Proteome profiling of enzalutamide-resistant cell lines and serum analysis identified ALCAM as marker of resistance in castration-resistant prostate cancer

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Abstract

Enzalutamide (ENZA) is a frequently used therapy in metastatic castration-resistant prostate cancer (mCRPC). Baseline or acquired resistance to ENZA have been observed, but the molecular mechanisms of resistance are poorly understood. We aimed to identify proteins involved in ENZA resistance and to find therapy-predictive serum markers. We performed comparative proteome analyses on ENZA-sensitive parental (LAPC4, DuCaP) and -resistant prostate cancer cell lines (LAPC4-ENZA, DuCaP-ENZA) using liquid chromatography tandem mass spectrometry (LC-MS/MS). The top four most promising candidate markers were selected using bioinformatic approaches. Serum concentrations of selected markers (ALCAM, AGR2, NDRG1, IDH1) were measured in pretreatment samples of 72 ENZA-treated mCRPC patients using ELISA. In addition, ALCAM serum levels were measured in 101 Abiraterone (ABI) and 100 Docetaxel (DOC)-treated mCRPC patients' baseline samples. Results were correlated with clinical and follow-up data. The functional role of ALCAM in ENZA resistance was assessed in vitro using siRNA. Our proteome analyses revealed 731 significantly differentially abundant proteins between ENZA-sensitive and -resistant cells and our filtering methods identified four biomarker candidates. Serum analyses of these proteins revealed only ALCAM to be associated with poor patient survival. Furthermore, higher baseline ALCAM levels were associated with poor survival in ABI- but not in DOC-treated patients. In LAPC4-ENZA resistant cells, ALCAM silencing by siRNA knockdown resulted in significantly enhanced ENZA sensitivity.
1 | INTRODUCTION

In the last decade, the management of metastatic castration-resistant prostate cancer (mCRPC) has significantly changed. Beside docetaxel (DOC) therapy, novel treatments became available for these patients including cabazitaxel,\(^1\) next-generation androgen receptor (AR) inhibitors (abiraterone (ABI),\(^2,3\) enzalutamide\(^4,5\)), immunotherapy (sipuleucel-T)\(^6\); PARP-inhibitors (olaparib, rucaparib\(^7,8\)), radiopharmaceuticals (radium-223)\(^9\) and radiogland therapy.\(^10\) Despite the obvious benefits achieved with these therapies, mCRPC remains a lethal disease with a poor clinical outcome.

Enzalutamide (ENZA) is a second-generation androgen receptor inhibitor, which has multiple effects on androgen signaling, including inhibition of ligand binding to AR, blockade of AR nuclear translocation and prevention of AR binding to DNA. In phase III studies, ENZA treatment significantly prolonged overall and progression-free survival of mCRPC patients in the pre- and postchemotherapy setting.\(^4,5\) Unfortunately, many patients show intrinsic or develop acquired resistance. Here, the authors used proteome analysis to identify proteins that contribute to enzalutamide resistance. By comparing protein expression in cells sensitive or resistant to enzalutamide, they created a list of 731 proteins of interest, which they later narrowed down to four potential biomarkers. Only ALCAM was associated with poor patient survival. Silencing ALCAM expression, they found, increased enzalutamide sensitivity.

Our analyses revealed that ALCAM serum levels may help to identify ENZA- and ABI-resistant patients and may thereby help to optimize future clinical decision-making. Our functional analyses suggest the possible involvement of ALCAM in ENZA resistance.

KEYWORDS
ALCAM, biomarker, enzalutamide, prostate cancer, proteome analysis

What's new?
Over the years, many new treatments have come on the market for metastatic castration-resistant prostate cancer, including enzalutamide, an androgen receptor inhibitor with multiple mechanisms of action. Still, patients often develop resistance. Here, the authors used proteome analysis to identify proteins that contribute to enzalutamide resistance. By comparing protein expression in cells sensitive or resistant to enzalutamide, they created a list of 731 proteins of interest, which they later narrowed down to four potential biomarkers. Only ALCAM was associated with poor patient survival. Silencing ALCAM expression, they found, increased enzalutamide sensitivity.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

Enzalutamide sensitive LAPC4 (RRID:CVCL\_4744), DuCaP (RRID: CVCL\_2025) and resistant LAPC4-ENZA and DuCaP-ENZA PC cell lines were a gift from Dr. Martin Puhr. ENZA resistance has been developed by the dose escalation method as described by Hoefer et al.\(^21\) LAPC4 and DuCaP cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France),
1% Glutamax (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Lonza, Basel, Switzerland). LAPC4 were further supplemented with 1 nM dihydrotestosterone (DHT). All cells were maintained at 37°C in 5% CO2 with humidified atmosphere. Medium was changed every third day. The parental cell lines as well as the ENZA-resistant subclones have recently been authenticated using short tandem repeat profiling (June 2021). All experiments were performed with mycoplasma-free cells. ENZA (MedChemExpress) was dissolved in DMSO as a 10 mM stock solution and stored at –80°C.

2.2 | Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

Proteome analyses were performed using the LC-MS/MS technique in order to identify differentially expressed proteins between ENZA-sensitive (LAPC4, DuCaP) and ENZA-resistant (LAPC4-ENZA, DuCaP-ENZA) cell lines. For the LC-MS/MS analysis, six technical replicates for each cell line were used. The significance of expression changes was tested by calculating an unpaired, Students’ t test. Details on sample preparation, LC-MS/MS and protein identification are described in Supplementary Materials and Methods.

2.3 | Gene ontology and KEGG pathway analysis

Gene Ontology (GO) biological process annotation and KEGG enrichment analysis was performed with using STRING (string-db.org). Proteins that were significantly up- and downregulated in ENZA-resistant cells, respectively, were analyzed separately. The enriched ontologies (Biological Process) were filtered (observed gene count ≥25, false discovery rate ≤0.0001, strength ≥0.25, background gene count ≤3000) and manually reviewed to remove redundant and inapplicable terms.

2.4 | Biomarker selection

In ENZA-resistant cells, proteins quantified by at least two unique peptides with significantly higher abundance than those of ENZA-sensitive cells at t-test P < .05 were considered as significantly differentially abundant (Figure S1). In order to identify the most promising proteins, we used three different approaches.

First, in order to identify potentially secreted proteins, we used a workflow, which applies the combination of existing prediction programs (SignalP 4.1, SecretemP 2.0, TargetP 1.1 and TMHMM 2.0) and databases (Uniprot, Human Protein Atlas, NCBI, ExoCarta).

Second, we performed a cross-reference analysis with a formerly published independent transcriptome dataset of ENZA-sensitive and -resistant prostate cancer cell lines in order to identify an overlap with our list of upregulated proteins.22

Third, we applied a pathway- and literature-based selection method by scoring molecular interactions (number of edges) according to the STRING database. In addition, we considered the proteins’ known oncological role according to literature as well as the availability of ELISA assays for later serum analyses.

2.5 | Patient cohort and sample

Pretreatment serum concentrations of selected proteins were measured in samples of 72 mCRPC patients who were treated with ENZA therapy as first or later line treatment between September 2013 and March 2016. As we found high baseline ALCAM levels to be associated with poor survival of ENZA-treated patients, we measured ALCAM concentrations also in baseline serum samples of ABI- and DOC-treated mCRPC patients. The ABI cohort included 101 mCRPC patients who underwent ABI therapy between November 2008 and May 2015. The DOC cohort included 100 mCRPC patients who received DOC chemotherapy between January 2013 and April 2019. Serum samples were collected directly before commencing ENZA, ABI and DOC treatment. In addition, for 64 ENZA-, 39 ABI- and 44 DOC-treated patients, serum samples at 3 months after initiation of therapy start were also available. Serum samples were collected at the Department of Urology at the Medical University of Vienna and at the Semmelweis University, Budapest. Blood samples were collected in 9 mL Vacuette tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and centrifuged for 10 min at room temperature with an Eppendorf 5702R centrifuge at 1500g within 3 h of venepuncture. Serum samples were immediately aliquoted and stored on –80°C until analysis. The study was performed in accordance with the ethical standards of the Helsinki Declaration and was approved by the ethical boards of the hospitals (TUKEB 55/2014, ECS 1986/2017). PSA response was defined, according to the Prostate Cancer Clinical Trials Working Group Criteria (PCWG) II, as at least 50% PSA decline from baseline during therapy.23

2.6 | Serum ELISA analyses

ALCAM serum levels were quantified in 420 serum samples of 273 patients by using the DuoSet ELISA assay (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Serum concentration of AGR2, IDH1 and NDRG1 were measured in 72 ENZA-treated patients by using ELISA assays (AGR2: SEC285Hu, IDH1: SEH839Hu, Cloud Clone Corp, USA; NDRG1: OKEH02359, Aviva System Biology Corp, San Diego; respectively). Absorbance was measured at 450 nm by a Multiscan FC Microplate Photometer (Thermo Fisher Scientific, Darmstadt, Germany).

2.7 | Western blot analysis

For Western Blot analysis, cells were lysed in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) containing 1:100 Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) according to the
manufacturer's instructions. A total of 20 μg protein per sample was loaded on bolt 4% to 12% Bis-Tris Plus gels (Thermo Fisher Scientific) for electrophoresis separation. Then, gels were blotted onto nitrocellulose membrane (iBlot 2 Transfer Stacks, Thermo Fisher Scientific) using iBlot 2 Gel Transfer Device. Gel electrophoresis and transfer was performed according to the manufacturer's instructions. Membranes were incubated in 5% nonfat dry milk (NFDM)/Tris-buffered saline with 0.1% Tween 20 detergent (TBST) for 1 h at room temperature. The following antibodies were used: anti-ALCAM antibody (1:10,000; Abcam, ab109215) with goat anti-rabbit secondary antibody (1:4,000; Abcam, ab6721); anti-GAPDH antibody (1:800; Abcam, ab8245) with goat anti-mouse secondary antibody (1:6,000, Abcam, ab6789). Antibody binding on the membrane were detected by SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific) and visualized on iBright FL1000 Imaging System (Thermo Fisher Scientific). To quantify protein relative amounts we used ImageJ software.

2.8 | Viability assay

Cell viability was detected by WST-1 assay (Roche) according to the manufacturer's protocol. 3500 cells per well were seeded in 100 μL medium onto 96 well plate. On the next day, the medium was changed to fresh medium containing the indicated concentrations of ENZA. Cells were incubated for 4 h at 37°C. On day 3, the old medium was replaced by 100 μL of fresh medium containing the appropriate amounts of ENZA. Cells were maintained at 37°C in 5% CO2 with humidified atmosphere. On the day 6, 10 μL of WST-1 solution was added into each well and cells were incubated for 4 h at 37°C with 5% CO2. Measurements were done in at least three independent biological experiments with three technical replicates.

2.9 | siRNA silencing

As ALCAM was not detectable either in DuCaP or in DuCaP-ENZA cell lines, siRNA-mediated ALCAM silencing was only performed in LAPC4 and LAPC4-ENZA cells. Cells were seeded in six well plate in 2 mL antibiotic-free RPMI-1640 medium, then transfected with 40 pmol siRNA using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, 13 778 150) and Opti-MEM Reduced Serum Medium (Gibco). We used SMARTpool on-target plus siRNA against ALCAM (Dharmacon, Lafayette, CO, Cat. No: L-004574-00-0005) and nontargeting control pool on-target plus siRNA (Dharmacon, D-0018101005). Medium was changed 8 h after transfection. Gene silencing was monitored by Western blot.

2.10 | Real-time quantitative PCR

Details of RT-qPCR are described in Supplementary Materials and Methods.

2.11 | In silico analysis of ALCAM gene expression

In silico analysis was performed by using TNMplot (http://www.tnmplot.com) and cBioPortal databases (https://www.cbioportal.org). TNMplot database were used to evaluate the expression of ALCAM in various cancers and to explore the ALCAM mRNA expression in normal, tumor and metastatic PC tissues. In order to analyze the correlation between ALCAM expression and clinicopathological data, we extracted mRNA expression data of 266 mCRPC patients and their genomic and clinical information from the TCGA using cBioPortal database.

2.12 | Statistical analysis

In the case of ELISAs, statistical analyses were done with the SPSS 26.0 (IBM, Chicago, Illinois) software. For independent group comparisons, the nonparametric two-sided Wilcoxon rank sum test (Mann-Whitney test) was used. Spearman's and Pearson's correlation were used for measure the degree of correlations between ALCAM serum levels and continuous clinical and laboratory parameters. Univariable survival analyses were done using both Kaplan-Meier curves with log-rank tests and univariable Cox regression analysis. For multivariable analysis, Cox proportional hazards regression modeling was used with parameters that were statistically significant in univariable analysis at P < .05. To determine the optimal cut-off value with the highest sensitivity and specificity for the prediction of death within 24 months, we used the nonparametric receiver operating characteristics (ROC) curves in which the value for sensitivity is plotted against false-positive rate (1-specificity). The concentrations with the highest specificity and sensitivity values (Youden index) were defined as optimal cut-offs. IC50 values were calculated by using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, California). In all tests, P values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Identification of proteins with differential expression between ENZA-sensitive and -resistant PC cells

LC-MS/MS proteome analyses revealed 385 (LAPC4 vs LAPC4-ENZA) and 346 (DuCaP vs DuCaP-ENZA) significantly differentially abundant (either increased or decreased) proteins in cells (Figures 1A and S1 and Tables S1 and S2). We focused on those proteins that were detected by at least two unique peptides and were significantly upregulated in ENZA-resistant cells (Figure S1).

Using the first selection method, we identified five (AGR2, PFKP, APOD, ALCAM and DYNLT3) potentially secreted proteins. Based on cross-reference analysis, we identified seven proteins (GALK2, PTGR1, SCIN, IDH1, AHCY, THOP1 and SF3B5). Our third, pathway- and literature-based selection method identified four proteins (AGR2, RHOA, NDRG1 and ALCAM).
Finally, identified targets were filtered by the availability of ELISA assays which selected four proteins (ALCAM, AGR2, IDH1 and NDRG1) for serum analyses.

3.2 | GO and KEGG pathway analysis

GO analysis found the highest number of enriched categories for proteins upregulated in LAPC4 cells (Figure S2). However, the most significantly enriched ontologies were corresponding to those proteins downregulated in LAPC4. For DuCaP more enriched categories were correspond to upregulated proteins although the numbers of analyzed proteins were not much different between up- and downregulated fractions. Some categories were homogeneously enriched for several of the analyzed fractions, such as ontologies associated with the metabolism of nucleic acids or its chemical synonyms. Accordingly, processes associated with gene expression and RNA processing were also significantly enriched.

Enrichment analysis of KEGG pathways resulted in complementary findings to GO analysis (Figure S3). Most enriched terms were found for proteins upregulated in LAPC4 (compared with downregulated ones for the gene ontology analysis). However, the most significant KEGG pathway was again found for proteins downregulated in LAPC4 (ie, Ribosome) and corresponded to processes associated to protein translation. Proteins upregulated in LAPC4 were associated with diverse metabolic pathways, such as carbon metabolism and amino acid metabolism. For DuCaP cells, metabolic pathways were enriched for both, up and downregulated proteins. Furthermore, upregulated proteins were enriched in pathways related to DNA replication and RNA processing, which was congruent with enriched gene ontologies.

3.3 | Selected protein levels in patients’ samples

3.3.1 | Patients’ characteristics

The median age in the ENZA cohort was 73 years (range: 56-89), the median pretreatment PSA value was 69.5 ng/mL. The primary endpoints of the analysis was overall survival (OS). 48 (66%) patients died during a median follow-up period of 19 months. 1-year survival was 65%, 2-years survival was 43% (Table 1).

In the ABI cohort, the median patients age was 71 years (range: 54-90), the median PSA value was 65.3 ng/mL. In this cohort 69 (68%) patients died during a median follow-up period of 21 months (Table 1).

The median age in DOC cohort was 70 years (range: 43-86), the median PSA value was 88.8 ng/mL. Seventy-eight (78%) of 100 DOC-
| Parameters                   | ENZA   | ABI    | DOC    |
|------------------------------|--------|--------|--------|
| **Baseline characteristics** |        |        |        |
| Total No. of patients        | 72     | 101    | 100    |
| Age (median)                 | 73 (56-89) | 71 (54-90) | 70 (43-86) |
| PSA (median; ng/mL)          | 69.48 (0.23-8422.0) | 65.30 (0.10-6785.0) | 88.77 (3.20-6115.41) |
| LDH (median; U/l)            | 200.0 (117.0-4930.0) | 212.5 (100.0-1180.0) | 261.0 (143.0-1123.0) |
| AP (median; U/l)             | 92.0 (32.0-591.0) | 93.0 (35.0-4149.0) | 224.0 (55.0-1204.0) |
| CRP (median; mg/L)           | 0.6 (0.02-10.8) | 0.4 (0.02-14.2) | 6.5 (0.4-264.1) |
| Hemoglobin (median; g/dL)    | 12.1 (6.2-168.0) | 11.7 (7.9-106.0) | 12.95 (8.3-15.2) |
| ECOG PS (%)                  |        |        |        |
| 0                            | 49 (68) | 57 (56) | 65 (65) |
| 1                            | 10 (14) | 16 (16) | 26 (26) |
| 2                            | 4 (6)   | 0       | 9 (9)   |
| Unknown                      | 9 (12)  | 28 (28) | 0       |
| Pain (%)                     |        |        |        |
| Yes                          | 19 (26) | 36 (35) | –       |
| No                           | 46 (64) | 50 (50) | –       |
| Unknown                      | 7 (10)  | 15 (15) | 100 (100) |
| Metastases (%)               |        |        |        |
| Bone                         | 61 (85) | 89 (89) | 94 (94) |
| LN (>2 cm)                   | 16 (22) | 12 (12) | 38 (38) |
| Visceral                     | 5 (7)   | 9 (9)   | 13 (13) |
| Primary local therapy (%)    |        |        |        |
| Prostatectomy                | 29 (40) | 48 (48) | 18 (18) |
| Radiation                    | 17 (24) | 21 (21) | 12 (12) |
| Therapy line (%)             |        |        |        |
| First line                   | 15 (20) | 38 (37) | 97 (97) |
| Second or later line         | 57 (80) | 60 (60) | 3 (3)   |
| Unknown                      | 0       | 3 (3)   | 0       |
| Follow-up characteristics    |        |        |        |
| Max. PSA decline* (%) >30    | 50 (69) | 73 (73) | 55 (55) |
| >50                          | 47 (65) | 65 (65) | 45 (45) |
| >90                          | 26 (36) | 33 (33) | 26 (26) |
| Subsequent therapy (%)       |        |        |        |
| No                           | 33 (46) | 49 (49) | 51 (51) |
| DOC                          | 5 (7)   | 15 (15) | –       |
| ABI                          | 5 (7)   | –       | 19 (19) |
| ENZA                         | –       | 33 (33) | 26 (26) |
| CABA                         | 25 (35) | 28 (28) | 7 (7)   |
| Xofigo                       | 11 (15) | 10 (10) | 6 (6)   |
| Lu-PSMA                      | 6 (8)   | 8 (8)   | 0       |
| Number of patients died (%)  | 48 (66) | 69 (69) | 78 (78) |
| OS median, months            | 19.9    | 21.6    | 22.9    |

Abbreviations: ABI, abiraterone; AP, alkaline phosphatase; CABA, cabazitaxel; CRP, C reactive protein; ENZA, enzalutamide; DOC, docetaxel; LDH, lactate dehydrogenase; LN, lymph node; Lu-PSMA, lutetium-prostate-specific membrane antigen; OS, overall survival; PS, performance status.

*In comparison to baseline PSA during the given therapy.
### TABLE 2  Association of baseline ALCAM levels with clinicopathological parameters in ENZA, ABI and DOC-treated patients

|      | ENZA n = 72 |      | ABI n = 101 |      | DOC n = 100 |
|------|-------------|------|-------------|------|-------------|
|      | ALCAM serum cc. (ng/mL) |      | ALCAM serum cc. (ng/mL) |      | ALCAM serum cc. (ng/mL) |
|      | Median (range) |      | Median (range) |      | Median (range) |
|      | P |      | P |      | P |
| Whole cohort | 127.8 (0-400.0) | 101 | 120.5 (45.2-400.0) | 100 | 114.2 (47.2-400.0) |
| Age (years) |      |      |      |      |      |
| ≤72 | 35 | 136.1 (82.3-400.0) | 56 | 121.4 (45.2-389.2) | 54 | 120.7 (51.9-400.0) |
| >72 | 37 | 123.6 (0-400.0) | 45 | 120.5 (55.1-400.0) | 46 | 113.0 (47.2-219.8) |
| Primary therapy |      |      |      |      |      |
| No | 28 | 133.7 (82.5-400.0) | 34 | 131.9 (55.1-400.0) | 71 | 129.0 (51.9-400.0) |
| Yes | 44 | 127.4 (0-400.0) | 67 | 119.9 (45.2-389.2) | 29 | 107.4 (47.2-219.8) |
| Line of above therapy |      |      |      |      |      |
| 1st line | 15 | 117.0 (82.3-400.0) | 38 | 131.9 (45.2-400.0) | 97 | 113.9 (47.2-400.0) |
| 2nd or later line | 57 | 136.1 (0-400.0) | 60 | 118.8 (49.6-389.2) | 3 | 135.0 (60.5-400.0) |
| Unknown | 0 |      | 3 |      | 0 |
| ECOG PS |      |      |      |      |      |
| 0 | 49 | 132.3 (0-400.0) | 57 | 115.1 (45.2-389.2) | 65 | 107.0 (47.2-315.9) |
| 1-2 | 14 | 122.4 (83.04-367.9) | 16 | 144.2 (56.8-400.0) | 35 | 135.0 (60.4-400.0) |
| Unknown | 9 |      | 28 |      | 0 |
| LN status |      |      |      |      |      |
| N⁻ | 56 | 131.9 (0-400.0) | 85 | 121.2 (45.2-400.0) | 62 | 113.0 (47.2-400.0) |
| N⁺ | 16 | 124.350 (92.2-256.1) | 15 | 118.1 (95.4-198.8) | 38 | 126.7 (51.8-400.0) |
| Unknown | 0 |      |      | 1 | |
| Visceral mets. |      |      |      |      |      |
| No | 67 | 128.0 (0-400.0) | 90 | 120.3 (45.2-400.0) | 87 | 111.2 (47.2-219.8) |
| Yes | 5 | 114.2 (83.5-153.9) | 9 | 136.4 (75.1-386.8) | 13 | 153.7 (67.9-242.8) |
| Unknown | 0 |      | 2 |      | 0 |
| Bone mets. |      |      |      |      |      |
| No | 11 | 114.6 (88.0-171.4) | 11 | 114.4 (56.8-138.9) | 6 | 100.9 (64.9-219.8) |
| Yes | 61 | 136.1 (0-400.0) | 89 | 122.6 (45.2-400.0) | 94 | 116.1 (47.2-400.0) |
| Unknown | 0 |      | 1 |      | 0 |
| PSA baseline |      |      |      |      |      |
| High* | 36 | 154.2 (82.5-400.0) | <.001 | 50 | 139.5 (52.9-400.0) | 50 | 156.7 (57.6-400.0) |
| Low* | 36 | 110.7 (0-208.6) | 51 | 114.9 (45.2-286.0) | 50 | 105.9 (47.2-240.3) |
| Unknown | 0 |      | 0 |      | 0 |
| LDH |      |      |      |      |      |
| >240 U/L | 23 | 157.3 (84.3-400.0) | .001 | 35 | 141.1 (67.9-400.0) | 15 | 151.2 (51.9-282.2) |
| <240 U/L | 46 | 117.5 (0-400.0) | 65 | 116.9 (45.2-291.1) | 12 | 105.5 (63.9-239.5) |
| Unknown | 3 |      | 1 |      | 73 |
| AP |      |      |      |      |      |
| >129 U/L | 22 | 157.1 (83.9-400.0) | .008 | 41 | 144.4 (52.9-389.2) | 21 | 133.9 (51.9-282.2) |
| <129 U/L | 47 | 119.5 (0-400.0) | 58 | 115.0 (45.2-400.0) | 10 | 111.8 (67.6-239.5) |
| Unknown | 3 |      | 2 |      | 69 |
| CRP |      |      |      |      |      |
| >5 mg/L | 7 | 184.3 (127.5-400.0) | .012 | 12 | 145.6 (68.8-198.8) | 18 | 136.5 (51.9-282.2) |
| <5 mg/L | 61 | 121.0 (0-400.0) | 88 | 120.0 (45.2-400.0) | 13 | 97.0 (57.6-223.2) |
| Unknown | 4 |      | 1 |      | 69 |

(Continues)
treated patients died during a median follow-up time of 23 months (Table 1).

3.3.2 | Correlations of clinicopathological parameters with serum levels

We found no significant association between the assessed marker levels and patients' age, primary local therapy or PSA response (Tables 2 and S3 and S4). In the ENZA cohort, IDH1 levels were decreased in bone metastatic cases ($P = .033$), while AGR2 levels were lower in visceral metastatic patients ($P = .010$). NDRG1 serum levels were significantly lower in men who had no PSA response ($P = .023$). ALCAM serum levels were significantly higher in patients who had high baseline PSA, LDH, AP and CRP levels ($P < .05$), while it were significantly lower in patients who responded with at least 30% PSA response to ENZA ($P = .045$; Table 2). In the ABI cohort, ALCAM levels were higher in patients who had pain ($P = .013$) and had high PSA, LDH and AP levels at baseline ($P < .05$; Tables 2 and S4). In the DOC and ABI cohort, ALCAM serum levels were significantly higher in men with poor ECOG status ($P = .015$ and $P = .007$; respectively). In all three therapy groups, Pearson's and Spearman's correlation analyses revealed a significant correlation between baseline ALCAM and PSA levels (Pearson $P < .001$; Spearman $P < .001$; Table S5).

3.3.3 | ROC analysis

For ROC analysis, OS were dichotomized at 24th month of survival. ROC analysis identified 146.6 ng/mL as an optimal cut-off value with the highest sensitivity (58.8%) and specificity (68.4%) for ALCAM in ENZA cohort. In ABI cohort ROC method found the cut-off value with the highest sensitivity (37.2%) and specificity (89.3%), which resulted 143.1 ng/mL as an optimal cut-off. In the ENZA&ABI cohort ROC analysis identified 137.7 ng/mL as an optimal cut-off value with the highest sensitivity (51.9%) and specificity (70.7%). In the DOC cohort, ROC method found 127.9 ng/mL as an optimal cut-off value with the highest sensitivity (46.2%) and specificity (59.1%). These cut-off values were further analyzed in subsequent survival analyses.

3.3.4 | Survival analyses

In the ENZA cohort, presence of bone or visceral metastases were associated with shorter OS ($P = .004$ and $P = .047$; respectively; Table 3). Patients who underwent second- or later-line of ENZA therapy had significantly worse survival ($P = .021$; Table 3). High PSA and ALCAM pretreatment serum levels were significantly associated with shorter OS ($P < .05$; Table 3).

In the ABI cohort, higher ECOG status, high baseline PSA and ALCAM serum levels were significantly associated with worse survival ($P < .005$; Table 3).

When combining the ENZA/ABI cohort, we found the presence of bone metastases, high PSA, AP, CRP, Hemoglobin and ALCAM pre-treatment serum levels to be significantly associated with shorter OS ($P < .005$; Table 3). In multivariable analysis, presence of bone metastases, high PSA, AP and ALCAM (>137.8 ng/mL) levels were independently associated with OS ($P < .005$; Table 4). In the ENZA&ABI first line treated subgroup, high baseline ALCAM levels were significantly associated with poor OS (Figure S4). Moreover, multivariable analysis identified high PSA and ALCAM (>131.9 ng/mL) levels as independent predictors of OS ($P = .041$ and $P = .002$; respectively; Table 4).
| Variables                  | ENZA Overall survival | ABI Overall survival | ENZA&ABI Overall survival | DOC Overall survival |
|----------------------------|-----------------------|----------------------|---------------------------|---------------------|
|                            | HR        | 95% CI      | P   | HR        | 95% CI      | P   | HR        | 95% CI      | P   | HR        | 95% CI      | P   |
| Age >72 years              | 1.038     | 0.589-1.837 | .898 | 1.612     | 0.984-2.641 | .058 | 1.335     | 0.921-1.934 | .127 | 1.204     | 0.768-1.886 | .419 |
| ECOG 2                    | 1.357     | 0.638-2.886 | .428 | 3.970     | 2.070-7.614 | <.001 | 1.601     | 0.504-5.087 | .425 | 2.128     | 1.349-3.357 | .001 |
| Visceral mets. pos.       | 3.511     | 1.017-12.122 | .047 | 1.731     | 0.788-3.803 | .171 | 0.912     | 0.365-2.280 | .844 | 1.567     | 0.860-2.856 | .143 |
| LN mets. pos.             | 0.622     | 0.290-1.330 | .221 | 1.627     | 0.864-3.131 | .145 | 1.026     | 0.626-1.680 | .920 | 0.942     | 0.592-1.498 | .800 |
| Bone mets. pos.           | 5.796     | 1.773-18.945 | .004 | 2.050     | 0.821-5.116 | .124 | 1.746     | 1.199-2.541 | .004 | 1.260     | 0.396-4.011 | .696 |
| Primary RPE pos.          | 0.779     | 0.512-1.651 | .919 | 0.775     | 0.481-1.250 | .296 | 0.822     | 0.569-1.188 | .296 | 1.144     | 0.648-2.017 | .643 |
| Primary RAD pos.          | 0.661     | 0.329-1.328 | .245 | 0.667     | 0.369-1.242 | .208 | 0.668     | 0.423-1.056 | .084 | 1.280     | 0.636-2.577 | .489 |
| Line of therapy 2nd or ltr.| 2.769     | 1.169-6.559 | .021 | 0.962     | 0.590-1.571 | .878 | 1.209     | 0.970-1.507 | .091 | 0.975     | 0.238-3.990 | .972 |
| PSA median *              | 3.696     | 2.036-6.709 | <.001 | 2.520     | 1.532-4.144 | <.001 | 2.986     | 2.039-4.373 | <.001 | 1.618     | 1.022-2.563 | .040 |
| PSA decline Yes           | 0.608     | 0.291-1.269 | .185 | 1.071     | 0.512-2.240 | .856 | 0.886     | 0.529-1.484 | .646 | 0.262     | 0.142-0.485 | <.001 |
| PSA decline >30%          | 0.740     | 0.385-1.419 | .364 | 0.560     | 0.317-0.990 | .046 | 0.623     | 0.407-0.953 | .029 | 0.467     | 0.286-0.764 | .002 |
| PSA decline >50%          | 0.735     | 0.394-1.373 | .335 | 0.555     | 0.327-0.943 | .029 | 0.626     | 0.419-0.934 | .022 | 0.521     | 0.319-0.852 | .009 |
| PSA decline >90%          | 0.471     | 0.255-0.870 | .016 | 0.623     | 0.375-1.036 | .068 | 0.559     | 0.379-0.826 | .003 | 0.561     | 0.318-0.989 | .046 |
| ALCAM median *            | 2.002     | 1.124-3.564 | .018 | 2.093     | 1.291-3.395 | .003 | 2.047     | 1.415-2.962 | <.001 | 1.483     | 0.940-2.341 | .091 |
| ALCAM (ROC) *             | 2.449     | 1.390-4.491 | .002 | 2.516     | 1.523-4.157 | <.001 | 2.658     | 1.883-3.856 | <.001 | 1.415     | 0.897-2.231 | .136 |
| LDH >240 U/L              | 2.945     | 1.599-5.424 | .001 | 2.173     | 1.315-3.593 | .002 | 2.477     | 1.683-3.645 | <.001 | 3.425     | 1.186-9.891 | .023 |
| AP >129 U/L               | 3.822     | 2.051-7.121 | <.001 | 2.892     | 1.736-4.818 | <.001 | 3.171     | 2.146-4.687 | <.001 | 1.802     | 0.710-4.575 | .215 |
| CRP >5 g/mL               | 3.268     | 1.222-8.737 | .018 | 2.448     | 1.196-5.013 | .014 | 2.700     | 1.521-4.792 | <.001 | 2.072     | 0.919-4.673 | .079 |
| Hemoglobin >13.5 g/dL     | 0.398     | 0.142-1.117 | .080 | 0.522     | 0.288-0.944 | .031 | 0.479     | 0.288-0.796 | .005 | 0.312     | 0.105-0.928 | .036 |
| AGR2 median               | 0.818     | 0.463-1.444 | .488 |          |          |       |          |          |       |          |          |       |
| AGR2 (ROC)                | 0.658     | 0.360-1.201 | .172 |          |          |       |          |          |       |          |          |       |
| IDH1 median               | 0.816     | 0.416-1.442 | .484 |          |          |       |          |          |       |          |          |       |
| IDH1 (ROC)                | 1.243     | 0.700-2.205 | .458 |          |          |       |          |          |       |          |          |       |
| NDRG1 median              | 1.083     | 0.609-1.926 | .786 |          |          |       |          |          |       |          |          |       |
| NDRG1 (ROC)               | 0.710     | 0.383-1.316 | .277 |          |          |       |          |          |       |          |          |       |

Note: Significant values are indicated in bold. *ENZA cohort: median (ng/mL) PSA: 69.45, ALCAM: 127.8/ALCAM ROC cut-off (ng/mL): 146.6; *ABI cohort: median (ng/mL) PSA: 65.3, ALCAM: 120.5/ALCAM ROC cut-off (ng/mL): 143.1; *ABISENZA cohort: median (ng/mL) PSA: 66.6, ALCAM: 122.4/ALCAM ROC cut off (ng/mL): 137.8; *DOC cohort: median (ng/mL) PSA: 88.02, ALCAM: 114.2/ALCAM ROC cut off (ng/mL): 127.95.

Abbreviations: AP, Alkaline phosphatase; CRP, C reactive protein; LN, lymph node; LDH, lactate dehydrogenase; RAD, radiation; RPE, radical prostatectomy.
In the DOC cohort, ECOG status (>1) and high baseline PSA levels were associated with poor OS, while ALCAM showed no significant association with OS (Table 3).

In the ENZA and ABI cohort, Kaplan-Meier OS curves revealed that higher baseline ALCAM levels (>146.6 and >143.1 ng/mL by ROC cut-off) are significantly associated with shorter OS ($P = .002$ and $P < .001$; respectively; Figure 1B). Moreover, we combined pre-treatment ALCAM and PSA serum levels and performed OS analysis by stratifying patients according to their received treatments (ENZA/ABI and DOC) in the whole cohort ($n = 273$). In addition, we performed subgroup analyses in first line treated patients and also in patients who received their last line of therapy. We found that in the low PSA and ALCAM levels group, ENZA/ABI-treated men had a significant better OS compared with those who received DOC therapy regardless of treatment line ($P \leq .001$; Figures S5, S6 and S7).

### 3.3.5 | Prognostic value of ALCAM levels changes during therapy

ALCAM level changes were calculated at 3 months after treatment start and were dichotomized as any increase, at least 30% and 50% increase. We found that patients with at least 30% increase in their ALCAM levels at 3 months had a significant shorter OS (ENZA cohort HR: 2.842; 95% CI 1.226-6.585; $P = .011$, ABI cohort HR: 2.710; 95% CI 1.114-6.595; $P = .022$; DOC cohort HR: 2.224; 95% CI 1.062-4.661; $P = .030$; respectively).

### 3.4 | Protein level of ALCAM in cell lines

Based on ELISA analyses, we selected ALCAM to assess its potential functional involvement in ENZA resistance. First, we compared

| ENZA&ABI cohort | Overall survival |
|-----------------|-----------------|
| **Any treatment lines** | HR  | 95% CI | $P$ |
| Bone mets. | 1.859 | 1.242-2.781 | .003 |
| PSA (median) > 66.6 ng/mL | 2.404 | 1.580-3.658 | <.001 |
| ALCAM (median) > 122.4 ng/mL | 1.423 | 0.942-2.150 | .094 |
| LDH > 240 U/L | 1.269 | 0.795-2.023 | .318 |
| CRP > 5 g/mL | 1.616 | 0.852-3.065 | .141 |
| AP > 129 U/L | 2.065 | 1.311-3.251 | .002 |
| Hemoglobin >13.5 g/dL | 0.743 | 0.418-1.320 | .311 |
| Bone mets. | 1.834 | 1.224-2.748 | .003 |
| PSA (median) > 66.6 ng/mL | 2.372 | 1.560-3.606 | <.001 |
| ALCAM (ROC) > 137.8 ng/mL | 1.833 | 1.213-2.771 | .004 |
| LDH > 240 U/L | 1.205 | 0.754-1.924 | .436 |
| CRP > 5 g/mL | 1.600 | 0.851-3.008 | .144 |
| AP > 129 U/L | 2.014 | 1.274-3.183 | .003 |
| Hemoglobin >13.5 g/dL | 0.769 | 0.433-1.368 | .372 |

| **First line treated patients** | |
|-----------------|-----------------|
| ALCAM (median) > 121.2 ng/mL | 2.606 | 1.093-6.213 | .031 |
| PSA (median) > 37 ng/mL | 2.466 | 1.029-5.907 | .043 |
| Hemoglobin >13.5 g/dL | 0.486 | 0.188-1.254 | .136 |
| CRP > 5 g/mL | 0.830 | 0.273-2.529 | .744 |
| AP > 129 U/L | 1.321 | 0.493-3.539 | .580 |
| ALCAM (ROC) > 131.9 ng/mL | 4.465 | 1.711-11.651 | .002 |
| PSA (median) > 37 ng/mL | 2.500 | 1.037-6.030 | .041 |
| Hemoglobin >13.5 g/dL | 0.508 | 0.202-1.277 | .150 |
| CRP > 5 g/mL | 0.735 | 0.244-2.213 | .584 |
| AP > 129 U/L | 0.911 | 0.327-2.532 | .857 |

Note: Significant values are indicated in bold.
Abbreviations: AP, alkaline phosphatase; CRP, C reactive protein; LDH, lactate dehydrogenase.
ALCAM expressions between ENZA-sensitive and -resistant (LAPC4-ENZA) cells. In line with our LC-MS/MS data—which found 1.39-fold higher expression of ALCAM in LAPC4-ENZA cells (compared with LAPC4)—Western-blot analysis found a 3.05-fold upregulation of ALCAM (Figure 2A,B).

3.5 | Functional analyses of ALCAM in PC cell lines

To investigate the functional role of ALCAM in ENZA resistance, we performed siRNA-mediated knockdown analysis. The siRNA transfection successfully silenced expression of ALCAM in LAPC4-ENZA and parental LAPC4 cells according to Western blot measurements (Figure 2C).

To examine ENZA treatment in ALCAM silenced cells, WST proliferation assay were performed with parental LAPC4 and LAPC4-ENZA cell lines. We found that ALCAM knock-down significantly enhanced ENZA-sensitivity in LAPC4-ENZA cells compared with the nontransfected cells (Figure 2D), while in the parental LAPC4 cells no similar effect could be observed (Figure 2E).

3.6 | Effect of ALCAM knock-down of expression of AR and their target genes in cell lines

To determine the possible effect of ALCAM knock-down to mRNA expression of AR and their target genes, PSA, FKBP5 and c-myc were measured using RT-qPCR. We found that AR, PSA, c-myc and FKBP5 were significantly upregulated in ENZA resistant LAPC4 cells compared with parental/ENZA sensitive cell lines. ALCAM knock-down did not significantly influenced AR, PSA and c-myc gene expression levels, while FKBP5 expression decreased significantly (Figure S8).

3.7 | Gene expression level of ALCAM and correlation with clinical data in databases

Using the TNMplot database, we compared ALCAM gene expressions between normal and tumor tissues in several organs and found ALCAM expression to be significantly elevated in breast, lung, renal, thyroid and prostate cancers compared with corresponding non-malignant tissues. Notably, we found the largest difference in ALCAM gene expression between normal and respective tumor tissues in
prostate and breast cancers (Figure S9). In prostate cancer, the highest ALCAM expressions were found in metastatic cases (Figure S9). To investigate the correlation between ALCAM mRNA expression and clinicopathological and follow-up data we applied the TCGA dataset using cBioPortal. This analysis revealed no significant association between ALCAM expressions and survival of mCRPC patients. In addition, high ALCAM mRNA expression was significantly associated with higher AR gene expression but lower neuroendocrine (NEPC) gene expression scores and absence of small cell histological variants (Figure S9).

4 | DISCUSSION

Several mechanisms and signaling pathways have been found to be implicated in ENZA resistance of PC, for example, the alterations of the AR, transdifferentiation to neuroendocrine tumors, glucocorticoid receptor overexpression, DNA-repair and PI3K pathway alterations.\(^1\)\(^{-\text{17,19}}\) Despite our increasing understanding of ENZA resistance, currently no markers predictive of ENZA resistance are available in clinical practice. Moreover, likely more than one molecular mechanism is involved in ENZA resistance. Therefore, our aim was to uncover proteins potentially involved in ENZA resistance and to test their value in the prediction of response to ENZA treatment when measured in serum samples of mCRPC patients. Using proteome analysis and applying three different hypothesis-free selection methods, we identified 14 candidate ENZA-resistant proteins. Four of the most promising proteins were determined in baseline serum samples of ENZA-treated mCRPC patients.

The first, “secreted protein” selection method identified ALCAM and Anterior gradient 2 (AGR2) proteins. AGR2 is a proto-oncogene that plays a functional role in angiogenesis, cell migration, cell growth and differentiation.\(^2\) In different cancer types, high AGR2 expression was associated with poor patients’ prognosis.\(^2\) The predictive value of AGR2 in PC was assessed for DOC and ENZA and revealed different roles for AGR2 between these therapies. Zhao et al\(^3\) showed that AGR2 is downregulated in DOC resistant cell lines and AGR2 silencing resulted in DOC resistance in PC3 cells, suggesting that AGR2 expressions are directly associated with DOC sensitivity. An opposite effect was observed for ENZA by Tang et al\(^3\) showing that inhibition of KDM1B caused AGR2 suppression which increased ENZA sensitivity of PC cells. Our present comparative proteome analysis showed conflicting results regarding the expression of AGR2; as it was strongly upregulated in LAPC4-ENZA-resistant cells, while it was significantly down regulated in DuCaP-ENZA cell lines. In addition, our serum analyses did not reveal ARG2 as serum marker of ENZA resistance.

The second “cross-reference” analysis compared our proteome results with a published transcriptomics dataset.\(^2\) Qian et al performed an integrative analysis of expression data of ENZA-sensitive and -resistant PC cell lines. Comparing their upregulated gene list with our proteome dataset, nine matching proteins could be identified. Of these, we selected isocitrate dehydrogenase 1 (IDH1) for ELISA analysis. IDH1 is a metabolic enzyme, which converts isocitrate to 2-oxoglutarate. According to the TCGA (The Cancer Genome Atlas) study in PC, IDH1 mutation carrier tumors represent a distinct molecular subclass of primary PCs. These mutations are enriched in early onset PCs and are associated with DNA hypermethylation.\(^3\) The role of IDH1 in ENZA resistance has not been evaluated yet. Our serum IDH1 analyses in ENZA-treated CRPC patients revealed lower IDH1 levels in bone metastatic patients but showed no associations with OS.

The third “pathway- and literature-based” selection method identified NDRG1 and ALCAM proteins. N-myc downstream regulated gene-1 (NDRG1) plays a role in cell growth, differentiation, signal transduction and is known as a tumor suppressor in various types of cancer including PC. However, some studies suggested NDRG1 as a proto-oncogene.\(^3\) In a gene expression analysis Zheng et al\(^4\) identified 12 hub genes including NDRG1 which are potentially associated with ENZA resistance, however its exact role in ENZA resistance has not been elucidated yet. In the present study, we found no correlation between NDRG1 serum levels and OS of ENZA-treated patients.

Activated leukocyte cell adhesion molecule (ALCAM), also called CD166, is a 100 to 105 kDa transmembrane glycoprotein which is a member of the immunoglobulin superfamily. ALCAM is an adhesion molecule, which can bind to the CD6 receptor resulting in a heterophilic interaction or can form an ALCAM-ALCAM homophilic dimer. It is involved in cell-cell adhesion, angiogenesis, cell migration, invasion as well as therapy resistance and tumor progression.\(^3\) Recent studies reported that high tissue expressions of ALCAM are correlated with poor survival in pancreatic, bladder and colorectal cancer.\(^3\) Recent studies reported that high tissue expressions of ALCAM are correlated with poor survival in prostate cancers (Figure S9). In PC, the highest ALCAM expressions were found in metastatic cases (Figure S9). In prostate cancer progression. ALCAM levels to be associated with higher Gleason-score, T-stage and PSA levels suggesting an association between increasing serum ALCAM concentrations during prostate cancer progression.

To address the molecular and clinical correlates of ALCAM in tissue samples, we conducted in silico analyses in published datasets comparing ALCAM mRNA levels between normal and tumor tissues in several organs and found the largest increase in prostate and breast cancers (Figure S9). In PC, the highest ALCAM expressions were found in metastatic cases (Figure S9). However, in mCRPC we found no significant association between ALCAM expressions and patients’ survival. In addition, high ALCAM mRNA expression was significantly associated with higher AR but lower neuroendocrine (NEPC) gene expression scores and absence of small cell histological variants (Figure S9). This suggest that ALCAM is rather involved in the
regulation of androgen signaling then in neuroendocrine differentiation. This notion may have clinical relevance suggesting that already established neuroendocrine serum markers such as chromogranin A and neuron-specific enolase as covering different resistance mechanisms might be well combined with ALCAM for the prediction of ENZA and ABI treatment.\textsuperscript{15} On the other hand, the correlation of ALCAM expression with AR gene expression score seems to be in line with current observations made in triple negative breast cancer where high ALCAM tissue protein expressions were shown to be associated with higher AR protein expression.\textsuperscript{46} However, in our experiment ALCAM knock-down did not consistently influence the expression of AR regulated genes, suggesting that ALCAM is coregulated with these genes rather than being their regulator.

The role of ALCAM in resistance to systemic therapy is poorly understood. Immunohistochemical analysis showed that tamoxifen resistant breast cancers have a higher ALCAM staining intensity compared with tamoxifen sensitive ones. Similar to our results, they showed that knockdown of ALCAM enhanced 4-hydroxytestosterone induced cell death in tamoxifen resistant breast cancer cell line.\textsuperscript{46} Darvishi et al.,\textsuperscript{47} in a further study described that ALCAM blockade resensitized resistant breast cancer cells to tamoxifen. The role of ALCAM in ENZA resistance of PC has not been revealed yet. In the present study, we found that higher pretreatment ALCAM levels are independently associated with shorter OS of ENZA-treated CRPC patients and further increase of serum ALCAM concentrations at 3 months of treatment was associated with shorter OS. These data suggest ALCAM as a potential biomarker for therapy monitoring in CRPC.

Our study has some potential limitations. First, as no ALCAM expression could be observed in DuCaP and DuCaP-ENZA cell lines (by LC-MS/MS and Western-blot) siRNA mediated ALCAM silencing could only be performed in one cell line pair (LAPC4 and LAPC4-ENZA). Second, we could only assess one cohort per treatment and could therefore not confirm our results in an independent validation cohort. As our serum analyses have been performed in a retrospective manner, selection bias between different treatment groups (eg, former local therapy, imbalanced numbers regarding therapy lines) may be present. Finally, as we did not assess other potential resistance markers (eg, AR-V7) we cannot concurrently evaluate the prognostic value of serum ALCAM in this context.

Taken together, serum ALCAM concentration is a potential serum marker of shorter survival in ENZA-treated patients and ALCAM seems to be functionally involved in ENZA resistance. Our further analyses in serum samples of ABI and DOC-treated patients revealed serum ALCAM as a predictor of OS in androgen signaling inhibitor-treated (ABI and ENZA) men but not in DOC chemotherapy-treated patients. This discriminating effect was even more obvious when ALCAM was combined with PSA (Figures S5,S6 and S7A,B). Moreover, based on subgroup analyses ALCAM levels to be able to identify patients deriving benefit from ENZA&ABI treatment regardless to the treatment line. Therefore, serum ALCAM analysis may discriminate between ENZA/ABI and DOC responsive patients. Our results however, need to be confirmed in larger studies. According to results of our functional analyses, we assume that ALCAM might be mechanistically involved in ENZA resistance and may therefore serve as a potential target for PC therapy. Further analyses are warranted to understand the exact role of ALCAM in ENZA and ABI resistance.

5 | CONCLUSION

In summary, our analyses revealed, for the first time, that high pretreatment serum ALCAM levels are independently associated with poor survival in ENZA- and ABI but not in DOC-treated CRPC patients. Therefore, ALCAM may help to identify ENZA- and ABI-resistant patients and thereby help to optimize therapeutic decision-making on type and timing of systemic therapy. However, this analysis has to be validated in larger independent patient cohort. Furthermore, our in vitro functional analyses showed that ALCAM gene silencing in ENZA-resistant cells leads to enhanced ENZA sensitivity.

AUTHOR CONTRIBUTIONS

The study reported in the paper has been performed by the authors, unless clearly specified in the text. Anita Csizmarik and Tibor Szarvas were the project administrator and wrote the original manuscript draft. Anita Csizmarik, Péter Nyirády, Boris Hadaschik and Tibor Szarvas conceptualized the study. Anita Csizmarik, Dávid Keresztes, Nikolett Nagy, Tibor Szarvas, József Lázár, Ilona Tornyai, László Takács, Gero Kramer, Sabina Sevcenco, Agnieszka Maj-Hes. and Zsolt Jurányi conducted the data curation. Anita Csizmarik, Dávid Keresztes, Thilo Bracht, Barbara SITEK, Kathrin Witzke, Ilona Tornyai, József Lázár and László Takács contributed to the methodology. Anita Csizmarik, Dávid Keresztes, Thilo Bracht and Tibor Szarvas created the visualizations. Dávid Keresztes, Nikolett Nagy, Thilo Bracht, Barbara SITEK, Kathrin Witzke, Martin Puh, Ilona Tornyai, József Lázár, László Takács, Gero Kramer, Sabina Sevcenco, Agnieszka Maj-Hes, Zsolt Jurányi, Boris Hadaschik, Péter Nyirády and Tibor Szarvas reviewed and edited the manuscript. Anita Csizmarik, Dávid Keresztes, Nikolett Nagy and Tibor Szarvas performed the formal analysis. Tibor Szarvas and Péter Nyirády supervised the project. Tibor Szarvas acquired funding.

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DATA AVAILABILITY STATEMENT
The raw mass spectrometry data generated in this study have been deposited in the PRIDE database with accession number PXD031847 (http://www.ebi.ac.uk/pride). Other data that support the findings of this study are available from the corresponding author upon request.

ETHICS STATEMENT
The study was performed in accordance with the ethical standards of the Helsinki Declaration and was approved by the ethical boards of the hospitals (TUKEB 55/2014, ECS 1986/2017). All patients signed consent to an institutional review board-approved protocol before sample collection.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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