurrent nature, compared with their non-malignant counterparts. Patients harboring *KLK11* mRNA-positive laryngeal tumors had a significantly decreased risk of death (141).

Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a form of leukemia, characterized by an excess of lymphoblasts, which are overproduced in bone marrow and infiltrate other organs. ALL is most common in childhood with a peak incidence at 2–5 years of age, and another peak in old age. The overall cure rate in children is about 80%, while about 45%–60% of adults have long-term DFS.

Epigenetic regulation of the *KLK10* gene by DNA methylation appears to be the central mechanism of *KLK10* silencing in ALL (64, 142, 143). A strong reduction of *KLK10* mRNA was noticed in B-precursor ALL cell lines and in 69% of diagnostic ALL samples, in comparison with *KLK10* mRNA levels detected in normal fresh bone marrow mononuclear cells. Methylation-specific PCR revealed that CpG islands of the KLK10 exon 3 were highly methylated in all ALL cell lines and in 133 of 222 (60%) samples from patients with ALL, in clear contrast to no *KLK10* methylation observed in normal cells. Interestingly, the methylation status of the promoter, of the 5′-UTR, and of the third exon of the *KLK10* gene was further associated with a poor prognosis in ALL and a higher chance of ALL patient relapse (64, 143). In a study examining the methylation status of several genes including *KLK10*, approximately 76% of patients with T-cell ALL had more than two methylated genes. Moreover, lack of methylation was demonstrated as a favorable prognosticator in ALL, suggesting a potential utility of the determination of the methylation status in assessing the risk of T-cell ALL patients (142).

Detection and quantification of KLKs in biological fluids

Various techniques have been used so far for the quantification of *KLK* mRNA and protein levels in tissue biopsies and/or biological fluids, including amniotic fluids, cervicovaginal fluids, follicular fluids, urine (9), cerebrospinal fluids, ascites fluid (76), and blood serum (98). Sandwich type KLK-specific ELISAs, with one monoclonal or polyclonal antibody used for capture and another polyclonal one used for detection have been developed for quantification and detection of each KLK in tissue extracts and biological fluid samples acquired for routine biochemical testing (9). For instance, protein concentrations of KLK6 and KLK10 in ovarian cancer ascites fluids were measured with ELISA-type immunoassays (144). A very recent study established, also, a reference interval for KLK6 serum levels in adults (145). Moreover, Vaisanen et al. developed a sensitive immunoassay with good specificity for the accurate determination of free and total human kallikrein 2 (KLK2) concentration in the male bloodstream, as this might improve the discrimination between prostate cancer and benign prostatic hyperplasia (146). tPSA and fPSA were also measured in the blood serum of patients with prostate cancer or BPH, using immunofluorometric techniques (147). Furthermore, time-resolved immunofluorometric assays with low detection limits and very good specificity have been developed for accurate determination of specific KLKs, such as KLK2 (148), KLK3 (149), KLK6, and KLK10 (150). In order to obtain information about the proportion of immunoreactive KLKs that represent active enzymes in biological fluids, assays using a serine proteinase-targeted activity-based probe coupled to antibody capture have been developed (76).

KLKs in personalized medicine

Given the heterogeneity of human malignancies, cancer patients stand to benefit enormously from personalized medicine. One of the most essential characteristics of cancer is that its pathobiology at each stage, from carcinogenesis to invasion and metastasis, is manifested via very dissimilar and difficult to predict patterns. One of the main goals of personalized medicine is to stratify patients that were initially given the same diagnosis into those who would not benefit from conventional chemotherapy and those who would benefit from being treated with anticancer drugs. Moreover, concerning the latter, personalized medicine should accurately define the chemotherapeutic schemes, including the selection of appropriate anticancer drugs as well as the dose at which each drug should be administered (151). Molecular profiling of tumors, based on DNA, mRNA, miRNA and/or protein signatures, can add to the existing information originating from the classical histological and/or morphological classification of tumors (tumor size, stage, grade, nodal status, presence or absence of distal metastasis), which often leads to erroneous decisions regarding treatment planning. Therefore, the discovery of novel biomarkers for the prediction and monitoring of cancer patient response to anticancer drug treatment constitutes a major clinical task.

KLKs are considered as very promising biomarkers for cancer personalized medicine, especially for predicting and monitoring of patients' response to chemotherapy, therefore opening up new horizons towards effective patient monitoring post-treatment. For instance, the combined molecular profile of the *KLK5* and *KLK11* genes has been proposed as a new potential molecular biomarker predicting treatment response of prostate cancer (152, 153). In more detail, treatment of PC-3 prostate cancer cells with mitoxantrone, etoposide, doxorubicin and/or carboplatin was shown to induce distinct alterations in the mRNA expression of *KLK5* and *KLK11* (153). *KLK5* mRNA expression was also dysregulated in DU145 prostate cancer cells after administration of the antineoplastic agents docetaxel and mitoxantrone. The same study postulated that the expression profile of KLK5 could serve as a putative biomarker for monitoring the treatment response in hormone refractory prostate cancer patients (152). According to our unpublished results, modulations of *KLK5* and *KLK14* mRNA levels were observed in BT-20 breast cancer cells during their apoptosis, induced by their exposure to chemotherapeutic drugs, such as epirubicin, docetaxel, and methotrexate. Furthermore, *KLK13* mRNA was modified in AGS gastric cancer cells up to 6-fold following their treatment with epirubicin and methotrexate (our unpublished data).

KLKs and multiparametric panels of biomarkers

During the last years, the potential of KLKs as cancer biomarker in a number of malignancies (e.g., KLK6 in ovarian cancer) along with the necessity for more reliable cancer biomarkers, has prompted the design of multiparametric models for identifying potential panels of biomarkers stemming from the KLK family as well as from other cancer-related families, and possessing greater sensitivity/specificity than existing biomarkers alone.

Perhaps the most prominent example comes from a very recent study, suggesting that a combined panel of *KLK6*, *KLK13*, and *CA125* mRNAs, is a more sensitive test to detect early stage ovarian cancer than *CA125* alone (154). Moreover, Zheng et al. developed a multiparametric strategy for predicting ovarian cancer progression and patients' response to chemotherapy, comprising clinical features and several biomarkers including some KLKs, as quantified in cytosolic extracts from ovarian tumors (155). Similarly, another very recent study provided strong evidence that a panel of serum biomarkers from the KLK family (and other families) can predict ovarian cancer patients' response to chemotherapy, DFS, and OS (156). A serum-based multiparametric panel of KLK biomarkers for NSCLC diagnosis with relatively good accuracy has also recently been developed by Planque et al. (131). Moreover, mRNA expression analysis of a panel of *KLK*s in colorectal cancer uncovered their combinatorial prognostic value and ability to predict disease outcome more efficiently than traditional clinical parameters (111).

Potential therapeutic uses of KLKs

Although primarily known for their clinical applicability as tumor biomarkers, KLKs represent also potential targets for therapeutic intervention, because of being implicated in many cancer-related processes, such as cell-growth regulation, angiogenesis, invasion and metastasis, and due to their capability to promote or inhibit neoplastic progression. Among distinct therapeutic approaches, exploitation and/or modulation of KLK proteolytic activity are the most attractive ones. For instance, KLK proteolytic activity has been exploited in the activation of prodrugs and in the development of cancer vaccines. Anticancer agents, such as doxorubicin, vinblastine and thapsigargin, have been coupled to a peptide carrier through a PSA-cleavable bond to target prostatic tumors (11). Active immunotherapy based on PSA-targeted recombinant vaccines has also gained ground in the battle against prostate cancer, during the last decade. Phase I and II clinical trials have been conducted to evaluate the safety of such vaccines as well as the prolongation of DFS and OS of prostate cancer patients (157, 158).

The downregulation of excessive KLK activity in cancer by small inhibitory compounds has only recently arrived on the cancer therapeutic scene. In fact, it is now well-known that dysregulation of KLK activity can lead to certain pathophysiological conditions. Consequently, inhibition of the excessive proteolytic activity of KLKs by small synthetic inhibitory compounds as well as by natural polypeptidic exogenous inhibitors might constitute a novel, challenging road under construction in cancer therapeutics (17, 159). For instance, a newly developed serine protease inhibitor, MDPK67b, is intended to treat asymptomatic hormone refractory prostate cancer patients with rising PSA (160). Another synthetic, peptide-based KLK1-specific inhibitor, FE999024, was shown to attenuate breast cancer cell invasion, in vitro (161). Besides to synthetic inhibitors of KLK activity, highly specific engineered antibodies could be used to block the proteolytic activity of certain KLKs; i.e., a monoclonal antibody against KLK13 was shown to inhibit the proteolytic activity of this latter, hence paving the way for a novel therapeutic application (162). Moreover, the identification of aptamers exhibiting high affinity for specific KLKs are expected to assist the development of therapeutic strategies. The very recent generation of DNA aptamers against KLK6 constitutes the most prominent example (163).

Conclusions

The potential of KLKs as diagnostic, prognostic, and treatment monitoring biomarkers in a wide spectrum of malignancies has been extensively investigated, mostly during the last decade. Dysregulation of *KLK* gene expression in malignant tissues, both at the mRNA and protein levels, along with aberrant KLK protein levels in blood circulation suggest the involvement of KLKs in the carcinogenesis and the metastatic process. The broad key role of KLKs in the pathobiology of human malignancies, the already proven applicability of PSA/KLK3 in routine clinical management of prostate cancer patients, and the promising results regarding the utility of other KLKs in clinical practice suggest further investigation of their clinical applicability as cancer biomarkers, alone or as components of multifactorial panels. On-going research efforts focusing on elucidation of KLK-mediated cascade pathways and substrate specificity of each KLK are expected to support clinical evaluation of more KLKs. Nevertheless, we should always bear in mind that cross-validation from independent research groups and additional corroboration from large-scale studies are indispensable for implementation of KLKs in clinical routine.

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Conflict of interest statement

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