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Analytical and Real-World Clinical Characterization of S2,3PSA% Test in MRI Fusion Targeted Prostate Biopsy Population

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Received: 7 January 2025 | **Revised:** 13 March 2025 | **Accepted:** 18 March 2025

Funding: This work was supported by the Japan Society for the Promotion of Science KAKENHI (Grant No. 24K12501).

Keywords: preanalytical stability | prostate cancer | prostate health index | prostate-specific antigen | ratio of α2,3-sialylated free PSA

ABSTRACT

Background: The α2,3-sialyl *N*-glycosylated free prostate-specific antigen ratio (S2,3PSA%) was approved in Japan as a prostate cancer (PCa) diagnostic test. We evaluated the analytical characterization and real-world diagnostic performance of S2,3PSA%.

Methods: The precision testing, dilution linearity, measurement sensitivity, preanalytical stability, and interferences of S2,3PSA, α2,6-sialyl *N*-glycosylated free PSA (S2,6PSA), and S2,3PSA% were performed. The diagnostic accuracy detecting PCa of S2,3PSA% was prospectively evaluated in 253 men (Cohort 1, vs. PI-RADS) and in 145 men (Cohort 2, vs. PI-RADS, *prostate health index*, *phi*) who scheduled MRI-targeted biopsy by area under the receiver operating characteristics curve (AUC).

Results: The precision of the S2,3PSA, S2,6PSA, and S2,3PSA% were all < 3.4% coefficient of variation. The dilution linearity of S2,3PSA had a correlation coefficient of 0.9949–0.9987. The detection limit of S2,3PSA and S2,6PSA was 0.044 and 0.029 ng/mL, respectively. Serum S2,3PSA and S2,6PSA concentrations were stable at 6°C for 24 h and −20°C for 90 days, while S2,3PSA% was unchanged at 6°C and −20°C for 90 days or under five freeze–thaw cycles. Serum S2,3PSA and S2,3PSA% were not affected by any interferences and drugs. In Cohort 1, AUC of S2,3PSA% (0.776, 95% CI 0.719–0.832) detecting PCa was comparable to that of PI-RADS (0.746, 0.685–0.807, $p = 0.7996$). In Cohort 2, AUC of S2,3PSA% detecting PCa (0.837, 0.774–0.901) was comparable to PI-RADS (0.779, 0.703–0.854, $p = 0.3037$), and *phi* (0.867, 0.809–0.926, $p = 0.3000$).

Conclusions: Analytical characteristics of S2,3PSA% perform well and the diagnostic performance of S2,3PSA% was comparable to *phi* and MRI.

Abbreviations: AUC, area under the receiver operating characteristics curve; CV, coefficient of variation; FNR, false negative rate; FPR, false positive rate; f-PSA, free PSA; F/T-PSA, f-PSA/t-PSA ratio; MRI-TBx, MRI targeted prostate biopsy; MRI-TBx(+), positive MRI targeted prostate biopsy; MRI-TBx(−), negative MRI targeted prostate biopsy; *phi*, prostate health index; PI-RADS, prostate imaging-reporting and data system; PSA, prostate-specific antigen; S2,3PSA, α2,3-sialyl *N*-glycosylated free PSA; S2,3PSA%, the ratio of S2,3PSA to sum of the S,3PSA and S2,6PSA; S2,6PSA, α2,6-sialyl *N*-glycosylated free PSA; SD, standard deviation; t-PSA, total PSA.

1 | Introduction

Prostate-specific antigen (PSA) is widely used as a biomarker for prostate cancer (PCa), but low cancer specificity limits its clinical utility, often leading to unnecessary biopsy, overdiagnosis, and overtreatment of low-grade PCa [1, 2]. Recently, prostate imaging-reporting and data system (PI-RADS) and biomarker such as *prostate health index* (*phi* = [−2]proPSA/f-PSA × √t-PSA), and aberrant glycosylated PSA such as the ratio of α2,3-sialyl *N*-glycosylated free PSA (S2,3PSA%) to the sum of S2,3PSA and α2,6-sialyl *N*-glycosylated free PSA (S2,6PSA) (Figure 1A) have been used to risk stratify men with suspected PCa before biopsy is recommended [3–11]. Based on these findings, FUJIFILM Wako Pure Chemical Corporation has launched μTASWako S2,3PSA%·i50 reagent for quantification of S2,3PSA% using microfluidic technology-based μTASWako i50 device in Japan on September 26, 2022 (Figure 1B) [7, 12, 13]. Subsequently, we have reported that the diagnostic performance of S2,3PSA% for PCa detection was superior to that of conventional strategies such as total PSA (t-PSA) or f-PSA/t-PSA ratio (F/T-PSA) (AUC: 0.727 vs. 0.517 or 0.679, respectively) in a multicenter retrospective cohort in t-PSA range 4–10 ng/mL using μTASWako S2,3PSA% assay [13]. Based on this, the S2,3PSA% was approved by the Ministry of Health, Labour and Welfare in Japan and started insurance coverage on February 1, 2024 as a secondary PCa screening test after the t-PSA test.

As described above, despite the clinical potential of S2,3PSA% to improve PCa detection rates compared to conventional methods, the comprehensive performance of assay reagent and preanalytical stability of S2,3PSA% measurements have not yet been published. Although there are no other reports of a head-to-head comparison of clinical performance between S2,3PSA% and *phi*, we have previously reported that the clinical performance of S2,3PSA% is comparable to PI-RADS [13]. There are several reports that a combination of *phi* and PI-RADS could reduce the unnecessary biopsy [14–16].

Therefore, we aimed to evaluate the precision, dilution linearity, sensitivity, preanalytical stability, interferences, and preanalytical stability to determine parameters for optimal sample handling. We also aimed to evaluate the real-world clinical outcomes of S2,3PSA% and PI-RADS or *phi* in a prospective observational MRI-targeted biopsy (MRI-TBx) cohort under the optimal specimen storage conditions determined in this study, using previously reported cutoff values in a head-to-head comparison.

2 | Methods

2.1 | S2,3PSA% and *phi* Measurement

The S2,3PSA% and S2,3PSA measurements were performed by a medical laboratory scientist at Hirosaki University blinded to each patient's clinical information (Figure 1B,C). μTASWako i50 device and reagents including μTASWako S2,3PSA%·i50 reagent, calibrator set, Control L, and Control H for S2,3PSA%, and μTASWako dilution solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were used for S2,3PSA% assay. The *phi* value was obtained using the laboratory testing service by sending frozen specimens at −80°C to BML Inc. (Tokyo, Japan).

The cutoff values for S2,3PSA% and *phi* were applied as S2,3PSA % ≥ 38.0% [13], *phi* ≥ 27.2 [17] with a sensitivity of 90% at t-PSA range from 4 to 10 ng/mL as described previously.

2.2 | Preparation of Pooled Serum for Evaluation of Precision, Dilution Linearity, Measurement Sensitivity, and Interference Analyses

The human-processed pooled serum (trinaBase) was obtained from Trina Bioactives (Naenikon, Switzerland). To stabilize the f-PSA concentration added to the serum, pooled serum was inactivating the protease inhibitors such as α1-antichymotrypsin or α2-macroglobulin by alkaline treatment (Japan Patent Application #61-070659) to avoid complex formation between the protease inhibitors and added f-PSA.

2.3 | Precision Testing

Precision testing at a single institute was evaluated according to the EP05-A3 protocol of the Clinical Laboratory Standard Institute (CLSI) [18]. Intra-assay variability for S2,3PSA and S2,3PSA% measurements were evaluated by two times duplicate measuring per day for 20 separate days using pooled sera-L prepared at 45.8% as S2,3PSA% containing 0.48 ng/mL S2,3PSA and pooled sera-H prepared at 34.2% as S2,3PSA% containing 1.63 ng/mL S2,3PSA. Each result was expressed as coefficients of variation (CV).

2.4 | Dilution Linearity Analysis

Dilution linearity analysis was evaluated according to the EP06 2nd Edition protocol of the CLSI [19]. Linearity of S2,3PSA concentrations was assessed by two dilution series of pooled sera containing two concentrations of S2,3PSA (Sample 1: 42 ng/mL and Sample 2: 7 ng/mL) prepared at ~48% as S2,3PSA %, each diluted in five steps using dedicated diluents. Linearity of S2,3PSA% was evaluated by two pooled serum samples with 20% and 80% of S2,3PSA% containing a sum of S2,3PSA and S2,6PSA concentration (1 or 50 ng/mL) were prepared, and then the 80% of S2,3PSA% sample was diluted five steps with the 20% of S2,3PSA% sample to prepare a dilution series of seven samples in total, respectively. Dilution linearity was confirmed using nonlinear regression analysis by plotting the mean of each diluted sample measured in duplicate.

2.5 | Measurement Sensitivity Analysis

Measurement sensitivity analysis was evaluated according to the EP17-A2 protocol of the CLSI [20]. 0.25, 0.05, 0.75, 1.00, 1.25, and 1.50 ng/mL of S2,3PSA or S2,6PSA each were prepared using pooled serum and dilutions and measured in triplicate for 3 days using two different reagent lots. The limit of detection (LoD) was evaluated by creating a precision profile with a cubic polynomial curve with the horizontal axis being the mean value of each

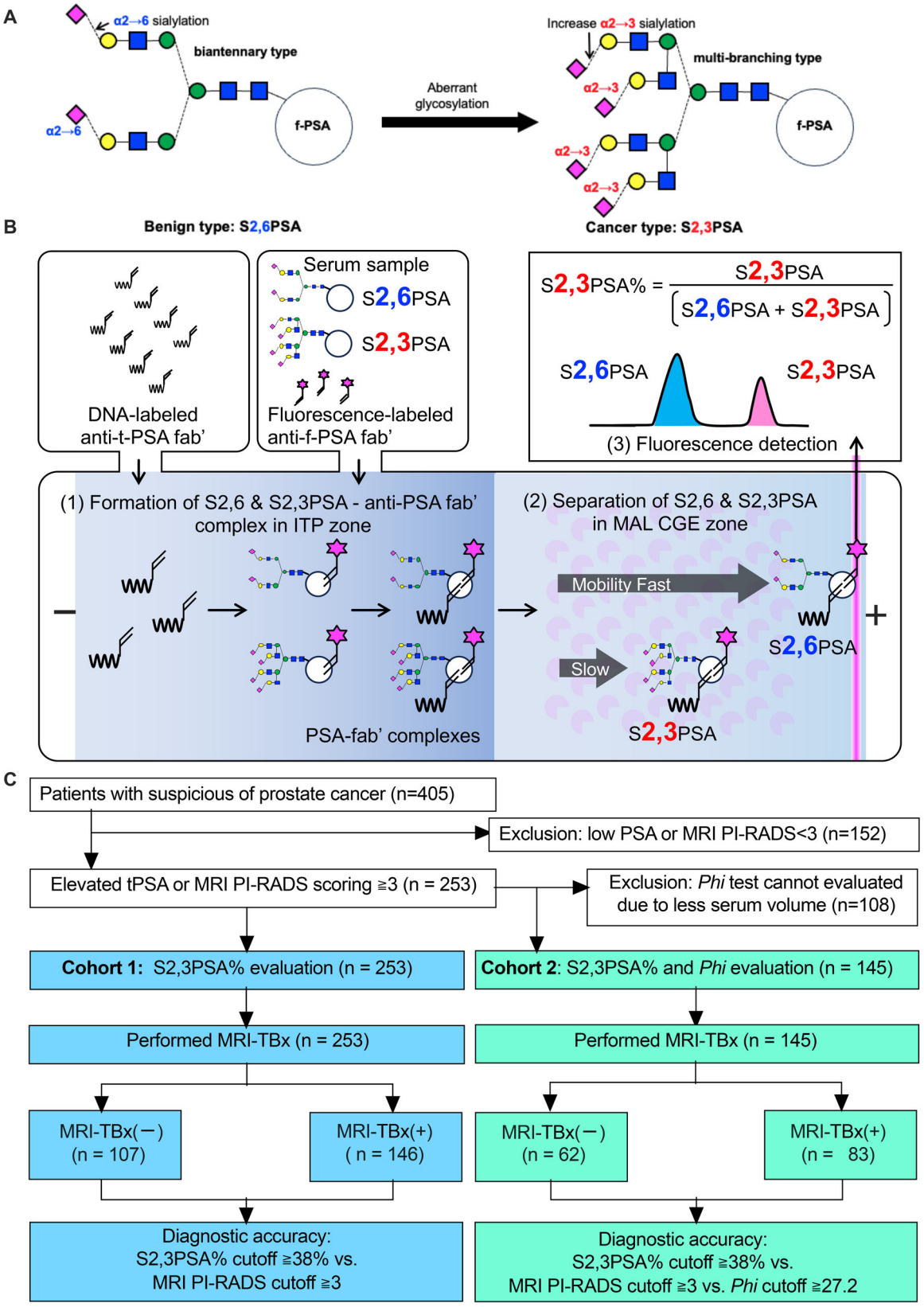

FIGURE 1 | (A) Prostate cancer-related aberrant *N*-glycosylation of PSA. (B) Schematic representation of S2,3PSA% test using μTASWako i50. (1) Formation of PSA-fab' complexes by isotachophoresis (ITP). (2) Separation of S2,3 and S2,6PSA-fab' complexes by *Maackia amurensis* lectin (MAL) containing capillary gel electrophoresis (CGE). (3) Fluorescence detection of S2,3 and S2,6PSA. (C) Flow diagram from study evaluating the accuracy of S2,3PSA%, PI-RADS, and *phi* testing for the diagnosis of prostate cancer in a prospective observational MRI-TBx cohort. [Color figure can be viewed at wileyonlinelibrary.com]

concentration sample and the vertical axis being the CV% value of the measurement.

2.6 | Preanalytical Stability in Whole Blood and Isolated Serum

Twenty patients who scheduled MRI-TBx in Hirosaki University Hospital from November 2022 to September 2023 were enrolled in this study. A total of 10 mL of blood was collected in the morning before the MRI-TBx and aliquoted as follows. To evaluate preanalytical stabilities in whole blood, blood was aliquoted in 400 μL portions and stored as whole blood at 25°C or 6°C for 1, 3, 8, and 24 h. To evaluate preanalytical stabilities in serum, blood was centrifuged after ~10 min of coagulation, and 1000 μL of collected serum was immediately measured for S2,3PSA, S2,6PSA, and S2,3PSA% as baseline values and aliquoted in 100 μL portions and stored at 6°C or −20°C for 1, 3, 8, 24 h, 2, 3, 7, 14, 30, and 90 days. To evaluate the serum sample stability under a total of five freeze–thaw cycles, the remaining 400 μL of serum was allowed to stand at −20°C for at least 1 h to ensure that it was completely frozen. Spontaneous thawing was left for 10 min at 25°C and vortexing for 1–2 s. Each specimen of S2,3PSA, S2,6PSA, and S2,3PSA% measurements was performed in duplicate. Data for the percentage recovery of S2,3PSA, S2,6PSA, and S2,3PSA% are expressed as mean (SD).

2.7 | Interference Studies

Two pooled serum samples with S2,3PSA% of approximately 35% and 45% were prepared for interference studies. Dilution series were prepared using pooled and non-added pooled sera with up to the maximum concentration of each additive shown in Figure 4 and measured in duplicate. The mean value was calculated to evaluate the measured value when the non-added sample was set at 100%. Interferents were assessed for ascorbic acid, hemoglobin, conjugated bilirubin (bilirubin C), free bilirubin (Bilirubin F), chyle, and rheumatoid factor (RF) including Interference check A Plus and Interference check RF Plus reagent (Sysmex Corporation, Tokyo, Japan). Drugs were assessed for dutasteride (Sawai Pharmaceutical Co. Ltd., Osaka, Japan), prazosin HCl (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan), leuprorelin acetate (Takeda Pharmaceutical Co. Ltd., Osaka, Japan), and docetaxel (FUJIFILM Wako Pure Chemical Corporation).

2.8 | Comparison of Diagnostic Performance With S2,3PSA% and PI-RADS or *phi* Test Who Scheduled MRI-TBx in Prospective Observational Study

As shown in Figure 1C, Cohort 1 prospectively enrolled 253 men scheduled for MRI-TBx at Hirosaki University between October 2018 and September 2024 to compare the diagnostic accuracy of prebiopsy S2,3PSA% and PI-RADS. Cohort 2 prospectively enrolled 145 men scheduled for MRI-TBx at Hirosaki University during the same duration as Cohort 1 to compare the diagnostic accuracy of prebiopsy S2,3PSA%, PI-RADS, and *phi* test. All prebiopsy blood was separated into serum within 1 h of blood collection and stored at −80°C. S2,3PSA% or *phi* measurement was performed by a medical laboratory scientist blinded to each patient's clinical information. In Cohort 2, 108 cases were excluded due to less serum volume for *phi* test measurement. All MRI examinations and index lesions scoring by PI-RADS ver. 2.1 [21] were assessed by two radiologists at Hirosaki University Hospital blinded to each patient's S2,3PSA% and *phi* values and MRI-TBx with systematic Bx was performed as described previously [13]. Men with positive and negative MRI-TBx represented as MRI-TBx(+) and MRI-TBx(−). The grade group (GG) according to the International Society of Urological Pathology guideline [22] for biopsy specimens was assessed by two histopathologists at Hirosaki University Hospital blinded to each patient's S2,3PSA% and *phi* values.

2.9 | Ethics Approval and Consent to Participate

This study was conducted according to the ethical standards of the Declaration of Helsinki and was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine (Approval Numbers, 2019-055, 2022-140, and 2023-021) (https://www.med.hirosaki-u.ac.jp/hospital/outline/resarch.html). Written informed consent was obtained from all patients.

2.10 | Statistical Analysis

All statistical calculations were performed using Graphpad Prism ver. 10.3.1 (GraphPad, San Diego, CA, USA), STATA/SE15.1 (StataCorp LLC, College Station, TX, USA). Comparisons between two groups were conducted using the Mann–Whitney *U* test. The preanalytical stability compared to baseline and each time point were analyzed two-way ANOVA Dunnett's test. The correlation analysis was analyzed using the nonparametric Spearman's rank-order correlation test. Diagnostic accuracy was quantified as the area under the receiver operating characteristic curve (AUC). p values of < 0.05 were used to denote statistical significance.

3 | Results

3.1 | Precision

Precision study showed that the CV of pooled sera-L in S2,3PSA was 3.4% (mean S2,3PSA value 0.44 ng/mL); S2,3PSA% was 1.3% (mean S2,3PSA% value 44.4%), and the CV of pooled sera-H in S2,3PSA was 2.1% (mean S2,3PSA value 0.44 ng/mL); S2,3PSA% was 0.9% (mean S2,3PSA% value 44.4%), respectively (Table 1).

3.2 | Dilution Linearity

The dilution linearity of S2,3PSA concentration in two distinct 48% of S2,3PSA% samples showed correlation coefficients of 0.9982 and 0.9975, respectively, and was obtained a linear line through the 1–42 ng/mL of S2,3PSA with an accuracy of 97%–105% at each dilution rate. The S2,3PSA% values were

TABLE 1 | Intra-assay variability for the S2,3PSA% assay.

			Pooled sera-L		Pooled sera-H	
			S2,3PSA	**S2,3PSA%**	**S2,3PSA**	**S2,3PSA%**
			0.48	**45.8**	**1.63**	**34.2**
Day	**Time**	***n***	**ng/mL**	**%**	**ng/mL**	**%**
1	a.m.	1	0.44	44.2	1.70	34.7
		2	0.43	43.5	1.69	34.3
	p.m.	1	0.43	43.6	1.66	34.1
		2	0.44	44.1	1.66	34.6
2	a.m.	1	0.46	44.8	1.68	34.7
		2	0.46	45.0	1.66	34.2
	p.m.	1	0.44	44.5	1.64	34.5
		2	0.44	45.3	1.63	34.3
3	a.m.	1	0.44	44.1	1.68	34.6
		2	0.44	44.4	1.69	34.9
	p.m.	1	0.44	44.7	1.68	34.8
		2	0.44	44.5	1.67	34.7
4	a.m.	1	0.43	43.8	1.68	34.8
		2	0.43	43.6	1.68	34.5
	p.m.	1	0.42	43.6	1.68	34.9
		2	0.43	44.2	1.63	34.8
5	a.m.	1	0.45	44.7	1.69	33.9
		2	0.45	44.4	1.69	34.3
	p.m.	1	0.44	44.5	1.67	34.2
		2	0.42	43.9	1.65	34.3
6	a.m.	1	0.44	44.6	1.49	34.0
		2	0.43	44.5	1.61	34.5
	p.m.	1	0.47	45.2	1.56	35.1
		2	0.46	45.2	1.55	34.9
7	a.m.	1	0.46	45.1	1.68	34.4
		2	0.48	45.4	1.68	34.1
	p.m.	1	0.46	44.6	1.67	34.7
		2	0.44	44.6	1.65	33.9
8	a.m.	1	0.44	44.6	1.62	34.3
		2	0.43	44.3	1.67	34.4
	p.m.	1	0.44	44.1	1.69	34.8
		2	0.43	43.6	1.66	34.6
9	a.m.	1	0.44	43.9	1.66	34.6
		2	0.43	43.7	1.66	34.4
	p.m.	1	0.46	44.8	1.67	34.4
		2	0.45	45.1	1.69	34.5
10	a.m.	1	0.46	44.8	1.70	34.8
		2	0.48	44.9	1.70	35.0
	p.m.	1	0.45	44.9	1.66	34.8
		2	0.45	44.4	1.68	34.7
11	a.m.	1	0.44	44.4	1.69	34.5

(Continues)

TABLE 1 | (Continued)

			Pooled sera-L		Pooled sera-H	
			S2,3PSA	**S2,3PSA%**	**S2,3PSA**	**S2,3PSA%**
			0.48	**45.8**	**1.63**	**34.2**
Day	**Time**	***n***	**ng/mL**	**%**	**ng/mL**	**%**
		2	0.44	44.8	1.65	34.3
	p.m.	1	0.44	44.2	1.68	34.6
		2	0.43	44.4	1.68	34.5
12	a.m.	1	0.43	43.5	1.70	34.3
		2	0.44	43.8	1.71	34.0
	p.m.	1	0.44	43.6	1.67	34.4
		2	0.42	43.3	1.65	34.4
13	a.m.	1	0.43	44.4	1.69	34.4
		2	0.43	44.2	1.70	34.7
	p.m	1	0.48	45.5	1.64	34.5
		2	0.47	45.4	1.67	34.9
14	a.m.	1	0.46	45.0	1.66	34.7
		2	0.45	44.8	1.68	34.7
	p.m.	1	0.47	45.1	1,69	34.9
		2	0.45	44.6	1,71	34.9
15	a.m.	1	0.43	43.7	1.64	34.1
		2	0.43	44.3	1.64	34.2
	p.m.	1	0.43	44.0	1.64	34.4
		2	0.42	44.8	1.62	34.4
16	a.m.	1	0.43	44.0	1.68	34.6
		2	0.43	44.0	1.67	34.5
	p.m.	1	0.43	43.8	1.69	34.8
		2	0.44	44.2	1.66	34.6
17	a.m.	1	0.44	43.9	1.69	35.2
		2	0.44	44.2	1.70	34.9
	p.m.	1	0.43	43.3	1.68	35.0
		2	0.43	43.9	1.68	35.1
18	a.m.	1	0.44	44.1	1.65	35.0
		2	0.43	44.2	1.67	35.4
	p.m.	1	0.47	45.3	1.66	34.6
		2	0.47	45.1	1.63	34.4
19	a.m.	1	0.44	44.6	1.62	34.0
		2	0.45	44.6	1.63	34.5
	p.m.	1	0.46	45.2	1.64	34.7
		2	0.45	44.9	1.64	34.4
20	a.m.	1	0.44	44.3	1.65	34.5
		2	0.43	43.7	1.65	34.5
	p.m.	1	0.44	43.8	1.70	35.0
		2	0.43	43.7	1.66	34.9
		Mean	0.44	44.4	1.66	34.6
		Max	0.48	45.5	1.71	35.4

(Continues)

TABLE 1 | (Continued)

Day	Time	*n*	Pooled sera-L S2,3PSA 0.48 ng/mL	Pooled sera-L S2,3PSA% 45.8 %	Pooled sera-H S2,3PSA 1.63 ng/mL	Pooled sera-H S2,3PSA% 34.2 %
		Min	0.42	43.3	1.49	33.9
		SD	0.015	0.56	0.035	0.31
		CV	3.4%	1.3%	2.1%	0.9%

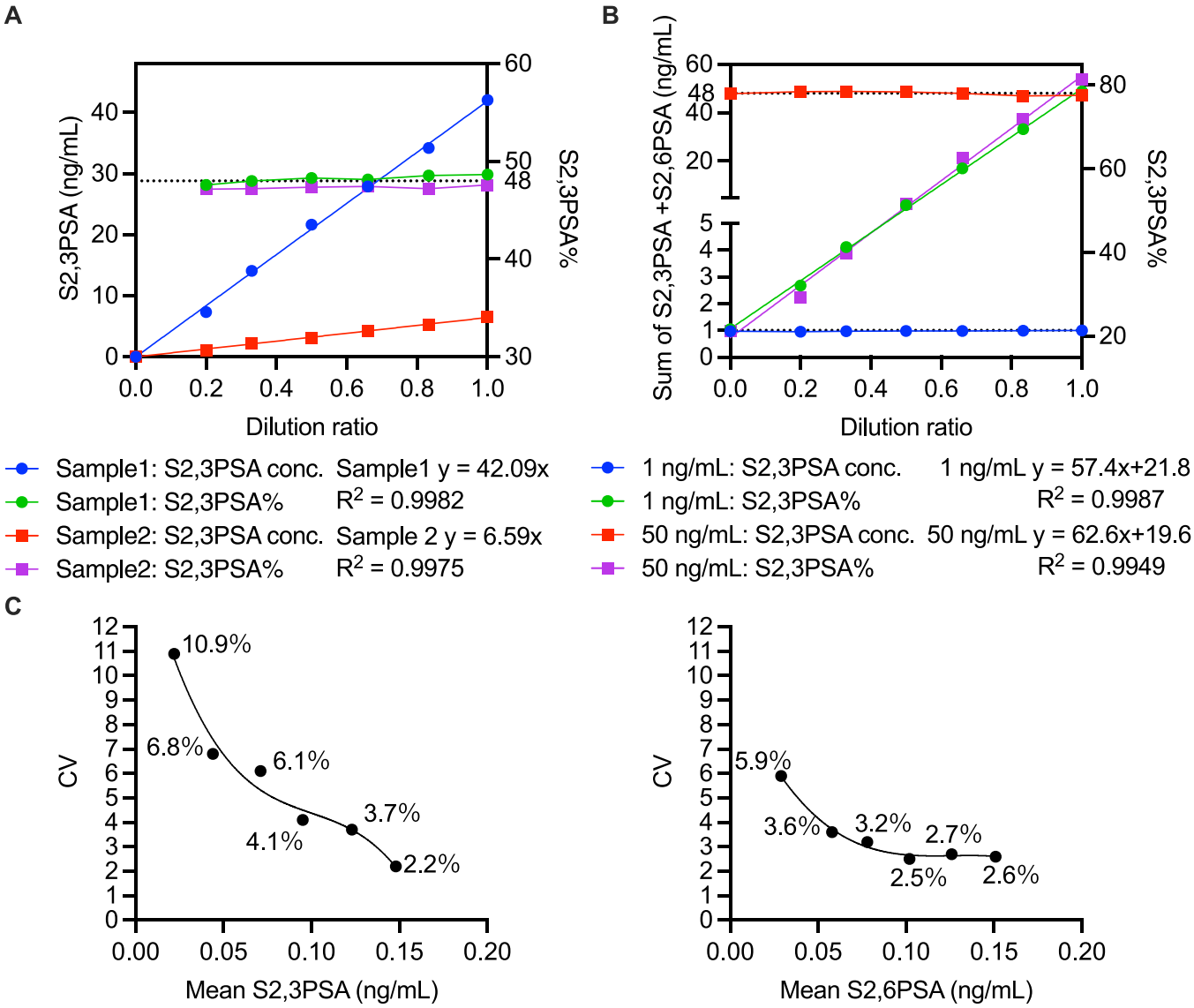

FIGURE 2 | (A) Dilution linearity of S2,3PSA concentration (ng/mL). (B) Dilution linearity of S2,3PSA%. (C) Precision profile of S2,3PSA (ng/mL) and S2,6PSA (ng/mL). [Color figure can be viewed at wileyonlinelibrary.com]

97%–100% more accurate than baseline at each dilution rate, suggesting that dilution of sample was not affect S2,3PSA% ratio (Figure 2A).

The dilution linearity of S2,3PSA% in two concentrations with the sum of S2,3PSA and S2,6PSA samples (total 1 and 48 ng/mL) showed correlation coefficients of 0.9987 and 0.9949, respectively, and was obtained a linear line through the 20%–80% of S2,3PSA% with an accuracy of 93%–103.0% at each dilution rate (Figure 2B).

3.3 | Measurement Sensitivity

The precision profile showed that S2,3PSA had a maximum CV of 6.8% at an average of 0.044 ng/mL of measurable values

(Figure 2C), which met the LoD as 0.05 ng/mL stated in the package insert of the μTASWako S2,3PSA% i50 reagent. Although the LoD for S2,6PSA is not stated in the package insert, the CV at an average of 0.029 ng/mL of measurements was 5.9%, suggesting that 0.029 ng/mL is a reasonable S2,6PSA LoD that would be within 10% CV (Figure 2C).

3.4 | Preanalytical Stability in Whole Blood

The patient characteristics for preanalytical stability are shown in Table 2. Of the 20 patients, 11 were MRI-TBx(+) and 9 were MRI-TBx(−), with a median prebiopsy t-PSA of 8.3 ng/mL (1.7–24.5 ng/mL). As shown in Figure 3A and Table 3, the mean percentage change (SD) of S2,3PSA and S2,6PSA in whole blood at 25°C for 1 h as compared to baseline values was 95.5% (4.3%) and 96.7% (3.0%), respectively, at 3 h was already to 91.6% (5.4%) and 92.8% (4.0%), respectively, and then decreased continuously with time to 81.0% (6.7%) and 82.6% (5.9%) at 24 h, respectively. The changes in the mean percentage change (SD) of S2,3PSA and S2,6PSA in whole blood at 6°C showed a trend similar to those at 25°C (Figure 3A). The S2,3PSA and S2,6PSA preanalytical stability in whole blood was favorable at time points within 1 h. Although the mean percentage change of S2,3PSA% in whole blood at 24 h at 25°C and 6°C as compared to baseline was 99.2% (1.5%) and 99.9% (1.6%), respectively, S2,3PSA and S2,6PSA concentrations decreased by ~19% during 24 h in whole blood (Figure 3A).

3.5 | Preanalytical Stability in Serum

As shown in Figure 3B and Table 3, the mean percentage change (SD) of S2,3PSA and S2,6PSA concentrations in serum at 6°C for 1 h as compared to baseline values was 99.2% (3.6%) and 99.3% (1.9%), respectively, at 3 h was to 97.7% (4.3%) and 98.8% (3.6%), respectively, at 8 h was to 96.1% (4.8%) and 96.7% (4.9%), respectively, and then decreased continuously with time to 93.5% (7.3%) and 93.9% (5.7%) at 24 h, respectively, at 30 days was 66.6% (13.7%) and 66.2% (13.6%), respectively, at 90 days was 84.6% (20.1%) and 83.1% (16.4%), respectively. The S2,3PSA% value remained almost unchanged after 90 days of storage at 99.7% (7.3%) relative to the immediate measurement (Figure 3B). Both S2,3PSA and S2,6PSA concentrations as compared to baseline values decreased to about 84% at 90 days, and since the percentage decrease in S2,3PSA and S2,6PSA concentrations were almost equal, it was S2,3PSA% were considered unchanged. Therefore, the S2,3PSA and S2,6PSA preanalytical stability in serum was favorable at time points within 8 h at 6°C. As shown in Figure 3B and Table 3, the mean percentage change (SD) of serum S2,3PSA and S2,6PSA concentrations and S2,3PSA% value under −20°C storage condition for 90 days as compared to baseline values was 100.2% (4.7%) and 100.3% (2.9%), respectively, showed better specimen stability than the refrigerated condition (84.6% (20.1%), 83.1% (16.4%), and 99.7% (7.3%), respectively).

TABLE 2 | Patient characteristics of preanalytical stability ($n = 20$).

Age (years)	72 (55–80)
t-PSA (ng/mL)	8.3 (1.7–24.5)
S2,3PSA (ng/mL) at baseline	0.51 (0.19–2.58)
S2,6PSA (ng/mL) at baseline	0.65 (0.26–4.73)
S2,3PSA% (%) at baseline	42.8 (30.1–61.8)
MRI-TBx(+)	11 (55%)
MRI-TBx(−)	9 (45%)

Note: All data presented median (range) or *n* (%).
Abbreviations: MRI-TBx = MRI targeted prostate biopsy, S2,6PSA = α2,6-sialylated f-PSA, S2,3PSA = α2,3-sialylated f-PSA, S2,3PSA% = S2,3PSA ratio, t-PSA = total PSA.

3.6 | Preanalytical Stability Under Repeated Freeze–Thaw Cycles

The mean percentage change (SD) of S2,3PSA, S2,6PSA, and S2,3PSA% in serum was very stable, 98.0% (5.2%), 98.7% (4.7%), and 99.8% (1.2%), respectively, after five freeze–thaw cycles compare to baseline values (Figure 3C and Table 3).

3.7 | Interference Studies

The percentage change in measured S2,3PSA and S2,3PSA% with the addition of interfering substances or drugs ranged from 104.3% to 114.4% and 96.3% to 98.8% with the addition of ascorbic acid, 95.7% to 109.1% and 98.9% to 100% with the addition of hemoglobin, 97.8% to 101.1% and 99.1% to 100.9% with the addition of bilirubin C, 97.8% to 104.4% and 99.1% to 101.4% with the addition of bilirubin F, 95.6% to 104.4% and 99.4% to 101.2% with the addition of chyle, 97.7% to 100.6% and 98.8% to 100.7% with the addition of RF, 97.0% to 100% and 99.1% to 100.9% with the addition of dutasteride, 95.1% to 100% and 95.6% to 101.6% with the addition of prazosin HCl, 95.1% to 103.1% and 97.7% to 100.6% with the addition of leuprorelin acetate, and 97.5% to 100% and 99.4% to 101.2% with the addition of docetaxel (Figure 4A–J).

3.8 | Diagnostic Performance of S2,3PSA% as Compared to PI-RADS or *phi*

We investigated the head-to-head diagnostic performance between S2,3PSA% and PI-RADS or *phi* in the prospective observational MRI-TBx cohort. The characteristics of Cohort 1, 253 patients, are shown in Table 4. Among the 107 MRI-TBx(−) patients, 55.1% ($n = 59$) had no evidence of malignancy in the biopsy specimen, 29.0% ($n = 31$) had prostatic hyperplasia, 7.5% ($n = 8$) had prostatitis, 8.4% ($n = 9$) had both prostatic hyperplasia and prostatitis in the biopsy specimen. Among the 146 patients with PCa, 27 were classified as GG1, and the remaining 119 patients with high-grade PCa were classified as GG2 (34, 23.3%), GG3 (16, 11.0%), GG4 (41, 28.1%), and GG5 (28, 19.2%). We found significant differences in age, prostate volume, t-PSA, PI-RADS, and S2,3PSA% levels between men with MRI-TBx(−) and MRI-TBx(+) ($p < 0.0001$, $p = 0.0005$, $p < 0.0001$, and $p < 0.0001$, respectively) (Table 4).

The AUC of S2,3PSA% for detection of PCa (0.776; 95% CI, 0.719–0.832) was higher than that of t-PSA (0.627; 95% CI,

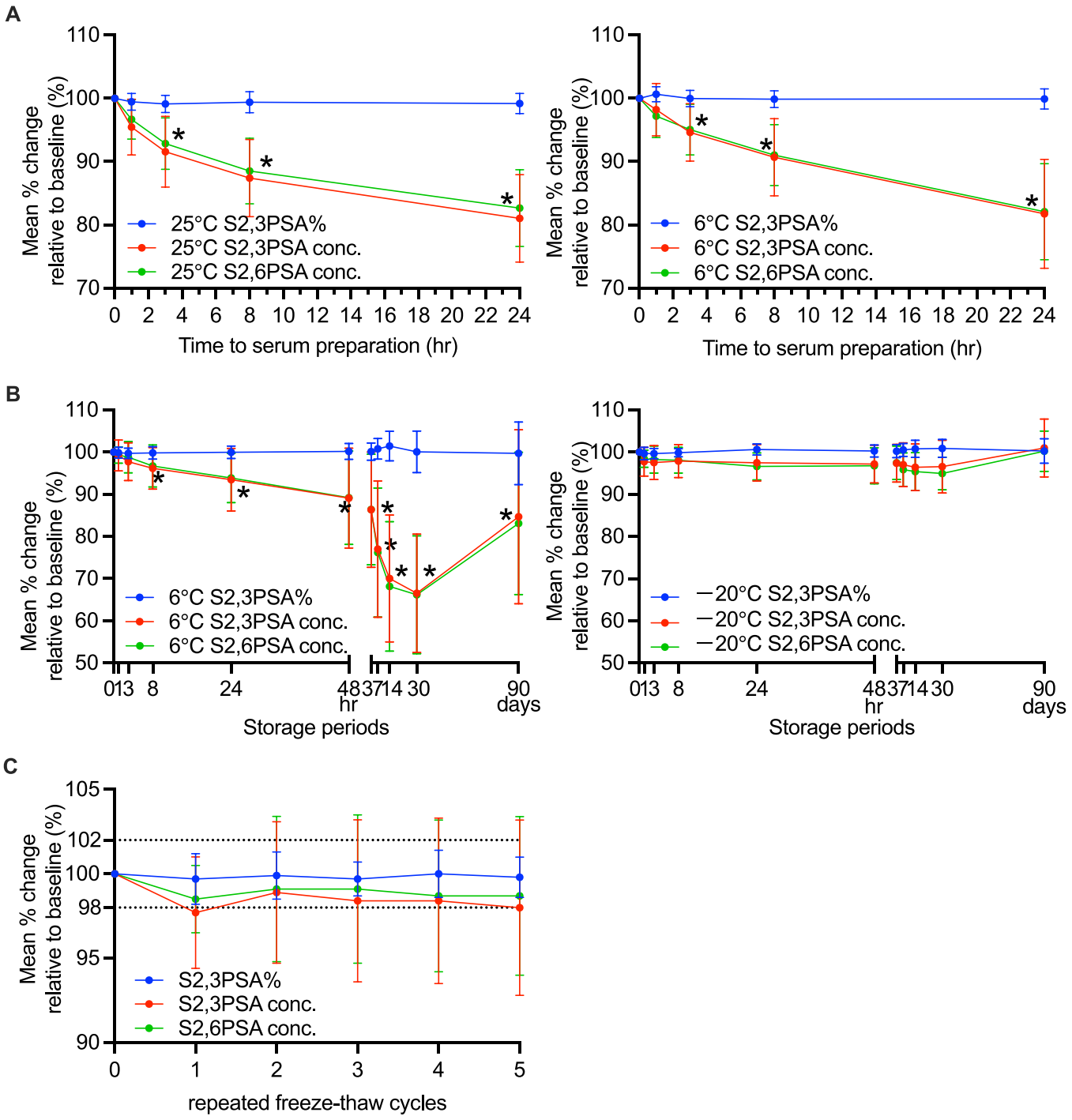

FIGURE 3 | Stability of S2,3PSA%, S2,3PSA (mg/mL) and S2,6PSA (ng/mL) in whole blood and in serum. (A) Time to serum preparation at 25°C and at 6°C, and (B) storage period in serum at 6°C and at −20°C and (C) at repeated freeze–thaw. *$p < 0.05$ compared with baseline. [Color figure can be viewed at wileyonlinelibrary.com]

0.559–0.696) ($P = 0.0010$), and comparable to PI-RADS (0.746; 95% CI, 0.685–0.807) ($p = 0.5312$) (Figure 5A,B and Table 5). When applying a cutoff value as described previously for each assays [13, 17], S2,3PSA% ≥ 38.0%, PI-RADS ≥ 3, and t-PSA ≥ 4 ng/mL, respectively, the sensitivity and specificity of S2,3PSA% were 84.2% and 43.0%, respectively, was higher specificity than those of PI-RADS (100% and 3.7%), and t-PSA (95.2% and 11.2%) (Table 5). The false positive rate (FPR) for S2,3PSA% was 57.0%, lower than for PI-RADS (96.3%) and t-PSA (88.8%) (Table 5). The false negative rate (FNR) for S2,3PSA% was 15.8%, higher than for PI-RADS (0%) and t-PSA (4.8%) (Table 5). When applying above cutoff value of each assay in PI-RADS3 cases, MRI-TBx(+) detection rate of S2,3PSA %-positive cases (39%, 15/38 cases) was higher than that of t-PSA-positive cases (32%, 17/53 cases) (Figure 5C). MRI-TBx (−) detection rate of S2,3PSA-negative cases (83%, 19/23 cases) was higher than that of t-PSA-negative cases (75%, 6/8) (Figure 5C). In PI-RADS ≥ 4 cases, MRI-TBx(+) detection rate of S2,3PSA%-positive cases (76%, 108/143 cases) was higher than that of t-PSA-positive cases (69%, 122/177 cases)

TABLE 3 | Percentage mean recovery under various storage conditions for S2,3PSA%.

	Whole blood storage under				Serum storage under			
S2,3PSA	**25°C**		**6°C**		**6°C**		**−20°C**	
0 h	100.0	(0.0)	100.0	(0.0)	100.0	(0.0)	100.0	(0.0)
1 h	95.5	(4.3)	98.2	(4.0)	99.2	(3.6)	97.7	(3.3)
3 h	91.6	(5.4)	94.6	(4.4)	97.7	(4.3)	97.5	(3.9)
8 h	87.4	(5.9)	90.7	(5.9)	96.1	(4.8)	97.9	(3.8)
24 h	81.0	(6.7)	81.7	(8.4)	93.5	(7.3)	97.5	(4.2)
2 days	—		—		89.1	(11.5)	97.2	(4.3)
3 days	—		—		86.4	(13.3)	97.4	(4.4)
7 days	—		—		77.0	(15.7)	97.0	(5.1)
14 days	—		—		70.0	(14.7)	96.4	(5.4)
30 days	—		—		66.6	(13.7)	96.6	(6.1)
90 days	—		—		84.6	(20.1)	101.0	(6.7)
S2,6PSA	**25°C**		**6°C**		**6°C**		**−20°C**	
0 h	100.0	(0.0)	100.0	(0.0)	100.0	(0.0)	100.0	(0.0)
1 h	96.7	(3.0)	97.2	(3.3)	99.3	(1.9)	98.5	(2.0)
3 h	92.8	(4.0)	95.1	(3.9)	98.8	(3.6)	98.3	(3.1)
8 h	88.5	(5.0)	91.0	(4.7)	96.7	(4.9)	98.1	(3.0)
24 h	82.6	(5.9)	82.1	(7.4)	93.9	(5.7)	96.6	(3.1)
2 days	—		—		89.2	(10.8)	96.8	(4.2)
3 days	—		—		86.4	(12.8)	97.5	(3.9)
7 days	—		—		76.2	(14.9)	95.9	(3.9)
14 days	—		—		68.1	(15.0)	95.4	(4.4)
30 days	—		—		66.2	(13.6)	94.9	(3.8)
90 days	—		—		83.1	(16.4)	100.2	(4.7)

Freeze–thaw cycles	**S2,3PSA**		**S2,6PSA**		**S2,3PSA%**	
Baseline	100.0	(0.0)	100.0	(0.0)	100.0	(0.0)
1	97.7	(3.3)	98.5	(2.0)	99.7	(1.5)
2	98.9	(4.2)	99.1	(4.3)	99.9	(1.4)
3	98.4	(4.8)	99.1	(4.4)	99.7	(1.0)
4	98.4	(4.9)	98.7	(4.5)	100.0	(1.4)
5	98.0	(5.2)	98.7	(4.7)	99.8	(1.2)

Note: All data presented as % recovery to baseline value, mean (SD).

(Figure 5C). MRI-TBx(−) detection rate of S2,3PSA-negative cases (58%, 26/45 cases) was higher than that of t-PSA-negative cases (55%, 6/11) (Figure 5C).

Although the Spearman's correlation coefficient between S2,3PSA% and GG (0.170; 95% CI, 0.003–0.328; $p = 0.0398$) and PI-RADS score and GG (0.101; 95% CI, −0.068 to 0.263; $p = 0.2272$) were both less correlated with GG, S2,3PSA% was more positively correlated with GG than PI-RADS score (Figure 5D and Table 6).

We further investigated the head-to-head diagnostic performance between S2,3PSA and *phi* or PI-RADS. The characteristics of Cohort 2, 145 patients, are shown in Table 4. Among the 62 MRI-TBx(−) patients, 54.8% ($n = 34$) had no evidence of malignancy in the biopsy specimen, 29.0% ($n = 18$) had prostatic hyperplasia, 6.5% ($n = 4$) had prostatitis, and 9.7% ($n = 6$) had both prostatic hyperplasia and prostatitis in the biopsy specimen. Among the 83 patients with PCa, 15 were classified as GG1, and the remaining 68 patients with high-grade PCa were classified as GG2 (18, 21.7%), GG3 (10, 12.0%), GG4 (24, 28.9%), and GG5 (16, 19.3%). We found significant differences in age, prostate volume, t-PSA, PI-RADS, *phi*, and S2,3PSA% levels between men with MRI-TBx(−) and MRI-TBx(+) ($p = 0.0201$, $p < 0.0001$, $p = 0.0002$, $p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively) (Table 4).

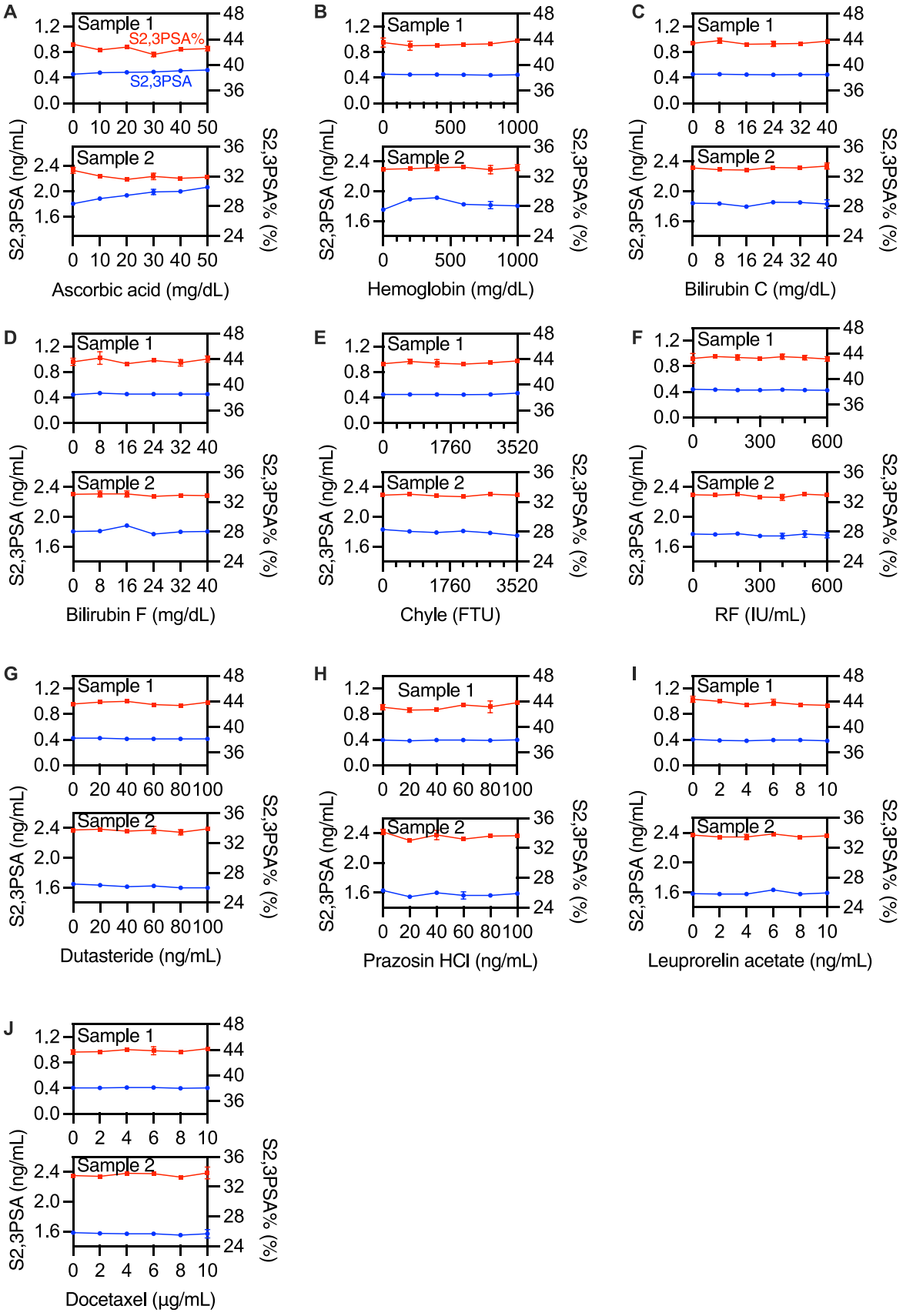

FIGURE 4 | Effect of S2,3PSA concentration (ng/mL) and S2,3PSA% by the presence of (A) 0–50 mg/dL ascorbic acid, (B) 0–1000 mg/dL hemoglobin, (C) 0–40 mg/dL bilirubin C, (D) 0–40 mg/dL bilirubin F, (E) 0–3520 FTU chyle, (F) 0–600 IU/mL rheumatoid factor, (G) 0–100 ng/mL dutasteride, (H) 0–100 ng/mL prazosin HCl, (I) 0–10 ng/mL leuprorelin acetate, and (J) 0–10 μg/mL docetaxel. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 4 | Patient characteristics of prospective observational study in MRI-TBx cohort.

Cohort 1 $n = 253$	**MRI-TBx(−) $n = 107$**	**MRI-TBx(+) $n = 146$**	***p* value**
Age	67 (62–72)	71 (66–75)	0.0012
Prostate volume	57.0 (37.8–76.3)	35.3 (25.0–48.1)	< 0.0001
t-PSA (ng/mL)	7.39 (5.08–11.10)	9.38 (6.23–16.60)	0.0005
PI-RADS	4 (3–4)	5 (4–5)	< 0.0001
S2,3PSA% (%)	39.7 (33.0–43.9)	49.0 (41.4–59.0)	< 0.0001
No malignancy *n*, (%)	59 (55.1%)	0 (0%)	
Prostatic hyperplasia *n*, (%)	31 (29.0%)	44 (30.1%)	
Prostatitis *n*, (%)	9 (8.4%)	9 (6.2%)	
Prostatic hyperplasia and prostatitis *n*, (%)	8 (7.5%)	3 (2.1%)	
PCa without benign disease	0 (0%)	89 (61.0%)	
PI-RADS *n*, (%)			
1	1 (0.9%)	0 (0%)	
2	3 (2.8%)	0 (0%)	
3	42 (39.3%)	19 (13.0%)	
4	47 (43.9%)	50 (34.2%)	
5	14 (13.1%)	77 (52.7%)	
Clinical T stage *n*, (%)			
T1c	87 (81.3%)	100 (68.5%)	
T2	13 (12.1%)	20 (13.7%)	
T3	4 (3.7%)	24 (16.4%)	
T4	0 (0%)	1 (0.7%)	
Unknown	3 (2.8%)	1 (0.7%)	
Grade group *n*, (%)			
GG1		27 (18.5%)	
GG2		34 (23.3%)	
GG3		16 (11.0%)	
GG4		41 (28.1%)	
GG5		28 (19.2%)	

Cohort 2 $n = 145$	**MRI-TBx (−) $n = 62$**	**MRI-TBx (+) $n = 83$**	***p* value**
Age	68 (63–72)	71 (66–76)	0.0201
Prostate volume	57.5 (42.7–78.0)	37.7 (24.3–46.4)	< 0.0001
t-PSA (ng/mL)	6.72 (4.74–10.6)	10.9 (6.18–17.8)	0.0002
PI-RADS	4 (3–4)	5 (4–5)	< 0.0001
phi	32.5 (24.5–44.9)	72.0 (46.5–104.0)	< 0.0001
S2,3PSA% (%)	37.4 (32.0–44.1)	52.4 (43.6–63.0)	< 0.0001
No malignancy *n*, (%)	34 (54.8%)	0 (0%)	

(Continues)

TABLE 4 | (Continued)

Cohort 2 $n = 145$	MRI-TBx (−) $n = 62$	MRI-TBx (+) $n = 83$	p value
Prostatic hyperplasia *n*, (%)	18 (29.0%)	27 (32.5%)	
Prostatitis *n*, (%)	4 (6.5%)	4 (4.8%)	
Prostatic hyperplasia and prostatitis *n*, (%)	6 (9.7%)	4 (4.8%)	
PCa without benign disease	0 (0%)	48 (57.8%)	
PI-RADS *n*, (%)			
1	1 (1.6%)	0 (0%)	
2	1 (1.6%)	0 (0%)	
3	26 (41.9%)	11 (13.3%)	
4	29 (46.8%)	26 (31.3%)	
5	5 (8.1%)	46 (55.4%)	
Clinical T stage *n*, (%)			
T1c	52 (83.9%)	54 (65.1%)	
T2	6 (9.7%)	11 (13.3%)	
T3	2 (3.2%)	17 (20.5%)	
T4	0 (0%)	1 (1.2%)	
Unknown	2 (3.2%)	0 (0%)	
Grade group *n*, (%)			
GG1		15 (18.1%)	
GG2		18 (21.7%)	
GG3		10 (12.0%)	
GG4		24 (28.9%)	
GG5		16 (19.3%)	

Note: All data presented median (interquartile range) or *n* (%).
Abbreviations: GG = grade group, MRI-TBx(−) = MRI targeted prostate biopsy negative, MRI-TBx(+) = MRI targeted prostate biopsy positive, *phi* = prostate health index, PI-RADS = prostate imaging-reporting and data system, S2,3PSA% = α2,3-sialylated f-PSA ratio, t-PSA = total PSA.

The AUC of S2,3PSA% for detection of PCa (0.837; 95% CI, 0.774–0.901) was higher than that of t-PSA (0.678; 95% CI, 0.592–0.765) ($p = 0.0040$), and comparable to PI-RADS (0.779; 95% CI, 0.703–0.854) ($p = 0.3037$), and *phi* (0.867; 95% CI, 0.809–0.926) ($p = 0.3000$) (Figure 6A,B and Table 5). When applying a cutoff value as described previously for each assays [13, 17], S2,3PSA % ≥ 38.0%, *phi* ≥ 27.2, PI-RADS ≥ 3, and t-PSA ≥ 4 ng/mL, respectively, the sensitivity and specificity of S2,3PSA% were 88.0% and 53.2%, respectively, which were higher specificity than *phi* (97.6% and 29.0%), PI-RADS (100% and 3.2%) and t-PSA (92.8% and 14.5%) (Table 5). The FPR for S2,3PSA% was 46.8%, lower than for *phi* (71.0%), PI-RADS (96.8%), and t-PSA (85.5%) (Table 2). The FNR for S2,3PSA% was 12.0%, higher than for *phi* (2.4%), PI-RADS (0%), and t-PSA (7.2%) (Table 5).

When applying above cutoff value of each assay in PI-RADS3 cases, MRI-TBx(+) detection rate of S2,3PSA%-positive cases (69%, 29/42 cases) was higher than those of t-PSA-positive cases (28%, 9/32 cases), *phi*(+) with PI-RADS3 (38%, 10/26) (Figure 6C). MRI-TBx(−) detection rate of S2,3PSA-negative cases (87%, 13/15 cases) was higher than that of t-PSA-negative cases (60%, 3/5) and comparable to that of *phi*-negative cases (91%, 10/11 cases) (Figure 6C). In PI-RADS ≥ 4 cases, MRI-TBx(+) detection rate of S2,3PSA%-positive cases (82%, 64/78 cases) was higher than those of t-PSA-positive cases (71%, 68/96 cases), *phi*-positive cases (73%, 71/97 cases) (Figure 6C). MRI-TBx(−) detection rate of S2,3PSA-negative cases (71%, 20/28 cases) was higher than that of t-PSA-negative cases (60%, 6/10) and lower than that of *phi*-negative (89%, 8/9 cases) (Figure 6C).

Spearman's correlation coefficient between S2,3PSA% and GG (0.264; 95% CI, 0.045–0.459; $p = 0.0157$) was more positively correlated than that of PI-RADS score (0.178; 95% CI, −0.046 to

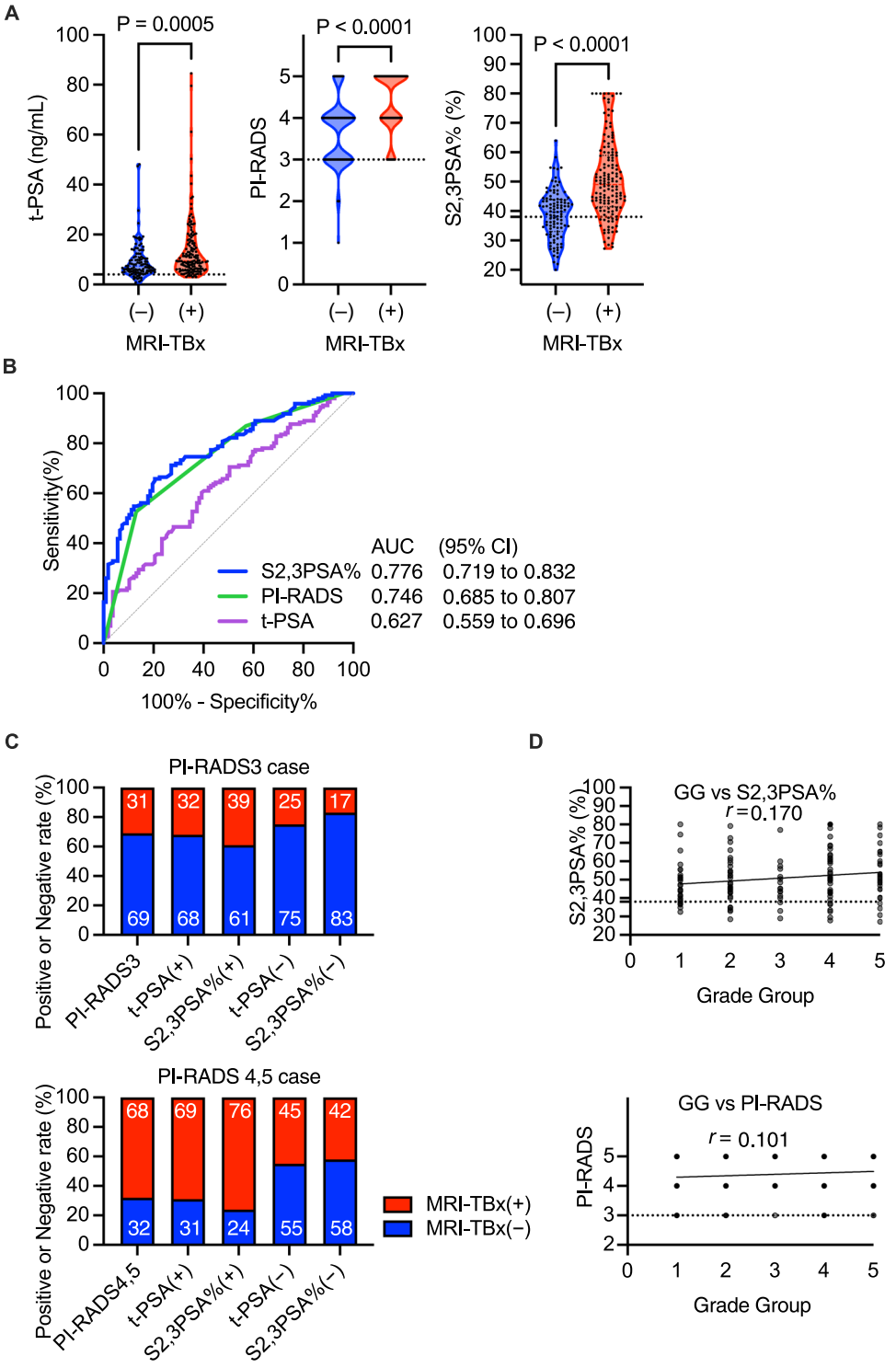

FIGURE 5 | (A) Violin plot and (B) AUC of t-PSA, PI-RADS and S2,3PSA% in the MRI-TBx(−) and MRI-TBx(+) patients with Cohort 1. (C) MRI-TBx(+) or MRI-TBx(−) rate of each assay combined with PI-RADS3 cases and each assay combined with PI-RADS≥ 4 cases. (D) The correlation between S2,3PSA% or PI-RADS and Grade group in the MRI-TBx(+) patients with Cohort 1. The dashed line indicates cutoff value of S2,3PSA% as ≥ 38.0% and PI-RADS as ≥ 3. [Color figure can be viewed at wileyonlinelibrary.com]

0.384; $p = 0.1080$), *phi* (0.207; 95% CI, −0.016 to 0.410; $p = 0.0608$) (Figure 6D and Table 6).

The AUC, specificity, PPV, NPV, FPR, and FNR of each assay when applying a cutoff value at 90% sensitivity in both Cohort 1 and Cohort 2 were shown in Supporting Information S1: Figure 1 and Supporting Information S2: Table 1.

4 | Discussion

Basic performances such as reproducibility, dilution linearity, measurement sensitivity, interference substances, and pre-analytical stability of μTASWako S2,3PSA% assay system showed favorable results. In particular, the reproducibility of S2,3PSA% value was limited to < 3.4% CV, which is considered

TABLE 5 | AUC, sensitivity, specificity, NPR, PPR, FPR, and FNR at the defined cutoff value of each assay in the prospective observational MRI-TBx cohort.

Cohort 1, $n = 253$	t-PSA	PI-RADS	S2,3PSA%
Cutoff	≥ 4.0 ng/mL	≥ 3	≥ 38.0%
AUC	0.627	0.746	0.776
95% CI	0.559–0.696	0.685–0.807	0.719–0.832
p vs. S2,3PSA%	0.0010	0.5312	
Sensitivity (%)	95.2	100.0	84.2
Specificity (%)	11.2	3.7	43.0
PPR (%)	59.4	58.6	66.8
NPR (%)	63.2	100.0	66.7
FPR (%)	88.8	96.3	57.0
FNR (%)	4.8	0.0	15.8

Cohort 2, $n = 145$	t-PSA	PI-RADS	*phi*	S2,3PSA%
Cutoff	≥ 4.0 ng/mL	≥ 3	≥ 27.2	≥ 38.0%
AUC	0.678	0.779	0.867	0.837
95% CI	0.592–0.765	0.703–0.854	0.809–0.926	0.774–0.901
p vs. S2,3PSA%	0.0040	0.3037	0.3000	
Sensitivity (%)	92.8	100.0	97.6	88.0
Specificity (%)	14.5	3.2	29.0	53.2
PPR (%)	59.2	58.0	64.8	71.6
NPR (%)	60.0	100.0	90.0	76.7
FPR (%)	85.5	96.8	71.0	46.8
FNR (%)	7.2	0.0	2.4	12.0

Abbreviations: AUC = area under the receiver operating characteristics curve, FNR = false negative rate, FPR = false positive rate, NPR = biopsy negative predictive rate, *phi* = prostate health index, PPR = biopsy positive predictive rate, PI-RADS = prostate imaging-reporting and data system, S2,3PSA% = α2,3-sialylated f-PSA ratio, t-PSA = total PSA.

TABLE 6 | Spearman's correlation coefficient between S2,3PSA% or PI-RADS or *phi* and grade group.

MRI-TBx(+) in Cohort 1	S2,3PSA% vs. GG	PI-RADS vs. GG
Spearman *r*	0.170	0.101
95% CI	0.003–0.328	−0.068 to 0.263
p value	0.0398	0.2272

MRI-TBx(+) in Cohort 2	S2,3PSA% vs. GG	PI-RADS vs. GG	*phi* vs. GG
Spearman *r*	0.264	0.178	0.207
95% CI	0.045–0.459	−0.046 to 0.384	−0.016 to 0.410
p value	0.0157	0.1080	0.0608

Abbreviations: GG = grade group, MRI-TBx(−) = MRI targeted prostate biopsy negative, MRI-TBx(+) = MRI targeted prostate biopsy positive, *phi* = prostate health index, PI-RADS = prostate imaging-reporting and data system, S2,3PSA % = α2,3-sialylated f-PSA ratio.

to have excellent reproducibility compared to the general enzyme immunological assay.

Preanalytical and analytical stability of tumor markers is essential for accurate cancer diagnosis [23]. In the present study, we characterized changes in S2,3PSA% values, which are useful in the diagnosis of PCa. The calculated S2,3PSA% results are influenced by the S2,3PSA or S2,6PSA values. S2,3PSA% took very stable at 3 months at 6°C and at −20°C, while S2,3PSA and S2,6PSA tend to decrease in a time-dependent manner after blood collection (Figure 3A,B). This suggests that the S2,3PSA% value is not affected by blood sample handling conditions shortly after blood collection, but whole blood samples should be centrifuged and separated into serum at least within 1 h after drawing blood to obtain accurate S2,3PSA and S2,6PSA results. This tendency is similar to the stability of f-PSA that was previously described [24, 25] because S2,3PSA and S2,6PSA were also f-PSA. Furthermore, since the effects of repeated freeze–thaw cycles on S2,3PSA, S2,6PSA, and S2,3PSA% values in serum were quite stable, it is recommended that the samples be stored at −20°C or below until measurement if stored for a long

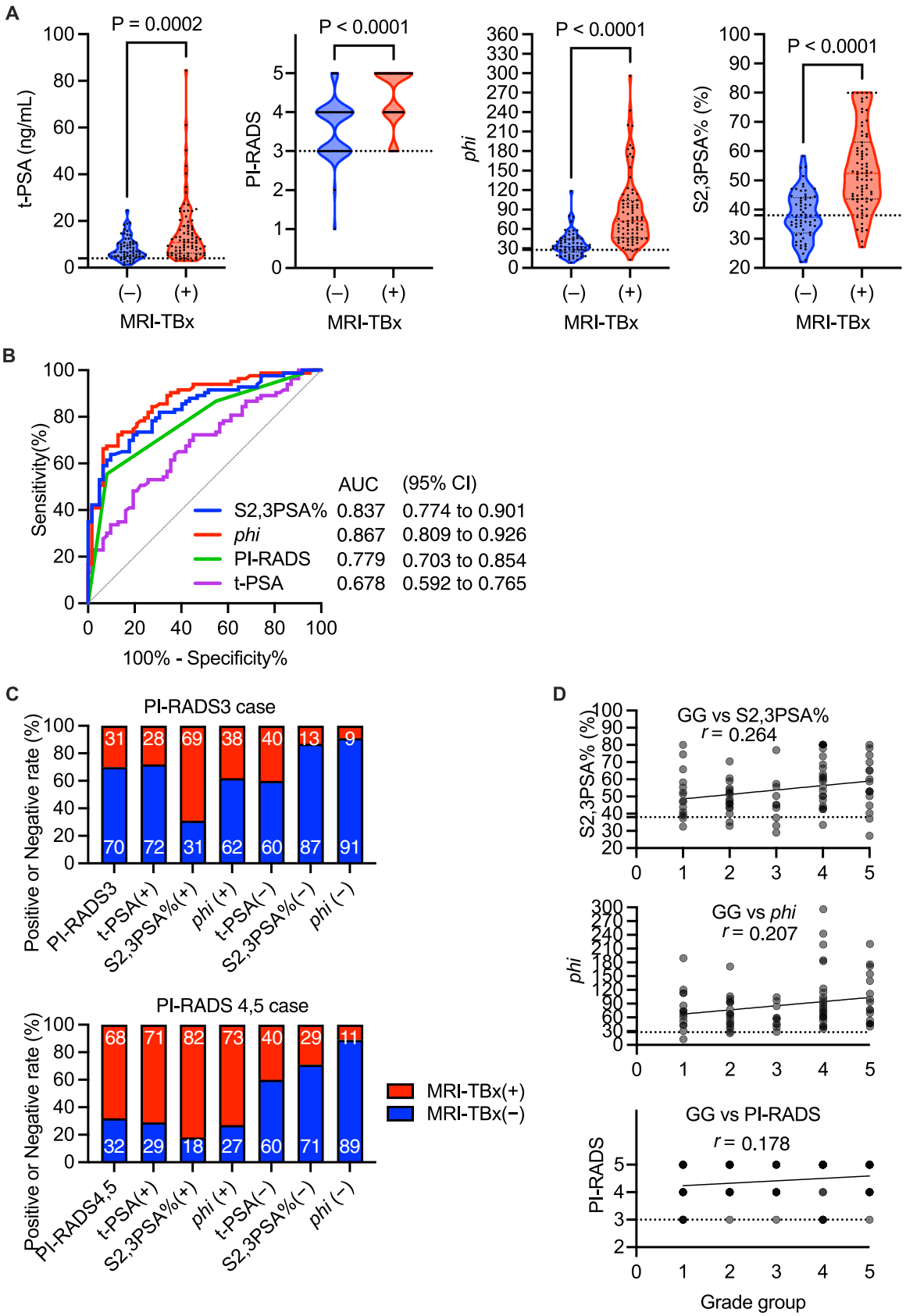

FIGURE 6 | (A) Violin plot and (B) AUC of t-PSA, PI-RADS, *phi* and S2,3PSA% in the MRI-TBx(−) and MRI-TBx(+) patients with Cohort 2. (C) MRI-TBx(+) or MRI-TBx(−) rate of each assay combined with PI-RADS3 cases and each assay combined with PI-RADS≥ 4 cases. (D) The correlation between S2,3PSA% or PI-RADS or *phi* and Grade Group in the MRI-TBx(+) patients with Cohort 2. The dashed line indicates cutoff value of S2,3PSA% as ≥ 38.0%, PI-RADS as ≥ 3, and *phi* as ≥ 27.2. [Color figure can be viewed at wileyonlinelibrary.com]

term. In addition, serum S2,3PSA and S2,3PSA% were not affected by drugs such as dutasteride, prezocin HCl, leuprorelin acetate, and docetaxel used in PCa treatment or common interfering substances. This was the first report of changes in S2,3PSA% including S2,3PSA concentration in clinical blood specimens under various handling conditions. These results showed that the preanalytical sample stability of S2,3PSA and S2,6PSA was similar to that of f-PSA and S2,3PSA% value was quite stable in the 24 h after blood collection under routine clinical handling conditions. On the other hand, [−2]pro-PSA in the serum is relatively unstable compared to t-PSA and f-PSA, and it has been reported that *phi* levels tend to increase by 5%–7% in the 1 h after blood collection under routine clinical handling conditions, which may lead to false positive results [26, 27]. Moreover, in routine clinical practice, the S2,3PSA% test is easier to handle specimens than *phi*, and is expected to reduce the workload of medical laboratory scientist.

The clinical utility of t-PSA is limited due to low cancer specificity. Among the different forms of f-PSA glycosylation isomer, S2,3PSA is a cancer-specific aberrant *N*-glycosylated f-PSA, and diagnostic accuracy of its ratio is much superior to t-PSA, F/T-PSA that can serve as a new screening marker [7, 10–13]. The prospective observational study showed that the diagnostic performance of S2,3PSA% was much superior to t-PSA, and comparable to that of PI-RADS and *phi* test in MRI-TBx cohort. When applying a cutoff value as described previously at 90% sensitivity for S2,3PSA% ≥ 38.0% [13] and *phi* ≥ 27.2, [17] respectively, the FPR of S2,3PSA% in Cohort 1 and Cohort 2 (57.0%, 46.8%) was much lower than that of PI-RADS (96.3%, 96.8) *phi* test (71.0%), suggesting S2,3PSA% test superior to PI-RADS or *phi* test in terms of reducing unnecessary biopsy.

Previously, we have already reported that the addition of S2,3PSA % to cases with PI-RADS3 or ≥ 4 cases improved the detection rate of biopsy negative and positive cases [13]. In the present study, we also obtained consistent results as reported previously. In particular, in Cohort 1 (Figure 5C), the MRI-TBx(−) detection rate (83% or 58%) of S2,3PSA% negative cases with PI-RADS3 or ≥ 4 reduced unnecessary biopsies by 8% or 3% compared to t-PSA negative cases with PI-RADS3 or ≥ 4 (75% or 55%). The MRI-TBx (+) detection rate (39% or 76%) of S2,3PSA% positive cases with PI-RADS3 or ≥ 4 reduced missing cancer by 7% or 7% compared to t-PSA positive cases with PI-RADS3 or ≥ 4 (32% or 69%). In Cohort 2 (Figure 6C), the MRI-TBx(−) detection rate (87% or 71%) of S2,3PSA% negative cases with PI-RADS3 or ≥ 4 reduced unnecessary biopsies by 27% or 11% compared to t-PSA negative cases with PI-RADS3 or ≥ 4 (60% or 60%). The MRI-TBx(+) detection rate (69% or 82%) of S2,3PSA% positive cases with PI-RADS3 or ≥ 4 reduced missing cancer by 41% or 11% compared to t-PSA positive cases with PI-RADS3 or ≥ 4 (28% or 71%). These results showed that the inclusion of S2,3PSA% and PI-RADS can further reduce unnecessary prostate biopsies. The correlation between S2,3PSA% level and Grade group was more positively correlated than those of *phi* level or PI-RADS score (Figures 5D and 6D). This result suggests that the S2,3PSA% level may have the potential to predict the aggressiveness of PCa than that of PI-RADS or *phi*. This was the first report for head-to-head comparison of clinical performance between S2,3PSA% and *phi* or PI-RADS. The limitation of this study is the relatively small sample size in a single-center prospective observational MRI-TBx cohort. Although there are limitations that invalidate comparisons between markers because different sensitivity values do not allow the comparison of specificity, the results of this study demonstrate the usefulness of S2,3PSA% with previously reported cutoff values in real-world clinical practice. For this reason, we were unable to draw further conclusions regarding the avoidable biopsy added value of S2,3PSA% in detecting PCa. Further large prospective interventional clinical trials using the S2,3PSA% will clarify the cost-effectiveness and clinical significance of the S2,3PSA% assay for avoiding MRI-TBx.

In conclusion, the measurement of S2,3PSA and S2,3PSA% in clinical specimens using μTASWako i50 performs well in terms of precision, dilution linearity, measurement sensitivity, interferences, and preanalytical stability of S2,3PSA%. The diagnostic performance of S2,3PSA% for the detection of PCa was superior to conventional strategies and comparable to the PI-RADS and *phi* test. These results suggest that the S2,3PSA% assay was suitable for the detection of aberrant glycosylated PSA in clinical specimens.

Author Contributions

The corresponding author takes full responsibility that all authors on this publication have met the following required criteria of eligibility for authorship: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved. Nobody who qualifies for authorship has been omitted from the list.

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Acknowledgments

The authors thank Mitsuharu Miyadate and Satomi Sakamoto, a technical assistant at Hirosaki University, for their invaluable help with the sample collection. This work was supported by the Japan Society for the Promotion of Science KAKENHI (Grant No. 24K12501).

Disclosure

Upon manuscript submission, all authors completed the author disclosure form. Tohru Yoneyama, Shingo Hatakeyama, and Chikara Ohyama have received payment or honoraria from FujiFilm corporation. The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, or final approval of manuscript.

Ethics Statement

This study was conducted according to the ethical standards of the Declaration of Helsinki and was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine (Approval Numbers, 2019-055, 2022-140, and 2023-021).

Consent

Written informed consent was obtained from all patients.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supporting Materials. Additional data related to this paper may be requested from the authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.