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Continued provision of WHO International Standards for total and free PSA: Content and commutability of replacement preparations

Jackie Ferguson[a], Dina Patel[b], Eleanor Atkinson[c], Peter Rigsby[c], Chris Burns[a,*]

[a] Biotherapeutics Group, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom
[b] UK NEQAS Immunology, Immunochemistry & Allergy, Northern General Hospital, PO Box 894, Sheffield S5 7YT, United Kingdom
[c] Biostatistics Group, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom

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ABSTRACT

Objectives: Replacements are required for the WHO International Standards (IS) for free PSA, coded 96/668 and total PSA (90:10), coded 96/670, which were established in 1999 to support efforts to harmonise PSA assays and address non-equimolarity. An important consideration is that the introduction of the replacements should have minimal impact on PSA measurements.

Design and methods: We report the development of a replacement strategy, informed by field assessment of preparations through an external quality assessment scheme and the subsequent evaluation of the candidate ISs in worldwide collaborative studies.

Results: By immunoassay, data from participants confirmed the value assigned to the current standards. Robust geometric mean estimates of the free PSA content of the candidate replacement for 96/668 coded 17/102 was 0.533 μg/ampoule (n = 21). The ratio of the content estimates of 17/102:96/668 was 0.516 (GCV 12.5%, n = 21). Robust geometric mean estimates of the total PSA content of the candidate replacement for 96/670, coded 17/100, was 0.505 μg/ampoule (n = 22). The ratio of the content estimates of 17/100:96/670 was 0.490 (GCV 5.3%, n = 22). Through concomitant measurement of a panel of 15 representative patient samples, the candidate ISs were shown to exhibit commutability with patient samples that was comparable with that of the current ISs.

Conclusion: On the basis of these results, the preparations coded 17/102 and 17/100 were established by the WHO Expert Committee on Biological Standardization as the 2nd ISs for free and total PSA (PSA-ACT + free PSA) respectively, with assigned contents of 0.53 μg/ampoule and 0.50 μg/ampoule.

1. Introduction

Measurement of serum concentrations of prostate specific antigen (PSA) by immunoassay continues to play an important role in the assessment of risk and monitoring of cancer of the prostate. Mature PSA is a 28.4 kDa kallikrein-related peptidase with a physiological role in liquefying seminal fluid [1]. In serum, the predominant, immunologically-detectable form of PSA is as a complex with α_1-antichymotrypsin (ACT), a serine protease inhibitor. Free PSA, which in serum is a population of different inactive forms of PSA, is also detectable despite the presence of a molar excess of ACT. The free PSA in serum includes populations that are not proteolytically active and forms that are internally cleaved [2,3]. Macroglobulin-associated PSA has also been detected in serum but is not considered to be immunoreactive in commercially-available assays [4].

Thus, clinical assays of PSA detect either total (PSA-ACT + free PSA) or free PSA forms and derived measurements of these such as free:total PSA ratio, PSA velocity and PSA density have been extensively evaluated for their predictive value [5]. The use of PSA-based prostate cancer population screening is controversial and meta-analyses have highlighted the risk of over-detection and subsequent over-treatment [6,7]. Also, it is recognised that the test for total PSA, used alone, has low specificity for prostate cancer detection at serum concentrations of 4–10 μg/L [8]. Historically, variability between different

Abbreviations: ALTM, all laboratory trimmed mean; EQA, external quality assessment; ECBS, Expert Committee on Biological Standardization; FDA, Food and Drug Administration; fPSA, free prostate specific antigen; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; PSA, Prostate specific antigen; PSA-ACT, PSA-α_1-antichymotrypsin; UK NEQAS, UK National External Quality Assessment Service; WHO, World Health Organization

* Corresponding author at: Biotherapeutics Group, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom.
E-mail address: chris.burns@nibsc.org (C. Burns).

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commercial methods further complicated efforts to interpret PSA values. The variability resulted in part, from non-uniform calibration and in some methods, non-equimolar recognition of the complexed and free PSA forms.

The need for standardization of PSA assays was recognised in the 1990s and prompted two conferences organised by Professor Thomas Stamey of Stanford University (Stanford, USA) [9]. Stamey proposed the use of ACT-complexed PSA (PSA-ACT) and free PSA, prepared in his laboratory from human seminal fluid, for the preparation of two 'Stanford calibrators' containing either free PSA or PSA in a 90:10 ratio of complexed:free PSA. Research by Stamey had defined the molar extinction coefficients at 280 nm of the complexed and free components by determining the amino acid content of nine representative amino acids in multiple lots of PSA-ACT or free PSA [10,11]. These coefficients were used to assign a protein concentration to the bulk preparations of PSA-ACT and free PSA from which calibrators containing 1 μg of PSA were prepared. The Stanford calibrators were adopted by manufacturers and a portion of each batch donated to WHO for establishment as the first International Standards (IS) for free PSA (96/668) and PSA (90:10) (96/670). The first WHO International Collaborative study demonstrated good agreement between the content determined by immunoassay and the assigned content of 1 μg total PSA per vial [12].

The impact of the introduction of these calibrators is shown in Fig. 1 which compares the variance (CV%) from external quality assessment (EQA) of total and free immunoassay measurements from 1992 to 2014 (Patel, D., UK NEQAS Pers. Comm.). Similarly, a survey conducted by the Japanese Urological Association in 1997 and repeated in 2000 showed an improvement in the equimolarity of the response to PSA-ACT and free PSA from a baseline where only 34% of assays were considered equimolar [13]. Prior to the availability of the WHO standards, the FDA-approved test from Hybritech used in-house calibrators. The Hybritech calibration is still used by some commercially-available immunoassays and it provides results that are around 16–25% higher than those from WHO calibrated assays. The impact of the different calibrations and the awareness of these by clinicians has been widely discussed, particularly regarding the accepted cut off, established using Hybritech-calibrated assays, of 4.0 μg/L total PSA [14–16]. The clinical impact of continued method-dependent biases has also been widely reported [17–23].

Stocks of the WHO IS for PSA (90:10), 96/670, are exhausted and those of 96/668 are depleting. To ensure continued provision of the ISs, the WHO endorsed projects proposed by NIBSC to prepare replacements. It is noted that for reference materials assigned in SI units, such as the PSA standards, the ultimate goal is to establish a reference system whereby a reference measurement procedure is used to establish a series of commutable, serum-based secondary reference materials traceable to a primary standard. The complexity of the PSA molecule, which is present in serum in both immunoreactive and non-immunoreactive complexed forms as well as multiple variants of the free form, currently precludes this approach. Therefore, our primary consideration for the preparation and value assignment of the replacements was to ensure that their introduction will have minimal impact on the values reported for patient samples. We report here, the development work which informed our strategy for the preparation of the replacement materials. Essential to the development of the candidate IS and reported here, was the evaluation of trial preparations in the EQA scheme for PSA organised by UK NEQAS Immunology, Immunochemistry & Allergy (Sheffield, UK). The candidate standards were then evaluated for content, commutability and stability through two WHO International Collaborative Studies. As part of the process to establish WHO IS, full details of the results of the collaborative studies are reported to the WHO Expert Committee on Biological Standardization (ECBS). The documents, WHO/BS/2018.2340 and WHO/BS/2018.2339 are available on-line [24,25].

2. Materials and methods

Non-complexing PSA without α-1-antichymotrypsin, purified from seminal fluid, (free PSA) was obtained from Scripps Laboratories (San Diego, USA, part no. 90024, Lot no. 2265702). The reported concentration of free PSA by absorbance at 280 nm was 1.08 mg/mL. Similarly, purified (≥95%) PSA-ACT complex (Scripps Laboratories, USA, part no. 90010, Lot no. 2445202) was provided with a reported total protein concentration of 1.57 mg/mL and a PSA concentration by Siemens Centaur immunoassay of 0.619 mg/mL. By Diasorin Liaison immunoassay, a comparable value of 0.661 mg/mL was recorded. Bovine serum albumin was obtained from EMD Millipore Corporation (Billerica, USA; Probumin, part no. 82–100). Human albumin was obtained from Bio Products Laboratory Ltd., (Elstree, UK). All other chemicals were supplied by Sigma-Aldrich (Dorset, UK). PSA and free PSA immunoassays were from Diasorin (Dartford, Kent), and Fujirebio (Gent, Belgium). Human serum samples were obtained from Cerba Specimen Services, Saint-Ouen l'Aumône, France and First Link UK, Wolverhampton, UK. These were diluted in normal male serum (First Link UK) in order to provide PSA concentrations within the range of most immunoassays.

2.1. Preparation and assessment of trial preparations

A 500 ng/mL stock solution of free PSA in formulation buffer comprising 20 mM sodium phosphate buffer pH 7.4, 150 mM sodium chloride, 10 g/L BSA was further diluted in 4.5% (v/v) human albumin in PBS to a free PSA concentration of 0.4 μg/L. Aliquots of the 0.4 μg/L preparation and an equivalent preparation of the 1st WHO IS for free PSA (96/668) were provided to globally-based participating laboratories of the UK NEQAS free PSA EQA scheme (n = 57). EQA participants were asked to measure the free PSA content of the sample using the same protocols as for their routine clinical analyses.

To assess preparations containing both PSA-ACT and free PSA, stock solutions of 500 ng/mL in terms of the relevant WHO IS were combined in volumetric ratios of 85:15, 90:10 and 92:8 PSA-ACT: free PSA. Preparations were diluted to 3 μg/L total PSA in 4.5% (v/v) human albumin in PBS and aliquots provided with those of the 1st WHO IS for PSA (90:10), also prepared at 3 μg/L, to participating laboratories of the UK NEQAS PSA EQA scheme (n = 270). Participants were asked to measure the total and free PSA concentration. The results submitted to the UK NEQAS PSA EQA scheme were analysed to produce the all laboratory trimmed mean (ALTM) which is a recalculated mean value after exclusion of all results outside 2 standard deviations from the all laboratory mean. The ALTM value allows comparison of mean values from different method groups in relation to the target value.

2.2. Preparation of a candidate standards

A 4200 mL volume of formulation buffer containing 500 ng/ml PSA in a 90:10 ratio of PSA-ACT:fPSA was prepared from stock solutions calibrated in terms of the current WHO IS (96/670 and 96/668, respectively). The bulk formulation was distributed into 3 mL glass ampoules at 1.0 mL per ampoule. After lyophilisation and sealing under nitrogen according to procedures described by WHO [26], the batch, coded 17/100, was stored at −20 °C. The total and free PSA immunoreactivity of 17/100 were compared to the current standard, 96/670, by ELISA (Fujirebio) and through the distribution of samples with a total PSA concentration of 4 μg/L to participants in the UK NEQAS EQA scheme for PSA, as described in section 2.1. Similarly, an 8420 mL volume of formulation buffer containing 500 ng/mL free PSA was used to prepare a batch of ampoules, coded 17/102.

2.3. WHO collaborative studies

The candidate preparations, 17/100 and 17/102, were assessed

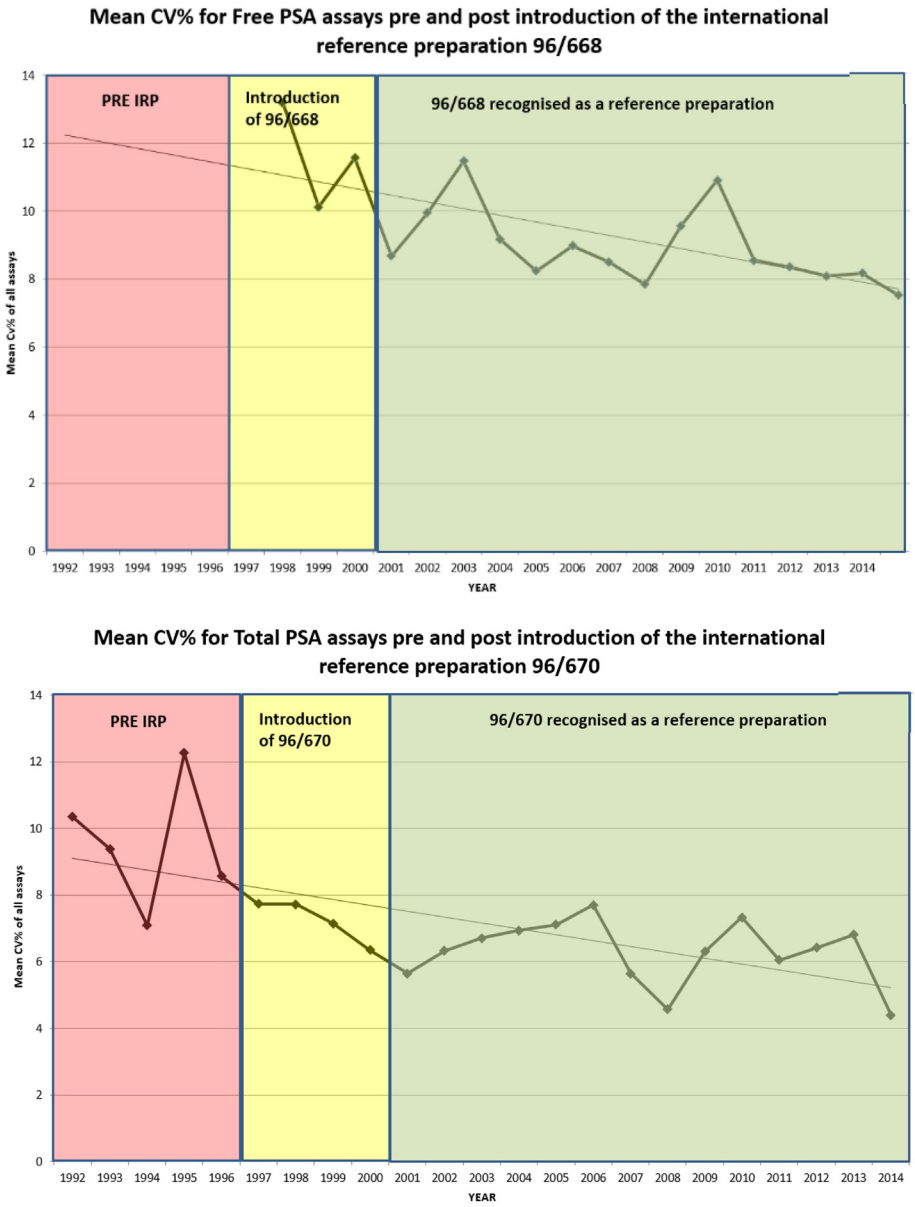

Fig. 1. Improvement in the inter-method variability (CV%) for immunoassays of total and free PSA following the introduction of WHO IS preparations 96/670 and 96/668, as compiled from EQA data (UK NEQAS EQA scheme for PSA) from 1992 to 2014. [IRP: International Reference Preparation].

Table 1
List of participants of the WHO International Collaborative Study in order of country.

Belgium	Dr Stefaan Marivoet Tosoh Europe N.V., Transportstraat 4, 3940 Tessenderlo.
China	Dr Yu Ting National Institutes for Food and Drug Control, Institute for Medical Devices Control, No. 2 Tiantan XiLi, Dongcheng District, Beijing, 100,050.
France	Dr Nathalie Ripoll, bioMerieux, Chemin de L'Orme, 69,280 Marcy L'Etoile.
Germany	Steffen Mueller, Roche Diagnostics GmbH, Nonnenwald 2, D-82377 Penzberg.
Ireland	Mel Costello, Abbott Ireland Diagnostics Division, Finisklin Business Park, Sligo, F91 VY44
Sweden	Maria Lundin, Fujirebio Diagnostics, Elof Lindälvs Gata 13, 41458 Göteborg
UK	Dr Jackie Ferguson and Katherine Partridge National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts, EN6 3QG.
UK	Dr Chris Thomas, Ortho-Clinical Diagnostics, Felindres Meadows, Pencoed, Bridgend, CF35 5PZ
USA	Ryan Masica Beckman Coulter Inc., 1000 Lake Hazeltine Drive, Chaska, MN 55318-1084.Dr
USA	Robert Pevere, Siemens Healthcare Diagnostics, 511 Benedict Avenue, Tarrytown, NY 10591

Table 2
Assessment of bulk free PSA, trial preparations of PSA-ACT and free PSA mixed in different volumetric ratios and candidate standards, 17/100, by participants in the UK NEQAS for PSA. [Distn code: year/month of each EQA distribution; ALTM: all laboratory trimmed mean].

Dist[n] code	n	Sample	Measurand	Target (μg/L)	ALTM (μg/L)	SD	CV(%)	%age free PSA
1611	57	96/668	Free PSA	0.4	0.42	0.03	7.1	n/a
1611	57	Bulk free PSA	Free PSA	0.4	0.44	0.04	8.7	n/a
168	278	96/670	Total PSA	3.0	2.8	0.2	6.2	n/a
168	57	96/670	Free PSA	n/a	0.41	0.02	4.9	14.6
168	278	85:15 (lyophilised)	Total PSA	3.0	2.9	0.2	6.4	n/a
168	57	85:15 (lyophilised)	Free PSA	n/a	0.50	0.03	6.3	17.2
1612	280	96/670	Total PSA	3.0	3.0	0.2	6.4	n/a
1612	57	96/670	Free PSA	n/a	0.42	0.03	7.2	14.0
1612	280	90:10 ratio (frozen)	Total PSA	3.0	3.0	0.2	6.5	n/a
1612	57	90:10 ratio (frozen)	Free PSA	n/a	0.38	0.03	6.7	12.7
1612	278	92:8 ratio (frozen)	Total PSA	3.0	3.0	0.2	7.2	n/a
1612	56	92:8 ratio (frozen)	Free PSA	n/a	0.32	0.02	6.9	10.7
1711	272	96/670	Total PSA	4.0	3.8	0.2	5.3	n/a
1711	52	96/670	Free PSA	n/a	0.55	0.03	6.2	14.5
1711	272	17/100 (candidate)	Total PSA	4.0	3.8	0.2	5.9	n/a
1711	52	17/100 (candidate)	Free PSA	n/a	0.52	0.03	5.7	13.7

through two WHO collaborative studies involving 10 laboratories in 8 countries (Table 1) with the objectives of assigning a value to each candidate and to evaluate their suitability and stability. Participants were provided with vials of the current standard (96/670 or 96/668), ampoules of the candidate preparations (17/100 or 17/102) and a panel of 15 human serum samples which contained total PSA concentrations ranging from 0.4–80 μg/L and free PSA concentrations ranging from 0.1–7.0 μg/L. A subset of participants were provided with thermal degradation samples in which ampoules had been incubated at 4°, 20°, 37° and 45 °C for 8 months for 17/100 and 7 months for 17/102.

Participants were asked to perform two independent immunoassays in which the PSA content of a dilution series of the current and candidate preparations was measured in triplicate alongside triplicate measurements of the PSA content of the serum samples. For 17/100, participants were asked to measure both the total and free PSA content of the current and candidate preparations. In both studies, participants were asked to prepare and measure the (nominal) test concentrations stated in the study protocol. This allowed determination of the reported, all-laboratory geometric mean values for each dilution of the reference materials, as required for the assessment of commutability. Ampouled preparations were coded such that the degradation samples were treated in the same manner as the candidate materials.

3. Statistical analysis

3.1. Assessment of the immunoreactivity of the current and candidate standards

For 17/100, analysis was performed using the reported PSA concentrations from the nominal concentration range of 2–64 μg/L only. For 17/102, the nominal concentration range was 0.5–8.0 μg/L. Results from an assay run were considered valid if the slope of the fitted regression line for $\log_{10}$ estimated concentration against $\log_{10}$ nominal concentration was in the range [0.90, 1.11] and the 90% confidence interval for the slope was within [0.80, 1.25]. Results from all valid assay runs were corrected for dilution factor and combined to generate unweighted geometric mean (GM) estimates for each laboratory and these laboratory means were used to calculate overall unweighted geometric mean estimates. Variability between laboratories has been expressed using geometric coefficients of variation (GCV = $\{10^{s}-1\} \times 100\%$ where s is the standard deviation of the $\log_{10}$ transformed estimates). Due to possible outliers and anomalous results, Huber's robust geometric mean was also calculated using the R package 'WRS2' [27].

3.2. Assessment of commutability

Commutability was assessed using a difference in bias approach. The reported results for the serum samples were $\log_{10}$ transformed for analysis to achieve approximately constant scatter over the range of concentrations used. A consensus value for the total or free PSA content of each sample was calculated as Huber's robust mean of laboratory means using the R package 'WRS2'. Bias values were then calculated for all reported results as the difference between the reported value and the study consensus value for that sample. For each individual laboratory and method, bias values were analysed using a linear mixed model with assay run included as a random effect and sample (96/670, 17/100 or serum, and 96/668, 17/102 or serum) included as a fixed effect. Calculations for this part were performed using Minitab 17 [28], with Dunnett's method used to estimate the difference in bias for the reference standards with serum samples.

To determine the commutability criteria, the standard deviation of the bias values for serum samples was calculated within each laboratory and a pooled value, s_P, was calculated across all laboratories. Possible commutability criteria representing the maximum acceptable difference in bias were then set as $\pm 2s_P$. Reference standards were considered commutable if the 90% confidence interval on the difference in bias was fully contained within the criteria.

To assess the stability of the candidate preparations, the relative immunoreactivities of the accelerated thermal degradation samples were used to fit an Arrhenius equation relating degradation rate to absolute temperature assuming first-order decay [29], and hence predict the degradation rates when stored at a range of temperatures.

4. Results

4.1. EQA assessment of trial preparations

Measurement of the free PSA concentration of the bulk free PSA stock diluted from the stated concentration of 1.08 mg/mL to a target concentration of 0.4 μg/L by 57 EQA participants demonstrated that the immunoreactivity of the bulk free PSA, as exemplified by the all-laboratory trimmed mean, was comparable that of the 1st WHO IS for free PSA, 96/668 (Table 2, Distribution 1611). EQA distributions were also used to assess trial ratios of PSA-ACT and free PSA in order to develop a suitable approach for the manufacture of a replacement standard for the 1st WHO IS for PSA (90:10). In both distributions 168 and 1612 (Table 2), the ALTM of the trial preparations for total PSA immunoreactivity were closely comparable to that of 96/670. From the methods contributed to these distributions, the mean free PSA content of the current standard, 96/670, expressed as a percentage of the total

PSA concentration ranged from 14.0–14.6%. Of the test preparations, the 90:10 ratio of PSA-ACT: free PSA, as prepared from IS-calibrated stock solutions of each component provided a percentage free PSA content of 12.7% (Table 2, distribution 1612).

4.2. Preparation and characterization of the candidate standards, 17/102 and 17/100

A total of 7209 ampoules, coded 17/102, of the candidate replacement for the 1st WHO IS for free PSA, 96/668, and 4125 ampoules, coded 17/100, of the candidate replacement for the 1st WHO IS for PSA (90:10), 96/670, were obtained. The quality control parameters for each candidate IS met the specifications required by WHO. Details are included in the reports of the collaborative studies submitted to the WHO Committee on Biological Standardization (ECBS) [24,25]. By ELISAs for total and free PSA (CanAg ELISA, Fujirebio), the total PSA content of 17/100 in terms of the current WHO IS for PSA (90:10) was 98.6% (95% CI: 95.9–101.4). The percentage free PSA content of the candidate standard 17/100 was determined as 15.7% (95% CI: 15.2–16.3) which was comparable to that observed using this method for the current standard, 96/670, of 15.8% (95% CI: 15.0–16.5). Agreement of the ALTMs for total PSA was also observed when samples of 96/670 and 17/100 with target concentrations of 4.0 μg/L total PSA were provided to participants in the UK NEQAS EQA scheme for PSA. As shown in distribution 1711 (Table 2), the ALTMs for both the current and candidate IS were 3.8 μg/L (17/100: CV 5.3%; 96/670: CV 5.9%, n = 272). The ALTMs for free PSA immunoreactivity were also in good agreement (0.55 μg/L (CV 6.2%, n = 52) for 96/670 and 0.52 μg/L (CV 5.7%, n = 52) for 17/100).

4.3. Assessment of the candidate standards by international collaborative studies

WHO collaborative studies were organised to assess the content, commutability and stability of the candidate replacement standards, 17/100 and 17/102. Details of valid individual laboratory estimates from both studies can be found in the reports submitted to the WHO ECBS [24,25] which are available on-line.

4.3.1. Value assignment, commutability and stability of 17/102

Participants in the collaborative study to evaluate a replacement for the 1st WHO IS for free PSA, 96/668, provided data from a total of 51 individual assays for free PSA from 24 methods (18 different methods). Five runs were excluded as the slope of the fitted regression line was outside of the range [0.90, 1.11] resulting in two methods being excluded [24]. A summary of estimates obtained for the current standard, 96/668 and the candidate 17/102, is shown in Fig. 2 with an outlier, identified using Grubbs' test [30], highlighted. Data from this laboratory method were excluded from both studies. Assessment of the content of the current standard, 96/668, confirmed that the geometric mean of the laboratory estimates for the fPSA content of 96/668 agreed with the assigned content of 1 μg/vial (1.057 μg/vial (95% CI 0.998–1.130, n = 21, GCV 15.9% with a robust geometric mean of 1.045 μg/vial) (Table 6). The geometric mean of the laboratory estimates for the fPSA content of 17/102 was 0.545 μg/ampoule (95% CI: 0.508–0.586, n = 21, GCV 17.0%) with a robust mean of 0.533 μg/ampoule. This value was comparable to the geometric mean of the estimates of the ratio of 17/102 to 96/668 which was 0.516 (95% CI: 0.489–0.544, n = 21, GCV% 12.5%) with a robust mean of 0.512.

For each of 19 methods included, the commutability of the current and candidate standards with patient samples was assessed by determining the difference between the mean bias determined for 15 serum samples and the bias determined for each dilution of the reference materials. The mean and 90% confidence interval for the differences in bias values for the reference standards of each method are summarised in Fig. 3. The criteria were calculated using $2s_P$ were ± 0.100, or 0.795 to 1.258 on the untransformed scale. As shown in Fig. 3, the commutability with patient samples of 96/668 and 17/102 are broadly comparable and the overall estimated difference in bias (with 90% confidence limits) is within the statistically-defined limits of commutability for 12 of the 19 valid methods submitted to the free PSA collaborative study. Individual estimates of the difference in bias were reported to WHO ECBS [24]. This assessment was supported by a comparison of the variability (GCV) of the reported mean serum estimates with values obtained when the free PSA concentration is expressed in terms of the current and candidate standard. Pooling the GCV values for all serum samples provided overall estimates of variability of 23.7% for the reported concentrations, 22.6% for values expressed in terms of the current standard, 96/668, and 24.1% for values expressed in terms of candidate, 17/102. (The GCV values for each serum sample were reported to WHO ECBS. [24]) Estimates of the immunoreactivity of ampoules stored at elevated temperatures were determined from data provided by four laboratories using five methods. The candidate standard, 17/102, was predicted to exhibit a loss of free PSA immunoreactivity per year of 0.096% when stored at −20 °C.

4.3.2. Value assignment, commutability and stability of 17/100

A similar approach was used to assess the total PSA content of 17/100, the candidate replacement for 96/670. Participants in the collaborative study provided data from a total of 47 individual assays from 23 methods (17 different methods) and data from all assays met the validity criteria [25]. One method was excluded as described above. A summary of estimates obtained for the current standard, 96/670 and the candidate 17/100 are shown in Fig. 2. The geometric mean of the laboratory estimates for the total PSA content of 96/670 confirmed the assigned value of 1 μg/vial (1.053 μg/vial (95% CI: 1.006–1.101, n = 22, GCV 10.7% with a robust geometric mean of 1.041 μg/vial)). For the candidate replacement, 17/100, the geometric mean of the laboratory estimates was 0.514 μg/ampoule (95% CI: 0.489–0.542, n = 22, GCV 12.3%) with a robust mean of 0.505 μg/ampoule. This value was supported by the geometric mean of the estimates of the ratio of 17/100 to 96/670 of 0.489 (95% CI: 0.478–0.500, n = 22, GCV 5.3%) with a robust mean of 0.490.

For the 19 methods included, the commutability of the current and candidate IS were assessed as described above with the limits calculated using $2s_P$ as ± 0.071, or 0.849 to 1.178 on the untransformed scale. The current and candidate materials, 96/670 and 17/100, respectively, were shown to have comparable commutability with patient samples. The overall estimated difference in bias (with 90% confidence limits) is within the statistically-defined limits of commutability for 14 of the 19 valid methods submitted to the total PSA collaborative study (Fig. 3). Individual estimates of the difference in bias were reported to WHO ECBS [25]. This commutability assessment was supported by the pooled variability (GCV) values for the total PSA concentration of the serum samples as reported, or when expressed in terms of the current (96/670) or candidate (96/668) standards which were 15.5%, 14.1% and 14.2%, respectively. The candidate standard, 17/100, was predicted to exhibit a loss of total PSA immunoreactivity per year of 0.028% when stored at −20 °C.

An additional element of the characterization of the candidate IS, 17/100, was the determination of the mean free PSA content determined from individual estimates calculated as a percentage of the corresponding laboratory estimate of total PSA. Data from two laboratories were excluded from the analysis as the slope of the fitted regression line was < 0.90 for both samples. The geometric means of the percentage of the free PSA content of 96/670 and 17/100 were comparable (96/670: 12.7% (95% CI: 11.9–13.5, n = 20, GCV 13.9% vs 17/100: 11.9% (95% CI: 11.0–12.9, n = 20, GCV 18.2%))). A robust geometric mean of the free PSA content of 17/100 was 12.1% whereas the content of 96/670 was unchanged.

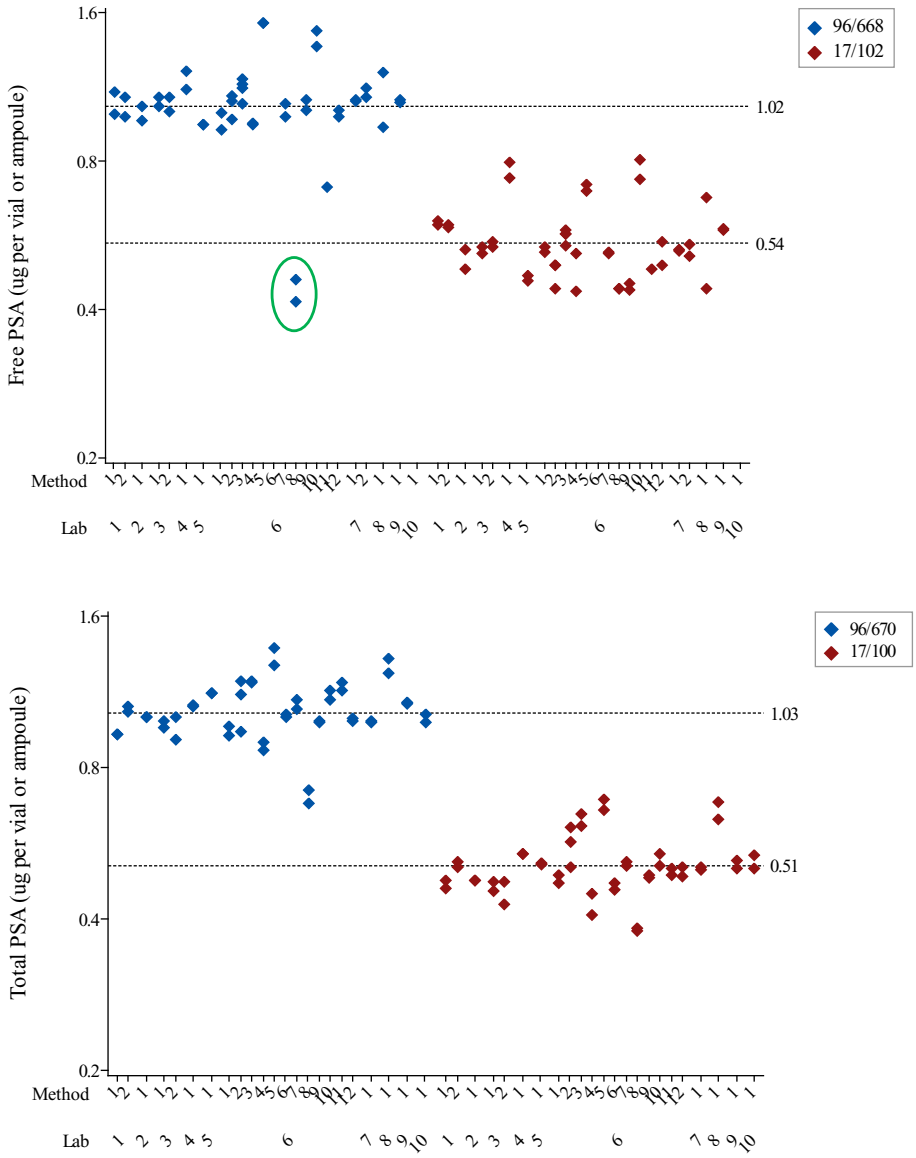

Fig. 2. Estimates of the free PSA content of 96/668 and candidate 17/102 (top) and the total PSA content of 96/670 and the candidate, 17/100 (bottom) as determined from each valid run by each laboratory-method as indicated along the x-axis. Data from Laboratory 6 [8] (circled) was identified as an outlier. Data from this laboratory was excluded from both studies.

5. Discussion

Replacement of the WHO IS standards for PSA (90:10), 96/670, and free PSA, 96/668, is required to ensure the continued availability of international standards to calibrate diagnostic immunoassays for total and free PSA. The current standards were established in 1999 using a portion of the batches of vials, known as the 'Stanford calibrators' which had been made available to users for 2–3 years prior to WHO establishment. The standards fulfilled a need to make available, universal, mass-assigned calibrators and, through the provision of the PSA (90:10) calibrator, aimed to address the issue of non-equimolarity. A key consideration in the development of replacements for these standards was to ensure that recalibration of immunoassays to the new preparations would not result in a change in the PSA measurements of patient samples.

The Stanford calibrators were prepared by Stamey and co-workers [9–11,31] from seminal fluid-derived PSA and assigned a mass value based on the absorbance of the bulk preparations at 280 nm with, for PSA-ACT, adjustment for the molar ratio of the PSA content in the complex. The molar extinction coefficients had been empirically defined by the assessment by amino acid analysis of earlier batches of PSA and PSA-ACT. The materials available for the preparation of the replacement standards were human seminal fluid-derived PSA, non-complexing with ACT (≥98% pure) and PSA-ACT complex (≥95% pure). PSA concentrations derived from the absorbance at 280 nm were reported by the supplier prior to the addition of sodium azide to 0.1% (v/v) and using the molar coefficients previously defined [9–11,31]. It was noted that the mass ratio-corrected, A_{280}-derived concentration for the PSA content of PSA-ACT of the bulk material was not in agreement with the immunoassay value by Siemens Centaur CP immunoassay (0.502 mg/mL versus 0.619 mg/mL, respectively) stated by the supplier. Measurement by Diasorin Liaison total PSA immunoassay also recorded a higher value.

To ensure that the replacement standards provided comparable immunoreactivity, it was important to obtain a field assessment of the materials. This was achieved through distribution of trial preparations, diluted to a clinically-relevant test concentrations, to members of the UK EQA scheme for PSA (total and free) organised by UK NEQAS

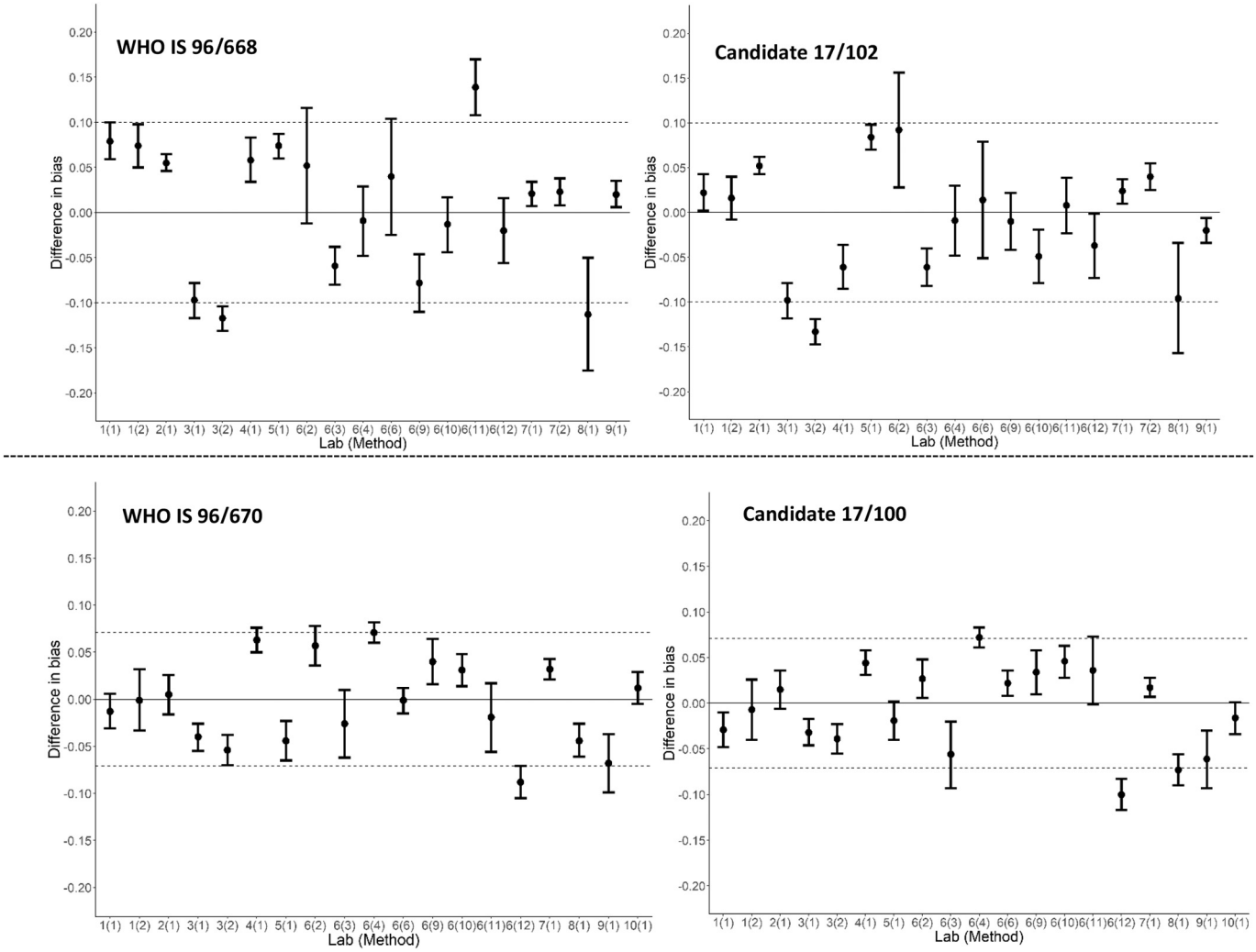

Fig. 3. Estimated mean difference in bias (with 90% confidence limits) between serum samples and the current IS 96/668 (top, left), candidate IS 17/102 (top, right), current IS 96/670 (bottom, left) and candidate IS 17/100 (bottom, right) for each laboratory and method as denoted on the x-axis. Solid horizontal line indicates no difference in bias from that observed for serum samples, dashed horizontal lines indicate maximum allowable difference in bias for commutable reference materials.

Immunology, Immunochemistry & Allergy. Although not part of the eventual calibration of the materials, this assessment informed our approach to the preparation of the replacements. The distribution of a dilution of the bulk free PSA to 57 laboratories (Table 2, distribution 1611) provided reassurance that the A_{280}-derived concentration provided comparable immunoreactivity to the same dilution of the current standard, 96/668. The distribution of preparations containing PSA-ACT and free PSA to over 270 laboratories (Table 2, distributions 168 and 1612) confirmed that calibration of stock solutions to the current WHO standards and combining in volumetric ratios provided preparations with a total PSA immunoreactivity that was closely comparable to that of the current standard, 96/670. Similarly, the agreement observed between the candidate standard, 17/100, and the current IS, 96/670, at a target concentration of 4 µg/L (Table 2, distribution 1711), supported the decision to proceed to a full evaluation of the material through collaborative study.

All WHO ISs are characterised through international collaborative studies to assign a value and to assess suitability and stability. The current standards are assigned in SI-units yet, as described earlier, calibration by a reference measurement procedure is currently not possible. Therefore, it was essential to first confirm the assigned content of the current standards by a robust geometric mean of reported estimates (Table 3 and Fig. 2) before using the same approach to assign a value to contents of the candidate materials. This provided content assignments

Table 3
Overall estimated PSA content of the current standards 96/668 and 96/670 (µg/vial), the candidate standards 17/102 and 17/100 (µg/ampoule) and the relative contents of the candidates to the relevant current standard.

Sample	GM	95% LCL	95% UCL	GCV	n	Robust GM
96/668	1.057	0.988	1.130	15.9%	21	1.045
17/102	0.545	0.508	0.586	17.0%	21	0.533
17/102:96/668	0.516	0.489	0.544	12.5%	21	0.512
96/670	1.053	1.006	1.101	10.7%	22	1.041
17/100	0.514	0.489	0.542	12.3%	22	0.505
17/100:96/670	0.489	0.478	0.500	5.3%	22	0.490

GM: Geometric Mean, LCL: Lower Confidence Limit, UCL: Upper Confidence Limit, GCV: Geometric Coefficient of Variation (%), n: number of estimates used in calculation.

of 0.50 µg/ampoule for 17/100 and 0.53 µg/ampoule for 17/102. The assigned values were supported by the robust geometric means of the estimates of the ratios of the candidate to current standard which were 0.512 for 17/102 and 0.490 for 17/100. In both studies, the geometric coefficient of variation (GCV) for the ratios was lower than the GCV for the mean of the estimates. This suggests that the current and candidate materials exhibit similar immunoreactivity in individual methods. Measurement of accelerated thermal degradation samples of each

standard also demonstrated that the candidates exhibited sufficient stability to be WHO IS.

Diagnostic reference materials require an assessment of commutability to evaluate if there is equivalence in the relationship between the assay response to dilutions of the reference material and the response to representative clinical samples. Serum samples (n = 15) prepared from the dilution of patient samples containing high concentrations of PSA were provided to each participant. Data were analysed by calculating the difference in bias, an approach based on the recommendation of the IFCC Working Group on Commutability [32]. For each test dilution of the reference material measured by a laboratory method, this approach allows the bias from the all-laboratory consensus value to be compared to the bias observed for the serum samples. The mean and 90% confidence interval for the difference in bias across all the reference material dilutions allows a method-to-method comparison of the likely impact of the new reference material as summarised in Fig. 3. The estimates and confidence intervals obtained for the difference in bias were comparable between the current and candidate standards which provides reassurance that the commutability of the candidate materials is likely to be similar to that of the current standards. There are limits to the scope of a commutability assessment within a collaborative study and no conclusions can be drawn regarding methods in which the commutability of the reference material was inconclusive or negative. It is anticipated that a manufacturer would conduct an in-house assessment of commutability using multiple reagent lots and performing runs on different days using different platforms and operators.

In both studies, the inter-laboratory variability (GCV%) determined for the PSA concentrations of each serum sample when measured in terms of kit, current and candidate standards, also suggests that the introduction of the replacements is unlikely to have a negative impact on the between-laboratory variability of PSA measurement. It is noted that there is higher variability in measurements of fPSA (GCV > 22–25%) compared to measurements of total PSA (GCV 14–16%). The multiple forms of fPSA may contribute to this variability. These include proenzyme forms which retain up to 7 amino acids as a leader sequence, internally cleaved forms and minor variants that appear intact but are enzymatically inactive due to structure and conformational changes [2,3]. A key element of the WHO approach is to provide a batch of ampoules with long term stability to prevent batch-to-batch variation in the PSA sub-form content as proposed by McJimpsey [33] to be a contributing factor to variability in calibration of manual immunoassays.

Inter- and intra-assay conformity is still a central goal for PSA measurement. The criteria which define treatment routes, as well as long term screening approaches, require interchangeability between commercial platforms. Despite the improvements driven by the introduction of the Stanford/WHO calibrators (Fig. 1), studies of method variability continue to be reported [17–22,34,35] suggesting that the clinical need for accuracy is not yet met by current assays. A mass spectrometry approach using tryptic digestion and quantification of signal peptides has been reported [36]. The method relies on an antibody extraction to ensure that the non-immunologically detectable form of PSA, PSA complexed to α_2-macroglobulin, is removed and the repeatability (CV of replicates) is not yet at the low levels achieved by automated immunoassays. It is therefore apparent that immunoassay detection is likely to remain the primary method for the determination of PSA concentration and the provision of the established WHO 2nd IS for PSA (total: PSA-ACT + free PSA), 17/100, and PSA (free), 17/102, available from NIBSC (www.nibsc.org), continue to support assay improvement and harmonization.

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