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Fifth-Generation Digital Immunoassay for Prostate-Specific Antigen by Single Molecule Array Technology

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BACKGROUND: Measurement of prostate-specific antigen (PSA) in prostate cancer patients following radical prostatectomy (RP) has been hindered by the limit of quantification of available assays. Because radical prostatectomy removes the tissue responsible for PSA production, postsurgical PSA is typically undetectable with current assay methods. Evidence suggests, however, that more sensitive determination of PSA status following RP could improve assessment of patient prognosis and response to treatment and better target secondary therapy for those who may benefit most. We developed an investigational digital immunoassay with a limit of quantification 2 logs lower than current ultrasensitive third-generation PSA assays.

METHODS: We developed reagents for a bead-based ELISA for use with high-density arrays of femtoliter-volume wells. Anti-PSA capture beads with immunocomplexes and associated enzyme labels were singulated within the wells of the arrays and interrogated for the presence of enzymatic product. We characterized analytical performance, compared its accuracy with a commercially available test, and analyzed longitudinal serum samples from a pilot study of 33 RP patients.

RESULTS: The assay exhibited a functional sensitivity (20% interassay CV) <0.05 pg/mL, total imprecision <10% from 1 to 50 pg/mL, and excellent agreement with the comparator method. All RP samples were well within the assay measurement capability. PSA concentrations following surgery were found to be predictive of prostate cancer recurrence risk over 5 years.

CONCLUSIONS: The robust 2-log improvement in limit of quantification relative to current ultrasensitive assays and the validated analytical performance of the assay allow for accurate assessment of PSA status after RP.

Measurement of prostate-specific antigen (PSA)[7] following radical prostatectomy (RP) has become standard practice for monitoring of prostate cancer recurrence. PSA is typically undetectable by most assay methods following surgery, and it is generally agreed that undetectable postsurgical PSA over time indicates a good prognosis *(1–3)*. Selection of individuals for potential postsurgical adjuvant therapy has been informed primarily by presurgical clinicopathologic information. Assessment of surgical and secondary treatment effectiveness has relied on monitoring for PSA rise using assays that are unable to measure PSA at very low concentrations. As long as PSA remains undetectable, the patient can be assured that there is no biochemical evidence of cancer recurrence. The less sensitive the assay, the longer this assurance is offered, although in fact, PSA could be rising but cannot be detected. The kinetics of rising PSA after RP can be fast or slow, and the period of undetectable PSA can be brief or on the order of years. Considerations of potential salvage therapies await the emergence of PSA above a reliably quantifiable threshold, generally 0.1 or 0.2 ng/mL (100 or 200 pg/mL). Practitioner response to reemergence of rising PSA after RP has depended on a number of factors, including the definition of biochemical recurrence (BCR), the time to biochemical failure and its potential clinical significance, clinicopathological aspects, and patient life expectancy *(4)*. Limited by the limit of quantification of PSA assay methods, BCR is generally defined as a confirmed PSA concentration of ≥0.2 ng/mL (200 pg/mL). Biochem-

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[7] Nonstandard abbreviations: PSA, prostate-specific antigen; RP, radical prostatectomy; BCR, biochemical recurrence; LoQ, limit of quantification; SiMoA, single molecule array; SβG, streptavidin:β-galactosidase; NCS, newborn calf serum; RGP, resorufin β-D-galactopyranoside; AEB, average number of label enzymes/bead; LoD, limit of detection; CLSI, Clinical and Laboratory Standards Institute; SRT, salvage radiation therapy; ART, adjuvant radiation treatment.

ical relapse occurs in up to 40% of patients after RP *(5–8)*, and a third of these patients develop metastatic disease with a 20% probability of dying within 10 years *(9, 10)*. Importantly, more clinical data are showing that early adjuvant and salvage radiation therapies after surgery have significantly improved patient outcomes *(11–14)*. However, current assays are unable to measure PSA at concentrations that could benefit patients with the earliest intervention and prospective selection for adjuvant treatment. A PSA assay with improved detection could inform a key question: Which patients are most likely to benefit, and for which patients would this represent overtreatment?

Ultrasensitive monitoring of postsurgical PSA was initially examined in the 1990s with the development of the first ultrasensitive immunoassays capable of measuring down to 10 pg/mL. A number of reports established the prognostic significance of serial ultrasensitive PSA measurements *(15–18)*. Vassilikos et al. *(17)* showed that an ultrasensitive method could detect PSA rises well before a first-generation assay could, providing potentially years of early warning. Ultrasensitive testing also elucidated different categories of recurrence: fast, slow, and apparent nonrecurrence *(17)*. Recently, an ultrasensitive RIA with a detection cutoff of 1 pg/mL was used to retrospectively explore the prognostic significance of the lowest postsurgical PSA (nadir PSA) for biochemical recurrence. Whereas PSA was undetectable in approximately half the patients, recurrence risk could nonetheless be stratified depending on nadir PSA concentration *(19)*. This result mirrored other studies *(20–24)*, indicating a clinical relevance of ultra-low PSA measurement for predicting long-term BCR-free survival.

Despite substantial data on the benefits of ultrasensitive PSA measurement, differences of opinion have persisted on the utility of such monitoring. The debate is due in part to differing views on the practical and clinical relevance of BCR, patient anxiety considerations, and both the analytical and biological noise associated with measuring ultra-low levels of PSA. It seems clear, however, that the inability to measure PSA <0.1 ng/mL (100 pg/mL) makes it more difficult to objectively assess patient response to surgical and adjuvant therapy in the timeliest manner, and it impedes identification of candidacy for salvage treatment during the critical period when residual cancer is most amenable to such treatment. The ability to accurately quantify PSA values for all RP patients could improve assessment of patient prognosis and response to treatment and better target secondary therapy to those who may benefit most.

Ultrasensitive PSA assays are referred to as third-generation because they are 2 logs more sensitive than the first generation of PSA RIAs. It is important to be clear that sensitivity (or ultrasensitivity) when referring to these assays is taken to mean functional day-to-day limit of quantification (LoQ), which is commonly defined as the lowest PSA concentration at which measurement variability over time does not exceed 20%. Thus, first-generation PSA assays exhibited LoQs of 0.5–1.0 ng/mL (500–1000 pg/mL), whereas third-generation assays are capable of detecting measurements as low as 10 pg/mL *(5)*. A fourth-generation assay would represent another 10-fold improvement (≤1 pg/mL), and fifth-generation performance would imply a LoQ of 0.1 pg/mL or better. Attempts to develop fourth-generation PSA assays have recently been reported. McDermed et al. *(25)* presented data from an immuno-PCR method with a reported LoQ of <1 pg/mL, but minimal analytical data have been published. Thaxton et al. *(26)* reported a gold nanoparticle biobarcode assay with a statistical detection limit of 0.3 pg/mL. Although the LoQ was not described, the assay was able to measure PSA in most RP specimens tested.

We previously reported preliminary data from a PSA assay based on a novel digital immunoassay technology using high-density arrays of femtoliter-volume wells and single-molecule counting *(27, 28)*. Here we report detailed analytical validation data from the assay demonstrating robust fifth-generation performance. The assay has a LoQ of <0.05 pg/mL, and reliably quantified serum PSA in all post-RP samples tested. The test can potentially be used to measure PSA in patients following primary and secondary therapy, improve BCR risk stratification, and better inform clinical decisions for use of secondary treatment.

Materials and Methods

SINGLE-MOLECULE ARRAYS

The single molecule array (SiMoA) technology *(27)* involves performing a paramagnetic bead–based ELISA, followed by isolation of individual capture beads in arrays of femtoliter-sized reaction wells. Singulation of capture beads within microwells permits buildup of fluorescent product from an enzyme label, so that signal from a single immunocomplex can be readily detected with a CCD camera. At very low PSA concentrations, Poisson statistics predict that bead-containing microwells in the array will contain either a single labeled PSA molecule or no PSA molecules, resulting in a digital signal of either "active" or "inactive" wells. At higher PSA concentrations, when all wells become occupied by at least 1 labeled PSA molecule, digital measurements transition to nondigital (analog) measurements of total fluorescence intensity. With single-molecule sensitivity, concentrations of labeling reagents can be lowered, resulting in reduced nonspecific background *(27)*. This effect enables high signal-

to-background ratios at extremely low analyte concentrations.

We prepared arrays of femtoliter-volume wells as described *(27)*. In brief, we polished the ends of bundles of 50 000 optical fibers with diamond lapping films and etched 1 end of each bundle in mild acid solution. Differential etch rates of the optical fiber core and cladding glass of the bundles causes 4.5 μm–diameter, 3.5 μm–deep wells to be formed, giving an array of 50 000 microwells across the bundle. We mounted optical fiber arrays in linear groups of 8 within glass holders for bead loading and imaging. We chose groups of 8 arrays to correspond with microtiter plate columns of 8 wells, which were used as rinse troughs for washing array surfaces after bead loading.

ELISA REAGENTS

We developed 3 reagents: paramagnetic PSA capture beads, biotinylated detector, and streptavidin:β-galactosidase (SβG) conjugate. The capture beads comprised a monoclonal anti-PSA antibody (BiosPacific) directed to amino acid residues 158–163. The antibody was covalently attached by standard coupling chemistry to 2.7-μm carboxy paramagnetic microbeads (Varian). The antibody-coated beads were diluted to a concentration of 5×10^6 beads/mL in Tris with a surfactant and BSA. Biotinylated detector reagent comprised a monoclonal anti-PSA antibody (BiosPacific) directed to amino acid residues 3–11. The antibody was biotinylated using standard methods and diluted to a concentration of 0.15 μg/mL in PBS/NCS [PBS diluent containing a surfactant and newborn calf serum (NCS)]. SβG was prepared by covalent conjugation of purified streptavidin (Thermo Scientific) and βG (Sigma) using standard coupling chemistry. For assay, we diluted aliquots of a concentrated SβG stock to 15 pmol/L in PBS/NCS with 1 mmol/L $MgCl_2$.

CALIBRATION

We calibrated the assay using WHO 90:10 PSA standards (National Institute for Biological Standards and Control). We prepared a stock PSA solution by dilution to 2 g/L in PBS/Tween-20 and assay calibrators by dilution of the stock solution in 25% NCS/PBS with Tween-20, EDTA, and ProClin 300. We prepared calibrators in a series from 0.1 to 100 pg/mL to emphasize quantification accuracy <100 pg/mL. Recovery studies indicated that use of NCS as a calibrator base gave equivalent accuracy to human serum (data not shown).

ELISA ASSAY

We conducted bead-sample incubations and labeling of immunocomplexes in conical 96-well plates (Axygen) as described *(27)*. In brief, the assay was performed in 3 steps, starting with analyte capture, incubation with biotinylated detector, and labeling of the immunocomplexes with SβG. After assay and bead collection with a magnet, beads were loaded onto the arrays for imaging in a loading buffer comprising PBS and 0.01% Tween-20, $MgCl_2$, and sucrose.

ARRAY IMAGING

We loaded beads from the ELISA onto the arrays as described *(27)*. Wells containing beads with labeled PSA were visualized by the hydrolysis of enzyme substrate [resorufin β-D-galactopyranoside (RGP), Invitrogen] by βG into fluorescent product. RGP was introduced to the wells during sealing of the arrays with a silicon gasket. Enzyme-containing wells were imaged by use of a fluorescence microscope fitted with a CCD camera. We analyzed the images to determine the average number of label enzymes/bead (AEB) as described *(28)*. At <70% active beads relative to total beads (low PSA), the signal output is a count of active beads corrected for a low statistical probability of multiple enzymes/bead *(29)*. At >70% active beads (higher PSA), the probability of multiple enzymes/bead increases, and mean fluorescence of the wells is converted to AEB based on the mean intensities of wells containing single enzymes determined at lower concentrations. The AEB unit thus works continuously across the digital and analog realms *(28)*.

RP PATIENTS

Under institutional review board approval, we obtained retrospective longitudinal serum samples from 20 RP patients without recurrence (BCR-free for 5 years) and 13 with biochemical recurrence from New York University (Urology Associates) and Johns Hopkins University and deidentified the samples. All study participants had undergone radical retropubic prostatectomy without neoadjuvant hormonal therapy. Targeted longitudinal sampling was a serum draw between 3 and 6 months after RP (nadir PSA), followed 3–6 months later by 2 subsequent draws separated by 3–6 months. Patients with positive lymph nodes at the time of surgery were excluded, as were patients who received neoadjuvant or adjuvant therapy before BCR. BCR was defined as either 2 consecutive PSA concentrations ≥0.2 ng/mL (200 pg/mL) after the initial collected sample or secondary treatment. Table 1 summarizes specific data for each patient.

SAMPLE HANDLING AND MEASUREMENT OF SERUM PSA

Specimens were stored at −70 °C until assayed. To limit effects of potential interferences, thawed samples were centrifuged at 9000*g* for 3–5 min and prediluted 1:4 in a diluent containing PBS with 0.01% Tween-20, heterophilic blocker, and EDTA before assay. Samples and calibrators were assayed in

Table 1. Patient data.

Patient	Age, years	Follow-up, years	Pre-RP PSA, ng/mL	Pathologic Gleason score	Pathologic stage	Postoperative treatment
193	54	7.8	4.8	3 + 3 = 6	pT2a	No
219	66	7.0	6.5	3 + 3 = 6	pT2b	No
120	65	8.2	5.9	4 + 4 = 8	pT2a	No
125	68	8.0	5.8	3 + 3 = 6	pT2a	No
335	67	7.2	4.9	3 + 3 = 6	pT2b	No
393	61	8.0	5.0	3 + 4 = 7	pT2b	No
157	70	6.9	5.5	3 + 4 = 7	pT2b	No
190	70	5.1	6.3	3 + 4 = 7	pT2b	No
394	46	3.8	5.7	3 + 3 = 6	pT2a	No
9458	61	10.0	15.0	3 + 2 = 5	pT2c	No
S569	61	10.1	6.9	3 + 4 = 7	pT2c	No
S1576	56	10.2	6.7	3 + 3 = 6	pT2a	No
S2278	56	10.4	4.1	5 + 4 = 9	pT2b	No
S9956	66	7.1	6.2	3 + 3 = 6	pT2c	No
A3181	63	6.4	1.5	3 + 3 = 6	pT2c	No
A6815	66	5.1	9.2	3 + 3 = 6	pT2a	No
9082	56	7.9	5.3	3 + 4 = 7	pT2c	No
8802	44	11.1	1.7	3 + 3 = 6	pT2c	No
5644	61	5.6	7.3	3 + 3 = 6	pT2a	No
9569	68	8.2	14.7	3 + 4 = 7	pT3a	No
172	62	0.5	10.0	3 + 4 = 7	pT3a	Yes
367A	61	2.0	4.3	4 + 3 = 7	pT2a	Yes
475A	63	1.0	6.5	4 + 3 = 7	pT3c	Yes
8929	61	1.9	11.2	4 + 5 = 9	pT3a	No
9908	56	3.0	13.4	3 + 2 = 5	pT2a	No
7288	59	2.0	10.0	3 + 4 = 7	pT3a	Yes
0138	62	2.0	8.7	3 + 4 = 7	pT3b	Yes
2209	57	1.2	6.6	3 + 4 = 7	pT3a	Yes
9645	46	4.4	12.2	3 + 4 = 7	pT3b	Yes
3710	57	5.3	27.9	4 + 5 = 9	pT3b	No
4789	59	6.0	7.3	4 + 5 = 9	pT2c	No
7795	67	1.1	3.5	3 + 3 = 6	pT2c	No
2049	69	0.5	5.6	4 + 3 = 7	pT3a	Yes

triplicate, and serial patient samples were tested within a single plate. Specimens above the highest calibrator were diluted 100-fold with the zero calibrator and reassayed.

Results

DOSE RESPONSE, LINEARITY, AND RECOVERY

Fig. 1 shows a representative dose response across a 3.5-log range. The assay demonstrated a highly linear response (R^2 = 0.999). In a study of 20 calibration curves over 10 days, the mean signal-to-noise ratio at 0.1 pg/mL was 4.33 (SD 0.76). Linearity, conducted with guidance from Clinical and Laboratory Standards Institute (CLSI) protocol EP6-A *(30)*, was evaluated with admixtures of female serum exhibiting relatively high and very low PSA concentrations (Fig. 1B). Linear (depicted) and third-order polynomial fit goodness was virtually identical (R^2 = 0.988 and 0.990, respectively). Percent deviation from linearity between the 2 models was within 5% across the range. Recovery of spiked PSA from serum in the absence and presence of

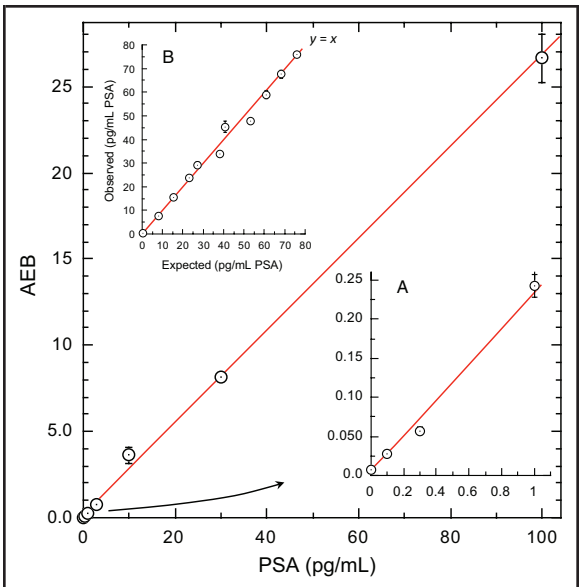

Fig. 1. Dose response and linearity of SiMoA PSA assay.

y Axis reflects the signal units of SiMoA technology (AEB). Fitting for optimal read-back used 4-parameter logistical regression.

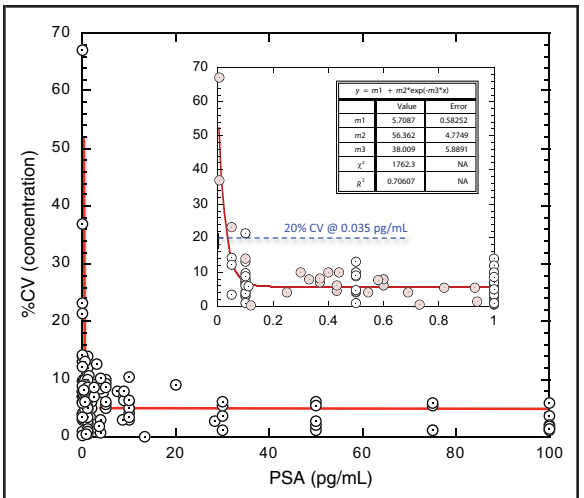

Fig. 2. LoQ of SiMoA PSA assay.

LoQ was estimated by a nonlinear power fit of sample replicate CVs across 6 weeks of testing. Female serum samples are highlighted in pink.

supplemented high concentrations of potential endogenous interferences [20 mg/dL (342 μmol/L) bilirubin, 1000 mg/dL (11.3 mmol/L) triglycerides, 12 g/dL (120 g/L) protein, 20 mg/dL (12.4 mmol/L) hemoglobin] was within 10% of expected.

SENSITIVITY

The analytical limit of detection (LoD) was estimated as 3 SDs above background. The LoD was calculated for each of 20 calibration runs from triplicate measurements of the zero calibrator and the lowest PSA-containing calibrator (0.1 pg/mL). The mean LoD was 0.028 pg/mL (SD 0.039 pg/mL). The LoQ was estimated from sample replicate CVs (n = 3) obtained across the assay range over 6 weeks. The resulting CV profile is depicted in Fig. 2. The replicates were obtained from repeated measurement of assay calibrators, controls, and female serum. CVs for the different sample types were not statistically different. The estimated LoQ was the concentration of PSA corresponding to a 20% CV. From the equation of the power fit, the LoQ was calculated as 0.035 pg/mL (SE 0.0340–0.0387 pg/mL).

REPRODUCIBILITY

We assessed reproducibility with guidance from CLSI EP5-A2 *(31)*. Four samples, consisting of 90:10 PSA spiked into 25% NCS, were assayed in triplicate in each of 2 separate runs per day for 10 days (n = 60 for each sample). The lowest sample was prepared near the estimated LoQ of 0.035 pg/mL. Because each reportable result is based on triplicate measurements, this protocol gave 2 results/day for each sample. The plate map was configured so that each PSA result spanned multiple columns, which meant that replicates included variation from different groups of arrays. We calculated PSA results from within-plate calibration curves. Thus, the overall study comprehended array-processing variation, calibration variation, and intraassay, interassay, and day-to-day variation. The results of the study (Fig. 3) showed that total CVs across all variation sources were <10% at PSA concentrations from 1 to 52 pg/mL. The total CV for the 0.04 pg/mL sample was 18.27%, consistent with the LoQ estimate (20% CV at 0.035 pg/mL).

ACCURACY

We assessed accuracy by comparison to a commercially available equimolar PSA method standardized with WHO reference material *(32)*. We assayed 40 serum samples from normal men and 8 serum samples from RP patients with PSA concentrations high enough for measurement in the comparator method (Advia Centaur, Siemens; LoD 0.1 ng/mL) with both methods (see Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue12). All samples were diluted 100-fold before testing with the SiMoA assay. The assays

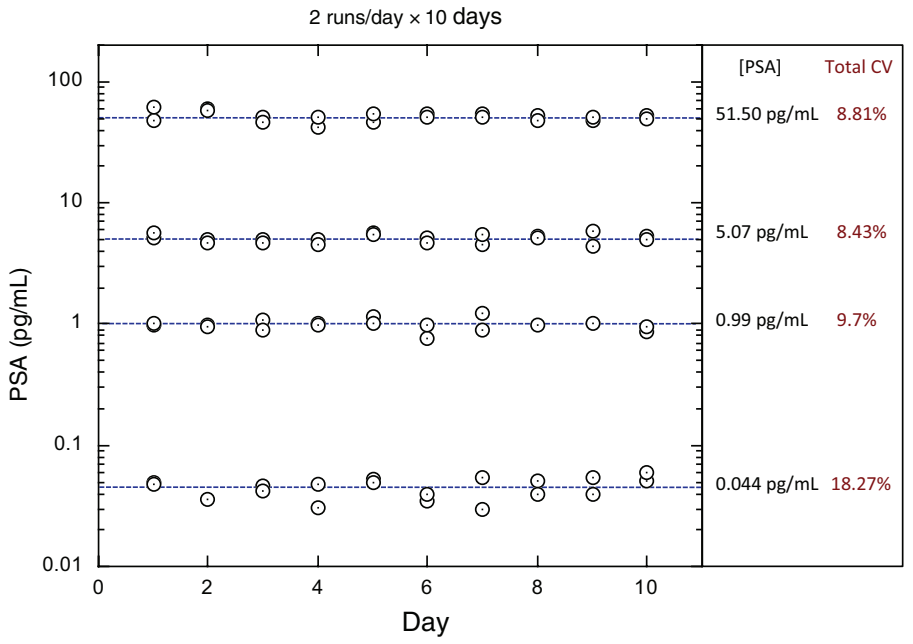

Fig. 3. Reproducibility of SiMoA PSA assay.

exhibited excellent agreement (R^2 = 0.970) with no significant bias throughout the range of results (0.17 to >13 ng/mL, mean bias 0.024 ng/mL).

CLINICAL SAMPLES

PSA results from all pilot study samples are shown in Fig. 4. Patient demographics, Gleason scores, and pathology stages were similar for recurrent (n = 13) and nonrecurrent (n = 20) groups (Table 1). Mean ages were 61.0 and 61.5 years for patients with and without recurrence, respectively. Gleason scores were 6–7 for the large majority of men (100% and 80% with and without recurrence, respectively), whereas pathology stage was fairly evenly distributed among pT2a, pT2b, and pT2c for both cohorts. Clinical stage was T1c for approximately half of men with recurrence and three-quarters of men without recurrence. The remaining men with recurrence were evenly distributed across T2a and T2b, while all (but 1) of the remaining patients without recurrence were T2a; 1 was classified T2b. The large majority of both groups exhibited negative margins (91% and 95% of men with and without recurrence, respectively.) Across all patients, approximately half of the initial PSA values were below the LoQ for commercially available third-generation assays (approximately 10 pg/mL). All samples were at least 10-fold above the LoQ of the SiMoA assay. Replicate CVs were consistent with the 10-day imprecision study.

Fig. 4 highlights the relationship between the nadir PSA and BCR. All patients with a nadir >10 pg/mL experienced biochemical relapse (red), and all patients with a nadir <1 pg/mL remained BCR-free for at least 5 years (black). Bifurcation of the data at 3.0 pg/mL resulted in a diagnostic sensitivity and specificity of 100% and 75%, respectively. This cut point is below the measurement capability of ultrasensitive PSA assays, therefore improvement in diagnostic sensitivity may be possible from reliable PSA quantification in the formally "undetectable" category. ROC analysis gave an area under the curve of 0.991, suggesting excellent discrimination between recurrent and nonrecurrent groups (see online Supplemental Fig. 2). Slopes of longitudinal PSA increases were also calculated as the median pairwise slope for each patient. According to a multivariate Cox proportional hazards model comprehending demographic, clinicopathologic, and PSA covariates, PSA nadir was a significant predictor of BCR-free survival ($P < 0.001$), whereas PSA slope was not a significant predictor in this study ($P = 0.264$) The assay's clinical performance is described in additional detail elsewhere *(33)*.

Fig. 5A highlights longitudinal data from 5-year BCR-free survivors from 1 of the clinical sites. All patients exhibited extremely low, stable PSA concentrations over the first year after surgery. Biological noise was minimal; for example, PSA values for patient 192 were 0.45, 0.51,

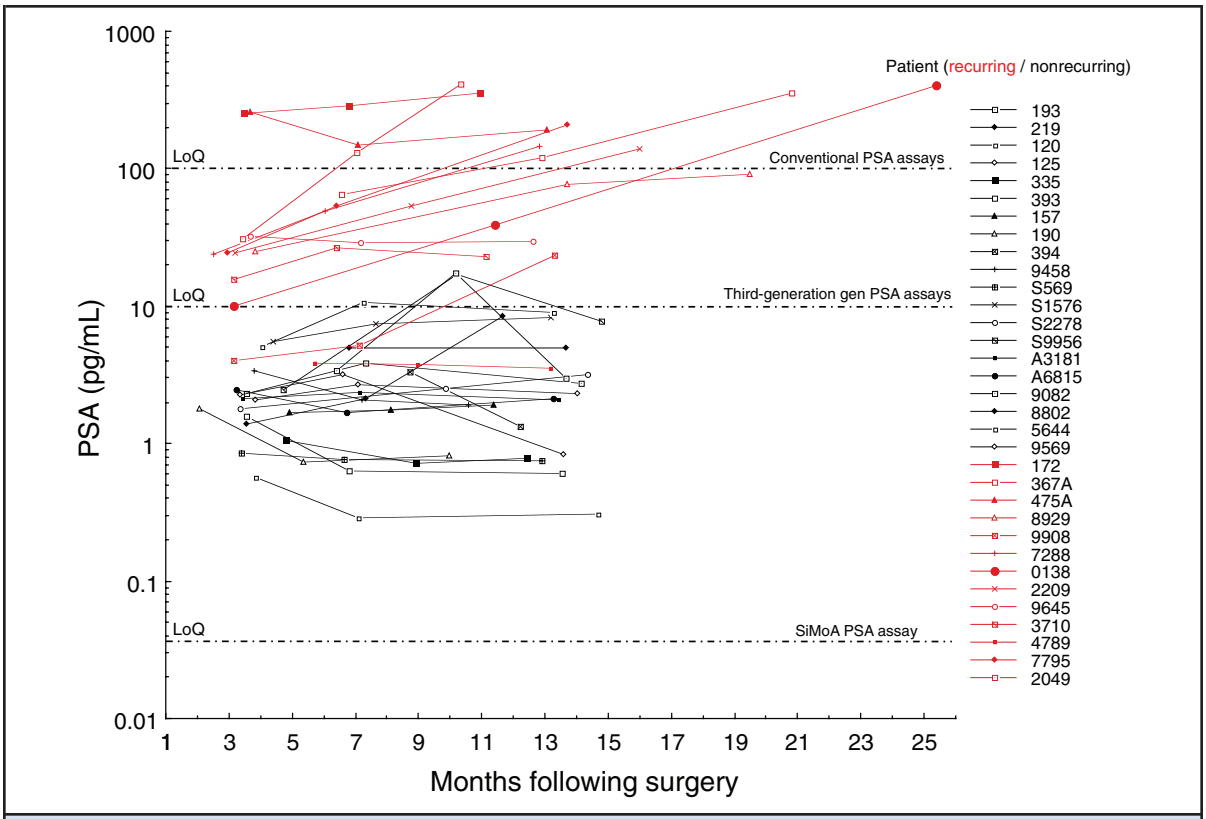

Fig. 4. Post-RP PSA results.
Longitudinal samples from 13 RP patients with recurrence (red) and 20 without (black). Horizontal lines depict LoQs.

and 0.34 pg/mL, a difference of only 0.17 pg/mL across 12 months (Fig. 5A inset). In contrast, there were other examples of patients without recurrence (patients S9956 and 9082, Fig. 4) exhibiting transient increases to >10 pg/mL, followed by PSA reduction back toward the nadir level. A similar phenomenon of lesser magnitude was noted in patients 193 and 125 (Fig. 5A). In contrast, patient 9908 (Fig. 5A red) exhibited a rapid upturn in PSA toward BCR from a similarly ultra-low PSA level. Because these patients exhibited similar presurgical clinicopathologies (T1c, Gleason 5–6, negative margins), factors contributing to successful remission in 1 patient in contrast to another at these ultra-low PSA concentrations may include surgical, biological, and immunological variables.

Fig. 5B contrasts the patients without recurrence in Fig. 5A with 3 examples of patients with recurrence. Whereas patient 9908 exhibited presurgical clinicopathology consistent with many patients without recurrence, patient 0138 exhibited less favorable pathology (T2b, pT3b with seminal vesicle invasion, Gleason 7). Perhaps not surprisingly, the patients with more aggressive recurrence (see Fig. 4) tended to have less favorable presurgical clinicopathologies.

Consistent with earlier reports *(17)*, the longitudinal profiles suggest 3 general categories of PSA kinetics following RP: fast rising, slow rising, and apparent nonrising. As shown here, however, very low nonrising PSA over a 1-year interval is no assurance of nonrecurrence. Patient 4789 exhibited highly stable, very low PSA values for 13 months after surgery (Fig. 5B inset), yet was diagnosed with BCR 5 years later. Unpredictable remission and kinetic characteristics may complicate use of PSA velocity following RP for prediction of long-term recurrence. The only safe generalization continues to be that the lower the nadir PSA, the more likely the patient will enjoy long-term BCR-free survival.

Discussion

The data presented here indicate that the SiMoA PSA assay defines a new analytical standard for PSA testing. Historically, acceptance of ultrasensitive PSA measurement has been inhibited by analytical variability, which has reduced the reliability of the information obtained from these assays. Monitoring PSA after RP is analytically demanding because it requires both high sensitivity and day-to-day reproducibility. Com-

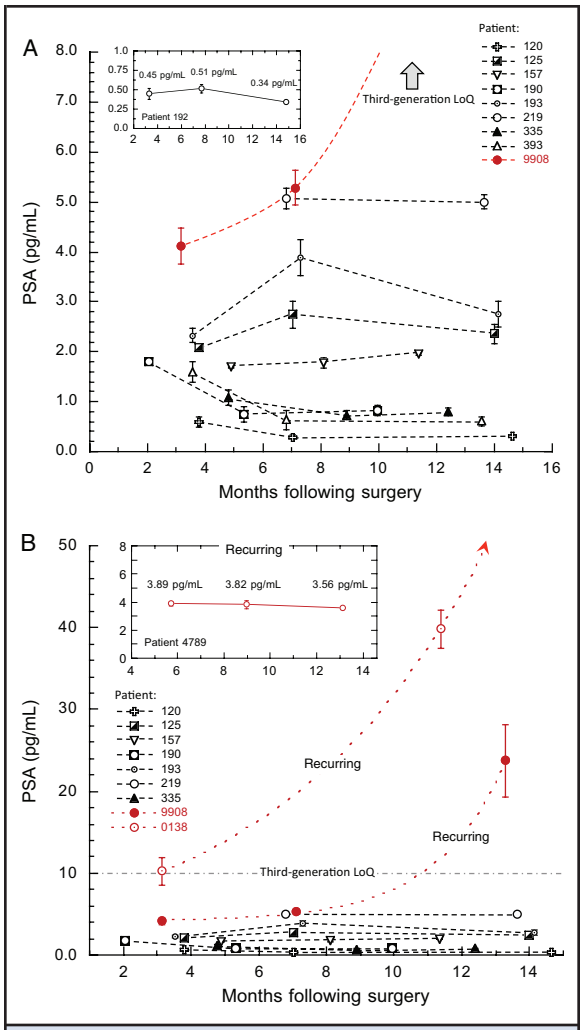

Fig. 5. Select longitudinal PSA trends.

(A), PSA results from patients without recurrence from 1 of the clinical sites. The early stages of BCR for patient 9908 (red) are also depicted. The LoQ of ultrasensitive PSA methods is off the scale (arrow). (B), Comparison of the same patients with 3 examples of patients with recurrence (red) on a broader scale. Exponential projections for the appearance of 200 pg/mL PSA for patients 9908 and 0138 (curved fits, $R^2 = 0.999$) were consistent with actual BCR. Inset depicts PSA results from a patient in remission whose disease later recurred.

pounding the difficulty has been confusion over "analytical sensitivity" (LoD) and true quantification sensitivity (LoQ). Assessing day-to-day reproducibility of results from ultra-low test samples is the most rigorous means of understanding an assay's limit of quantification. This report demonstrates analytically acceptable day-to-day reproducibility in the subpicogram range, low enough for reliable quantification of PSA in RP patients.

Robust fifth-generation measurement of PSA in all RP patients has the potential to impact management of prostate cancer in a number of clinically significant ways. Reports showing the prognostic value of nadir PSA suggest a category of patients may be identified that represents an extremely low likelihood for BCR. Data from the pilot study reported here and elsewhere *(33)* indicate that a subgroup of patients below the detection limit of current methods were recurrence-free after 5 years. As reflected in Fig. 5, PSA concentrations appear to be biochemically stable for men without recurrence. The current practice—hoping that PSA remains undetectable with analytically less sensitive detection methods—could be supplanted with reliable data indicating a highly favorable status. Positively discerning these patients with precise measurement of their PSA concentrations could improve delineation of an ultra–low-risk category with statistically powered follow-up studies.

PSA trends measured with fifth-generation sensitivity could provide the earliest possible indicator of potential aggressive BCR, with marked potential improvement in early warning time relative to current PSA methods, including third-generation methods. As shown in Fig. 5B, an exponential rise in PSA would not have been measured by a third-generation assay in patient 9908 for 11 months after surgery. Salvage radiation therapy (SRT) is more effective if administered earlier in the cancer recurrence *(13, 14)*. Stephenson et al. *(14)* found that postsurgical PSA concentrations before SRT were a highly significant predictor of disease progression, with more favorable outcomes observed at lower PSA concentrations, indicating that intervention with lower cancer burden before systemic dissemination leads to improved outcome. With this therapy model, intervention at the earliest sign of recurrence is most likely to lead to the most favorable outcome.

Reliably measuring PSA in every RP patient with fifth-generation sensitivity could also provide additional guidance on who may benefit most from adjuvant radiation treatment (ART). Evidence is growing of significant increases in overall and cancer-specific survival after ART *(34, 35)*. Only about a third of patients who have had RP develop BCR, however, and about a third of this subset develop metastases *(34)*. Which patients would benefit from ART and which patients would be overtreated remains unclear. Lower-risk pathology with nadir PSA in an ultra-low-risk group might represent a cohort for whom ART represents overtreatment. Higher-risk pathology with high nadir could be a group most likely to benefit from ART. Treatment decisions for patients between these 2

groups could be better informed by highly reliable postsurgical PSA data.

Clinical studies are needed to further examine the utility of high-resolution postsurgical PSA status. Fifth-generation sensitivity could provide more timely and reliable data around primary treatment effectiveness, monitoring intervals, selection for secondary treatment, and additional therapies based on effectiveness monitoring.

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