

Research Article

# Clinical Relevance of Viable Circulating Tumor Cells detected by PSA-EPISPOT prior Trans-rectal Prostate Biopsy

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**Citation:** Thibaut Murez, et al. Clinical Relevance of Viable Circulating Tumor Cells detected by PSA-EPISPOT prior Trans-rectal Prostate Biopsy. Cancer Research Frontiers. 2015 Sep; 1(3): 288-298. doi: 10.17980/2015.288

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**Competing Interests:** The authors declare that there are no competing interests.

Received Oct 5, 2015; Revised Dec 14, 2015; Accepted Dec 18, 2015. Published Dec 23, 2015

## Abstract

**Background:** Accurate new tools are advocated to help clinical decisions from screening to follow-up and salvage-treatment of prostate cancer. We report here the clinical relevance of the PSA-EPISPOT assay for circulating tumor cells (CTCs) detection prior to immediate prostate biopsy.

**Patients and Methods:** One hundred and eleven patients selected to undergo prostate biopsy based on conventional triggering markers were recruited between 2002 and 2006. CTCs in the peripheral blood were detected by the fluoroPSA-EPISPOT assay. Peripheral blood was sampled before prostate biopsy. CTC enumeration was performed with an EpCAM-independent enrichment method followed by the fluoroPSA-EPISPOT assay that detects only viable PSA-secreting CTCs.

**Results:** Sixty-three patients were negative biopsy and 48 were positive. Median follow-up was 69.5 months [0.8 – 115.8]. Viable CTCs were detected in 12/63 negative biopsy patients (19%) and 23/48 positive biopsy patients (47.9%). CTC mean count was significantly higher in positive biopsy patients ( $2 \pm 3.1$ ) than in negative biopsy patients ( $0.7 \pm 1.9$ ;  $p=0.0015$ ). PSA-EPISPOT characteristics were respectively 47.92%, 80.95%, 65.71%, 67.11%, 66.67% for sensitivity, specificity, positive and negative predictive value and accuracy. PSA-EPISPOT was better than random to predict positive biopsy but not different from total PSA. Its relation to other markers made the PSA-EPISPOT assay not eligible to multivariate logistic regression.

**Conclusion:** This report indicates that PSA-EPISPOT technique was able to detect CTCs in patients screened for prostate cancer. Despite interesting characteristics, it was not sensitive enough to prevent each unnecessary prostate biopsy. Further analyses are mandatory to assess the prognosis value of the PSA-EPISPOT assay in positive biopsy patients.

**Keywords:** circulating tumor cell, prostate cancer, screening, biomarker, diagnosis

## Introduction

Prostate cancer is a major health issue as it represents the second worldwide cancer. A 89% and 93% increase of incidence and specific-mortality are expected by 2030 (1). Relevant screening and staging

tools are still lacking. The benefit of a PSA-based prostate cancer-screening program is controversial (2, 3) and could lead up to a 50% overdiagnosis (4). Clinical decisions need more accurate tools than D'Amico's seminal risk classification which is

known to be heterogeneous among contemporary patients (5).

CTCs could be one relevant tool as it provides a real-time liquid biopsy based on a simple blood sample (6). Cancers would be able to spread these tumor cells through blood circulation from early stages. The issue is the ability to detect a single cell of interest out of hundreds of thousands of blood cells (7).

Many assays can nowadays detect CTCs through immunology or molecular biology (8). Many pitfalls have burdened CTC enrichment or detection processes inducing conflicting results (9). Thus, qualifying CTCs as a relevant biomarker needs a strict methodology, otherwise their detection will remain a marginal test in clinical practice (7, 10).

Among immunology-based assays, the EPISPOT assay has the feature to only detect viable cells (11) through a protein secretion detection (which is PSA in that case) following negative enrichment of the blood sample and a 24-48 hours cell culture phase. Based on a limited number of unsorted prostate cancer patients, sensitivity, specificity, positive predictive and negative predictive value were respectively 69.4%, 100%, 100% and 85.7% (12). We expected here to confirm these characteristics among potentially localized prostate cancer patients and to define its clinical relevance.

## Patients and Methods

### Study design

The aim of this study was to explore fluoroPSA-EPISPOT assay ability to predict prostate cancer on immediate TRUS biopsy. Between 2002 and 2006, in a single urology center (Beausoleil clinic, Montpellier, France), each patient selected to undergo transrectal ultrasound guided (TRUS) biopsy according to the ERPC French arm criterions (13) received oral and written information concerning the fluoroPSA-EPISPOT assay for CTC detection. Patients who gave their signed consent were included and the data were analysed anonymously as authorized by the ethics review board. Selection criterions for their first or repeated biopsy were a high total PSA level (threshold 4 ng/mL) and/or an abnormal digital rectal examination (DRE) without prior prostate cancer diagnosis. The assays results did not change clinical decisions. Patients then underwent TRUS biopsies according to the sextant technique by their usual urologist (XR, BS, AF, SAH). Each core was sent to the pathologist in a single bottle containing formalin. Biopsies were considered positive when prostate adenocarcinoma was observed by the uro-pathologist. Negative biopsy

patients may have undergone new biopsies later but no additional blood sample for CTC detection has been performed.

### Isolation and CTC detection

For CTC detection, the fluoroPSA-EPISPOT assay was achieved as previously described (12) in a CTC dedicated lab (LCCRH laboratory, UMC Montpellier). Each time, blood sample has been performed before patients had their prostate biopsy. Eighteen milliliters of peripheral blood were collected in EDTA tubes and stored at room temperature until sample was processed (<24 hours after collection). Viable CTCs were first enriched via a depletion of the hematopoietic CD45<sup>+</sup> cells (RosetteSep, StemCell Technology, Vancouver, Canada) and defined as PSA-secreting cells (PSA-SC).

### Statistical analysis

Patients' files were retrospectively reviewed when available. Incomplete files were excluded when the patient or his general practitioner could not be reached. An Access 2007<sup>®</sup> (Microsoft, Redmond, WA, USA) blinded database was built to gather medical, clinical and chemical history.

Statistical analyses were performed using SAS<sup>®</sup> v9.3 (SAS Institute Inc., Cary, NC, USA) and figures were generated using SPSS v18 (SPSS Inc, Chicago, IL, USA). fluoroPSA-EPISPOT counts were not normally distributed according to the Kolmogorov-Smirnov test. Patients demographics were compared using the chi<sup>2</sup> or Fisher exact test concerning categorical variables and using Kruskal-Wallis test concerning continuous variables. Youden's test was used to assess the more relevant fluoroPSA-EPISPOT count threshold. Logistic regression used the binary Logit model, with step-by-step selection method. All tests were double-sided using a 5%  $\alpha$  risk and 95% confidence interval.

## Results

### Patients' demographics

One hundred eleven patients' files were available for analysis: 48 had immediate positive biopsy without evidence of metastasis, and 63 had negative biopsies (Table 1 and Fig. 1). The median follow-up was 69.5 [0.8 – 115.8] months. Ten of the 63 negative biopsy patients were diagnosed for a prostate cancer during their follow-up needing repeated prostate biopsies. These ten patients had similar characteristics compared to the 53 confirmed negative biopsy patients except concerning number of biopsy rounds which was higher in the secondary positive biopsy

**Table 1.** Patients' demographics and biological results

	Negative prostate biopsy group n = 63 (56.8 %)	Positive prostate biopsy group n = 48 (43.2 %)	Total n = 111	p
Age (years) <sup>¥</sup>	64.6 ± 6.8	68.8 ± 7.2	66.4 ± 7.2	0.0010
Follow-up (months) <sup>¥</sup>	69.2 [0.8 – 115.8]	69.8 [1.2 – 81.6]	69.5 [0.8 – 115.8]	0.3319
CCI <sup>∞</sup>				0.0075
0	1 (1.6 %)	0	1 (0.9 %)	
1	6 (9.5 %)	4 (8.3 %)	10 (9 %)	
2	33 (52.4 %)	8 (16.7%)	41 (36.9 %)	
3	13 (20.6%)	14 (29.2%)	27 (24.3 %)	
4	6 (9.5%)	9 (18.8%)	15 (13.5 %)	
5	3 (4.8 %)	6 (12.5 %)	9 (8.1 %)	
6	1 (1.6 %)	0	1 (0.9 %)	
7	0	3 (6.3%)	3 (2.7 %)	
8	0	0	0	
9	0	1 (2.1 %)	1 (0.9 %)	
Prostate volume (cc) <sup>¥</sup>	50.7 ± 27.3	48 ± 33.3	50 ± 29,7	0.2201
LUTS history <sup>∞</sup>	24 (38.1 %)	10 (20.8 %)	34 (30.6 %)	0.0506
Prostate cancer family history <sup>∞</sup>	3 (4.8 %)	5 (10.4 %)	8 (7.2 %)	0.2537
Biopsy cores <sup>¥</sup>	9.8 ± 2.4	9.0 ± 2.4	9.5 ± 2.4	0.1064
Repeat biopsy status <sup>∞</sup>	22 (34.9 %)	10 (20.8 %)	32 (28.8 %)	0.1045
Total biopsy rounds <sup>¥</sup>	2.0 ± 1.1	1.2 ± 0.5	1.7 ± 1.0	<0.0001
Total PSA (ng/mL) <sup>¥</sup>	6.8 [1.8 – 17.7]	11.0 [3.9 – 87.0]	11.7 [1.8 – 87.0]	<0.0001
Free to total PSA ratio (%) <sup>¥</sup>	17.0 [6 – 33]	10.6 [7 – 77]	16.0 [6 – 77]	0.0608
PSA-V (ng/mL/year) <sup>¥</sup>	0.5 [-36 – 9]	2.0 [-151 – 63]	0.9 [-151 – 63]	0.0053
PSA-DT (months) <sup>¥</sup>	52.3 [-122 – 1910]	31.9 [-32 – 567]	40.5 [-122 – 1910]	0.2289
PSA-D (ng/mL/cc) <sup>¥</sup>	0.2 [0.04 – 0.65]	0.3 [0.08 – 2.05]	0.2 [0.04 – 2.05]	<0.0001
Blood sample volume (mL) <sup>¥</sup>	17.3 ± 3.2	18.2 ± 3.4	17.7 ± 3.3	0.1590
PSA-EPISPOT count <sup>¥</sup>	0.7 ± 1.9	2.0 ± 3.1	1.3 ± 2.8	0.0015

**Abbreviations:** CCI, Charlson Comorbidity Index; cc, cm<sup>3</sup>; LUTS, Lower Urinary Tract Symptoms; PSA-V, PSA Velocity; PSA-DT, PSA Doubling Time; PSA-D, PSA Density.

**Statistical analysis:** categorical variables were compared through khi<sup>2</sup> or Fisher test (¥) and continuous variables through kruskall-wallis analysis (∞).

patients (**Supplemental Data 1**). We thus assumed prostate cancer was not detectable at the time of fluoroPSA-EPISPOT assay and kept the 63 patients as a single group. The mean number of biopsy cores was 9.5 ± 2.4.

Patients with a positive prostate biopsy were older (68.8 ± 7.2 vs 64.6 ± 6.8 years old,  $p=0.001$ ). Age difference was the only Charlson Index comorbidity that differed depending on the presence of prostate cancer. Most patients had a 0 to 4 CCI. Positive

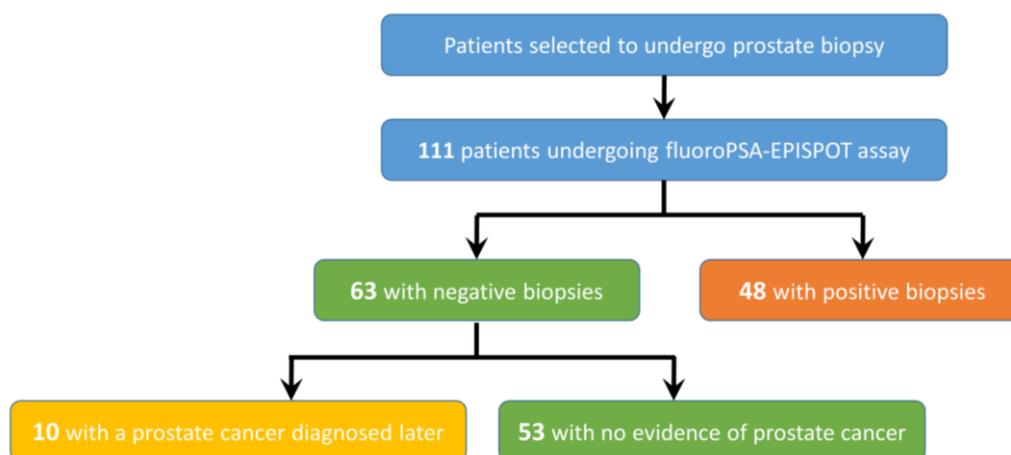


Figure 1. Patient flow-chart

biopsy patients had a 1-point shift because of the age difference.

While mean prostate volume was similar in both groups ( $50 \pm 29.7$  cc), there was a trend for negative biopsy patients to have more LUTS (20.8% vs 38.1%,  $p=0.0506$ ). Familial history of prostate cancer was rarely recorded and it was not associated to a positive prostate biopsy ( $p=0.25374$ ). Negative biopsy patients underwent more prostate biopsies during their medical history and their follow-up ( $1.2 \pm 0.5$  vs  $2 \pm 1.1$   $p<0.0001$ ). Repeated versus first biopsy status and number of biopsy cores were similar in both groups (respectively 28.8% and  $9.5 \pm 2.4$ ).

### Biological results

Median total PSA was higher in positive biopsy group (11 ng/mL [3.9 – 87.0] vs 6.8 [1.8 – 17.7],  $p<0.0001$ ) like was the PSA velocity (PSA-V) (2 ng/mL/year [-151 – 63] vs 0.5 [-36 – 9],  $p=0.0053$ ) and the PSA density (PSA-D) (0.3 ng/mL/cm<sup>3</sup> [0.08 – 2.05] vs 0.2 [0.04 – 0.65],  $p<0.0001$ ). Free to total PSA ratio was lower in the positive biopsy group but these results were not significantly different (11 % [7 – 77] vs 17 [6 – 33],  $p=0.0608$ ). The PSA doubling time (PSA-DT) was similar in both groups (median of 41 months).

The mean blood sample volume analyzed was  $17.7 \pm 3.3$  mL. CTCs were detected in 12 out of 63 (19%) negative biopsy patients and 23 out of 48 (47.9%) positive biopsy patients. FluoroPSA-EPISPOT (Fig. 2) count was respectively  $2 \pm 3.1$  and  $0.7 \pm 1.9$  among positive and negative biopsies patients ( $p=0.0015$ ). Youden's test identified 1 spot as the more relevant threshold to distinguish patients with a negative or a positive prostate biopsy. Thus, fluoroPSA-EPISPOT characteristics were

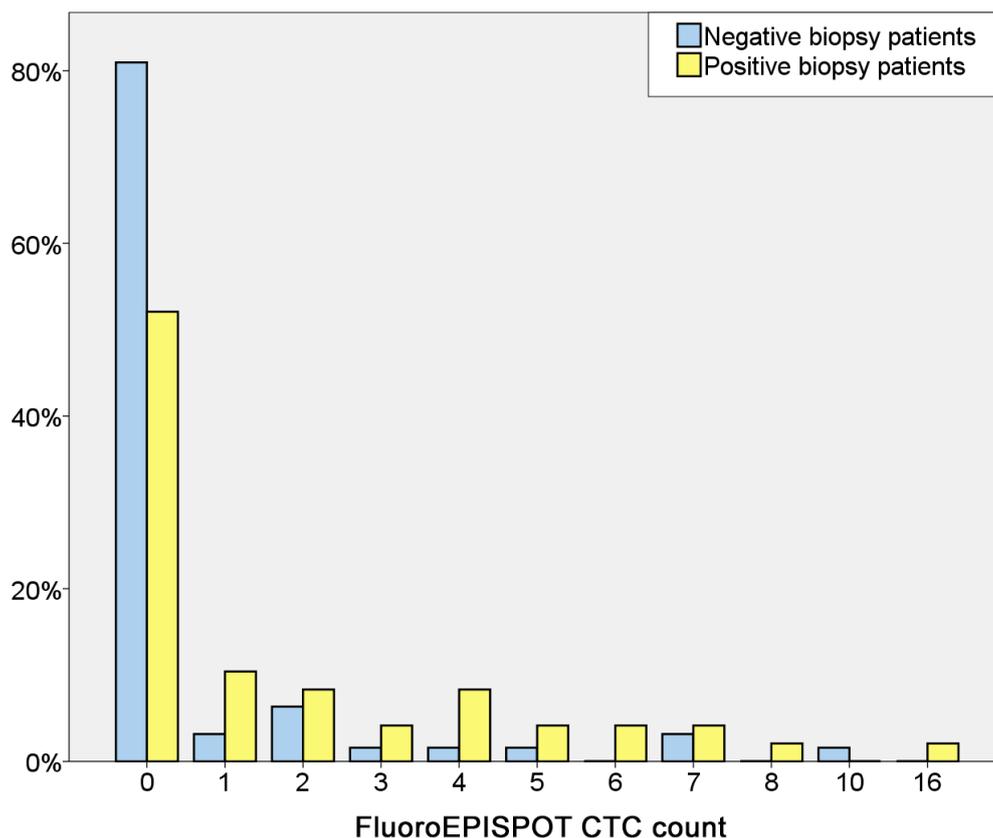
respectively 47.92%, 80.95%, 65.71%, 67.11%, 66.67% for sensitivity, specificity, positive and negative predictive value and accuracy. In addition, there was no association between CTC status and biopsy Gleason score ( $p=0.499$ ) or D'Amico classification ( $p=0.1911$ ) in immediate prostate cancer patients (data not shown).

Moreover, a second biopsy has been performed in all the patients who had a first negative biopsy. A prostate cancer was diagnosed in 10 out of 12 (83.3%) CTC positive / immediate biopsy negative patients. There was a median of 34.1 [2.7 – 71.8] months between fluoroPSA-EPISPOT assay and prostate cancer diagnosis. There was no significant difference between immediate and secondary cancers concerning D'Amico classification and biopsy Gleason score except for stage, which was lower in secondary cancers ( $p=0.018$ ).

### Logistic regression

Each variable that had a less than 0.2  $p$  was included in our multivariate model. Only 4 reached statistical significance using their median as cut-off. Age above 66.8 years and total PSA above 8.1 ng/mL were the highest risk factors (OR respectively 3.77 [1.24 – 11.45] and 8.52 [2.37 – 30.63]) whereas prostate volume above 40 cc and more than one round of prostate biopsies were protective conditions (0.21 [0.06 – 0.77] and 0.11 [0.03 – 0.41]). FluoroPSA-EPISPOT count seemed to be dependent to one of these variables. Those 5 variables distribution is presented as boxplots on Fig. 3.

When analyzing the assay characteristics using area under the ROC curve (Fig. 4), the fluoroPSA-EPISPOT assay was statistically different from random (AUC 0.646 [0.557 – 0.734],  $p=0.0012$ ) such



**Figure 2. FluoroPSA-EPISPOT counts distribution depending on biopsy outcome.** CTC count is represented on the x-axis, and relative count on the y-axis.

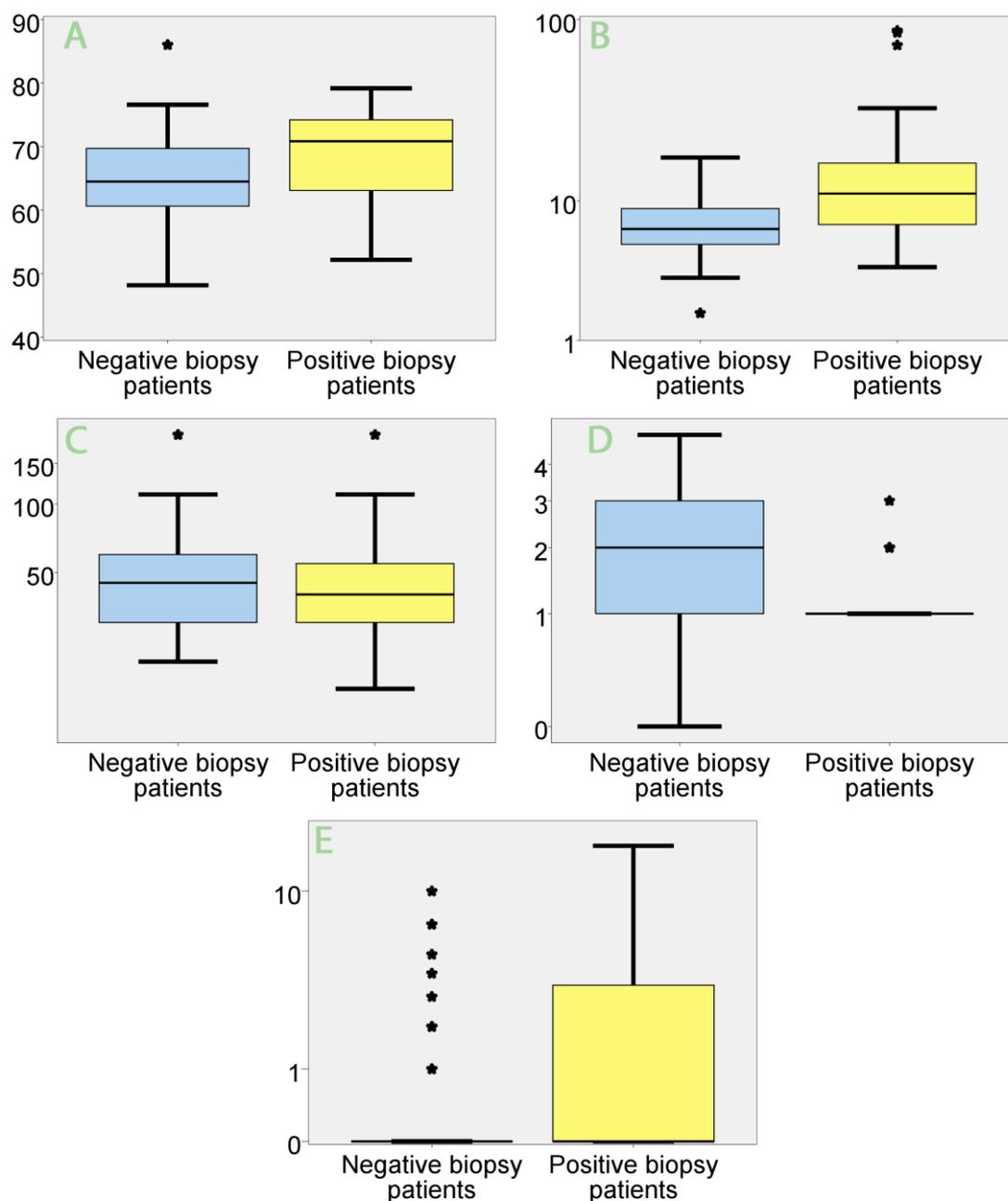
as total PSA (0.729 [0.627 – 0.831],  $p < 0.0001$ ). fluoroPSA-EPISPOT AUC did not differ from total PSA's one ( $p = 0.222$ ).

### Discussion

This study is the first long-term analysis following the fluoroPSA-EPISPOT assay evaluation on a big cohort of patients undergoing TRUS biopsies. A long follow-up was mandatory to expect relevant groups of patients as it lowers false-negative biopsy probability. This issue was all the more relevant as the mean biopsy core number was lower than expected by current guidelines (14). The actual biopsy core number reflected the evolution of the French urology association guidelines during the inclusion period.

Charlson Comorbidity Index (CCI) was related to prostate cancer risk but seemed to be linked to other patients' demographics. Age is a major component of the CCI (15) and may here be the only relevant one. The issue about age is its narrow range in a screened prostate cancer population (16) making it a poor screening tool.

Patients undergoing more than one prostate biopsy seemed to have a lower risk of prostate cancer. This conclusion is concordant with literature such as Djavan *et al.* analysis. In his study, the positive biopsy risk lowered while the round of biopsy increased, being respectively 22%, 10%, 5% et 4% at first, second, third and fourth round. Moreover, organ-confined pathological stage dramatically increased in the meantime from 58% to 100% (17), raising the over-diagnosis and over-treatment issues caused by non-cancer specific conditions triggering prostate biopsies. These concerns may contribute to explain the low relevance of fluoroPSA-EPISPOT patients as our sample mixed first round and subsequent round positive biopsy patients. Unlike several CTC studies, we explored patients' outcomes in immediate negative patients and reported secondary cancers. We found this population had lower stage disease, as patients undergoing several biopsy rounds. We analyzed patients and disease's characteristics and decided not to split patients in 3 groups (immediate positive biopsy, secondary positive biopsy, confirmed negative biopsy) as our

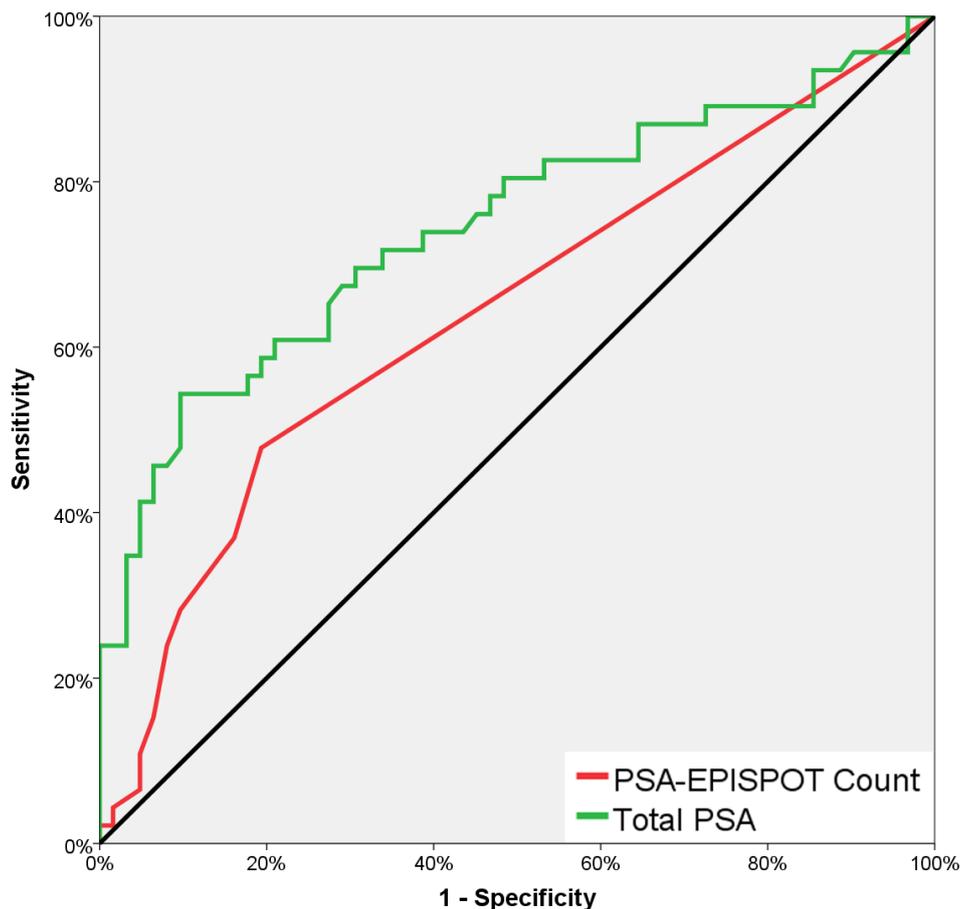


**Figure 3. Boxplots' distribution of age (A), PSA (B), prostate volume (C), biopsy round (D) and PSA-EPISPOT counts (E), based on a logarithmic scale (B, C, D, E) or linear scale (A). Outliers are plotted as individual points (represented as black stars).**

primary goal was to assess fluoroPSA-EPISPOT ability to predict immediate biopsy result. Our conclusions may thus be discussed based on false-negative prostate biopsy issue.

Despite its high relation to prostate cancer diagnosis, total PSA suffered from an important overlap between positive and negative biopsies patients. As shown on **Fig. 3**, we observed the same

conclusion concerning age, prostate volume and the number of repeated biopsy. This overlapping phenomenon was already described by Briganti *et al.* (18), explaining the poor specificity of conventional prostate cancer screening and staging tools. We were expecting less overlapping with the fluoroPSA-EPISPOT assay, however 11 patients with negative biopsies showed the presence of viable CTCs. No



**Figure 4. PSA-EPISPOT counts and total PSA ROC for predicting prostate cancer on biopsies.**

threshold adjustment could improve specificity without impairing sensitivity, which is an important issue in a screening area. Despite these considerations, specificity remained excellent (81%). Sensitivity seemed lower than in Alix-Panabieres *et al.* (12) study (47.9% vs 69.4%) but was in fact similar when considering only localized disease patients in this seminal report (41.67%, unpublished data). Our statistical analysis advocated a 1 CTC threshold to define negative or positive test. One would expect low CTC counts in a localized disease area (19) but it raises sensitivity issues. More than one CTC threshold was required for prognosis assessment using CellSearch® in the metastatic prostate cancer field (20) and may circumvent stochastic detection of rare events described based on Poisson statistics (7).

Sensitivity and specificity are important issues concerning CTC detection assays. Blood sampling conditions need to be strictly controlled as higher positive CTC ratios were observed following prostate

resection (21), biopsies (22), infection (23), or surgical manipulation without impairing prognosis (24). None of these conditions was reported here to explain CTC detection and a long follow-up makes unlikely prostate cancer misdetection. False positive due to illegitimate or ectopic transcription was a known pitfall of molecular biology techniques (9, 20). We were expecting fluoroPSA-EPISPOT to solve this issue as (i) it requires PSA-secreting viable cells and shouldn't detect apoptotic or not prostatic cells (25), and (ii) systematic negative and positive controls were achieved. An explanation could be the retrospective approach that may lead to an unknown disturbing event prior to blood sampling and advocates a prospective study. Unlike other techniques, fluoroPSA-EPISPOT didn't allow cultured cells retrieval for characterization (11), however the current improvement of a new fluoroPSA-EPISPOT assay will overcome this point and will allow CTC molecular characterization at the single cell level. On the other side, false negative

issues have also been reported when dealing with CTCs. One explanation could be the enrichment process when positive techniques are used. Despite it allows the purest sampling, phenotype modifications can induce selection failure as it has been described through epithelial-to-mesenchymal transition (7, 9, 10, 20, 26). This pitfall can also result from cell-surface marker antigens occlusion by platelets (26) or *in vitro* previous antibodies (27). Samples processing delay may also be an important issue as our technique is based on living PSA-secreting cells. However, even if we collected all blood samples in a short time (<24h) to analyze viable CTCs, we can still imagine that we faced certain variability in the viability of CTCs when analyzing them immediately after the blood draw or at 24h of shipment. A new prospective validation with a bigger cohort of patients is mandatory to confirm these results.

Previous studies on localized prostate cancer and CTC detection reported sensitivity ranging from 0 (28) to 80.3% (29) when using molecular biology techniques and 20.6 (30) to 100% (31) when using immunology. Specificity ranged from 33.3 (32) to 100% (33). Most of these studies were feasibility ones based on limited number of patients. Nowadays, there's no common technique and each research team applies its own one using proper selection of enrichment and detection methods (CTC characteristics definition). Thus, no comparison can be done between studies and techniques (7, 10, 34-36). Dedicated comparing studies are scarce but mandatory to assess correlation or superiority as Farace *et al.* did in metastatic cancers (37). The monocentric retrospective approach raises the issue of selecting patients for prostate biopsies. Here, we report a PSA-AUC of 0.729, which is high, compared to the 0.530 – 0.830 range reported by Louie *et al.* in their meta-analysis (38). Implied urologists did not report nomogram or risk calculator usage. These data may thus reflect their experience in mental synthesis of many clinical and biological variables. This high

PSA-AUC result may have impaired fluoroPSA-EPISPOT discrimination value.

### Conclusion

This study reports the first long-term analysis of the fluoroPSA-EPISPOT assay characteristics for detection of viable CTCs when blood samples have been done before prostate biopsies in a large cohort of patients undergoing TRUS biopsies. We observed lower overlapping between positive and negative biopsy patients than with conventional markers. This point is of utmost importance in a screening cohort where age and PSA range is narrow. In this retrospective cohort, sensitivity was not sufficient to prevent efficiently prostate biopsy in patients without evidence of disease. Despite its relation to prostate cancer, the PSA-EPISPOT assay seemed to be linked to another conventional marker and did not succeed logistic regression. Thus, we cannot advocate this CTC detection technique as the only assay triggering biopsies. Further analyses are mandatory to assess the PSA-EPISPOT prognosis value in patients with a positive biopsy.

### Acknowledgments

The authors thank the FEDER, the Region Languedoc Roussillon, the INCA and the DGOS supported this work through grants.

### Abbreviations

AUC	Area Under the Curve
CCI	Charlson Comorbidity Index;
CTC	Circulating Tumor Cell;
DRE	Digital Rectal Examination;
EPISPOT	EPithelial ImmunoSPOT;
LUTS	Low Urinary Tract Symptoms;
PSA	Prostate Specific Antigen;
PSA-D	PSA Density;
PSA-DT	PSA Doubling Time;
PSA-V	PSA Velocity;
TRUS	TransRectal UltraSound;

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**Supplemental Data 1** – Patients’ demographics and biological results comparison between confirmed negative biopsies patients and secondary positive patients.

	Confirmed negative prostate biopsy group n = 53 (47.7%)	Secondary positive prostate biopsy group n = 10 (9%)	Immediate positive prostate biopsy group N=48 (43.2%)	Total n = 111	p
Age (years) ¥	64.2 ± 6.3	66.6 ± 8.9	68.8 ± 7.2	66.4 ± 7.2	0.0034* 0.4750¶
Follow-up (months) ¥	69.2 [0.8 – 115.8]	68.0 [ 8.7 – 93.1]	69.8 [1.2 – 81.6]	69.5 [0.8 – 115.8]	0.5934* 0.6650¶
CCI <sup>∞</sup>					
0	1 (1.9%)	0	0	1 (0.9 %)	0.0071* 0.1760¶
1	6 (11.3%)	0	4 (8.3 %)	10 (9 %)	
2	27 (50.9%)	6 (60%)	8 (16.7%)	41 (36.9 %)	
3	10 (18.9%)	3 (30%)	14 (29.2%)	27 (24.3 %)	
4	6 (11.3%)	0	9 (18.8%)	15 (13.5 %)	
5	3 (5.7%)	0	6 (12.5 %)	9 (8.1 %)	
6	0	1 (10%)	0	1 (0.9 %)	
7	0	0	3 (6.3%)	3 (2.7 %)	
8	0	0	0	0	
9	0	0	1 (2.1 %)	1 (0.9 %)	
Prostate volume (cc) ¥	52 ± 29.2	45 ± 14.7	48 ± 33.3	50 ± 29,7	0.4577* 0.7610¶
LUTS history <sup>∞</sup>	21 (39.6%)	3 (30%)	10 (20.8 %)	34 (30.6 %)	0.1233* 0.5650¶
Prostate cancer family history <sup>∞</sup>	2 (3.8%)	1 (10%)	5 (10.4 %)	8 (7.2 %)	0.4086* 0.3960¶
Biopsy cores ¥	9.8 ± 2.5	10 ± 1.6	9.0 ± 2.4	9.5 ± 2.4	0.2599* 0.8030¶
Repeat biopsy status <sup>∞</sup>	18 (34%)	4 (40%)	10 (20.8 %)	32 (28.8 %)	0.2485* 0.7130¶
Total biopsy rounds ¥	1.9 ± 1.1	2.6 ± 0.8	1.2 ± 0.5	1.7 ± 1.0	<0.0001* 0.0280¶
Total PSA (ng/mL) ¥	6.8 [1.8 – 17]	6.4 [4.7 – 17.7]	11.0 [3.9 – 87.0]	11.7 [1.8 – 87.0]	< 0.0001* 0.8490¶
Free to total PSA ratio (%) ¥	17 [6 – 33]	16 [10 – 27]	10.6 [7 – 77]	16.0 [6 – 77]	0.1724* 0.9120¶
PSA-V (ng/mL/year) ¥	0.5 [-36 – 9]	0.7 [0 – 1.6]	2.0 [-151 – 63]	0.9 [-151 – 63]	0.0206* 0.8370¶
PSA-DT (months) ¥	52 [-122 – 539]	59 [40 – 1910]	31.9 [-32 – 567]	40.5 [-122 – 1910]	0.1630* 0.2320¶
PSA-D (ng/mL/cc) ¥	0.2 [0.04 – 0.65]	0.2 [0.11 – 0.35]	0.3 [0.08 – 2.05]	0.2 [0.04 – 2.05]	0.0003* 0.5340¶
Blood sample volume (mL) ¥	17.2 ± 3.3	17.4 ± 3.1	18.2 ± 3.4	17.7 ± 3.3	0.3693* 0.8340¶
PSA-EPISPOT count ¥	0.9 ± 2.1	0.1 ± 0.3	2.0 ± 3.1	1.3 ± 2.8	0.0047* 0.3510¶

**Abbreviations:** CCI, Charlson Comorbidity Index; cc, cm<sup>3</sup>; LUTS, Lower Urinary Tract Symptoms; PSA-V, PSA Velocity; PSA-DT, PSA Doubling Time; PSA-D, PSA Density.

**Statistical analysis:** categorical variables were compared through khi<sup>2</sup> or Fisher test (¥) and continuous variables through kruskall-wallis analysis (∞). *p* result was given marked with “\*” concerning all patients’ categories and with “¶” for comparison between confirmed negative biopsy and secondary positive biopsy.