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Research Paper

Improved diagnostic performance of a phage display antibody-assisted intact free PSA assay as used in the four kallikrein concept applied to the IMPROD/multi-IMPROD study

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ABSTRACT

Introduction: Multi-kallikrein immunoassays are widely used to overcome the limitations of PSA in identifying potentially aggressive prostate cancer. In this study, we report the diagnostic performance of a mutant antibody-assisted assay for intact free PSA (iPSA) in combination with the commonly used four-kallikrein approach.
Methods: Immunoassays for total PSA (T), free PSA (F), iPSA using wild-type (I-W) or mutant 4D4 antibody (I-MC), total hK2 (T-hK2), and free hK2 (F-hK2) were used to assess perioperative plasma samples (n = 310). The individual parameters alone, in different ratios or in the four-kallikrein combinations were specifically analyzed in patients with prostate gland volume lower or equal and above the median (38 mL).
Results: In the low prostate gland volume group, T and the two hK2s alone provided superior separation of the two groups over all other parameters. The ratio of calculated nicked PSA (F-I) to T using I-MC separated the two groups significantly. In the higher gland volume group, calculated nicked/T using both I-MC and I-W provided identical separations superior to the conventional F/T ratio. In the whole material, F/T and CN/T ratios performed similarly superseding any single parameter.
In the four-kallikrein Logistic Regression analysis, the I-MC (AUC, 0.75) provides clearly better results than I-W (AUC, 0.71) in the group of lower gland volumes. In terms of Odds Ratios, I-MC overall provides improvements over I-W in the whole cohort and the two groups.
Conclusions: The I-MC assay demonstrates modest but clear improvement in differentiating benign and low-grade prostate cancer from clinically significant cancers, particularly in patients with less than median gland volume. This is an effect of the mutant antibody and its improved affinity enabling an alternative assay format providing robust and accurate analytical test performance.

1. Introduction

Prostate-specific antigen (PSA) remains an extensively used biomarker for prostate cancer, despite widely recognized limitations such as low diagnostic accuracy and overdiagnosis of indolent disease. A major line of improvement on the diagnostic performance of PSA is the

Abbreviations: PSA, Prostate-specific antigen; iPSA or I, Intact PSA; tPSA or T, Total PSA; fPSA or F, Free PSA; T-hK2, Total human glandular kallikrein 2; F-hK2, Free human glandular kallikrein 2; nPSA or N, Nicked PSA; CN, Calculated nPSA; Mab, Monoclonal antibody; GS, Gleason score; Wt, Wild-type; AUC, Area under the curve; PV, Prostate gland volume.

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measurement of sub-forms of PSA or combining it with the other prostate-specific human glandular kallikrein (hK2). Prostate Health Index (*PHI*) from Beckman Coulter and a four-kallikrein panel, 4Kscore, from OPKO Health are presently widely used.

The discovery of free PSA in the early nineties to complement the measurement of total PSA is a cornerstone for improving the performance in distinguishing between the age-associated increases in benign hyperplastic conditions and cancer [1]. Further molecular heterogeneity of circulating free PSA is shown with a unique monoclonal antibody (Mab 4D4), which does not recognize PSA with cleavage at Lys145/Lys146 [2]. The iPSA assay, which uses Mab 4D4 for detection with an fPSA-specific capture antibody (5A10), measures any circulating fPSA isoforms that lack the Lys145/Lys146 cleavage. It detects both precursor forms of PSA (proPSA) and mature PSA not complexed with serpins [3,4]. The internally cleaved form or nicked free PSA (nPSA) concentrations have been calculated by subtracting iPSA concentrations from fPSA concentrations, which has the strongest relationship with prostate gland volume (PV) [5]. iPSA has become an established part both in the four kallikrein investigational concepts as well as the commercial 4Kscore [6,7].

Several limitations of the 4D4 antibody have been addressed in previous work [8], such as a high off-rate dissociation causing decreased assay sensitivity, linearity, and stability in the low standard range. Following the cloning of the 4D4 antibody, a mutant library was created utilizing the phage display technique. Several 4D4 mutants with better affinity and lower off-rate dissociation were identified while maintaining the original iPSA specificity. The mutant L3-2 Fab in optimized versions of the iPSA assay yielded several-fold improvements of the detection limit compared with wild-type 4D4 Fab. Using the mutant L3-2 Fab as tracer, as blocker or for capturing, several new assay constructs for iPSA and internally nicked PSA were compared to the original wild-type assisted iPSA assay using a small cohort of samples with a suspicion of harboring prostate cancer. The results overall suggested improved diagnostic performance of the new constructs, especially when the biotinylated mutant 4D4 was used for capturing together with Mab 5A10 as the tracer.

The aim of this study was to expand the clinical cohort for evaluation of the I-MC assay when mutant 4D4 used as the capture antibody relative to the original iPSA (I-W) reference assays widely in clinical use. As variation of free PSA is most evident in differently sized gland volumes, we specially sought to evaluate the performance in two subsets of patients based on the gland volumes ($\leq$ or $>$ median volumes). In our previous study [9] iPSA was compared to free and total PSA alone. In this study we compared the diagnostic performance also to two assay constructs of hK2, "total hK2" which is the standard component of the multi-kallikrein approach or 4KScore but also to the free from of hK2 [10,11].

2. Materials and methods

2.1. Clinical samples

Plasma samples (n = 310) were prospectively obtained from a cohort of 61 male patients (registered IMPROD trial, ClinicalTrials.gov Identifier NCT01864135) and a cohort of 249 male patients ages from 18 to 85 years between 2015 and 2018, designed to evaluate the accuracy of MRI and biomarkers in the diagnosis of prostate cancer (registered Multi-IMPROD trial, ClinicalTrials.gov Identifier NCT02241122) as previously described [9,12,13]. The study included men with clinical suspicion of prostate cancer due to serum PSA higher than 2.5 ng/mL or abnormal digital rectal examination. After signed informed consent, all participants underwent anatomical magnetic resonance imaging (MRI) and diffusion weighted imaging at 1.5/3 Tesla magnetic field using surface coils to non-invasively predict the presence or absence of prostate cancer followed by TRUS-guided biopsy. All patients had systematic biopsies (6 + 6), and in case of a suspected lesion in MRI, two cognitively targeted biopsies were obtained from the lesions for biomarker research. All samples were stored at − 80 °C.

2.2. The antibodies, reagents, and instrumentation

Monoclonal antibodies of the different assays have been described elsewhere [2,5,8,10,14–18] and were labeled with Europium chelate or biotinylated as previously described [11]. Streptavidin-coated microtiter plates, assay buffer and wash buffer concentrate were from Kaivogen, Turku, Finland. Enhancement solution was prepared at the Department of Biotechnology, University of Turku, Finland as described previously [8]. Time-resolved fluorescence signal from Europium was measured with Victor 1420 multilabel counter was from Perkin-Elmer Life Sciences, Wallac, Turku, Finland.

2.3. Assay procedures

The tPSA (T) and fPSA (F) concentrations were measured using in-house immunoassays as described previously [10]. Total-hK2 (T-hK2) and Free-hK2 (F-hK2) assays were performed as described previously [10]. The two iPSA (I-W and I-MC) assays were performed as described previously [8]. Calculated nPSA (CN) was obtained by subtracting the I-W and I-MC concentrations from total fPSA concentration as described elsewhere [19].

2.4. Statistical analysis

In patients' characteristics (Table 1), median and interquartile range (first quartile and third quartile) are presented for age, PSA, and prostate volume. ISUP Gleason Grade Group [20] is provided as frequency and proportion using the whole cohort. For the analyses, the patient's Gleason score (GS) was used as the "ground truth" and dichotomized into benign/GS = 6 and GS $\geq$ 7.

To evaluate the ability of each variable in detecting GS $\geq$ 7, the area under the ROC (receiver operating characteristic) curve (AUC) with the corresponding 95 % confidence intervals (CI) was computed using the DeLong method [21,22]. In addition, the Mann-Whitney *U* test was performed to assess the statistical significance of the difference between benign/GS = 6 and GS $\geq$ 7.

In order to analyze the combination of variables, logistic regression models were fitted. The results of the analyses are presented with odds ratios (OR), 95 % CI, and p-value. The prediction performance of a model was evaluated by computing the mean AUC and Standard Deviation (SD) over hold-out cross-validation repeated 10,000 times. The hold-out cross-validation consisted in randomly splitting the data into 70 % for training the model and 30 % for testing.

The data analysis was performed using Python v. 3.6. Specifically, the Python Statsmodels module [23] was used for the statistical and logistic regression analyses. Hold-out cross-validation was implemented using Scikit-learn v. 0.20.0 [24]. Results with a p-value < 0.05 are reported as significant. However, when performing multiple comparisons, the Bonferroni correction (0.05/ number of comparisons) is also considered to determine the significance level.

3. Results

Table 1 summarizes information about the patient cohort of the 163 Benign and low-grade cancer (Benign/GS = 6) and 147 clinically significant cancer (GS $\geq$ 7) patients. Of the measured parameters only tPSA significantly discriminated the two groups, with T-hK2 and I-MC trending towards significance. Calculated nicked PSA based on the two iPSA constructs significantly and equally separated the two groups, whereas measured fPSA did not. The mutant-based iPSA measured lower concentrations than the reference iPSA assay in both groups. All the calculated nPSA ratios to tPSA including the F/T ratio as well as all the proportion of iPSA to fPSA separated the 2 groups highly significantly (p

Table 1
Clinical characteristics, Median (interquartile range Q1 – Q3) and p-Values of different plasma PSA forms and ratios (n = 310).

Parameter	Benign/GS = 6 n = 163	GS ≥ 7 n = 147	p-Value
Age, years	62 (57, 67)	67 (61, 70)	<0.0001[b]
T, ng/mL	8.45 (6.06, 11.9)	10.16 (7.87, 13.77)	0.0003[b]
F, ng/mL	1.19 (0.76, 1.83)	1.09 (0.75, 1.55)	0.2224
I-W, ng/mL	0.55 (0.38, 0.76)	0.57 (0.38, 0.78)	0.6211
I-MC, ng/mL	0.40 (0.27, 0.60)	0.44 (0.31, 0.65)	0.0667
T-hK2, ng/mL	0.09 (0.06, 0.16)	0.11 (0.07, 0.17)	0.0659
F-hK2, ng/mL	0.04 (0.03, 0.07)	0.05 (0.04, 0.07)	0.1053
CN(I-W), ng/mL	0.60 (0.32, 1.2)	0.50 (0.29, 0.79)	0.0106[a]
CN(I-MC), ng/mL	0.77 (0.46, 1.42)	0.65 (0.39, 1.01)	0.0164[a]
F/T	0.15 (0.11, 0.2)	0.11 (0.08, 0.14)	<0.0001[b]
CN(I-W)/T	0.08 (0.05, 0.12)	0.05 (0.03, 0.07)	<0.0001[b]
CN(I-MC)/T	0.1 (0.07, 0.13)	0.06 (0.04, 0.09)	<0.0001[b]
I-W/F	0.44 (0.36, 0.58)	0.53 (0.44, 0.64)	<0.0001[b]
I-MC/F	0.34 (0.26, 0.41)	0.41 (0.34, 0.50)	<0.0001[b]
[c]Gleason Grade Group, n (%)			
Benign	115 (37)		
1 (GS 3 + 3)	48 (16)		
2 (GS 3 + 4)	66 (21)		
3 (GS 4 + 3)	36 (12)		
4 (GS 4 + 4, 3 + 5, 5 + 3)	32 (10)		
5 (GS 4 + 5, 5 + 4)	13 (4)		

GS, Gleason score.
[a] Significance at the 0.05 level.
[b] Significance at the Bonferroni-adjusted 0.004 level.
[c] ISUP Gleason Grade Group [20].

< 0.0001).

Table 2 shows the results of ROC analyses of Benign/GS = 6 vs GS ≥ 7 for single parameters and their ratios in the whole cohort (n = 310) and subgroups with PV lower or equal (n = 157) and higher (n = 153) than median (38 mL). In the low PV group, measured tPSA and the two hK2 assays provided superior separation of the two groups (AUC 0.69–0.71, p < 0.0001). Of the free PSA forms, I-MC provided the best separation (AUC 0.67, p = 0.0002) clearly superseding that of fPSA (AUC 0.61). Of the calculated ratios, those based on I-MC provided the best separations (AUC 0.63–0.65, p = 0.006 and 0.002) whereas those based on the I-W did not reach significance. In the high PV group, the separation provided by tPSA was clearly inferior to that of the low PV group and the two hK2 assays provided no discrimination of the two groups. Likewise, the two measured iPSA constructs provided no separation, whereas the calculated nicked concentrations provided modest but significant separation (AUC 0.60). The CN/T ratios for both iPSA constructs however provided the best and identical separation (AUC 0.71, p < 0.0001) of the two groups. AUC provided by F/T was 0.67 (p = 0.0004).

We further analyzed a logistic regression model of tPSA, fPSA and T-hK2 in combination with either I-W or I-MC as described in Materials and Methods with the whole cohort and the two gland volume-based cohorts. In the whole cohort, when comparing Benign/GS = 6 with GS ≥ 7, the corresponding AUCs were 0.74 and 0.76 (Fig. 1a) thus a modest improvement was obtained with I-MC. The contribution of I-MC (OR, 33.25; p < 0.001) to the model was highly increased compared to I-W (OR, 4.66; p = 0.009) (Table 3).

In the low PV group, corresponding AUCs were 0.71 to 0.75 (Fig. 1b). Whereas the contribution to the model by I-W did not reach significance (OR, 2.30; p = 0.334), the odds ratio for I-MC was highly increased reaching significance (p = 0.011) (Table 3). In the high PV group, the AUCs of I-W and I-MC were 0.71 and 0.72 (Fig. 1c). Both I-W and I-MC contributed significantly to the model (OR, 6.37; p = 0.028 and OR, 19.06; p = 0.008) (Table 3). Of note in the high-volume group, is the lack of contribution by T-hK2 to the model.

The three ROC curves are shown in Fig. 1. It is evident that the improvement provided by the I-MC assay instead of the original wild-type 4D4 based reference iPSA assay is overall modest in the whole cohort and in the group with higher gland volumes. However, in the group with PV less than the median, the mutant 4D4 I-MC assay construct provides a clear improvement. At an 80 percent specificity, the sensitivity was increased by 10 percent to 52 percent.

4. Discussion

In this study we have evaluated a technically improved iPSA assay construct centrally utilizing a recombinant antibody fragment of the original 4D4 Mab that was subjected to directed mutagenesis with the

Table 2
AUCs and p-Values of different plasma PSA forms and ratios with Benign/GS = 6 vs GS ≥ 7, PV ≤ 38 mL and PV > 38 mL groups.

Parameter	Benign/GS = 6 (n = 163) vs GS ≥ 7 (n = 147)		Benign/GS = 6 (n = 67) vs GS ≥ 7 (n = 90) PV ≤ 38 mL		Benign/GS = 6 (n = 96) vs GS ≥ 7 (n = 57) PV > 38 mL	
	AUC (95 % CI)	p-Value	AUC (95 % CI)	p-Value	AUC (95 % CI)	p-Value
Age	0.65 (0.59–0.71)	<0.0001[b]	0.69 (0.61–0.77)	<0.0001[b]	0.67 (0.58–0.76)	0.001[b]
PV			0.54 (0.45–0.63)	0.359	0.59 (0.50–0.68)	0.061
T	0.62 (0.56–0.68)	0.0003[b]	0.70 (0.61–0.79)	<0.0001[b]	0.60 (0.51–0.69)	0.039[a]
F	0.54 (0.48–0.60)	0.2224	0.61 (0.52–0.70)	0.017[a]	0.56 (0.46–0.66)	0.230
I-W	0.52 (0.46–0.58)	0.6211	0.62 (0.53–0.71)	0.010[a]	0.50 (0.40–0.60)	0.991
I-MC	0.56 (0.50–0.62)	0.0667	0.67 (0.58–0.76)	0.0002[b]	0.55 (0.45–0.65)	0.293
T-hK2	0.56 (0.50–0.62)	0.0659	0.69 (0.61–0.77)	<0.0001[b]	0.53 (0.43–0.63)	0.603
F-hK2	0.55 (0.49–0.61)	0.1053	0.71 (0.63–0.79)	<0.0001[b]	0.51 (0.41–0.61)	0.768
CN(I-W)	0.58 (0.52–0.64)	0.0106[a]	0.56 (0.47–0.65)	0.169	0.60 (0.51–0.69)	0.032[a]
CN(I-MC)	0.58 (0.52–0.64)	0.0164[a]	0.56 (0.47–0.65)	0.239	0.60 (0.51–0.69)	0.047[a]
F/T	0.69 (0.63–0.75)	<0.0001[b]	0.61 (0.52–0.70)	0.019[a]	0.67 (0.58–0.76)	0.0004[b]
CN(I-W)/T	0.69 (0.63–0.75)	<0.0001[b]	0.57 (0.47–0.67)	0.156	0.71 (0.63–0.79)	<0.0001[b]
CN(I-MC)/T	0.71 (0.65–0.77)	<0.0001[b]	0.63 (0.54–0.72)	0.006[a]	0.71 (0.62–0.80)	<0.0001[b]
I-W/F	0.63 (0.57–0.69)	<0.0001[b]	0.53 (0.43–0.63)	0.508	0.65 (0.56–0.74)	0.002[b]
I-MC/F	0.68 (0.62–0.74)	<0.0001[b]	0.65 (0.56–0.74)	0.002[b]	0.66 (0.57–0.75)	0.001[b]

[a] Significance at the 0.05 level.
[b] Significance at the Bonferroni-adjusted 0.004 level.

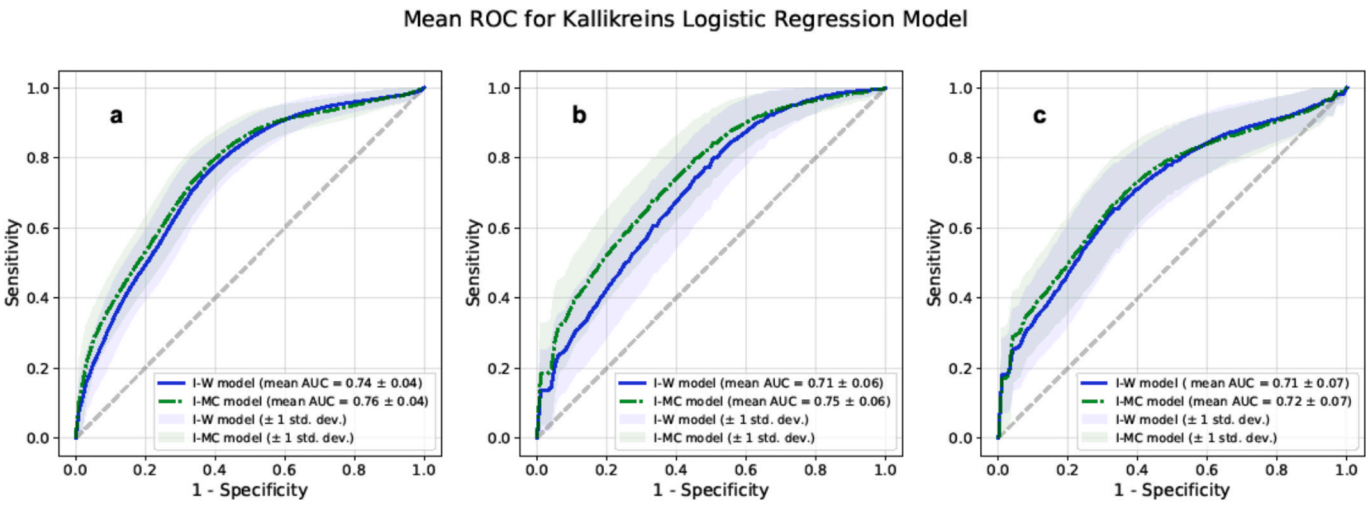

Fig. 1. Mean ROC curves and mean AUC obtained by performing holdout cross-validation (30 % test and 70 % training) 10,000 times to assess the two Kallikreins Logistic Regression models. a) whole cohort b) gland volume ≤ 38 mL and c) gland volume > 38 mL.

Table 3
Logistic Regression model analysis with Benign/GS = 6 vs GS ≥ 7, PV ≤ 38 mL and PV > 38 mL groups.

Parameter	Benign/GS = 6 (n = 163) vs GS ≥ 7 (n = 147)			Benign/GS = 6 (n = 67) vs GS ≥ 7 (n = 90) PV ≤ 38 mL			Benign/GS = 6 (n = 96) vs GS ≥ 7 (n = 57) PV > 38 mL		
	OR (95 % CI)	p-Value	AUC (SD)[b]	OR (95 % CI)	p-Value	AUC (SD)[b]	OR (95 % CI)	p-Value	AUC (SD)[b]
Intercept	0.20 (0.10–0.41)	<0.001[a]		0.18 (0.07–0.47)	0.001[a]		0.16 (0.05–0.47)	0.001[a]	
T	1.28 (1.17–1.39)	<0.001[a]		1.24 (1.09–1.41)	0.001[a]		1.26 (1.11–1.42)	<0.001[a]	
F	0.13 (0.07–0.27)	<0.001[a]		0.24 (0.07–0.84)	0.026[a]		0.16 (0.06–0.39)	<0.001[a]	
T-hK2	526.20 (9.49–29166.56)	0.002[a]		51170.06 (35.74—7.33e + 07)	0.003[a]		71.87 (0.38–13530. 44)	0.110	
I-W	4.66 (1.46–14.92)	0.009[a]		2.30 (0.42 – 12.50)	0.334		6.37 (1.23–33.04)	0.028[a]	
Model + I-W			**0.74 (0.04)**			**0.71 (0.06)**			**0.71 (0.07)**
Intercept	0.18 (0.09–0.37)	<0.001[a]		0.14 (0.05–0.38)	<0.001[a]		0.15 (0.05–0.45)	0.001[a]	
T	1.27 (1.16–1.38)	<0.001[a]		1.23 (1.08–1.41)	0.002[a]		1.23 (1.09–1.40)	0.001[a]	
F	0.10 (0.05–0.20)	<0.001[a]		0.11 (0.03–0.43)	0.002[a]		0.15 (0.06–0.37)	<0.001[a]	
T-hK2	264.58 (4.65–15046.60)	0.007[a]		15052.77 (7.67–2.95e + 07)	0.013[a]		76.01 (0.50–11584.81)	0.091	
I-MC	33.25 (5.36–206.44)	<0.001[a]		83.65 (2.72–2572.00)	0.011[a]		19.06 (2.17–167.45)	0.008[a]	
Model + I-MC			**0.76 (0.04)**			**0.75 (0.06)**			**0.72 (0.07)**

model = T + F + T-hK2.
[a] Significance at the 0.05 level.
[b] Holdout Cross-Validation Mean AUC (SD).

intention to solve certain observed technical insufficiencies such as a high off-rate dissociation causing decreased assay sensitivity, linearity, and stability in the low standard range regarding its use in the original assay using 4D4 Mab. The phage display generated mutant antibody fragment decisively solved these problems and also enabled a superior assay design as the site specifically biotinylated Fab fragment was used as a highly effective capture. From a previous evaluation we selected the most promising assay construct tested, using the L3-2 mutant for capturing with Mab 5A10 as a tracer. In this study we expanded the sample cohort to 310 patient samples (163 benign/GS = 6 and 147 GS ≥ 7) and set out to validate the new I-MC assay in a logistic regression model consisting of tPSA, fPSA, hK2 and iPSA.

Our results with the whole cohort show modest improvement using the mutant based iPSA assays over reference iPSA (AUC 0.76 vs 0.74). In the group of patients with gland volumes above 38 mL, the two assays performed highly similarly. In contrast, the mutant based iPSA performed clearly better than the reference iPSA (AUC 0.75 vs 0.71) in the group with gland volumes ≤ 38 mL both in a univariate comparison as well as in the logistic regression model. We suggest that this is a consequence of the enhanced accuracy and precision made possible by the better binding characteristics we originally sought to achieve by the directed mutation efforts performed to the binding cleft of wild-type 4D4 antibody and the use of *in vivo* and site- specifically biotinylated Fab fragment for capturing of iPSA.

In contrast to our previous study [9] we also evaluated the performance of a free hK2 assay concept [10] in the model instead of the total hK2 used in the four-kallikrein model. The two hK2 assay constructs perform in a highly identical manner. Of note is that both hK2 constructs perform in an excellent way in the low gland volume group but very poorly in the group with gland volumes above median. Nevertheless, the contribution to the whole cohort of the total hK2 assay still remains very clear.

This study highlights a promising potential of the novel I-MC assay to enhance the accuracy of prostate cancer diagnosis, particularly in patients with smaller prostate volumes. Although the mutant antibody still is equal to the original 4D4 Mab regarding its active site (binding to a

site in the one-chain PSA being absent in internally cleaved PSA, the form being elevated particularly in high volume glands), it enables the design of a new assay concept. The modest but clear improvement in the ≤ 38 mL gland volume groups we interpret as a consequence of the improved overall robustness and test accuracy enabled by the enhanced affinity compared to the original wild-type antibody. The development of the I-MC assay highlights its potential for integration into diagnostic multi-marker /multi-kallikrein algorithms for prostate cancer.

5. Conclusions

The iPSA assay construct using 4D4 L3-2 mutant as a capture offers an improvement in separating the group of benign and low-grade cancers from clinically significant cancers especially in patients with lower prostate gland volumes. This is an effect of the mutant antibody enabling an alternative assay format providing more robust and accurate analytical test performance.

CRediT authorship contribution statement

Md. Ferdhos L. Khan: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ileana M. Perez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation. **Henna Kekki:** Writing – review & editing, Methodology, Investigation, Data curation. **Pekka Taimen:** Writing – review & editing, Resources. **Peter J. Boström:** Writing – review & editing, Resources, Investigation. **Ivan Jambor:** Writing – review & editing, Resources. **Otto Ettala:** Writing – review & editing, Resources. **Tapio Pahikkala:** Writing – review & editing, Formal analysis. **Kim Pettersson:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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