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Clinical significance of the LacdiNAc-glycosylated prostate-specific antigen assay for prostate cancer detection

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Abstract

To reduce unnecessary prostate biopsies (Pbx), better discrimination is needed. To identify clinically significant prostate cancer (CSPC) we determined the performance of LacdiNAc-glycosylated prostate-specific antigen (LDN-PSA) and LDN-PSA normalized by prostate volume (LDN-PSAD). We retrospectively measured LDN-PSA, total PSA (tPSA), and free PSA/tPSA (F/T PSA) values in 718 men who underwent a Pbx in 3 academic urology clinics in Japan and Canada (Pbx cohort) and in 174 PC patients who subsequently underwent radical prostatectomy in Australia (preop-PSA cohort). The assays were evaluated using the area under the receiver operating characteristics curve (AUC) and decision curve analyses to discriminate CSPC. In the Pbx cohort, LDN-PSAD (AUC 0.860) provided significantly better clinical performance for discriminating CSPC compared with LDN-PSA (AUC 0.827, $P = 0.0024$), PSAD (AUC 0.809, $P < 0.0001$), tPSA (AUC 0.712, $P < 0.0001$), and F/T PSA (AUC 0.661, $P < 0.0001$). The

Abbreviations: β4GALNT3, β1,4 N-acetylgalactosyltransferase 3; β4GALNT4, β1,4 N-acetylgalactosyltransferase 4; ACTB, β-actin; ASPC, active surveillance eligible prostate cancer; AUC, area under receiver operating characteristic curve; CSPC, clinically significant prostate cancer; ddPCR, Droplet Digital PCR; DRE, digital rectal examination; EPI, epithelial cells in urine; FFPE, formalin-fixed paraffin-embedded; F/T PSA, free PSA/total PSA; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; GS, Gleason Score; HGPC, high grade prostate cancer; IQR, interquartile range; LDN, LacdiNAc, GalNAcβ1-4GlcNAc; LDN-PSAD, LDN-PSA normalized by prostate volume; LDN-PSA, LacdiNAc-glycosylated prostate-specific antigen; LVI, lymphovascular invasion; MiPS, Mi-Prostate Score; Pbx, prostate biopsy; PC, prostate cancer; PCA3, prostate cancer antigen 3 gene; PNI, perineural invasion; preop-PSA, preoperative PSA baseline; PRIAS, Prostate Cancer Research International Active Surveillance; PSA, prostate-specific antigen; pT, pathological stage; RM, resection margin; ROC, receiver operating characteristics curve; RP, radical prostatectomy; SPFS, surface plasmon field-enhanced fluorescence spectroscopy; SV, seminal invasion; tPSA, total PSA.

decision curve analysis showed that using a risk threshold of 20% and adding LDN-PSA and LDN-PSAD to the base model (age, digital rectal examination status, tPSA, and F/T PSA) permitted avoidance of even more biopsies without missing CSPC (9.89% and 18.11%, respectively vs 2.23% [base model]). In the preop-PSA cohort, LDN-PSA values positively correlated with tumor volume and tPSA and were significantly higher in pT3, pathological Gleason score ≥ 7. Limitations include limited sample size, retrospective nature, and no family history information prior to biopsy. LacdiNAc-glycosylated PSA is significantly better than the conventional PSA test in identifying patients with CSPC. This study was approved by the ethics committee of each institution ("The Study about Carbohydrate Structure Change in Urological Disease"; approval no. 2014-195).

KEYWORDS
biomarker, clinically significant prostate cancer, LacdiNAc, *N*-glycan, prostate-specific antigen

1 | INTRODUCTION

In a large subpopulation, clinically localized low-grade PC will remain indolent over the patient's lifetime[1,2]; consequently, the most important issues resulting from PC screening are overdiagnosis and overtreatment.[3,4] Several randomized clinical trials have strongly suggested that intermediate- to high-risk cancers with GS of 7-10 benefit from aggressive therapy, such as radiotherapy or RP, by reducing mortality.[5-8] Active surveillance is proposed for low-risk PC patients who meet the PRIAS criteria, 42%-80% of active surveillance patients experience a GS upgrade after RP[9-12]; therefore, the most efficient early detection strategy for PC would be to identify CSPC inexpensively before MRI to more effectively triage those men needing to proceed to Pbx.

Several assays provide prognostic information for HGPC (GS ≥ 7) at Pbx, such as the serum assays (Prostate Health Index and 4Kscore),[13-15] the DRE urine genetic tests (PCA3 and SelectMDx),[16] the tPSA plus urinary PCA3 tests (MiPS),[17] and first catch urine genetic test (EPI).[18] The reported AUC to evaluate the accuracy of predicting HGPC (GS ≥ 7) of these 6 assays ranged from 0.730 to 0.870, outperforming tPSA which has an AUC of 0.718.[13,19,20]

We previously established an SPFS-based immunoassay system to detect PC-associated nonreducing terminal LacdiNAc (LDN, GalNAcβ1-4GlcNAc) structure carrying LDN-PSA in serum[21,22] (Figure S1). A previous training cohort study on tPSA ≤ 20 ng/mL at initial Pbx (n = 442) reported that the diagnostic performance of LDN-PSA (AUC 0.795) outperformed that of tPSA (AUC 0.718).[20] In the present study, we retrospectively evaluated the diagnostic performance and clinical significance of LDN-PSA and LDN-PSAD in a Pbx multi-institutional cohort and in a single institutional preop-PSA cohort.

2 | MATERIALS AND METHODS

2.1 | Study design and assessments

A flow diagram of this observational study is shown in Figure 1. We evaluated the diagnostic performance of LDN-PSA and LDN-PSAD, and compared their performance with that of tPSA, F/T PSA, and PSAD in determining overall PC, CSPC, and HGPC at Pbx. A Pbx cohort enrolled 718 patients who received a Pbx at Hirosaki University (Hirosaki, Japan), Tohoku University (Sendai, Japan), or McMaster University (Hamilton, Canada) between June 2010 and August 2017. Eligible participants comprised men ≥ 40 years old who received Pbx. Men with a history of invasive treatment for prostatic hyperplasia or who were taking medication that had an effect on tPSA levels 6 months before serum collection were excluded. Histopathology for the Pbx cohort was reviewed by a histopathologist at each institution blinded to each patient's LDN-PSA status. Active surveillance eligible prostate cancer was defined according to PRIAS criteria (tPSA < 10 ng/mL, PSAD ≤ 0.2, Pbx GS 3 + 3, or clinical stage 2b or lower). We also evaluated the correlation between preoperative LDN-PSA value and several prognostic factors including tumor volume, pT, GS, PNI status, LVI status, SV status, and RM status in the preop-PSA cohort. A preop-PSA cohort enrolled 174 patients with PC who underwent RP at Royal Brisbane and Women's Hospital (Brisbane, Australia) between January 2010 and January 2015. Histopathology for the RP cohort was reviewed centrally by a histopathologist blinded to each patient's LDN-PSA status. All serum samples were stored at −80°C until use. Furthermore, 17 FFPE prostate sections obtained from patients who underwent RP at Hirosaki University were used to evaluate the levels of LDN-PSA and LDN-glycan synthesis-related glycosyltransferase gene expression in tissues. This study was carried out in accordance with the ethical standards of the Declaration of Helsinki and was approved by the ethics committee of each institution ("The Study about Carbohydrate Structure Change in Urological Disease"; approval no. 2014-195). Informed consent was obtained from all patients.

2.2 | LacdiNAc-glycosylated PSA and LDN-PSAD tests

Serum LDN-PSA (mU/mL) was measured using SPFS-based immunoassay system as previously described.[20] LDN-PSAD (mU/mL/cm^3) was

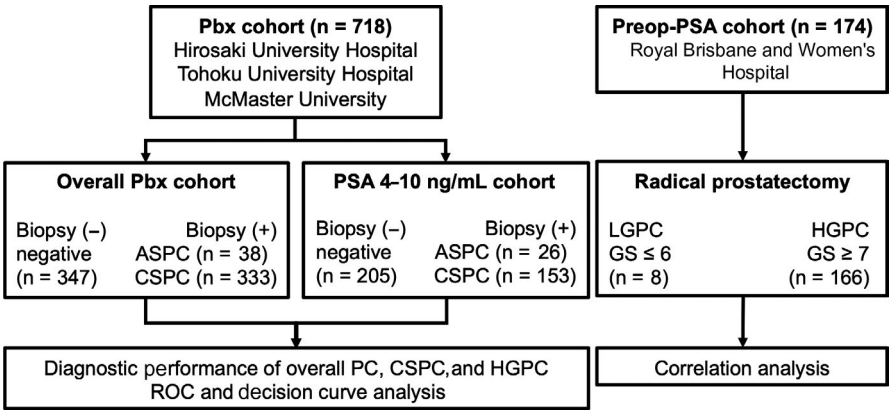


FIGURE 1 Flow diagram of this retrospective observational study of a prostate biopsy (Pbx) cohort of 718 patients with biopsy negative (no prostate cancer [PC]) or biopsy positive PC who underwent Pbx at Hirosaki University (Hirosaki, Japan), Tohoku University (Sendai, Japan), or McMaster University (Hamilton, Canada) between June 2010 and August 2017. Of those with PC (n = 371), 38 were classified as the active surveillance-eligible PCa (ASPC) group according to Prostate Cancer Research International Active Surveillance criteria, and the remaining 333 PC patients were classified as having clinically significant PC (CSPC). A preoperative prostate-specific antigen baseline (preop-PSA) cohort enrolled 174 patients with PC who underwent radical prostatectomy at the Royal Brisbane and Women's Hospital (Brisbane, Australia) between January 2010 and January 2015. GS, Gleason Score, HGPC, high grade PC; LGPC, low grade PC; ROC, receiver operating characteristic

calculated by dividing the LDN-PSA value by the prostate volume, as measured by transrectal ultrasonography. Serum tPSA and fPSA were measured using Architect i1000 (Abbott Japan, Tokyo, Japan).

2.3 | Quantification of *β4GALNT3* and *β4GALNT4* expression and LDN-PSA FFPE prostate benign and tumor tissues

Total RNA and total protein were extracted from benign tissue and each Gleason pattern of tumor tissue that was macrodissected from 20-μm thickness FFPE prostate section in 17 patients who underwent radical prostatectomy at Hirosaki University. Total RNA from FFPE tissue was extracted using Pure Link FFPE RNA isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was synthesized from 0.5 μg total RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer's instructions. All reagents and equipment used for ddPCR were from Bio-Rad Laboratories (Hercules, CA, USA). cDNA (10 ng) was mixed with 10 μL of 2× ddPCR Supermix for probes (No dUTP) (Bio-Rad Laboratories), 1 μL 20× target primers/probe mix (FAM) (Bio-rad Laboratories) or 20× reference primers/probe (HEX) (Bio-Rad Laboratories) and nuclease-free water to a total reaction volume of 20 μL. The entire reaction mix was then loaded into a sample well of a DG8 cartridge for the QX200/QX100 droplet generator. Then 70 μL droplet generation oil was added for probes into the oil wells of the cartridge, according to the QX200/QX100 droplet generator instruction manual. After droplet generation, the droplets were transferred to a 96-well plate and sealed. Thermal cycling was carried out on the droplets using the Veriti Thermal Cycler (Thermo Fisher Scientific) according to the following protocol: enzyme activation at 95°C for 10 minutes, denaturation at 94°C for 30 seconds, followed by annealing/extension at 60°C for 1 minute (40 cycles), enzyme deactivation at 98°C for 10 minutes, followed by hold at 4°C. The ramp rate was set at 2°C/s, the heated lid to 105°C and the sample volume at 40 μL. After thermal cycling, the absolute gene expression level per well for the probes and reference genes were determined using a QX200/QX100 droplet reader and quantitated using QuantaSoft software (Bio-Rad Laboratories). For analysis of the gene expression data, we assumed a normal distribution. The gene expression values (absolute copy number) for each sample were normalized to the housekeeping gene *ACTB*. The PCR probes for human *β4GALNT3* (unique assay ID: dHsaCPE5056467), human *β4GALNT4* (unique assay ID: dHsaCPE5027332), and human β-actin (*ACTB*) (unique assay ID: dHsaCPE5190200) were purchased from the PrimePCR ddPCR Expression Probe Assay (Bio-Rad). Total protein from FFPE tissue was extracted by using the Formalin Fixed Paraffin Embedded Protein Isolation Kit (ITSI-Biosciences, Johnstown, PA, USA). To eliminate SDS, total protein solution was further treated by using SDS-eliminant reagent (ATTO, Tokyo, Japan). The LDN-PSA (mU/mL) of SDS-free protein solution from each tissue was measured using an SPFS-based immunoassay system as previously described.[20] Total PSA levels were measured using Architect i1000 (Abbott Japan, Tokyo, Japan).

2.4 | Statistical analyses

All statistical calculations were undertaken using GraphPad Prism 8 (GraphPad, San Diego, CA, USA), XLSTAT-Biomed (Addinsoft, New York, NY, USA), and R software version 3.5.2 (R Foundation for Statistical Computing; available on: http//www.r-project.org/). For non-normally distributed model, the Mann-Whitney *U* test was used to analyze intergroup differences and the Kruskal-Wallis test was used to analyze multiple group differences. The predictive accuracy was

quantified as the area under the ROC curves. The clinical net benefit of the diagnostic base model, which included age, tPSA, DRE status, and F/T PSA, with and without prostate volume, LDN-PSA, or LDN-PSAD for prediction of overall PC, CSPC, and HGPC in the Pbx cohort was evaluated by decision curve analysis.[23] To prove the significance of LDN-PSA or LDN-PSAD, multivariate logistic regression analysis calculations were carried out using XLSTAT-Biomed (Addinsoft) (Document S1). To evaluate the correlations between LDN-PSA and tPSA, F/T PSA, and tumor volume in the preop-PSA cohort, a correlation coefficient was analyzed using the nonparametric Spearman's rank order correlation test. $P < 0.05$ was considered statistically significant.

3 | RESULTS

The characteristics of the Pbx cohort (n = 718) and 384 patients belonging to the subgroup with 4-10 ng/mL tPSA are shown in Table 1. Of those with PC (n = 371), 38 cases, all with GS 6, were classified as ASPC and the remaining 333 cases were classified as CSPC. Of these, 19 (5.7%) had GS 6, 145 (43.4%) had GS 7, and 169 (50.6%) had GS ≥ 8. The age was significantly different in biopsy negative vs CSPC ($P < 0.0001$), but not significantly different in biopsy negative vs ASPC ($P = 0.319$) and ASPC vs CSPC ($P = 0.178$). Digital rectal examination status and the levels of prostate volume, LDN-PSA, and LDN-PSAD were significantly different in biopsy negative vs CSPC (all $P < 0.0001$) and ASPC vs CSPC (all $P < 0.0001$) but not significantly different in biopsy negative vs ASPC ($P = 0.450$, $P = 0.306$, $P = 0.361$, $P = 0.800$, respectively). The tPSA and PSAD levels were significantly different in biopsy negative vs ASPC ($P < 0.0001$), biopsy negative vs CSPC ($P < 0.0001$) and ASPC vs CSPC ($P < 0.0001$). The F/T PSA level was significantly different in biopsy negative vs ASPC ($P = 0.009$) and biopsy negative vs CSPC ($P < 0.0001$), but not significantly different in ASPC vs CSPC ($P = 0.301$).

The characteristics of the Pbx cohort belonging to the subgroup with 4-10 ng/mL tPSA (n = 384) are shown in Table 1. Out of the 179 patients with PC, 26 patients, all with GS 6 were in the ASPC group. Out of the 153 patients with CSPC, 9 (5.9%) had GS 6, 90 (58.8%) had GS 7, and 54 (35.3%) had GS ≥ 8. The age was significantly different in biopsy negative vs CSPC ($P = 0.005$), but not significantly different in biopsy negative vs ASPC ($P = 0.155$) and ASPC vs CSPC ($P = 0.988$). The DRE status, prostate volume, and levels of LDN-PSA and LDN-PSAD were significantly different in biopsy negative vs CSPC (all $P < 0.0001$) and ASPC vs CSPC ($P = 0.009$, $P < 0.0001$, $P = 0.002$, and $P < 0.0001$, respectively) but not significantly different in biopsy negative vs ASPC ($P = 0.570$, $P = 0.186$, $P = 0.068$, and $P = 0.612$, respectively). The tPSA level was significantly different in biopsy negative vs ASPC ($P = 0.010$) and ASPC vs CSPC ($P = 0.001$), but not significantly different in biopsy negative vs CSPC ($P = 0.074$). The F/T PSA level was significantly different in biopsy negative vs CSPC ($P < 0.0001$) and biopsy negative vs ASPC ($P = 0.036$), but not significantly different in ASPC vs CSPC ($P = 0.954$). The PSAD level was significantly different in biopsy negative vs ASPC ($P = 0.008$), biopsy negative vs CSPC ($P < 0.0001$) and ASPC vs CSPC ($P < 0.0001$).

In the Pbx cohort, LDN-PSAD levels in CSPC (median, 5.58 mU/mL/cm^3, [interquartile range (IQR) 3.10-13.70]) and LDN-PSA levels in CSPC (median, 150.7 mU/mL [89.6-326.6]) were significantly higher than those in biopsy negative men (median, 1.70 mU/mL/cm^3 [1.12-2.58] and median, 67.2 mU/mL [50.5-91.0], respectively) and ASPC (median, 1.78 mU/mL/cm^3 [1.77-2.80] and median, 76.7 mU/mL [56.5-90.1], respectively), whereas F/T PSA could not clearly discriminate ASPC from CSPC (Figure 2A, Table 1). The AUC of the LDN-PSAD for discriminating overall PC (AUC 0.825; 95% confidence interval [CI], 0.795-0.856) provided significantly better clinical performance compared with LDN-PSA (AUC 0.801; 95% CI, 0.769-0.832, $P = 0.0026$), tPSA (AUC 0.654; 95% CI, 0.615-0.694, $P < 0.0001$), F/T PSA (AUC 0.668; 95% CI, 0.629-0.707, $P < 0.0001$), and PSAD (AUC 0.745; 95% CI, 0.709-0.781, $P < 0.0001$) (Figure 2B, Table 2), and the AUC of LDN-PSAD for discriminating CSPC (AUC 0.860; 95% CI, 0.830-0.890) was significantly higher than those of LDN-PSA (AUC 0.827; 95% CI, 0.795-0.860; $P = 0.0024$), tPSA (AUC 0.712; 95% CI, 0.673-0.752, $P < 0.0001$), F/T PSA (AUC 0.661; 95% CI, 0.618-0.703, $P < 0.0001$), and PSAD (AUC 0.809; 95% CI, 0.776-0.842, $P < 0.0001$) (Figure 2B, Table 2). Furthermore, the AUC of LDN-PSAD for discriminating HGPC (0.857; 95% CI, 0.826-0.889) showed significantly better performance compared with LDN-PSA (AUC 0.823; 95% CI, 0.789-0.858, $P = 0.0016$), PSAD (AUC 0.798; 95% CI, 0.762-0.834, $P < 0.0001$), tPSA (AUC 0.699; 95% CI, 0.657-0.741, $P < 0.0001$), and F/T PSA (AUC 0.657; 95% CI, 0.613-0.701, $P < 0.0001$). At a preset 90% sensitivity, the specificities of LDN-PSAD to detect overall PC, CSPC, and HGPC (41.2%, 62.9% and 61.1%, respectively) and LDN-PSA (40.6%, 48.6%, and 49.3%, respectively) were much higher than those of tPSA (21.6%, 27.0%, and 25.5%, respectively), and F/T PSA (25.9%, 28.3%, and 27.7%, respectively), and higher than those of PSAD (31.1%, 44.6%, and 46.8%, respectively) (Table 2).

In the PSA gray zone cohort (subgroup of patients with 4-10 ng/mL tPSA), LDN-PSAD levels of CSPC (median, 4.42 mU/mL/cm^3 [IQR 2.53-6.39]) and LDN-PSA levels of CSPC (median, 104.2 mU/mL [78.0-173.1]) were significantly higher than those in biopsy negative men (median, 1.64 mU/mL/cm^3 [1.12-2.55] and median, 66.2 mU/mL [50.4-86.3], respectively) and ASPC (median, 1.96 mU/mL/cm^3 [1.38-2.92] and median, 81.5 mU/mL [61.4-96.6], respectively), whereas tPSA and F/T PSA could not clearly discriminate ASPC from CSPC and/or biopsy negative (Figure 2C, Table 1). The AUC of the LDN-PSAD for discriminating overall PC (AUC 0.780; 95% CI, 0.731-0.829) provided significantly better clinical performance compared with LDN-PSA (AUC 0.747; 95% CI, 0.695-0.799, $P = 0.047$), tPSA (AUC 0.524; 95% CI, 0.462-0.586, $P < 0.0001$), F/T PSA (AUC 0.627; 95% CI, 0.567-0.686, $P < 0.0001$), and PSAD (AUC 0.682; 95% CI, 0.624-0.732, $P < 0.0001$) (Figure 2D, Table 2). The AUC of LDN-PSAD for discriminating CSPC (AUC 0.820; 95% CI, 0.771-0.870) was significantly higher than those of LDN-PSA (AUC 0.761; 95% CI, 0.705-0.817, $P = 0.0006$), tPSA (AUC 0.572; 95% CI, 0.506-0.638, $P < 0.0001$), F/T PSA (AUC 0.613; 95% CI, 0.548-0.678, $P < 0.0001$), and PSAD (AUC 0.754; 95% CI, 0.698-0.810, $P = 0.0011$) (Figure 2D, Table 2). Furthermore, LDN-PSAD for discriminating HGPC also had

TABLE 1 Characteristics of 718 men who underwent a prostate biopsy and a subgroup of 384 men with 4-10 ng/mL total prostate-specific antigen (tPSA)

Biopsy outcome	Negative (a)	ASPC (b)	CSPC (c)	*P* value		
All (n = 718)	**(n = 347)**	**(n = 38)**	**(n = 333)**	**a vs b**	**a vs c**	**b vs c**
Median age (IQR)	66 (61.0-72.0)	67 (64.5-73.3)	70 (65.0-74.0)	0.319	<0.0001	0.178
DRE status normal/abnormal	303/44	33/5	178/156	0.450	<0.0001	0.0001
Median P vol., cm^3 (IQR)	40.1 (28.4-53.1)	41.8 (33.8-47.4)	27.1 (20.2-36.9)	0.306	<0.0001	<0.0001
Median tPSA, ng/mL (IQR)	6.38 (4.67-9.31)	4.51 (4.67-9.31)	10 (6.42-15.59)	<0.0001	<0.0001	<0.0001
Median F/T PSA, % (IQR)	25.9 (16.9-38.5)	17.3 (14.9-29.4)	17.7 (11.6-26.5)	0.009	<0.0001	0.301
Median PSAD, ng/mL/cm^3 (IQR)	0.17 (0.10-0.25)	0.11 (0.09-0.16)	0.36 (0.22-0.66)	<0.0001	<0.0001	<0.0001
Median LDN-PSA, mU/mL (IQR)	67.2 (50.5-91.0)	76.7 (56.5-90.1)	150.7 (89.6-326.6)	0.361	<0.0001	<0.0001
Median LDN-PSAD, mU/mL/cm^3 (IQR)	1.70 (1.12-2.58)	1.78 (1.77-2.80)	5.58 (3.10-13.70)	0.800	<0.0001	<0.0001
Clinical T stage		n (%)	n (%)			
1c		32 (84.2)	172 (51.5)			
2a		5 (13.2)	47 (14.1)			
2b		1 (2.6)	36 (10.8)			
2c-3		0 (0.0)	73 (21.9)			
4		0 (0.0)	5 (1.5)			
Prostate biopsy GS sum		n (%)	n (%)			
GS 6		38 (100.0)	19 (5.7)			
GS 7		0 (0.0)	145 (43.4)			
GS 8		0 (0.0)	45 (13.5)			
GS 9		0 (0.0)	117 (35.0)			
GS 10		0 (0.0)	7 (2.1)			
Biopsy outcome	**Negative (a)**	**ASPC (b)**	**CSPC (c)**	**P value**		
PSA4–10 (n = 384)	**(n = 205)**	**(n = 26)**	**(n = 153)**	**a vs b**	**a vs c**	**b vs c**
Median age (IQR)	66 (61.0-71.0)	67.5 (65.0-73.8)	68 (63.0-73.0)	0.155	0.005	0.988
DRE status normal/abnormal	183/22	22/4	96/57	0.570	<0.0001	0.009
Median P vol., cm^3 (IQR)	39.2 (30.6-52.2)	45.0 (35.5-50.0)	26.0 (20.0-36.8)	0.186	<0.0001	<0.0001
Median tPSA, ng/mL (IQR)	6.16 (5.15-7.56)	5.15 (4.49-6.47)	6.60 (5.27-8.30)	0.010	0.074	0.001
Median F/T PSA, % (IQR)	24.7 (16.7-35.6)	17.1 (14.9-28.0)	18.4 (13.0-27.1)	0.036	<0.0001	0.954
Median PSAD, ng/mL/cm^3 (IQR)	0.16 (0.11-0.22)	0.13 (0.10-0.17)	0.24 (0.19-0.33)	0.008	<0.0001	<0.0001
Median LDN-PSA, mU/mL (IQR)	66.2 (54.0-86.3)	81.5 (61.4-96.6)	104.2 (78.0-173.1)	0.068	<0.0001	0.002
Median LDN-PSAD, mU/mL/cm^3 (IQR)	1.64 (1.12-2.55)	1.96 (1.38-2.92)	4.42 (2.53-6.39)	0.612	<0.0001	<0.0001
Clinical T stage		n (%)	n (%)			
1c		22 (84.6)	93 (60.8)			
2a		2 (7.7)	30 (19.6)			
2b		2 (7.7)	8 (5.2)			
2c-3		0 (0.0)	20 (13.1)			
Prostate biopsy GS sum		n (%)	n (%)			
GS 6		26 (100.0)	9 (5.9)			
GS 7		0 (0.0)	90 (58.8)			
GS 8		0 (0.0)	23 (15.0)			
GS 9		0 (0.0)	31 (20.3)			

ASPC, active surveillance eligible prostate cancer; CSPC, clinically significant prostate cancer; DRE, digital rectal examination; F/T PSA, free PSA/tPSA; GS, Gleason Score; IQR, interquartile range; LDN-PSA, LacdiNAc-glycosylated PSA; LDN-PSAD, LDN-PSA normalized by prostate volume; PSAD, PSA normalized by prostate volume; P vol., prostate volume.

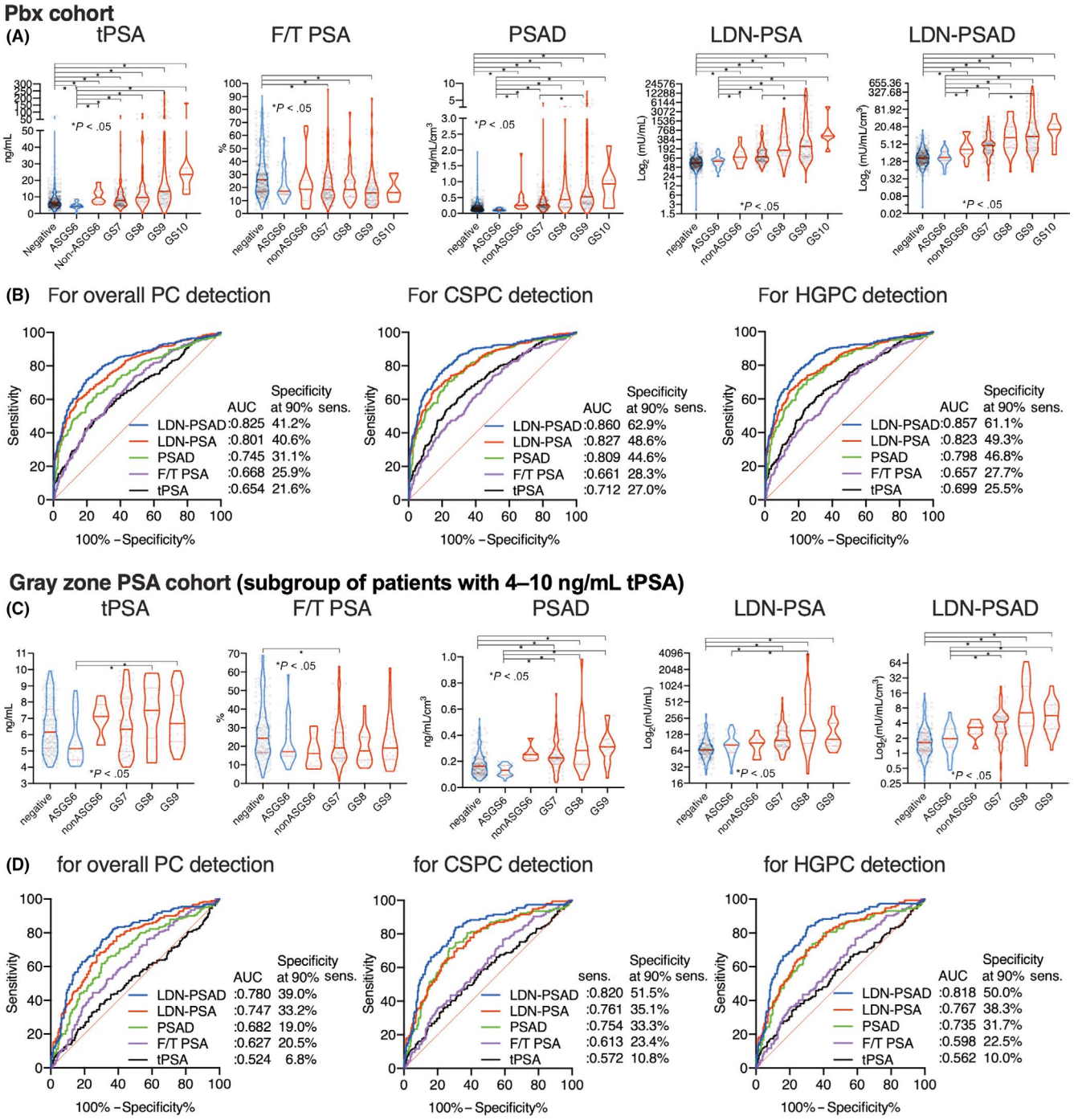


FIGURE 2 Serum levels and receiver operating characteristic (ROC) curve analysis of LacdiNAc-glycosylated prostate-specific antigen (LDN-PSA), LDN-PSA normalized by prostate volume (LDN-PSAD), total (t)PSA, free PSA/tPSA (F/T PSA) ratio, and PSAD at prostate biopsy (Pbx) in patients diagnosed with prostate cancer (PC) or not. A, Violin plot of each test in overall Pbx cohort. Each PC group was classified as active surveillance-eligible Gleason sum 6 (ASGS6), non-AS-eligible GS6 (nonASGS6), GS7, GS8, GS9, and GS10. B, Violin plot of each test in gray zone PSA cohort (subgroup of patients with 4-10 ng/mL tPSA). PC group was classified as ASGS6, non-ASGS6, GS7, GS8, and GS9. Dashed red lines outline the interquartile range (IQR) of each test value. Solid red line represents the median of each test value. Multiple group differences were analyzed using the Kruskal-Wallis test for non-normally distributed models. C, Receiver operating characteristic (ROC) curves of overall PC, clinically significant (CS) PC (except for ASGS6 PC) and high grade (HG) PC (GS ≥ 7 PC) prediction accuracy of tPSA, fPSA/tPSA (F/T PSA), PSAD, LDN-PSA, and LDN-PSAD in the overall Pbx cohort. D, ROC curves of overall PC, CSPC, and HGPC prediction accuracy of tPSA, fPSA/tPSA (F/T PSA), PSAD, LDN-PSA, and LDN-PSAD in a cohort with PSA range 4-10 ng/mL

the largest AUC (0.818; 95% CI, 0.767-0.869) and provided significantly better clinical performance compared with LDN-PSA (AUC 0.767; 95% CI, 0.710-0.824, $P = 0.0033$), tPSA (AUC 0.562; 95% CI, 0.493-0.631, $P < 0.0001$), F/T PSA (AUC 0.598; 95% CI, 0.531-0.665, $P < 0.0001$), and PSAD (AUC 0.735; 95% CI, 0.683-0.788, $P = 0.0001$). At a preset 90% sensitivity, the specificities of LDN-PSAD to detect

TABLE 2 Specificity at 90% sensitivity of each assay in 718 men who underwent a prostate biopsy and in a subgroup of 384 men with 4-10 ng/mL total prostate-specific antigen (tPSA) (PSA gray zone cohort)

Overall cohort	tPSA	F/T PSA	PSAD	LDN-PSA	LDN-PSAD
Overall PC detection					
Cut-off	4.3 ng/mL	37.90%	0.118 ng/mL/cm^3	62.0 mU/mL	1.491 mU/mL/cm^3
AUC (95% CI); *P* (vs LDN-PSAD)	0.654 (0.615-0.694); *P* < 0.0001	0.668 (0.629-0.707); *P* < 0.0001	0.745 (0.709-0.781); *P* < 0.0001	0.801 (0.769-0.832); *P* = 0.0026	0.825 (0.795-0.856)
PPV, %	55.1	56.5	58.2	61.9	62.1
NPV, %	67	70.9	74.9	79.2	79.4
Specificity, % (95% CI)	21.6 (17.3-25.9)	25.9 (21.3-30.5)	31.1 (26.3-36.0)	40.6 (35.5-45.8)	41.2 (36.0-46.4)
CSPC detection					
Cut-off	4.64 ng/mL	36.40%	0.153 ng/mL/cm^3	66.8 mU/mL	2.060 mU/mL/cm^3
AUC (95% CI); *P* (vs LDN-PSAD)	0.712 (0.673-0.752); *P* < 0.0001	0.661 (0.618-0.703); *P* < 0.0001	0.809 (0.776-0.842); *P* < 0.0001	0.827 (0.795-0.860); *P* = 0.0024	0.860 (0.830-0.890)
PPV, %	51.6	52.1	60.3	60.2	67.7
NPV, %	75.9	76.8	84.7	85	88
Specificity, % (95% CI)	27 (22.6-31.4)	28.3 (23.8-32.8)	44.6 (39.7-49.6)	48.6 (43.6-53.6)	62.9 (58.0-67.7)
HGPC detection					
Cut-off	4.60 ng/mL	36.20%	0.152 ng/mL/cm^3	68.3 mU/mL	2.084 mU/mL/cm^3
AUC (95%CI); *P* (vs LDN-PSAD)	0.699 (0.657-0.741); *P* < 0.0001	0.657 (0.613-0.701); *P* < 0.0001	0.798 (0.762-0.834); *P* < 0.0001	0.823 (0.789-0.858); *P* = 0.0016	0.857 (0.826-0.889)
PPV, %	48.5	49.2	56.7	58	64.3
NPV, %	76.9	78.3	85.5	86.5	88.8
Specificity, % (95% CI)	25.5 (21.2-29.7)	27.7 (23.4-32.1)	46.8 (41.9-51.6)	49.3 (44.4-54.1)	61.1 (56.4-65.9)
PSA gray zone cohort LDN--PSA	**tPSA - LDN-PSAD**	**F/T PSA**		**PSAD**	
Cut-off	4.42 ng/mL	37.80%	0.102 ng/mL/cm^3	57.3 mU/mL	1.375 mU/mL/cm^3
AUC (95% CI); *P* (vs LDN-PSAD)	0.524 (0.462-0.586); *P* < 0.0001	0.627 (0.567-0.686); *P* < 0.0001	0.682 (0.624-0.732); *P* < 0.0001	0.747 (0.695-0.799); *P* = 0.047	0.78 (0.731-0.829)
PPV, %	45.7	49.7	47.9	54	56.3
NPV, %	43.8	70	62.5	79.1	81.6
Specificity, % (95% CI)	6.8 (3.4-10.3)	20.5 (15.0-26.0)	19.0 (14.2-25.0)	33.2 (26.7-39.6)	39 (32.3-45.7)
CSPC detection					
Cut-off	4.51 ng/mL	36.00%	0.126 ng/mL/cm^3	59.8 mU/mL	1.710 mU/mL/cm^3
AUC (95%CI); *P* (vs LDN-PSAD)	0.572 (0.506-0.638); *P* < 0.0001	0.613 (0.548-0.678); *P* < 0.0001	0.754 (0.698-0.810); *P* = 0.0011	0.761 (0.705-0.817); *P* = 0.0006	0.820 (0.771-0.870)
PPV, %	40.1	43.8	47.1	47.9	55.2
NPV, %	62.5	78.3	82.8	84.4	88.8
Specificity, % (95% CI)	10.8 (7.4-15.5)	23.4 (18.0-28.8)	33.3 (27.3-39.4)	34.2 (28.4-40.5)	51.5 (44.7-57.5)
HGPC detection					
Cut-off	4.51 ng/mL	36.10%	0.124 ng/mL/cm^3	61.7 mU/mL	1.710 mU/mL/cm^3
AUC (95% CI); *P* (vs LDN-PSAD)	0.562 (0.493-0.631); *P* < 0.0001	0.598 (0.531-0.665); *P* < 0.0001	0.735 (0.683-0.788); *P* = 0.0001	0.767 (0.710-0.824); *P* = 0.0033	0.818 (0.767-0.869)
PPV, %	37.6	41.1	44.0	46.8	52
NPV, %	63.2	79.4	83.5	86.8	89.6
Specificity, % (95% CI)	10 (6.2-13.8)	22.5 (17.2-27.8)	31.7 (25.8-37.6)	38.3 (32.2-44.5)	50 (43.7-56.3)

AUC, area under the receiver operating characteristic curve; CI, confidence interval; CSPC, clinically significant PC; F/T PSA, free PSA/tPSA; HGPC, high grade PC; LDN-PSA, LacdiNAc-glycosylated PSA; LDN-PSAD, LDN-PSA normalized by prostate volume; NPV, negative predictive value; PC, prostate cancer; PPV, positive predictive value; PSAD, PSA normalized by prostate volume.

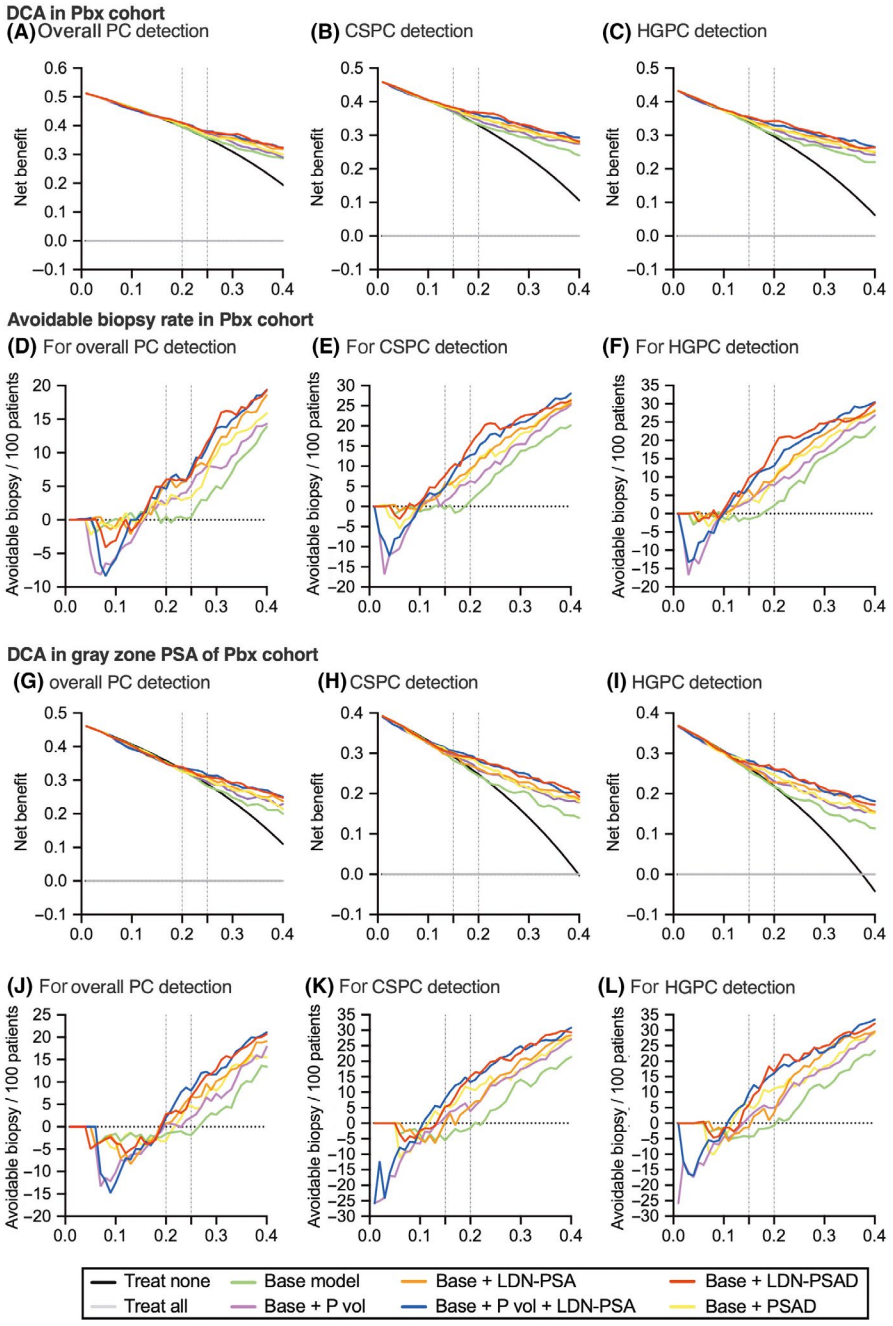


FIGURE 3 *Comparison of decision curve analyses (DCA) of net benefit for a relevant risk threshold of a base model (age + digital rectal examination status + total prostate-specific antigen [tPSA] + free PSA/tPSA [F/T] PSA), base model + prostate volume, base model + PSA normalized by prostate volume (PSAD), base model + LacdiNAc-glycosylated PSA (LDN-PSA), base model + LDN-PSA + prostate volume, and base model + LacdiNAc-glycosylated PSAD (LDN-PSAD). A,G, DCA showing net benefit for carrying out biopsy in men at risk for overall prostate cancer (PC) in 718 men who underwent a prostate biopsy (Pbx cohort) (A) and in the gray zone PSA cohort (subgroup of patients with 4–10 ng/mL tPSA) (G). B,H, DCA showing net benefit for carrying out biopsy in men at risk for clinically significant PC (CSPC) in the Pbx cohort (B) and gray zone PSA cohort (H). C,I, DCA showing net benefit for carrying out biopsy in men at risk for high grade PC (HGPC) in the Pbx cohort (C) and gray zone PSA cohort (I). D,J, Avoidable biopsy rate per 100 patients without missing overall PC in the Pbx cohort (D) and gray zone PSA cohort (J). E,K, Avoidable biopsy rate per 100 patients without missing CSPC in the Pbx cohort (E) and gray zone PSA cohort (K). F,I Avoidable biopsy rate per 100 patients without missing CSPC in the Pbx cohort (F) and gray zone PSA cohort (I). DCA plots were developed using the rmda package of R statistical software*

overall PC, CSPC, and HGPC (39.0%, 51.5%, and 50.0%, respectively) and LDN-PSA (33.2%, 34.2%, and 38.3%, respectively) were much higher than those of tPSA (6.8%, 10.8%, and 10.0%, respectively) and F/T PSA (20.5%, 23.4%, and 22.5%, respectively), and higher than those of PSAD (19.0%, 33.3%, and 31.7%, respectively) (Table 2).

Decision curve analyses predicting overall PC, CSPC, and HGPC in the Pbx cohort revealed that the base model (which included age,

TABLE 3 Net benefit and avoidable biopsies for the diagnostic model compared to the treat all strategy to biopsy every patient for different risk thresholds in a cohort of 718 men who underwent a prostate biopsy (Pbx)

		Risk threshold (%) of overall cohort						In patients with 4.0-10.0 ng/mL tPSA					
	Diagnostic model	10	15	20	25	30	35	10	15	20	25	30	35
Net benefit for detecting overall PC	Base model	0.461	0.433	0.394	0.362	0.331	0.307	0.407	0.370	0.327	0.286	0.254	0.221
	Base + P vol.	0.457	0.431	0.402	0.377	0.341	0.316	0.407	0.360	0.332	0.303	0.265	0.252
	Base + PSAD	0.46	0.43	0.4	0.37	0.35	0.33	0.4	0.37	0.32	0.3	0.27	0.25
	Base + LDN-PSA	0.462	0.432	0.406	0.374	0.359	0.337	0.404	0.362	0.333	0.306	0.279	0.261
	Base + P vol. + LDN-PSA	0.456	0.435	0.405	0.383	0.360	0.343	0.399	0.363	0.343	0.326	0.294	0.280
	Base + LDN-PSAD	0.460	0.435	0.407	0.387	0.370	0.345	0.404	0.368	0.338	0.317	0.296	0.272
Pbx avoided per 100 patients without missing overall PC	Base model	−9.74	1.07	−8.36	1.81	4.92	9.37	0.00	−0.87	−2.34	−0.52	3.91	7.96
	Base + P vol.	−5.29	−2.79	2.37	6.27	7.24	11.02	0.00	−6.60	2.22	4.43	6.34	13.65
	Base + PSAD	−1.25	−0.28	2.23	3.34	9.75	13.15	−4.95	−3.91	−3.13	4.69	8.07	13.65
	Base + LDN-PSA	−5.57	6.50	4.04	5.57	11.47	14.96	−2.34	−5.73	0.26	5.47	9.64	15.29
	Base + P vol. + LDN-PSA	−5.99	2.18	3.76	8.22	11.84	16.06	−7.03	−4.51	4.17	11.20	13.11	18.75
	Base + LDN-PSAD	−2.92	2.04	4.46	9.33	14.16	16.37	−2.34	−2.17	2.08	8.59	13.63	17.26
	Diagnostic model	10	15	20	25	30	35	10	15	20	25	30	35
Net benefit for detecting CSPC	Base model	0.404	0.368	0.335	0.315	0.288	0.268	0.332	0.285	0.245	0.212	0.183	0.161
	Base + P vol.	0.403	0.374	0.345	0.325	0.304	0.290	0.326	0.297	0.266	0.235	0.212	0.195
	Base + PSAD	0.4	0.38	0.35	0.33	0.31	0.3	0.33	0.3	0.28	0.24	0.21	0.19
	Base + LDN-PSA	0.405	0.379	0.354	0.333	0.308	0.294	0.332	0.290	0.261	0.244	0.221	0.211
	Base + P vol. + LDN-PSA	0.403	0.384	0.365	0.344	0.320	0.312	0.326	0.311	0.283	0.262	0.236	0.210
	Base + LDN-PSAD	0.405	0.385	0.375	0.341	0.323	0.303	0.330	0.301	0.292	0.257	0.241	0.220
Pbx avoided per 100 patients without missing CSPC	Base model	−0.14	−0.46	2.23	9.05	12.81	17.21	0.00	−3.91	−1.04	4.17	9.81	16.11
	Base + P vol.	−0.84	2.46	6.13	11.84	16.39	21.43	−5.21	2.78	7.29	11.20	16.75	22.36
	Base + PSAD	0.42	4.69	8.77	12.40	17.92	22.54	−3.39	5.73	11.20	12.76	16.84	21.88
	Base + LDN-PSA	1.11	5.29	9.89	14.48	17.22	22.12	0.00	−1.48	5.21	13.80	18.66	25.37
	Base + P vol. + LDN-PSA	−0.83	8.26	14.21	17.83	20.06	25.49	−5.47	10.50	14.06	19.27	22.31	25.22
	Base + LDN-PSAD	0.42	8.87	18.11	16.71	20.84	23.72	−1.56	4.86	13.54	17.71	23.35	27.39
	Diagnostic model	10	15	20	25	30	35	10	15	20	25	30	35

(Continues)

TABLE 3 (Continued)

		Risk threshold (%) of overall cohort						In patients with 4.0-10.0 ng/mL tPSA					
	Diagnostic model	10	15	20	25	30	35	10	15	20	25	30	35
Net benefit for detecting HGPC	Base model	0.375	0.335	0.303	0.285	0.265	0.237	0.306	0.259	0.217	0.191	0.155	0.143
	Base + P vol.	0.374	0.345	0.314	0.295	0.277	0.259	0.300	0.271	0.238	0.209	0.179	0.169
	Base + PSAD	0.37	0.35	0.32	0.3	0.28	0.27	0.300	0.274	0.247	0.209	0.186	0.177
	Base + LDN-PSA	0.374	0.345	0.322	0.305	0.289	0.267	0.306	0.265	0.236	0.220	0.208	0.180
	Base + P vol. + LDN-PSA	0.375	0.354	0.334	0.311	0.300	0.283	0.302	0.283	0.258	0.237	0.209	0.201
	Base + LDN-PSAD	0.374	0.356	0.343	0.318	0.297	0.269	0.3	0.28	0.27	0.24	0.21	0.18
Pbx avoided per 100 patients without missing HGPC	Base model	−0.14	−1.49	2.37	10.45	16.16	19.06	0.00	−3.47	−0.78	7.29	11.20	19.42
	Base + P vol.	−0.84	3.99	6.96	13.64	18.85	23.20	−4.95	3.73	7.81	12.76	16.84	24.22
	Base + PSAD	−2.37	4.04	9.61	15.88	19.31	24.29	−4.69	5.47	11.20	12.76	18.40	25.78
	Base + LDN-PSA	−0.84	3.85	10.17	16.71	21.68	24.69	0.00	0.09	6.77	15.89	23.44	26.38
	Base + P vol. + LDN-PSA	0.42	8.82	14.90	18.38	24.33	27.70	−3.65	10.33	15.89	21.09	23.70	30.25
	Base + LDN-PSAD	−0.42	10.07	18.52	20.33	23.54	24.97	−1.56	7.99	20.31	20.83	24.57	26.82

Base model: age, digital rectal examination status, total prostate-specific antigen (tPSA), and free PSA/tPSA (F/T PSA).CSPC, clinically significant prostate cancer; HGPC, high grade PC; LDN-PSA, LacdiNAc-glycosylated PSA; LDN-PSAD, LDN-PSA normalized by prostate volume; PC, prostate cancer; PSAD, PSA normalized by prostate volume; P vol., prostate volume.

DRE status, tPSA, and F/T PSA) combined with LDN-PSAD had the largest net benefit for overall PC prediction at greater than 20% risk threshold, and for CSPC and HGPC prediction at greater than 15% risk threshold (Figure 3A-C, Table 3). At the 25% risk threshold, the rate of Pbx avoided without missing overall PC of the base model combined with LDN-PSAD (9.33%) and LDN-PSA (5.57%) significantly improved the base model (1.81%) and base model combined with PSAD (3.34%) (Table 3). At the 20% risk threshold, the rate of Pbx avoided without missing CSPC or HGPC of base model combined with LDN-PSAD (18.11% and 18.52%, respectively) and combined with LDN-PSA (9.89% and 10.17%, respectively) significantly improved compared with the base model (2.23% and 2.37%, respectively) and also improved compared with the base model combined with PSAD (8.77% and 9.61%, respectively) (Table 3). In the PSA gray zone cohort, the base model combined with LDN-PSAD also provided the largest net benefit for overall PC prediction at greater than 20% risk threshold, for CSPC and HGPC prediction at greater than 15% risk threshold (Figure 3D-F and Table 3). At 25% risk threshold, the rate of Pbx avoided without missing overall PC of the base model combined with LDN-PSAD (8.59%) and LDN-PSA (5.47%) significantly improved the base model (−0.52%) and base model combined with PSAD (4.69%) (Table 3). At the 20% risk threshold, the rate of Pbx avoided without missing CSPC or HGPC of the base model combined with LDN-PSAD (13.54% and 20.31%, respectively) also significantly improved compared with the base model (−1.04% and −0.78%, respectively), the base model combined with LDN-PSA (5.21% and 6.77%, respectively), and the base model combined with PSAD (11.20% and 11.20%, respectively) (Table 3). These results suggested that the base model combined with LDN-PSAD is the best option for detecting overall PC, CSPC, and HGPC at any PSA range.

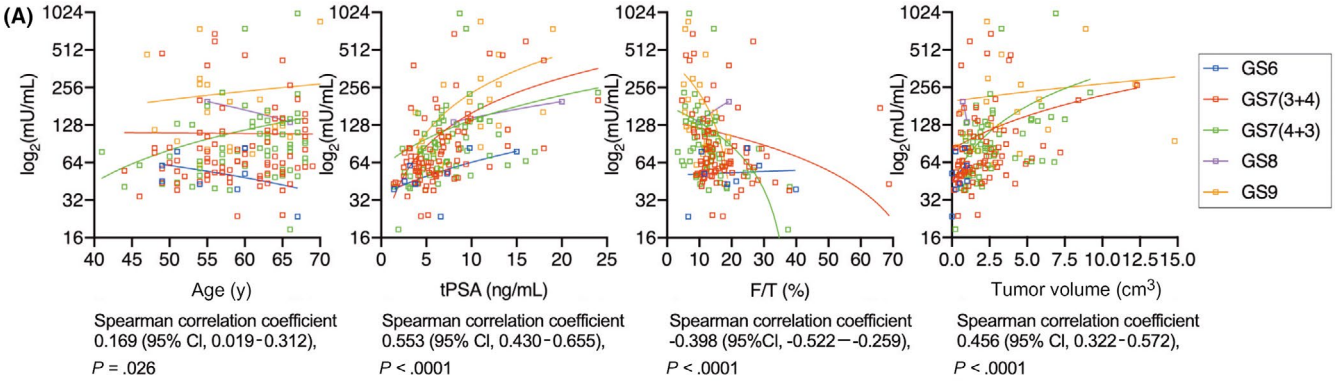
To evaluate the significance of LDN-PSA or LDN-PSAD, we undertook multivariate logistic regression analyses (Table S1). The odds ratio of LDN-PSAD for detection of overall PC (1.439; 95% CI, 1.251-1.655, $P < 0.0001$) and CSPC (1.492; 95% CI, 1.286-1.730, $P < 0.0001$) much superior to those of PSAD (1.176; 95% CI, 0.450-3.069, $P = 0.7411$ for overall PC) and (3.162; 95% CI, 0.998-10.016, $P = 0.0503$ for CSPC). The odds ratio of LDN-PSA for detection of overall PC (1.004; 95% CI, 0.998-1.009, $P = 0.1735$) and CSPC (1.003; 95% CI, 0.998-1.008, $P = 0.2900$) were comparable to those of PSAD (1.176; 95% CI, 0.450-3.069, $P = 0.7411$ for overall PC) and (3.162; 95% CI, 0.998-10.016, $P = 0.0503$ for CSPC). These results suggested that LDN-PSAD is a strong predictor of overall PC and CSPC detection.

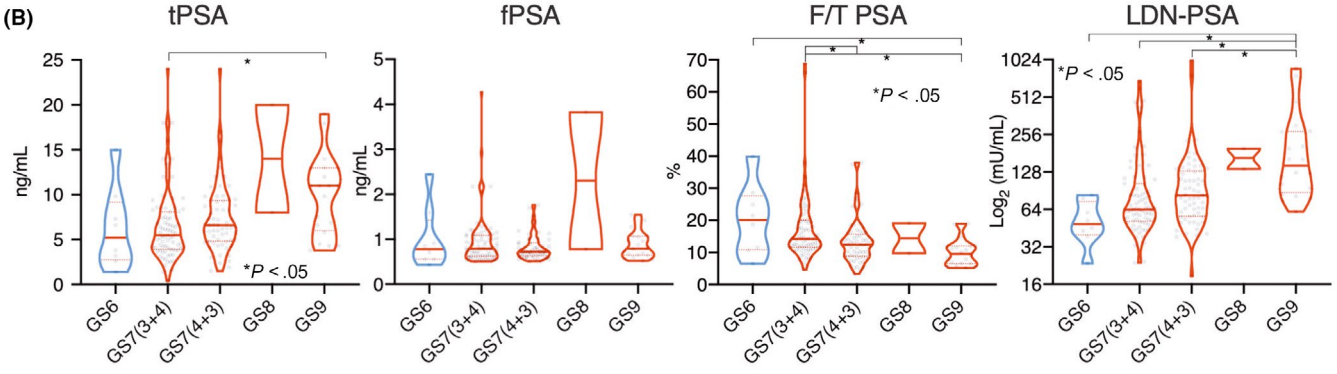
The characteristics of 174 patients in the preoperative baseline PSA cohort are shown in Table 4. The preoperative LDN-PSA levels were positively correlated with tumor volume (Spearman correlation coefficient 0.456; 95% CI, 0.322-0.572, $P < 0.0001$) and tPSA (0.553; 95% CI, 0.430-0.655, $P < 0.0001$). Low LDN-PSA level (≤100 mU/mL) cases tended to lower tumor volume (≤2.0 cm^3) and GS ≤ 7. The LDN-PSA levels were negatively correlated with F/T PSA (−0.398; 95% CI, −0.522 to −0.259, $P < 0.0001$) but did not strongly correlate with patient age (0.169; 95% CI, 0.019-0.312, $P = 0.026$) (Figure 4A). Levels of LDN-PSA at GS 3 + 4 (median, 64.0 mU/mL [IQR 52.1-98.6]), GS 4 + 3 (median, 82.5 mU/mL [56.7-126.2]), GS 8 (median, 166.2 mU/mL [150.6-181.8]), and GS 9 (median, 144.3 mU/mL [92.4-269.7]) were higher than those in patients with GS 6 (median, 48.7 mU/mL [42.0-65.0]), whereas tPSA and F/T PSA did not clearly discriminate PC GS 6 patients from PC GS ≥ 7 patients (Figure 4B). The LDN-PSA levels in pT3 patients (median, 102.3 mU/mL [72.0-174.5]) were also significantly higher than those in patients with pT2ab (median, 59.9 mU/mL [49.0-111.8]) and pT2c (median, 70.3 mU/mL [54.8-92.0]), whereas the tPSA test could not clearly discriminate between patients with pT3 and pT2 (Figure 4C). The LDN-PSA levels in patients with positive SV, LVI, or RM were significantly higher than those in patients with negative SV, LVI, or RM, respectively (Figure 4D-F).

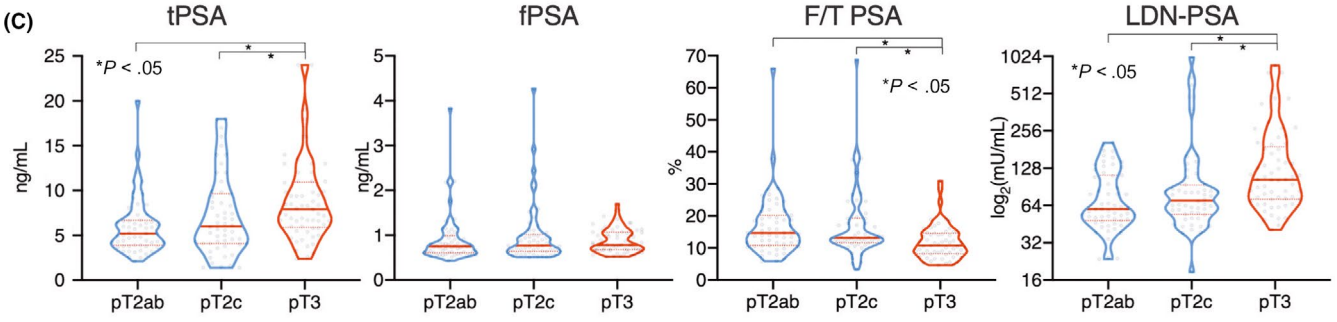
TABLE 4 Characteristics of preoperative baseline prostate-specific antigen (PSA) cohort

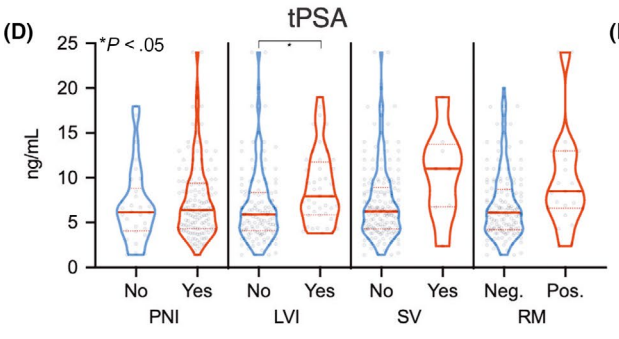
Variable	Median	(IQR)
Total (n = 174) pre-operative baseline serum		
Age, years	60	(55.0-65.0)
Tumor volume, cm^3	1.8	(0.91-2.92)
tPSA, ng/mL	6.4	(4.30-9.38)
F/T PSA, %	12.9	(10.1-17.8)
LDN-PSA, mU/mL	78.7	(54.6-128.0)
	n	(%)
Pathological GS sum after RP		
GS 6	8	4.6
GS 7 (3 + 4)	80	46.0
GS 7 (4 + 3)	64	36.8
GS 8	2	1.1
GS 9	20	11.5
Pathological stage		
pT2a,b	59	33.9
pT2c	54	31.0
pT3	61	35.1
Perineural invasion		
Yes	144	82.8
No	30	17.2
Seminal vesicle invasion		
Yes	8	4.6
No	166	95.4
Lymphovascular invasion		
Yes	44	25.3
No	130	74.7
Resection margin		
Positive	23	13.2
Negative	151	86.8

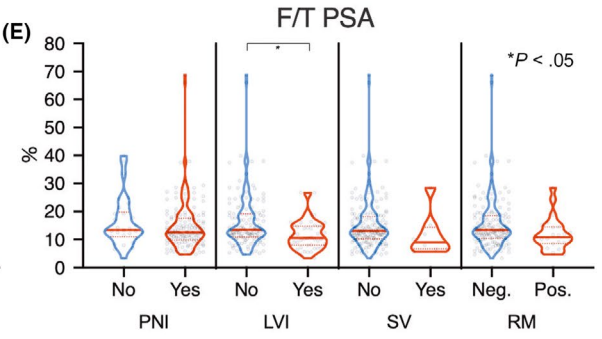
F/T PSA, free PSA/total PSA; GS, Gleason Score; IQR, interquartile range; LDN-PSA, LacdiNAc-glycosylated PSA; PSA, prostate-specific antigen; RP, radical prostatectomy; tPSA, total PSA.


(A)
log2(mU/mL)
Age (y)
tPSA (ng/mL)
F/T (%)
Tumor volume (cm3)
GS6
GS7(3+4)
GS7(4+3)
GS8
GS9
Spearman correlation coefficient 0.169 (95% CI, 0.019-0.312), P = .026
Spearman correlation coefficient 0.553 (95% CI, 0.430-0.655), P < .0001
Spearman correlation coefficient -0.398 (95%CI, -0.522—0.259), P < .0001
Spearman correlation coefficient 0.456 (95% CI, 0.322-0.572), P < .0001


(B)
tPSA
fPSA
F/T PSA
LDN-PSA
ng/mL
%
Log2 (mU/mL)
*P < .05
GS6
GS7(3+4)
GS7(4+3)
GS8
GS9


(C)
tPSA
fPSA
F/T PSA
LDN-PSA
ng/mL
%
log2(mU/mL)
*P < .05
pT2ab
pT2c
pT3


(D)
tPSA
ng/mL
*P < .05
No
Yes
PNI
LVI
SV
Neg.
Pos.
RM


(E)
F/T PSA
%
*P < .05
No
Yes
PNI
LVI
SV
Neg.
Pos.
RM

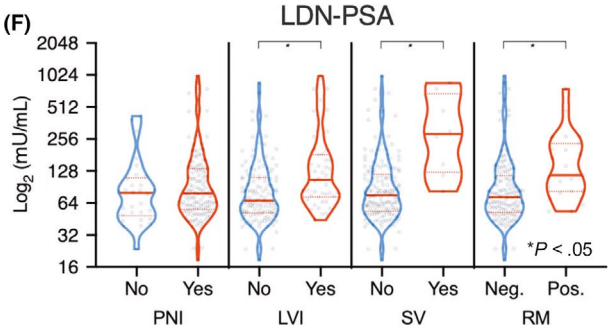

(F)
LDN-PSA
Log2 (mU/mL)
*P < .05
No
Yes
PNI
LVI
SV
Neg.
Pos.
RM

FIGURE 4 Correlation between LacdiNAc-glycosylated prostate-specific antigen (LDN-PSA) levels and pathological parameters in preoperative baseline serum. A, Correlation between LDN-PSA levels and age, tumor volume, total (t)PSA levels, and free (f)PSA/tPSA (F/T PSA). Open square with blue line represents Gleason Score (GS) 6 cases, open square with red line represent GS 7 (3 + 4) cases, open square with green line represents GS 7 (4 + 3) cases, open square with purple line represents GS 8, and open square with yellow line represents GS 9 cases. B, Serum levels of LDN-PSA, tPSA, and F/T classified by the sum of pathological GS after radical prostatectomy. C, Serum levels of LDN-PSA, tPSA, and F/T PSA classified by pathological stage (pT) after radical prostatectomy. D, Serum levels of tPSA classified by the status of perineural invasion (PNI), lymphovascular invasion (LVI), seminal invasion (SV), and resection margin (RM). E, Serum levels of F/T PSA classified by the status of PNI, LVI, SV, and RM. F, Serum levels of LDN-PSA classified by the status of PNI, LVI, SV, and RM. (B-F). Dashed red line in violin plot outlines the interquartile range of each test value. Red line in violin plot represents the median of each test value. Multiple group differences were analyzed using the Kruskal-Wallis test for non-normally distributed models

Furthermore, to determine whether benign or prostate cancer tissues contributed to aberrantly glycosylated LDN-PSA, we evaluated the expression level of LDN-glycan synthesis-related *β4GALNT3* and *β4GALNT4* gene expression and LDN-PSA/tPSA level in prostate sections obtained from patients who underwent RP at Hirosaki University (Figure 5A, Table 5). We found that the gene expression

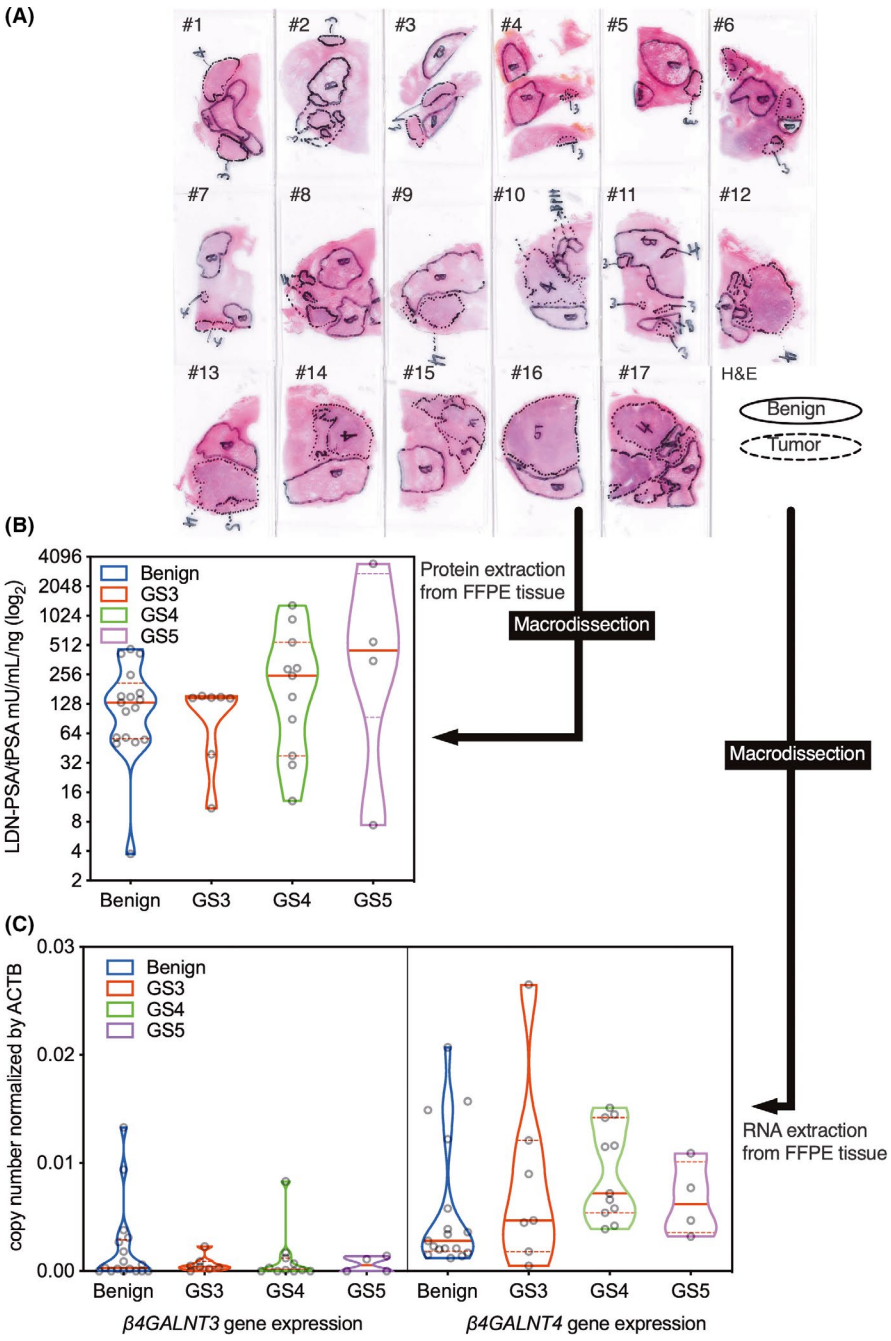


FIGURE 5 *LacdiNAc-glycosylated prostate-specific antigen (LDN-PSA)/total PSA (t)PSA level and LDN-glycan synthesis-related β4GALNT3 and β4GALNT4 gene expression in formalin-fixed paraffin-embedded (FFPE) prostate benign and tumor tissues in 17 patients who underwent radical prostatectomy in Hirosaki University (Hirosaki, Japan). A, Total RNA and total protein were extracted from benign tissue and each Gleason pattern of tumor tissue that was macrodissected from 20-μm thickness FFPE prostate section indicated by the areas marked with a solid and dashed outline, respectively. B, Levels of the LDN-PSA/tPSA in the benign tissue and tumor tissues with Gleason pattern 3-5. C, Levels of β4GALNT3 and β4GALNT4 gene expression in benign and tumor tissues with Gleason pattern 3-5. Dashed red line in the violin plots outlines the interquartile range of each test value; solid red line represents the median of each test value*

TABLE 5 Characteristics of formalin-fixed paraffin-embedded (FFPE) prostate sections from 17 patients who underwent radical prostatectomy at Hirosaki University (Hirosaki, Japan)

FFPE section no.	Gleason pattern	Age, years	Macrodisected tissue area, mm^2	Macrodisected tissue volume, mm^3	tPSA, ng/mL in tissue	LDN-PSA, mU/mL in tissue	LDN-PSA/tPSA, mU/mL/ng in tissue	*β4GALNT3* per *ACTB* copy number	*β4GALNT4* per *ACTB* copy number
1	Benign	68	84.01	1.68	1.60	132.39	82.74	0.0038	0.0122
	3		31.57	0.63	1.68	148.90	88.63	0.0009	0.0005
	4		51.06	1.02	5.20	297.87	57.28	0.0012	0.0054
2	Benign	69	66.86	1.34	4.22	117.30	27.80	0.0031	0.0034
	3		44.71	0.89	4.94	146.16	29.59	0.0003	0.0047
3	Benign	72	92.10	1.84	2.80	153.05	54.66	0.0018	0.0058
	3		49.98	1.00	2.34	147.38	62.98	0.0000	0.0265
4	Benign	74	84.64	1.69	0.43	51.92	121.60	0.0027	0.0023
	3		12.33	0.25	0.26	39.01	150.03	0.0023	0.0018
5	Benign	74	116.91	2.34	11.38	253.70	22.29	0.0002	0.0017
	3		17.21	0.34	1.48	10.98	7.42	0.0002	0.0121
6	Benign	62	73.02	1.46	1.54	50.33	32.68	0.0006	0.0015
	3		84.68	1.69	6.10	154.53	25.33	0.0005	0.0045
7	Benign	78	83.74	1.67	5.47	418.54	76.52	0.0133	0.0149
	4		22.65	0.45	2.60	89.24	34.32	0.0000	0.0039
8	Benign	58	137.09	2.74	4.73	465.92	98.50	0.0000	0.0036
	4		39.36	0.79	2.66	290.58	109.24	0.0000	0.0066
9	Benign	73	156.17	3.12	1.31	165.95	126.68	0.0094	0.0157
	4		61.05	1.22	2.68	248.66	92.78	0.0083	0.0116
10	Benign	61	76.57	1.53	0.08	3.76	47.03	0.0000	0.0012
	4		122.58	2.45	0.10	13.02	130.16	0.0017	0.0151
11	Benign	71	187.75	3.75	9.88	420.55	42.57	0.0002	0.0207
	3		33.18	0.66	4.00	150.03	37.51	0.0004	0.0090
	4		5.02	0.10	1.24	30.43	24.54	0.0000	0.0058
12	Benign	70	48.12	0.96	0.58	58.03	100.05	0.0000	0.0028
	4		177.13	3.54	41.63	1304.00	31.32	0.0007	0.0072
13	Benign	74	97.35	1.95	0.98	107.49	109.68	0.0000	0.0021
	4		131.46	2.63	2.93	937.44	319.94	0.0000	0.0145
	5		30.19	0.60	0.08	7.41	92.57	0.0014	0.0109

TABLE 5 (Continued)

FFPE section no.	Gleason pattern	Age, years	Macrodisected tissue area, mm^2	Macrodisected tissue volume, mm^3	tPSA, ng/mL in tissue	LDN-PSA, mU/mL in tissue	LDN-PSA/tPSA, mU/mL/ng in tissue	*β4GALNT3* per *ACTB* copy number	*β4GALNT4* per *ACTB* copy number
14	Benign	64	146.83	2.94	4.74	151.03	31.86	0.0000	0.0020
	4		114.32	2.29	11.22	545.86	48.65	0.0001	0.0142
	5		36.34	0.73	5.76	352.69	61.23	0.0011	0.0047
15	Benign	73	102.05	2.04	4.56	140.83	30.88	0.0009	0.0039
	4		82.61	1.65	1.00	150.72	150.72	0.0001	0.0115
	5		68.49	1.37	1.96	553.11	282.20	0.0000	0.0032
16	Benign	63	89.62	1.79	0.94	55.34	58.87	0.0000	0.0014
	5		267.44	5.35	2.64	3481.34	1318.69	0.0000	0.0077
17	Benign	63	97.99	1.96	0.52	58.26	112.05	0.0003	0.0021
	4		265.07	5.30	0.22	37.81	171.86	0.0003	0.0042

LDN-PSA, LacdiNAc-glycosylated prostate-specific antigen; tPSA, total prostate-specific antigen.

of *β4GALNT4* and LDN-PSA/tPSA level was increased in Gleason pattern 4 and 5 tissues compared to benign (Figure 5B,C, Table 5).

4 | DISCUSSION

More than 2 million transrectal ultrasonography-guided Pbx procedures are carried out every year in the USA and Europe following tPSA levels ≥ 4.0 ng/mL and/or DRE findings with patient characteristics, such as age, race, family history, and ethnicity, also taken into consideration.[24] These diagnostic procedures and factors, including Pbx, are costly and can be associated with pain, anxiety, and complications, such as an increased risk of infection.[24,25] Two recent studies have reported a decline in the incidence of early stage PC and a reduced rate of PSA screening in men less than 75 years old after the 2012 United States Preventive Services Task Force recommendation.[26,27] Consequently, the tPSA-based PC screening strategy has been changed and now includes the use of MRI to target HGPC and to avoid detection of low-grade cancer, retaining the potential to continue to reduce mortality but to avoid harm from overdetection of indolent PC.

We and others previously reported that LDN-PSA in serum is significantly increased in PC,[21,28] especially HGPC with GS ≥ 7[20] and that the amount of LDN-glycan on PC tissue is positively correlated with higher GS and an independent risk factor of PSA recurrence.[20] Furthermore, we found that LDN-PSA/tPSA level and LDN-glycan synthesis-related *β4GALNT4* gene expression was increased in higher Gleason pattern tissues (Figure 5B,C), suggesting that LDN-glycan synthesis on PSA was increased in aggressive tumors. LacdiNAc GalNAcβ1-4GlcNAc glycan expression has been reported in other cancers. LacdiNAc GalNAcβ1-4GlcNAc in *N*-glycans significantly decreases during progression of human breast cancer and transfection with β4GALNT4 reduced breast cancer cell growth in vitro.[29,30] In contrast, the enhanced expression of LDN glycan has been shown to be associated with the progression of human prostate, ovarian, colon, and liver cancers.[31-33] Of note, in colon cancer, *β4GALNT3* gene expression was upregulated in colonospheres and modulated cancer stemness through the epidermal growth factor receptor signaling pathway.[34] This indicates that the function of LDN-glycan that is synthesized by *β4GALNT3* and *β4GALNT4* genes is cancer type-specific and complicated. Although the biological function of LDN-glycan on PC tissue has not yet been fully understood, LDN glycan on PC tissue might be involved in PC stemness-related signal transduction and LDN-PSA could be useful as a diagnostic and preoperative prognostic biomarker. Further molecular biological studies would clarify the biological significance of LDN-glycan synthesis for PC progression. In this study, we found that the levels of LDN-PSA and LDN-PSAD were predictive of CSPC patients with a negative predictive value of 84.7%-88.3%, positive predictive value of 53.1%-60.3%, and a specificity of 45.3%-61.7% at 90% sensitivity in the Pbx cohort. The diagnostic accuracy of both LDN-PSA (AUC 0.827) and LDN-PSAD (AUC 0.860) significantly improved predicting CSPC over that of tPSA (AUC 0.712), F/T PSA (AUC 0.661),

and PSAD (AUC 0.809). We also found that including LDN-PSA or LDN-PSAD in a multivariate decision curve base model resulted in a significant increase in its accuracy for predicting overall PC, CSPC, and HGPC in patients without missing any cancer (Figure 3, Table 3). Furthermore, we found that the LDN-PSA levels in the Pbx cohort (Asian and Canadian) were increased in HGPC (GS ≥ 7) over that of low-grade ASPC (Figure 2) and the preoperative LDN-PSA levels in a preop-PSA baseline cohort (n = 174) in Australia (Caucasian only) also positively correlated with tPSA levels and tumor volumes. Furthermore, higher LDN-PSA levels correlated with GS ≥ 7 and SV, LVI, or RM positive PC patients (Figure 4). Interestingly and consistent with previously reported findings, a low tumor volume case (≤2.0 cm^3) was also observed to have a very low LDN-PSA level. These results suggest that the level of LDN-PSA reflects tumor aggressiveness and this was not significantly different among races. Therefore, LDN-PSA might predict HGPC before RP and could play a role in replacing tPSA as an initial screening test as well as in monitoring men under active surveillance. We will continue to evaluate the association with pathologic features of RP specimens in a larger prospective cohort.

Although several marker assays (Prostate Health Index, 4KScore, PCA3, MiPS, SelectMDx, and EPI) and MRI have reported promising results for the prediction of high-grade PC,[13,35,36] these biomarkers have not yet been approved in Japan. In this study, we found that the inclusion of LDN-PSA or LDN-PSAD in a decision curve base model (tPSA + F/T PSA + age + DRE status) resulted in a significant increase in its net benefit for detecting overall PC, CSPC, and HGPC in patients at any PSA range in a multicenter Pbx cohort (n = 718, Asian plus Canadian). These results suggest that the diagnostic performance and clinical utility of LDN-PSA and LDN-PSAD outperformed the base model. Limitations include limited sample size, retrospective nature, no family history, and no Prostate Imaging-Reporting and Data system (PI-RADS) information prior to biopsy and no data regarding the abovementioned biomarkers. Further prospective clinical trials using LDN-PSA combined with new biomarkers would further clarify the cost-effectiveness and diagnostic performance of the LDN-PSA assay.

Although our study was relatively small and retrospective, it did not influence the main results. Aberrantly glycosylated LDN-PSA and LDN-PSAD at Pbx is useful for providing a clinical index for active surveillance as well as for discriminating HGPC with GS ≥ 7. Thus, both LDN-PSA and LDN-PSAD could reduce overdiagnosis and overtreatment of PC patients.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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