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**LIPIDOMIC PROFILING IN PATIENTS WITH METASTATIC
CASTRATION-RESISTANT PROSTATE CANCER**

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XXXVII CICLO

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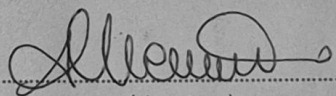
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1 INTRODUCTION

1.1 Prostate Cancer as a Major Health Concern

Prostate cancer (PCa) is the most commonly diagnosed cancer in Italy by incidence, ranking third in terms of mortality, and represents 18.5% of all cancers diagnosed in men. In 2020, the AIRTUM association estimated around 36,000 new cases, with approximately 563,960 men in Italy currently living with a PCa diagnosis. Around 7,200 deaths were projected for 2021 (1). The five-year overall survival (OS) rate for prostate cancer patients, regardless of diagnosis stage, is approximately 91%. This survival rate has improved over the years, largely due to earlier detection and the widespread use of PSA screening. Nevertheless, when the cancer is diagnosed at advanced stage, the five-year OS is about 30%.

Multiple randomized studies have demonstrated that androgen-receptor signaling inhibitors (ARSi) and chemotherapy provide notable survival advantages in metastatic (m) PCa. However, actual survival outcomes for mPCa patients remain disappointing, with a median survival of approximately 30 months (2).

1.2 Metastatic castration-resistant prostate cancer

The primary systemic therapy for both metastatic and non-metastatic prostate cancer is androgen-deprivation therapy (ADT) that can be surgically achieved or through the use of Luteinizing Hormone-Releasing Hormone (LH-RH) analogs, agonists or antagonists. The length of time that ADT remains effective can range from several months to years, which characterizes the hormone-sensitive prostate cancer (HSPC) phase. However, prolonged ADT eventually leads to disease progression, despite castration, resulting in a condition known as castration-resistant prostate cancer (CRPC).

Metastatic CRPC (mCRPC) is identified by biochemical, radiographic, or clinical worsening despite testosterone levels being at castration levels (<50 ng/mL or <1.7 mmol/L).

The criteria for choosing the treatment for mCRPC patients are performance status, comorbid conditions, age, eligibility for chemotherapy, drug interactions and drug approval and reimbursement.

In Italy, approved therapies for mCRPC include abiraterone acetate with prednisone, docetaxel, enzalutamide, cabazitaxel, olaparib (for BRCA-mutated patients) and radium-223.

1.2.1 Upfront therapy for mCRPC

Docetaxel and new hormonal agents such as abiraterone acetate and enzalutamide have shown significant survival benefits for not-pretreated mCRPC patients and are all considered standard options (3-5) as shown in **Table 1**.

Most enrolled patients had only received ADT as prior therapy and this does not reflect the current treatment landscape, where patients commonly receive ARSi or chemotherapy in addition to ADT for metastatic HSPC (mHSPC) or nonmetastatic CRPC (nmCRPC). It remains unclear to what extent the clinical benefits observed in phase III trials for mCRPC would apply today after prior exposure to these agents. Potential cross-resistance between treatments is not fully understood and could significantly affect patient outcomes.

Currently, there is no formal randomized trial directly comparing chemotherapy and ARSi as first-line treatments for mCRPC. The significant variation in median OS observed in the control arms of the TAX-327 (16.5 months), COU-AA-302 (30.3 months), and PREVAIL (31 months) studies suggests that these trials involved different patient populations, making cross-trial comparisons of efficacy inappropriate. In a large, real-world observational study, patients who received first-line ARSi had longer progression-free survival (PFS) than those treated with docetaxel, but no difference in OS was noted (6). Furthermore, patients with poorer baseline prognostic characteristics were more likely to be treated with first-line docetaxel. Similar findings were reported in a sub-analysis of the prospective PROREPAIR-B trial (7)

The longer PFS seen with ARSi compared to chemotherapy may be due to continuous ARSi exposure versus limited docetaxel exposure. Retrospective data suggest a short ADT response predicts poor ARSi response (8), while docetaxel remains effective in early castration resistance (9). The FIRSTANA trial found no survival difference between docetaxel and cabazitaxel as first-line mCRPC therapies, with cabazitaxel being better tolerated at 20 mg/m² (10). However, since the trial aimed to demonstrate cabazitaxel's superiority in OS, it was not approved as a first-line option.

It is unclear which treatment is best for mCRPC patients who previously received docetaxel for mHSPC (Figure 1a). The GETUG-AFU-15 trial showed limited benefit from docetaxel rechallenge in these patients, with only 14% achieving a PSA decline $\geq 50\%$ (11). In contrast, abiraterone and enzalutamide remain effective for these patients. A phase II study found cabazitaxel provided greater clinical benefit than ARSi (80% vs. 62%, $P = 0.039$) in ARSi-naïve

mCRPC patients with poor prognostic factors (12). While no difference in PSA declines or radiographic response was observed, cabazitaxel led to stable disease in 75% of patients, compared to 56% for ARSi ($p = 0.083$).

Chemotherapy is a viable first-line option for mCRPC patients who previously received ARSi in the mHSPC setting (Figure 1b), and for nmCRPC patients progressing on ARSi. Cross-resistance between ARSi and chemotherapy has been noted, and the sequence of two ARSi treatments is often discouraged (13). In the SPARTAN trial, a sequence of apalutamide → abiraterone improved PFS2 compared to placebo → abiraterone, but this result was likely driven by apalutamide's superior PFS in first-line nmCRPC.

A phase II trial compared abiraterone acetate → enzalutamide (group A) to enzalutamide → abiraterone acetate (group B) in newly diagnosed mCRPC patients (14). Group A had a longer time to second PSA progression (19.3 vs. 15.2 months, HR 0.66) and better PSA response to second-line therapy (26% vs. 4%, $p < 0.0001$). A trend towards improved overall survival was also observed in group A (28.8 vs. 24.7 months, HR 0.79, $p = 0.23$). A systematic review supports abiraterone followed by enzalutamide as an optimal treatment sequence for mCRPC, regardless of prior docetaxel use (15). However, a recent study of 3,174 chemotherapy-naïve mCRPC patients found opposing results (16).

Around half of the patients in these cohorts received only one line of treatment, with many discontinuing the therapy without further active treatment. About 25% of patients switched from one ARSi to another, with 23% ($n = 282$) moving from enzalutamide to abiraterone, and 26% ($n = 504$) from abiraterone to enzalutamide. When analyzing the entire population receiving first-line therapy, enzalutamide-treated patients showed significantly better OS compared to those on abiraterone (HR 0.84, 95% CI 0.76–0.94). For those who stayed on first-line therapy, enzalutamide led to improved OS compared to abiraterone (HR 0.71, 95% CI 0.62–0.82). Furthermore, enzalutamide patients who crossed over to abiraterone had similar OS to abiraterone patients who switched to enzalutamide (HR 0.91, 95% CI 0.74–1.13). An indirect comparison from phase 3 trials showed no significant OS difference between abiraterone and enzalutamide, whether before or after chemotherapy for mCRPC (17). However, enzalutamide performed better in terms of time to PSA progression, radiographic progression-free survival (PFS), and PSA response rate.

These results point to a methodological concern: to avoid selection bias, the full population of first-line treatment recipients should be considered when determining the best sequencing

approach. The outcomes of those who receive only first-line therapy can greatly influence the results, and sequencing analyses should not only focus on patients who receive multiple lines of treatment. In conclusion, larger randomized trials are needed to determine whether choosing between enzalutamide or abiraterone as first-line therapy significantly affects outcomes for mCRPC patients.

The differing side effect profiles of abiraterone and enzalutamide may influence treatment selection for some patients, though both are generally well tolerated.

Chemotherapy continues to be a valid treatment option, and more data are needed to compare the outcomes of ARSi versus chemotherapy more comprehensively.

TABLE 1 ADVANCEMENTS THROUGH PROSPECTIVE RANDOMIZED TRIALS IN mCRPC.

Setting	Name of the trial	Population	Exp arm	Control arm	N Exp/Contr	Primary Endpoint	FU (mo)	mOS (mo) Exp/Contr	HR (95% CI)	Ref.
1 st line mCRPC	TAX 327	With or without symptoms	Doce + P	Mitoxantro ne + P	335/337	OS	NA	19.2/16.3	0.79 (0.67-0.93)	(5)
	COU-AA-302	A/midly symptomatic pre-doce; no visceral mtx	AA + P + ADT	Placebo + P + ADT	546/542	rPFS, OS	49.2	34.7/30.3	0.81 (0.70-0.93)	(3)
	PREVAI L	A/midly symptomatic pre-doce	Enza + ADT	Placebo + ADT	872/845	rPFS, OS	69	36/31	0.83 (0.75-0.93)	(18)
	IMPAC T	A/midly symptomatic pre-/post-doce; Gleason ≤7; no visceral mtx	Sipuleucel -T + ADT	Placebo + ADT	341/171	OS	34.1	25.8/21.7	0.78 (0.61-0.98)	(19)
	IPAtent ial150	A/midly symptomatic	AA + P + ipataserti b	AA + P + placebo	547/554	(bio)r PFS	19	NE/NE	NE	(20)
≥2 nd line mCRPC	COU-AA-301	Post-doce	AA + P	Placebo + P	797/398	OS	20.2	15.8/11.2	0.74 (0.64-0.86)	(21)
	TROPIC	Post-doce	Cabazitax el + P	Mitoxantro ne + P	378/377	OS	25.5	NA/NA	0.72 (0.61-0.84)	(22)
	AFFIR M	Post-doce	Enza	Placebo	800/399	OS	14.4	18.4/13.6	0.63 (0.53-0.75)	(23)
	ALSYM PCA	Pre- and post-doce or unfit for doce; bone mtx and no visceral mtx	Radium-223	Placebo	614/307	OS	NA	14.9/11.3	0.70 (0.58-0.83)	(24)

CARD	Post-docetaxel and post-ARSi	Cabazitaxel	AA+P/Enza	129/126	IPFS	9.2	13.6/11	0.64 (0.46-0.89)	(25)
PROFO UND	Post-ARSi and pre-/post-taxane	Olaparib	AA+P/Enza	162/83*	(bio)IPFS	21	19.1/14.7*	0.69 (0.50-0.97)*	(26)
VISION	Post-ARSi and 1-2 taxanes	LuPSMA	Standard of care	551/280	rPFS, OS	20.9	15.3/11.3	0.62 (0.52-0.74)	(27)

AA: abiraterone acetate; ADT: Androgen Deprivation Therapy; (bio): biomarker-defined population; ARSi: androgen-receptor signalling inhibitors; CI: Confidence Interval; Doce: docetaxel; Enza: enzalutamide; Exp: experimental; HR: Hazard Ratio; IPFS: image-guided progression-free survival; LuPSMA: Lutetium-177-PSMA-617; mCRPC: Metastatic Castration Resistant Prostate Cancer; mOS: median overall survival; mo: months; mtz: metastases; NA: not available; P: prednisone; Ref: references; rPFS: Radiographic progression-free survival; *Results from BRCA1, BRCA2, ATM alterations Cohort.

1.2.2 Choosing Subsequent Treatment Options for mCRPC

Cabazitaxel, abiraterone acetate, enzalutamide and radium-223 have all demonstrated significant improvements in OS for patients with mCRPC after prior docetaxel treatment (21-23,25,28) (Table 1). However, no direct head-to-head comparisons of these treatments are available. In patients with mCRPC who were previously treated with docetaxel, enzalutamide showed lower PSA response rates than in chemotherapy-naïve patients (78% vs 54%) (23,29). Similarly, an analysis from the COU-AA-302 trial showed that patients receiving docetaxel after abiraterone had a reduced benefit compared to those who received docetaxel in the first-line setting (30,31). Choosing between chemotherapy and androgen receptor signaling inhibitors remains a crucial decision, especially in patients who have received either docetaxel or ARSi as initial therapy. Preclinical and clinical evidence suggests varying degrees of cross-resistance between abiraterone and enzalutamide, as well as between ARSi and docetaxel (13,32,33), while cabazitaxel continues to demonstrate efficacy even in patients previously treated with both chemotherapy and ARSi (34,35). The phase III CARD trial demonstrated that cabazitaxel is the optimal treatment for patients who experience disease progression on an ARSi following prior docetaxel therapy (25). In this study, 255 patients with mCRPC, who had progressed within 12 months of receiving an ARSi (either abiraterone or enzalutamide) after docetaxel, were randomized to either cabazitaxel or the alternative ARSi. Cabazitaxel significantly improved imaging-based progression-free survival (PFS) (HR 0.54, 95% CI 0.40-0.73) and OS (13.6 vs. 11.0 months, HR 0.64, 95% CI 0.46-0.89) compared to the alternative ARSi. A post hoc analysis confirmed the superiority of cabazitaxel over the ARSi, regardless of whether abiraterone or enzalutamide was used during the trial. Retrospective data further suggest that

patients with early progression on first-line ARSi benefit more from second-line chemotherapy in terms of response rates and time to PSA progression, compared to those receiving another ARSi (36). In an indirect comparison, PSA response rates after a second ARSi in the control arms of the CARD (13.5%) and PROFOUND (8%) trials were much lower than those observed in post-docetaxel patients treated with abiraterone (38%) or enzalutamide (54%) in the COU-AA-301 and AFFIRM trials (23,25,26,37). Additionally, data from the PLATO trial control arm, where patients received abiraterone acetate after first-line enzalutamide, were particularly discouraging, with a median time to PSA progression of only 2.8 months and just 2% of patients achieving a PSA response of $\geq 50\%$ (38). A retrospective study showed that enzalutamide still had some efficacy (21% of patients with a PSA decline $\geq 50\%$) in those who had previously been treated with docetaxel and abiraterone acetate, and could be considered for patients who are not suitable candidates for cabazitaxel (39).

1.2.3 Radium-223: Its Contribution to mCRPC Management

Radium-223 is an intravenous radiopharmaceutical that binds to bone mineral hydroxyapatite in areas of high bone turnover. In the phase III ALSYMPCA study (24), six cycles of radium-223 at 50 kBq/kg significantly improved OS (HR 0.70) and delayed symptomatic skeletal events (SSE) (HR 0.66) compared to placebo in mCRPC patients with symptomatic bone metastases (Table 1). Prior docetaxel use was associated with higher thrombocytopenia rates but did not affect radium-223's efficacy (40). In the Expanded Access Program, a single-arm study examined the safety and efficacy of radium-223, including patients with asymptomatic disease, and allowed for the combination of radium-223 with abiraterone or enzalutamide (41). Radium-223 was found to be safe, with a median OS of 16 months. Notably, patients receiving the combination of radium-223 with an ARSi showed significantly longer OS compared to those treated with radium-223 alone. These findings generated interest in potential radium-223 combinations. However, the ERA-223 trial (42), which tested the combination of abiraterone and radium-223, was stopped early due to increased fractures and deaths in the treatment group. The combination did not improve survival (HR 1.2) and caused more fractures, particularly osteoporotic ones (49% vs 17%). Notably, approximately 60% of participants in this trial did not receive bone protective agents. This led to updates in other ongoing trials, such as PEACE-3, requiring bone protective agents. Updated findings from the

trial revealed that bone protective agents significantly reduced the incidence of fractures at 12 months in those treated with the combination (37.1% vs 2.7%) and in those treated with enzalutamide alone (15.6% vs 2.6%) (43). The use of radium-223 is approved by the European Medicines Agency (EMA) for patients with mCRPC, symptomatic bone metastases, and no visceral disease, after failure of at least two prior treatments or for those ineligible for further systemic therapies. Based on the CARD trial results (25), radium-223 should be considered after cabazitaxel in patients with bone-predominant disease, unless they are ineligible or refuse chemotherapy.

1.2.4 Lutetium-177-PSMA-617 in the near future

Lutetium-177-prostate-specific membrane antigen (PSMA)-617 (LuPSMA) is an experimental radioligand therapy being explored for mCRPC patients (44). LuPSMA targets PSMA, a protein commonly expressed in prostate cancer cells, including metastatic sites, and delivers β -particle radiation. The phase II TheraP trial investigated LuPSMA in mCRPC patients for whom cabazitaxel was considered the next treatment option (45). A total of 291 men were screened, and 200 were eligible based on PET imaging and randomized to receive either cabazitaxel or LuPSMA. LuPSMA showed a higher PSA response (66% vs. 37%, $p < 0.0001$) and fewer grade 3 or 4 adverse events (33% vs. 53%) compared to cabazitaxel. The phase III VISION study (27, Table 2) examined LuPSMA in mCRPC patients who had previously received at least one ARSi and one taxane. Among the 1179 patients screened, 86.6% met the PSMA-positive imaging criteria, and 82.9% were randomized. The study showed that LuPSMA significantly prolonged OS (median 15.3 vs. 11.3 months, HR 0.62) and radiographic PFS (median 8.7 vs. 3.4 months, HR 0.4). Based on these findings, LuPSMA has been proposed as a new standard of care for patients with pretreated mCRPC. Additionally, a pivotal trial is exploring the use of LuPSMA as a metastasis-directed therapy after surgery and radiation in low-volume metastatic hormone-sensitive prostate cancer (mHSPC), with an ongoing randomized phase II study in this setting (46).

1.2.5 Combination Therapies in mCRPC

Various treatment combinations have been explored for advanced prostate cancer, with mixed results. In mHSPC, the ENZAMET trial showed that enzalutamide plus ADT extended OS

compared to ADT plus a first-generation antiandrogen (47). However, combining enzalutamide with docetaxel improved clinical PFS but did not show an OS benefit (HR 0.90, 95% CI 0.62–1.31). Similar results were found in the TITAN trial with apalutamide (48). The PEACE-1 trial showed a PFS and an OS benefit (HR 0.50 95% CI 0.40-0.62 and (HR 0.75 95% CI 0.59-0.95, respectively) from adding abiraterone to docetaxel (49). The ARASENS trial investigated darolutamide plus ADT and docetaxel showing a statistically significant reduction in the risk of death by 32.5% (50).

In mCRPC, phase III trials of abiraterone combined with enzalutamide (ALLIANCE A031201) or apalutamide (ACIS) showed significant PFS improvement but no OS benefit (51, 52). The CHEIRON trial also found improved PFS with enzalutamide plus docetaxel (53). The CHAARTED2 trial is evaluating the addition of cabazitaxel to abiraterone (54). The IPATENTIAL 150 trial showed that adding ipatasertib to abiraterone improved radiographic PFS but did not improve OS for patients with PTEN loss in IHC (20). Other combinations, including radium-223, olaparib, and immunotherapies, have shown activity in mCRPC, but OS benefit data are still needed to determine their role in treatment (55-58).

1.3 Biomarkers for Prognosis and Prediction in mCRPC

In PCa, various prognostic factors have been recognized, including clinical elements such as age, performance status and comorbidities, as well as tumor-related characteristics like Gleason score, mitotic index, extracapsular spread, seminal vesicle invasion, PSA levels, and the metastatic stage. In mHSPC, disease volume plays a crucial role in predicting the potential benefit from chemotherapy. Despite this, most of these factors do not predict how patients will respond to treatment, and they are not particularly useful for selecting specific therapies. Molecular changes, particularly in genes that drive tumor growth, are frequently seen in prostate cancer and could be valuable as predictive biomarkers for tailoring treatment strategies. These genetic alterations can help detect primary resistance early on, thus avoiding unnecessary side effects. Some of these mutations are inherited, while others develop during cancer progression or as a result of the selective pressures exerted by treatments. Notable examples include androgen receptor (AR) amplification, mutations, or splice variants. In addition, some resistance mechanisms bypass AR signaling entirely by activating alternative biological pathways (64). Table 2 outlines the proposed molecular biomarkers for advanced prostate cancer. Several DNA damage repair (DDR) genes have been confirmed as useful in identifying patients

who might benefit from poly ADP-ribose polymerase (PARP) inhibitors. Moreover, the AKT inhibitor ipatasertib has demonstrated effectiveness in patients with PTEN loss. These biomarker-driven approaches are anticipated to become standard practice, but their impact on treatment sequencing and response is still unclear and will be studied in the coming years.

TABLE 2 NEW FRONTIERS IN PROGNOSTIC AND PREDICTIVE BIOMARKERS FOR mCRPC

Biomarker	Source	Drugs	Studies	Phase III trials
DDR (<i>BRCA1/2, ATM, PALB2</i> and other genes)	PMBC, tumor tissue or ctDNA	Olaparib Rucaparib Talazoparib Niraparib	Phase 2 TOPARP (60) Phase 2 TRITON-2 (61) Phase 2 TALAPRO-1 (62) Phase 2 GALAHAD (63)	PROFOUND (26,55) PROpel (64)* KEYLINK-010 (65)* TRITON-3 (66)* CASPAR (67)* TALAPRO-2 (68)* MAGNITUDE (69)*
PTEN loss	Tumor tissue	Ipatasertib	Phase 2 A. Martin study (70)	IPATential150 (71)
AR-V7	CTCs	ARSi	PROPHCY biomarker study (72)	
Molecular subtype Luminal A Luminal B Basal	Tumor tissue	Apalutamide Docetaxel	SPARTAN (73) and TITAN (74) (biomarker analyses) CHAARTED (75) (biomarker analysis)	
Others MSI-h/MMRd CDK12 deficiency SPOP mutations RB1 loss TP53 alterations TMPRSS2	Tumor tissue	ARSi ICI	<i>Explorative analyses</i>	

ARSi: androgen receptor signaling inhibitors; AR-V7: androgen-receptor variant 7; CTC: circulating tumor cells; ctDNA: circulating tumor DNA; DDR: DNA damage response (genes); ICI: immune checkpoint inhibitors; mCRPC: metastatic castration-resistant prostate cancer; MSI-h/MMRd: microsatellite instability-high/mismatch repair deficient; PMBC: peripheral blood mononuclear cells. *Ongoing trial.

1.3.1 Genes Involved in DNA Damage Repair (DDR)

Alterations in DDR genes have gained significant attention in prostate cancer research due to their prognostic and predictive implications (76). These defects are observed in 8-17% of metastatic patients, with BRCA2 mutations being the most common, both in somatic and germline DNA (77, 78). Germline BRCA2 mutations correlate with aggressive disease and poor outcomes (79, 80), and the PROREPAIR-B study highlighted their negative prognostic impact. Moreover, germline BRCA2 status has been shown to influence treatment response, especially

in selecting first-line therapy (ARSi vs. taxane) for mCRPC patients (81). The BRCA2men study aims to validate BRCA2 mutations as a predictive biomarker for therapy selection (82).

The PROFOUND study recently confirmed the predictive value of DDR gene defects in mCRPC patients progressing on enzalutamide or abiraterone. Patients were randomized to receive olaparib or the physician's choice of enzalutamide or abiraterone (control) (83).

Patients with alterations in BRCA1, BRCA2, or ATM, who were randomized to receive olaparib, showed significantly improved PFS and OS (26, 55). A subgroup analysis indicated that patients with BRCA alterations derived the greatest benefit, while those with ATM mutations showed less clear improvements in PFS (HR: 1.04, 95% CI 0.61–1.87) and OS (HR: 0.93, 95% CI 0.53–1.75) (84). Notably, some experts underline that the use of an ARSi in the control arm after progression on an ARSi represents an important limit of the PROFOUND trial and they caution against using a second ARSi after progression on an ARSi due to potential cross-resistance, with the CARD trial suggesting cabazitaxel as the preferred standard for these patients (25). The TOPARP-B phase II trial investigated biomarkers predicting response to olaparib, revealing that BRCA1/2 mutations—both germline and somatic—offered similar clinical benefits, with homozygous BRCA deletions showing the greatest response. The loss of ATM protein and RAD51 foci were also associated with better outcomes, suggesting RAD51 as a functional biomarker for identifying tumors sensitive to PARP inhibition (85). In the TRITON 2 phase II trial, mCRPC patients with BRCA1/2 mutations who progressed after ARSi and taxane therapy responded well to rucaparib, with higher efficacy in germline versus somatic BRCA mutations and in biallelic versus monoallelic mutations (61). This suggests a class effect of PARP inhibitors favoring BRCA2 alterations over BRCA1 in mCRPC (86). Similarly, the TALAPRO-1 trial demonstrated that talazoparib was effective in mCRPC patients with DDR defects, particularly those with BRCA1/2 mutations (62). The GALAHAD trial is further assessing niraparib in patients with biallelic DDR defects (63). Overall, PARP inhibitors like olaparib show significant benefit for pretreated mCRPC patients with DDR gene alterations, particularly BRCA1/2 mutations. Ongoing trials are investigating their role in combination with ARSi at earlier stages, leveraging the relationship between AR function and PARP1 activity. The PROpel Phase III trial evaluated olaparib combined with abiraterone in first-line mCRPC treatment, expanding the use of PARP inhibitors to broader patient populations. The trial showed that abiraterone plus olaparib prolonged OS by > 7 m vs standard-of-care abiraterone (64). The TALAPRO-2 trial also demonstrated that Talazoparib plus enzalutamide improves OS in mCRPC

patients, including those with HRR gene mutations, compared to enzalutamide monotherapy. Similarly, the Phase 3 MAGNITUDE Study showed a trend toward Improvement in OS in mCRPC Patients with BRCA alterations treated with Niraparib and Abiraterone Acetate Plus Prednisone. Lastly, the CASPAR trial is also exploring the combination with enzalutamide plus rucaparib (67-69).

1.3.2 Androgen-receptor signaling

Numerous studies suggest that alterations in the androgen receptor (AR) pathway play a crucial role in resistance mechanisms in mCRPC. Variations in AR copy number in plasma DNA are linked to poorer outcomes in mCRPC patients treated with ARSi (89). Additionally, AR gain in plasma DNA correlates with worse prognosis in patients treated with docetaxel. However, patients with AR gain appear to benefit more from taxane treatment than from ARSi therapy (90, 91). The androgen receptor variant 7 (AR-V7) has been identified as a potential predictor of poor response to ARSi treatments such as abiraterone acetate and enzalutamide. Antonarakis et al. first demonstrated that the presence of AR-V7 is associated with resistance to ARSi (92). Interestingly, AR-V7 does not seem to contribute to resistance against taxane-based chemotherapy, and its detection may even decrease after treatment with taxanes (93-95). In the PROPHECY trial, 118 men with mCRPC starting treatment with abiraterone or enzalutamide were evaluated for AR-V7 presence (72). AR-V7 detection, using both Johns Hopkins and Epic AR-V7 assays, was independently linked to shorter progression-free survival (PFS) and OS, with AR-V7-positive patients showing fewer prostate-specific antigen (PSA) or soft tissue responses. However, no randomized trials have conclusively shown that chemotherapy offers a survival benefit over ARSi for AR-V7-positive patients, raising questions about the predictive and clinical value of AR-V7. AR-V7 is rarely detected in patients starting first-line treatment for mCRPC (3-8%) after androgen-deprivation therapy, but its prevalence increases with each subsequent treatment line (96, 97). Although AR-V7 could help guide decisions regarding second-line ARSi therapy for pretreated patients, further research is required to confirm its clinical utility.

1.3.3 PTEN loss and PI3K alterations

Around 50% of mCRPC patients show PTEN loss, leading to hyper-activation of the PI3K/AKT pathway, which is associated with poorer prognosis and reduced response to ARSi (98, 99). A Phase II study tested the combination of the AKT inhibitor ipatasertib and abiraterone versus abiraterone alone in mCRPC patients previously treated with docetaxel (70). The combination significantly improved radiographic PFS, with greater benefits seen in PTEN-loss tumors. These findings led to the Phase III IPATential150 trial, comparing ipatasertib plus abiraterone to abiraterone alone as first-line therapy for mCRPC (71, 100). In the PTEN-loss subgroup, the combination showed a significant PFS benefit (18.5 vs 16.5 months, HR 0.77, 95% CI 0.61–0.98, $p=0.034$), though no significant difference was observed in the overall population. Subgroup analysis indicated that prior taxane therapy may impact the benefit of ipatasertib. A biomarker analysis revealed that patients with PTEN loss and alterations in PIK3CA/AKT1/PTEN had greater PFS benefit from ipatasertib than those without detectable alterations (101). These results suggest that ipatasertib plus abiraterone could be a promising first-line treatment for mCRPC with PI3K/AKT pathway alterations.

1.3.4 Differentiating Basal and Luminal Prostate Cancer

The PAM50 gene expression classifier, which categorizes breast cancer into luminal A, luminal B, HER2, and basal subtypes, was applied by Zhao and colleagues to prostate cancer samples, identifying luminal A, luminal B, and basal subtypes (102). They found that luminal B prostate cancer had the poorest clinical outcomes, followed by basal and luminal A. Both luminal-like subtypes showed increased AR expression and signaling, but only luminal B was significantly linked to postoperative response to ADT. Similar results were seen in the CHAARTED trial, where the luminal B subtype had shorter OS with ADT alone compared to the basal subtype, confirming its negative prognostic value. However, luminal B patients treated with ADT plus docetaxel had significant improvement in time to castration-resistance and OS, while basal subtype showed no OS benefit from the combination, even in high-volume disease patients. Luminal subtype also responded better to ARSi than basal subtype. In the Phase III SPARTAN trial (apalutamide in nmCRPC), more than 50% of patients achieved $\geq 90\%$ PSA reduction with apalutamide. The PSA decline was more rapid and profound in patients with the luminal subtype, and OS improvement also favored the luminal subtype (HR 0.43, 95% CI

0.19-1, $p=0.051$) compared to the basal subtype (HR 0.67, 95% CI 0.40-1.14, $p=0.14$) (73). In contrast, in the TITAN trial sub-analysis (apalutamide in mHSPC), apalutamide significantly prolonged radiographic PFS in basal subtype (HR 0.31 95% CI 0.16-0.62, $p=0.0008$), but no significant difference was observed in the luminal subtype (HR 0.74, 95% CI 0.40-1.36, $p=0.33$) (74). The distinct settings of mHSPC vs mCRPC might explain these discrepancies. Importantly, these biomarker analyses were conducted on diagnostic biopsies from patients receiving treatment in later stages, during castration resistance, and metastatic site characteristics may differ from primary tumors. Overall, these findings suggest that luminal versus basal classification could help identify patients most likely to benefit from ARSi and docetaxel, though prospective studies are needed to confirm its predictive value.

1.3.5 The Aggressive-Variant Phenotype in Prostate Cancer

Aggressive-variant prostate cancer (AVPC) refers to AR-independent anaplastic forms of prostate cancer that progress rapidly, show poor therapeutic response, and have a poor prognosis (103). Many AVPC cases display neuroendocrine features (NEPC), but some do not exhibit the typical morphology or immunohistochemical markers of neuroendocrine differentiation. AVPC cells may either develop de novo or more commonly emerge through divergent clonal evolution from castration-resistant adenocarcinoma cells (104). Chemotherapy and ARSi create selective pressure that fosters the emergence of these resistant clones, often seen in advanced stages of castration resistance. Clinically, AVPC is characterized by one or more of the following features (103, 105, 106): a) histologic evidence of small-cell NEPC; b) exclusive visceral metastases; c) radiographically predominant lytic bone metastases; d) bulky lymphadenopathy or large high-grade tumor mass in the prostate/pelvis; e) low PSA at presentation plus high-volume bone metastases; f) presence of neuroendocrine markers in serum or histology, plus elevated serum LDH, hypercalcemia, or CEA, with no other causes; g) rapid progression to androgen independence after hormonal therapy, with or without neuroendocrine markers. AVPC responds well to platinum-based chemotherapy, though responses are often short-lived (105). The NCCN guidelines recommend chemotherapy regimens such as cisplatin/etoposide, carboplatin/etoposide, and docetaxel/carboplatin for patients with small-cell or NEPC (107). A phase II study of the AURKA inhibitor alisertib in metastatic NEPC patients did not meet its primary endpoint of improved

PFS, but tumors with N-myc and Aurora-A overactivity responded exceptionally, including complete resolution of liver metastases and prolonged stable disease (108). Ongoing trials are exploring immunotherapy, PARP inhibitors, and EZH2 inhibitors in AVPC and NEPC patients (109). For AVPC patients without small-cell or NEPC histology, there is no consensus on the best first-line treatment. At the 2019 Advanced Prostate Cancer Consensus Conference (APCCC), 75% of panelists supported adding docetaxel to ADT, 16% favored platinum-based combination therapy, and 9% suggested adding an ARSi. The impact of first-line platinum-based chemotherapy on the efficacy of subsequent treatments, such as PARP inhibitors, docetaxel, or ARSi, remains unclear and warrants further research.

1.3.6 Other biomarkers

Due to its tissue-agnostic approval by the U.S. FDA, patients with prostate cancer exhibiting microsatellite instability (MSI) or deficiencies in mismatch repair may benefit from pembrolizumab treatment (110). In a study by Abida et al., 3.1% of 1033 patients with sufficient tumor quality for MSI analysis had MSI-high or mismatch repair-deficient prostate cancer, with 7 carrying a pathogenic germline mutation in a Lynch syndrome-related gene (110). Of the 11 patients who received anti-PD1/PD-L1 therapy, 6 (54.5%) showed a greater than 50% reduction in PSA levels, and 4 displayed radiographic responses. However, none of the six patients with tumor responses in the Phase II KEYNOTE-199 study had MSI, indicating other factors may play a role in response to immunotherapy (56). Notably, 11% of patients with BRCA or ATM mutations responded to pembrolizumab, compared to just 3% of those without DDR alterations. Additionally, patients with CDK12 deficiencies may have a better response to anti-PD-1 checkpoint inhibitors (111,112). SPOP mutations have been associated with improved outcomes when treated with abiraterone acetate (113). RB1 mutations, which become more common after selective treatment pressure, are linked to worse prognosis and progression-free survival in mCRPC patients treated with enzalutamide (114). Patients with RB1 mutations also had worse clinical outcomes. Furthermore, alterations in RB1 and TP53 were found to be linked to shorter treatment durations with abiraterone or enzalutamide (116), and the concurrent loss of tumor suppressor genes like TP53, PTEN, and RB1 may drive more aggressive disease and a higher likelihood of relapse (117).

1.3.7 Overcoming Biomarker and Diagnostic Obstacles

In the PROFOUND trial, 4425 patients were initially enrolled, and 4047 had tumor tissue available for testing. Of these, 2792 (69%) were successfully sequenced, but only 162 patients (3.7% of initial enrollment) had germline or somatic alterations in BRCA1, BRCA2, or ATM. This highlights the limitations of tumor tissue analysis. To expand the pool of patients who could benefit from biomarker-driven treatments, an increase in sequencing success rates or the adoption of liquid biopsy methods is needed. ctDNA has been shown to effectively identify driver DNA alterations found in metastatic tissue for most mCRPC patients (118). In the PROFOUND trial, a strong correlation between tumor tissue and circulating tumor DNA (ctDNA) was observed, supporting ctDNA testing as a minimally invasive approach to identify DDR-altered mCRPC patients (119). ctDNA can detect somatic mutations, copy-number variations, and structural changes in metastatic disease that are predictive of therapy response. However, ctDNA-based genotyping is influenced by various technical and biological factors, complicating its clinical use (120). The ctDNA fraction is crucial for assay detection sensitivity and specificity, and low ctDNA% can limit the identification of clinically relevant alterations, such as those in TP53, BRCA2, PTEN, RB1, and AR (120). Thus, both ctDNA and tumor tissue analysis have their strengths and weaknesses, and they are likely to complement each other in the future of precision oncology. The next challenge will be developing more reliable and accessible assays to detect specific biomolecular alterations in cancer patients.

1.4 Lipidomics

Lipids are hydrophobic compounds that are crucial for various biological functions and are essential to the structural composition of normal cells. The field of lipidomics focuses on investigating lipids and their interactions within biological systems (87). In cancer, lipid metabolism is often disrupted, with cells utilizing either lipogenic or lipolytic pathways to enhance proliferation, survival, and the capacity to migrate and form metastases (121, 122). Altered lipid profiles, or dyslipidemia, have been linked to cancer development through several mechanisms (123, 124). Hypoxia is known to contribute to lipid dysregulation, impacting membrane integrity, immune system function, and fostering abnormal angiogenesis (125). In lung cancer, distinct lipid profiles have been associated with the variation among different subtypes of the disease (126). Additionally, higher plasma levels of

cholesterol and lipoproteins have been implicated in the progression of breast and ovarian cancers (134).

1.4.1 Exploring Lipidomics in Prostate Cancer

Recent findings support the idea that PCa is associated with altered lipid metabolism (128,129). Obese men show a higher likelihood of developing aggressive forms of PCa and face an increased risk of prostate cancer-specific mortality (130). Prostate cancer cells exhibit elevated rates of lipid synthesis and breakdown, alongside changes in cholesterol and phospholipid metabolism (131). Furthermore, studies have suggested that the transition from hormone-sensitive to castration-resistant PCa involves disruptions in lipid metabolism, including increased intratumoral levels of polyunsaturated fatty acids (132). The expression of AR-V7 appears to be pivotal in reactivating lipid biosynthesis in CRPC, indicating the critical role this variant plays in regulating lipid metabolism in the context of CRPC (129). Fatty acid synthase (FASN), a crucial enzyme involved in lipid production, has been identified as one of the top overexpressed genes in AR-V7-driven CRPC metastases (133). Newer research indicates a reciprocal regulation between FASN and AR-V7, suggesting that targeting FASN could be a strategy to counteract AR-V7 and potentially overcome resistance to treatments like enzalutamide and abiraterone (134). Another study observed that 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), a key enzyme in the cholesterol synthesis pathway, was upregulated in enzalutamide-resistant PCa cell lines. The use of simvastatin, an HMGCR inhibitor, inhibited AR synthesis and suppressed tumor growth both in vitro and in vivo (135). Additionally, evidence suggests that statins may lower the risk of metastatic PCa and reduce prostate cancer-related mortality through their effects on cholesterol and lipid metabolism (136).

Given this, a thorough investigation of lipid metabolism in PCa could offer valuable insights into tumor development, progression, resistance to treatment, and potentially provide biomarkers for predicting patient responses to therapies. Several studies have examined specific lipid molecules as potential biomarkers for diagnosing PCa, with some exploring their role in predicting outcomes during treatments with ARSi or chemotherapy (137-139). For example, enzalutamide has been shown to induce significant remodeling of lipid profiles across various phospholipid classes, shifting from storage lipids to increased membrane lipid

desaturation and elongation of acyl chains, with these changes correlating with the activity of enzalutamide (140).

1.4.2 Three-lipid signature and prostate cancer

A team from Australia conducted an in-depth lipid profiling study on men with mCRPC, showing that higher concentrations of sphingolipids like ceramide and sphingomyelin were linked to poorer OS (141). The researchers employed liquid chromatography-tandem mass spectrometry to analyze plasma samples from a primary cohort of 96 mCRPC patients (Figure 2), and their findings were later confirmed in a secondary Phase 2 cohort of 63 patients. Through unsupervised analysis of lipid profiles, which included 323 lipid species, they identified two distinct patient subgroups with significantly different survival outcomes (HR 2.31, 95% CI 1.44–3.68, $p=0.0005$). Notably, 46 lipids, primarily sphingolipids, were found to be independently prognostic, with higher levels correlating to worse prognosis. From these results, the researchers developed a three-lipid signature—ceramide d18:1/24:1, sphingomyelin d18:2/16:0, and phosphatidylcholine 16:0/16:0—which was associated with shorter survival in the validation cohort (HR 4.8, 95% CI 2.06–11.1, $p=0.0003$). This lipid signature remained a significant prognostic indicator even when adjusted for clinical-pathological factors and metabolic characteristics.

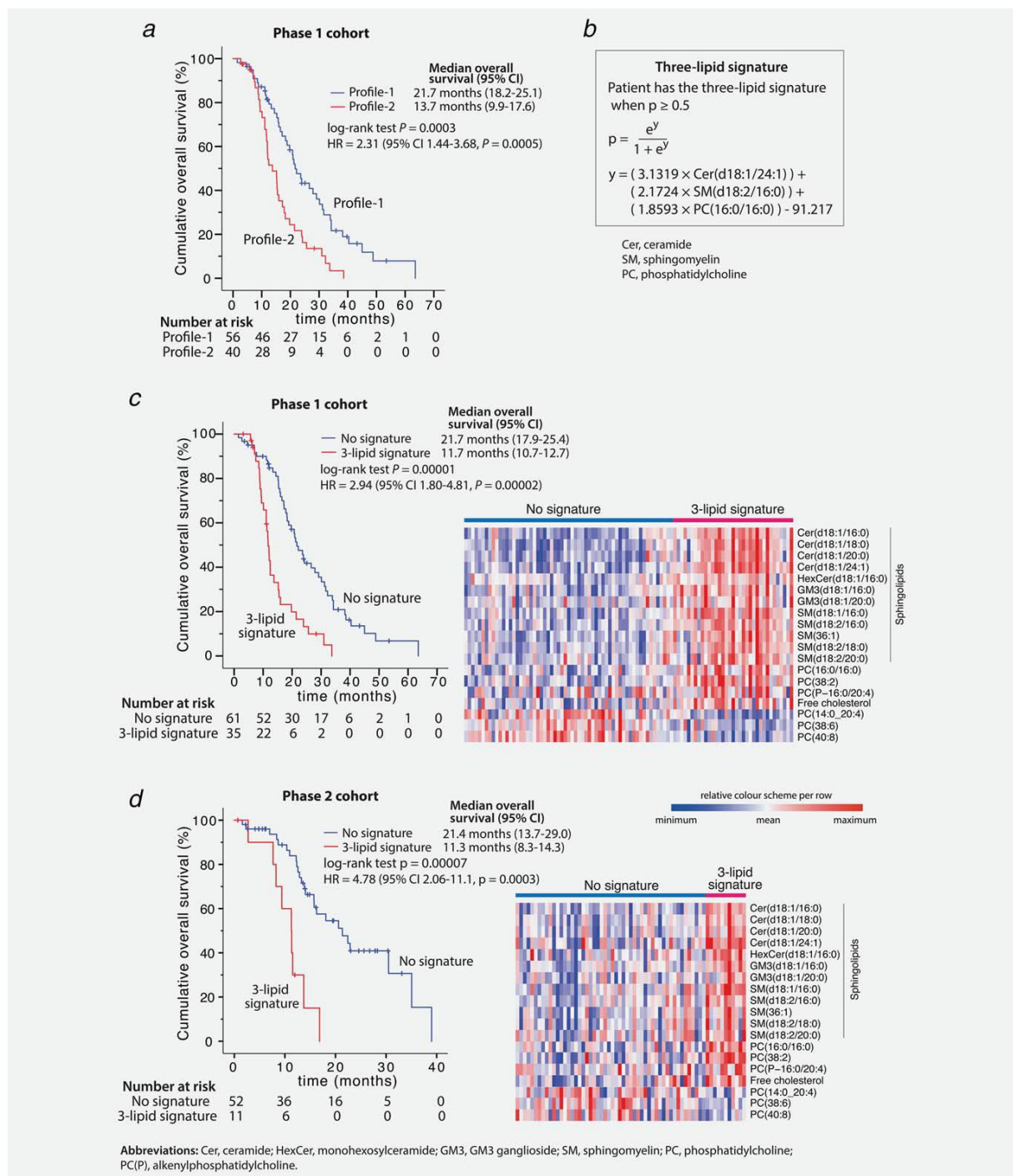


FIGURE 1 PROGNOSTIC BASELINE PLASMA LIPID LEVELS: SURVIVAL CURVES, LIPID SIGNATURES, AND HEATMAP ANALYSIS.

(a) Survival plots for the phase 1 discovery cohort, categorized based on latent class analysis of initial lipidomic profiles; (b) three-lipid signature representing normalized baseline lipid levels; (c) survival plots for the phase 1 discovery cohort, classified according to the three-lipid signature, along with a heatmap depicting 19 prognostic lipids validated in phase 2; (d) survival plots for the phase 2 validation cohort, grouped by the three-lipid signature, accompanied by a heatmap showing the 19 validated prognostic lipids (148).

Moreover, the prognostic relevance of 19 of the 46 lipids initially found in the discovery cohort was corroborated in the validation cohort. (Table 3).

Lipid	Phase 1 cohort			Phase 2 cohort		
	Hazard ratio	95% CI	p-value	Hazard ratio	95% CI	p-value
Cer(d18:1/16:0)	2.20	1.34–3.60	0.003	10.20	3.19–32.6	0.0003
Cer(d18:1/18:0)	1.74	1.24–2.45	0.002	4.45	2.16–9.19	0.00006
Cer(d18:1/20:0)	1.86	1.23–2.83	0.005	5.24	2.09–13.2	0.0005
Cer(d18:1/24:1)	2.56	1.51–4.35	0.0007	2.90	1.13–7.46	0.02
HexCer(d18:1/16:0)	2.37	1.47–3.83	0.0003	2.75	1.26–6.00	0.01
GM3(d18:1/16:0)	2.94	1.70–5.05	0.0001	5.60	1.73–18.1	0.004
GM3(d18:1/20:0)	1.79	1.16–2.76	0.009	3.29	1.19–9.08	0.02
SM(d18:1/16:0)	3.51	1.49–8.28	0.004	9.99	2.51–39.7	0.0009
SM(d18:2/16:0)	4.82	2.04–11.4	0.0004	4.36	1.11–17.2	0.03
SM(36:1)	2.29	1.28–4.10	0.007	4.25	1.74–10.4	0.002
SM(d18:2/18:0)	2.11	1.20–3.70	0.01	3.94	1.55–10.1	0.004
SM(d18:2/20:0)	2.23	1.18–4.19	0.01	3.20	1.13–9.10	0.02
PC(16:0/16:0)	4.72	1.93–11.6	0.0007	12.51	2.68–58.3	0.0007
PC(38:2)	2.60	1.30–5.19	0.006	3.40	1.56–7.38	0.003
PC(P-16:0/20:4)	2.06	1.09–3.88	0.02	4.99	1.53–16.3	0.006
Free cholesterol	4.06	1.33–12.4	0.01	7.86	1.40–44.1	0.02
PC(38:6)	0.52	0.31–0.88	0.01	0.21	0.07–0.61	0.005
PC(14:0_20:4)	0.64	0.45–0.92	0.02	0.45	0.23–0.88	0.02
PC 40:8	0.41	0.23–0.75	0.005	0.21	0.07–0.69	0.01

Abbreviations: Cer: ceramide; HexCer: monohexosylceramide; GM3: GM3 ganglioside; SM: sphingomyelin; PC: phosphatidylcholine; PC(P): alkenylphosphatidylcholine.

TABLE 3 UNIVARIABLE COX REGRESSION ANALYSIS OF 19 PROGNOSTIC LIPIDS AND THEIR HAZARD RATIOS BASED ON BASELINE PLASMA LEVELS (148)

The Australian team conducted a follow-up study to extensively profile the circulating lipidome throughout the progression of prostate cancer, including localized PCa, mHSPC, and mCRPC (142). They found that lipid profiles with high ceramide levels were linked to metastatic recurrence in localized PCa (HR 5.80, 95% CI 3.04–11.1, $P = 1 \times 10^{-6}$) and earlier failure of testosterone suppression in mHSPC (HR 3.70, 95% CI 1.37–10.0, $P = 0.01$). Notably, the prognostic value of circulating lipid profiles in localized PCa was independent of conventional clinic-pathological and metabolic variables.

Additionally, the three-lipid signature was reassessed in the discovery cohort with extended follow-up and continued to show prognostic relevance (Figure 3). In this cohort, all patients had received docetaxel as first-line therapy for mCRPC, and those with the three-lipid signature experienced a shorter time to PSA progression (HR 1.67, 95% CI 1.14–2.44, $P = 0.01$).

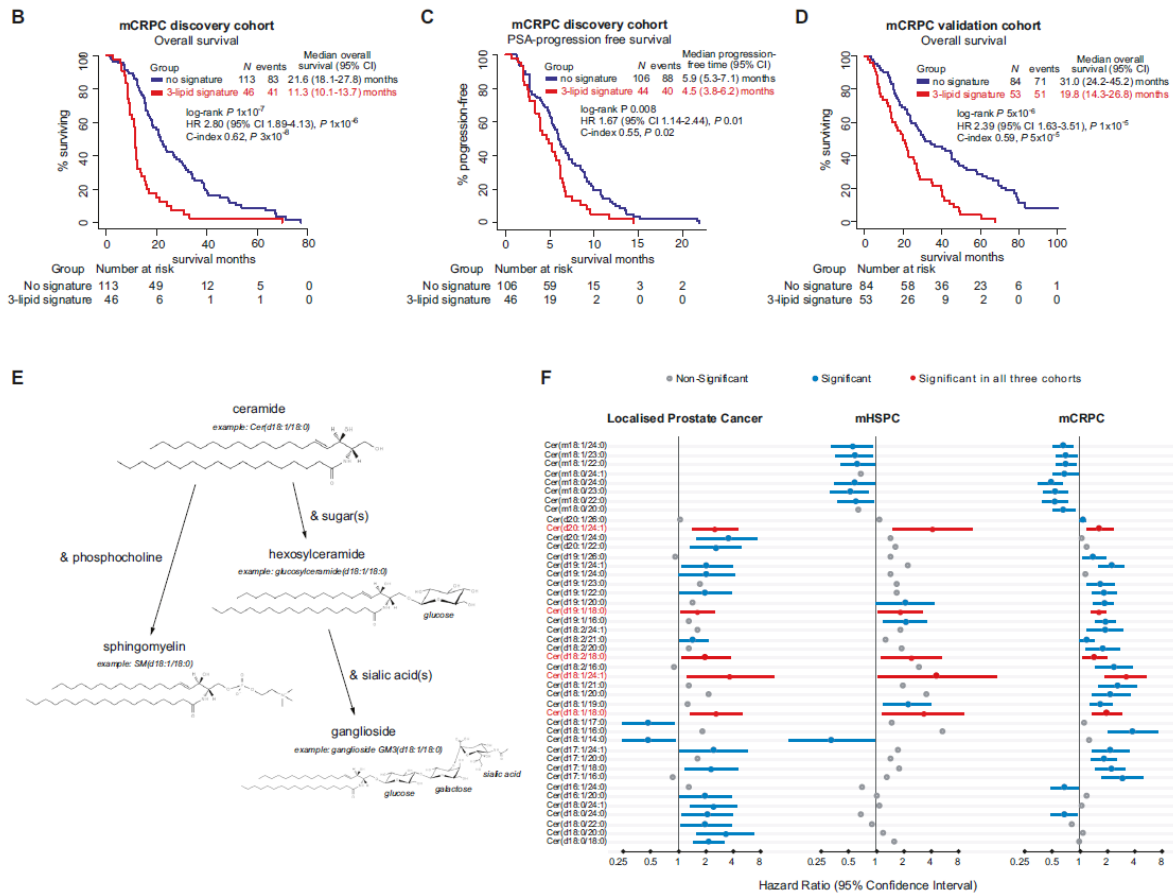


FIGURE 2 A PROGNOSTIC 3-LIPID SIGNATURE AND ITS ASSOCIATION WITH CERAMIDE SPECIES IN mCRPC.

OS (B) and PSA progression-free curves (C) of discovery cohort classified by the 3-lipid signature; OS curves of validation cohort classified by the 3-lipid signature (D); metabolism of ceramide and other sphingolipids (E); forest plots of the HR of ceramide species that are prognostic in localised PCa, mHSPC or mCRPC validation cohorts (F) (149).

In the validation cohort, 275 lipid species were found to have a strong association with OS. The top 20 lipids identified were predominantly ceramide, sphingomyelin, and acylcarnitine species. Notably, ceramide (d18:1/24:1) alone showed a comparable prognostic value to the three-lipid signature (HR 3.2 (95% CI 1.88–5.40, $P = 4 \times 10^{-5}$) in univariate analysis); however, the three-lipid signature was more effective in predicting 1-year survival.

In a subsequent investigation, plasma lipidomic profiling and cell-free DNA (cfDNA) sequencing were conducted on 106 men with mCRPC beginning treatment with docetaxel,

A Landscape of somatic aberrations in the discovery cohort



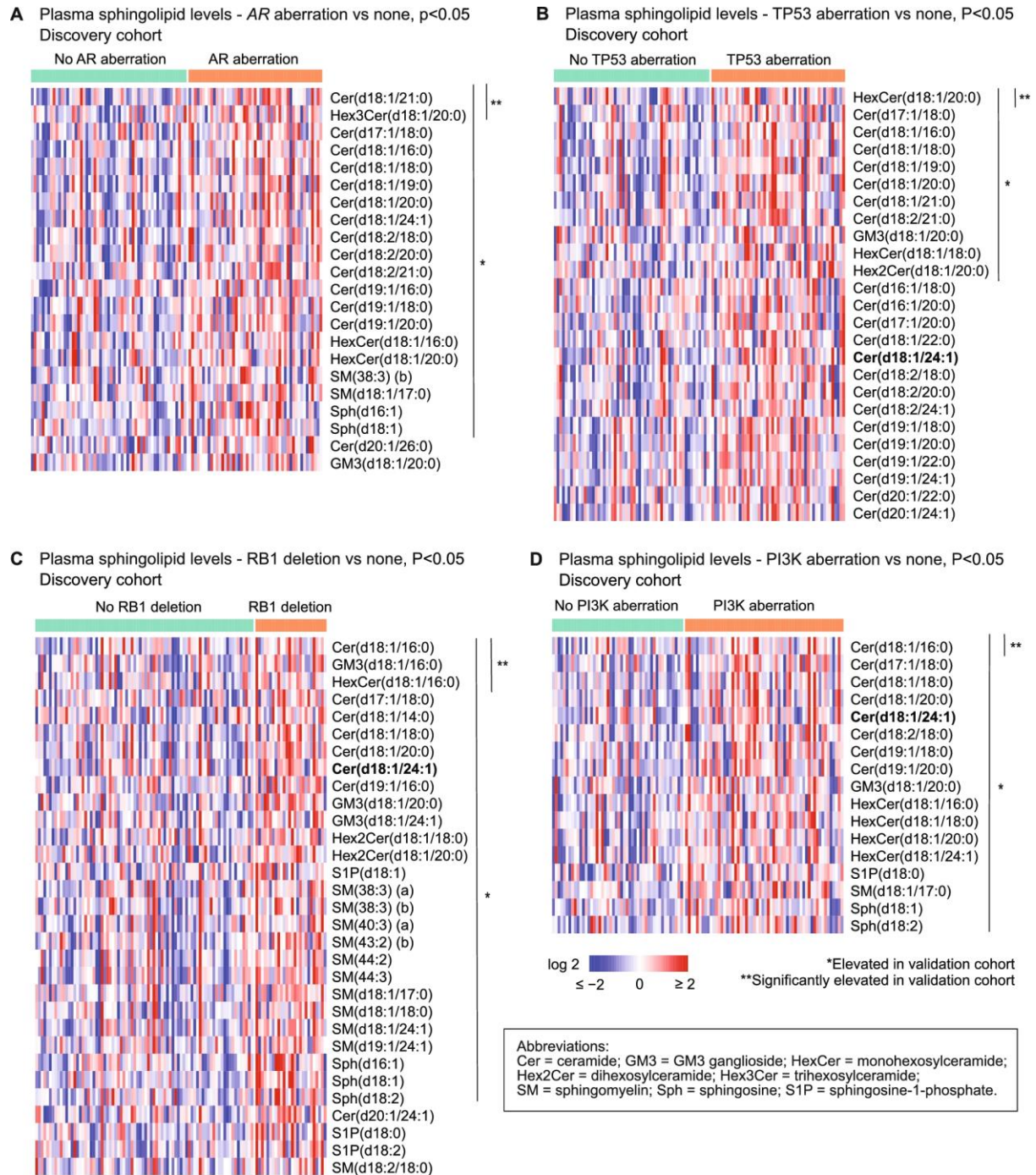


FIGURE 4 LIPIDOMIC AND GENETIC ABERRATIONS: THEIR COMBINED EFFECT ON CLINICAL OUTCOMES IN MCRPC (150).

The presence of the three-lipid signature was linked to an increased frequency of somatic mutations in key pathways, including AR, TP53, cell cycle regulation, PI3K, DNA repair, mismatch repair (MMR), and WNT. Additionally, genomic diversity was heightened in individuals with the three-lipid signature (Figure 5). Higher levels of circulating sphingolipids were particularly correlated with AR mutations, TP53 alterations, RB1 deletions, and

disruptions in the PI3K pathway in both patient cohorts. Approximately 20 sphingolipids were found to be significantly elevated in individuals with any AR mutation, compared to those without. Furthermore, elevated sphingolipid levels were notably associated with TP53 mutations, RB1 deletions, and PI3K pathway disruptions. However, no significant link was observed between increased sphingolipid levels and mutations in DNA repair genes (BRCA1/2, ATM, CHEK2), MMR genes (MLH1, MSH2, MSH6), or the WNT pathway (APC, CTNNB1), suggesting that not all genetic mutations are associated with the unfavorable lipidomic profile. In multivariate analysis, both the presence of AR mutations and the three-lipid signature were independently correlated with worse OS compared to individuals without these characteristics, in both the discovery and validation cohorts. This relationship with shortened OS was also seen when combining the three-lipid signature with TP53 mutations, RB1 deletions, or PI3K alterations. Additionally, elevated sphingolipid levels were linked to AVPC in both cohorts. Men with both the three-lipid signature and AVPC had significantly shorter OS in comparison to those without either factor, with a median survival of approximately 12 months versus over 2 years for those without these signatures.

Ceramide metabolism has been associated with cancer and various other pathological conditions (144). Sphingolipids in circulation are primarily produced by the liver, transported via lipoproteins, and can increase in response to systemic inflammation (145). However, some of these sphingolipids may also be derived from the tumor itself, as prostate cancer cells express relevant biosynthetic enzymes, many of which are linked to poor prognosis. Moreover, prostate cancer cell-derived exosomes are notably enriched in sphingolipids (146).

2 Published data

We have recently published data related to this topic, which is also the focus of this thesis (147). This study aimed to explore lipidomic profiles in mCRPC patients to identify lipid species that could serve as predictors of prognosis and treatment response. Plasma samples were collected from two groups: patients beginning first-line treatment (1L, n = 29) and those who had received at least two prior lines of treatment (> 2L, n = 19), including androgen-receptor signaling inhibitors and taxanes. Using an untargeted lipidomic approach, 789 plasma lipids were identified and quantified. Notably, 75 lipid species showed significant dysregulation in the > 2L group compared to the 1L group, with 63 species being elevated and 12 being

reduced. Elevated levels of acylcarnitines, diacylglycerols, phosphatidylethanolamines, triacylglycerols, and ceramides were observed in the > 2L cohort. Some lipids, such as CAR 14:0, CAR 24:1, Cer d18:1/16:0, and C18 Cer, were significantly associated with overall survival (OS) in univariate analysis.

3 STUDY OBJECTIVES

3.1 Primary endpoint

To assess the differences in the lipidomic profiles between heavily pretreated mCRPC patients (>2L cohort) and those undergoing first-line treatment for mCRPC (1L).

3.2 Secondary endpoints

To examine the prognostic and predictive value of lipid species that are differentially expressed in patients with >2L treatment history compared to those with 1L treatment.

To assess the prognostic impact of the previously identified 3-lipid signature in our mCRPC patient cohort.

4 MATERIALS AND METHODS

4.1 Patients' population

Patients with mCRPC who were either starting first-line treatment (1L cohort) or had received at least two previous lines of treatment, including one ARSi and one chemotherapy regimen (2L cohort), were invited to take part in this study.

Following consent, blood samples were collected from the patients, who were then prospectively monitored with PSA evaluations every 4-6 weeks, continuing until death or until the study's cutoff date of June 2022

4.2 Sample Collection and Handling

Plasma lipids were extracted using a biphasic extraction procedure. A 30 μ L plasma sample was mixed with 225 μ L of cold methanol (MeOH) containing a mix of deuterated standards (Splash Lipidomix®). The mixture was briefly stirred for 10 seconds, followed by the addition of 750 μ L of cold MTBE, with another 10-second stirring step. The tube was then

placed in a thermomixer at 4°C and stirred for 6 minutes at 2000 rpm. Afterward, 188 µL of water was added, followed by vortexing for 10 seconds and centrifugation for 2 minutes at 14,000 rpm at 4°C. The resulting supernatant (300 µL) was collected and evaporated using a SpeedVac. The dried extract was then reconstituted in 50 µL of a 9:1 methanol/toluene solution, with the internal standard CUDA added at 12.5 ng/mL.

4.3 Liquid chromatography – mass spectrometry analysis

The reconstituted samples were analyzed using a Vanquish UHPLC system coupled with an Orbitrap Q-Exactive Plus. Lipid separation was done on a reversed-phase column (Hypersil Gold™ 150 × 2.1 mm), with a flow rate of 0.260 mL/min at 45 °C. The mobile phase for positive ESI mode consisted of acetonitrile/water with ammonium formate and formic acid, and for negative ESI mode, ammonium acetate was used. The gradient for separation varied from 30% to 100% B over 20 minutes. Mass spectrometry was performed in both positive and negative ion modes, with source voltages of 3.5 kV and 2.8 kV, respectively. Data acquisition was in ddMS2 mode, with full-scan MS spectra collected at a resolution of 70,000, and MS/MS fragmentation at 17,500 resolution. The injection volume was 3 µL, with regular calibration to ensure accurate mass analysis.

4.4 Data processing

The raw data from the untargeted analysis were processed using MSDIAL software (version 4.24) for peak detection, MS2 data deconvolution, compound identification, and peak alignment across samples. An 85% identification threshold was applied based on retention time, *m/z*, isotopic pattern, and MS/MS similarity scores. Features related to internal standards were excluded and analyzed in Skyline for reproducibility. The dataset, including *m/z* values, retention time, peak area, and aligned files, was exported as an Excel file and reviewed for missing or misregistered signals. For quantification, peak areas for each lipid were summed, normalized with a deuterated internal standard, and concentrations estimated in nmol/mL. Statistical analysis was conducted using MetaboAnalyst 4.0 and pathway analysis with Lipea software. The data have been deposited in the EMBL-EBI MetaboLights database (MTBLS1866).

4.5 Quality control

Retention time consistency, mass accuracy, and intensity are critical for reliable lipidomic analysis using LC-MS. To ensure quality control, pooled samples were analyzed before the batch, at the start of the batch, and at the end of the batch. Additionally, blank samples were included to detect any residual interference. Internal standards were added directly to plasma samples, including various analyte classes at levels suited for plasma (Avanti SPLASH Lipidomix), along with an internal standard (CUDA) prior to the LC-MS analysis. As the assays were conducted over an extended period, the pooled samples used for quality control were prepared from plasma of individuals not involved in the study, aiming to maintain the integrity of patient samples and avoid unnecessary freeze-thaw cycles. Instrumental variability was assessed by calculating the coefficient of variation (CV%) of internal standards across both individual samples and pooled quality control samples.

4.6 Evaluation of Treatment Response and Clinical Outcomes

X-Tile software was employed to determine optimal cut-off points for outcome prediction and to identify lipid species whose plasma concentrations correlated with the hazard risk for overall survival (OS) (154).

Survival analysis was performed using Kaplan-Meier curves, followed by comparisons using the log-rank test. Variables found to have a significant prognostic impact were included in multivariate Cox regression models to assess the independent prognostic value of individual lipid species. Biochemical response was defined as a reduction in PSA levels of 50% or more from baseline. Descriptive statistics were applied to evaluate treatment responses based on the circulating levels of specific lipid species.

5 RESULTS

5.1 Patients' characteristics

The participants in this study were patients with mCRPC. The 1L group consisted of patients beginning first-line treatment for mCRPC, while the >2L group comprised patients who had undergone at least two previous lines of treatment, including at least one ARSi and docetaxel. A total of 48 patients were eligible for inclusion in the analysis.

The first cohort (1L) included 29 patients. The baseline characteristics of these patients are summarized in Table 4. The median age was 75 years (range 56-84). The median baseline PSA value was 13.2 ng/mL, with values ranging from 0.3 ng/mL to 564.9 ng/mL. The median LDH level was 220 U/L, ranging from 138 U/L to 628 U/L. Bone metastases were present in 22 out of 29 patients (75.9%), while only 3 out of 29 patients (10.3%) had visceral metastases. Additionally, 18 out of 29 patients (62.1%) had multiple metastatic sites.

The second cohort (>2L) included 19 patients, with a median age of 70 years (range 58-84). The median PSA value for this group was 90.5 ng/mL, with values ranging from 3.9 ng/mL to 4668.0 ng/mL. The median LDH level was 234 U/L, with a range from 121 U/L to 2735 U/L. Bone metastases were observed in 84.2%, while visceral metastases were present in 31.6%. 68.4% in the >2L group had multiple metastatic sites.

Variables	1L N = 29	>2L N = 19	Total N = 48
Median age, ys	75	70	73.5
(range)	(56-84)	(58-84)	(56-84)
Median PSA, ng/mL	13.2	90.5	33.0
(range)	(0.3-564.9)	(3.9–4668.0)	(0.3-4688.0)
Median LDH, U/L	220	234	228
(range)	(138-628)	(121-2735)	(121-2735)
Bone metastases			
Absent	7 (24.1%)	3 (15.8%)	10 (20.8%)
Present	22 (75.9%)	16 (84.2%)	38 (79.2%)
Visceral metastases			
Absent	26 (89.7%)	13 (68.4%)	39 (81.2%)
Present	3 (10.3%)	6 (31.6%)	9 (18.8%)
Number of metastatic sites			
=1 site	11 (37.9%)	6 (31.6%)	17 (35.4%)
>1 site	18 (62.1%)	13 (68.4%)	31 (64.6%)

TABLE 4 PATIENTS BASELINE CHARACTERISTICS.

1L= patients starting a first-line treatment for mCRPC; >2L= pretreated patients' cohort.

5.2 Discovery lipidomic analysis (>2L vs. 1L patients)

A total of 789 circulating lipids were measured in the plasma samples of 48 patients using LC-MS/MS. We analyzed the lipidomic profiles of mCRPC patients who had been treated with multiple prior therapies (>2L) and compared them to those receiving first-line treatment (1L). The volcano plot illustrates the differences in lipid expression between the >2L and 1L groups. Red dots highlight lipids that were upregulated in the >2L cohort, while blue dots represent lipids that were downregulated. A total of 56 lipids showed increased expression (fold change > 1.3, p-value < 0.05), and 12 lipids were found to be downregulated (fold change < 0.75, p-value < 0.05).

Additionally, the heatmap shown in Figure 6 indicates that the lipids most abundantly expressed in the >2L cohort include triacylglycerols (TG), diacylglycerols (DG), phosphatidylethanolamines (PE), and ceramides (Cer).

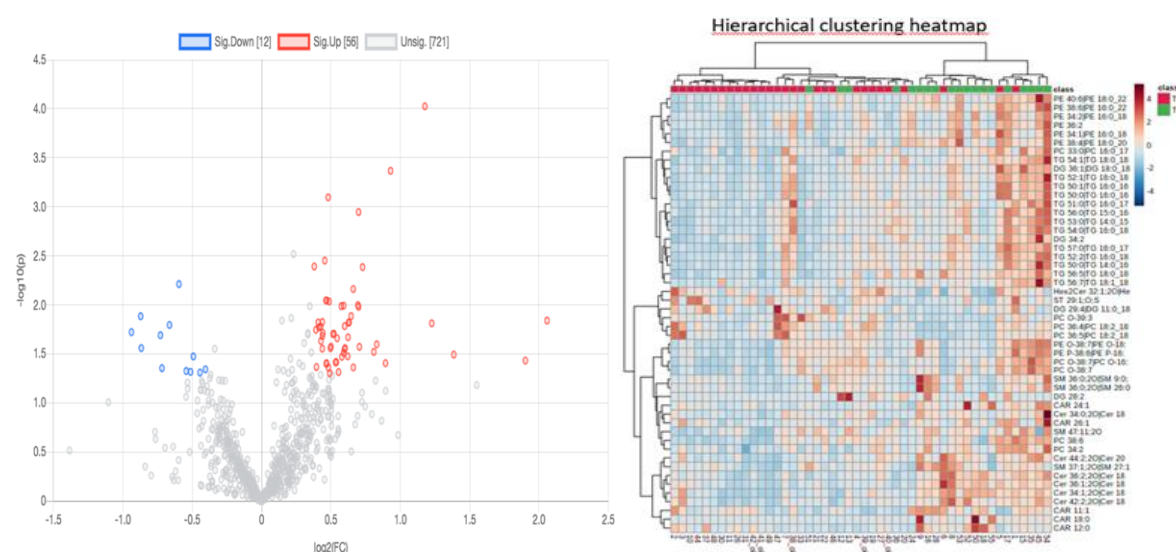


FIGURE 5 ANALYSIS OF LIPID SPECIES ALTERED IN >2L VERSUS 1L MCRPC PATIENTS

On the left, the volcano plot highlights lipids that are overexpressed (shown in red), underexpressed (12 in blue), and those with no significant change (depicted in grey) when comparing the >2L and 1L cohorts. On the right, the heatmap illustrates the 68 lipids that show differential expression. In the first row, red squares denote patients from the 1L group, while green squares correspond to patients from the >2L group.

Overall, 63 lipid species were found to be overexpressed in >2L cohort compared to 1L cohort, with a FC ≥ 1.2 (Table 5), and 12 were found to be underexpressed (Table 6).

LIPID SPECIES	FC	log2(FC)	p value
DG 28:2	4,162 8	2,0576	0,01439
CAR 14:0	3,736 6	1,9017	0,03680 1
CAR 20:1	2,612 9	1,3856	0,03197 4
CAR 18:0	2,340 2	1,2267	0,01535 2
PE 40:6 PE 18:0_22:6	2,261 8	1,1774	9,44E-05
PE 38:6 PE 16:0_22:6	1,905	0,92982	0,00042 9
CAR 18:1	1,857 7	0,89351	0,03911
TG 56:7 TG 18:1_18:2_20:4	1,777 9	0,83018	0,02515 3
TG 56:7 TG 16:0_18:1_22:6	1,752 1	0,80912	0,03002 1
Cer 36:2;2O Cer 18:2;2O/18:0	1,656 5	0,72814	0,00410 4
CAR 12:0	1,630 3	0,70517	0,02664
SM 36:0;2O SM 26:0;2O/10:0_SM 36:0;2O	1,623 9	0,6995	0,01043 7
CAR 24:1	1,623 6	0,69916	0,00112 7
SM 36:0;2O SM 9:0;2O/27:0	1,617 9	0,69413	0,01000 8
TG 52:0 TG 16:0_18:0_18:0	1,581 4	0,66117	0,04322 2
PE 34:1 PE 16:0_18:1	1,581	0,66088	0,00687 3
Cer 34:0;2O Cer 18:0;2O/16:0	1,564	0,64525	0,01296 4
TG 52:1 TG 16:0_18:0_18:1	1,549 2	0,63157	0,01499 8
TG 50:1 TG 16:0_16:0_18:1	1,544	0,62666	0,01513 5
TG 50:0 TG 16:0_16:0_18:0	1,54	0,62298	0,02129 7
TG 51:1 TG 16:0_17:0_18:1	1,538 1	0,62118	0,03332 5
DG 36:1 DG 18:0_18:1	1,518 2	0,6024	0,02745

PE 34:2 PE 16:0_18:2	1,514 3	0,59866	0,01641 8
CAR 20:0	1,513	0,59741	0,02812 6
DG 34:2	1,507 4	0,59203	0,01013 8
TG 50:2 TG 16:0_16:1_18:1	1,506 9	0,59156	0,03063 5
TG 53:1 TG 17:0_18:0_18:1	1,493 3	0,57847	0,03367 7
Cer 36:1;20 Cer 18:1;20/18:0	1,489 5	0,57484	0,01026 3
TG 51:2 TG 16:0_17:1_18:1	1,469 1	0,55493	0,04823
TG 50:0 TG 14:0_16:0_20:0	1,458 9	0,54492	0,02179 7
SM 42:2;2°	1,453 8	0,5398	0,03814 3
PE 36:2 PE 18:0_18:2	1,446 6	0,53269	0,03839 2
PE 36:2	1,435 7	0,5218	0,01956 1
TG 52:2 TG 16:0_18:1_18:1	1,428 8	0,5148	0,01979 8
TG 54:1 TG 18:0_18:0_18:1	1,414 9	0,50066	0,02656 5
TG 52:3 TG 16:0_18:1_18:2	1,410 5	0,49625	0,02750 1
PC O-40:10	1,405 9	0,49152	0,04951
PE O-38:7 PE O-18:2_20:5	1,401 4	0,48684	0,00918
TG 55:1 TG 18:0_19:0_18:1	1,396 7	0,48198	0,04346 7
Cer 34:1;20 Cer 18:1;20/16:0	1,394 9	0,48011	0,00079 8
PE 38:4 PE 18:0_20:4	1,384 1	0,46895	0,00898 7
TG 49:0 TG 15:0_16:0_18:0	1,383 6	0,46839	0,03919 3
PE 36:4 PE 16:0_20:4	1,381 8	0,46655	0,03963 9
Cer 44:2;20 Cer 20:1;20/24:1	1,379 4	0,46401	0,00897 4
Cer 42:2;20 Cer 18:1;20/24:1	1,371 2	0,45543	0,00352 1

TG 51:0 TG 16:0_17:0_18:0	1,358 3	0,44175	0,01929 5
SM 37:1;2O SM 27:1;2O/10:0	1,355	0,43828	0,02067 9
PC O-38:7 PC O-16:1_22:6	1,354 7	0,43794	0,01487 8
TG 56:6 TG 16:0_18:1_22:5	1,354 6	0,43784	0,02781 3
PE P-38:6 PE P-16:0_22:6	1,345 1	0,42772	0,02326 3
PC O-38:7	1,343 8	0,42627	0,01665 2
TG 56:5 TG 18:0_18:1_20:4	1,332 1	0,41369	0,01688 2
CAR 26:1	1,327	0,40819	0,01504 1
SM 34:0;2O SM 10:0;2O/24:0	1,315 6	0,39569	0,04283 9
CAR 11:1	1,311 8	0,39155	0,01788 4
TG 53:0 TG 14:0_15:0_24:0	1,301 4	0,38007	0,00404 9
TG 58:0 TG 16:0_17:0_25:0	1,282	0,35834	0,03039 3
TG 56:0 TG 15:0_16:0_25:0	1,272 6	0,34775	0,01026 9
PC 33:0 PC 16:0_17:0	1,260 7	0,33421	0,01992 2
TG 54:0 TG 16:0_18:0_20:0	1,260 6	0,33407	0,01933 7
TG 57:0 TG 16:0_17:0_24:0	1,236 9	0,30674	0,02440 9
TG 56:4 TG 18:0_18:1_20:3	1,228	0,29626	0,04197 1
SM 36:2;2O SM 16:1;2O/20:1	1,207 3	0,2718	0,03924

TABLE 5 LIST OF 63 OVEREXPRESSED LIPIDS IN >2L COMPARED TO 1L PATIENTS, BASED ON FOLD CHANGE (FC).

Table shows FC, log₂(FC) and p-value. DG= diacylglycerol; CAR= carnitine; PE = phosphatidylethanolamine; TG = triacylglycerol; Cer = ceramide; SM = sphingomyelin.

LIPID SPECIES	FC	log ₂ (FC)	p value
PC O-39:3	0,52201	-0,93784	0,018891

ST 29:1;O;S	0,54664	-0,87134	0,013015
PC 36:5 PC 18:2_18:3	0,54818	-0,86728	0,027465
PC 36:4 PC 18:2_18:2	0,60335	-0,72894	0,020285
PC O-44:8	0,60795	-0,71797	0,043982
DG 29:4 DG 11:0_18:4	0,63116	-0,66393	0,015955
Hex2Cer 32:1;2O Hex2Cer 18:1;2O/14:0	0,66182	-0,59548	0,006116
SM 30:2;2O	0,6865	-0,54266	0,046993
LPC 18:2/0:0	0,70137	-0,51175	0,047975
DG 37:7	0,71118	-0,49172	0,033409
LPE 18:1	0,73527	-0,44365	0,048796
PC 37:2 PC 19:0_18:2	0,75556	-0,40438	0,045237
DG 30:6	0,79132	-0,33766	0,027953

TABLE 6 LIST OF 12 LIPIDS WITH DECREASED EXPRESSION IN >2L COMPARED TO 1L PATIENTS, RANKED BY FOLD CHANGE (FC).

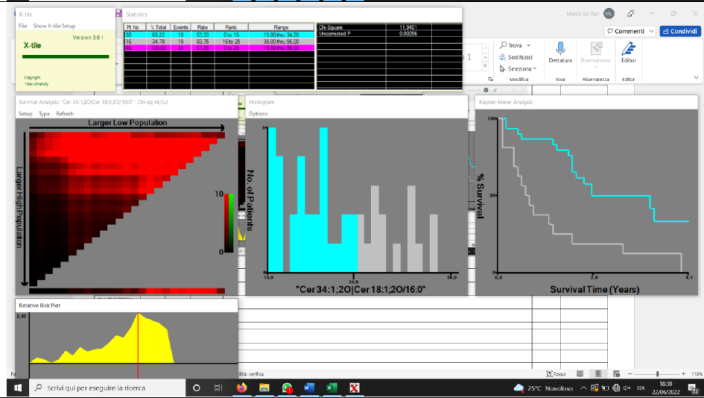
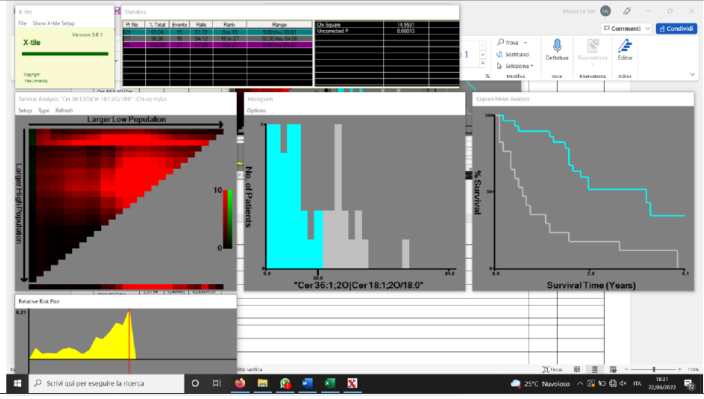
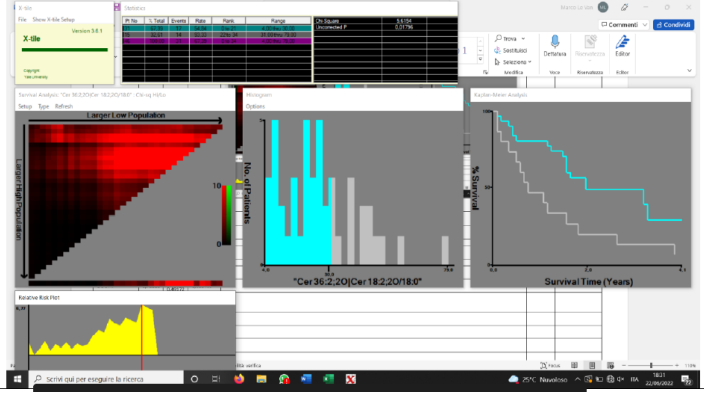
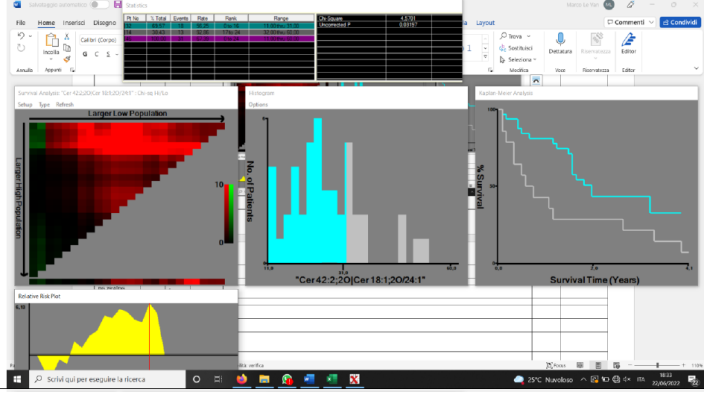
Table shows FC, log₂(FC) and p-value. DG= diacylglycerol; Hex2Cer= dihexosylceramide; LPC=lysophosphatidylcholine; LPE=lysophosphatidylethanolamine; PC: phosphatidylcholine; PE = phosphatidylethanolamine; SM = sphingomyelin; ST= sterols.

5.2.1 Investigating the Relationship Between Lipid Species and Prognosis: An Exploratory Analysis

We employed X-Tile to determine the optimal cut-off based on outcomes and to identify lipid species whose plasma levels correlated with the hazard risk for OS (154). Among the various dysregulated lipids identified, we observed that the plasma concentrations of certain lipid species increased in proportion to the risk of death, showing a significant association with OS when using the determined cut-off (Figure 6):

- **Cer 34:1;2O|Cer 18:1;2O/16:0**
- **Cer 36:1;2O|Cer 18:1;2O/18:0**
- **Cer 36:2;2O|Cer 18:2;2O/18:0**
- **Cer 42:2;2O|Cer 18:1;2O/24:1**
- **Cer 44:2;2O|Cer 20:1;2O/24:1**

Notably, Cer 42:2;2O|Cer 18:1;2O/24:1 was the same lipid that was part of the previously identified 3-lipid signature (149). Plasma levels of the under-expressed lipids, however, did not show a proportional correlation with OS.

Cer 34:1:20 Cer 18:1:20 16:0	1,3949	0,48011	0,00079806		7.47	0.00056
Cer 36:1:20 Cer 18:1:20 18:0	1,4895	0,57484	0,010263		8.21	0.00013
Cer 36:2:20 Cer 18:2:20 18:0	1,6565	0,72814	0,0041042		6.77	0.0179
Cer 42:2:20 Cer 18:1:20 24:1	1,3712	0,45543	0,0035211		6.13	0.0319

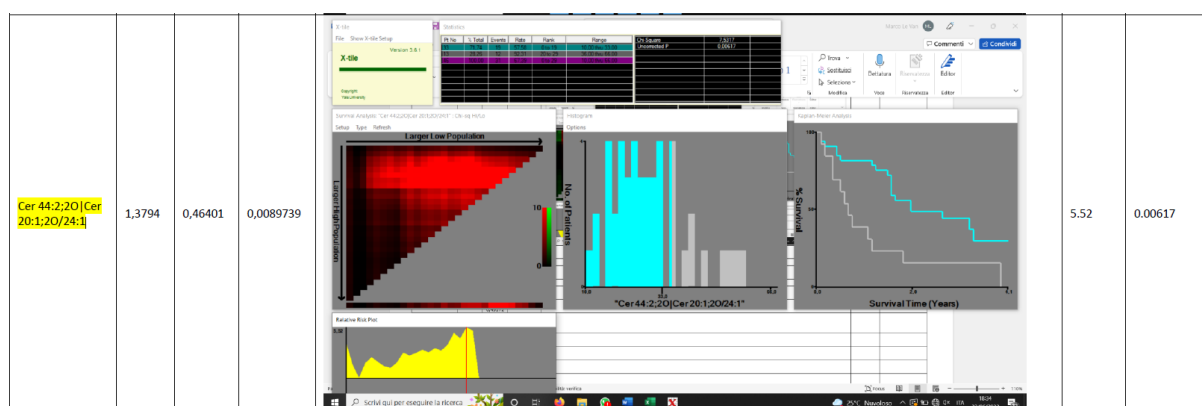


FIGURE 6 LIPID SPECIES WHOSE PLASMA VALUES INCREASED PROPORTIONALLY WITH THE HAZARD RISK OF OS

5.2.2 Relationship Between Cer 36:1;20|Cer 18:1;20/18:0 and Clinical Outcomes

The five ceramide species identified in the discovery analysis were further assessed through multivariate analysis to account for the potential confounding effects of key prognostic variables, particularly the treatment line. This initial analysis revealed differential lipid expression in patients from the >2L cohort compared to the 1L cohort. As patients in the >2L group generally had poorer survival outcomes than those in the 1L group, it was plausible that the lipid species found to be elevated in the >2L cohort would correlate with OS. Among the ceramides identified, Cer 36:1;20|Cer 18:1;20/18:0 (as denoted in Figure 8) emerged as the only lipid significantly associated with prognosis in univariate analysis (Figure 9). This relationship remained statistically significant even after adjusting for baseline PSA levels and treatment line. Higher plasma levels of this ceramide were linked to a HR for OS of 3.3 (95% CI 1.4-7.8, $p = 0.007$) when compared to patients with lower values (Figure 10).

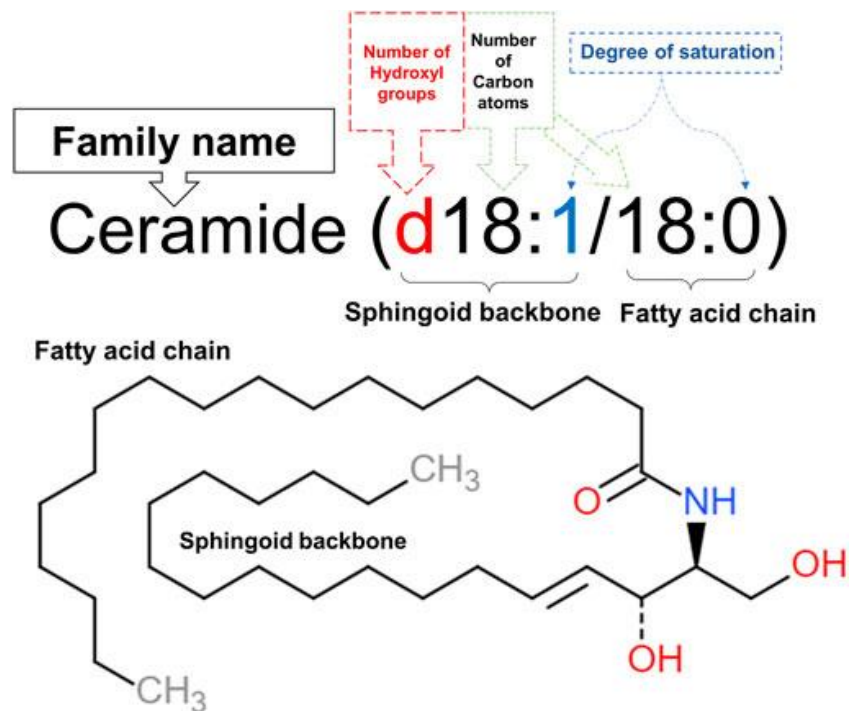


FIGURE 7 NOMENCLATURE OF C18:0 CERAMIDE

The first segment of the name (d18:1) refers to an 18-carbon chain with one double bond in its sphingoid backbone, along with two hydroxyl groups. This sphingosine chain is linked to a saturated fatty acid chain, denoted by the second part of the name (18:0), via an amide bond (155)..

The median OS was 6 months (95% CI, 2.1-9.9) for patients with low plasma levels, while it was 39 months (95% CI, 16.1-61.9) for those with high plasma levels. The plasma threshold for this differentiation was set at 30 ng/mL.

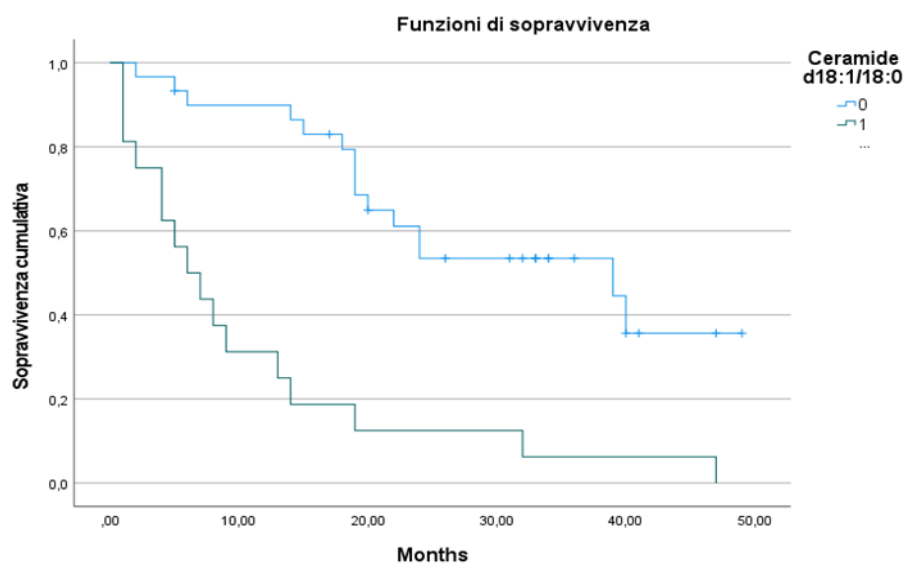


FIGURE 8 UNIVARIATE ANALYSIS OF CER 36:1;20 | CER 18:1;20/18:0

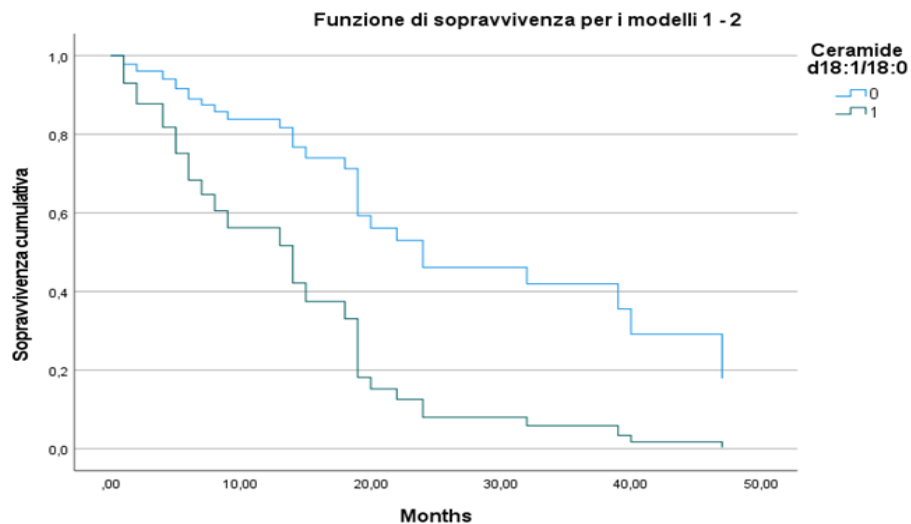


FIGURE 9 MULTIVARIATE ANALYSIS OF CER 36:1;20|CER 18:1;20/18:0

Notably, the subgroup analysis, despite lacking sufficient statistical power for significance, still indicated an unfavorable correlation between the levels of Cer 36:1;20|Cer 18:1;20/18:0 and patient survival, independent of the treatment line. In the 1L cohort, the Kaplan-Meier curve revealed a median survival of 14 months for patients with low ceramide levels, compared to 39 months for those with high ceramide levels (p -value = 0.098) (Figure 11).

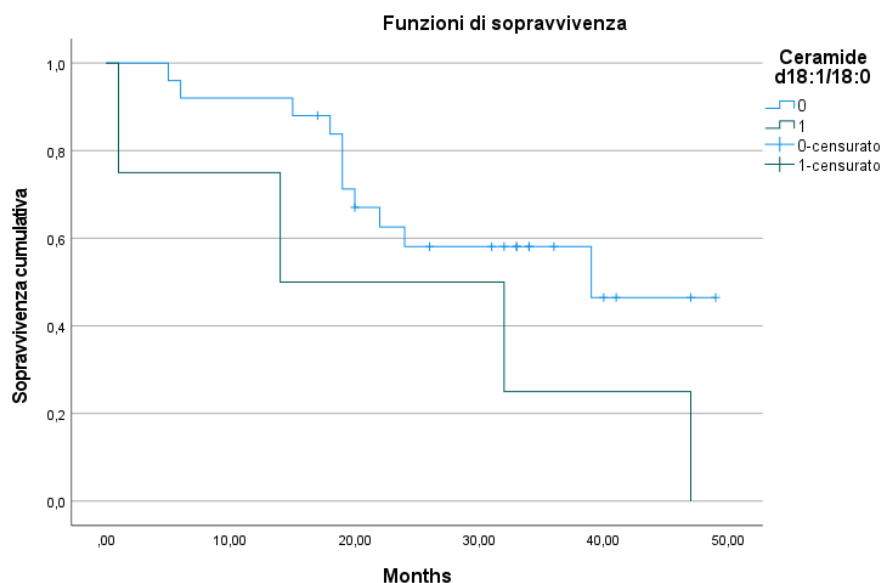


FIGURE 10 UNIVARIATE ANALYSIS OF CER 36:1;20|CER 18:1;20/18:0 IN 1L COHORT

In the >2L group, the Kaplan-Meier survival curve demonstrated a median survival of 5 months for patients with low ceramide levels, in contrast to 24 months for those with high ceramide levels (p-value = 0.025) (Figure 12).

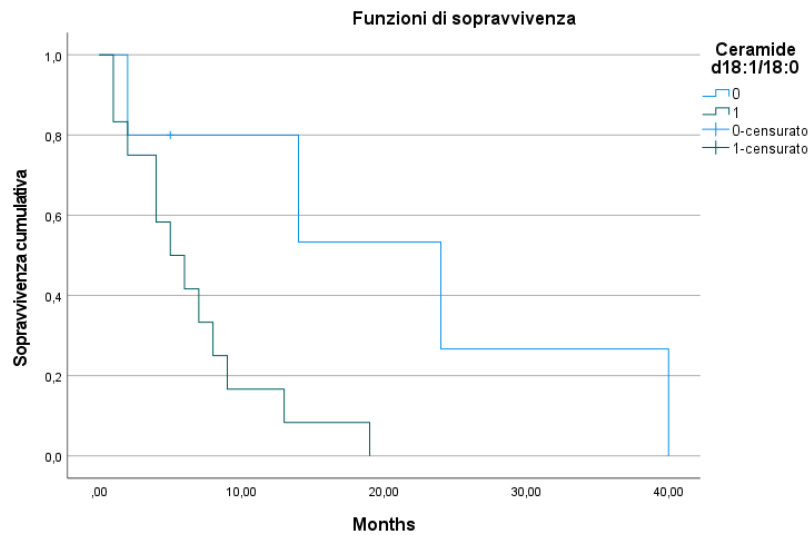


FIGURE 11 UNIVARIATE ANALYSIS OF CER 36:1;20 | CER 18:1;20/18:0 IN >2L COHORT

The graph presented in Figure 13 illustrates how the risk of death varies between populations based on different cut-off values of Cer 36:1;20 | Cer 18:1;20/18:0. For the cut-off chosen in this study (marked by the red line), the risk between the two cohorts peaks at 8.21 (p-value = 0.00013).

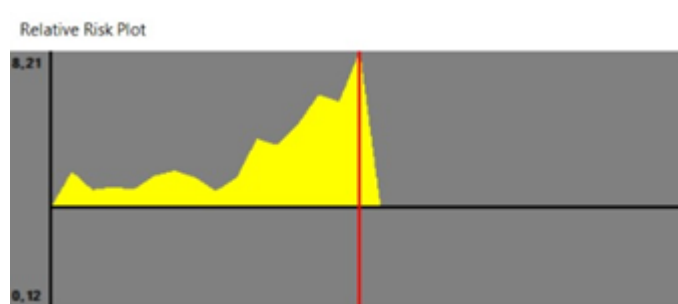


FIGURE 12 RELATIVE RISK PLOT OF CER 36:1;20 | CER 18:1;20/18:0

5.2.3 Correlation Between Cer 36:1;20|Cer 18:1;20/18:0 and PSA Treatment Response

Finally, the relationship between plasma levels of Cer 36:1;20|Cer 18:1;20/18:0 and treatment response was analyzed (Figure 14). Treatment response was defined as a PSA50, which is a reduction of 50% or more in PSA values. The waterfall plot displayed below illustrates this: patients with elevated ceramide levels (>30 ng/mL) are marked in red, while those with lower ceramide levels (< 30 ng/mL) are indicated in blue.

In total, 19 out of 28 patients (67.9%) with low ceramide levels experienced a 50% reduction in PSA, whereas only 6 out of 14 patients (42.9%) with high ceramide levels achieved a similar decrease in PSA. Among the patients who started first-line ARSi treatment, 1 of 2 patients with elevated ceramide levels reached PSA50. For those who began first-line docetaxel treatment with high Cer 36:1;20|Cer 18:1;20/18:0 levels, both patients (2 of 2) achieved PSA50

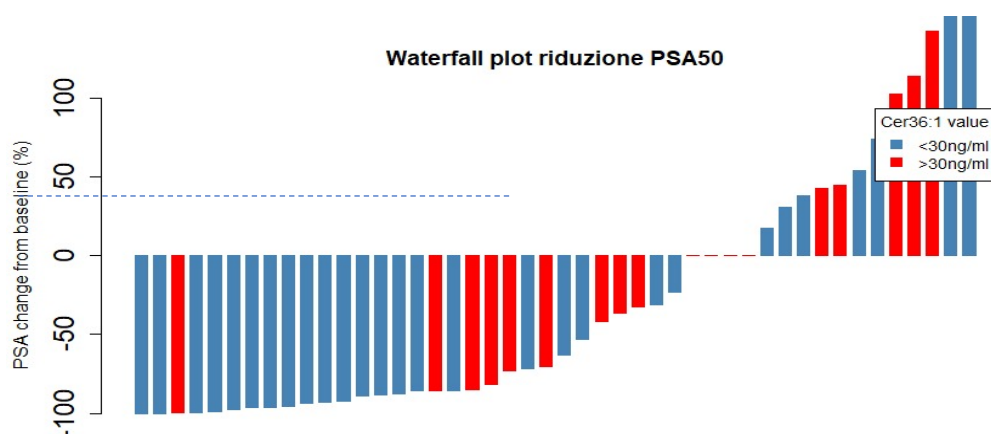


FIGURE 13 WATERFALL PLOT OF PSA RESPONSE ACCORDING TO CERAMIDE VALUES

5.3 Three-lipid signature and association with clinical outcome

We also investigated the link between clinical outcomes and three lipids from the previously established 3-lipid signature: ceramide 42:2;20|Cer 18:1;20/24:1, sphingomyelin 34:2;20|SM 18:2;20/16:0, and phosphatidylcholine 32:0|PC 16:0_16:0 (as described in paragraph 1.4.2) (148,149). Receiver operating characteristic (ROC) curves were generated for

overall survival (OS) using these lipids. The ROC curve for ceramide d18:1/24:1 (Figure 15) yielded an area under the curve (AUC) of 0.771 (95% CI, 0.623–0.894). For sphingomyelin d18:2;20/16:0 (Figure 16), the AUC was 0.577 (95% CI, 0.423-0.747). The ROC curve for phosphatidylcholine 16:0/16:0 (Figure 17) showed an AUC of 0.637 (95% CI, 0.467-0.775).

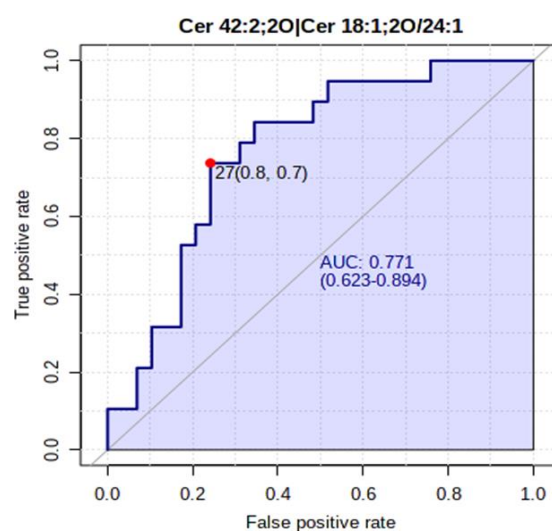


FIGURE 14 ROC CURVE OF CER 42:2;20 | CER 18:1;20/24:1, WITH OPTIMAL CUT-OFF POINT

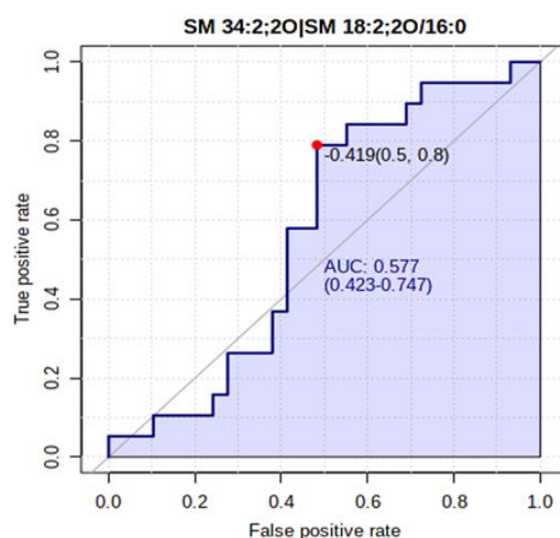


FIGURE 15 ROC CURVE OF SM 34:2;20 | SM 18:2;20/16:0 WITH OPTIMAL CUT-OFF POINT

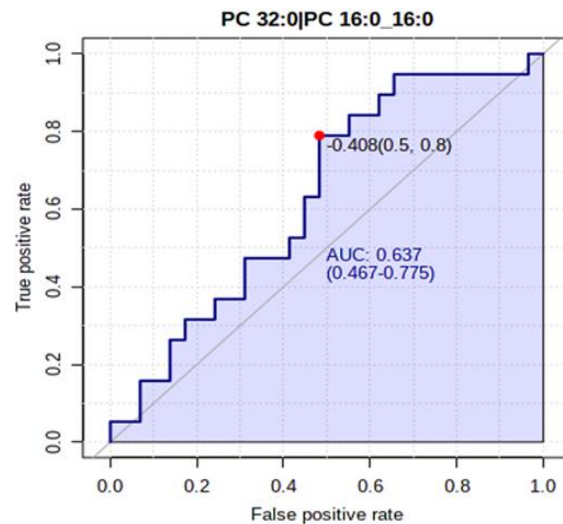


FIGURE 16 ROC CURVE OF PC 32:0|PC 16:0_16:0 WITH OPTIMAL CUT-OFF POINT

5.3.1 Association of 3-lipid signature with OS

Kaplan-Meier survival curves were generated to examine the relationship between the lipids in the 3-lipid signature and OS. In the univariate analysis, Cer 42:2;20|Cer 18:1;20/24:1 revealed that patients with elevated ceramide levels had poorer survival outcomes compared to those with lower levels (Figure 18). The median OS was 7 months for patients with high ceramide levels (95% CI, 17.7-30.3), while those with lower levels had a median OS of 24 months (95% CI, 1.5-12.5). A ceramide concentration of 31 ng/mL was used as the threshold to differentiate between high and low levels. The log-rank test confirmed this finding as statistically significant (p-value = 0.025).

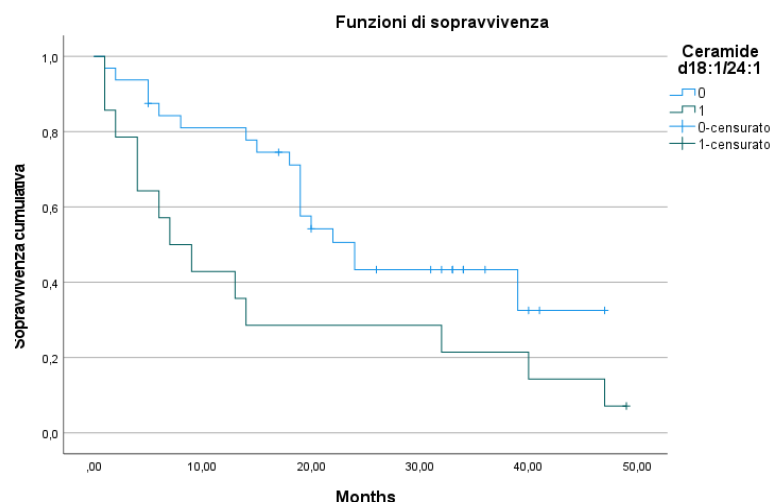


FIGURE 17 UNIVARIATE ANALYSIS FOR OS OF CER 42:2;20|CER 18:1;20/24:1

Nevertheless, the multivariate analysis, which accounted for treatment line and baseline PSA, did not reveal a statistically significant correlation with overall survival (HR = 1.4, 95% CI 0.7-3.1, p-value = 0.347) (Figure 19).

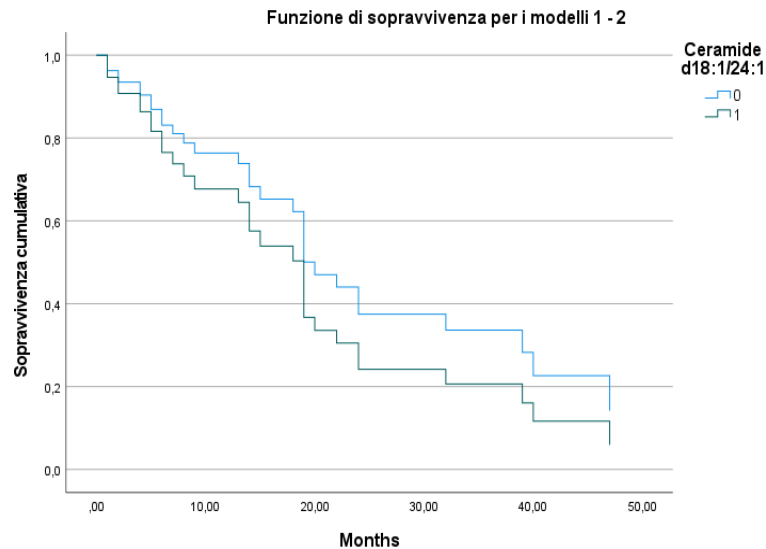


FIGURE 18 MULTIVARIATE ANALYSIS FOR OS OF CER 42:2;20 | CER 18:1;20/24:1

No correlation was found between plasma levels of sphingomyelin 34:2;20 | SM 18:2;20/16:0 and overall survival (Figure 20). Patients with high sphingomyelin levels had a median OS of 22 months (95% CI, 17.6-20.4), while those with low levels had a median OS of 19 months (95% CI, 14.8-23.2). The threshold used to categorize high and low sphingomyelin levels was 5.5 ng/mL, but the t-test did not yield a statistically significant result (p-value = 0.982).

Furthermore, multivariate analysis of sphingomyelin 34:2;20 | SM 18:2;20/16:0, after adjusting for treatment line and baseline PSA, confirmed no significant association with OS (HR = 0.6, 95% CI, 0.2-1.4, p-value = 0.224) (Figure 21).

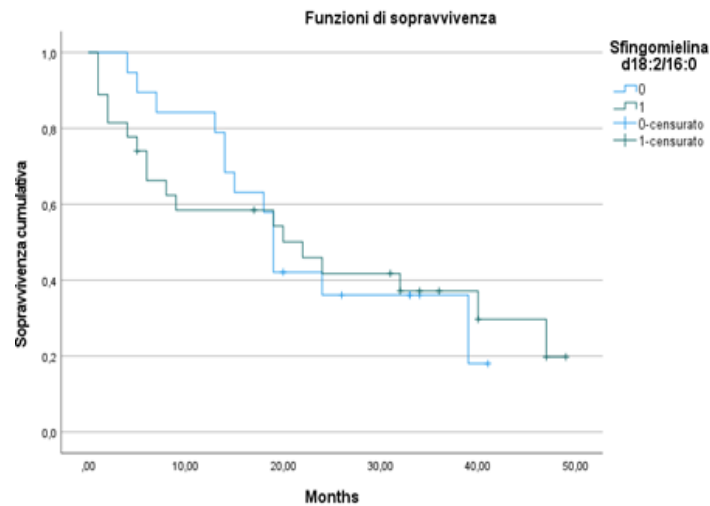


FIGURE 19 UNIVARIATE ANALYSIS FOR OS OF SM 34:2;20|SM 18:2;20/16:0

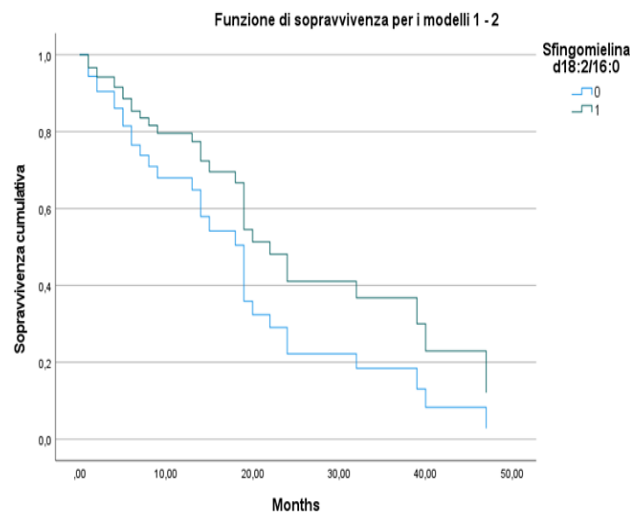


FIGURE 20 MULTIVARIATE ANALYSIS FOR OS OF SM 34:2;20|SM 18:2;20/16:0

We also found no statistically significant relationship between plasma levels of phosphatidylcholine 32:0|PC 16:0_16:0 and overall survival (Figure 22). The median OS was 32 months in patients with elevated phosphatidylcholine levels (95% CI, 9.5-54.5) and 19 months in those with lower levels (95% CI, 14.8-23.2). The cut-off value for determining high or low phosphatidylcholine levels was set at 27.5 ng/mL. The t-test result was not statistically significant (p-value = 0.371). Additionally, the multivariate analysis, adjusted for treatment

line and baseline PSA, further confirmed the lack of a significant association with overall survival (HR = 1.9, 95% CI 0.8-4.2, p-value = 0.102) (Figure 23).

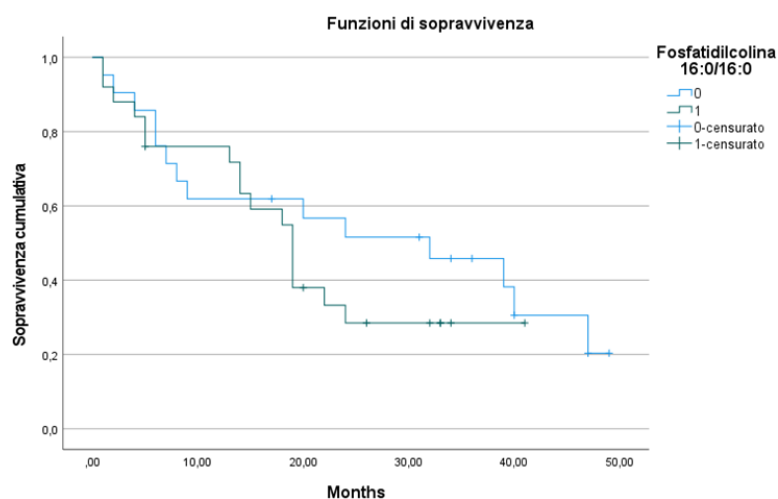


FIGURE 21 UNIVARIATE ANALYSIS FOR OS OF PC 32:0|PC 16:0_16:0

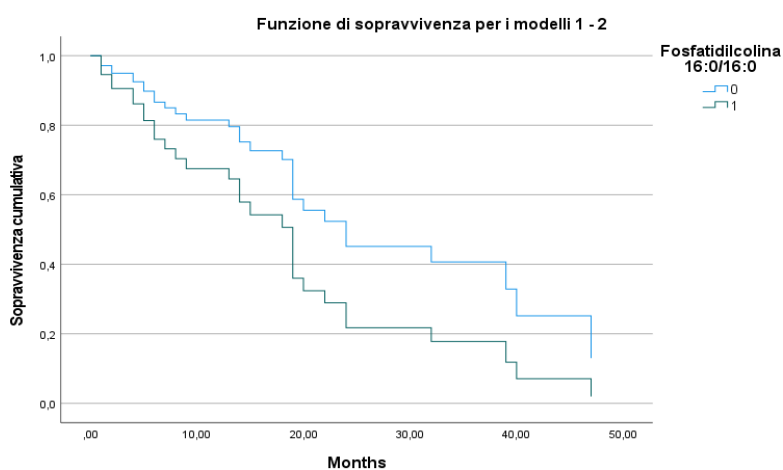


FIGURE 22 MULTIVARIATE ANALYSIS FOR OS OF PC 32:0|PC 16:0_16:0

6 DISCUSSION

A variety of prognostic and predictive factors have been investigated to help identify PCa patients at higher risk for progression or death. In the context of mCRPC, alterations in DNA repair genes, such as BRCA1 and BRCA2, have been established as critical biomarkers to assist in treatment decisions (55). Nevertheless, there remains a substantial clinical gap in identifying further prognostic and predictive factors. Various investigations have been carried out to examine the concentrations of particular lipid species in patients with mCRPC, aiming to assess their possible role as prognostic and predictive indicators.

Several studies by an Australian research group (141-143) have provided key insights into lipid species as potential biomarkers in mCRPC. The first study analyzed lipidomic profiles in 96 mCRPC patients, identifying two subgroups with distinct survival outcomes. A three-lipid prognostic signature, consisting of ceramide d18:1/24:1, sphingomyelin d18:2/16:0, and phosphatidylcholine 16:0/16:0, was developed, and it was successfully validated in a cohort of 63 patients, identifying those with poorer survival (HR 4.78, 95% CI 2.06–11.1). Additionally, 19 of 46 prognostic lipids, including various ceramides and sphingomyelins, were further validated.

In a subsequent study, the researchers analyzed lipid profiles from pre-treatment plasma samples of patients with localized PCa (N = 389), mHSPC (N = 44), and mCRPC (N = 137). Elevated ceramide levels were linked to metastatic relapse in localized PCa (HR 5.80, 95% CI 3.04–11.1), earlier failure of testosterone suppression in mHSPC (HR 3.70, 95% CI 1.37–10.0), and reduced overall survival (HR 2.54, 95% CI 1.73–3.72) in mCRPC. Importantly, the lipid profiles' prognostic significance in localized PCa was independent of clinicopathological and metabolic factors, and the three-lipid signature was confirmed in the mCRPC cohort (HR 2.39, 95% CI 1.63–3.51).

In the third study conducted by the Australian research team, plasma lipidomic profiling and cell-free DNA (cfDNA) sequencing were carried out on 106 men with mCRPC beginning treatment with docetaxel, cabazitaxel, abiraterone, or enzalutamide (discovery cohort), as well as 94 mCRPC patients starting docetaxel (validation cohort) (143). The three-lipid signature was found to be associated with reduced OS in both groups. Increased levels of sphingolipids, particularly ceramides, were connected to genetic mutations in AR, TP53, RB1,

PI3K, and AVPC in mCRPC. Additionally, the combination of lipid and genetic alterations was predictive of worse outcomes.

Building on these lipidomic findings in prostate cancer, our study aimed to examine the lipidomic profiles of mCRPC patients in order to identify lipid species that could serve as new prognostic and predictive biomarkers. The study included 48 mCRPC patients, divided into two groups: those starting first-line treatment (1L cohort, n=29) and those who had already received two or more lines of treatment (≥ 2 L cohort, n=19).

A total of 789 lipids were examined and identified, and a comparison was made between the lipid levels in the 1L and >2 L cohorts. This initial analysis revealed 63 lipids that were overexpressed (fold change > 1.2 , p-value < 0.05) in the >2 L cohort compared to the 1L cohort, and 12 lipids that were underexpressed (fold change < 0.75 , p-value < 0.05). Among the overexpressed lipids, ceramide d18:1/24:1, which was part of the previously established three-lipid signature (142), stood out.

Among the lipids that were dysregulated, we observed that specific ceramides, including Cer 34:1;2O|Cer 18:1;2O/16:0, Cer 36:1;2O|Cer 18:1;2O/18:0, Cer 36:2;2O|Cer 18:2;2O/18:0, Cer 42:2;2O|Cer 18:1;2O/24:1, and Cer 44:2;2O|Cer 20:1;2O/24:1, were strongly linked to a higher risk of death from any cause and were significantly associated with OS when an appropriate cutoff point was applied. On the other hand, the under-expressed lipids did not show a clear proportional relationship with OS.

Among the identified lipids, Ceramide 36:1;2O|Cer 18:1;2O/18:0 was the only one to retain statistical significance after multivariate analysis, which accounted for baseline PSA levels and treatment line. While this ceramide was not part of the three-lipid signature, it had been identified as a prognostic lipid in a study by Lin et al. (141). Patients with elevated levels of Cer 18:1/18:0 had a significantly higher risk of mortality compared to those with lower levels (HR=3.3, 95% CI 1.4–7.8, p-value = 0.007), with a median OS of 6 months (95% CI, 2.1–9.9) versus 39 months (95% CI, 16.1–61.9) for the lower levels. Subgroup analysis confirmed this unfavorable correlation between Cer 18:1/18:0 levels and survival, regardless of the treatment line.

We also investigated the connection between Cer 18:1/18:0 levels and treatment response. Among patients with higher ceramide levels, 42.9% achieved a PSA50 response, while 67.9% of those with lower ceramide levels did. However, due to patient diversity and the small sample size, further studies are needed to better evaluate the potential predictive role of this

ceramide. Concerning the three-lipid signature, we found no correlation between sphingomyelin d18:2/16:0 or phosphatidylcholine 16:0/16:0 and OS in our mCRPC cohort. Although ceramide d18:1/24:1 showed a significant association with OS in univariate analysis, this link was not sustained in multivariate analysis.

These results align with the idea that altered lipid metabolism and elevated circulating ceramide levels are linked to poorer outcomes in patients at various stages of PCa. While ceramides are typically seen as having anti-tumor properties by inducing senescence and inhibiting cancer cell growth, some research indicates that their impact depends on the specific context and the downstream effectors involved, which can either support or hinder tumor growth (150).

Ceramides can be categorized by the length of their acyl chain into long-chain (C14:0–C20:0), very-long-chain (C22:0–C26:0), and ultra-long-chain (>26 carbons) ceramides. The biological effects of ceramides vary based on the length of their chains. In our study, both long-chain and very-long-chain ceramides were found to be prognostic, with elevated levels correlating with worse prognosis.

The enzyme acid ceramidase (AC) appears to play a key role in the distinct functions of ceramides. AC significantly altered the expression of different ceramide species without changing their total levels. In DU145 cells overexpressing AC, we detected reduced levels of long-chain ceramides (C14-C20) and increased levels of very-long-chain ceramides (C24, C24:1). This shift was associated with higher cellular proliferation, migration, and tumorigenicity in vivo, effects that were reversed by either pharmacological or genetic inhibition of AC (151,152).

Long-chain ceramides may enhance the aggressiveness of PCa through their metabolite, sphingosine-1-phosphate (S1P). S1P is generated through enzymatic processes involving AC and sphingosine kinase (SPHK), both of which are overexpressed and more active in prostate cancer (PCa) cells (153,154). Increased SPHK expression in localized PCa has been linked to disease progression (142). S1P plays a crucial role in promoting cancer cell proliferation, survival, and metastasis, and it also affects lymphocyte movement by binding to specific S1P receptors on both immune and cancer cells (155). In mice lacking SPNS2, the transporter responsible for S1P in lymphoid tissues, metastatic colonization is significantly reduced (156).

In addition, the enhanced ceramide-S1P signaling pathway may contribute to resistance to ARSi, particularly when there is AR gain. In patients with mCRPC, AR gain combined with upregulation of sphingolipid-related genes was linked to shorter durations of ARSi treatment (151). Inhibiting sphingosine kinase 2 (SPHK2), one of the isoforms responsible for generating S1P, effectively decreased CRPC cell proliferation and xenograft tumor growth by targeting both the AR and the oncogene MYC (158). In vitro studies have shown that resistance to enzalutamide in androgen-independent cells could be reversed by using SPHK inhibitors (157). Clinical trials are currently investigating SPHK inhibitors, such as ABC294640, for cancer treatment. ABC294640 has completed a Phase I trial for advanced solid tumors (NCT01488513) and is undergoing Phase IIA trials for cholangiocarcinoma (NCT03377179). Furthermore, ceramides act as activators of phospholipase A2 (PLA2), an enzyme that releases arachidonic acid, which is subsequently transformed into prostaglandins. These molecules are involved in processes such as inflammation, immune response, and the regulation of tumor growth. Increased prostaglandin levels, including PGE2, are associated with enhanced PCa cell proliferation and invasion. This effect can be reversed by the use of cyclooxygenase (COX) inhibitors, indicating the role of PGE2 in the progression of PCa (160,161). Altered ceramide metabolism in PCa can also be affected by manipulating the metabolic environment of the host. Diets high in fat have been shown to increase circulating ceramide levels (162), which may promote inflammation and metastasis via S1P signaling, as observed in a breast cancer mouse model (163). Notably, this metabolic disruption can be corrected with pharmacological interventions. Studies in cardiovascular diseases and obesity have demonstrated that elevated ceramide levels can be reduced using cholesterol-lowering drugs, such as statins and PCSK9 inhibitors (164,165), or through physical activity (166). Overall, targeting sphingolipid metabolism, whether in the tumor or in the host, represents a promising therapeutic strategy that could enhance outcomes for PCa patients.

7 CONCLUSIONS

In summary, we examined the prognostic and predictive value of lipid species in mCRPC patients using an untargeted lipidomic approach with mass spectrometry and liquid chromatography. Our findings confirmed that long-chain and very-long-chain ceramides are

prognostic markers, with ceramide 18:1;18:0 showing independent prognostic value related OS. The three-lipid signature did not show significant prognostic value, except for ceramide d18:1/24:1, which was associated with OS in univariate analysis. Further research is needed to validate ceramide 18:1;18:0 and explore the predictive role of long-chain ceramides in clinical settings..

8 LIST OF PUBLICATIONS

- 1 Caffo O, Basso U, Cattrini C,**Mennitto A**, ... et al. Development and validation of prognostic model for metastatic castration-resistant prostate cancer patients treated with first-line abiraterone or enzalutamide. November 2024. Clinical Genitourinary Cancer. DOI:[10.1016/j.clgc.2024.102265](https://doi.org/10.1016/j.clgc.2024.102265)
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