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LIPIDOMIC PROFILING IN PATIENTS WITH METASTATIC CASTRATION-RESISTANT PROSTATE CANCER (mCRPC)

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1 INTRODUCTION

1.1 Prostate cancer as a health problem

Prostate cancer (PCa) represents the first neoplasm in Italy by incidence, the third by mortality and 18.5% of all tumors diagnosed in men. In 2020, according to the AIRTUM association, around 36,000 new diagnoses were estimated and there are currently 563,960 men in Italy with a diagnosis of prostate cancer. About 7,200 deaths have been estimated for 2021 (1). Also in the USA, PCa is the most frequent neoplasm in males and the second leading cause of death because of cancer, with 34,500 people dying of PCa (2).

The 5-year overall survival (OS) of patients with prostate cancer, irrespective of stage at diagnosis, is around 91%. It has also been observed that OS of patients with prostate cancer is constantly improving. The main factor related to this improvement is the diagnostic anticipation at earlier stages and the progressive widespread of PSA screening. However, if the tumor is diagnosed in the metastatic phase, 5-year OS drops drastically to about 30%.

Several randomized trials demonstrated that both chemotherapy and androgen-receptor signaling inhibitors (ARSi) can provide a significant survival benefit in metastatic (m) PCa. However, the real-world survival outcomes of patients with mPCa remain poor with a median survival of about 30 months (3).

In 2020, we performed an analysis of the U.S. Surveillance, Epidemiology, and End Results (SEER) database to assess survival improvements in patients with mPCa over time (3). As shown in **Figure 1**, we demonstrated that survival has not changed substantially in recent year, despite the advent of several new therapeutic agents. Although health insurance policies might have affected the extensive use of drugs in patients managed in the U.S., our analysis highlights that mPCa remains an incurable disease, characterized by poor prognosis.

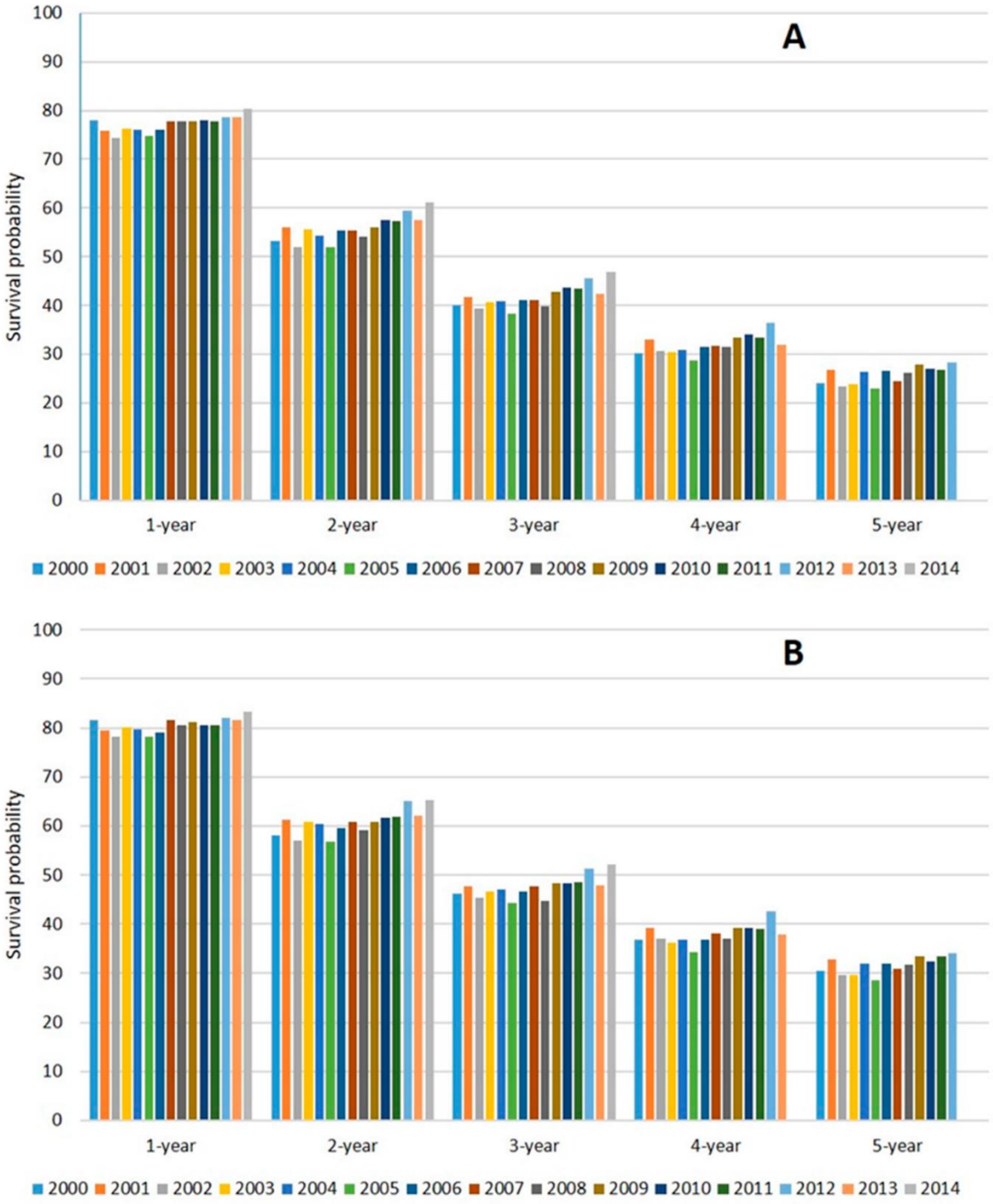


FIGURE 1 AGE-STANDARDIZED 1- TO 5-YEAR OS (A) AND CSS (B) OF PATIENTS ACCORDING TO YEAR OF DIAGNOSIS.

1.2 Metastatic castration-resistant prostate cancer (mCRPC)

The initial systemic treatment of both metastatic and nonmetastatic patients is represented by androgen-deprivation therapy (ADT). ADT can be obtained by the use of Luteinizing Hormone Releasing Hormone (LH-RH) analogous, agonists or antagonist, or by surgical castration. The duration of response to ADT can last from months to many years, and this disease stage is known as hormone-sensitive prostate cancer (HSPC). The long-term exposure to ADT eventually results in disease progression despite castration, a clinical condition known as castration-resistant prostate cancer (CRPC).

Metastatic castration-resistant prostate cancer (mCRPC) is defined by biochemical, radiographic or clinical progression, despite castration serum testosterone levels (< 50 ng/mL or <1.7 mmol/L). Biochemical disease progression should be documented by three consecutive PSA elevations at least one week apart resulting in two 50% elevations from the lowest value over time (nadir) and a PSA > 2 ng/ mL (4). Radiographic progression requires the appearance of two or more new bone lesions on bone scintigraphy or a soft tissue lesion according to the RECIST criteria (Response Evaluation Criteria in Solid Tumours) (5). The choice of treatment for patients mCRPC depends on several factors, including patient age, performance status, concurrent comorbidities, eligibility to chemotherapy, drug interactions, previous treatments for metastatic HSPC, nonmetastatic CRPC and mCRPC, quality of response to previous treatments, cross-resistance between drugs, specific genetic alterations (microsatellite instability/mismatch repair defects or DNA repair deficiencies), local drugs approvals and reimbursement.

Agents approved for the treatment of mCRPC in Europe are: docetaxel, abiraterone acetate plus prednisone, enzalutamide, cabazitaxel, radium-223 and olaparib (in patients with *BRCA* mutations).

1.2.1 First-line treatment for mCRPC

Docetaxel (TAX-327 trial), abiraterone acetate (COU-AA-302 trial) and enzalutamide (PREVAIL trial) have all shown a significant survival benefit as first-line therapies for mCRPC, and are considered standard options as initial therapy (6-8) **Table 1.** The current interpretation of these trials is challenging, as enrolled patients had mainly received ADT as prior therapy.

This is not an updated scenario, in which patients are used to receive ARSi or chemotherapy in addition to ADT for mHSPC or nmCRPC. It is not known to what extent the clinical benefit observed in the phase 3 trials of mCRPC would be observed nowadays after treatment with these agents in prior settings. Potential cross-resistance between agents is not fully understood and could significantly affect patients' outcomes. The current median OS from first-line therapy is likely lower than that reported in the pivotal COU-AA-302 and PREVAIL trials, since patients are now experiencing longer time in the mHSPC or nmCRPC stages of the disease.

No formal randomized comparison between chemotherapy and ARSi is currently available in the first-line setting of mCRPC. The marked difference 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) trials suggests that different patient populations were investigated, and cross-trial efficacy comparisons are inappropriate. In a large, real-world, observational study, patients treated with first-line ARSi experienced longer times to progression than those treated with docetaxel, but there was no difference in terms of OS (9); additionally, patients with worse baseline prognostic features were more likely to receive first-line docetaxel. Similar results were observed in a sub-analysis of the prospective PROREPAIR-B study (10). The longer PFS observed in patients treated with ARSi compared to those treated with chemotherapy might be related to the different exposure to treatment, which is continuous with ARSi and limited with docetaxel. Some retrospective data suggests that a short duration of response to prior treatment with ADT predicts for poor response to ARSi (11), whereas docetaxel seems to retain its efficacy in patients experiencing early castration-resistance (12). Of note, no difference in survival was observed when comparing docetaxel with cabazitaxel as first-line mCRPC therapy in the FIRSTANA trial, and cabazitaxel seemed to be better tolerated than docetaxel at the dosage of 20 mg/m2 (13). However, the trial was designed to demonstrate the superiority in terms of OS – not non-inferiority – of cabazitaxel over docetaxel, thus cabazitaxel was not approved as a first-line option for mCRPC.

It remains unclear which might be the best treatment for mCRPC patients who have received prior treatment with docetaxel for mHSPC (Figure 1a). Data from the GETUG-AFU-15 trial showed that the benefit from docetaxel rechallange in mCRPC is limited in patients who have previously received docetaxel in mHSPC, as assessed by a PSA decline ≥50% obtained

only in 14% of patients (14). Conversely, abiraterone or enzalutamide seem to retain efficacy in these patients. However, according to a phase II study, cabazitaxel showed a greater clinical benefit compared to ARSi (80% versus 62%, P = 0.039) in patients with ARSi-naive mCRPC and poor prognosis features (presence of liver metastases, progression to mCRPC after <12 months of ADT, or ≥4 of 6 clinical criteria), who were allowed to receive docetaxel in mHSPC or mCRPC (15). Patients who achieved stable disease for longer than 12 weeks were 75% for cabazitaxel and 56% for ARSi (p = 0.083), whereas there was no difference in terms of radiographic response rate or confirmed PSA decline ≥50%.

Chemotherapy appears to be a reasonable option for the first-line mCRPC treatment of eligible patients who have previously received ARSi in mHSPC setting (Figure 1b). Similarly, chemotherapy appears to be an appropriate option for patients with nmCRPC who are progressing during treatment with ARSi in that setting. Data suggest that cross-resistance may occur between different ARSi and the sequence including two sequential ARSi is often discouraged. However, clinical data of cross-resistance between ARSi and chemotherapy have also been reported (16). The analyses from the SPARTAN trial in nmCRPC, where up to 80% of patients received abiraterone at progression, reported a benefit in PFS2 for patients in the apalutamide -> abiraterone over the placebo -> abiraterone sequence. However, these results must be interpreted with caution, since most of the benefit is likely to be driven by the superior PFS of apalutamide over placebo in first-line nmCRPC, and outcome analyses restricted to patients that received second-line therapy in mCRPC setting are lacking.

Regarding the choice of first-line ARSi, a phase II crossover trial investigated the best sequence between abiraterone acetate -> enzalutamide (group A) vs enzalutamide -> abiraterone acetate (group B) for the first-line treatment of 202 patients with newly-diagnosed mCRPC (17). Time to second PSA progression was longer in group A than in group B (median 19.3 vs 15.2 months, HR 0.66, 95% CI 0.45–0.97); PSA responses to second-line therapy were seen in 26 (36%) of 73 patients for enzalutamide and 3 (4%) of 75 for abiraterone ($\chi 2$ p<0.0001). A trend for increased OS in group A compared to group B was also observed (28.8 vs 24.7 months, HR 0.79, 95% CI 0.54–1.16, p=0.23). A recent systematic review and meta-analysis of nonrandomized retrospective and prospective studies supports the notion that a sequencing strategy of abiraterone acetate followed by enzalutamide would be the most appropriate option to maximize the benefit of treatments in mCRPC, regardless of the previous use of docetaxel (18). However, a recent retrospective study analyzed the outcomes

of 3174 patients with chemotherapy-naive mCRPC treated with first-line enzalutamide or abiraterone acetate, achieving opposite results (19). Approximately half of patients in these cohorts received one line of treatment only, of these about a half stopped treatment without receiving any other active treatment. Globally, about one-quarter of patients crossed over from a first-line ARSi to receive the alternative ARSi; 23% (n = 282) crossed over from enzalutamide to abiraterone, and 26% (n = 504) crossed over from abiraterone to enzalutamide. By analyzing the entire population of patients who received a first-line treatment, those who received enzalutamide had significantly better OS compared to those who were treated with abiraterone (HR 0.84, 95% CI 0.76-0.94). For patients who remained on first line-therapy only, enzalutamide-treated patients had improved OS versus abiraterone-treated patients (HR 0.71, 95% CI 0.62-0.82). In addition, enzalutamide-treated patients who crossed over to abiraterone had a comparable OS compared to abiraterone-treated patients who crossed over to enzalutamide (HR 0.91, 95% CI 0.74-1.13). An indirect comparison was performed using data from the phase 3 trials and it did not identify a statistically significant difference in OS between abiraterone and enzalutamide both pre- and post-chemotherapy for mCRPC (20). However, the authors found that enzalutamide may better outperform control arms in terms of time to PSA progression, radiographic PFS, and PSA response rate.

Overall, these data highlights a methodological issue: the entire population of patients who start a first-line treatment should be analyzed to determine the best first-line approach in a sequencing perspective, in order to avoid a selection bias. The outcome of patients who only receive a first-line treatment can significantly affect the final results, and sequencing analyses should not be only restricted to patients who receive two or more lines. In conclusion, randomized studies with a greater sample size are needed to understand whether the first-line choice between enzalutamide or abiraterone would significantly affect the outcome of patients with mCRPC. The different toxicity profile of abiraterone and enzalutamide may assist during the treatment selection in some men with mCRPC, although they are both generally well tolerated and safe in the vast majority of patients. Chemotherapy remains a valid treatment option and more data are still needed to adequately compare the outcomes of patients treated with ARSi vs chemotherapy.

TABLE 1 PROSPECTIVE RANDOMIZED CLINICAL TRIALS IN MCRPC.

Setting	Name of the trial	Population	Exp arm	Control arm	N Exp/ Cont	Primary Endpoint	FU (mo)	mOS (mo) Exp/Contr	HR (95% CI)	Ref.
1st line mCRPC	**TAX 327**	With or without symptoms	Doce + P	Mitoxantrone + P	335/337	OS	NA	19.2/16.3	0.79 (0.67-0.93)	(8)
	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)	(6)
	PREVAIL	A/midly symptomatic pre-doce	Enza + ADT	Placebo + ADT	872/845	rPFS, OS	69	36/31	0.83 (0.75-0.93)	(21)
	IMPACT	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)	(22)
	IPAtential150	A/midly symptomatic	AA + P + ipataserti b	AA + P + placebo	547/554	(bio)rPFS	19	NE/NE	NE	(23)
≥2nd 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)	(24)
	TROPIC	Post-doce	Cabazitaxel + P	Mitoxantrone + P	378/377	OS	25.5	NA/NA	0.72 (0.61-0.84)	(25)
	AFFIRM	Post-doce	Enza	Placebo	800/399	OS	14.4	18.4/13.6	0.63 (0.53-0.75)	(26)
	ALSYMPCA	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)	(27)
	CARD	Post-doce and post-ARSi	Cabazitaxel	AA+P/Enza	129/126	IPFS	9.2	13.6/11	0.64 (0.46-0.89)	(28)
	PROFOUND	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)*	(29)
	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)	(30)

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; mtx: metastases; NA: not available; P: prednisone; Ref; references; rPFS: Radiographic progression-free survival; *Results from *BRCA*1, *BRCA*2, *ATM* alterations Cohort.

1.2.2 Selection of subsequent lines for mCRPC

Cabazitaxel (TROPIC trial), abiraterone acetate (COU-AA-301 trial), enzalutamide (AFFIRM), and radium-223 (ALSYMPCA trial) have demonstrated a significant improvement in OS after treatment with docetaxel in mCRPC setting (24,26,27,31) (Table 1). However, no direct comparison among these agents is available. PSA response rates observed with enzalutamide in post-docetaxel mCRPC were lower than that observed in chemo-naïve mCRPC (78% vs 54%) (26,32). Similarly, the analysis of patients included in the COU-AA-302 trial who received docetaxel after abiraterone, consistently with different retrospective series, seem to suggest that the benefit of second-line docetaxel is lower than that observed in patients who received it in first-line (33,34). The choice between chemotherapy and ARSi remains critical both in patients who have received docetaxel and in those who have received ARSi in first-line. As previously mentioned, preclinical and clinical data suggest a variable degree of cross-resistance of abiraterone with enzalutamide, but also of ARSi with docetaxel (16,35,36); cabazitaxel, on the other hand, retains its clinical activity in patients pretreated with both chemotherapy and ARSi (37,38). The phase III CARD trial has established that treatment with cabazitaxel is the best choice for patients who experience progression during an ARSi after having received docetaxel (28). In this study, 255 patients with mCRPC, who were previously treated with docetaxel and had progression within 12 months while receiving an ARSi (abiraterone or enzalutamide), received cabazitaxel or the alternative ARSi. Cabazitaxel showed significantly increased imaging-based 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 other ARSi. A post hoc analysis confirmed the superiority of cabazitaxel over the ARSi regardless of whether abiraterone or enzalutamide was received during the trial. Retrospective data also support the notion that patients with early progression on first-line ARSi show increased response rates and time to PSA progression after treatment with second-line chemotherapy compared to the alternative ARSi (39). As indirect comparison, the PSA response rates of a second ARSi after ARSi in the control arms of the CARD (13.5%) and PROFOUND (8%) trials are clearly inferior compared to those observed in post-docetaxel patients treated with abiraterone (38%) or enzalutamide (54%) included in the COU-AA-301 or AFFIRM trials (26,28,29,40). Data from the control arm of the PLATO trial, in which patients received abiraterone acetate after first-line enzalutamide, are quite discouraging, with a median time to PSA progression of only 2.8 months and a PSA

response ≥ 50% observed in 2% of patients (41). A retrospective study showed that enzalutamide has some activity (21% of patients with PSA decline ≥ 50%) in patients pretreated with docetaxel and abiraterone acetate, and this ARSi could be offered to those patients who are not suitable for cabazitaxel (42).

1.2.3 The role of Radium-223

Radium-223 is an intravenous alpha-emitting radiotherapeutic drug that mimics calcium and binds to bone mineral hydroxyapatite in areas of high bone turnover. In the phase III ALSYMPCA trial, six cycles of radium-223 at 50 kBq/kg prolonged OS (HR 0.70 95% CI 0.58-0.83) and delayed time to first symptomatic skeletal event (SSE) compared to placebo (HR 0.66 95% CI 0.54-0.77) in mCRPC patients with symptomatic bone metastases (no visceral disease, soft tissue disease >2 cm or less than two bone metastases) (Table 1). Patients had either received docetaxel or were deemed ineligible or refused docetaxel; no patients had received abiraterone or enzalutamide (27). Prior docetaxel was associated with higher rates of thrombocytopenia, but it did not appear to impair radium-223 efficacy (43). A significant proportion of patients received docetaxel at progression, and chemotherapy after radium-223 was shown to be active, with manageable side effects (44). In the Expanded Access Program, the safety and activity of radium-223 was examined in a single-arm cohort of patients, including those with asymptomatic disease, and the combination of radium-223 with abiraterone or enzalutamide was allowed (45). Radium-223 was found to be safe, with a median OS of 16 months. Interestingly, patients receiving the combination of radium-223 with ARSi experienced a significantly longer OS compared to those receiving radium-223 alone. These results led to increased interest in potential combinations of radium-223. However, the ERA-223 trial, a phase III randomized trial that compared abiraterone plus radium-223 with abiraterone alone in first-line mCRPC patients, was prematurely unblinded due to the high occurrence of bone fractures and deaths in the treatment arm of the trial. The combination of abiraterone and radium-223 was not shown to increase survival (HR 1.2, 95% CI 0.95-1.51). In addition, although the rate of SRE events was not different between arms, a higher rate of fractures (18% vs 9%), mainly osteoporotic fractures (49% vs 17%), was observed in the treatment arm. Of note, approximately 60% of patients included in the trial were not receiving bone protective agents (46). These results led to the amendment of the other ongoing clinical

trials, such as the PEACE-3 phase III trial, comparing radium-223 plus enzalutamide with enzalutamide in first-line mCRPC, to mandate the use of bone protective agents in all patients. Updated results on the incidence of fractures in patients treated before and after the amendment showed that the use of bone protective agents significantly reduced the 12-month fracture incidence in patients treated with the combination (37.1% vs 2.7%), and also in patients treated with enzalutamide alone (15.6% vs 2.6%) (47). According to the European Medicines Agency (EMA), the use of radium-223 is restricted for the treatment of men with mCRPC, symptomatic bone metastases and no known visceral metastases, who are in progression after at least two prior lines of systemic therapy for mCRPC, or ineligible for any available systemic mCRPC treatment (48). Conversely, no restriction per line is included in the U.S. National Comprehensive Cancer Network Guidelines (NCCN). In view of the OS benefit with cabazitaxel as third-line therapy in the CARD trial (28), radium-223 should be reserved as post-cabazitaxel therapy for patients with bone-predominant disease, unless deemed ineligible or refusing chemotherapy.

1.2.4 The advent of Lutetium-177-PSMA-617

Lutetium-177-prostate-specific membrane antigen (PSMA)-617 (LuPSMA) is an investigational radioligand therapy that has been investigated for patients with mCRPC (49). LuPSMA binds with high affinity to PSMA, which is commonly expressed in prostate cancer including metastatic lesions, delivering ß-particle radiation. The phase II TheraP trial enrolled patients with mCRPC for whom cabazitaxel was considered the next appropriate standard treatment (50). Patients underwent gallium-68 Ga-PSMA-11 and 18fluoro-deoxy-glucose (FDG) PET-CT scans. PET eligibility criteria for the trial were PSMA-positive disease, and no sites of metastatic disease with discordant FDG-positive and PSMA-negative findings. Overall, 291 men were screened, of these 200 were eligible on PET imaging and were randomized to receive cabazitaxel or LuPSMA. Compared with cabazitaxel, Lu-PSMA led to a higher PSA response (66% vs 37%, $p<0.0001$) and fewer grade 3 or 4 adverse events (33% vs 53%). The results of the phase 3 VISION study involving patients with mCRPC treated with LuPSMA were recently presented at the ASCO Congress 2021 (30) (Table 2). In this study, men previously treated with at least one ARSi and one taxane were randomized to receive LuPSMA plus standard of care vs standard of care alone. Of note, eligible patients had at least one PSMA-

positive metastatic lesion and no PSMA-negative metastatic lesions. In addition, protocol-permitted standard of care excluded chemotherapy, immunotherapy, radium-223 and investigational drugs. Of 1179 patients screened, 86.6% met the imaging criteria for PSMA-positive mCRPC and 82.9% were randomized. Initially, 56% of early drop-out was noted in control arm before receiving study treatment and measures were implemented through enrolment to reduce control-arm drop-out rate (final early drop-out 16.3% in control vs 4.2% in treatment arms). Compared to standard-of care alone, LuPSMA significantly prolonged OS (median 15.3 vs 11.3 months, HR 0.62 95%CI 0.52-0.74) and radiographic PFS (median 8.7 vs 3.4 months, HR 0.40 99.2% CI 0.29- 0.57). Overall, this treatment was safe and tolerable. However, a significant proportion of patients experienced grade 3-5 bone marrow suppression (23.4% vs 6.8% in placebo) and 39.3% of patients treated with LuPSMA reported all grades dry mouth, nausea and vomiting. Based on these data, the authors have acclaimed the adoption of LuPSMA as a new standard of care for pretreated patients with mCRPC. A pivotal trial has also opened the door for the use of LuPSMA as metastastis-directed therapy after surgery and external beam radiotherapy in patients with low-volume mHSPC, and a randomized controlled multicenter phase II study is ongoing in this setting (51).

1.2.5 Treatment combinations

In an attempt to maximize benefit, a number of combinations of agents with seemingly non-overlapping mechanisms of action have been studied in advanced prostate cancer. Combinations, for instance, of different ARSi with chemotherapy in mHSPC have been pursued, with conflicting results. The ENZAMET trial demonstrated that the addition of enzalutamide to ADT prolonged OS compared to ADT plus a first-generation antiandrogen (52). Concomitant treatment with docetaxel was also allowed. In the prespecified subgroup analysis, the use of enzalutamide in combination with docetaxel was associated with significant improvement in clinical PFS (HR 0.48 95% CI 0.37–0.62), but the hazard ratio was suggestive for no OS benefit (HR 0.90, 95% CI 0.62–1.31). Of note, no evidence of heterogeneity of effect according to docetaxel use was found (adjusted p=0.14), and this result should be interpreted with caution. Similar data were observed in the post-hoc analysis of the TITAN trial of apalutamide in mHSPC (53). Only 11% of patients had received prior treatment with docetaxel and such subgroup analyses are purely exploratory. In these

patients treated with chemotherapy, the benefit of adding apalutamide was consistent with the overall population in terms of radiographic PFS (HR 0.47 95% CI 0.22-1.01), but it was unclear in terms of OS (HR 1.27 95% CI 0.52-3.09). The recently presented results of the PEACE-1 trial also confirmed the potential benefit of adding abiraterone acetate to docetaxel in terms of radiographic PFS (HR 0.50 95% CI 0.40-0.62; no interaction for docetaxel use) (54); however, data on OS are still missing and will likely be needed in order to establish the clinical relevance of this combination. The ARASENS trial, a randomized, double-blind, placebo-controlled, phase III trial is currently evaluating the AR antagonist darolutamide plus standard ADT plus docetaxel (55).

In the mCRPC setting, two phase III trials evaluated the combination of abiraterone with the antiandrogens enzalutamide (ALLIANCE A031201) and apalutamide (ACIS trial) compared with ARSi alone as first-line mCRPC treatment. Both abiraterone plus enzalutamide (HR: 0.70 95% CI 0.67-0.72) and abiraterone plus apalutamide (HR: 0.69, 95% CI 0.58-0.83) showed a significant benefit in terms of radiographic PFS over ARSi monotherapy, but no OS benefit (56,57). The combination of enzalutamide and docetaxel was shown to increase PFS over docetaxel alone as first line-therapy for mCRPC in the phase II CHEIRON trial (58). Currently, the randomized phase II CHAARTED2 trial is actively recruiting mCRPC patients, who received prior docetaxel chemotherapy for high volume mHSPC, to receive abiraterone acetate with or without cabazitaxel (59). In the recently presented IPATENTIAL 150 phase III study, the combination of abiraterone and the *PI3K* inhibitor ipatasertib was shown to increase radiographic PFS compared to abiraterone alone as first-line mCRPC therapy in patients with loss of *PTEN*; OS data are awaited to define the role of this combination in the treatment of mCRPC (23). A number of different combinations of hormonal and chemotherapeutic agents with other agents such as radiopharmaceuticals (radium-223), PARP inhibitors (olaparib) or immunotherapeutic agents (nivolumab, pembrolizumab) have reported clinical activity in mCRPC (60-63). Evidence of an OS benefit in randomized trials is required to determine their role in the treatment of advanced prostate cancer.

1.3 Prognostic biomarkers with predictive value in mCRPC

Several prognostic factors have been identified in patients with PCa. Prognostic clinical factors related to patients include age, performance status and pre-existing comorbidities. Factors related to tumor include Gleason score, mitotic index, extracapsular extension, seminal vesicle invasion, PSA levels and metastatic stage. In patients with metastatic hormone-sensitive prostate cancer (mHSPC) disease volume is relevant and can predict benefit from chemotherapy. However, the majority of these prognostic factors do not show predictive value of response to therapies and cannot guide the selection to specific therapies.

Biomolecular alterations, including alterations in tumor driving genes, have been observed in patients with prostate cancer. Some of these molecular alterations could be explored as predictive biomarkers for planning treatment to early identify primary resistance, avoiding useless toxicity to patients. In some cases, these alterations involve inherited or spontaneously acquired gene mutations in the germline.

More frequently, alterations are acquired at somatic level during the oncogenesis and/or cancer progression or they could arise or be enriched as result of the selective pressure induced by treatments. Examples of molecular alterations associated to mechanisms of treatment resistance that could be helpful in castration-resistant disease to select the appropriate therapy include androgen receptor (AR) amplification, mutation, or splice variants. Other resistance mechanisms bypass AR by exploiting alternative signaling and metabolic pathways (64). **Table 2** summarizes the evidence for proposed molecular biomarkers in advanced prostate cancer. Some DNA damage and response genes (DDR) have been clinically validated as biomarkers for selecting patients who are sensitive to poly ADP-ribose polymerase (PARP) inhibition. Similarly, the *AKT* inhibitor ipatasertib has demonstrated significant activity in patients with *PTEN* loss. These biomarker-driven treatments are going to be implemented in routine clinical practice. However, to what extent these treatments will affect the sequencing and response of other therapies is largely unknown and it will be object of investigation in the next future.

TABLE 2 PROMISING PROGNOSTIC AND PREDICTIVE BIOMARKERS IN MCRPC

Biomarker	Source	Drugs	Studies	Phase III trials
DDR (*BRCA1/2, ATM, PALB2* and other genes)	PMBC, tumor tissue or ctDNA	Olaparib Rucaparib Talazoparib Niraparib	Phase 2 TOPARP (65) Phase 2 TRITON-2 (66) Phase 2 TALAPRO-1 (67) Phase 2 GALAHAD (68)	PROFOUND (29,60) PROpel (69)* KEYLINK-010 (70)* TRITON-3 (71)* CASPAR (72)* TALAPRO-2 (73)* MAGNITUDE (74)*
***PTEN* loss**	Tumor tissue	Ipatasertib	Phase 2 A. Martin study (75)	IPATential150 (76)
AR-V7	CTCs	ARSi	PROPHECY biomarker study (77)	
Molecular subtype Luminal A Luminal B Basal	Tumor tissue	Apalutamide Docetaxel	SPARTAN (78) and TITAN (79) (biomarker analyses) CHAARTED (80) (biomarker analysis)	
Others **MSI-h/MMRd** ***CDK12* deficiency** ***SPOP* mutations** ***RB1* loss** ***TP53* alterations** **TMPRSS2**	Tumor tissue	ARSi ICI	*Explorative analyses*	

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; PBMC: peripheral blood mononuclear cells. *Ongoing trial.

1.3.1 DDR genes

Alterations in DDR genes have been recently became a field of major interest in prostate cancer research, given their potential prognostic and predictive implications (81). DDR defects have been encountered in the germline of 8-17% of patients with metastatic disease (82-84). *BRCA*2 gene alterations are the most common DDR event both in the somatic- and germline (82,85).

Germline *BRCA*2 mutations have been associated with aggressive disease and poor clinical outcomes (86,87). The PROREPAIR-B study has shown that the detection of germline *BRCA*2 alterations has negative prognostic significance. Additionally, a significant interaction between germinal *BRCA*2 status and treatment type (ARSi versus taxane therapy) has been observed, suggesting that *BRCA*2 might be a valid biomarker during the selection of the first-line treatment choice in patients with mCRPC (88). The *BRCA*2men study aims to validate germline *BRCA*2 alterations as a predictive biomarker for the selection of ARSi or taxanes as

first-line of therapy (89). Importantly, the PROFOUND study has recently established the predictive value of certain DDR genes defects in patients with mCRPC whose disease had progressed during previous treatment with enzalutamide, abiraterone, or both (29,60). Patients were randomized to receive olaparib or the physician's choice of enzalutamide or abiraterone (control). 65% of patients had also received prior taxane therapy. Treatment with olaparib significantly prolonged the PFS and OS of patients with at least one alteration in *BRCA*1, *BRCA*2, or *ATM*, establishing the first validated biomarker in patients with prostate cancer. The subgroup analysis of PFS and OS favored olaparib irrespective of prior taxane use (90). The gene subgroup analysis suggested that patients with *BRCA* alterations are those who derive the greatest benefit from olaparib, whereas those with *ATM* alterations showed unclear PFS (HR: 1.04, 95% CI 0.61–1.87) and OS benefit (HR: 0.93, 95%CI 0.53–1.75) (91). Of note, many experts acknowledge that the use of a second ARSi in the control arm after progression on an ARSi represents an important limit of the PROFOUND trial, as the sequence ARSi -> ARSi is not generally advised, due to possible emergence of cross-resistance and reduced activity. In addition, based on the CARD trial, cabazitaxel should be the standard of care for these patients (28). A recent study investigated potential biomarkers associated with benefit during treatment with olaparib in patients enrolled in the TOPARP-B phase II trial (92). *BRCA*1/2 germline and somatic pathogenic mutations were associated with similar benefit from olaparib; greater benefit was observed in patients with homozygous *BRCA* deletion. Biallelic, but not mono-allelic, *PALB2* deleterious alterations were associated with clinical benefit. In addition, loss of *ATM* protein by immunohistochemistry associated with better outcome. Of note, loss of RAD51 foci, a functional biomarker of homologous recombination repair (HRR) function, was primarily found in tumors with biallelic *BRCA*1/2 and *PALB2* alterations, and the authors have suggested that RAD51 assay could help identify less-common genomic variants impacting HRR function that sensitize to PARP inhibition.

In the phase II TRITON 2 trial, patients with mCRPC and *BRCA*1/2 alterations who had progressed after one to two lines of ARSi and one taxane-based chemotherapy for mCRPC were treated with rucaparib (66). Complete response rates and confirmed PSA response rate were 43.5% and 54.8%, respectively. According to PSA response, the efficacy of rucaparib was apparently greater in patients with germline versus somatic *BRCA*1/2 mutations, in biallelic versus monoallelic mutations, and in homozygous deletions versus other deleterious mutations. In addition, the efficacy of rucaparib was greater in patients with *BRCA*2- versus

*BRCA*1-altered mCRPC, as assessed by PSA50 response rates, overall response rates, and median radiographic PFS estimates. Of note, this apparent discrepancy in PARP inhibitor sensitivity between patients with *BRCA*1- and *BRCA*2-mutated mCRPC seems to be a class effect of PARP inhibitors in prostate cancer (93). Taza and colleagues found that PARP inhibitor activity was diminished in *BRCA*1- versus *BRCA*2-altered mCRPC in a cohort of 123 *BRCA*1/2-altered mCRPC patients receiving PARP inhibitor, and this differential activity was not explained by mutation origin (germline vs somatic) or allelic status (mono- vs biallelic) (94). The phase II TALAPRO-1 trial reported results from treatment with talazoparib in patients with mCRPC and associated DDR defects, who had progressed after ARSi and taxane (67). Overall response rates were 44% in patients harboring *BRCA*1/2 alterations, 33% in *PALB2* and 12% in *ATM*, whereas the complete response rates were 76% in *BRCA*1/2, 50% in *PALB2* and 28% in *ATM*. The phase II GALAHAD trial is assessing niraparib in patients with mCRPC and biallelic DDR defects with disease progression on taxane and ARSi (68). At the interim analysis, niraparib showed an overall response rate of 41% and a complete response rate of 63% in *BRCA* carriers, with durable responses, particularly in biallelic *BRCA* mutation carriers.

We could conclude that olaparib and other PARP-inhibitors as monotherapy showed significant benefit in patients with pretreated mCRPC and alterations in DDR, especially in those with *BRCA*1/2 alterations. However, ongoing studies are assessing the role of these agents in combination with ARSi at earlier stages of mCRPC, given the strict relationship between PARP1 activity and AR function. It is also hypothesized that the co-blockade of PARP1 and AR using could be active regardless of DDR deficiency status. A phase II trial of olaparib in combination with abiraterone in post-docetaxel mCRPC showed a significant improvement in terms of radiographic PFS with the combination compared to abiraterone alone (95). The ongoing PROpel Phase III trial is testing olaparib as a first-line treatment for patients with mCRPC in combination with abiraterone versus abiraterone alone irrespective of DDR status, and could extend the use of this agents in unselected populations of patients with mCRPC (69). The phase 3 CASPAR trial is ongoing to assess the combination of enzalutamide with rucaparib as first-line treatment of mCRPC (72). The phase III TALAPRO-2 trial is ongoing to evaluate the efficacy of talazoparib combined with enzalutamide for the first-line of mCRPC (73). Similarly, the phase III MAGNITUDE trial is ongoing to assess the efficacy of niraparib in combination with abiraterone acetate as first-line treatment of mCRPC in patients with DDR alterations (74).

1.3.2 AR pathway

Several studies support the notion that alterations in AR pathway represent an important driver of resistance in the context of mCRPC. Circulating AR copy number variations (CNV) in plasma DNA are associated with worse outcome in patients with mCRPC treated with ARSi (96). AR gain in plasma DNA is also associated with worse outcome in docetaxel-treated mCRPC patients, but AR-gained patients seem to derive greater benefit from treatment with taxanes than with ARSi (97,98).

The androgen-receptor variant 7 (AR-V7) has been proposed to predict for poor response to treatment with ARSi, such as abiraterone acetate or enzalutamide. Antonarakis and colleagues firstly showed that the detection of this AR variant was associated with treatment resistance to ARSi (99). Interestingly, AR-V7 did not seem to be associated with resistance to taxane-based chemotherapy and potential reversion of AR-V7 detection was observed after taxane treatment (100-102). In the PROPHECY trial, 118 men with mCRPC who were starting abiraterone or enzalutamide were enrolled to assess the role of AR-V7 (77). AR-V7 detection by both the Johns Hopkins and Epic AR-V7 assays was independently associated with shorter PFS and OS, and patients with AR-V7–positive mCRPC had fewer confirmed prostate-specific antigen responses or soft tissue responses. However, no randomized trial has ever demonstrated that alternative treatment with chemotherapy in AR-V7–positive patients could clearly translate into a survival benefit, and the potential confounding prognostic effect of AR-V7 have made into question its predictive value and its clinical utility. AR-V7 is rarely detected in patients who are starting a first-line treatment for mCRPC after androgen-deprivation therapy (3-8%), but its prevalence progressively increases with the number of treatment lines received for mCRPC (103,104). This biomarker could be useful to determine the utility of a second ARSi in pretreated patients, but its clinical implementation still needs further studies.

1.3.3 PTEN loss and PI3K alterations

About a half of patients with mCRPC show loss of the *AKT* phosphatase *PTEN*, with hyper-activation of the oncogenic *PI3K*/*AKT* signaling (105). These patients show worse prognosis and reduced benefit from treatment with ARSi (106). The phase II A. Martin study assessed the activity of the *AKT* inhibitor ipatasertib plus abiraterone vs abiraterone alone in

patients with mCRPC after docetaxel chemotherapy (75). The radiographic PFS was prolonged in the ipatasertib cohort, with similar trends in OS and time-to-PSA progression; in addition, a larger radiographic PFS prolongation for the combination was demonstrated in *PTEN*-loss tumors. Based on these data, the phase III IPATential150 trial assessed the efficacy ipatasertib in combination with abiraterone compared to abiraterone alone for the first-line treatment of patients with mCRPC (76,107). The co-primary endpoints were radiographic PFS in the *PTEN*-loss-by-immunohistochemistry population and in the intention-to-treat population. Of 1101 patients enrolled in this study, 521 (47%) harbored *PTEN* loss. In patients with *PTEN* loss, the combination arm with ipatasertib achieved significantly superior radiographic PFS (18.5 vs 16.5 months, HR 0.77, 95% CI 0.61-0.98, p=0.034) and antitumor activity compared to the placebo arm. However, the improvement of radiographic PFS in the ITT population was not statistically significant. The subgroup analysis of the IPATential150 trial suggests that prior treatment with taxanes may influence the benefit induced by ipatasertib in patients with *PTEN* loss. A biomarkers analysis of the IPATential150 trial also showed that patients with *PTEN* loss and with genomic alterations in *PIK3CA*/AKT1/*PTEN* by next generation sequencing had a larger magnitude of radiographic PFS benefit with ipatasertib than patients with no detectable alterations (108). These results support the notion that ipatasertib plus abiraterone is a valid treatment option for first-line mCRPC with *PI3K*/*AKT* pathway alterations.

1.3.4 Basal versus luminal prostate cancer

The PAM50 is a well-known gene expression classifier that categorizes breast cancer into luminal A, luminal B, HER2, and basal subtypes. Zhao and colleagues applied this classifier to subtype prostate cancer samples into luminal A, luminal B and basal subtypes (109). The authors found that luminal B prostate cancers had the poorest clinical outcomes, followed by basal, and luminal A. Although both luminal-like subtypes were associated with increased AR expression and signaling, only luminal B prostate cancers were significantly associated with postoperative response to ADT. Similar results were observed with chemotherapy in patients included in the CHAARTED trial (80). In the control arm with ADT alone, luminal B subtype was associated with shorter OS compared to basal subtype, confirming the negative prognostic significance of luminal B subtype. However, patients with luminal B subtype treated with ADT plus docetaxel showed significant improvement in time to castration-resistance and OS,

whereas basal subtype showed no OS benefit from ADT plus docetaxel, included patients with high-volume disease. Luminal subtype also seems to better respond to ARSi compared to basal subtype. Regardless of basal/luminal subtype, > 50% of patients enrolled in the phase III SPARTAN trial (apalutamide in nmCRPC) achieved ≥ 90% reduction in PSA with apalutamide. However, PSA decline was deepest and most rapid in patients with luminal subtype. Similarly, the OS improvement with apalutamide seemed to favor patients with luminal subtype (HR 0.43, 95% CI 0.19-1, p=0.051) compared to basal subtype (HR 0.67, 95% CI 0.40-1.14, p=0.14) (78). Conversely, in the sub-analysis of the TITAN trial (apalutamide in mHSPC), apalutamide determined significant prolongation of radiographic PFS in basal molecular subtype (HR 0.31 95% CI 0.16-0.62, p=0.0008), whereas no significant difference was seen in luminal subtype (HR 0.74, 95% CI 0.40-1.36, p=0.33) (79). It is unclear whether the distinct setting (mHSPC vs mCRPC) might explain these discordant results. Importantly, these biomarkers analyses were performed in diagnostic biopsies, included patients that received these treatments in later stages during castration-resistance. The molecular characteristics of metastatic sites might differ from that of primary tumors, therefore caution should be used when interpreting these analyses. Overall, these data suggest that luminal versus basal classification may be useful to select patients who are expected to derive the greatest benefit from ARSi and docetaxel. However, prospective biomarker-driven studies are needed to determine the real potential predictive impact of this classification.

1.3.5 Aggressive-variant prostate cancer

Aggressive-variant prostate cancer (AVPC) refers to AR-independent anaplastic forms of prostate cancer that are characterized by a rapidly progressive disease, weak response to therapies and poor prognosis (110). Many of these tumors are prostate cancers with neuroendocrine features (NEPC), but some of these cases do not show typical morphology or immunohistochemical profiles of neuroendocrine differentiation. AVPC cells can arise de novo or, more commonly, be the result of divergent clonal evolution from one or more castration-resistant adenocarcinoma cells (111). The selective pressure induced by chemotherapy and ARSi favors the emergence of such resistant clones, which are commonly found in the advances stages of castration-resistance. AVPC is clinically characterized by at least one of these features (110,112,113): a) histologic evidence of small-cell NEPC; b) presence of

exclusively visceral metastases; c) radiographically predominant lytic bone metastases; d) bulky lymphadenopathy or bulky high-grade tumor mass in prostate/pelvis; e) low PSA at initial presentation plus high volume bone metastases; f) presence of neuroendocrine markers on histology or in serum plus any of the following in the absence of other causes: elevated serum LDH, malignant hypercalcemia, elevated serum CEA; g) short interval to androgen-independent progression following the initiation of hormonal therapy with or without the presence of neuroendocrine markers.

AVPC shows a high response rate, generally of short duration, to platinum-based chemotherapy (112). The NCCN guidelines currently recommend to use chemotherapy with cisplatin/etoposide, carboplatin/etoposide, and docetaxel/carboplatin as first or subsequent treatments for patients with small-cell or NEPC (114). A phase II study investigated the use of the AURKA inhibitor alisertib in patients with metastatic NEPC (115). Although the trial did not meet its primary endpoint of improved PFS, tumors suggestive of N-myc and Aurora-A overactivity showed exceptional responses, including complete resolution of liver metastases and prolonged stable disease. Many trials are currently ongoing in patients with AVPC and NEPC to test the activity of immunotherapy, PARP inhibitors and EZH2 inhibitors in these patients (116).

For patients with AVPC (excluding those with small-cell or NEPC histology) there is no consensus for the optimal first-line treatment. At the Advanced Prostate Cancer Consensus Conference (APCCC) 2019, 75% of panellists voted to add docetaxel to ADT, 16% voted to add platinum-based combination therapy, and 9% voted to add an ARSi. Finally, the potential effect of a first-line platinum-based chemotherapy on the efficacy of subsequent treatments such as PARP inhibitors, docetaxel or ARSi is largely unknown, and requires further studies.

1.3.6 Other biomarkers

Given its tissue-agnostic approval by the U.S. Food and Drug Administration (FDA), patients with microsatellite instability or mismatch repair-deficient prostate cancer tumors might benefit from treatment with pembrolizumab (117). In the study by Abida and colleagues, among 1033 patients who had adequate tumor quality for microsatellite instability (MSI) analysis, 32 (3.1%) had MSI-high/mismatch-deficient prostate cancer, and 7 of them had a pathogenic germline mutation in a Lynch syndrome-associated gene (117). Six of eleven

patients (54.5%) who received anti-programmed cell death protein 1 (PD1)/ligand 1 (PD-L1) therapy had a >50% decline in PSA levels, and 4 of them had radiographic responses. However, none of the six patients with tumor response included in the Phase II KEYNOTE-199 study of pembrolizumab in mCRPC were found to have microsatellite instability, suggesting that other mechanisms could be also involved in favoring response to immunotherapy (61). Of interest, 2/19 patients (11%) with *BRCA* or *ATM* aberrations included in this trial showed response to pembrolizumab, compared to 4/124 (3%) of those without alterations in DDR. Data also suggest that a proportion of patients with *CDK12* deficiency may respond favorably to anti-PD-1 checkpoint inhibitors (118,119). *SPOP* mutations have been suggested to predict for response to abiraterone acetate (120). *RB1* aberrations increase in prevalence after treatment-selective pressure (121); patients with mCRPC treated with enzalutamide and concurrent *RB1* alterations showed worse clinical outcomes and worse progression-free survival (122). A study also found that alterations in *RB1* and *TP53* are associated with shorter time on treatment with abiraterone or enzalutamide (123). Another study also suggested that the cooperative loss of two or more tumor suppressor genes, including *TP53*, *PTEN*, and *RB1*, may drive more aggressive disease and increased risk of relapse (124).

1.3.7 Biomarkers and diagnostic challenges

Of 4425 patients initially enrolled in the PROFOUND trial, 4047 patients had tumor tissue available for testing, of these 2792 (69%) were successfully sequenced, and only 162 patients (3.7% from initial enrollment) were found to harbor germline or somatic alterations in these *BRCA*1, *BRCA*2 or *ATM*. These data show the important limits of tumor tissue analysis. An increase in the sequencing success rate or the implementation of liquid biopsy approaches are necessary to enlarge the number of patients who could benefit from biomarker-driven treatments. It has been shown that ctDNA can sufficiently identify all driver DNA alterations found in matched metastatic tissue in the majority of patients with mCRPC (125). Data from PROFOUND trial found a high concordance between tumor tissue and circulating tumor DNA (ctDNA), supporting the development of ctDNA testing as a minimally invasive method to identify patients with DDR-altered mCRPC (126). In metastatic disease, ctDNA can identify somatic mutations, copy-number variations, and structural rearrangements that are predictive of response to therapies. However, multiple technical and biological variables can

confound the ctDNA-based genotyping, complicating the implementation of ctDNA into clinical practice (127). The ctDNA fraction (ctDNA%) strongly influences assay detection sensitivity and specificity for different genomic events and it is a critical variable during the interpretation of patient results. For example, copy number variations in *TP53*, *BRCA*2, *PTEN*, *RB1*, and AR all have clinical relevance in mCRPC, but these alterations are not always possible to identify in samples with low ctDNA% (127). Therefore, both ctDNA and tumor tissue analysis show advantages and constraints, and are likely to become more complementary than competing in the era of precision oncology. The development of more accurate and feasible assays to easily detect the presence of specific biomolecular alteration in patients with cancer will be the challenge of the next decades.

1.4 Lipidomics

Lipids are hydrophobic molecules widely involved in several biological processes, and play a central role in the architecture of normal cells. Lipidomics is the branch of science who studies lipids and their interacting patterns in biological systems (94). Lipids' metabolism is usually dysregulated in cancer cells, as they can use lipogenic or lipolytic pathways to promote cell proliferation, survival, or to gain the ability to migrate and metastasize (128,129). It is known that dyslipidemia can promote tumorigenesis through different pathogenic mechanisms (130,131). Hypoxia can play a role in lipid dysregulation in cancer, contributing to alter the membrane structure, dysregulating the immune response, and promoting aberrant angiogenesis (132). In lung cancer, specific lipidomic profiles seem to explain the heterogeneity of different lung cancers' subtypes (133). It has also been reported that increased levels of cholesterol and lipoproteins in plasma could have a role in breast and ovarian cancer progression (134).

1.4.1 Lipidomics in prostate cancer

Current evidence supports the notion that PCa is characterized by dysregulated lipid metabolism (135,136). Higher incidence of aggressive PCa and prostate cancer-specific mortality are observed in obese men (137). Prostate cancer cells show increased lipid lipogenesis and lipolysis, and altered metabolism of cholesterol and phospholipids (138). In addition, preclinical data showed that the transition from hormone-sensitive to castration-resistant PCa is characterized by alterations in lipid metabolism, including increased

intratumoral levels of essential polyunsaturated fatty acids (139). Increased expression of AR-V7 seems to be crucial for the reactivation of the lipid synthesis in CRPC, suggesting a key role of this splicing variant in regulating lipid metabolism in the CRPC setting (136). The fatty acid synthase (FASN), a key lipogenic enzyme, was found among the top ten genes overexpressed in AR-V7-driven CRPC metastases (140). Recent data have also uncovered the existence of a reciprocal modulation between FASN and AR-V7, and it has been proposed that FASN inhibition could be an approach to indirectly antagonize AR-V7 and potentially overcome resistance to enzalutamide and abiraterone (141). Another study found that 3-hydroxy-3-methyl-glutaryl–CoA reductase (HMGCR), a key enzyme in the cholesterol synthesis pathway, was elevated in enzalutamide-resistant PCa cell lines and that simvastatin, a HMGCR inhibitor, blocked AR synthesis and inhibited growth in vitro and in vivo (142). Several data support the notion that statins may reduce risk of metastatic PCa and PCa mortality, acting on cholesterol and lipid metabolism (143).

Therefore, in-depth delineation of lipid metabolism in PCa is significant to open new insights into prostate tumorigenesis, progression, resistance to therapies and provide potential biomarkers to predict treatment response. Many researchers explored the ability of specific lipid molecular species to serve as biomarkers for the diagnosis of PCa, and some data regarding the potential prognostic and predictive role of specific lipid species during treatment with ARSi or chemotherapy are also available (144-146). For example, enzalutamide has shown to induce extensive lipid remodelling of all major phospholipid classes at the expense of storage lipids, leading to increased desaturation and acyl chain length of membrane lipids and, conversely, significant associations were found between phospholipid profile and activity of enzalutamide (147).

1.4.2 Three-lipid signature and prostate cancer

An Australian group undertook comprehensive plasma lipid profiling in men with mCRPC, demonstrating that higher levels of sphingolipids such as ceramide and sphingomyelin species were associated with shorter OS (148). Lipidomic profiling by liquid chromatography-tandem mass spectrometry was performed on plasma samples from a discovery cohort of 96 mCRPC patients **(Figure 2)**. Results were then validated in an independent Phase 2 cohort of 63 patients. Unsupervised analysis of lipidomic profiles (323 lipid species) classified the

discovery cohort into two patient subgroups with significant survival differences (HR 2.31, 95% CI 1.44–3.68, p=0.0005). Levels of 46 lipids, predominantly sphingolipids, were individually prognostic and higher levels were associated with poor prognosis. The authors also derived a prognostic three-lipid signature that included ceramide d18:1/24:1, sphingomyelin d18:2/16:0 and phosphatidylcholine 16:0/16:0. This signature was associated with shorter survival in the validation cohort (HR 4.8, 95% CI2.06–11.1, p=0.0003), and was an independent prognostic factor when modelled with clinic-pathological factors or metabolic characteristics.

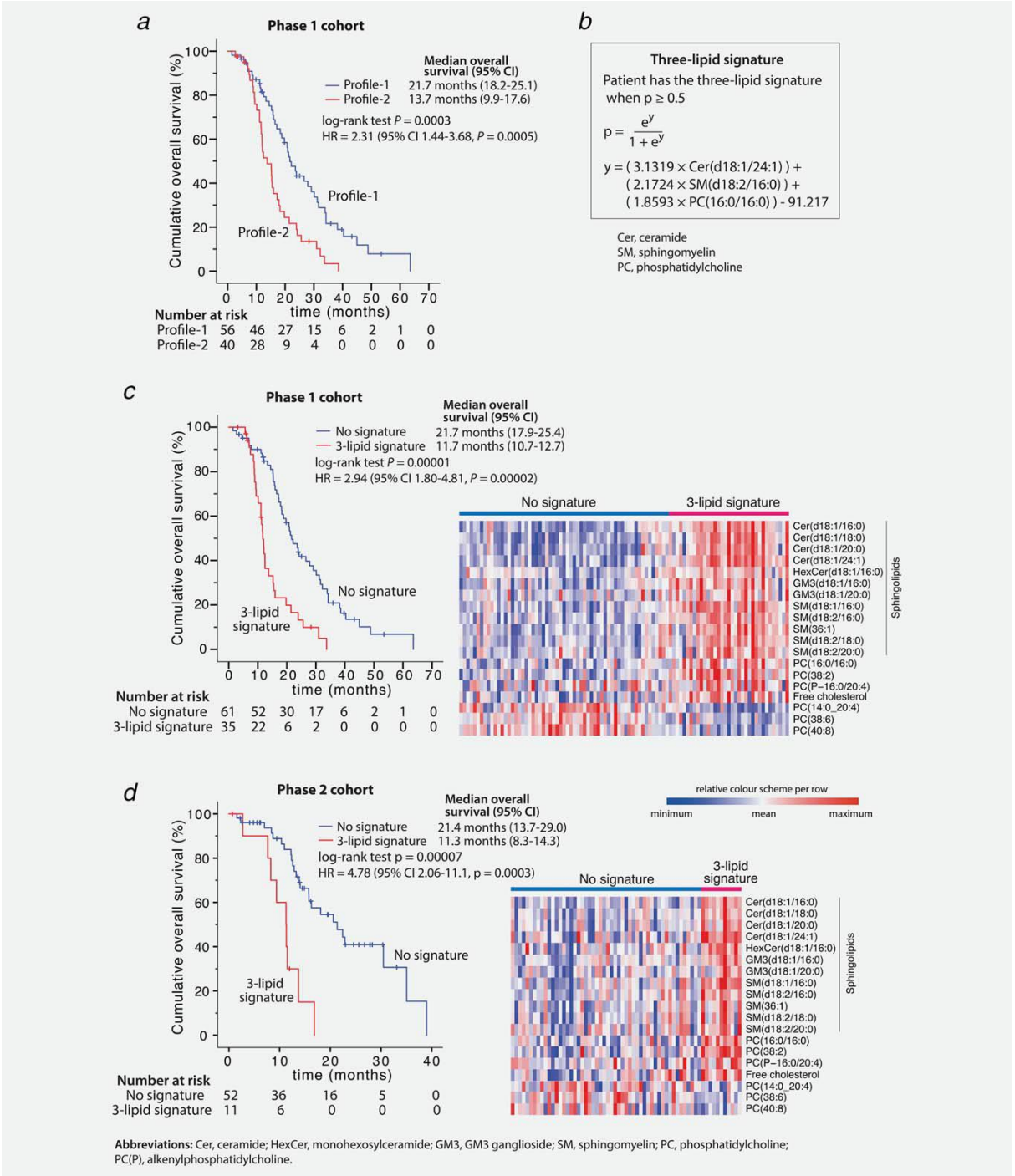


FIGURE 2 SURVIVAL CURVES, LIPID SIGNATURE AND HEATMAPS OF PROGNOSTIC BASELINE PLASMA LIPID LEVELS.

(a) survival curves of phase 1 discovery cohort classified by latent class analysis of baseline lipidomic profile; (b) three-lipid signature of normalised baseline lipid levels; (c) survival curves of phase 1 discovery cohort classified by the three-lipid signature, and heatmap of 19 prognostic lipids validated in phase 2; (d) survival curves of phase 2 validation cohort classified by the three-lipid signature, and heatmap of the 19 validated prognostic lipids (148)

In addition, the prognostic value of 19 of 46 lipids previously identified in the discovery cohort was confirmed in the validation cohort **(Table 3)**.

	Phase 1 cohort			Phase 2 cohort		
Lipid	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 HAZARD RATIO OF BASELINE PLASMA LEVELS OF 19 VALIDATED PROGNOSTIC LIPIDS, ANALYSED AS CONTINUOUS VARIABLES IN UNIVARIABLE COX REGRESSION (148)

The Australian group performed a subsequent study to comprehensively profile the circulating lipidome across the natural history of PC spanning localised PCa, mHSPC and mCRPC (149). Circulating lipid profiles featuring elevated levels of ceramide species were associated with metastatic relapse in localized PCa (HR 5.80, 95% CI 3.04–11.1, P = 1 × 10−6) and earlier testosterone suppression failure in mHSPC (HR 3.70, 95% CI 1.37–10.0, P = 0.01).

The prognostic significance of circulating lipid profiles in localized PC was independent of standard clinic-pathological and metabolic factors.

The circulating 3-lipid signature was also re-analyzed in the discovery cohort with additional follow-up and retained prognostic ability **(Figure 3)**. In this cohort, all patients had received docetaxel as first-line mCRPC therapy and those with the 3-lipid signature had a shorter time to PSA progression (HR 1.67, 95% CI1.14–2.44, P = 0.01).

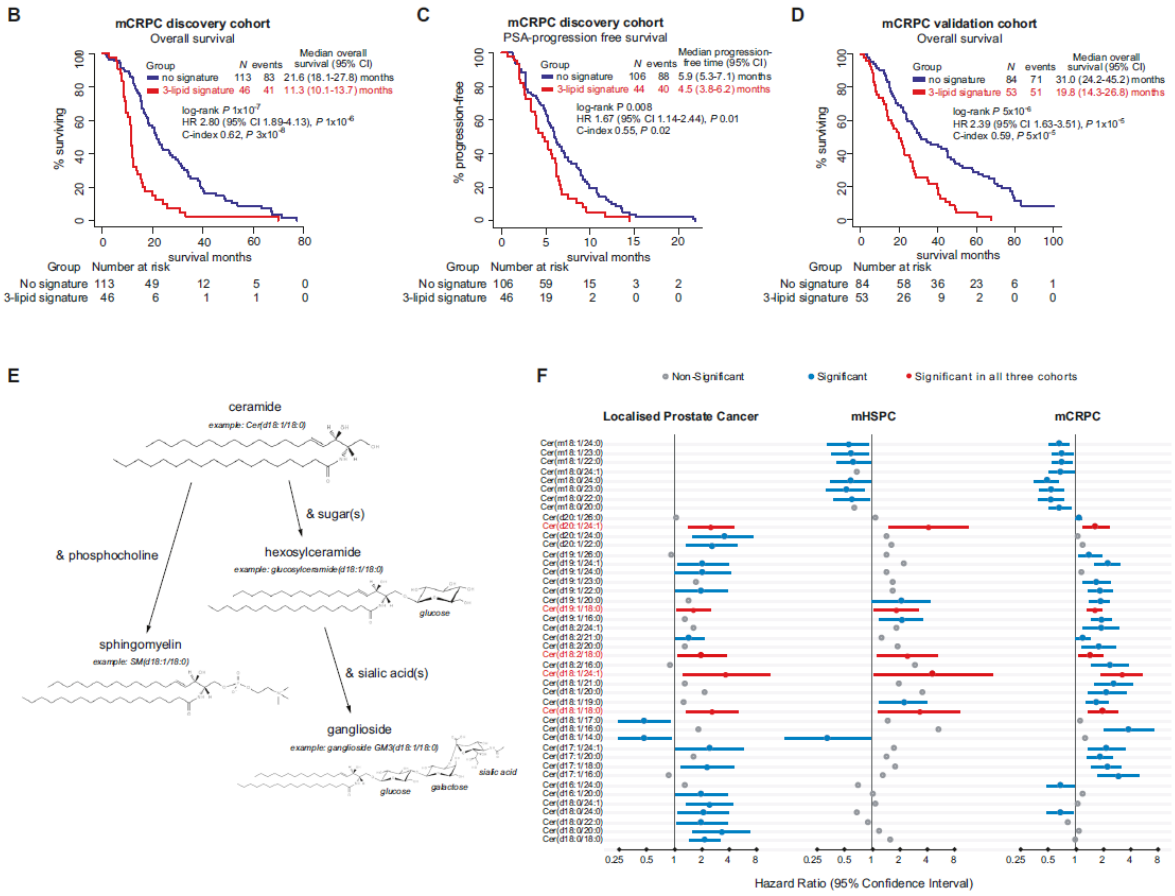


FIGURE 3 PROGNOSTIC MCRPC 3-LIPID SIGNATURE AND CERAMIDE SPECIES.

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

In the validation cohort, the levels of 275 lipids were significantly associated with OS. The top 20 significant lipids mainly consisted of species of ceramide, sphingomyelin and acylcarnitine. Of note, ceramide (d18:1/24:1) alone was comparable to the 3-lipid signature

(HR 3.2 (95% CI 1.88–5.40, P = 4 × 10−5) on univariate analysis; however, the 3-lipid signature performed better in the prediction of 1-year survival.

A subsequent study performed plasma lipidomic analysis and cell-free DNA (cfDNA) sequencing on 106 men with mCRPC initiating docetaxel, cabazitaxel, abiraterone or enzalutamide (discovery cohort) and 94 men with mCRPC initiating docetaxel (validation cohort) (150) **(Figure 4)**.

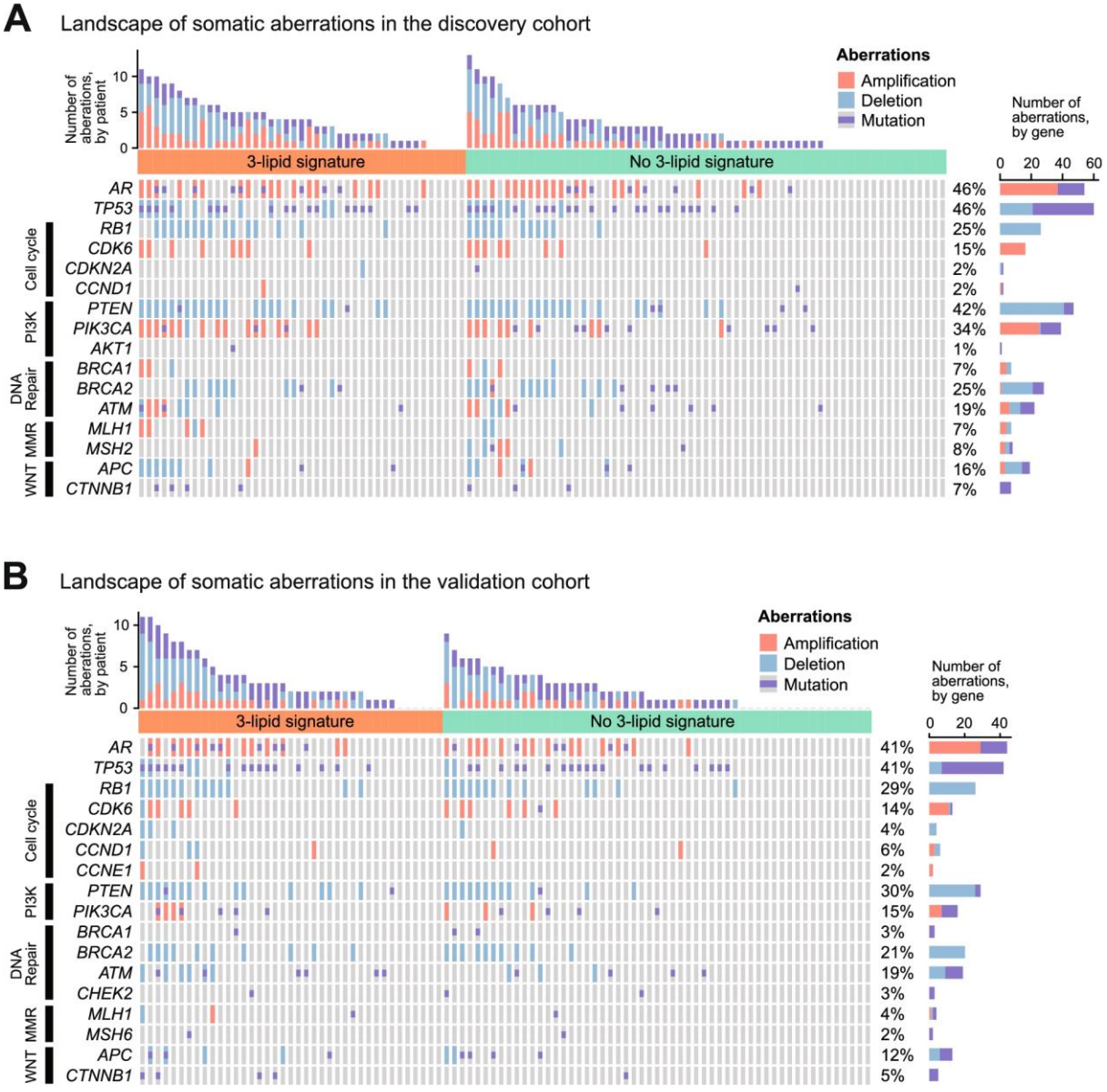


FIGURE 4 LANDSCAPE OF SOMATIC ABERRATIONS IN THE A DISCOVERY COHORT AND B VALIDATION COHORT (150)

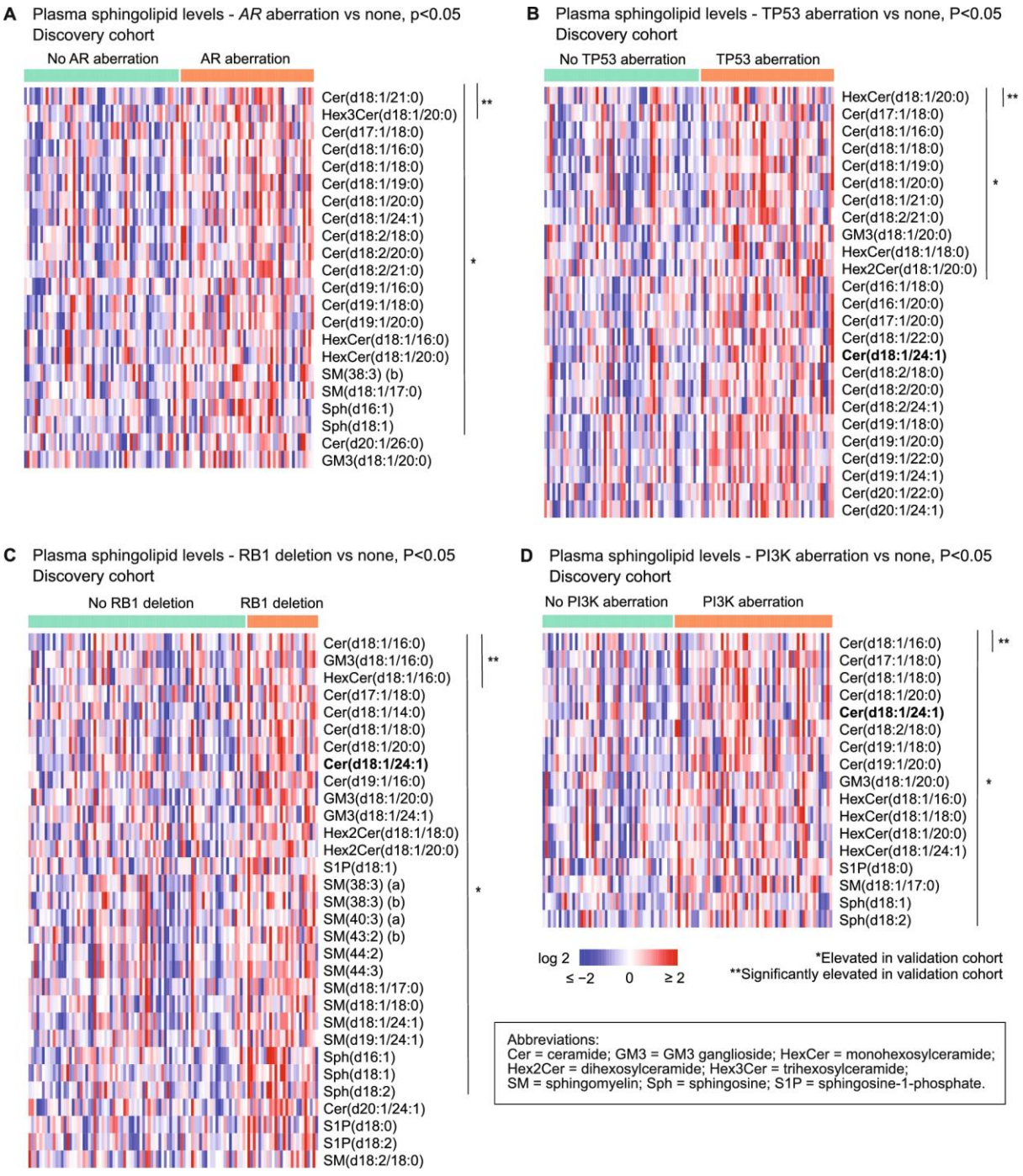


FIGURE 5 COMBINED IMPACT OF LIPIDOMIC AND GENETIC ABERRATIONS ON CLINICAL OUTCOMES IN METASTATIC CASTRATION-RESISTANT PROSTATE CANCER (150).

The overall frequency of somatic aberrations within the *AR*, *TP53*, cell cycle, *PI3K*, DNA repair, mismatch repair (MMR) and *WNT* pathways was increased in men with the 3-lipid signature, and increased genomic heterogeneity was associated with the presence of the 3-lipid signature **(Figure 5)**. Elevated circulating sphingolipids were associated with *AR*

aberrations, *TP53* aberrations, *RB1* deletion and *PI3K* pathway aberrations in both cohorts. About 20 sphingolipids were significantly elevated in men with any *AR* aberration compared to men without, and a significant number of sphingolipids were significantly elevated in men with *TP53* aberrations, *RB1* deletion or *PI3K* aberrations. Aberrations in the DNA repair pathway (*BRCA1/2*, *ATM*, *CHEK2*), MMR genes (*MLH1*, *MSH2*, *MSH6*) or *WNT* pathway (*APC*, *CTNNB1*) were not significantly associated with elevated circulating sphingolipids in either cohort, demonstrating that not all genotypes are associated with the poor prognostic metabolic profile.

In multivariate analysis with clinic-pathologic factors, presence of an *AR* aberration and/or the 3-lipid signature was independently associated with worse OS compared to men with neither characteristic in both discovery and validation cohorts. The association with shorter OS was also seen with the *TP53* aberration and/or 3-lipid signature combination, the *RB1* deletion and/or the 3-lipid signature combination and the *PI3K* and 3-lipid signature combination. In addition, elevated circulating sphingolipids were associated with aggressive-variant prostate cancer (AVPC) in both cohorts. Men with the combination of 3-lipid signature and AVPC had significantly shorter OS in both cohorts, with median survival of ~12 months compared to > 2 years for men with neither signature.

Ceramides metabolism has been implicated in cancer and other pathological conditions (151). Circulating sphingolipids are mainly derived from the liver, transported in lipoprotein pools, and can be increased by systemic inflammation (152). However, some circulating sphingolipids may originate from the tumor, given that PCa cells express the relevant biosynthetic enzymes of which some are associated with poorer PC outcomes. Exosomes secreted by PCa cells are also enriched in sphingolipids (153).

2 STUDY OBJECTIVES

2.1 Primary objective

To analyze the lipidomic profile of highly pretreated mCRPC patients (>2L cohort) compared to patients starting a first-line for mCRPC (1L).

2.2 Secondary objectives

- To explore the prognostic and predictive potential of lipid species differentially expressed in >2L compared to 1L patients.
- To test the prognostic effect of the previously reported 3-lipid signature in our cohort of patients with mCRPC.

3 MATERIALS AND METHODS

3.1 Sample collection and patients' population

Patients with mCRPC treated at the IRCCS Policlinico San Martino hospital in Genoa, who were starting first-line treatment for mCRPC (cohort 1L) or who had already been treated with at least two lines for mCRPC – including at least one ARSi and one chemotherapy regimen – (cohort 2L) were invited to participate in this study.

Informed consent was obtained from all patients, after the approval of the study protocol by the Local Ethics Committee (P.R. 505REG2015).

After informed consent, patients had a blood draw and were prospectively followed up with PSA assessments every 4-6 weeks, until death or a cut-off date of December 31, 2018. Survival update was performed in June 2022.

3.2 Sample preparation

The extraction of plasma lipids was carried out with a biphasic method: 30 µL of plasma were introduced into a test tube and extracted with 225 µL of cold MeOH, containing a combination of deuterated standards (Splash Lipidomix®). The solution was then stirred for 10 seconds, then 750 µL of cold MTBE were added and stirred for 10 seconds. The tube was then placed in a thermomixer at 4°C and stirred for 6 minutes at 2000 rpm. After that, 188 µL of water was added and the tube was vortexed for 10 s and then centrifuged for 2 minutes at 14,000 rpm at 4°C. Finally, 300 µL of supernatant was collected and evaporated with a SpeedVac. The dried sample was replenished with 50 µL of a 9:1 MeOH/Toluene solution containing the internal standard CUDA (12.5 ng/mL).

3.3 Liquid chromatography – mass spectrometry analysis

Reconstituted samples were tested with a Vanquish UHPLC system (Thermo Scientific, Rodano, Italy) paired with an Orbitrap Q-Exactive Plus (Thermo Scientific, Rodano, Italy). Lipid separation was achieved with a reversed phase column (Hypersil Gold™ 150 × 2.1 mm, particle size 1.9 µm), the column was maintained at 45 °C with a flow rate of 0.260 mL /min. Mobile phase A for ESI mode positive consisted of 60:40 (v/v) acetonitrile/water with ammonium formate (10 mmol) and 0.1% formic acid, while mobile phase B was 90:10 isopropanol/acetonitrile (v/v) with ammonium formate (10 mmol) and 0.1% formic acid, while in the negative ESI mode the organic solvents for both mobile phases were the same as in the positive with the exception of using ammonium acetate (10 mmol) as a mobile phase modifier. The gradient used was as follows: 0-2 minutes from 30% to 43% B, 2-2.1 minutes from 43% to 55% B, 2.1-12 minutes from 55% to 65% B, 12-18 minutes at 65% to 85% B, 18-20 minutes at 85% to 100% B; 100% B was held for 5 minutes and then the column was allowed to equilibrate to 30% B for another 5 minutes. Total running time was 30 minutes.

The mass spectrometry analysis was performed in both positive ion and negative ion modes. The source voltage was maintained at 3.5 kV in positive ion mode and 2.8 kV in negative ion mode. All other interface settings were identical for the two analysis types. The capillary temperature, jacket gas flow, and auxiliary gas flow were set at 320°C, 40 arb, and 3 arb, respectively. The S-lens has been adjusted to 50 rf. Data were collected in a data-dependent top 10 scan mode (ddMS2). MS full-scan Survey spectra (mass range m/z 80-1200) were acquired with resolution R=70,000 and target AGC 1×106. MS/MS fragmentation was performed using high energy c-trap dissociation (HCD) with R=17,500 resolution and 1×105 AGC target. The step normalized collision energy (NCE) was set to 15, 30 and 45 respectively. The injection volume was 3 µL. For accurate mass-based analysis, regular Lockmass and inter-run calibrations were used. An exclusion list for background ions was generated by testing the same procedural sample, for both positive and negative ESI modes.

3.4 Data processing

Raw data acquired from untargeted analysis were processed with MSDIAL software (Yokohama City, Kanagawa, Japan), version 4.24. The procedure included peak detection, MS2 data deconvolution, compound identification, and peak alignment across all samples. An 85%

cut off was chosen for the identification: this value is based on 6 different similarity scores: 1 for retention time, 1 for m/z, 1 for isotopic pattern and 3 for MS/MS (dot product, inverted dot product and presence). Peaks corresponding to internal standards were removed from the features detected by MS-Dial and were analyzed in the Skyline program to evaluate reproducibility. The dataset containing the m/z values, retention time, peak area, and annotation of aligned files was exported as an Excel file and manually checked for signals from gaps or misregistrations. For quantification, the peak area for the different molecular species detected for each particular lipid was combined (e.g., [M + NH4]+ and [M + Na]+ for TG) followed by normalization using the deuterated internal standard for each lipid class. To obtain an estimated concentration expressed in nmol/mL (plasma), the normalized areas were multiplied by the concentration of the internal standard. An in-house library of standards was also used for lipid identification.

The MetaboAnalyst 4.0 software (www.metaboanalyst.org) was used for the statistical analysis, while the Lipea software (https://lipea.biotec.tu-dresden.de/home) was used for the path analysis. The data provided in this article has been deposited in the EMBL-EBI MetaboLights database under the identifier MTBLS1866.

3.5 Quality control

Retention time stability, mass accuracy, and intensity are essential in LC-MS-based lipidomics analysis. Quality control was ensured by analyzing pooled samples before batch, at the beginning of the batch, and at the end of the batch; entering blank spaces to check for residual interference; using internal standards, directly in plasma samples, which include a series of analyte classes at levels appropriate for the plasma (Avanti SPLASH Lipidomix) and an internal standard (CUDA) prior to LC-MS analysis. Because the assays were performed over a long period of time, the pooled samples were created using plasma from subjects not included in this study, as we wanted to preserve the quality of the patient samples and avoid unnecessary freeze-thaw cycles. Instrument variability was determined by calculating the percent coefficient of variation (CV%) of the internal standards in each sample and pooled quality control samples.

3.6 Therapy response and outcome assessments

X-Tile was used to optimize outcome-based cut-point and to identify lipid species whose plasma values increased or decreased proportionally with the hazard risk of OS (154).

Survival curves were constructed with the Kaplan-Meier method, and then compared with the log-rank test. Variables with significant prognostic effect were entered into multivariate Cox models, in order to explore the independent prognostic effect of specific lipid species. Biochemical response was defined as a 50% or greater decrease from baseline PSA values. Descriptive statistics were employed to evaluate response to treatments based on circulating levels of specific lipid species

4 RESULTS

4.1 Patients' characteristics

Patients involved in this study were mCRPC patients. 1L were starting a first-line treatment for mCRPC, whereas >2L were pretreated patients with at least two lines for mCRPC including at least ARSi and docetaxel. The total number of patients suitable for this analysis was 48.

In the first cohort (1L), 29 patients were included. **Table 4** summarizes patients' baseline characteristics. Patients had: median age of 75 years (range 56-84); a median value of PSA, measured at baseline, of 13.2 ng/mL, ranging from 0.3 ng/mL to 564.9 ng/mL; a median LDH value of 220 U/L, ranging from 138 U/L to 628 U/L; bone metastases were present in 22 patients out of 29 (75.9%) of patients in cohort 1L; only 3 of 29 (10.3%) patients presented visceral metastases; 18 patients out of 29 (62.1%) had more than one metastatic site.

In the >2L cohort, 19 patients were included, with a median age of 70 years (range 58-84). Patients had: a median PSA value of 90.5 ng/mL, with a range from 3.9 ng/mL to 4668.0 ng/mL; a median LDH of 234 U/L, ranging from 121 U/L to 2735 U/L; bone metastases were present in 16 out of 19 (84.2%); visceral metastases were present in 6 of 19 patients (31.6%); 13 patients out of 19 (68.4%) patients had more than one metastatic site.

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.

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

Using LC-MS/MS, a total of 789 circulating lipids were quantified in the plasma of the 48 patients involved in this analysis.

We compared the lipidomic profile of pretreated (>2L) mCRPC patients with those initiating a first-line treatment (1L).

The volcano plot shows the differential expression between >2L and 1L cohorts. The red dots identify overexpressed lipids in the >2L cohort, whereas blue dots represent the under-expressed lipids in the same cohort. 56 lipids were overexpressed (fold change > 1.3, p-value < 0.05), whereas 12 were downregulated (fold change < 0.75, p -value < 0.05).

The heatmap in **Figure 6** shows that lipid species more expressed in the >2L group were triacylglycerols (TG), diacylglycerols (DG), phosphatidylethanolamines (PE) and ceramides (Cer).

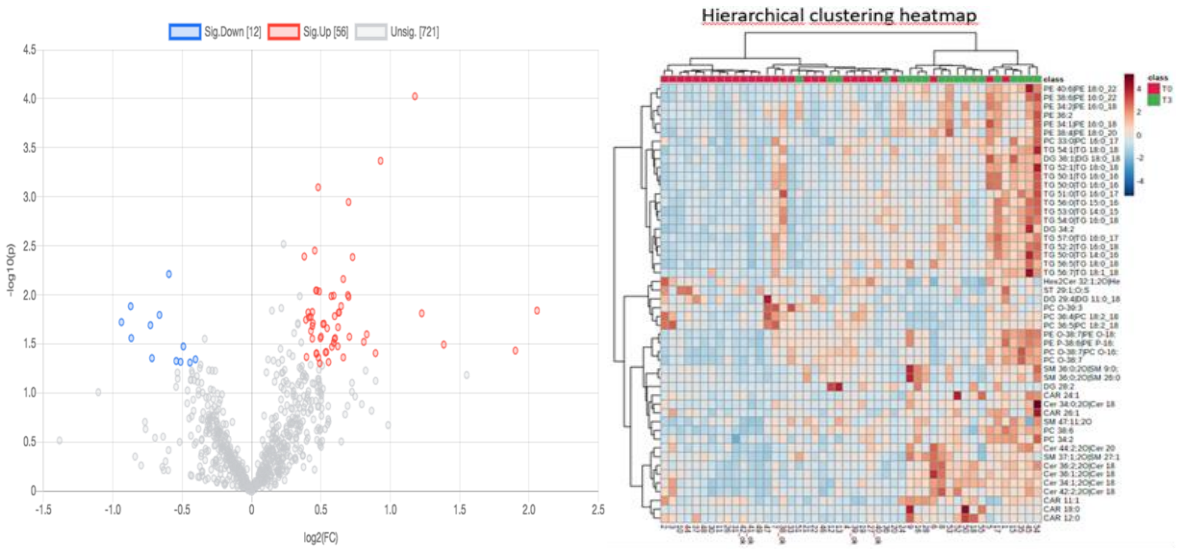


FIGURE 6 LIPID SPECIES DIFFERENTIALLY EXPRESSED IN >2L COMPARED TO 1L PATIENTS.

On the left, volcano plot: overexpressed lipids (in red), downregulated lipids (12, in blue), and non-significant lipids (in grey) in >2L vs. 1L. On the right, the heatmap of the 68 differentially expressed lipids. in the first row, red squares indicate patients belonging to the 1L cohort, whereas green squares the patients belonging to the >2L cohort.

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	4,1628	2,0576	0,01439
CAR 14:0	3,7366	1,9017	0,036801
CAR 20:1	2,6129	1,3856	0,031974
CAR 18:0	2,3402	1,2267	0,015352
PE 40:6|PE 18:0_22:6	2,2618	1,1774	9,44E-05
PE 38:6|PE 16:0_22:6	1,905	0,92982	0,000429
CAR 18:1	1,8577	0,89351	0,03911
TG 56:7|TG 18:1_18:2_20:4	1,7779	0,83018	0,025153
TG 56:7|TG 16:0_18:1_22:6	1,7521	0,80912	0,030021
Cer 36:2;2O|Cer 18:2;2O/18:0	1,6565	0,72814	0,004104
CAR 12:0	1,6303	0,70517	0,02664
SM 36:0;2O|SM 26:0;2O/10:0_SM 36:0;2O	1,6239	0,6995	0,010437
CAR 24:1	1,6236	0,69916	0,001127
SM 36:0;2O|SM 9:0;2O/27:0	1,6179	0,69413	0,010008
TG 52:0|TG 16:0_18:0_18:0	1,5814	0,66117	0,043222
PE 34:1|PE 16:0_18:1	1,581	0,66088	0,006873
Cer 34:0;2O|Cer 18:0;2O/16:0	1,564	0,64525	0,012964
TG 52:1|TG 16:0_18:0_18:1	1,5492	0,63157	0,014998
TG 50:1|TG 16:0_16:0_18:1	1,544	0,62666	0,015135
TG 50:0|TG 16:0_16:0_18:0	1,54	0,62298	0,021297
TG 51:1|TG 16:0_17:0_18:1	1,5381	0,62118	0,033325
DG 36:1|DG 18:0_18:1	1,5182	0,6024	0,02745
PE 34:2|PE 16:0_18:2	1,5143	0,59866	0,016418
CAR 20:0	1,513	0,59741	0,028126
DG 34:2	1,5074	0,59203	0,010138
TG 50:2|TG 16:0_16:1_18:1	1,5069	0,59156	0,030635
TG 53:1|TG 17:0_18:0_18:1	1,4933	0,57847	0,033677
Cer 36:1;2O|Cer 18:1;2O/18:0	1,4895	0,57484	0,010263
TG 51:2|TG 16:0_17:1_18:1	1,4691	0,55493	0,04823
TG 50:0|TG 14:0_16:0_20:0	1,4589	0,54492	0,021797
SM 42:2;2O	1,4538	0,5398	0,038143
PE 36:2|PE 18:0_18:2	1,4466	0,53269	0,038392
PE 36:2	1,4357	0,5218	0,019561
TG 52:2|TG 16:0_18:1_18:1	1,4288	0,5148	0,019798
TG 54:1|TG 18:0_18:0_18:1	1,4149	0,50066	0,026565
TG 52:3|TG 16:0_18:1_18:2	1,4105	0,49625	0,027501
PC O-40:10	1,4059	0,49152	0,04951
PE O-38:7|PE O-18:2_20:5	1,4014	0,48684	0,00918
TG 55:1|TG 18:0_19:0_18:1	1,3967	0,48198	0,043467
Cer 34:1;2O|Cer 18:1;2O/16:0	1,3949	0,48011	0,000798
PE 38:4|PE 18:0_20:4	1,3841	0,46895	0,008987
TG 49:0|TG 15:0_16:0_18:0	1,3836	0,46839	0,039193

PE 36:4|PE 16:0_20:4	1,3818	0,46655	0,039639
Cer 44:2;2O|Cer 20:1;2O/24:1	1,3794	0,46401	0,008974
Cer 42:2;2O|Cer 18:1;2O/24:1	1,3712	0,45543	0,003521
TG 51:0|TG 16:0_17:0_18:0	1,3583	0,44175	0,019295
SM 37:1;2O|SM 27:1;2O/10:0	1,355	0,43828	0,020679
PC O-38:7|PC O-16:1_22:6	1,3547	0,43794	0,014878
TG 56:6|TG 16:0_18:1_22:5	1,3546	0,43784	0,027813
PE P-38:6|PE P-16:0_22:6	1,3451	0,42772	0,023263
PC O-38:7	1,3438	0,42627	0,016652
TG 56:5|TG 18:0_18:1_20:4	1,3321	0,41369	0,016882
CAR 26:1	1,327	0,40819	0,015041
SM 34:0;2O|SM 10:0;2O/24:0	1,3156	0,39569	0,042839
CAR 11:1	1,3118	0,39155	0,017884
TG 53:0|TG 14:0_15:0_24:0	1,3014	0,38007	0,004049
TG 58:0|TG 16:0_17:0_25:0	1,282	0,35834	0,030393
TG 56:0|TG 15:0_16:0_25:0	1,2726	0,34775	0,010269
PC 33:0|PC 16:0_17:0	1,2607	0,33421	0,019922
TG 54:0|TG 16:0_18:0_20:0	1,2606	0,33407	0,019337
TG 57:0|TG 16:0_17:0_24:0	1,2369	0,30674	0,024409
TG 56:4|TG 18:0_18:1_20:3	1,228	0,29626	0,041971
SM 36:2;2O|SM 16:1;2O/20:1	1,2073	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	log2(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 UNDEREXPRESSED LIPIDS IN >2L COMPARED TO 1L PATIENTS, BASED 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.

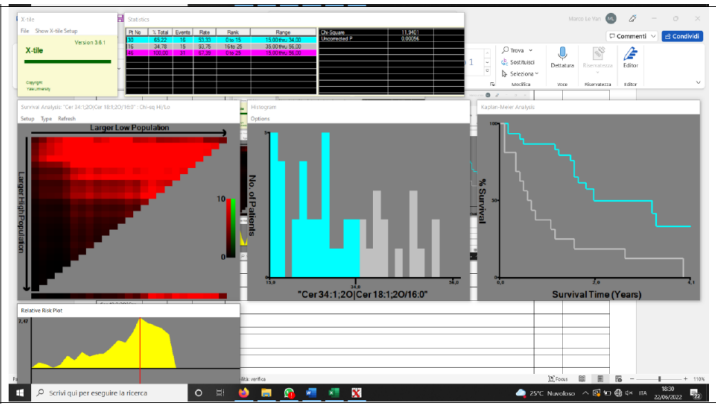
4.2.1 Explorative analysis to assess the association of lipid species with prognosis

We used X-Tile to optimize outcome-based cut-point and to identify lipid species whose plasma values increased or decreased proportionally with the hazard risk of OS (154).

Among all deregulated lipids identified above, we found that plasma values of the following lipid species increased proportionally with the risk of death and were significantly associated with OS using an appropriate cut-point (**Figure 7**):

- **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**

Of significant interest, Cer 42:2;2O|Cer 18:1;2O/24:1 was the same lipid included in the previously reported 3-lipid signature (149). Plasma values of under-expressed lipids did not show proportional association with OS.

Cer 34:1;2O|Cer 18:1;2O/16:0	1,3949	0,48011	0,00079806		7.47	0.00056

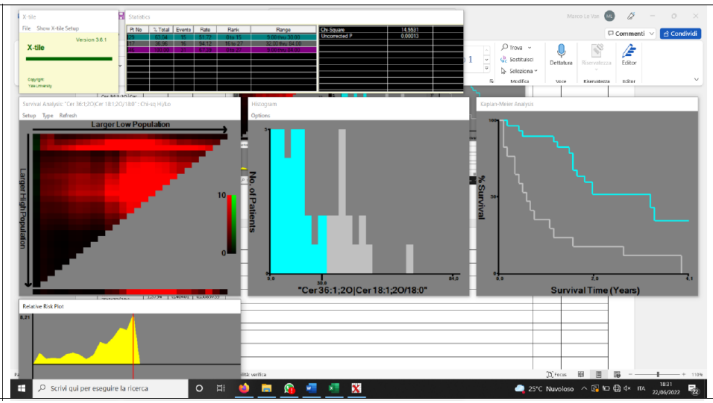
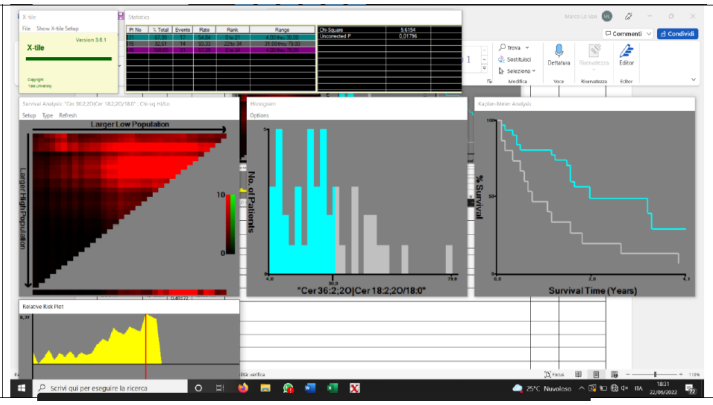
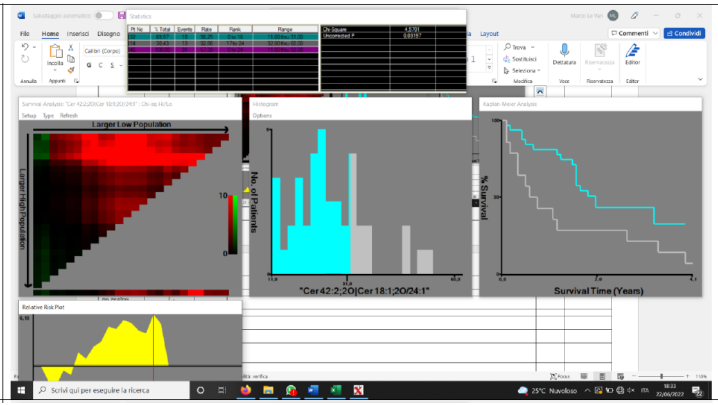
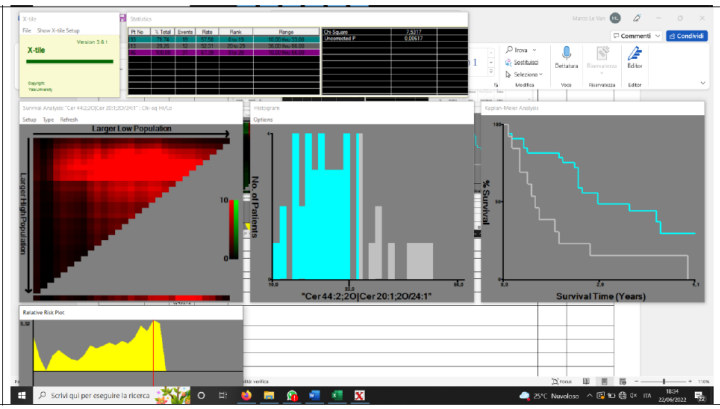
Cer 36:1;2O|Cer 18:1;2O/18:0	1,4895	0,57484	0,010263		8.21	0.00013
Cer 36:2;2O|Cer 18:2;2O/18:0	1,6565	0,72814	0,0041042		6.77	0.0179
Cer 42:2;2O|Cer 18:1;2O/24:1	1,3712	0,45543	0,0035211		6.13	0.0319
Cer 44:2;2O|Cer 20:1;2O/24:1	1,3794	0,46401	0,0089739		5.52	0.00617

FIGURE 7 LIPID SPECIES WHOSE PLASMA VALUES INCREASED PROPORTIONALLY WITH THE HAZARD RISK OF OS

4.2.2 Association of Cer 36:1;2O|Cer 18:1;2O/18:0 with clinical outcome

The five ceramides identified in the discovery analysis were tested in multivariate analysis, to exclude the interference of significant prognostic variables, in particular the line of treatment.

In fact, this discovery analysis identified lipid species differentially expressed in >2L patients compared to 1L patients. Overall, >2L patients show intrinsic reduced survival compared to 1L patients and it was likely that species identified in >2L cohort showed association with OS, because higher values of lipids were found in >2L patients compared to 1L patients.

Cer 36:1;2O|Cer 18:1;2O/18:0 (nomenclature in **Figure 8**) was the only lipid species that was associated with prognosis in univariate analysis (**Figure 9**) and retained the statistical significance after adjustment for basal PSA and line of treatment. Patients with higher plasma values showed an HR for OS of 3.3 (95% CI 1.4-7.8, p-value = 0.007) compared to those with lower values (**Figure 10**).

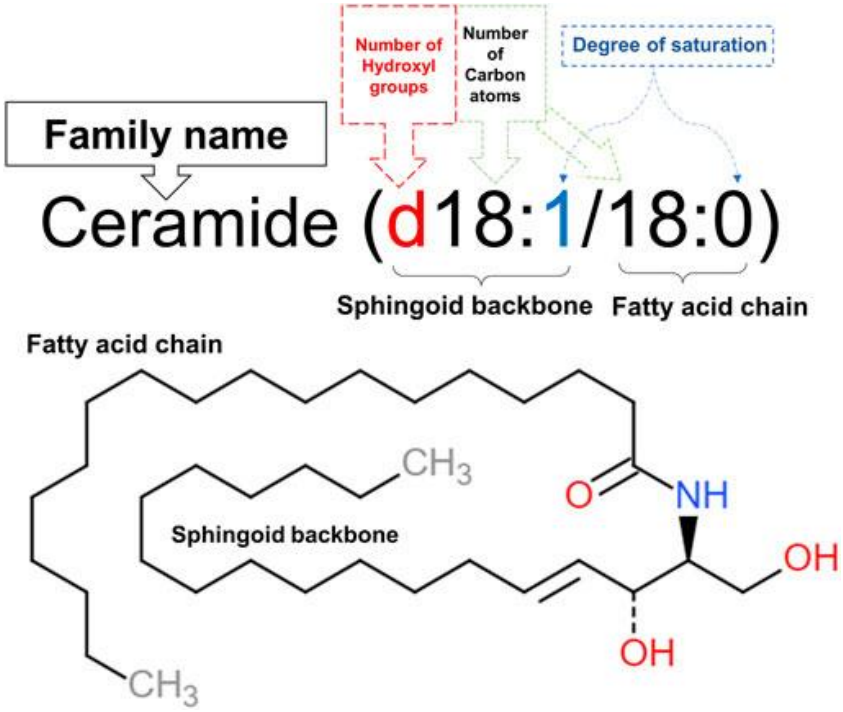


FIGURE 8 NOMENCLATURE OF C18:0 CERAMIDE

The first part of the name (d18:1) denotes the 18 carbon atoms, having one double bond in its sphingoid backbone along with two hydroxyl groups. This sphingosine chain is attached to a saturated fatty acid chain, represented by the second part of the name (18:0), through an amide bond (155).

Median OS was 6 months (CI 95%, 2.1-9.9) compared to 39 months (CI 95%, 16.1-61.9) in patients with high plasma levels. The identified plasma cut-off was identified in 30 ng/mL.

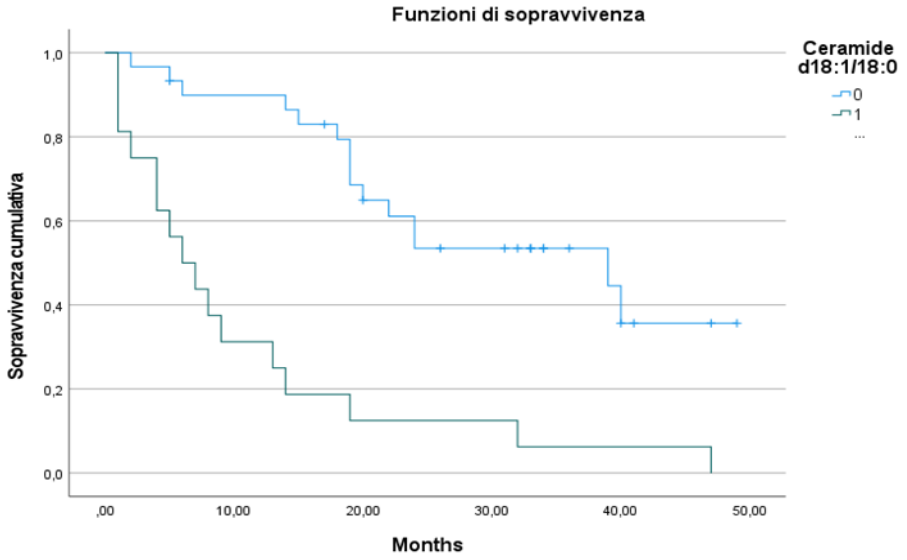


FIGURE 9 UNIVARIATE ANALYSIS OF CER 36:1;2O|CER 18:1;2O/18:0

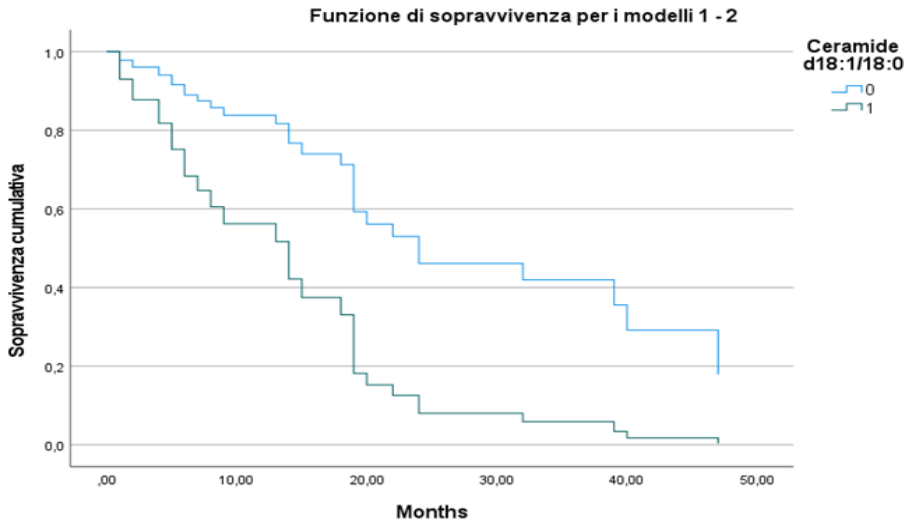


FIGURE 10 MULTIVARIATE ANALYSIS OF CER 36:1;2O|CER 18:1;2O/18:0

Of interest, the subgroup analysis, even in the absence of adequate power and statistical significance, confirmed an unfavorable association between Cer 36:1;2O|Cer 18:1;2O/18:0 levels and patients' survival, regardless of the line of treatment. In 1L cohort, the Kaplan-Meier curve showed a median survival of 14 months compared to 39 months for subjects with high vs. low ceramide levels, respectively (p-value = 0.098) (**Figure 11**).

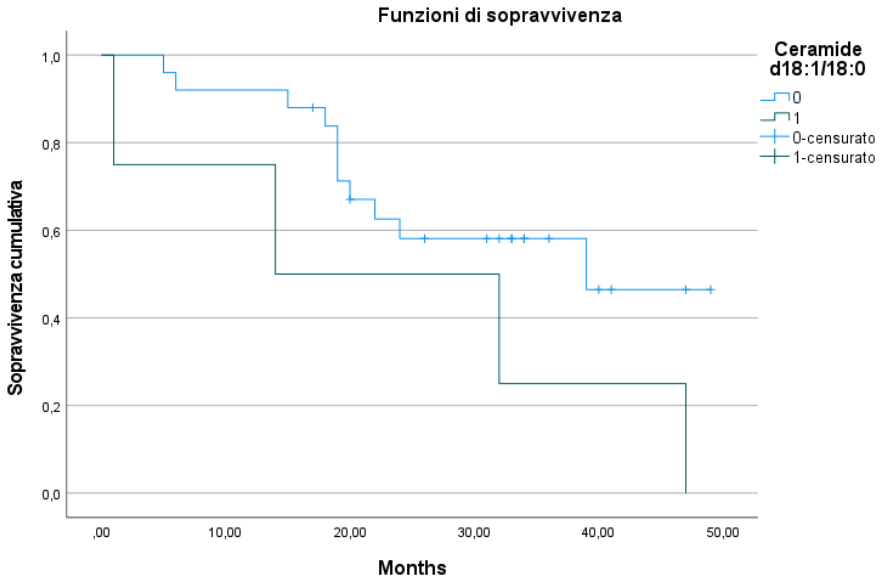


FIGURE 11 UNIVARIATE ANALYSIS OF CER 36:1;2O|CER 18:1;2O/18:0 IN 1L COHORT

In >2L group, the Kaplan-Meier survival curve showed a median survival of 5 months compared to 24 months for patients with high and low ceramide levels, respectively (p-value = 0.025) **(Figure 12)**.

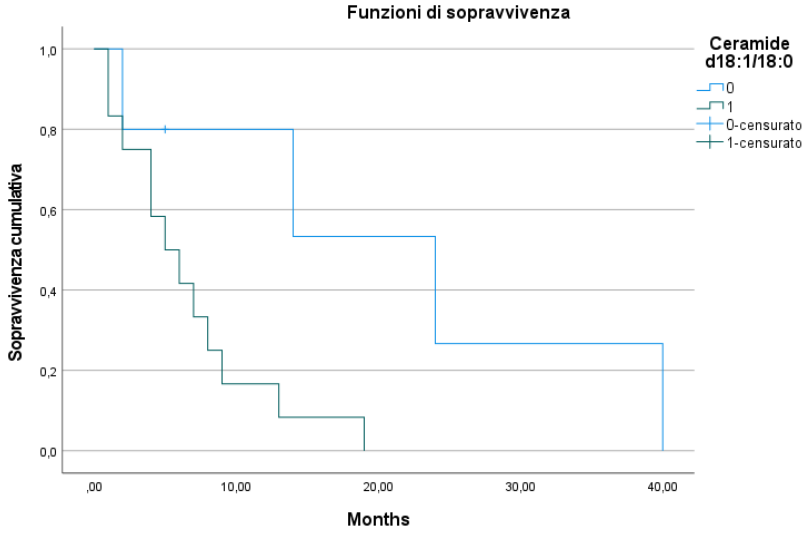


FIGURE 12 UNIVARIATE ANALYSIS OF CER 36:1;2O|CER 18:1;2O/18:0 IN >2L COHORT

The graph reported in **Figure 13** shows the variation in the risk of death between populations based on the different cut-offs of Cer 36:1;2O|Cer 18:1;2O/18:0. For the cut-off

selected in this study (indicated by the red line in the figure), the risk between the two cohorts reaches a maximum of 8.21 (p-value = 0.00013).

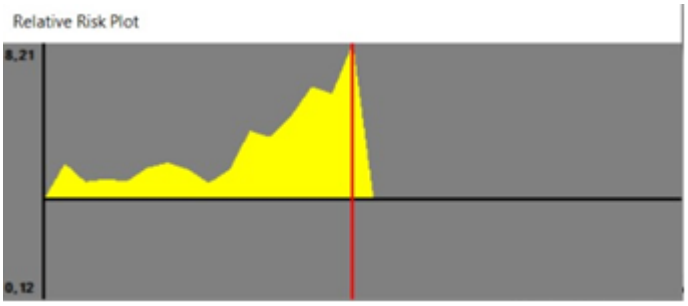


FIGURE 13 RELATIVE RISK PLOT OF CER 36:1;2O|CER 18:1;2O/18:0

4.2.3 Association of Cer 36:1;2O|Cer 18:1;2O/18:0 with PSA response

Ultimately, the correlation between plasma Cer 36:1;2O|Cer 18:1;2O/18:0 levels and response to treatment was evaluated (**Figure 14**). Response to treatment was assessed through the achievement of PSA50 (50% or greater reduction in PSA values). In the waterfall plot below, patients with high levels of circulating ceramide (>30 ng/mL) are shown in red, whereas patients with low levels of ceramide (< 30 ng/mL) are shown in blue.

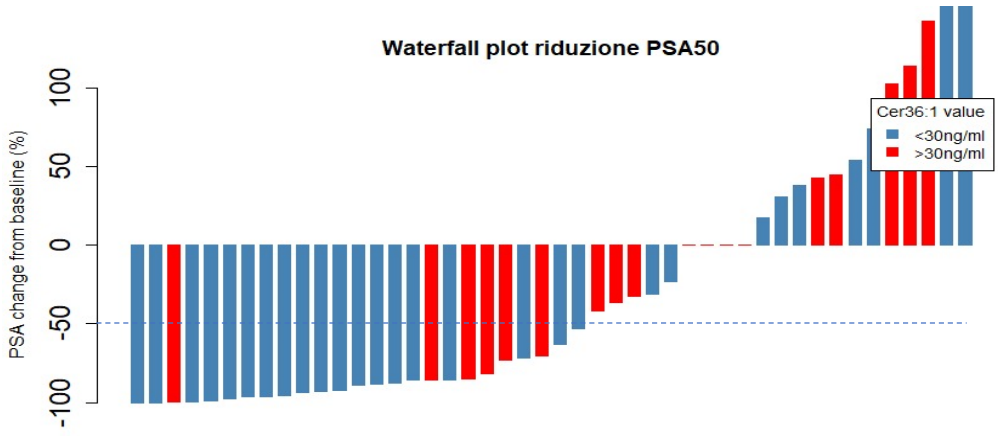


FIGURE 14 WATERFALL PLOT OF PSA RESPONSE ACCORDING TO CERAMIDE VALUES

Overall, 19 of 28 patients (67.9%) who had low ceramide levels achieved a 50% reduction in PSA; conversely, only 6 out of 14 patients (42.9%) who had high ceramide levels achieved a 50% reduction in PSA. Of patients who initiated on first-line ARSi, 1 of 2 with elevated ceramide levels achieved PSA50. Of two patients who started first-line docetaxel with elevated Cer 36:1;2O|Cer 18:1;2O/18:0 levels, 2 of 2 achieved PSA50.

4.3 Three-lipid signature and association with clinical outcome

We also explored the association with clinical outcome of 3 lipids included in the previously validated 3-lipid signature, namely ceramide 42:2;2O|Cer 18:1;2O/24:1, sphingomyelin 34:2;2O|SM 18:2;2O/16:0 and phosphatidylcholine 32:0|PC 16:0_16:0 (see paragraph 1.4.2) (148,149).

The ROC curves for OS of these lipids were plotted. The ROC curve of ceramide d18:1/24:1 (**Figure 15**) showed an area under the curve of 0.771 (95% CI, 0.623–0.894). The ROC curve of sphingomyelin d18:2;2O/16:0 (**Figure 16**) showed an area under the curve of 0.577 (95% CI, 0.423-0.747). The ROC curve of phosphatidylcholine 16:0/16:0 (**Figure 17**) showed an area under the curve of 0.637 (95% CI, 0.467-0.775).

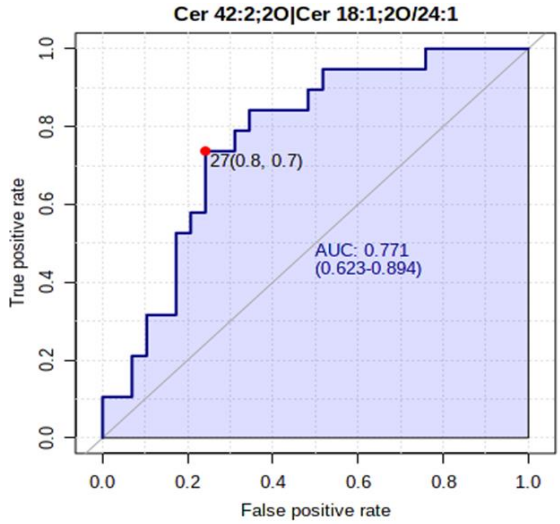


FIGURE 15 ROC CURVE OF CER 42:2;2O|CER 18:1;2O/24:1, WITH OPTIMAL CUT-OFF POINT

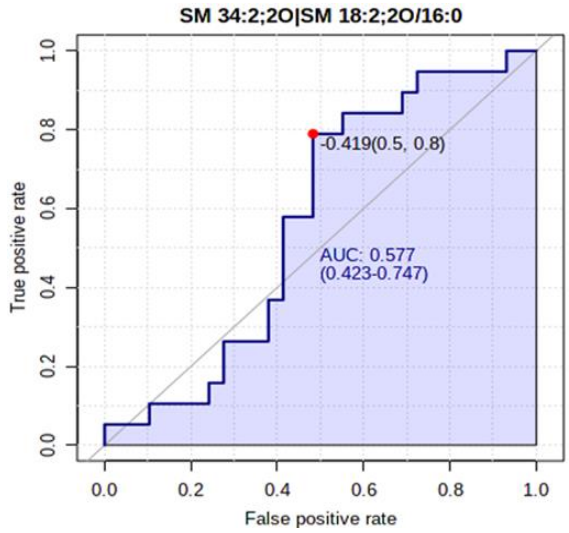


FIGURE 16 ROC CURVE OF SM 34:2;2O|SM 18:2;2O/16:0 WITH OPTIMAL CUT-OFF POINT

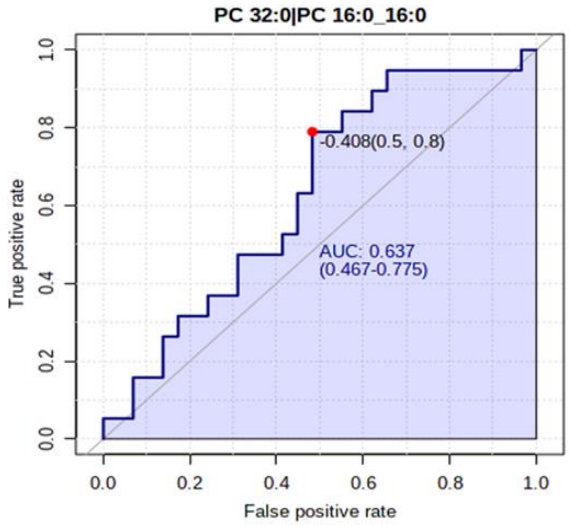


FIGURE 17 ROC CURVE OF PC 32:0|PC 16:0_16:0 WITH OPTIMAL CUT-OFF POINT

4.3.1 Association of 3-lipid signature with OS

Kaplan-Meier curves were constructed to assess the association of lipids included in the 3-lipid signature with OS.

The univariate analysis of Cer 42:2;2O|Cer 18:1;2O/24:1 showed that patients with higher levels of ceramide had worse survival compared to those with lower levels (**Figure 18**). Median OS was 7 months for those with high levels (95% CI, 17.7-30.3) and 24 months for

those with low levels (95% CI, 1.5-12.5). The cut-off used to determine high or low ceramide level was 31 ng/mL. The result of the log-rank test was statistically significant (p-value = 0.025).

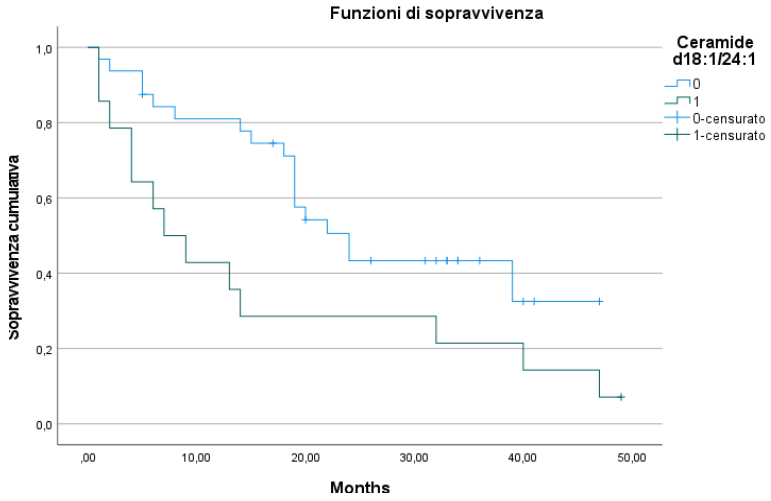


FIGURE 18 UNIVARIATE ANALYSIS FOR OS OF CER 42:2;2O|CER 18:1;2O/24:1

However, the multivariate analysis, after adjustment for treatment line and baseline PSA, showed no statistically significant association with OS (HR= 1.4, 95% CI 0.7-3.1, p-value = 0.347) (**Figure 19**).

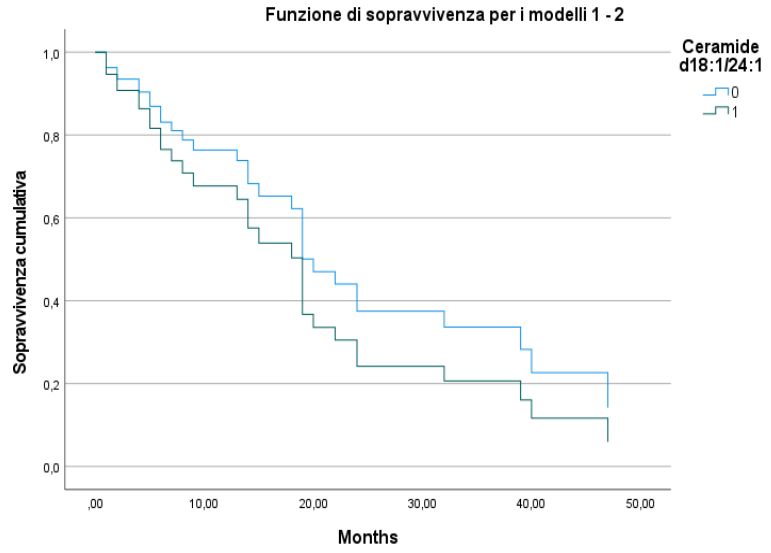


FIGURE 19 MULTIVARIATE ANALYSIS FOR OS OF CER 42:2;2O|CER 18:1;2O/24:1

We did not find any association between plasma values of sphingomyelin 34:2;2O|SM 18:2;2O/16:0 and OS (**Figure 20**). The median OS was 22 months in patients with high sphingomyelin levels (95% CI, 17.6-20.4) and 19 months in those with low sphingomyelin levels (95% CI, 14.8-23.2). The cut-off used as a threshold to determine high or low sphingomyelin level was 5.5 ng/mL. T-test was not statistically significant (p-value = 0.982).

Multivariate analysis of sphingomyelin 34:2;2O|SM 18:2;2O/16:0, adjusted for line of treatment and baseline PSA, confirmed no statistically association with OS (HR= 0.6, 95% CI, 0.2-1.4, p-value = 0.224) (**Figure 21**).

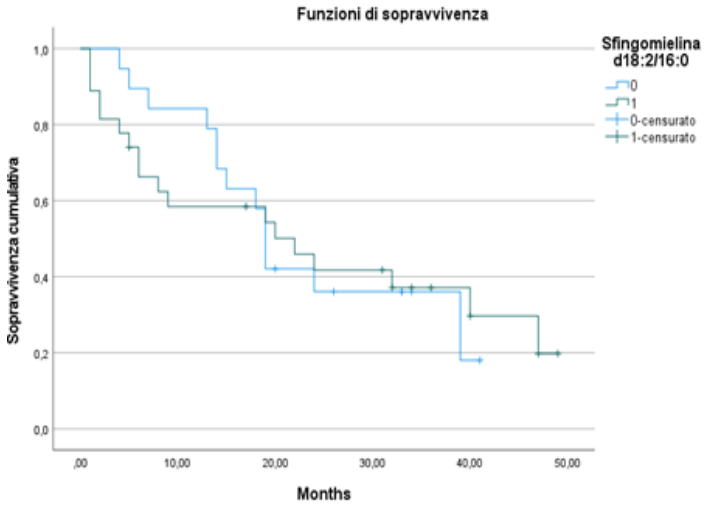


FIGURE 20 UNIVARIATE ANALYSIS FOR OS OF SM 34:2;2O|SM 18:2;2O/16:0

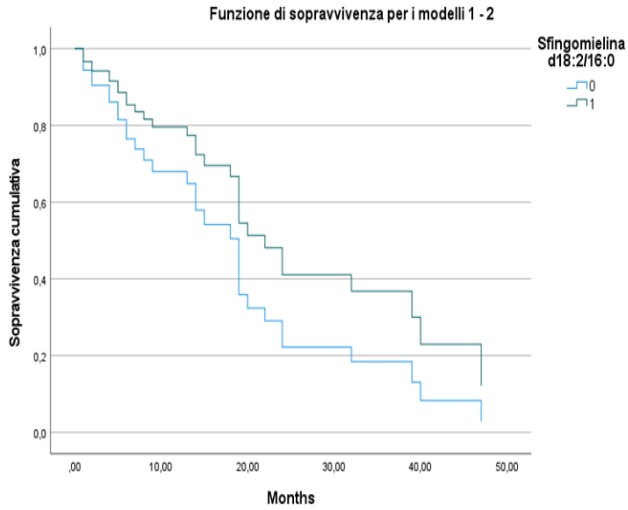


FIGURE 21 MULTIVARIATE ANALYSIS FOR OS OF SM 34:2;2O|SM 18:2;2O/16:0

We also did not find any statistically significant association between plasma values of phosphatidylcholine 32:0|PC 16:0_16:0 and OS (**Figure 22**). The median OS was 32 months in patients with high phosphatidylcholine levels (CI 95%, 9.5-54.5) and 19 months in those with low sphingomyelin levels (CI 95%, 14.8- 23.2). The cut-off used as a threshold to determine high or low sphingomyelin level was 27.5 ng/mL. T-test was not statistically significant (p-value = 0.371).

Multivariate analysis of phosphatidylcholine 32:0|PC 16:0_16:0, adjusted for line of treatment and baseline PSA, confirmed no statistically association with OS (HR=1.9, 0.8-4.2, p-value = 0.102) (**Figure 23**).

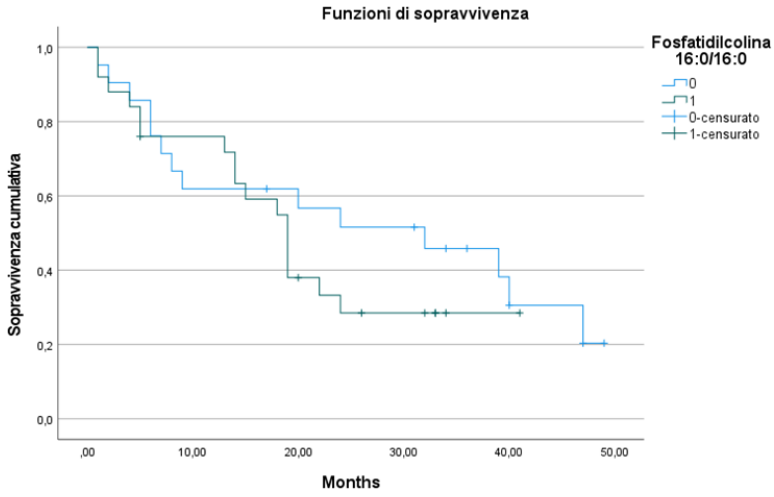


FIGURE 22 UNIVARIATE ANALYSIS FOR OS OF PC 32:0|PC 16:0_16:0

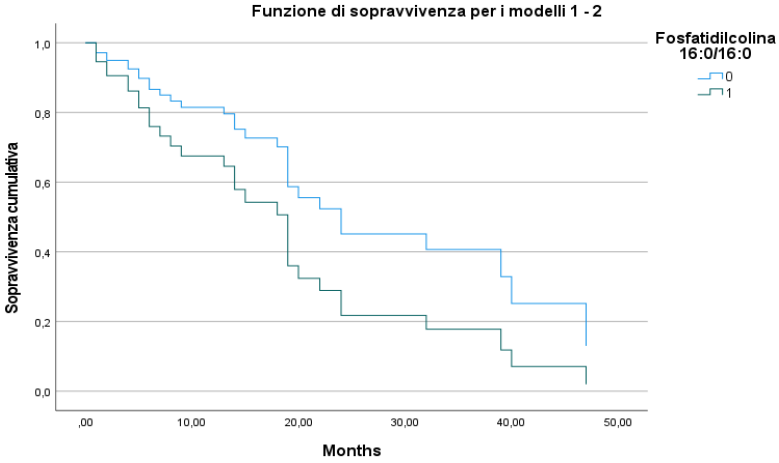


FIGURE 23 MULTIVARIATE ANALYSIS FOR OS OF PC 32:0|PC 16:0_16:0

5 DISCUSSION

Multiple prognostic and predictive factors have been investigated to identify patients with PCa at increased risk of progression or death. In the case of metastatic castration-resistant disease, only alterations in DNA damage and response genes, in particular *BRCA1* and *BRCA2*, have been validated to guide therapeutic choices (60). Therefore, it remains a clinical unmet need to identify new prognostic and predictive factors. Some studies have been already conducted to assess the levels of lipid species in patients affected by mCRPC, in order to explore their prognostic and predictive significance. In this regard, the most interesting studies were reported by an Australian research group (148-150) (see also paragraph 1.4.2).

The first study explored the role of lipids in a discovery cohort of 96 patients affected by mCRPC (148). Unsupervised analysis of lipidomic profiles identified two patient subgroups with significant survival differences. Overall, 46 lipids, predominantly sphingolipids, were individually prognostic and higher levels were associated with poor prognosis. From this discovery cohort, the authors also derived a prognostic three-lipid signature that included ceramide d18:1/24:1, sphingomyelin d18:2/16:0 and phosphatidylcholine 16:0/16:0. This signature and the 46 individually prognostic lipids were then tested in a validation cohort that included 63 patients (148). The 3-lipid signature was validated, confirming its ability to identify patients with poor survival (11.3 vs. 21.4 months; HR 4.78, 95% CI 2.06–11.1). In addition, 19 of 46 prognostic lipids previously identified were also validated. These lipids included four ceramides [Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/24:1)], monohexosylceramide (d18:1/16:0), GM3 gangliosides, free cholesterol, sphingomyelins and phosphatidylcholines.

In a subsequent study, the authors performed a comprehensive lipidomic analysis on pre-treatment plasma samples from patients with localized PCa (N = 389), mHSPC (N = 44), and mCRPC (validation cohort, N = 137) (149). Circulating lipid profiles characterized by elevated levels of ceramides were associated with metastatic relapse in localized PCa (HR 5.80, 95% CI 3.04–11.1), earlier testosterone suppression failure in mHSPC (HR 3.70, 95% CI 1.37–10.0), and shorter OS in mCRPC (HR 2.54, 95% CI 1.73–3.72). The prognostic significance of circulating lipid profiles in localized PCa was independent of clinic-pathological and metabolic

factors. In addition, the 3-lipid signature was verified in the mCRPC validation cohort (HR 2.39, 95% CI 1.63–3.51).

In the third study from the Australian group, plasma lipidomic analysis and cell-free DNA (cfDNA) sequencing was performed in 106 men with mCRPC starting docetaxel, cabazitaxel, abiraterone or enzalutamide (discovery cohort) and 94 men with mCRPC starting docetaxel (validation cohort) (150). The 3-lipid signature was associated with shorter OS in the discovery and validation cohorts. Elevated circulating sphingolipids, especially ceramides, were associated with *AR*, *TP53*, *RB1*, *PI3K* and aggressive-variant prostate cancer (AVPC) aberrations in mCRPC, and the combination of lipid and genetic alterations predicted for worse prognosis.

In the light of the interesting discoveries made in the field of lipidomic analyses applied to PCa, the main objective of our study was to analyze the lipidomic profile of patients affected by mCRPC, in order to identify lipid species that could serve as new prognostic and predictive biomarkers, as well as to validate the previously proposed 3-lipid signature. Our study involved 48 patients with mCRPC who were going to start a first-line treatment for mCRPC (1L cohort – n=29) or who had already received two or more lines of treatment for mCRPC (>2L cohort – n=19).

A total of 789 lipids were analyzed and identified, and a comparison between lipid levels in 1L and >2L cohorts was performed. From this preliminary investigation, we identified 63 overexpressed lipids (fold change > 1.2 and p-value < 0.05) in the >2L cohort compared to 1L cohort, and 12 downregulated lipids (fold change < 0.75 and p -value < 0.05). We highlight that ceramide d18:1/24:1, previously included in the 3-lipid signature (149), was also significantly overexpressed in our highly pretreated patients.

Among all deregulated lipids identified above, we found that plasma values of specific ceramides (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) increased proportionally with the risk of death for any cause and were significantly associated with OS using an appropriate cut-point. Conversely, plasma values of under-expressed lipids did not show proportional association with OS.

Cer 36:1;2O|Cer 18:1;2O/18:0 was the only lipid species that retained the statistical significance after adjustment for basal PSA and line of treatment in multivariate analysis. This ceramide was not included in the 3-lipid signature, but it was reported among the individually prognostic lipids in the study performed by Lin and colleagues (148). Patients with higher levels of Cer 18:1/18:0 showed a significantly increased risk of death 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 (CI 95%, 2.1-9.9) compared to 39 months (CI 95%, 16.1-61.9), respectively. Of interest, the subgroup analysis confirmed an unfavorable association between Cer 18:1/18:0 levels and patients' OS in both 1L and >2L cohorts separately analyzed. We also explored the association between Cer 18:1/18:0 and response to treatments. Overall, 42.9% of patients with high ceramide levels achieved PSA50, compared to 67.9% with low ceramide levels. However, given high patients' heterogeneity and small sample size, further investigations are needed to assess the potential predictive value of this ceramide.

Regarding the 3-lipid signature, we did not identify any association between sphingomyelin d18:2/16:0 or phosphatidylcholine 16:0/16:0 and OS in our cohort of mCRPC patients. Ceramide d18:1/24:1 showed a statistically significant association with OS in univariate analysis, however this association was not confirmed in multivariate analysis.

Overall, all these data are consistent with the assumption that deregulated lipid metabolism and elevated circulating ceramide levels are associated with poor outcomes in patients with mCRPC. Classically, ceramides have anti-tumorigenic functions, inducing senescence and growth inhibition in cancer. However, some studies suggest that ceramide effects are context dependent and rely on downstream effectors, which can both promote or inhibit tumor growth (156). Depending on the length of their acyl side chain, all ceramides can be grouped as long-chain (C14:0-C20:0), very-long-chain (C22:0-C26:0) and ultra-long-chain (>26 carbons). Different ceramide length often results in distinct biological activity. In our study, we found that both long- and very-long-chain ceramides can have prognostic significance, and higher values are associated with poor prognosis.

The enzyme acid ceramidase (AC) might affect the different roles of ceramides. In preclinical models, AC significantly altered the expression of ceramide species without affecting the total levels. In AC-overexpressing DU145 cells, low levels of C14-C20 (long-chain) and elevated levels of C24, C24:1 (very-long-chain) ceramides were detected. This was

associated with increased proliferation, migration and tumorigenicity in vivo, which were reversed by pharmacological or genetic AC inhibition (157,158).

Long chain ceramides may promote aggressive PCa through their metabolite, sphingosine-1-phosphate (S1P). S1P is produced by a series of enzymatic reactions involving AC and sphingosine kinase (SPHK), which both show high expression and activity in PCa cancers (159,160). Elevated SPHK gene expression in localized PCa is associated with disease progression (149). S1P can promote cancer cell proliferation, survival and metastasis; it can also regulate lymphocyte trafficking by acting on S1P-specific receptors present on immune and cancer cells (161). Mice lacking SPNS2, the lymphoid tissue-specific transporter of S1P, show reduced metastatic colonization (162). Enhanced ceramide-S1P signalling may mediate ARSi resistance induced by AR gain, as men with mCRPC had significantly shorter ARSi treatment duration if their tumours had AR gain in combination with increased expression of sphingolipid genes (163).

These data support the rationale to explore new therapeutic targets in patients with PCa. Pharmacological inhibition (with ABC294640) of SPHK2, one of the two SPHK isoforms that catalyzes the synthesis of S1P from sphingosine, effectively reduced CRPC cell proliferation and xenograft tumor growth by targeting AR and the oncogene MYC (164). In vitro experiments also showed that de novo resistance to enzalutamide in androgen-independent cells can be reversed with SPHK inhibitors in vitro (163). SPHK inhibitors (165), are being tested in patients with cancer: ABC294640 completed a Phase I trial for advanced solid tumors (NCT01488513) and is undergoing Phase IIA clinical trials for cholangiocarcinoma (NCT03377179). Ceramides are also activators of PLA2, an enzyme that releases arachidonic acid for subsequent conversion to prostaglandins, molecules involved in inflammation, immunity, and tumor growth modulation. Increased levels of prostaglandins, like PGE2, are associated with enhanced PCa proliferation and invasion, which can be reversed by the use of cyclooxygenases (COX) inhibitors, suggesting the involvement of PGE2 in PCa progression (166,167).

Aberrant ceramide metabolism in PCa could be finally modulated by targeting the metabolic environment of the host. High-fat feeding increases circulating ceramides (168), and promoted inflammation and metastasis through S1P signalling in a breast cancer mouse model (169). Importantly, this metabolic state can be pharmacologically normalised;

cardiovascular and obesity studies demonstrate that elevated circulating ceramides can be decreased using cholesterol-lowering drugs (statins and PCSK9 inhibitors) (170,171) and exercise (172).

In summary, our data confirm that specific lipid species, in particular ceramides, show a prognostic and potentially predictive value in patients with mCPRC. Our results also pave the way and rationale for targeting 'host' or tumor sphingolipid metabolism in patients with PCa.

6 CONCLUSIONS

In this study, we explored the prognostic and predictive value of several lipid species in patients with mCRPC by using an untargeted lipidomic approach that combined mass spectrometry techniques with liquid chromatography (LC-MS/MS).

We confirmed that long- and very-long chain ceramides show prognostic significance in patients with mCRPC and could serve as new clinical biomarkers. We found that Cer 18:1;18:0 had independent prognostic capacity in our cohort of patients, being associated with OS after adjustment for relevant confounding factors.

Lipids included in the previously reported 3-lipid signature had not statistically significant prognostic significance in our cohort, except for ceramide d18:1/24:1 that was associated with patient's OS in univariate analysis, but not in multivariate analysis.

In the light of these results, further studies are needed to validate the prognostic significance of Cer 18:1;18:0 and to explore the predictive value of long-chain ceramides.

Finally, literature data support the notion that targeting sphingolipid metabolism is a feasible approach that could be tested in patients with mCRPC and may lead to the discovery of new active drugs in PCa.

7 APPENDIX

7.1 Abbreviations

AC = Acid Ceramidase
ADT = Androgen Deprivation Therapy
AR = Androgen Receptor
ARFL = Androgen Receptor Full Length
ARSi = Androgen Receptor Signaling inhibitor
AR-V7 = Androgen Receptor Variant 7
Cer = Ceramide
cfDNA = cell-free DNA
CI = Confidence Interval
CTC = Circulating Tumor Cells
EMA = European Medicines Agency
ESI = ElectroSpray Ionization
FABP = Fatty Acid Binding Protein
FDA = Food and Drug Administration
HILIC = Hidrophilic Interaction Liquid Chromatography
HPLC/UHPLC = High Performance Liquid Chromatography / Ultra High Performance Liquid Chromatography
HR = Hazard Ratio
LC-MS/MS = Liquid Chromatography – Mass Spectrometry
mCRPC = metastatic Castration Resistance Prostate Cancer
mHSPC = metastatic Hormone Sensitive Prostate Cancer
OS = Overall Survival
PC = Phosphatidylcholine
PCa = Prostate Cancer
PS = Performance Status
PSA = Prostate Specific Antigen
PSA50 = >50% PSA decline after treatment start
QoL = Quality of Life
rPFS = radiographic Progression Free Survival
SM = Sphingomyelin
SPHK = Sphingosine Kinase
S1P = Sphingosine-1-Phosphate

7.2 Summary of figures

7.3 Summary of tables

8 BIBLIOGRAPHY

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