Original Research

A mechanistic insight into the anti-metastatic role of the prostate specific antigen

Francesco Pellegrino\textsuperscript{a,b,1}, Arianna Coghi\textsuperscript{b,1}, Giovanni Lavorgna\textsuperscript{b}, Walter Cazzaniga\textsuperscript{a}, Edoardo Guazzoni\textsuperscript{c}, Irene Locatelli\textsuperscript{b}, Isabella Villa\textsuperscript{d}, Simona Bolamperti\textsuperscript{d}, Nadia Finocchio\textsuperscript{b}, Massimo Alfano\textsuperscript{b}, Roberta Luciano\textsuperscript{e}, Alberto Briganti\textsuperscript{a,f}, Francesco Montorsi\textsuperscript{a,f}, Andrea Salonia\textsuperscript{a,f,1}, Ilaria Cavarretta\textsuperscript{b,1,*}

\textsuperscript{a} Department of Urology, IRCCS San Raffaele Hospital, Milan, Italy
\textsuperscript{b} Division of Experimental Oncology/Unit of Urology, IRCCS San Raffaele Hospital, Urological Research Institute, Via Olgettina, 60, Milan 20132, Italy
\textsuperscript{c} Operative Unit of Orthopedics and Traumatology, Department of Clinical-Surgical Sciences, Diagnostics and Pediatrics, Fondazione IRCCS Polichinico San Matteo, Pavia, Italy
\textsuperscript{d} Bone Metabolism Unit, Division of Genetics and Cell Biology, IRCCS San Raffaele Scientific Institute, Milan, Italy
\textsuperscript{e} Pathology Unit, IRCCS San Raffaele Hospital, Milan, Italy
\textsuperscript{f} Vita-Salute San Raffaele University, Milan, Italy
\textsuperscript{1} These authors contributed equally to this work.

\textsuperscript{*} Corresponding author. E-mail address: cavarretta.ilaria@hsr.it (I. Cavarretta).

\begin{abstract}
Aim: Since its discovery Prostate Specific Antigen (PSA), also referred to as kallikrein-3 (KLK3), has been used as standard circulating biomarker for prostate cancer (PCa). However, its specificity remains not adequate and its mechanism of action still elusive. Therefore, deciphering PSA role throughout PCa-pathobiology would be relevant in improving both cancer diagnosis and outcome prediction. We investigated the possible role played by PSA on/in the tumor microenvironment and over the first steps of cancer invasion.

Methods: Fresh PCa-specimens and cell lines were used for \textit{ex-vivo/in-vitro} invasion assays and assessment of prostate tissue-PSA (tPSA), type 1 collagen (COL1A1) and \textit{\beta}1-integrin expression. Tissue Cancer Genome Atlas (TCGA) and Decipher\textsuperscript{®} datasets were considered to estimate tPSA clinical relevance.

Results: A more precise, inverse, correspondence between tPSA and clinical/pathological parameters was found than for circulating PSA. KLK3 combined with Gleason grade and pathologic stage, better predicted cancer-related mortality. Consistently, we demonstrated that PSA inhibits prostate extracellular-matrix (ECM) invasion by PCa cells. As for the mechanism of action, we provided novel information that PSA is able to cleave COL1A1, a main component of the ECM. Finally, \textit{\beta}1-integrin, a crucial COL1A1 transducing-receptor involved in tumor adhesion/invasion, resulted to be downregulated in PCa specimens with higher levels of tPSA.

Conclusions: By interfering with type 1 collagen and its downstream targets, PSA may hamper adhesion and path of the cancer cells through ECM and their migration ability, thus explaining the inverse correlation highlighted between prostate tPSA levels and clinically significant disease.
\end{abstract}

Introduction

Despite advances in cancer prevention, diagnosis and treatment, prostate cancer (PCa) remains one of the leading causes of male cancer death in Western countries [1,2]. Since its discovery in the ’80, the prostate specific antigen (PSA) has been considered the marker for excellence for PCa [3,4], and therefore used as a paradigm of reference basically in all studies on novel biomarkers. Nevertheless, despite the high accuracy of nomograms including PSA in predicting clinical outcomes, the percentage of misdiagnosis remains significant [5–7], with heavy consequences in terms of morbidity, mortality and social costs. Surprisingly, in the face of thousands of publications on PSA as a biomarker, only few studies addressed its role in terms of PCa pathobiology [8–13]. Conversely, deciphering the biological role of PSA
throughout cancer progression may help to more comprehensively understand its clinical significance and limitations with a benefit in terms of cancer diagnosis and outcome prediction. PSA, also referred to as kallikrein-3 (KLK3), belongs to the family of glandular kallikrein-related peptidases with serine protease activity [14]. It is secreted from the apical end of luminal epithelial cells, stored at high concentrations in the prostatic collecting ducts and normally released into the seminal fluid. Its main physiological role consists in maintaining semen fluidity by cutting semenogelin I and II. In the case of PCs, the alteration of the histological structure of the prostate causes a remarkable spillover of PSA into the surrounding stroma [15,16], where its levels may exceed of several orders of magnitude the levels of circulating PSA (cPSA). In this context, PSA has been reported to be active [16]. PSA may cleave also additional and different target molecules such as IGFBP-3, PTH-related protein, latent TGF-beta 2 and extracellular matrix (ECM) components (i.e., fibronectin, collagen IV and XXIII, laminin and galectin-3) [8,13,16-18]. Overall, these observations prompted us to investigate the possible consequences of high prostate tissue-associated PSA (tPSA) levels toward type 1 collagen (COL1A1), one of the most abundant components of the prostate ECM, and toward PCs invasion. We hypothesize that, by altering the tumor microenvironment structure/composition, PSA activity on tumor ECM substrates has an impact on the PCs cell ability to enter the metastatic process. Of relevance, we also compared the net benefit of including tPSA rather than cPSA in the prediction of clinically significant PCs.

Materials and methods

Datasets

In case of the Tissue Cancer Genome Atlas (TCGA) dataset [19], normalized expression data and clinical data were taken from the Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/), whereas, in the case of the Decipher® dataset [20], access to data was obtained directly from Decipher Biosciences (Veracyte, San Diego, CA).

Statistical analysis

The Pearson correlation coefficient between KLK3 mRNA expression and several clinical parameters was calculated both considering TCGA and Decipher® dataset by using the R software (https://www.r-project.org). Selected clinical variables were: primary and secondary Gleason score (then transformed into Gleason Grading Groups, GGG); pathological T-stage (pT); and, preoperative cPSA. Descriptive statistics, univariable (UVA) and multivariable (MVA) logistic regression analyses were performed to evaluate the association between KLK3 expression and specific outcomes. Covariates for the multivariable models were identified with a stepwise forward selection approach and consisted of cPSA, pT, GGG and prostate tissue-KLK3. ROC curves and AUC calculation were used to quantify the discrimination ability of the different models. Decision Curve Analysis was calculated from the multivariable models to evaluate the net benefit of using KLK3 expression to predict a specific outcome. In vitro experiments were repeated at least three times. KLK3 mRNA expression was expressed as RPKMs (Reads Per Kilo Million). GraphPad Prism 8 (La Jolla, CA, USA) was used to create graphical representation and for statistical evaluation. The Mann-Whitney U test was applied to assess statistical significance. All tests were run using a significance level (p-value) set to 0.05.

Biobank specimens collection/processing

Prostate specimens were collected from patients undergoing radical prostatectomy (RP) for PCs at a single tertiary-referral academic institute. The protocol was approved by the Institutional Ethical Committee (Protocol URBBAN, 01.09.2010). All patients signed an informed consent agreeing to deliver their own anonymous information for future studies. Specimens were either vital frozen in the absence of serum to obtain ECM or immediately snap frozen in liquid nitrogen to obtain protein lysates. Seminal fluid was collected from healthy voluntary men, processed immediately by centrifugation at 5000 rpm and stored at −80 °C.

Reagents and prostate cell lines

All reagents and chemicals were from Thermo-Fisher Scientific Inc. (Waltham, Massachusetts, USA), unless otherwise stated. The human epithelial prostate cell lines RWPE-1 and RWPE-2 (RRID:CVCL_3791 and CVCL_3792) and the human metastatic MDaPca2b (RRID:CVCL_4748) and PC3 (RRID:CVCL_4748) cell lines were purchased from ATCC and maintained as previously described [21,22]. RWPE-2 were maintained as RWPE-1. All cell lines were regularly tested for mycoplasma presence by amplifying a specific mycoplasma sequence; the primers utilized in the Polymerase Chain Reaction were ACTCTACGGGAGCCAGCAGCAGTAGT (Forward) and TGCACTCTGTGAACCTTACCT (Reverse). PSA was from ExBio (Vestec, Czech Republic) and Merck Life Science. PSA antibody was from Santa Cruz (Heidelberg, Germany). Rabbit anti-integrin β1 was from Abcam (Milan, Italy). Secondary antibodies were from Biorad (Hercules, California, USA) and Cell Signaling Technologies (Danvers, MA, USA).

Western blot

Human prostate tissues were lysed by sonication (50% power; 15 pulses/cycles punctuated by a 1 s pause for a total of 1 min and 15 s). Cell lysate was obtained as previously described [23]. Equal amounts of proteins were resolved by SDS-PAGE and transferred onto PVDF membranes. After blocking, membranes were incubated overnight at 4 °C with the primary antibodies, followed by 1 h incubation with secondary antibodies. Signals were detected using the ECL method, according to the manufacturer protocol.

Tissue decellularization

Prostate tissue ECMs were obtained from RP specimens, previously marked by the pathologist as either tumoral or non-tumoral. Specimens were decellularized by using a protocol previously described [21,24] and then utilized in co-culture experiments.

EX vivo-in vitro 3D model of prostate stroma invasion

Prostate stroma invasion by prostate cells was evaluated in direct co-culture experiments, as previously described [21] by using non-tumoral prostate tissue-derived ECM, as a model of prostate tumor microenvironment, and the above described cell lines.

PSA activity

PSA activity on COL1A1 substrate was assessed by incubating either PSA or seminal fluid with the different substrates in PBS, for 22 h at 37 °C. The substrates were: human placenta-derived COL1A1 (Rockland Tebu-bio, Limerick, PA, USA), prostate ECM lysates and osteoblast cell lysates.

Primary osteoblast cultures

Description is reported in Supplementary Material and Methods.

Invasion assays with boyden chambers

Description is reported in Supplementary Material and Methods.
PSA elisa

Description is reported in Supplementary Material and Methods.

Guilt by association approach

Description is reported in Supplementary Material and Methods.

Results

PSA cleaves COL1A1

As type 1 collagen is one of the most abundant ECM components, we tested the action of PSA on this substrate. In order to recapitulate the extremely high PSA levels accumulating in the tumor microenvironment during the first steps of invasion, we incubated the substrate, human COL1A1, with human seminal fluid (SF), which contains large amounts of PSA. We observed that SF completely degraded the substrate (Fig. 1A). This action was abrogated by an anti-PSA antibody, thus demonstrating that the effect is due to the PSA present in the human SF and not to other enzymes. PSA activity on COL1A1 was also verified on more complex substrates. In particular, a pool of SF from 6 different healthy subjects was able to cut the COL1A1 present in the ECM derived from human peripheral prostate tissue (Fig. 1B) and from human osteoblast primary cell cultures (Suppl. Fig. 1A). We finally tested the commercially available PSA considered in our next in-vitro assays, demonstrating it does cleave COL1A1 (Suppl. Fig. 1B). To verify whether COL1A1 is a substrate of PSA also in vivo, we analyzed endogenous COL1A1 expression and degradation in 25 PCa resections from our institutional biobank in relation to their prostate tPSA content. We observed that COL1A1 is a substrate of PSA also in vitro, demonstrating it does cleave COL1A1 (Suppl. Fig. 1A). We finally tested the action of PSA on this substrate. In order to recapitulate the extremely high PSA levels accumulating in the tumor microenvironment during the first steps of invasion, we incubated the substrate, human COL1A1, with human seminal fluid (SF), which contains large amounts of PSA. We observed that SF completely degraded the substrate (Fig. 1A). This action was abrogated by an anti-PSA antibody, thus demonstrating that the effect is due to the PSA present in the human SF and not to other enzymes. PSA activity on COL1A1 was also verified on more complex substrates. In particular, a pool of SF from 6 different healthy subjects was able to cut the COL1A1 present in the ECM derived from human peripheral prostate tissue (Fig. 1B) and from human osteoblast primary cell cultures (Suppl. Fig. 1A). We finally tested the commercially available PSA considered in our next in-vitro assays, demonstrating it does cleave COL1A1 (Suppl. Fig. 1B). To verify whether COL1A1 is a substrate of PSA also in vivo, we analyzed endogenous COL1A1 expression and degradation in 25 PCa resections from our institutional biobank in relation to their prostate tPSA content. We observed that COL1A1 is a substrate of PSA also in vitro, demonstrating it does cleave COL1A1 (Suppl. Fig. 1A).

Table 1

| Pathological features | n. specimens/patients | Preop. cPSA levels ng/ml (mean ± ES) | Statistical Significance of preop. cPSA levels |
|-----------------------|-----------------------|-------------------------------------|-----------------------------------------------|
| GS < 4 + 3            | 5                     | 7.39 ± 1.60                         |                                               |
| GS 4 + 3              | 9                     | 12.67 ± 3.60                        |                                               |
| GS 8                  | 1                     | 2                                   |                                               |
| GS 9                  | 11                    | 16.41 ± 6.51                        |                                               |
| < pT3a                | 6                     | 4.39 ± 0.97                         |                                               |
| pT3a                  | 7                     | 11.66 ± 4.11                        |                                               |
| > pT3a                | 13                    | 17.36 ± *                          | ** vs <pT3a; * vs ≤ pT3a                     |
| pN0                   | 18                    | 8.16 ± 1.79                        |                                               |
| pN1                   | 8                     | 23.33 ± *                          | * vs N0                                        |

Abbreviations: cPSA: circulating serum PSA; pN0: without lymph node invasion; pN1: with Lymph node invasion

Tissue KLK3 gene expression inversely correlates to clinicopathological features and collagen expression

In order to confirm our findings on a wider case-study, we analyzed the well-established cohort of PCa patients of TCGA [19] and of the more...
Fig. 2. Protein expression levels of tPSA and COL1A1 in human PCa specimens. PSA and COL1A1 levels were quantified by western blot analysis in surgical resections from RP. Specimens are from our institutional biobank. Equal micrograms of tissue lysate were loaded. (A) Representative blot images showing COL1A1 expression from samples with different PSA levels. COL1A1 and PSA were quantified on the same blot. (B) Plots showing quantification of COL1A1 and cleavage products of COL1A1 according to PSA levels. (C) Table showing number of cases, pathological features and statistical differences among groups with low or high PSA expression. (D) Plots showing protein PSA levels, normalized to the level of actin, according to grade, stage and lymph node positivity (LN1). Mean values ± SEM are reported. *p < 0.05; **p < 0.01; ****p < 0.0001 Mann-Whitney test.
recent Decipher® dataset [20], which considers several more metastatic cases. In order to be used for calculation of the correlation coefficient, categorical variables 'pathologic T' and 'clinical T' were converted to numerical variables by using a progressive numbering. In TCGA, we observed a significant inverse correlation between KLK3 gene expression and the number of positive LNs, c-stage (cT), p-stage (pT), and Gleason score (GS) (Table 2). In the Decipher®, in addition to LNI, stages and GS, a significant correlation between KLK3 and preoperative cPSA, biochemical recurrence (BCR), metastases (MET), time to MET, PCa-related specific mortality (PCSM) and time to PCSM was also observed (Table 3). Thereof, we focused on the Decipher® dataset and further investigated the correlation and predictive value of KLK3 expression on PCa progression/adverse outcomes. Descriptive characteristics of the entire cohort are summarized in Suppl. Table 1. Prostate tissue-KLK3 expression levels were significantly lower among patients with LNI, BCR, MET and for those who died from PCa (Table 4A,b). At UVA logistic regression analysis, KLK3 expression emerged to be associated with BCR, MET and PCSM, while no significant association was observed with nodal status (Table 5). The discriminative ability of each individual UVA model was graphically displayed with ROC curves (Fig. 3A); the calculated AUC were comparable for Gleason Grade Group (GGG), pT and KLK3 expression and were higher than the AUC obtained for preoperative cPSA for each of the analyzed outcome. At MVA logistic regression analysis, KLK3 expression emerged as a significant predictor of MET and PCSM (Table 6). Likewise, ROC curves and AUC were calculated for both MVA models (i.e., with and without KLK3); hence, adding KLK3 expression as a covariate improved the model performance in terms of MET and PCSM prediction, while no effect was observed on BCR (Suppl. Fig. 2). Decision-curve-analysis showed the net benefit of including KLK3 expression to predict PCSM in comparison to not including KLK3 gene expression (Fig. 3B). Finally, we highlighted an inverse correlation between KLK3 mRNA and several collagens including COL1A1 (Suppl. Table 2).

**PSA inhibits PCa invasion in vitro**

We questioned whether PSA action on different stromal substrates, including COL1A1, may affect PCa cell escape from the primary tumor site toward the future metastatic niche. In this context, as an index of local invasion, we considered PCa cell movement through the ECM...
Table 2

Correlation of prostate KLK3 mRNA expression and preoperative circulating serum PSA levels to clinical parameters in patients with primary prostate tumors from the TCGA dataset (n = 497).

|           | r KLK3 | Preop. cPSA | p KLK3 | Preop. cPSA | n KLK3 | Preop. cPSA |
|-----------|--------|-------------|--------|-------------|--------|-------------|
| KLK3      | 0.0119 | 1/0         | 0.3453 | 0/0         | 0.034  | 0/0         |

Abbreviations: cPSA: circulating serum PSA; LN: lymph nodes; H&E: hematoxylin and eosin; numeric pathologic T: numeric pathologic T stage; numeric clinical T: numeric clinical T stage.

Table 3

Correlation of prostate KLK3 mRNA expression to clinical parameters in tumors from Decipher® (n = 6577).

|           | r KLK3 | Preop. cPSA | p KLK3 | Preop. cPSA | n KLK3 | Preop. cPSA |
|-----------|--------|-------------|--------|-------------|--------|-------------|
| pathgs p  | 0.1409 | 0.1211      | 6.12E-26| 7.77E-12    | 6076   | 3861        |
| pathgs s  | 0.0918 | 0.0467      | 1.32E-10| 2.58E-01    | 6076   | 3861        |
| preop cpsa| 0.0470 | 1.0000      | 2.51E-02| 0.00E+00    | 5994   | 5994        |
| age       | 0.0636 | 0.0005      | 1.00E+00| 0.06E+01    | 6577   | 4347        |
| svi       | 0.1784 | 0.2177      | 2.71E-44| 1.13E-46    | 6365   | 4242        |
| epe       | 0.0747 | 0.1269      | 3.32E-07| 1.02E-16    | 6384   | 4252        |
| sm        | 0.0102 | 0.0772      | 1.00E+00| 3.84E-07    | 6487   | 4310        |
| lni       | 0.0106 | 0.1928      | 5.65E-14| 3.12E-34    | 5863   | 3975        |
| clings p  | 0.0167 | 0.2017      | 4.40E-11| 3.72E-16    | 2000   | 1600        |
| clings s  | 0.0184 | 0.1426      | 1.40E-04| 1.04E+08    | 2000   | 1600        |
| bcr       | 0.1980 | 0.1278      | 1.45E-13| 9.06E-07    | 1626   | 1468        |
| bcr time  | 0.0312 | -0.1098     | 1.00E+00| 3.33E-05    | 1578   | 1422        |
| met       | 0.0163 | 0.1206      | 2.00E-09| 3.66E-06    | 1626   | 1468        |
| met time  | 0.0290 | 0.1164      | 1.23E-12| 2.62E-05    | 1447   | 1298        |
| psa       | 0.0231 | 0.0427      | 3.05E-15| 1.24E-01    | 1315   | 1297        |
| capra s   | 0.0690 | 0.0535      | 1.00E+00| 2.40E-06    | 1062   | 1062        |
| with      | 0.0231 | 0.1935      | 5.15E-14| 6.53E-12    | 1261   | 1239        |
| rt        | 0.0736 | 0.0994      | 1.00E+00| 7.80E-01    | 910    | 888         |
| rt s      | 0.0053 | 0.0340      | 1.00E+00| 1.20E-01    | 1446   | 1421        |
| KLK3      | 0.9900 | 0.0070      | 1.00E+00| 2.73E-04    | 8626   | 5994        |
| TotalPathGS| 0.7072 | 0.1186      | 0.05E-40| 1.44E-13    | 6076   | 3861        |
| TotalClinGS| 0.7678 | 0.2256      | 1.63E-15| 6.64E-20    | 2000   | 1600        |
| Numeric p  | 0.8111 | 0.1692      | 1.14E-45| 1.13E-26    | 6034   | 3939        |
| Numeric c  | 0.9614 | 0.1793      | 2.82E-02| 1.04E-09    | 1277   | 1143        |

Abbreviations: bpathgs p: primary pathological Gleason score; pathgs s: secondary pathological Gleason score; preop_psa: preoperative serum PSA; svi: seminal venular invasion; epe: extra prostatic extension; sm: surgical margins; lni: lymph node invasion; clings p: primary clinical Gleason score; clings s: secondary clinical Gleason score; bcr: biochemical recurrence (0=no; 1=yes); bcr time: time from RP to biochemical recurrence; met: metastasis (0=no; 1=yes); met time: time from RP to metastasis; psa: prostate specific antigen (0=no; 1=yes); psa time: time from RP to psa; capra s: CAPRA-S nomogram score; adt: androgen deprivation therapy received (0=no; 1=yes); rt: radiotherapy received (0=no; 1=yes); rt a: adjuvant radiation received (0=no; 1=yes); rt s: salvage radiation received (0=no; 1=yes); r t a: adjuvant rt received (0=no; 1=yes); r t s: salvage rt received (0=no; 1=yes). PathGS: pathological Gleason score; ClinGS: clinical Gleason score; pstage: pathological tumor stage; cstage: clinical tumor stage.
tPSA protein expression and low-to-null COL1A1 expression, paralleled human PCa specimens, demonstrating a correspondence between higher migration and invasion [26–28]. KLK3, in fact, we showed a decreased expression of KLK3 in tumors with low tPSA levels (Suppl. Table 4), which further supports the relevance of tPSA content on the structure and function of the ECM in dictating disease progression. Whether these correlations are causative, direct, or indirect deserves to be elucidated in future studies; however, our in vitro activity tests clearly suggest PSA does actively participate into this ECM-remodeling.

In parallel, we demonstrated that PSA hampers invasive abilities of PCa cells throughout both human prostate-derived ECM and Matrigel supports. This is true for both endogenous PSA (inferred by the comparison between the PSA- PCa3 and the PSA+ MDAca2b cells) and exogenous PSA levels (inferred by the comparison between vehicle- and PSA-treated cell lines), thus excluding possible misinterpretations due to the different PCa cell phenotype. This seems counterintuitive observation, where PSA suppresses tumor cell migration even though it is cleaving ECM sustrates, becomes understandable if we consider the complexity of the ECM composition and the multiplicity of the signals arising from the tumor microenvironment. COL1A1, is, in fact, not only a direct, or indirect deserves to be elucidated in future studies; however, our in vitro activity tests clearly suggest PSA does actively participate into this ECM-remodeling.

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Here, PSA was found to inhibit the invasive ability of RWPE-2 cells toward the osteoblasts (Suppl. Fig. 3A,B) and toward the conditioned medium from osteoblasts (Suppl. Fig. 3C). Also in this model, PC3 depicted greater ability to invade than MDApc2b cells (Suppl. Fig. 3D, E). In order to get insight into the mechanisms linking tPSA levels/activity, the altered COL1A1 expression/cleavage and the impaired PCa cell invasive ability, we looked at the β1-integrin, which is the main collagen 1 transducing receptor regulating cancer cell adhesion, migration and invasion [26–28]. By western blot analyses we found decreased levels of β1-integrin in correspondence of higher tPSA levels and COL1A1 cleavage or lower COL1A1 levels (Suppl. Table 4), which further supports the relevance of tPSA content on the structure and function of the ECM in dictating disease progression. Whether these correlations are causative, direct, or indirect deserves to be elucidated in future studies; however, our in vitro activity tests clearly suggest PSA does actively participate into this ECM-remodeling.

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Discussion

Current findings provide novel evidence that COL1A1 is an additional tissue-substrate for PSA. As for in vivo tPSA activity, we utilized human PCa specimens, demonstrating a correspondence between higher tPSA protein expression and low-to-null COL1A1 expression, paralleled by high COL1A1 cleavage; this envisages PSA per se is enzymatically active in the tumor milieu. Notably, PSA activity measured in prostate tissue has been previously reported to be inversely correlated to tumor aggressiveness and PCa cell invasive abilities [16,29–31]. In addition, our dataset analysis highlighted a consistent inverse correlation between KLK3 and several types of collagens, including COL1A1. The inverse correlation among KLK3 expression and such a broad range of collagens, together with additional PSA-substrates (as IGFBP-3, r = -0.376 p = 3.35E-18; nidogen 1, r = -0.335 p = 1.66E-14; laminin, r = -0.345 p = 3.28E-15; fibronectin, 1 r = -0.154 p = 7.7E-4;) suggests a more than a marginal action of PSA in the tumor microenvironment. By employing a ‘guilt by association’ approach [32], we indeed observed a consistent and consisting enrichment in pathways related to cell movement, invasion, matrix remodeling, and TGF-beta signaling among patients with low KLK3 levels (Suppl. Table 4), which further supports the relevance of tPSA content on the structure and function of the ECM in dictating disease progression. Whether these correlations are causative, direct, or indirect deserves to be elucidated in future studies; however, our in vitro activity tests clearly suggest PSA does actively participate into this ECM-remodeling.
also demonstrated that adding tPSA in the model eventually improved the ability to predict PCSM of Gleason-grade and pT. In both datasets and our human samples, tPSA did not parallel cPSA levels. In contrast, this limit is, however, mitigated by the additional analysis of the 2 PCa specimens (25 specimens) processed and analyzed in this study.

The presence of higher PSA levels. As regard to the limits of this study, it must be mentioned that the use of PCa tissues rather than PCa-derived ECM did not allow us to associate the extent of PSA spillover in the tumor stroma to the extent of COL1A1 expression/cleavage. However, the grade and stage of our biobank specimens infer the brake of the basal membrane with the consequent leakage of PSA and tumor cell invasion. This is true also for the dataset of PCa tissues rather than PCa-derived ECM did not allow us to associate.

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**Table 4B**

Descriptive statistics between patients’ groups stratified according to the different outcomes (MET, PCSM).

|                  | No MET | MET | p      | No PCSM | PCSM | p      |
|------------------|--------|-----|--------|---------|------|--------|
| **No. patients (%)** | 382 (65.5) | 201 (34.5) |        | 494 (84.7) | 89 (15.3) | < 0.001 |
| **KLG3**<br>Median (IQR) | 3.8 (3.3–4.2) | 3.5 (2.9–4.0) | < 0.001 | 3.8 (3.3–4.1) | 3.1 (2.7–3.8) | < 0.001 |
| GGG no. (%) |        |        |        |        |       |        |
| < 2             | 211 (55.2) | 38 (18.9) | < 0.001 | 237 (48.0) | 12 (13.5) | < 0.001 |
| 3               | 68 (17.4) | 39 (19.4) |        | 92 (18.6) | 15 (16.9) |        |
| 4               | 42 (11.0) | 30 (14.9) |        | 60 (12.1) | 12 (13.5) |        |
| 5               | 61 (16.0) | 94 (46.8) |        | 105 (21.3) | 50 (56.2) |        |
| **pT stage**   |        |        |        |        |       |        |
| pT2             | 181 (47.4) | 50 (24.9) | < 0.001 | 208 (42.1) | 23 (25.8) | < 0.001 |
| pT3a            | 135 (35.3) | 61 (30.3) |        | 171 (34.6) | 25 (28.1) |        |
| pT3b            | 66 (17.3) | 90 (44.8) |        | 115 (23.3) | 41 (46.1) |        |
| **Preop. cPSA (ng/ml)** |        |        | 0.5 |        | 8.8 (5.8–13.8) | 9.1 (6.1–15.0) | < 0.001 |
| Median (IQR)    | 8.7 (5.8–13.5) | 9.3 (6.1–15.0) |        | 8.8 (5.8–13.8) | 9.1 (6.1–15.0) | < 0.001 |
| **LNI no. (%)** |        |        |        |        |       |        |
| No              | 346 (90.6) | 143 (71.1) | < 0.001 | 427 (86.4) | 62 (69.7) | < 0.001 |
| Yes             | 36 (9.4) | 58 (28.9) |        | 67 (13.6) | 27 (30.3) |        |
| **BCR no. (%)** |        |        |        |        |       |        |
| No              | 235 (61.5) | 1 (0.5) | < 0.001 | 236 (47.8) | 0 (0.0) | < 0.001 |
| Yes             | 147 (38.5) | 200 (99.5) |        | 258 (52.2) | 89 (100.0) | < 0.001 |
| **Time to BCR (months)** |        |        |        |        |       |        |
| Median (IQR)    | 72.0 (50.7–112.8) | 13.3 (12.0–30.8) | < 0.001 | 60 (25.7–101.2) | 12 (12.0–24.0) | < 0.001 |
| **Metastasis no. (%)** |        |        |        |        |       |        |
| No              | 382 (100.0) | 112 (55.7) | < 0.001 | – | – | – |
| Yes             | 0 (0.0) | 89 (44.3) |        | – | – | – |
| **Time to PCSM (months)** |        |        |        |        |       |        |
| Median (IQR)    | 96.0 (69.5–131.6) | 84.0 (56.3–123.5) | 0.003 | – | – | – |

Statistical test: Kruskal-Wallis; significance level set: 0.05.

Metastases (MET) and Prostate Cancer Specific Mortality (PCSM) were coded as categorical variables with two levels (0 or 1). Data regarding time to the specific outcome were also obtained. **Abbreviations:** LNI (lymph node involvement at surgery); BCR (Biochemical recurrence); PCSM (Prostate cancer specific mortality).

**Table 5**

UVA logistic regression analysis (Decipher®).

|          | BCR OR (95% CI) | p    | Metastasis OR (95% CI) | p    | PCSM OR (95% CI) | p     |
|----------|----------------|------|------------------------|------|-----------------|-------|
| **KLG3** | 0.75 (0.6–0.91) | 0.006 | 0.61 (0.49–0.74) | < 0.001 | 0.52 (0.41–0.66) | < 0.001 |
| **PSA pre** | 1 (0.99–1.01) | 0.958 | 1.01 (0.99–1.02) | 0.296 | 1.01 (0.99–1.02) | 0.44 |
| **pT stage**<br>Median (IQR) |        |        |        |        |       |        |
| pT2      | 1.86 (1.27–2.75) | 0.002 | 1.64 (1.06–2.53) | 0.027 | 1.64 (0.72–2.43) | 0.027 |
| pT3a     | 4.08 (2.6–6.5) | < 0.001 | 4.94 (3.18–7.67) | < 0.001 | 4.94 (1.86–5.71) | < 0.001 |
| GGG      | 1.3 (0.83–2.06) | 0.257 | 1.3 (1.89–5.39) | 0.257 | 1.3 (1.46–7.27) | 0.257 |
| GGGG     | 1.25 (0.74–2.13) | 0.408 | 1.25 (2.21–7.12) | 0.408 | 1.25 (1.68–9.32) | 0.408 |
| GGGG     | 2.81 (1.82–4.4) | < 0.001 | 2.81 (3.38–13.86) | < 0.001 | 2.81 (4.96–19.18) | < 0.001 |

**Abbreviations:** BCR (Biochemical recurrence); PCSM (Prostate cancer specific mortality).

...they were inversely associated. In particular, a more precise correspondence of tPSA over cPSA levels with the aggressiveness of the disease did emerge.

To understand the inverse correlations between tissue KLG3/PSA and tumor pathological features it is important to recall, first, that PSA is not a tumor specific marker but, rather, an organ specific marker (almost exclusively expressed by the prostate gland). Indeed, the increase of PSA in the blood does not necessarily indicate the presence of malignant prostate tumors. Second, it must be considered that PSA is a differentiation marker and PSA tumors are, in their nature, more aggressive than PSA+ positive tumors [15, 36]. This also explains, at least in part, the association of lower PSA levels to higher Gleason grade. Third, prostate cancer cells have been reported to express equal or even lower levels of PSA compared to normal prostate epithelial cells [15, 37–40]. In line with this, we also observed lower PSA expression in the tumor surgical resections compared to the non-tumor resections (Suppl. Fig. 4).
Fig. 3. ROC curves analysis (A) and Decision Curve Analysis (B) on Decipher® dataset. (A) ROC curves analysis demonstrating the discriminative ability of a univariable model for KLK3 expression, preoperative PSA blood levels (PSA), pathological T stage (pT stage) and Gleason grade group (GGG) to predict biochemical recurrence (BCR), development of metastasis (MET) and PCa specific mortality (PCSM); (B) Decision curve analysis (DCA) demonstrating the net benefit associated with use of KLK3 expression on MVA models to predict PCSM in comparison to MVA without KLK3 expression.

Table 6
MVA model with KLK3 (Decipher®).

|         | BCR OR (95% CI) | p-value | Metastasis OR (95% CI) | p-value | PCSM OR (95% CI) | p-value |
|---------|-----------------|---------|------------------------|---------|-----------------|---------|
| KLK3    | 0.85 (0.69–1.06) | 0.154   | 0.72 (0.57–0.91)       | 0.007   | 0.6 (0.46–0.78) | < 0.001 |
| PSA     | 1 (0.99–1.02)   | 0.983   | 1.01 (1.1–1.03)        | 0.205   | 0.6 (0.46–0.78) | 0.327   |
| pT stage|                 |         |                        |         |                 |         |
| pT2     | Ref (-)         |         |                        |         |                 |         |
| pT3a    | 1.79 (1.2–2.66) | 0.004   | 1.53 (0.95–2.46)       | 0.079   | 0.6 (0.46–0.78) | 0.619   |
| pT3b    | 3.48 (2.19–5.6) | < 0.001 | 3.92 (2.42–6.41)       | < 0.001 | 1.01 (0.99–1.02) | 0.014   |
| GGG     |                 |         |                        |         |                 |         |
| GGG < 2 | Ref (-)         |         |                        |         |                 |         |
| GGG3    | 1.13 (0.71–1.82) | 0.605   | 2.91 (1.69–5.06)       | < 0.001 | 0.6 (0.46–0.78) | 0.01    |
| GGG4    | 1.18 (0.68–2.05) | 0.561   | 3.88 (2.1–7.18)        | < 0.001 | 1.01 (0.99–1.02) | 0.005   |
| GGG5    | 2.2 (1.39–3.5)  | 0.001   | 6.86 (4.22–11.36)      | < 0.001 | 1.18 (0.62–2.24) | < 0.001 |

Abbreviations: BCR (Biochemical recurrence); PCSM (Prostate cancer specific mortality).
Finally, our findings demonstrating an anti-metastatic role of PSA, also help to understand the inverse correlation between tPSA and tumor progression.

The reliability of our findings is supported by Stege and colleagues, who were able to demonstrate the superiority of bioptic tPSA (rather than the cPSA) in predicting the outcome of endocrine treatment in metastatic PCa patients [41]. In addition, Bonk and colleagues recently assessed the prognostic/diagnostic utility of PSA-immunostaining by analyzing large tissue microarrays; they found a link between reduced PSA staining intensity with unfavorable tumor phenotype and poor prognosis [35]. However, it must be noticed that both methods as well as the outcome of our study are not of immediate clinical translation as they are based on subjective, semiquantitative assessments of PSA thresholds. Future studies should aim to establish reproducible cut-offs, which must be defined according to specific clinical/pathological parameters [42]. Regardless of the feasibility to immediately translate...
tPSA quantification to the clinical practice, current findings are of major relevance since they clarified at least one of the mechanisms underneath the correlation between higher tPSA levels and a lower tumor malignancy, with all its clinical implications. Moreover, having recognized a protective role of tPSA over the steps of PCa invasion may also have important speculative implications in terms of future choice or refinement of neoadjuvant treatments.

Conclusions

Together with previous evidences [34,37], our data demonstrate that tPSA content and its activity in the tumor microenvironment are inversely correlated to cPSA and to tumor progression. Likewise, we provide novel evidence that tPSA can cleave also COL1A1, one of the most abundant ECM proteins, and interfere with the integrin-mediated matrisome signaling, thus being potentially able to control cells path through the ECM and their migration ability. Moreover, we demonstrated that tPSA levels improve PCSM prediction, which is of high clinical relevance for its consequences in terms of tailored treatment choices. Therefore, a deeper knowledge of PSA role throughout PCa pathobiology is relevant to make the most of this biomarker in terms of disease prognosis.

CRediT authorship contribution statement

Francesco Pellegrino: Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. Arianna Coghi: Investigation, Visualization. Giovanni Lavorgna: Formal analysis, Investigation, Methodology, Validation, Visualization. Walter Cazzaniga: Formal analysis, Investigation, Methodology, Visualization. Edoardo Guazzoni: Investigation, Methodology. Irene Locatelli: Investigation, Project administration, Validation, Visualization. Isabella Villa: Investigation, Methodology. Simona Bolamperi: Investigation, Validation. Nadia Finocchio: Visualization. Massimo Alfano: Methodology. Roberta Luciano: Investigation, Methodology. Alberto Briganti: Validation. Francesco Montorsi: Resources. Andrea Salonia: Conceptualization, Resources, Project administration, Resources, Writing – review & editing. Ilaria Cavarretta: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

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