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TOPIC PAPER

Discovery and validation of a serum microRNA signature to characterize oligo- and polymetastatic prostate cancer: not ready for prime time

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Abstract

Purpose Patients with oligometastatic prostate cancer (PC) may benefit from metastasis-directed therapy (MDT), delaying disease progression and the start of palliative systemic treatment. However, a significant proportion of oligometastatic PC patients progress to polymetastatic PC within a year following MDT, suggesting an underestimation of the metastatic load by current staging modalities. Molecular markers could help to identify true oligometastatic patients eligible for MDT.

Methods Patients with asymptomatic biochemical recurrence following primary PC treatment were classified as oligo- or polymetastatic based on 18^{F}-choline PET/CT imaging. Oligometastatic patients had up to three metastases at baseline and did not progress to more than three lesions following MDT or surveillance within 1 year of diagnosis of metastases. Polymetastatic patients had > 3 metastases at baseline or developed > 3 metastases within 1 year following imaging. A model aiming to prospectively distinguish oligo- and polymetastatic PC patients was trained using clinicopathological parameters and serum-derived microRNA expression profiles from a discovery cohort of 20 oligometastatic and 20 polymetastatic PC patients. To confirm the models predictive performance, it was applied on biomarker data obtained from an independent validation cohort of 44 patients with oligometastatic and 39 patients with polymetastatic disease.

Results Oligometastatic PC patients had a more favorable prognosis compared to polymetastatic ones, as defined by a significantly longer median CRPC-free survival (not reached versus 38 months; 95% confidence interval 31–45 months with $P < 0.001$). Despite the good performance of a predictive model trained on the discovery cohort, with an AUC of 0.833 (0.693–0.973; 95% CI) and a sensitivity of 0.894 (0.714–1.000; 95% CI) for oligometastatic disease, none of the miRNA targets were found to be differentially expressed between oligo- and polymetastatic PC patients in the signature validation cohort. The multivariate model had an AUC of 0.393 (0.534 after cross-validation) and therefore, no predictive ability.

Conclusions Although PC patients with oligometastatic disease had a more favorable prognosis, no serum-derived biomarkers allowing for prospective discrimination of oligo- and polymetastatic prostate cancer patients could be identified.

Keywords Prostate cancer · Oligometastasis · miRNA · Serum · Biomarker · Machine learning

Introduction

The oligometastatic state, an intermediate state of metastatic disease, has been described by Hellman and Weichselbaum since 1995 [1]. This metastatic state consists of low-volume disease, typically defined as the presence of up to 3–5 metastases [2]. Currently, there is increasing evidence that the prognosis of this low-volume state in prostate cancer (PC) could be more favorable compared to polymetastatic disease [3–7]. Moreover, a metastasis-directed treatment (MDT) approach could potentially control metastatic spread and

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enhance overall survival in oligometastatic PC patients [1, 7–10]. Several retrospective studies have already suggested that MDT could delay further disease progression and defer palliative androgen deprivation therapy (ADT) [2, 11]. In a phase II prospective randomized trial by Ost et al., MDT was associated with a longer ADT-free survival compared to surveillance alone [12]. With the emerging use of whole-body magnetic resonance imaging and ^{18}F- and ^{11}C-choline or ^{68}Ga-prostate-specific membrane antigen (PSMA) positron-emission tomography/computed tomography (PET/CT), detection rates of metastatic disease have improved in patients with early biochemical recurrence [13, 14]. Nonetheless, detection rates of ^{68}Ga PSMA-PET/CT for metastatic PC recurrence increase in parallel with rising serum prostate-specific antigen (PSA) levels [15]. Considering this rationale, it is important to state that for some patients, the definition of oligometastatic PC is in fact an underestimation of the true metastatic load or disease kinetics, especially at low PSA levels. A metastasis-directed approach in these patients could result in early disease recurrence, making this a less valuable treatment option for them [16]. This stresses the need for biomarkers to accurately capture the ongoing disease process and to select patients that might benefit from MDT. As oligometastatic PC is considered as an intermediate state between localized and widespread metastatic disease, differences in genetic and molecular background might play a role in the progression to an oligometastatic, and differentiation away from a polymetastatic phenotype [17]. There is a growing body of evidence to support that adaptive communication between cancer cells and their local and distant environment through microRNAs (miRNAs) drives metastatic progression. These small noncoding RNAs of 18–24 nucleotides in length control gene expression post-transcriptionally and regulate multiple steps in the metastatic cascade [18]. The aim of this study was to find an effective stratification model for metastatic PC patients, allowing to prospectively distinguish oligometastatic from polymetastatic cases by a combination of clinicopathological parameters and a liquid biopsy-derived miRNA signature.

Materials and methods

Patients

This study was conducted after approval by the Ghent University Hospital ethics committee (EC2013/1004) and in accordance to the guidelines and regulations of the Helsinki Declaration. Participants had given written informed consent. Patients were eligible in case of an asymptomatic biochemical recurrence following primary prostate cancer treatment (radical prostatectomy, RP, and/or radiotherapy, RT) as defined by the EAU guidelines [19], WHO performance status 0–1, testosterone level > 50 ng/ml and no active treatment with any products known to influence PSA levels. Patients with up to three metastatic lesions (any N1/M1) on ^{18}F-choline PET/CT were considered as oligometastatic in case they did not progress to more than three lesions following MDT or surveillance within 1 year of diagnosis of metastasis. Patients developing > 3 metastases within 1 year of ^{18}F-choline PET/CT or diagnosed with > 3 metastases at time of first ^{18}F-choline PET/CT were considered polymetastatic.

Study design and sample collection

This was a prospective biomarker discovery and validation study evaluating the ability of a serum-derived miRNA signature to discriminate oligometastatic prostate cancer patients from polymetastatic patients. The study comprised:

1. A discovery phase performing small RNA sequencing and signature establishment.
2. A technology switch to targeted RT-qPCR and signature optimization.
3. A signature validation phase.

The discovery patient cohort included serum samples of 40 patients: 20 patients with polymetastatic disease and 20 patients with oligometastatic disease. The validation cohort included serum samples of 83 patients: 39 patients with polymetastatic diseased and 44 patients with oligometastatic disease.

Full description of sample collection and processing details according to the Biospecimen Reporting for Improved Study Quality (BRISQ) recommendations [20] can be found in the Supplementary Methods and Supplementary Appendix 1.

MicroRNA gene expression analysis

MicroRNA gene expression analysis was performed by Biogazelle (Zwijnaarde, Belgium). RNA was isolated using the miRNeasy serum/plasma kit (Qiagen, Hilden, Germany) using 200 µl serum following the manufacturer's instructions. The RNA concentration was determined using the Nanodrop 2000 UV–Vis spectrophotometer (Thermo Fisher Scientific). Descriptions of the methods used for small RNA sequencing and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) are provided in the Supplementary Methods. RT-qPCR analyses were run in duplicate for each patient.

Transcriptomic data analysis

Transcriptomic data analysis was performed by DNAlytics (Louvain-la-Neuve, Belgium) using R 3.2.3. Prior to the analysis, pre-processing was performed by removing uninformative miRNA variables from the dataset. Two clinical variables, *PSA* and *PSA* doubling time, were log transformed to normality. For qPCR measurements, missing and too high Cq values were replaced by 30. ΔCq values were computed by averaging Cq values between duplicates and normalizing them by removing the mean Cq value of three reference genes (hsa-miR-532-5p, hsa-miR-425-5p and hsa-miR-30e-5p).

Outlier analysis, aiming at discarding patients with a profile strongly different from the rest of the cohort, was performed by running principal component analyses on the miRNA and on the clinical data. To assess whether variables were differentially expressed between the oligometastatic and polymetastatic groups, univariate tests were performed. Non-parametric Wilcoxon's tests were applied on continuous variables. A Pearson χ^2 test was computed for the Risk group variable. The resulting *P* values were corrected for multiple testing by application of Benjamini–Hochberg's method. To assess the predictive potential of the included variables, multivariate analysis was performed considering two classes of predictive models: support vector machines (SVM) and random forests (RF). Variables were ranked a priori with respect to their potential discriminative power and predictive models were then learned from a selection of s top-ranked variables, with *s* varying between 1 and the total number of available variables. Ensemble of Wilcoxon's tests (WilcoxE), ensemble of SVMs (SvmE) and feature importance from RF (RFimp) were the considered variable ranking methods. Because the number of available samples was limited, a resampling procedure was used that repeatedly learned models on part of the data and predicted the labels of the samples that were not used during learning. This evaluation procedure is described in the Supplementary Methods. Predictive performances were reported in terms of area under the ROC curve (AUC), accuracy and balanced classification rate (BCR). In addition, the stability of feature selection, measured by Kuncheva's index, was assessed. Predictions for individual patient samples were calculated as the ratio of the number of correct predictions over the number of times it appeared in a test set in the resampling procedure. The most promising predictive models were tuned to optimize the sensitivity to oligometastatic cases with different specificity/sensitivity trade-offs. The choice of the decision threshold was integrated to the model learning.

Power analysis was performed to determine the validation study sample size to prove the sensitivity and the specificity of the selected predictive model above 0.70 and 0.20, respectively, for a power of 80% (alpha value: 0.05).

Statistical analysis

Statistical analysis of clinical patient characteristics and outcomes was performed using SPSS 25.0. A two sided P value < 0.05 indicated statistical significance. Descriptive statistics were used to define the study population. Mann–Whitney *U* test and Fisher's exact test were used to compare continuous and categorical variables, respectively, between oligo- and polymetastatic patient groups. Kaplan–Meier method with log-rank statistics was used to analyze the difference in metastatic castration resistant prostate cancer-free survival (CRPC-FS) between both groups. Time to event was calculated starting from the date of oligo- or polymetastatic PC recurrence.

Results

Clinical patient characteristics and outcomes

In total, 64 oligometastatic and 59 polymetastatic PC patients were included in the study cohort. Patient and tumor characteristics are presented in Table 1. Median time to CRPC was not reached versus 38 months (95% CI 31–45 months; $P < 0.001$) for oligometastatic and polymetastatic PC patients, respectively, indicating a prognostic difference between both patient groups and concordance of patient selection with the biological definition of oligo- and polymetastatic disease (Fig. 1a).

Biomarker discovery

Discovery data generation

Whole genome microRNA expression profiling was performed on 40 serum samples from PC patients (20 oligometastatic and 20 polymetastatic cases), using small RNA sequencing technology. In total, 1431 mature canonical miRNAs were detected across all samples. On average 587 miRNAs were detected per sample with a lower detection rate for three samples (supplementary Tables 1–4 and supplementary Figure 1). Pair-wise sample–sample correlation was calculated on normalized miRNA expression values and the pair-wise sample Pearson correlation coefficients were subsequently visualized in a heat map. The samples clustered in two main groups, but these did not correspond to the sample classification (supplementary Figure 2). For differential miRNA expression analysis, miRNAs expressed in 80% of samples of either one of both analyzed patient subgroups were selected. Subsequently, differentially expressed miRNAs were

Table 1 Patient and tumor characteristics

	Total ($n=123$)	Oligometastatic ($n=64$)	Polymetastatic ($n=59$)	P value
Age at diagnosis, years				
Median (IQR)	61 (57–66)	60 (56–65)	63 (59–69)	**0.024**
PSA at diagnosis, ng/mL				
Median (IQR)	11.0 (6.8–20)	10.5 (7.4–17.9)	11.0 (6–22.6)	0.937
Gleason score, *n* (%)				
≤6	22 (18)	11 (17)	11 (19)	> 0.9
7	54 (44)	29 (46)	25 (43)	0.856
≥8	44 (37)	23 (37)	21 (36)	> 0.9
Type of primary treatment, *n* (%)				
RP only	20 (16)	15 (23)	5 (9)	**0.029**
RT only	24 (20)	6 (9)	18 (31)	**0.005**
RP and RT	77 (63)	43 (67)	34 (58)	0.351
ADT only	1 (1)	0 (0)	1 (2)	0.480
ADT at primary treatment, *n* (%)				
No	66 (56)	43 (68)	23 (41)	**0.003**
Yes	53 (44)	20 (32)	33 (59)	
PSA at recurrence, ng/mL				
Median (IQR)	4.0 (1.9–9.5)	2.7 (1.2–5.9)	6.7 (3.3–15)	**< 0.001**
PSA-DT at recurrence, ng/mL				
Median (IQR)	6.0 (3.8–10.1)	6.8 (4.2–11.7)	5.1 (3.3–8.5)	**0.023**

ADT androgen deprivation therapy, *IQR* interquartile range, *PSA* prostate-specific antigen, *PSA-DT* PSA doubling time, *RP* radical prostatectomy, *RT* radiotherapy

Bold values indicate statistically significant ($P < 0.05$)

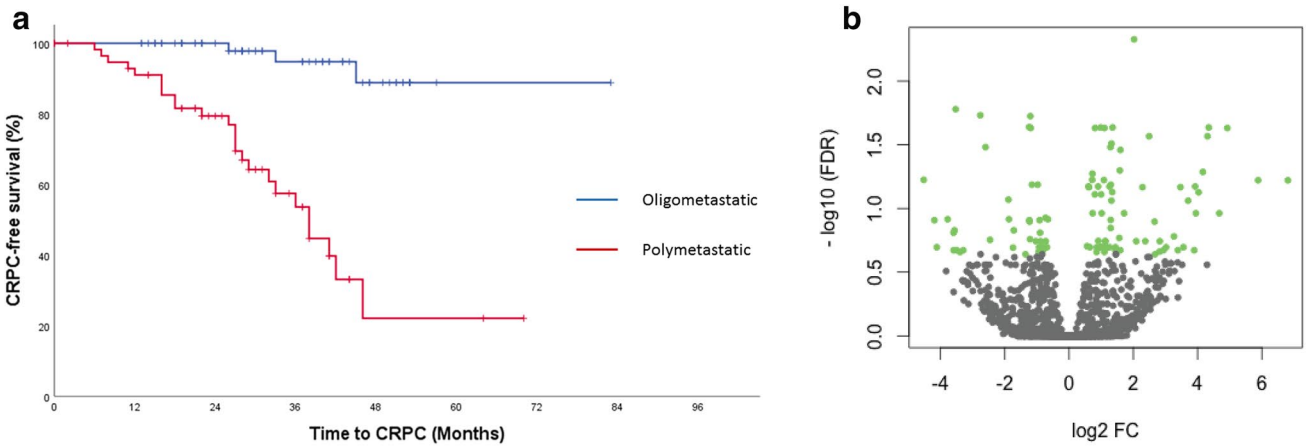

Fig. 1 **a** (Left) Kaplan–Meier plot with log-rank statistics for CRPC-FS. Median time to CRPC for the oligometastatic and polymetastatic patient cohort was not reached versus 38 months (95% CI 31–45 months; $P < 0.001$), respectively. **b** (Right) differential miRNA expression analysis. Volcano plot showing −log10 FDR in function of the log2 FC in gene expression. A positive log2 FC indicates higher expression in polymetastatic versus oligometastatic samples. Green points indicate significantly differential expressed miRNAs at P value < 0.01. *CI* confidence interval, *CRPC-FS* castration-resistant prostate cancer-free survival, *FC* fold change, *FDR* false discovery rate

identified (supplementary Table 5). No miRNAs were found to be differentially expressed at a false discovery rate (FDR) < 0.05, while 112 miRNAs were differentially expressed at a P value < 0.01 (Fig. 1b).

Signature establishment

During pre-processing, 1451 uninformative miRNA variables were removed from the dataset prior to the analyses,

leaving 1137 miRNA variables out of the 2588 in the original data. Four clinical variables (*Gleason score, Risk group, PSA and PSA doubling time*) were included in the analysis. *PSA* and *PSA doubling time* were log transformed to normality (supplementary Figure 3a, b). Because of their different miRNA profiles, three outlier samples were discarded from the multivariate analyses (supplementary Figure 4a). No samples were excluded based on the PCA projection of the four clinical variables (supplementary Figure 4b).

After univariate descriptive analysis and correction for multiple testing, 50 miRNA variables were shown to be significantly differential expressed between oligometastatic and polymetastatic patients (supplementary Table 6). To assess the predictive potential of the included variables, multivariate analysis was performed. Predictive performances for each kind of model are reported in supplementary Figure 5a–c. Random Forest was shown to have the best predictive performances (AUC of 86%) when using 14 variables from RFimp. Its AUC was 85% when using 100 variables from WilcoxE, a more stable selection method (Table 2 and supplementary Figure 6). A consensus signature of 100 variables was selected with WilcoxE to perform the switch to RT-qPCR technology (supplementary Table 7). All 50 significant variables from the univariate analysis were part of this signature. Individual predictive performances of the models for all patients are shown in supplementary Figure 7 and are consistent with the PCA projection.

Technology switch to RT-qPCR

The established signature was transferred to a targeted RT-qPCR platform. The log10 transformed normalized miRNA expression values (supplementary Table 10) of the discovery samples were used for subsequent signature optimization. After removal of four patients due to poor qPCR quality (supplementary Tables 8, 9) or classification as outliers (supplementary Figure 8), univariate analysis highlighted 15 variables showing significant differences between oligo- and polymetastatic patients (supplementary Table 11). The distributions of these variables were fully consistent between qPCR and RNA seq data. Multivariate predictive models optimizing the sensitivity to oligometastatic patients were produced with different specificity/sensitivity trade-offs (supplementary Figure 9 and supplementary Table 12). The final prognostic model, a Random Forest with ten predictors selected by Wilcoxon tests (supplementary Table 13), reached a sensitivity of 0.894 and offered predictive performances of 83.3% AUC (Table 3 and supplementary Figure 10). Using all 50 variables did not lower this performance. In addition, a model including only the clinical parameters, without the microRNA signature, did not perform significantly better than random [AUC 0.575 (0.367–0.782)], demonstrating a significant impact of the serum microRNA signature on the predictive performance of the model.

Biomarker validation

Following signature optimization, the expression of the retained candidate miRNA biomarkers was profiled on a second, independent validation sample cohort (44 oligometastatic cases and 39 polymetastatic cases; supplementary Tables 14–17). After correction for multiple testing and removal of three outliers (supplementary Figure 11), no predictor differed significantly between both groups (supplementary Table 18). In contrast, in the discovery cohort, 14 genes and PSA doubling time were significantly different after correction for multiple testing. Subsequently, the

Table 3 Prognostic performances (95% CI) of a Random Forest model with ten variables obtained on qPCR data, selected with WilcoxE and evaluated by multiple resampling

AUC	0.833 (0.693–0.973)
BCR	0.693 (0.550–0.836)
Sensitivity	0.894 (0.714–1.000)
Specificity	0.492 (0.203–0.782)
NPV	0.867 (0.657–1.000)
PPV	0.664 (0.517–0.812)

AUC area under the curve, *BCR* balanced classification rate, *CI* confidence interval, *NPV* negative predictive value, *PPV* positive predictive value, *WilcoxE* ensemble of Wilcoxon's tests

Table 2 Predictive performances with RF and SVM

	# Variables	AUC	BCR	ACC	Selection	Stability
RF	14	0.864	0.763	0.771	RF_{imp}	0.469
	100	0.853	0.777	0.787	WilcoxE	0.637
SVM	14	0.856	0.785	0.792	SvmE	0.466
	100	0.804	0.752	0.759	WilcoxE	0.637

For each kind of models, two results are reported: one for the variable selection method leading to the best AUC, and one using 100 variables selected by WilcoxE

ACC accuracy, *AUC* area under the curve, *BCR* balanced classification rate, *RF* random forest, *SVM* support vector machines, *WilcoxE* ensemble of Wilcoxon's tests

previously established model was applied on the validation data. Compared to the discovery cohort, more patients in the validation cohort had a Gleason score of 6. In addition, risk score distribution and ranges of ΔCq values of several genes were different between cohorts (supplementary appendix 3). Due to these differences, per-gene centering of ΔCq values was performed prior to application of the model. Nonetheless, the good predictive performance obtained on the discovery cohort was not confirmed and the multivariate model had no predictive ability (Table 4 and Fig. 2a). Given the poor results obtained, an attempt was made to retune a model of the same family on the validation cohort and evaluate it with cross-validation. Performances were not significantly better compared to a random model (Table 4 and Fig. 2b).

Discussion

Oligometastatic PC, as a phenotype with an incompletely developed metastatic potential and slow natural history, is an increasingly recognized concept in oncology. As suggested by retrospective data, and recently confirmed by a prospective randomized trial, these patients are eligible for MDT, delaying additional clinical progression [2, 12]. However, contemporary imaging methods often underestimate the metastatic burden, with 30% of patients treated with MDT progressing to polymetastatic disease within 1 year. The aim of this study was to investigate if *true* oligometastatic PC patients could be differentiated from patients with polymetastatic disease using clinicopathological parameters and a circulating miRNA signature. In previous work by Lussier et al., analysis of disparate primary and metastatic cancer tissue samples revealed differential miRNA expression profiles corresponding to the metastatic progression rate. Distinct miRNAs, associated with tumor-suppressive activity, were upregulated in oligometastatic lesions, suggesting their potential involvement in the metastatic cascade leading to the development of an oligometastatic phenotype [21, 22]. In addition, three molecular subtypes of colorectal cancer liver metastasis were identified through transcriptomic profiling of miRNA and mRNA networks, complementing clinical risk stratification for the identification of patients with potentially curable oligometastatic disease that might benefit from MDT [23]. In contrast to these findings, the multivariate model in the current study, trained with clinical parameters and serum-derived small RNA sequencing data, had no predictive ability to prospectively distinguish oligo- and polymetastatic PC cancer patients in the validation cohort. The range of expression values between the discovery and validation cohort changed for some of the miRNA targets and none of the 41 miRNA targets was differentially expressed between oligo- and polymetastatic PC patients in the validation cohort. It was not possible to retune a model of the same family which would achieve satisfying performance. Although showing much promise as potential

Table 4 Prognostic performances applied and cross-validated on the validation cohort

	Applied on validation cohort	Cross-validated on validation cohort
Sensitivity	0.841	0.939
Specificity	0.077	0.080
AUC	0.393	0.534

AUC area under the curve

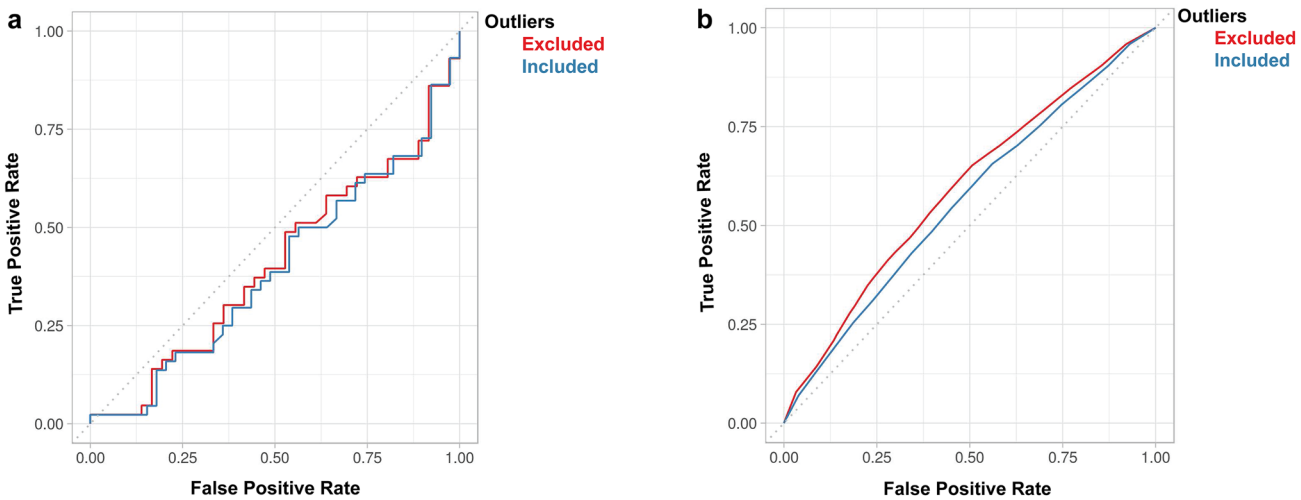

Fig. 2 **a** (Left) ROC curves of a random forest with ten features selected by Wilcoxon tests, applied on the validation cohort. **b** (Right) ROC curves of a retuned model, cross-validated on the validation cohort

disease biomarkers, miRNAs in biological fluids are very challenging to measure accurately. Technical and pre-analytical factors may have a profound influence on miRNA levels. This might result in biases not reflecting the biological state of the samples. Although standard operating procedures for sample collection, handling and storage were carefully followed, some pre-analytical variables might account for the inability to validate the current biomarker discovery findings. Serum degradation associated with storage time or freeze-thaws can impact downstream omics analyses [24, 25]. Most importantly, miRNA is being released by platelets and blood cells during the coagulation process, possibly influencing the bona fide circulating miRNA repertoire in serum samples [26].

In summary, the good predictive performance of a combined clinicopathological and serum-derived miRNA model to prospectively distinguish oligo- and polymetastatic PC patients, obtained on a discovery cohort, was not confirmed. Further research is needed to assess whether oligometastatic prostate cancer can be characterized by a distinct miRNA signature, traceable by a liquid biopsy approach. Meanwhile, the phase 2 ORIOLE trial, through analysis of circulating tumor cells and circulating tumor DNA, might provide additional insights on the biology of the oligometastatic state [27].

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