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# Prognostic Role of Prostate-specific Antigen Isoforms and Their Early Kinetics in Patients With Metastatic Castration-resistant Prostate Cancer Receiving New Generation Androgen Receptor Targeted Agents

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## Abstract

*Background/Aim:* New generation androgen receptor-targeting agents (ARTA) have been in the spotlight for their efficacy in metastatic castration-resistant prostate cancer (mCRPC). Prostate-specific antigen (PSA) represents one of the most commonly used serum cancer biomarkers worldwide. The present retrospective study focused on the prognostic role of serum PSA isoforms and their early dynamics in mCRPC patients treated with abiraterone acetate (ABI) or enzalutamide (ENZ).

*Patients and Methods:* The association between outcomes of 334 mCRPC patients treated with ABI or ENZ and the levels of serum total PSA (tPSA), free PSA (fPSA), [-2]proPSA and the Prostate Health Index (PHI) at baseline and one month after treatment initiation was analyzed retrospectively.

*Results:* In the multivariable Cox proportional hazards models, baseline tPSA>50 μg/l ($p$<0.001), and [-2]proPSA>300 ng/l ($p$=0.017) remained independent significant factors associated with inferior OS, while baseline fPSA>1.75 μg/l ($p$=0.050) and Δ [-2]proPSA >–50% approached statistical significance ($p$=0.062). The results of ROC analyses assessing the ability

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of baseline tPSA, fPSA, and [-2]proPSA to predict mortality within two years showed area under the curve (AUC) values of 0.709, 0.685, and 0.740, respectively. Among the subgroup with baseline tPSA≤20.0 μg/l, the results of ROC analyses for baseline tPSA, fPSA and [-2]proPSA showed AUC values of 0.441, 0.682, and 0.688, respectively.
*Conclusion:* Our results suggest a significant correlation between pretreatment serum levels of tPSA and [-2]proPSA with OS in mCRPC patients receiving ARTA.

**Keywords:** Castration-resistant prostate cancer, ARTA, enzalutamide, abiraterone acetate, PSA, free PSA, (-2)proPSA.

## Introduction

Prostate cancer (PC) is the second most common cancer in men (1, 2). It poses significant challenges for both patients and healthcare providers. Metastatic castration-resistant prostate cancer (mCRPC) is one of the most challenging forms of prostate cancer among its advanced stages. This is an advanced prostate cancer characterized by resistance to initial androgen deprivation therapy (ADT) and by strong metastatic activity. Notwithstanding therapeutic advances, mCRPC remains challenging to treat due to its aggressive nature and limited treatment options. In recent years, there has been significant progress in understanding the genomic landscape and biological functions of prostate cancer. This has led to the development of novel therapeutics. New generation androgen receptor-targeting agents (ARTA) such as abiraterone acetate, enzalutamide, apalutamide, and darolutamide have been in the spotlight for their efficacy in mCRPC. Prostate-specific antigen (PSA) represents one of the most commonly used serum cancer biomarkers worldwide. It has been used for decades to diagnose, prognosticate, and monitor treatment in patients with PC. However, PSA has several important limitations, especially in mCRPC. Historically, post-treatment changes in PSA levels have not been shown to have a robust association with survival of mCRPC patients and have not been qualified as an endpoint to support regulatory approval. Previously, the low diagnostic specificity of total PSA (tPSA) for detecting prostate cancer was improved by identifying two major molecular subforms of PSA, including free PSA (fPSA), an unbound fraction, and a form complexed with the protease inhibitor alpha-1-antichimotrypsin (3). Further research led to detection of precursor forms of fPSA, known as proPSA. This fraction could be further differentiated by the detection of truncated forms that are more resistant to activation into mature PSA. In particular, [-2]proPSA is the most consistent of these truncated forms (4). In the current clinical practice, [-2]proPSA has an important role as a diagnostic serum biomarker, especially in combination with tPSA and fPSA to calculate the Prostate Health Index (PHI), which significantly improves prostate cancer detection (5). In addition, as the variables increased with increasing Gleason score, there was evidence of an association with highly aggressive forms of PC (6, 7). While the role of the above PSA isoforms is well established in PC diagnostics, their prognostic role in patients with mCRPC remains underexplored.

In the present retrospective study we focused on the prognostic role of serum PSA isoforms and their early kinetics in mCRPC patients treated with abiraterone acetate (ABI) or enzalutamide (ENZ).

## Patients and Methods

*Study design*. Data from mCRPC patients receiving ABI or ENZ were retrospectively analyzed. We assessed the association between the outcome of patients and serum total PSA (tPSA), free PSA (fPSA), [-2]proPSA and the PHI at baseline and one month after treatment initiation. Clinical data were extracted from the hospital information

system. The protocol of the study and the informed consent form for participants were approved by the Ethical Committee of the Faculty of Medicine and University Hospital in Pilsen on April 6, 2023 (No. 155/23) and complied with the International Ethical Guidelines for Biomedical Research, the Declaration of Helsinki, and local laws. Informed consent was obtained from all the participants.

*Patients and treatment*. Patients with histologically confirmed mCRPC were treated between 2007 and 2023 at the Department of Oncology and Radiotherapeutics, University Hospital in Pilsen, Czech Republic. ABI (Zytiga, Janssen Pharmaceuticals Co., Beerse, Belgium) was administered orally in the standard approved schedule (1,000 mg daily) in combination with prednisone (10 mg daily). ENZ (Xtandi, Astellas Pharma Inc., Tokyo, Japan) was administered orally in the standard approved dose (160 mg daily). The therapy was continued until disease progression, unacceptable toxicity, or patient refusal. Routine clinical checks including physical examination and biochemical laboratory tests were performed each month. Radiographic controls using computed tomography (CT) or positron emission tomography-CT (PET/CT) or PET-magnetic resonance (PET/MR) were performed every three to six months.

*Assessment of PSA isoforms*. Peripheral blood was drawn using VACUETTE Z Serum Sep tubes (Greiner Bio-One, Kremsmünster, Austria) and allowed to clot. Serum was separated within three hours of blood collection and analyzed. ACCESS chemiluminescent assays (Beckman Coulter, Brea, CA, USA) were used for the measurement of tPSA, fPSA and [-2]proPSA. The assessed biomarkers included measured biomarkers: tPSA, fPSA, [-2]proPSA, and calculated parameters: free/total PSA ratio and PHI. The calculation of free/total PSA ratio and PHI was performed using the formulas: free/total PSA ratio=free PSA/total PSA and PHI=([−2] proPSA/freePSA)×√tPSA. The prognostic role of these biomarkers was evaluated based on their baseline values and their relative change in percentage after one month of therapy [(*after one month* - *baseline*)/*baseline*, denoted by "Δ… (%)"]. The data were analyzed for the overall patient population and, additionally, for the subgroup of patients with relatively low baseline tPSA, defined as ≤20 μg/l.

*Statistical analysis*. Standard descriptive statistics and frequencies were used to characterize the data set. Overall survival (OS) was defined as the time from the date of treatment initiation until the date of death. Patients still alive at the end of observation were censored at the date of the last follow-up. OS was estimated using the Kaplan-Meier method by point estimates and two-sided 95% confidence intervals; two-group comparisons were performed using the Gehan-Wilcoxon test. Univariable Cox proportional hazards model was used to assess the effects of continuous biomarker levels on OS, while multivariable version of this model was then used to verify the prognostic independence of selected markers in the context of other common clinical factors. Threshold values for Multivariate Cox and Kaplan-Meier analysis were determined by plotting the Cox-Mantel *p*-value for all possible threshold values and manually selecting rounded cut-off points that produced the smallest *p*-values, thereby leading to the maximally significant separation of groups. The ability of marker levels to predict death within two years of treatment initiation was quantified using the area under curve (AUC) of the receiver operating characteristic (ROC). The median follow-up time was estimated using the inverse Kaplan-Meier method. The level of statistical significance was set at $\alpha=0.05$ and all reported *p*-values are two-tailed. The statistical analysis was performed using STATISTICA (Version 12; StatSoft, Inc., TuIsa, OK, USA) and MATLAB (R2021a, The MathWorks Inc., Natick, MA, USA).

## Results

*Patient characteristics*. In total, 334 mCRPC patients were included in our study and their baseline clinical characteristics are summarized in Table I.

Table I. *Baseline patient characteristics.*

| Characteristic, n (%) | All patients (*n*=334) |
|---|---|
| Age at treatment initiation (yrs) | |
| Median (range) | 72.7 (51.5-98.8) |
| tPSA at treatment initiation | |
| Median (range) | 24.5 (0-2,006) |
| ≤20 μg/l | 148 (44.6%) |
| >20 μg/l | 184 (55.4%) |
| Not available | 2 |
| Gleason score | |
| 3-6 | 45 (14.8%) |
| 7 | 96 (31.6%) |
| 8-10 | 163 (53.6%) |
| Not available | 30 |
| Therapy | |
| Enzalutamide | 179 (53.6%) |
| Abiraterone acetate | 155 (46.4%) |
| Synchronous metastases | |
| Yes | 153 (45.8%) |
| No | 181 (44.2%) |
| Previous prostatectomy | |
| Yes | 64 (19.2%) |
| No | 270 (80.8%) |
| Previous radiotherapy | |
| Yes | 141 (42.2%) |
| No | 193 (57.8%) |
| Metastatic sites (non-exclusive) | |
| Lymph nodes | 233 (70.0%) |
| Bone | 283 (85.0%) |
| Visceral | 49 (14.7%) |
| Not specified | 1 |

tPSA: Total prostate-specific antigen.

Table II. *Univariable Cox proportional hazards model evaluating impact of the assessed serum biomarkers on overall survival.*

| Predictor | Overall survival | *p*-Value |
|---|---|---|
| All patients | | |
| Baseline tPSA | 1.001 (1.000–1.001) | **0.018** |
| Baseline fPSA | 1.152 (1.025–1.296) | **0.018** |
| Baseline free/total PSA | 1.015 (0.996–1.035) | 0.113 |
| Baseline (-2)proPSA | 1.001 (1.000–1.001) | **0.006** |
| Baseline Prostate Health Index (PHI) | 1.002 (0.997–1.006) | 0.478 |
| Δ tPSA (%) | 1.024 (0.999–1.049) | 0.057 |
| Δ fPSA (%) | 1.278 (0.423–3.862) | 0.663 |
| Δ free/total PSA | 0.621 (0.166–2.325) | 0.479 |
| Δ (-2)proPSA (%) | 1.286 (1.031–1.604) | **0.026** |
| Δ PHI (%) | 0.846 (0.241–2.975) | 0.795 |
| Only patients with baseline tPSA ≤20 μg/l | | |
| Baseline tPSA | 1.028 (0.978–1.079) | 0.279 |
| Baseline fPSA | 1.262 (1.032–1.543) | **0.024** |
| Baseline free/total PSA | 1.030 (1.008–1.054) | **0.008** |
| Baseline (-2)proPSA | 1.005 (1.001–1.009) | **0.021** |
| Baseline PHI | 1.004 (0.999–1.009) | 0.090 |
| Δ tPSA (%) | 1.032 (1.001–1.064) | **0.041** |
| Δ fPSA (%) | 1.431 (0.489–4.185) | 0.513 |
| Δ free/total PSA | 0.645 (0.182–2.287) | 0.497 |
| Δ (-2)proPSA (%) | 1.313 (0.961–1.795) | 0.087 |
| Δ PHI (%) | 0.908 (0.277–2.970) | 0.873 |

PSA: Prostate-specific antigen; PHI: prostate health index; tPSA: total PSA; fPSA: free PSA. Statistically significant *p*-values are shown in bold.

*Patient survival*. Median OS for the whole cohort was 30.9 months (95%CI=24.4-35.3). At the time of data analysis, 161 (48.2%) patients died and the median follow-up time was 30.7 months.

The univariable Cox proportional hazards model evaluating the impact of the assessed serum biomarkers on OS revealed that the baseline levels of tPSA ($p$=0.018, HR per unit increase=1.001, 95%CI=1.000-1.001), fPSA ($p$=0.018, HR per unit increase=1.152, 95%CI=1.025-1.296), [-2]proPSA ($p$=0.006, HR per unit increase=1.001, 95%CI=1.000-1.001), and Δ [-2]proPSA ($p$=0.026, HR per unit increase=1.286, 95%CI=1.031-1.604) were significant predictors. Among the subgroup with baseline tPSA ≤20.0 μg/l, the results of the univariable Cox model revealed that the baseline fPSA ($p$=0.024, HR per unit increase=1.262, 95%CI=1.032-1.543), fPSA/tPSA ratio ($p$=0.008, HR per unit increase=1.030, 95%CI=1.008-1.054), [-2]proPSA ($p$=0.021, HR per unit increase=1.005, 95%CI=1.001-1.009), and ΔtPSA ($p$=0.041, HR per unit increase=1.032, 95%CI=1.001-1.064) were significant predictors for OS. The results of the univariable Cox model are summarized in Table II and the Kaplan-Meier curves are shown in Figure 1. In the multivariable Cox proportional hazards models, baseline tPSA >50 μg/l ($p$<0.001, HR=2.511, 95%CI=1.757-3.587), and [-2]proPSA >300 ng/l ($p$=0.017, HR=2.283, 95%CI= 1.161-4.493) remained independent significant factors associated with inferior OS, while baseline fPSA>1.75 μg/l ($p$=0.050, HR=2.291, 95%CI=1.000-5.249) and Δ [-2]proPSA >–50% approached statistical significance ($p$=0.062, HR=2.170, 95%CI=0.960-4.904) (Table III).

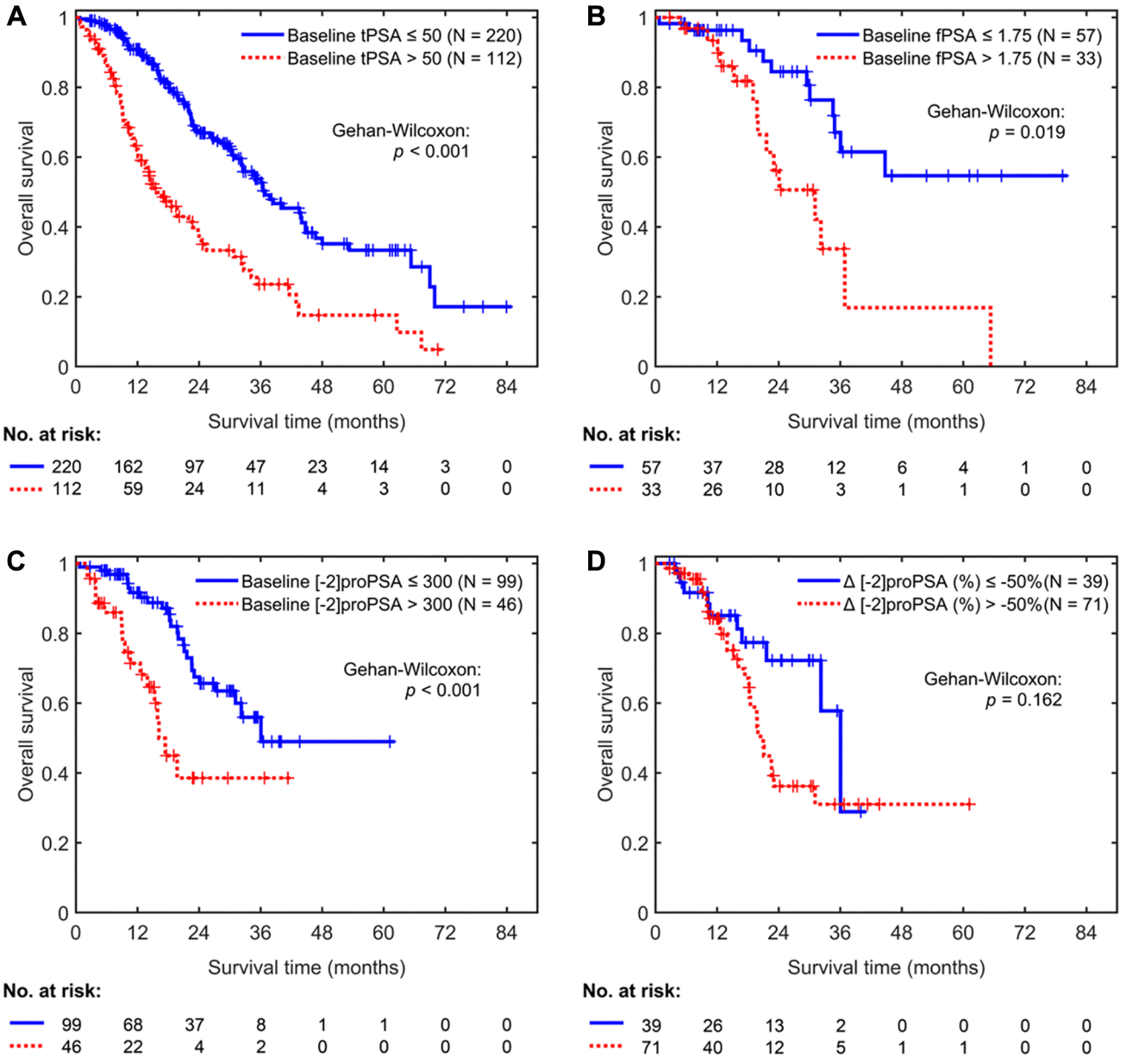


Figure 1. *Overall survival according to the baseline total prostate-specific antigen (tPSA) (A), free PSA (fPSA) (B), [-2]proPSA (C) and early change (Δ [-2]proPSA, %) (D).*

*ROC analyses.* The results of ROC analyses assessing the ability of baseline tPSA, fPSA, and [-2]proPSA to predict mortality within two years showed AUC values of 0.709, 0.685, and 0.740, respectively. Among the subgroup with baseline tPSA≤20.0 μg/l, the results of ROC analyses for baseline tPSA, fPSA, and [-2]proPSA showed AUC values of 0.441, 0.682, and 0.688, respectively. The results of the ROC analyses are summarized in Table IV; selected ROC curves for baseline tPSA, fPSA, and [-2]proPSA are shown in Figure 2.

Table III. *Multivariable Cox proportional hazards model evaluating impact of selected serum biomarkers on overall survival (OS).*

| Variable | Category | OS model with Baseline total PSA (*n*=299) | | OS model with Baseline free PSA (*n*=87) | | OS model with Baseline (-2)proPSA (*n*=138) | | OS model with Δ (-2)proPSA (%) (*n*=104) | |
|---|---|---|---|---|---|---|---|---|---|
| | | HR (95% CI) | *p*-value | HR (95% CI) | *p*-value | HR (95% CI) | *p*-value | HR (95% CI) | *p*-value |
| Age | | | 0.701 | | 0.616 | | 0.186 | | 0.315 |
| | <70 years | 1 | | 1 | | 1 | | 1 | |
| | ≥70 years | 1.073 (0.748-1.540) | | 1.248 (0.526-2.959) | | 1.589 (0.801-3.153) | | 1.494 (0.683-3.269) | |
| Line of therapy for mCRPC | | | 0.200 | | 0.184 | | 0.162 | | 0.260 |
| | 1st | 1 | | 1 | | 1 | | 1 | |
| | 2nd or higher | 1.290 (0.874-1.905) | | 2.127 (0.698-6.484) | | 1.813 (0.787-4.175) | | 1.668 (0.684-4.069) | |
| Gleason score (GS) | | | **0.002** | | 0.888 | | **0.000** | | **0.003** |
| | GS≤7 | 1 | | 1 | | 1 | | 1 | |
| | GS>7 | 1.735 (1.227-2.455) | | 1.063 (0.453-2.495) | | 3.691 (1.779-7.660) | | 3.169 (1.468-6.842) | |
| Synchronous metastases | | | 0.054 | | 0.596 | | **0.008** | | **0.034** |
| | No | 1 | | 1 | | 1 | | 1 | |
| | Yes | 1.426 (0.994-2.046) | | 1.281 (0.513-3.200) | | 2.580 (1.274-5.222) | | 2.353 (1.069-5.180) | |
| Visceral metastases | | | **0.004** | | 0.317 | | 0.264 | | 0.370 |
| | No | 1 | | 1 | | 1 | | 1 | |
| | Yes | 1.897 (1.225-2.936) | | 0.553 (0.174-1.763) | | 1.648 (0.686-3.958) | | 1.508 (0.614-3.703) | |
| Therapy | | | 0.388 | | 0.189 | | 0.390 | | 0.926 |
| | Abiraterone ac. | 1 | | 1 | | 1 | | 1 | |
| | Enzalutamide | 0.858 (0.606-1.214) | | 0.537 (0.213-1.359) | | 0.751 (0.391–1.442) | | 1.034 (0.508–2.105) | |
| Baseline tPSA | | | **<0.001** | | – | | – | | – |
| | ≤50 μg/l | 1 | | – | | – | | – | |
| | >50 μg/l | 2.511 (1.757-3.587) | | – | | – | | – | |
| Baseline fPSA | | | – | | 0.050 | | – | | – |
| | ≤1.75 μg/l | – | | 1 | | – | | – | |
| | >1.75 μg/l | – | | 2.291 (1.000-5.249) | | – | | – | |
| Baseline (-2)proPSA | | | – | | – | | **0.017** | | – |
| | ≤300 ng/l | – | | – | | 1 | | – | |
| | >300 ng/l | – | | – | | 2.283 (1.161-4.493) | | – | |
| Δ (-2)proPSA (%) | | | – | | – | | – | | 0.063 |
| | ≤50% | – | | – | | – | | 1 | |
| | >50% | – | | – | | – | | 2.1703 (0.9605-4.9040) | |

mCRPC: Metastatic castration-resistant prostate cancer; PSA: prostate-specific antigen; CI: confidence interval; HR: hazard ratio; tPSA: total PSA; fPSA: free PSA. Statistically significant *p*-values are shown in bold.

## Discussion

The results of our study suggest that baseline tPSA and [-2]proPSA levels are independent predictors of OS in mCRPC patients treated with ARTA. We did not find a significant association between OS and early change (after one month of treatment) in either tPSA or PSA isoforms including fPSA, [-2]proPSA, or PHI. A non-significant trend

Table IV. *Results of the ROC analyses for baseline tPSA, fPSA, and (-2)proPSA.*

| Death within two years<br>All patients | |
|---|---|
| | AUC |
| Baseline tPSA | 0.709 |
| Baseline fPSA | 0.685 |
| Baseline (-2)proPSA | 0.740 |
| Only patients with baseline tPSA ≤20.0 μg/l | |
| | AUC |
| Baseline tPSA | 0.559 |
| Baseline fPSA | 0.682 |
| Baseline (-2)proPSA | 0.688 |

PSA: Prostate-specific antigen; tPSA: total PSA; fPSA: free PSA.

for baseline fPSA and the early change of [-2]proPSA was observed. Additionally, in the univariate analysis, baseline fPSA and [-2]proPSA levels showed significant association with OS, in contrast to baseline tPSA in a subgroup of patients with low baseline tPSA (≤20 μg/l).

The clinical management of mCRPC is constantly evolving and the landscape of systemic therapies has improved dramatically in recent years. The advent of ARTA revolutionized the treatment of mCRPC, leading to a significant improvement of patient survival and quality of life. Furthermore, several combination regimens composed of poly-ADP ribose polymerase (PARP) inhibitors (PARPi) and ARTA have recently emerged. These combinations represent abiraterone acetate plus olaparib and enzalutamide plus talazoparib (8, 9). The expansion of therapeutic options is leading to an urgent need to find effective prognostic biomarkers that can be used to estimate the aggressiveness of the disease and to personalize the treatment. Although tPSA has been used for decades as a key serum biomarker in the diagnosis, follow-up and prognosis of PC patients, its specific role in patients with mCRPC is limited. Therefore, research on prognostic biomarkers for mCRPC is of great interest in the uro-oncology community. In the present study, we focused on the prognostic role of baseline and early changes in PSA isoforms as candidate prognostic serum biomarkers, alongside tPSA, an established serum biomarker in PC. We observed a significant association between baseline [-2]proPSA and OS, which is in agreement with data from a prospective study including 98 CRPC patients treated with enzalutamide reported by Miyazawa *et al.* Their results also showed a significant correlation between baseline PHI and OS, which was not confirmed by our results (10). In the present study, we also performed a univariate analysis focused on a subgroup of patients with low baseline tPSA and the results showed a significant association between baseline [-2]proPSA, fPSA, and free/total PSA with OS, whereas baseline tPSA did not show a significant association with OS. These interesting data suggest a higher utility of PSA isoforms in predicting OS, in contrast to the lack of a prognostic role for tPSA in mCRPC patients with low baseline tPSA. This can be clearly seen from the results of the ROC analyses focusing on the ability to predict mortality within two years. However, we were not able to perform a multivariate analysis due to the limited number of patients. Our results confirmed the prognostic role of baseline tPSA that has been previously reported by others (11-14). Several retrospective studies confirmed that mCRPC patients achieving a 50% decline in tPSA from the baseline levels derive a survival benefit compared with those who did not achieve such tPSA reduction. However, with conflicting data published to date, the role of early tPSA kinetics in predicting OS during ARTA treatment remains uncertain (14-19). Early tPSA kinetics does not always indicate which patients may still benefit from ARTA treatment. Therefore, tPSA kinetics could not be validated as an independent prognostic marker of treatment response in the registration clinical trials (20, 21). In addition, an early rise in tPSA (PSA flare) during the first three months of treatment with ARTA, followed by a delayed decline, occurs in approximately 10% of mCRPC patients treated with ARTA and it was found to be a prognostic factor for improved survival (22). In daily clinical practice, it is often difficult to differentiate between a PSA flare and a continuous rise, which may lead to

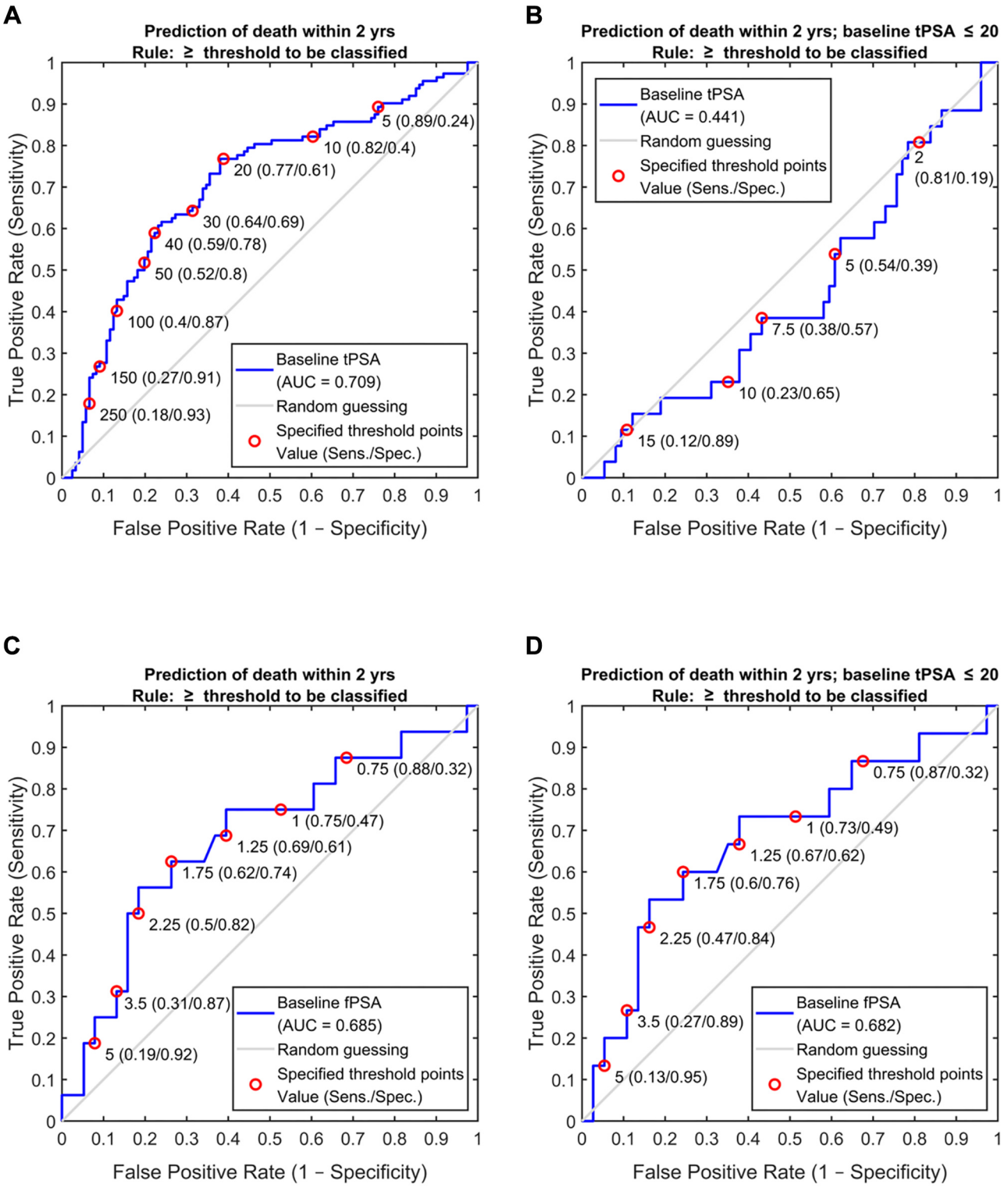


Figure 2. *Continued*

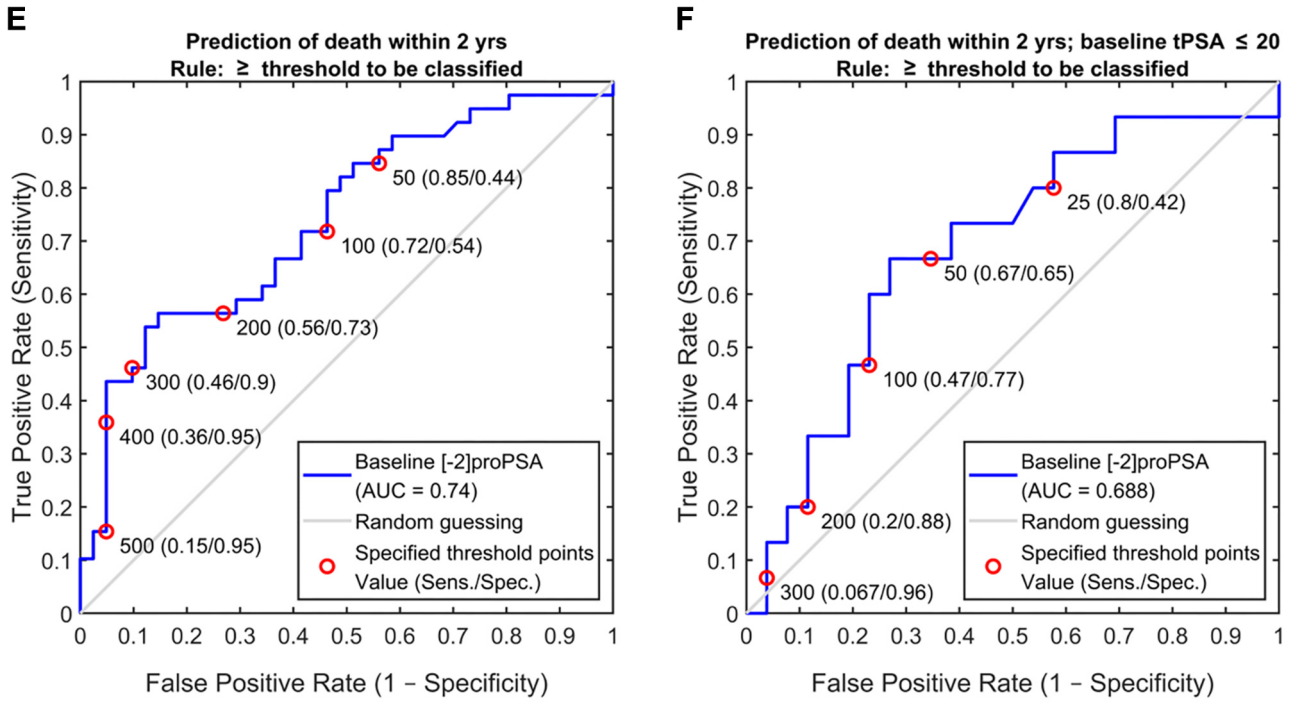


Figure 2. *Receiver operating characteristics (ROC) showing prediction of mortality within two years for baseline total prostate-specific antigen (tPSA), free PSA (fPSA), and [-2]proPSA in all patients (A, C, E) and separately in the subgroup of those with baseline tPSA ≤20 μg/l (B, D, F).*

premature discontinuation of therapy or unnecessary waiting for a delayed tPSA decline, which in many cases could result in a treatment beyond true progression. In our study, we did not observe a significant association between early tPSA kinetics and OS, which is in agreement with several previous studies (19). The prognostic role of the kinetics of PSA isoforms as an alternative to tPSA in mCRPC patients has been underexplored. Promising results suggesting a prognostic role of early kinetics of fPSA and [-2]proPSA in mCRPC patients treated with Abiraterone acetate have been reported by Schlack *et al.* They analyzed the change in PSA isoforms between baseline and 8-12 weeks after the initiation of therapy in 25 patients and their results showed a significant correlation of the relative median change in fPSA ($p$=0.03) and [-2]proPSA ($p$=0.05) with PFS (15). In our study, we observed a significant association of early [-2]proPSA kinetics with OS in a univariate analysis, showing a better prognosis in patients with a decrease of 50% or more; however, the results of the multivariate analysis were beyond statistical significance ($p$=0.063). Nevertheless, our results support further research into the role of PSA isoform kinetics in prognostication and therapy monitoring in patients with mCRPC receiving ARTA. On the other hand, our results show that the role of such biomarkers is currently limited, especially in the area of therapy monitoring, and support further research into alternative blood-based biomarkers. The most promising may be the methods focusing on circulating tumor cells and/or circulating tumor DNA (23-26).

Major limitations of the present study include its retrospective design and the relatively limited sample size, especially when divided into different subgroups. Nevertheless, our study included the largest cohort of mCRPC patients treated with ARTA in which the prognostic role of PSA isoforms has been evaluated. The

results of our study may have been influenced by the early measurement of serum biomarker kinetics shortly after treatment initiation.

In conclusion, the results of our study suggest a significant correlation between pretreatment serum levels of tPSA and [-2]proPSA with OS in mCRPC patients receiving ARTA. However, while we support further research into the role of PSA isoform kinetics, our results suggest that their role, particularly in therapy monitoring, may be limited. Therefore, the search for other blood-based biomarkers, especially in the field of liquid biopsy, is warranted.

## Conflicts of Interest

OF received honoraria from Novartis, Janssen, Merck, and Pfizer for consultations and lectures unrelated to this project. JF has received honoraria from Astra Zeneca, Roche, and Novartis for consultations and lectures unrelated to this project. MH received honoraria from Merck Sharp & Dohme for consultations and lectures unrelated to this project. PH, HK, MT, DŠ, PS, RK, JW, and OT declare that they have no conflicts of interest that might be relevant to the contents of this manuscript.

## Authors' Contributions

OF designed the study, collected clinical data, and wrote the manuscript with support from PH, HK, MK, MH, DŠ, PS, JF, RK, JW, and OT; PH performed statistical analyses.

## Acknowledgements

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