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Predictions for the future of kallikrein-related peptidases in molecular diagnostics

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Kallikrein-related peptidases (KLKs) form a cancer-related ensemble of serine proteases. This multigene family hosts the most widely used cancer biomarker that is PSA-KLK3, with millions of tests performed annually worldwide. The present report provides an overview of the biomarker potential of the extended KLK family (KLK1-KLK15) in various disease settings and envisages approaches that could lead to additional KLK-driven applications in future molecular diagnostics. Particular focus is given on the inclusion of KLKs into multifaceted cancer biomarker panels that provide enhanced diagnostic, prognostic and/or predictive accuracy in several human malignancies. Such panels have been described so far for prostate, ovarian, lung and colorectal cancers. The role of KLKs as biomarkers in non-malignant disease settings, such as Alzheimer's disease and multiple sclerosis, is also commented upon. Predictions are given on the challenges and future directions regarding clinically oriented KLK research.

KEYWORDS: biomarker panel • cancer diagnosis • cancer prognosis • kallikrein-related peptidases • KLK • molecular tumor marker • neurodegenerative diseases • ovarian cancer • prostate cancer • PSA

Kallikrein-related peptidases: serine proteases with extensive implication in human (patho)physiology & unique biomarker capabilities

Prostate-specific antigen (PSA) or kallikrein-related peptidase 3 (KLK3) is the most widely recognized member of the tissue kallikrein and kallikrein-related peptidases gene family (*KLKs*) [1]. PSA-KLK3 has been broadly used as a prostate cancer biomarker for almost three decades [2–6].

KLKs, located on chromosome 19q13.3–q13.4, comprise 15 gene members (*KLK1–KLK15*) [1] that have been suggested, in a plethora of studies, as promising disease biomarkers [7–9]. Efforts have also been made in order to evaluate the therapeutic potential of *KLKs* through various approaches that include engineered inhibitors, prodrugs activation and *KLK*-driven immunotherapy [10,11].

Perhaps, the continuously reported broad role of *KLKs* as biomarkers is a reflection of the important functions they serve in human physiology. *KLKs* share notable similarities at the levels of gene/protein structure (five coding

exons that encode for inactive zymogens), protein activity (trypsin or chymotrypsin like) and biological regulation of their gene expression (by steroid hormones, methylation, histone modification, miRNAs) and enzyme activity (cleavage activation, inhibition by endogenous molecules) [12–14]. Under physiological conditions, *KLKs* act on their substrates, which include growth factors, hormones, proteases and cell signaling molecules, in order to orchestrate important body functions such as skin homeostasis, blood pressure regulation, semen liquefaction and neuronal plasticity [13–16]. Looking at the other side of the coin, deregulation of *KLK* function can trigger the manifestation of severe diseases. *KLKs* have been primarily studied as molecules that can influence the initiation and progression of human malignancies [9–12,16]. Nonetheless, their involvement has also been thoroughly investigated in neurodegenerative/neuroinflammatory disorders [15–18] and, particularly, skin diseases [19–22].

The present report provides an overview of the *KLK* biomarker potential in various disease settings and envisages approaches that increase

the probabilities of introducing additional KLK-driven applications in future molecular diagnostics. Expected challenges and predicted directions in clinically oriented KLK research are also commented upon.

KLKs as cancer biomarkers: from PSA–KLK3 to the extended KLK family

Clinical oncology has been radically evolved after the incorporation of biological tumor markers in the management of human malignancies. Representative examples of the few tumor biomarkers that have succeeded in reaching routine clinical practice include the *HER2* gene amplification and OncotypeDx gene expression panel tests as predictive biomarkers for breast cancer, CA125 for ovarian cancer monitoring, *BRAF* mutation assessment for melanoma and PSA testing for prostate cancer screening and monitoring [23,24].

PSA: a successful, yet controversial, cancer biomarker

The influence of PSA testing on clinical decision making, both in a positive and negative sense, is so unique that in the history of oncology, it is logical to categorize prostate cancer management in a pre-PSA period, the current PSA period, as well as a post-PSA period, which is agonizingly anticipated [23,25].

The first studies describing the usefulness of PSA as a prostate cancer biomarker showed its utility as a disease monitoring and recurrence prediction tool and in 1986 received US FDA approval for this use [4,26]. The use of PSA measurements still remains the gold standard in defining biochemical relapse and thus disease recurrence [27]. However, after its initial approval, PSA testing was also applied for diagnostic purposes. In the following years, detection of prostate cancer increased spectacularly in the USA. The broad application of PSA testing as a screening strategy in asymptomatic men (FDA approved for this use in 1994) led to an impressive decrease in the cases diagnosed with advanced disease [28,29]. Nonetheless, all these important benefits came with a price. PSA serum levels are increased not only in clinically relevant prostate cancer, but also in every lesion that alters prostate gland's architecture including benign conditions and clinically indolent cancer. The widespread use of PSA testing led to more men with benign conditions being biopsied and to more men with no life-threatening tumors undergoing unnecessary radical treatment [23,30,31]. Two large-scale trials that investigated the effect of PSA screening *per se* in reducing prostate cancer mortality (Prostate, Lung, Colorectal, and Ovarian [PLCO] cancer screening trial and European Randomized Study of Screening for Prostate Cancer [ERSPC]) produced conflicting results. The PLCO trial [32], which was later criticized due to contamination of the unscreened arm, reported no benefit from screening, whereas ESPRC [33] reported a decrease in prostate cancer mortality [23,30]. The abovementioned data led the US Preventive Services Task Force to recommend against PSA screening, a decision that provoked extensive discussion and heavy criticism [23,34,35]. It seems that PSA testing as we know it may have gone full

circle and its initial use for disease monitoring happens to be also the most suitable. The introduction of additional biomarkers that would limit the PSA-driven overdiagnosis/overtreatment and that could identify patients who will indeed benefit from hormonal and chemotherapy represents a clinical necessity. A systematic, multiphase procedure is needed in order for novel prostate cancer biomarkers to reach clinical practice. This carefully designed process should begin from the initial evidence-based discovery stage and continue with rigorous validation phases, including independent cohorts, retrospective and prospective studies, before finally reaching large-scale population trials [36].

Normalizing PSA to the prostate gland volume, determining changes in PSA measurements over time, measuring percentage of free PSA (%fPSA), complexed PSA, as well as the use of the more recently (2012) FDA-approved tests, such as the PCA3 urine test and the combined serum measurements of -2proPSA, free PSA and total PSA (calculated as prostate health index), have been proposed as next steps for improving the biomarker capabilities of PSA. Artificial neural networks, nomograms and logistic regression models can also help toward this direction [23,37–39]. Despite the reported improvements in the diagnostic performance of PSA by the aforementioned approaches, it is believed that a more radical paradigm shift is still needed.

As discussed in detail below (in 'KLKs in cancer biomarker panels: A more realistic and promising approach for routine clinical practice' section), the introduction of novel molecular markers belonging to the extended KLK family, through a biomarker panel strategy, could provide considerable improvements for prostate cancer screening.

Beyond PSA: the capacity of individual KLKs as biomarkers for human malignancies

Papers published on the association of KLKs with human malignancies (1933–2012) have been calculated to amount to the astonishing sum of 28,744 [40]. Every single member of the *KLK* gene family has been evaluated and proposed as a meaningful serum and/or tissue marker for at least one human malignancy [8,9].

Recently published review articles thoroughly describe the biomarker potential of the *KLK* gene family in urogenital and reproductive organ malignancies [41,42], gastrointestinal malignancies [43], lung, brain, head and neck cancers, and acute lymphoblastic leukemia [7–9]. A book volume dedicated to the biomarker potential of KLKs for cancer diagnosis, prognosis and treatment response prediction/monitoring has also been published [40]. A brief overview of this information is conveyed in TABLE 1.

The characterization of the extended cancer-related *KLK* family, including several alternative mRNA transcripts and protein isoforms, the comprehensive expression profiling of *KLKs* at different levels (mRNA, protein) and different disease settings (malignant and nonmalignant), as well as the significant improvements in the sensitivity and specificity of molecular

Table 1. The biomarker potential of individual KLKs in human malignancies.

Cancer type	Clinical relevance	Ref.
Prostate	Diagnosis: KLK2, KLK3, KLK11 Prognosis/Prediction: KLK2, KLK3, KLK4, KLK5, KLK11, KLK14, KLK15	[41]
Ovarian	Diagnosis: KLK5, KLK6, KLK8, KLK10, KLK11, KLK13, KLK14 Prognosis/prediction: KLK4, KLK5, KLK6, KLK7, KLK8, KLK9, KLK10, KLK11, KLK13, KLK14, KLK15	[41,42,55]
Breast	Diagnosis: KLK3, KLK5, KLK10, KLK14 Prognosis/Prediction: KLK3, KLK4, KLK5, KLK7, KLK9, KLK10, KLK12, KLK13, KLK14, KLK15	[41,104]
Lung	Diagnosis: KLK5, KLK7, KLK8, KLK10, KLK11, KLK12, KLK13, KLK14 Prognosis: KLK6, KLK8, KLK11, KLK13	[8,105]
Colorectal	Prognosis: KLK4, KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13, KLK14	[43]
Gastric	Diagnosis: KLK6 Prognosis: KLK6, KLK10, KLK11, KLK12, KLK13	[43,106]
Endometrial	Diagnosis: KLK6, KLK10 Prognosis: KLK6	[42]
Cervical	KLK7 (increases with the severity of lesions)	[42]
Testicular	Prognosis: KLK5	[42]
Kidney	Prognosis: KLK6	[42]
Urinary bladder	KLK5, KLK6, KLK9 (increased in invasive tumors)	[107]
Head and neck	Prognosis: KLK4, KLK7, KLK11	[8]
Pancreatic	Prognosis: KLK6, KLK7, KLK10	[9,43]
Intracranial tumors	Prognosis: KLK6, KLK7	[9,108]

KLK: Kallikrein-related peptidases.

assays and reagents for KLK determination, raised expectations that the detailed information of *KLK* genes and proteins will trigger the identification of several potential tumor markers. The initial hopes and predictions have been fulfilled to a considerable extent, but still need to be realized into everyday clinical decision making.

KLKs in cancer biomarker panels: a more realistic & promising approach for routine clinical practice

A series of genomic and proteomic analyses have robustly demonstrated that cancer is an extremely heterogeneous disease in terms of morphological and biological features, clinical disease manifestation and progression, as well as response to currently available therapeutics [44]. Thus, it would be reasonable to foresee that no single biomarker will be ever capable of providing accurate enough information for cancer diagnosis, prognosis or treatment response prediction. The current trend for maximizing the clinical effectiveness of biomarkers, and thus compensating for their low incorporation rates into clinical practice, is the identification of combinatorial biomarker panels. KLKs have already been identified as valuable composites of such panels for prostate, ovarian, lung and colorectal cancers.

Perhaps, the most intriguing and well-validated data derived from a series of studies regarding the identification of a four-kallikrein serum panel consisting of total, free and intact PSA, as well as KLK2, for prostate cancer management. The above-mentioned 'kallikrein panel' can effectively predict the result of a prostate biopsy in both unscreened and previously screened men. The application of the 'kallikrein panel' could appropriately tackle the overdiagnosis and overtreatment complications deriving from PSA-based screening. It is a straightforward approach that makes use of routine laboratory tests and current standard of care in order to reduce the number of unnecessary prostate biopsies, and accompanying complications, with enhanced sensitivity (especially in high-grade tumors) and specificity (reduced detection rates of clinically indolent low-grade tumors) [45–47]. Moreover, statistical models based on the 'kallikrein panel' could effectively replace invasive procedures, such as transrectal ultrasonography and digital rectal examination, in predicting prostate biopsy outcome [48]. Another essential problem that the 'kallikrein panel' addresses is the economic impact of repeated unnecessary prostate biopsies that cannot be overlooked during the times of global economic crisis. A future application of the 'kallikrein panel' in routine diagnostics would reduce the unnecessary biopsies performed in

the USA up to 56%, without compromising standards of care; this translates to approximately US\$1.12 billion savings per year for the US health care system [49].

Multivariate models and/or artificial neural networks combining serum KLK2, %fPSA and PSA levels could provide, to some extent, an improved discrimination between CaP and BPH patients. Similar approaches using serum KLK11, %fPSA, MIC-1, MIF, prostate volume or age can also enhance BPH and CaP discrimination. Logistic regression models have demonstrated the potential of serum IGF-1/fPSA ratio and PSP94-fPSA combination for the differential diagnosis of benign and malignant prostate tumors [38]. The incorporation of a number of single nucleotide polymorphisms (SNPs) in a nomogram also containing PSA, %fPSA, age and other clinical can improve the positive predictive value of PSA testing [50].

Regarding ovarian cancer, initial studies had demonstrated that serum KLK6 [51] and KLK10 [52] measurements can be used to enhance the diagnostic sensitivity of CA125 in early disease stages. A later study revealed improved diagnostic properties for the combination of serum KLK10 and CA125 over CA125 alone [53]. Additionally, combinations between serum KLK6 and KLK10 with CA125 and age provided improvements in the discriminatory accuracy for patients harboring ovarian tumors compared with single marker use [54]. A panel of eight KLKs (KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13 and KLK14) determined in effusion samples produces remarkable areas under the curve (AUCs) of 0.99 and 0.96 for the discrimination of cancer from benign cases and ovarian cancer from other malignancies, respectively [55]. Performed in tissue samples, a panel of *KLK6*, *KLK13* and *MUC16* (encoding for CA125) mRNA levels increases the detection sensitivity for early stage ovarian cancer and the negative predictive value compared with *MUC16* alone [56]. In cytosolic extracts, the combination of CA125, B7-H4, KLK4, KLK5, KLK7, KLK8 and KLK11 and the combination of CA125, KLK8, KLK10 and KLK13 protein levels can effectively distinguish between ovarian cancer and benign tumors, and between primary ovarian cancer and primary tumors metastatic to the ovary, respectively [57].

KLK-based biomarker panels with prognostic significance can also be proven extremely helpful in ovarian cancer decision making. A combination of KLK6 and KLK13 tissue protein levels, ascites volume and nuclear grade can accurately predict (AUC = 0.833) the presence of residual tumor after surgery and thus instruct the administration of preoperative chemotherapy [58]. The combined determination of KLK13, KLK6, KLK8 protein levels in ovarian cytosolic extracts, disease stage and debulking status can efficiently identify patients' response to chemotherapy (AUC = 0.91). Similarly, impressive predictive capabilities have been identified regarding 1-year progression-free survival (AUC = 0.90) for a combination of KLK6, KLK8, KLK11, KLK1 and clinical variables (i.e., stage, debulking success, chemotherapy response), and 5-year progression-free survival (AUC = 0.93) for a

combination of KLK6, KLK7, KLK11, KLK14, B7-H4 and clinical variables [57]. Serum-based multiparametric panels have also been constructed and could offer valuable prognostic information regarding 1-year survival (KLK7, KLK10, B7-H4 and Spondin-2), 1-year disease progression (CA125, KLK7, KLK8 and Spondin-2) and chemotherapy response (CA125, KLK5 and KLK7) [59].

In non-small-cell lung cancer (NSCLC), the study of Planque *et al.* has demonstrated the diagnostic capabilities (AUC = 0.90) of combined KLK4, KLK8, KLK10, KLK11, KLK12, KLK13 and KLK14 serum measurements for the discrimination between NSCLC patients and healthy individuals. Another model that includes serum KLK8, KLK11, KLK12, KLK13, KLK1 as well as gender and smoking produced an AUC of 0.92 [60]. Several combinatory assessments of *KLK* mRNA expression levels can provide important prognostic information regarding the overall survival (OS) of NSCLC patients. The *KLK8* alternative transcript 4/*KLK11* mRNA ratio in lung cancer is a strong independent predictor of poor OS, showing better prognostic performance even from clinical stage [61].

In colorectal cancer, the combination of tissue protein levels of KLK14, and KLK8, KLK10, KLK14, KLK15, with clinical parameters (age and TNM stage) leads to an increased predictive potential of 1-year and 5-year OS, respectively, compared with clinical parameters alone [62].

Regulation of KLK expression by miRNAs & DNA methylation: epigenetic mechanisms with great translational potential

Determinations of DNA methylation status and miRNA expression levels are regarded as valuable biological tumor markers. They can be easily assessed in body fluids, such as blood or urine, as well as in archival material (formalin-fixed paraffin-embedded tissues) that is accompanied by invaluable clinical follow-up information [63–65].

The miRNA–KLK axis of interaction has been described so far in prostate cancer [66], ovarian cancer [67] and renal cell carcinoma [68]. Several miRNAs that are deregulated in the aforementioned malignancies are predicted to target *KLKs*. The experimentally validated interactions include those between miR-331-3p and *KLK4*, miR-143 and *KLK10*, miR-224 and both *KLK1* and *KLK10*, let-7f and both *KLK6* and *KLK10*, miR-516a and *KLK10*, as well as between members of the miR-99 family and *KLK3* [14,69]. In ovarian and prostate cancers, a significant negative correlation between the expression levels of miRNAs and their target *KLKs* has been documented [14,66,69]. For example, a negative correlation between the expression levels of miR-224 and its predicted target *KLK15* has been reported in prostate cancer [69]. Interestingly, both *KLK15* and miR-224 have been repeatedly proposed by independent researchers as important biomarkers of unfavorable and favorable, respectively, prognosis for prostate cancer patients [70–73]. We believe that the combination of miRNA and *KLK* expression into informative

diagnostic/prognostic scores can constitute a novel approach in stratifying and translating the heterogeneity of human malignancies to meaningful clinical information.

The *KLK* locus contains multiple CpG islands, and DNA methylation represents a suggested mechanism of *KLK* transcriptional regulation for certain gene members (e.g., *KLK6*, *KLK10*, *KLK1*, *KLK11*, *KLK12* and *KLK13*). The decreased expression of certain *KLKs* observed in some human malignancies (e.g., *KLK6* in breast cancer, *KLK10* in NSCLC) is closely associated with CpG island hypermethylation [14,74]. *KLK10* exon 3 methylation is associated with poor prognosis in acute lymphoblastic leukemia and breast cancer, while it is also considered as an indicator of advanced disease in NSCLC [14]. A recent study shows that low *KLK10* methylation levels are associated with biochemical relapse in prostate cancer patients [75].

There is even more to KLKs than their tumor marker potential: the role of KLKs as biomarkers in nonmalignant diseases

Without doubt, most of the studies regarding the biomarker capacity of KLKs concern human malignancies. Nonetheless, studies addressing the potential role of KLKs in the molecular diagnostics of nonmalignant diseases are also intriguing.

The deregulation of KLK levels has been comprehensively reported in nonmalignant diseases of the CNS [11,76]. Serum and/or cerebrospinal fluid (CSF) KLK levels could be considered as biomarkers for major CNS diseases.

In Alzheimer's disease (AD), CSF levels of KLK10 increase, while those of KLK7 decrease. Lower KLK7 CSF levels are associated with the presence of ApoE4 alleles, a risk factor of AD [77]. Both whole blood and CSF levels of KLK6 were initially reported to be increased in AD patients compared with normal controls [78]. Contrariwise, it has been recently shown that patients with synucleinopathy showed lower KLK6 CSF levels compared with controls and AD patients; no difference was found in KLK6 CSF levels between AD and control samples in the same study [79]. KLK6 CSF and plasma levels are positively correlated with age in normal individuals, and this association is lost [80] or even inverted [81] in AD patients. Decreased KLK6 CSF levels have been proposed as a risk factor for AD development [80]. Interestingly, KLK6 plasma concentrations can be used for the discrimination between AD patients and individuals without neurodegenerative dementia [81], as well as for calculating the risk for progression of mild cognitive impairment to dementia with vascular component or AD [82]. Furthermore, patients with frontotemporal dementia exhibit decreased KLK6, KLK7 and KLK10 CSF levels compared with control subjects [77].

Serum KLK6 levels are increased after traumatic brain injury [83] and in postpolio syndrome [84], whereas they are decreased in a life-threatening condition known as aneurysmal subarachnoid hemorrhage, with the lowest levels measured in patients with worse outcome [85].

Aberrant KLK levels are also found in multiple sclerosis (MS). Interestingly, KLK6 participates directly in the

pathogenesis of MS by cleaving basic myelin proteins [17,76]. An initial study has shown that serum KLK1 and KLK6 levels are elevated in MS patients and are associated with secondary progressive MS and expanded disability status scale scores [86]. Patients diagnosed with advanced MS exhibit elevated KLK6 CSF levels compared with neurological controls [87]. On the contrary, three recent proteomic studies have shown that KLK6 is significantly decreased in the CSF of patients with relapsing–remitting MS compared with other neurological disease controls [88–90]. Significantly increased anti-KLK11 antibody levels (82% sensitivity, 94% specificity) are found in the serum of patients diagnosed with Sjögren's syndrome, another autoimmune-mediated disease [91].

One of the well-studied physiological roles of KLKs is their central contribution in maintaining skin homeostasis and desquamation (mainly for KLK5, KLK7 and KLK14). Studies on Netherton syndrome, a rare genetic skin disease linked with mutations in the *SPINK5* gene encoding for the inhibitor LEKTI that blocks KLK activity, have shown that the lack of LEKTI causes KLK overactivation, over-desquamation of corneocytes, inflammation and ultimately severe skin dysfunction [19–22,92]. Regarding the biomarker capacity of KLKs in skin diseases, they may hold promise as biomarkers for psoriasis. KLK6 and KLK8 are increased in psoriatic arthritis synovial fluid compared with noninflammatory osteoarthritis. KLK8 serum levels are also elevated in psoriatic disease and are independently associated with cutaneous psoriasis activity, but not with arthritic disease activity. Consequently, KLK8 could constitute a novel surrogate molecular marker for estimating cutaneous psoriasis severity and monitoring therapeutic response [93]. KLK10 and KLK13 serum levels are also correlated with the severity of skin lesions in psoriasis. Interestingly, KLK5 and KLK11 concentrations are lower in the serum of psoriasis vulgaris and arthropathic psoriasis patients compared with normal individuals and are significantly decreased after therapy [94]. As far as atopic dermatitis is concerned, serum KLK8 levels are increased, whereas KLK5 and KLK11 levels are decreased [95]; a *KLK7* polymorphism (AACC insertion in the 3'-UTR) has also been associated with this disease [96].

KLK SNPs: biomarker potential deriving from yet another facet of KLKs association with human pathology

A plethora of data demonstrates the linkage between SNPs within the *KLK* locus and a wide variety of diseases [96]. In the nonmalignant setting, *KLK1* SNPs are associated with hypertension (rs3212816, rs5517), cerebral hemorrhage (rs5517) and cardiovascular disease (rs5515), a *KLK2* SNP is related to male infertility (rs2664155), a *KLK4* mutation has been identified as causing event of a tooth disorder (mutation: g.2142 G > A, Trp153Stop) and certain *KLK8* SNPs have been linked with intracranial aneurysm (rs1722561, rs1701946) [96] and bipolar disorder (rs1612902) [97]. Prostate cancer has been the cornerstone of *KLK*-related SNPs research, perhaps due to the importance of PSA. *KLK2*

(rs198977, rs2664155, rs1506684), *KLK3* (e.g., rs266882, rs1058205, rs266870, rs2659122), *KLK4* (e.g., rs7248321, rs1654551, rs1701927, rs806019) *KLK10* (rs3745535) and *KLK12* (rs3865443) SNPs have been associated with prostate cancer risk, *KLK3* (e.g., rs266882, rs2735839, rs17632542), *KLK4* (rs198968), *KLK14* (rs17728459, rs35287116) and *KLK15* (rs2659056) SNPs with disease aggressiveness, two *KLK3* SNPs (rs61752561, rs2735839) with patients' survival and a *KLK2* SNP (rs198977) with biochemical recurrence. Likewise, *KLK2* (rs198977) and *KLK4* (rs806019) SNPs have been associated with breast cancer risk, a *KLK3* SNP was related to less aggressive breast cancer (rs11575894) as well as *KLK3* (rs11084033) and *KLK15* (rs266851) SNPs were linked with ovarian cancer survival [96,98–100].

Even though individual *KLK* SNPs may not be likely to be used as a standalone test in future molecular diagnostics, the multifactorial approach seems, once again, to be more realistic. The combination of *KLK* SNPs with these of other loci and/or with other conventional clinicopathological data may form the basis of a rapid, easily performed DNA-based clinical test. Toward this direction, it has already been shown that a *KLK2* SNP (rs198977) can be combined with clinical data or serum *KLK2* levels in order to improve the prediction of biochemical relapse [96] or to enhance prostate cancer detection [100], respectively. Other *KLK* SNPs can also aid in prostate cancer risk prediction and therapeutic decision making through their incorporation into multifactorial models [101].

Expert commentary

The use of PSA measurements as a prostate cancer biomarker was a major breakthrough for the practice of oncology. Nonetheless, its extensive use as screening tool in asymptomatic men introduced complexities in prostate cancer management. Without intending to overlook the benefits that PSA has provided regarding the detection of prostate cancer in early stages, it should be noted that PSA-driven overdiagnosis and overtreatment are issues that still remain to be tackled [23,30]. The current challenge is to identify multifactorial biomarker panels that will provide clear advantages over individual biomarkers such as PSA. The application of *KLK*-based biomarker panels could assist in the decision-making processes primarily for prostate cancer and, in the long term, for other malignancies as well.

Data from the 'four-kallikrein panel' approach are very encouraging toward the reduction of unnecessary prostate biopsies [45–49]. Ovarian, lung and colorectal cancer management could also be enhanced by multifactorial biomarker panels that include multiple *KLK* members [54–62]. The amount of *KLK*-derived information that can be combined is enormous and has been made available through decades of, still ongoing, translational research. *KLK* expression (mRNA and protein levels), *KLK*-targeting miRNAs, SNPs found within the *KLK* locus and the methylation status of *KLK* genes are examples of detailed molecular data that could be combined with traditional clinicopathological

factors. This multifaceted approach can enhance the diagnostic, prognostic, predictive and/or treatment monitoring potency of the one-sided procedures that are currently followed throughout cancer patients' management. Well-designed and established assays are already available for *KLK* assessment at the protein (ELISA, immunohistochemistry), RNA (qPCRs for mRNA and *KLK*-targeting miRNAs) and DNA (quantitative methylation methods, sequencing for SNPs) levels. These easily performed assays can be cost-effectively used in routine clinical practice. Interestingly, *KLK* levels can be also measured in the serum and CSF of patients suffering from nonmalignant diseases. This has opened new opportunities for the consideration of *KLKs* as biomarkers for major neurodegenerative and neuroinflammatory diseases such as AD and MS [78,81,86,87].

Five-year view

It is rather unlikely that any single-biomarker test could address the heterogeneity observed, in terms of biological and clinical manifestation, in most human malignancies. Combining multiple biomarkers with conventional, yet strong, indicators can significantly improve the accuracy of molecular diagnostics. The FDA clearance of the multifactorial ROMA score and OVA1 panel for ovarian cancer enhances this notion further [24].

The significance of several *KLK* members as individual biomarkers has been corroborated by independent research groups [7,8,12,41,42]. Nonetheless, the need for multicentric large-scale validation still exists. We believe that there is a niche for additional *KLKs* in the treatment decision making of cancer patients and that this can be achieved through their integration in well-defined biomarker panels. Based on the repeatedly validated and straightforward results that have been reported [45–49], it is highly probable that the 'four-kallikrein panel' will be eventually introduced in clinical practice for prostate cancer. The recent FDA approvals for prostate health index (based on serum-2pro-, total- and free PSA measurements) and urinary PCA3 score highlight the intense medical need for improving decisions regarding prostate cancer management.

Current and future trends in *KLK* research, such as the elucidation of miRNA–*KLKs* axis of interaction, deciphering the *KLK*-methylation patterns, characterization of *KLK* SNPs, discovery of novel *KLK* mRNA transcripts, determination of glycosylated *KLK* isoforms – especially for *KLK3* [102] and *KLK6* [103] – could help in transforming the envisagement of the incorporation of additional *KLKs* in future molecular diagnostics into a clinical reality.

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Key issues

- The Kallikrein-related peptidase (KLK) gene family encodes for 15 secreted serine proteases, many of which have been described as cancer-related molecules.
- KLK3 (also known as prostate-specific antigen [PSA]) represents the most renowned member of the KLK family. The wide application of PSA as a biomarker for prostate cancer screening has provided notable benefits, such as the detection of prostate cancer at early stages and a, much debated, decrease in mortality rates.
- The benefits of PSA testing came with the complications of overdiagnosis and overtreatment. Thus, novel prostate cancer biomarkers should be introduced in order to reduce the amount of unnecessary biopsies.
- Individual KLK members have been described as biomarkers for the majority of human malignancies including prostate, ovarian, breast, lung and colorectal cancers.
- The incorporation of KLKs into multifaceted biomarker panels provides enhanced diagnostic, prognostic and predictive accuracy in several human malignancies. The most successful example is that of the 'four-kallikrein panel' for prostate cancer, which has great dynamics for introduction into future clinical practice.
- Multifactorial KLK-driven biomarker panels have also been proposed for ovarian, lung and colorectal cancer decision making.
- KLK-derived information that could be combined in biomarker panels includes data from the KLK expression profiling (mRNA and/or protein levels), KLK-targeting miRNAs, SNPs found within the KLK locus and methylation status of KLK genes.
- KLKs are also regarded as promising biomarkers for nonmalignant diseases such as Alzheimer's disease and multiple sclerosis.

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