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Technical Report

Pre- and post-centrifugation stability of total and free prostate specific antigen samples at room temperature storage conditions

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Abstract

Objectives: Considering the recommendations of literature, it was important to note the potential for differences in pre- and post-analytical storage conditions at room temperature between total and free prostate-specific antigen. The aim of our study was to establish whether it would be appropriate to align the pre- and post-analytical times for the determination of free prostate-specific antigen (fPSA) with those for total prostate-specific antigen (tPSA).

Methods: Two blood samples were taken from 48 male patients aged 60 to 84. One specimen was centrifuged within one hour of collection. Each sample was tested immediately for total and free PSA. The second blood sample was kept at room temperature for 12 hours before being tested and then reanalyzed 24 hours after blood sampling. Serum specimens were analyzed on the Roche Cobas E801.

Results: There were no notable alterations in any PSA forms (p=0.866 and 0.971) or calculated ratios (Kappa=1) for the blood sample that was stored at room temperature for 12 hours prior to processing. Furthermore, all forms of PSA demonstrated stability (p=0.956 and 0.901), and fPSA/tPSA ratios showed good agreement in serum for up to 24 hours at room temperature (Kappa=1).

Conclusion: It would be beneficial to extend the pre- and post-analysis times of fPSA to align them with those of tPSA. Following the elevated tPSA discovery, investigating fPSA could be more streamlined, offering an improved patient management solution.

Keywords: fPSA/tPSA ratios, free prostate-specific antigen, *in vitro* stability, total prostate specific antigenstorage

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Prostate-specific antigen (PSA) is a glycoprotein with a molecular weight of 30,000–34,000 Dalton. It has a close structural relationship to the glandular kallikreins and functions as a serine proteinase [1]. In the blood, the proteolytic activity of PSA is inhibited by the formation of irreversible complexes linked to inhibitors such as alpha-1-antichymotrypsin, alpha-2-macroglobulin and other proteins of the acute phase of inflammation [2]. Furthermore, PSA is present in the bloodstream in a free form (fPSA) that has lost its proteolytic activity [3]. In 1991, JE Oesterling [4] showed that the total PSA (tPSA) assay lacked the necessary sensitivity and specificity to be an ideal tool for screening or early diagnosis. The fPSA/tPSA ratio has been shown in several studies to improve sensitivity and specificity in patients with tPSA levels in the grey zone of 4–10 ng/mL [5–8]. It is important to note that pre- and post-analytical factors related to sample handling and storage can affect the values of all the molecular forms of PSA [9]. If the tPSA is sufficiently stable to allow whole blood samples to remain at room temperature for 24 hours before serum separation [10], the level of stability of f-PSA varies between 3 and 8 hours [11]. Some authors have indicated that there is a 5% reduction in fPSA after whole blood is stored for 8 hours at room temper-

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ature, with an 8% decrease observed after 24 hours [12]. It is important to note that the accuracy of the biomarkers fPSA/tPSA ratio is contingent upon the correct application of stability conditions. The objective of the present study was to examine the impact of storage at room temperature on free and total prostate-specific antigen (PSA) levels in blood samples collected 12 hours ago and in serum separated from clotted blood stored at room temperature for varying periods.

Materials and Methods

Ethical approval

The laboratory investigations were conducted in accordance with the EU General Data Protection Regulation (EU Regulation 2016/679 and Directive 95/46/EC) and French data protection legislation (Law 78–17 of 1978, as amended by Decree 2019–536 of May 29, 2019). In accordance with Article 6 of the European Parliament and Council Directive 95/46/EC of 24 October 1995 and Article 2 of the French Data Protection Act of 6 January 1978 and Decree 2019–536 of 29 May 2019, a review by an ethics committee is not required for the secondary use of samples collected for healthcare purposes. In such cases, the use of human biological material for medical or scientific purposes other than those for which it was originally collected is permitted (article L.1211-2 of the French Public Health Code). The procedures followed were in accordance with the French Public Health Code. The database is registered with the French National Commission on Informatics and Liberty (CNIL) under record No. 2073511v0.

Sample size

In quantitative studies, the sample size is a critical factor in obtaining reliable data. The final size of the sample will depend on how precise (accurate) it needs to be, what the budget is, and all the other practical issues.

The following formula was used to calculate the required sample size for our study:

$s = z^2 \times p (1 - p) / m^2$, with:

- "s" was the sample size retained.
- "z" was the confidence level according to reduced centered normal distribution for 99% confidence (z=2.575).
- "p" was the estimated proportion f-PSA analyzed in the laboratory the characteristic (2% or 0.02).
- "m" was the margin of error (we might want to know the true proportion to within 5% or 0.05).

According to this formula, the size of the population tested should be approximately 50 patients.

Subjects and collection tubes

Two 5ml coagulated blood samples were taken (by single venipuncture) from 48 male patients aged 60 to 84, who were attending the laboratory for a blood test. All samples were collected using BD Vacutainer® SST™ II (ref 367957) tubes with Gel and Clot Activator.

One of the specimens (Sample "1") was centrifuged within 1 hour upon collection (at 2000 g for 10 min) and the serum fraction separated. Each sample "1" was assayed immediately for tPSA and fPSA.

The second blood specimen (Sample 2) was left undisturbed on the laboratory bench at room temperature for 12 hours prior to processing. Following storage, the sample was centrifuged at 2000g for 10 minutes, after which the serum fraction was assayed immediately for tPSA and fPSA. The stability of total and free PSA in coagulated blood during a 12-hour period at room temperature (15°–25°C) was evaluated by comparing sample 1 with sample 2, designated "time-12h." To evaluate the stability of tPSA and fPSA in serum at room temperature, each serum fraction of sample 2 was reanalyzed 24 hours after the blood collection. The stability of total and free PSA in serum at room temperature (15°–25°C) was assessed by comparing sample 1 with sample 2, designated "time-24h".

Instruments

The total and free PSA levels were determined using the Roche Cobas E801 system (Roche Diagnostics, Mannheim, Germany). The total and free PSA were determined using the ECLIA method. The expanded uncertainty for the total and free PSA were estimated at 12.4% (with a 95% confidence interval between 8.8% and 21.1%) and 8.6% (with a 95% confidence interval between 6.0% and 15.1%), respectively. The samples were analyzed in the different runs using the same instrument which had been verified according to the accreditation criteria of ISO 15189 [13].

Statistical analysis

The conformity of the numerical values to a normal distribution was evaluated using a Shapiro–Wilk test, which demonstrated that the distribution of results was non-parametric. The comparison between populations was evaluated using a Mann–Whitney test ($p<0.05$ was considered significantly different). To illustrate the impact of the results according to the methods used, boxplots have been used.

The analytical agreements between sample 1 and sample 2 (time-12h and time-24h) were analysed using a scatter plot with a Passing-Bablok regression analysis. The correlations were evaluated using the Pearson correlation test, with a p-value of less than 0.05 considered statistically significant. In the regression analysis, the limits of agreement were plotted according to the desirable total error formula developed by Callum G. Frazer [14], using the biological variation of total and free PSA defined by Carobene et al. [15]. The acceptable accuracy for tPSA and fPSA should fall within the total allowable error range of ±15.2% and ±14.0%, respectively.

Agreement results versus fPSA/tPSA ratio

For all samples with tPSA levels of 2.6 ng/mL or above (this limit accounts for the margin of error inherent to the measurement process at a value of 3 ng/mL), the results of the pre- and post-centrifugation phases were evaluated in comparison to the

Table 1. Statistical description of the tPSA and fPSA results according to different time periods.

	tPSA					fPSA				
	Sample "1" (time <1h)	Sample "2" designated "time-12h"	Sample "2" designated "time-24h"	Mann-Whitney test "time<1h" versus "time-12h"	Mann-Whitney test "time<1h" versus "time-24h"	Sample "1" (time <1h)	Sample "2" designated "time-12h"	Sample "2" designated "time-24h"	Mann-Whitney test "time<1h" versus "time-12h"	Mann-Whitney test "time<1h" versus "time-24h"
Nb. specimens	48	48	48	p=0.866	p=0.956	48	48	48	p=0.971	p=0.901
Minimum	0.014	0.014	0.014			0.020	0.020	0.020		
Maximum	25.7	25.2	25.2			4.670	4.300	4.230		
1st quartile	0.401	0.400	0.411			0.155	0.158	0.150		
Médian	0.900	0.922	0.910			0.260	0.260	0.255		
3rd quartile	2.855	2.9575	2.9725			0.473	0.460	0.455		
Mean	2.680	2.717	2.715			0.507	0.496	0.491		
Variance (n-1)	24.374	24.195	24.842			0.651	0.580	0.576		
SD (n-1)	4.937	4.918	4.984			0.807	0.761	0.759		

tPSA: Total prostate-specific antigen; fPSA: Free prostate-specific antigen; SD: Standard deviation

Table 2. The agreement of fPSA/tPSA ratio using a threshold of 0.23 according to different time periods

	fPSA/tPSA ratio (time-12h)		fPSA/tPSA ratio (time-24h)	
	<0.23	≥0.23	<0.23	≥0.23
fPSA/tPSA ratio (time <1h)				
<0.23	12	0	12	0
≥0.23	0	3	0	3
Kappa [95% CI]	1.00 (1.00–1.00)		1.00 (1.00–1.00)	

tPSA: Total prostate-specific antigen; fPSA: Free prostate-specific antigen; CI: Confidence interval.

fPSA/tPSA ratio using a pathological decision threshold of 0.23, as defined by de la Taille et al. [16]. The kappa coefficients with a 95% CI were calculated to estimate the agreement of evidence and recommendation levels between all paired samples [17]. According to Landis and Koch [18], kappa coefficients can be interpreted as one of the following six degrees of agreement: Poor (κ<0), slight (0.01–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), and almost perfect (0.81–1.00).

Results

The study cohort comprised 48 patients aged between 60 and 84 years, median age 74 (1st quartile 68, 3rd quartile 77). Table 1 provides a statistical overview of the results. The median values for tPSA and fPSA for the population of specimens (Sample 1) that were centrifuged and analyzed within one hour of collection were 0.900 ng/mL and 0.260 ng/mL, respectively. A total of 15 patients from the 48-patient cohort presented with total prostate-specific antigen (tPSA) levels above 2.6 ng/mL. These subjects were included for the purpose of evaluating the Kappa concordance rate for the fPSA/tPSA proportion.

Effect of delayed centrifugation 12 hours after collection

The median values for tPSA and fPSA in the specimens (sample 2, designated "time-12h.") left undisturbed on the laboratory bench at room temperature for 12 hours prior to processing and analysis were 0.922 ng/mL and 0.260 ng/mL, respectively. The data showed no statistically significant differences for tPSA and fPSA with p-values of 0.866 and 0.971, respectively. The box plot showed a notable degree of convergence between the two measurement periods (Fig. 1a, b). For tPSA, the Pearson correlation coefficient (r) was significant and strong (r=1.000 [p<0.001; 95% confidence interval [CI]: 0.992–1.007]). The regression slope was 0.996 (p<0.001; 95% CI: 0.989–1.003) and the y-intercept was 0.048 (p<0.001; 95% CI: 0.006–0.089). Regarding fPSA, the Pearson correlation coefficient (r) was significant and strong (r=0.999 [p<0.001; 95% confidence interval [CI]: 0.987–1.011]). The regression slope was 0.943 (p<0.001; 95% CI: 0.932–0.954) and the y-intercept was 0.018 (p<0.001; 95% CI: 0.008–0.029). The regression analysis revealed no analytical discordance within the allowable

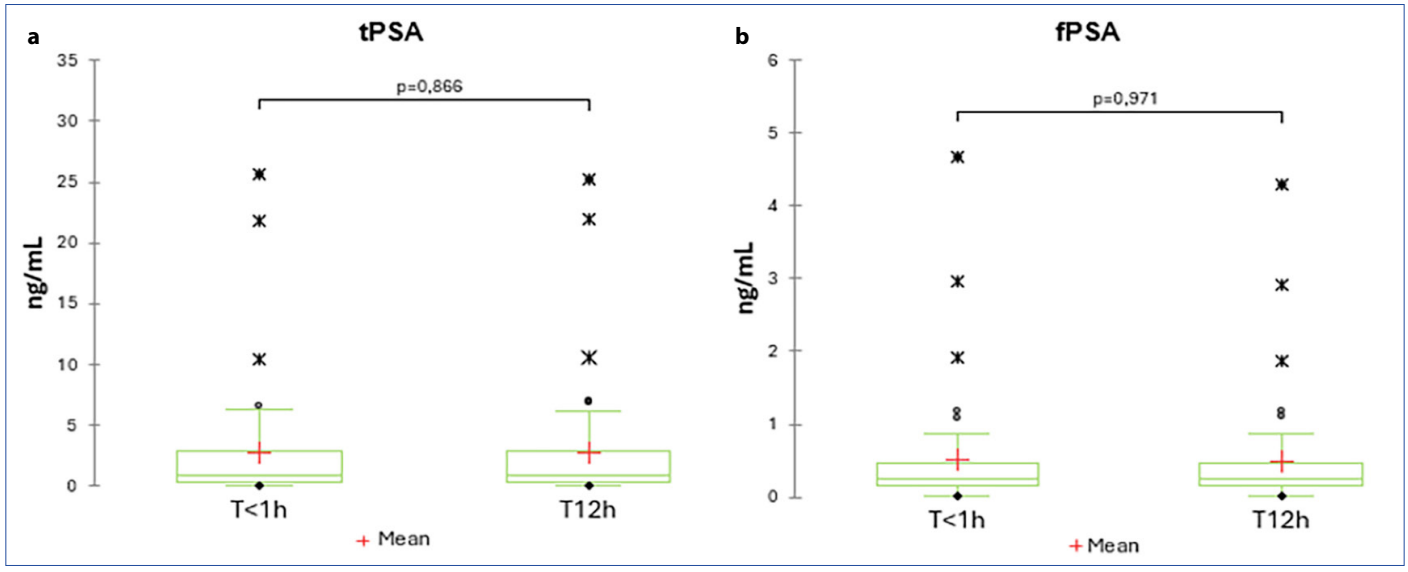

Figure 1. Boxplots of tPSA and fPSA results between samples analyzed within an hour of collection and samples kept for 12 hours before centrifugation.

tPSA: Total prostate-specific antigen; fPSA: Free prostate-specific antigen.

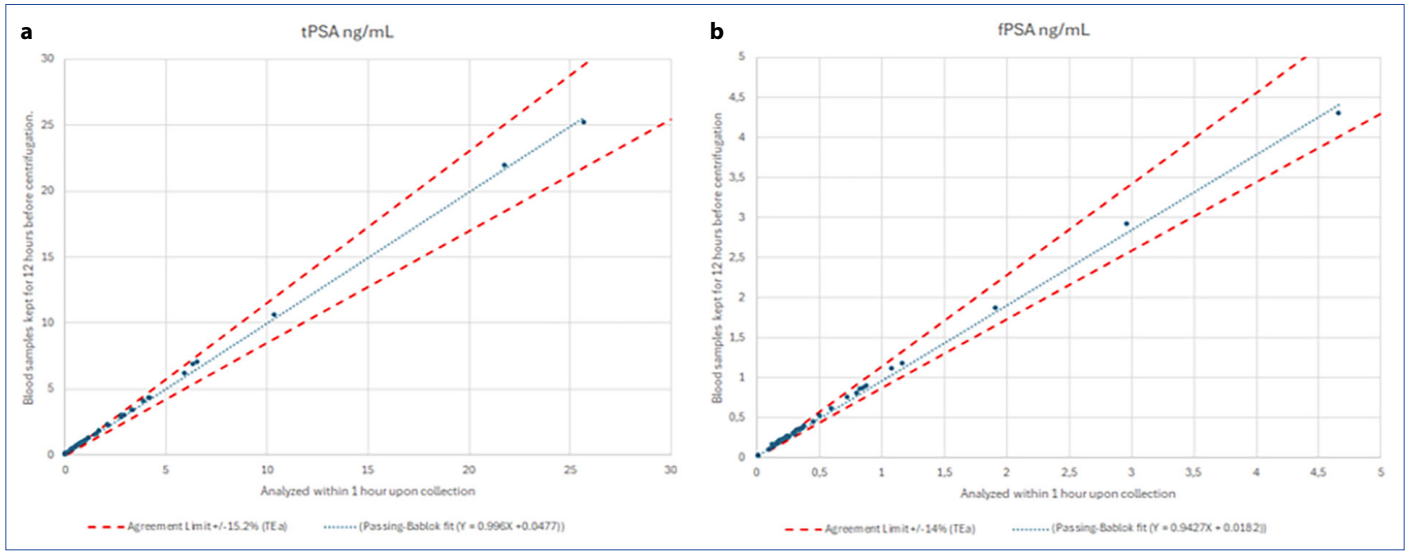

Figure 2. Comparison of tPSA and fPSA results between samples analyzed within an hour of collection and samples kept for 12 hours before centrifugation.

total error range (Fig. 2a, b). An interpretation based on the fPSA/tPSA ratio with a decision threshold of 0.23 yielded perfect agreement (Kappa=1.00) and is detailed in Table 2.

Effect of stability 24 hours after collection in serum

The median values for tPSA and fPSA in the specimens (sample 2, designated "time-24h") that were left undisturbed on the laboratory bench at room temperature for 12 hours before centrifugation and reanalyzed 24 hours after the sample collection were 0.910 and 0.255, respectively. The data showed no statistically significant differences for tPSA and fPSA with p-values of 0.956 and 0.901, respectively. The box plot showed a notable degree of convergence between the two measurement periods (Fig. 3a, b). For tPSA, the Pearson correlation coefficient (r) was significant and strong (r=0.999 [p<0.001; 95% confidence interval [CI]: 0.990–1.009]). The regression slope was 1.009 (p<0.001; 95% CI: 0.999–1.019) and the y-intercept was 0.011 (p<0.001; 95% CI: -0.044–0.065). Regarding fPSA, the Pearson correlation coefficient (r) was significant and strong (r=0.998 [p<0.001; 95% confidence interval [CI]: 0.981–1.016]). The regression slope was 0.939 (p<0.001; 95% CI: 0.922–0.955) and the y-intercept was 0.015 (p<0.001; 95% CI: 0.000–0.031). The regression analysis revealed no analytical discordance within the allowable total error range (Fig. 4a, b). An analysis based on the fPSA/tPSA ratio with a decision threshold of 0.23 resulted in perfect agreement (Kappa=1.00), as shown in Table 2.

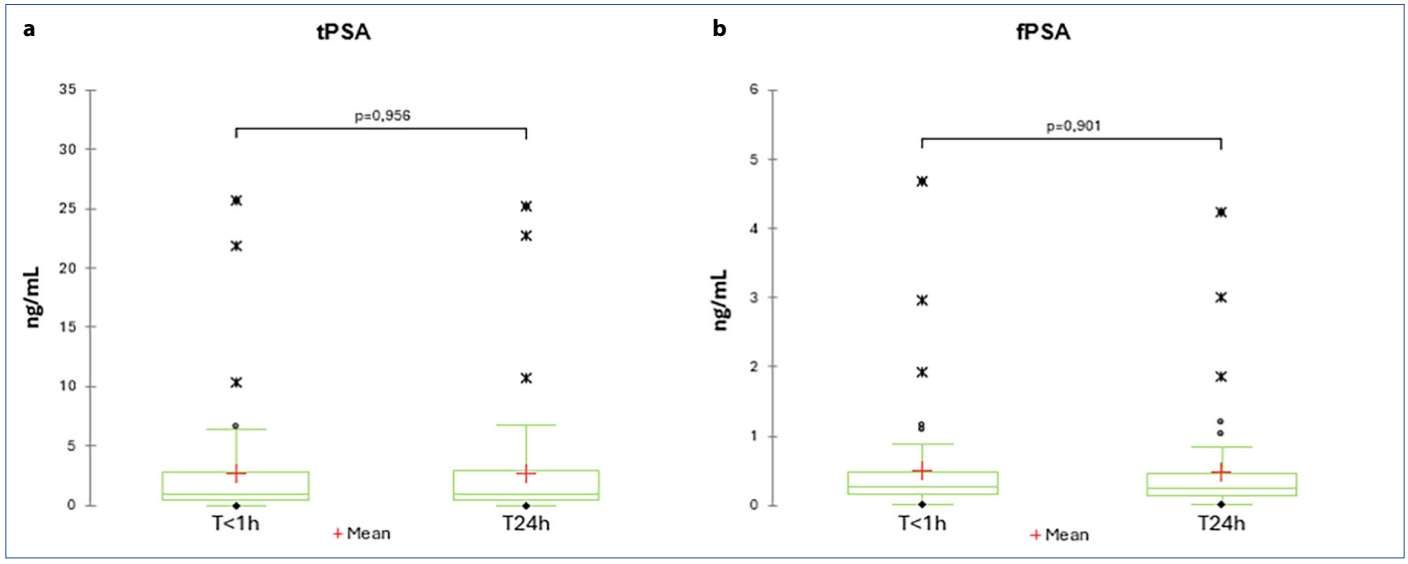

Figure 3. Boxplots of tPSA and fPSA results between samples analyzed within an hour of collection and samples kept for 12 hours before centrifugation and reanalyzed 24 hours after venipuncture.

tPSA: Total prostate-specific antigen; fPSA: Free prostate-specific antigen.

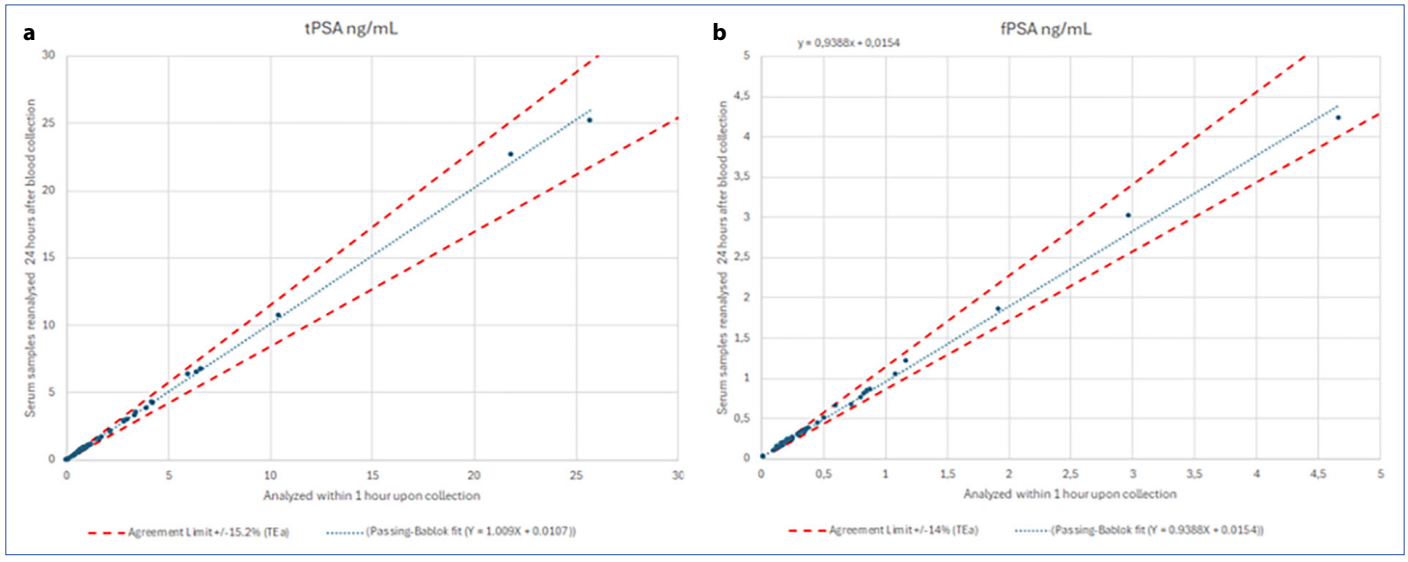

Figure 4. Comparison of tPSA and fPSA results between samples analyzed within an hour of collection and samples kept for 12 hours before centrifugation and reanalyzed 24 hours after venipuncture.

Discussion

Several studies have investigated the short- and long-term stability of tPSA and fPSA assays since their introduction in the late 1980s [19–21]. In line with the results of this study, other reports have shown that tPSA levels are relatively stable when stored at room temperature for a period of 24 hours [22, 23]. Regarding fPSA stability at room temperature, there is a lack of consensus in the literature on this topic, as the findings of different studies vary considerably. Piironen et al. [24] and Jung et al. [12] showed the rapid loss of fPSA in the presence of clotted blood cells could be due to complex formation with α2-macroglobulin (PSA-AMG), which occurs faster than the complex formation with α1-antichymotrypsin (PSA-ACT). In accordance with the recommendations of these authors, serum preparation should be conducted promptly, within five hours of venipuncture, and fPSA analysis should be completed within eight hours to ensure optimal stability of PSA variants and eliminate pre-analytical variables as sources of variability. Kumari et al. [23] reported excellent stability *in vitro* of fPSA at room temperature. The authors reported that clotted blood stored at room temperature for 24 hours showed no decline in fPSA. This finding is at odds with the results of other studies, which have demonstrated a decrease in free PSA when separation is delayed [12, 24].

The objective of our study was to gain a comprehensive understanding of the room temperature stability characteristics

of free and total PSA from a pre- and post-analytical perspective. Our study corroborates the findings of previous research, confirming the stability of tPSA. Furthermore, it offers valuable insights into the routine use of fPSA. Whole blood samples may be kept at room temperature for up to 12 hours before processing. The fPSA results of subsequent assays on serum samples stored at room temperature remain unaffected until 24 hours post-blood collection.

Since 2010, the number of laboratories in France conducting these analyses has decreased significantly. It is standard practice for blood samples to be collected at a peripheral laboratory or a healthcare practitioner's office, which are geographically distant from the technical laboratory responsible for analyzing the samples. This necessitates the transportation of samples, resulting in a notable increase in the time required for the pre-analysis phase. Our results will ensure that samples are handled correctly, both in the field and in the laboratory, and guarantee their accurate evaluation.

According to scientific guidelines [25–27], it is not recommended that free PSA be used as a first-line test. The free PSA/total PSA ratio is a useful indicator for assessing the risk of prostate cancer in patients with tPSA levels between 4 and 10 ng/mL. Considering this, it would be beneficial to extend the pre- and post-analysis times of fPSA to align them with those of tPSA. Following the elevated tPSA discovery, investigating fPSA could be more streamlined, offering an improved patient management solution.

Nevertheless, it was observed that most of the results of t-PSA falling within the range of 0.4 and 2.9 ng/mL were a limiting factor. Further investigations should be conducted to examine a greater number of patients with tPSA 4–10 ng/mL and also more patients with tPSA above 10 ng/mL.

Conclusion

Regarding the stability of free PSA, our results indicate that samples may be stored on the clot at room temperature for up to 12 hours, with the option of reanalysis up to 24 hours after blood collection.

Informed Consent: Informed consent was obtained from all participants.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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