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## Development and Independent Validation of a Prognostic Gene Expression Signature Based on RB1, PTEN, and TP53 in Metastatic Hormone-sensitive Prostate Cancer Patients

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### Abstract

**Background:** Androgen deprivation therapy (ADT) with docetaxel (D) and/or antiandrogen receptor therapies (ARTs) are the standard therapies in metastatic hormone-sensitive prostate cancer (mHSPC). Alterations in the tumor suppressor genes (TSGs) *RB1*, *PTEN*, and *TP53* are associated with an aggressive evolution and treatment resistance in castration-resistant prostate cancer (CRPC).

**Objective:** To study the clinical implications of TSG mRNA expression in mHSPC patients.

**Design, setting, and participants:** This is a multicenter retrospective biomarker study in mHSPC patients. TSG<sub>low</sub> status was defined when two or more out of the three TSGs presented low RNA expression by nCounter in formalin-fixed paraffin-embedded samples and TSG<sub>wt</sub> for the remaining cases. The microarray data from the CHARTED trial were analyzed as an independent validation cohort.

**Outcome measurements and statistical analysis:** Molecular data were correlated with CRPC-free survival (CRPC-FS) and overall survival (OS) by the Kaplan-Meier method and multivariate Cox analysis.

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Biomarkers  
Androgen deprivation therapy  
Docetaxel  
CHAARTED trial

**Results and limitations:** A total of 226 patients were included, of whom 218 were eligible: 93 were treated with ADT and 125 with ADT + D; 75.7% presented de novo stage IV and 67.9% high-volume disease. TSG<sub>low</sub> (19.2%) was independently correlated with shorter CRPC-FS (hazard ratio [HR] 1.8,  $p = 0.002$ ) and OS (HR 2,  $p = 0.002$ ). In the CHAARTED trial, TSG<sub>low</sub> was independently correlated with lower CRPC-FS (HR 2.2,  $p = 0.02$ ); no differences in clinical outcomes according to treatment were observed in TSG<sub>low</sub> patients, while a significant benefit was observed for ADT + D in the TSG<sub>wt</sub> group for CRPC-FS (HR 0.4,  $p < 0.001$ ) and OS (HR 0.4,  $p = 0.001$ ). However, no interaction was observed between TSG signature and treatment in either series. Study limitations are the retrospective design, small sample size, and lack of inclusion of patients treated with ADT + ART.

**Conclusions:** TSG<sub>low</sub> expression correlates with adverse outcomes in patients with mHSPC. The investigation of new therapeutic strategies in these patients is warranted.

**Patient summary:** The low RNA expression of tumor suppressor genes in the tumors is correlated with adverse outcomes in patients with metastatic hormone-sensitive prostate cancer.

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## 1. Introduction

Prostate cancer is ranked as second in cancer incidence and the fifth cause of cancer death in men [1]. Androgen deprivation therapy (ADT) with docetaxel (D) or antiandrogen receptor therapies (ARTs) are the standard upfront treatments in metastatic hormone-sensitive prostate cancer (mHSPC) [2–8]. Moreover, the addition of ARTs to ADT + D, with either darolutamide or abiraterone, has also shown survival benefits in patients with synchronous mHSPC regardless of the risk and volume of disease [9–11]. However, treatment selection remains a challenge and biomarkers are needed.

Alterations in the tumor suppressor genes (TSGs) *RB1*, *PTEN*, and *TP53* have been associated with the development of aggressive variant prostate cancer and neuroendocrine (NE) dedifferentiation in castration-resistant prostate cancer (CRPC) [12]. These variants usually appear after antiandrogen therapies, are defined by distinct clinical features and androgen receptor (AR)-independent progression, and are associated with reduced response to conventional therapies, poor prognosis, and more sensitivity to platinum [13].

The role of TSG alterations in mHSPC is less well defined. In this context, exome mutations at least in one gene can be detected in about 30% of patients [14,15]. Moreover, genomic alterations in two or more TSGs in men with HSPC and CRPC are associated with poor clinical outcomes [13].

The transcriptional profile of primary tumors may determine a distinct clinical evolution and treatment benefit of mHSPC patients. A subanalysis of the CHAARTED clinical trial [2], which compared ADT + D versus ADT alone treatments in mHSPC, identified that patients with a luminal B molecular subtype benefited from the addition of D to ADT, in contrast to the basal subtype. More recently, a molecular analysis of the ADT-treated patient cohorts with or without abiraterone from the STAMPEDE trial identified several prognostic transcriptional signatures, as low mRNA expression of *PTEN* or *TP53* [16].

In a prior study in mHSPC patients treated with ADT + D, we found that the low TSG expression signature correlated

with lower overall survival (OS). Moreover, patients with lowest tertile expression of at least two TSGs presented shorter CRPC-free survival (CRPC-FS) and OS [17].

In the current study, we define and further validate a TSG<sub>low</sub> signature in a larger series of mHSPC patients with extended follow-up, and explore its potential value for treatment selection. Additionally, we independently validate these results through an *in silico* analysis of molecular data from patients included in the phase 3 CHAARTED trial [18].

## 2. Patients and methods

Complete details are given in the [Supplementary material](#).

### 2.1. Design, patients, and samples

We present a multicenter retrospective biomarker study in patients with mHSPC from ten hospitals in Spain. The key inclusion criteria were as described previously [17]. The study was conducted according to the principles of the Declaration of Helsinki, and it was approved by the institutional ethics committees of all participating centers. Informed consent was obtained from all patients. Treatment for mHSPC was ADT alone (ie, luteinizing hormone-releasing hormone analogs) or ADT in combination with D (75 mg/m<sup>2</sup> every 21 d for six cycles).

The primary endpoint was to correlate TSG mRNA expression with CRPC-FS. The secondary endpoints were to correlate TSG mRNA expression and OS, to study the correlation between loss-of-function exome mutations with mRNA expression and immunohistochemistry (IHC), and to explore the impact of the determination of TSGs through different techniques on clinical outcomes.

### 2.2. Gene expression panel design

We configured a gene expression nCounter panel (Nanostring Technologies, Seattle, WA, USA) of 184 genes [17]. Here, we present the data focused on the TSG signature and also explore the expression of the full-length AR.

### 2.3. Bioinformatics and statistical analysis

Tertiles were applied to transformed (*z* score) nCounter gene expression data from an exploratory series to establish the cutoff for *RB1*, *PTEN*, and *TP53* expression, and categorize the samples as high-, mid-, or low-expression groups for each gene. These cutoffs were then applied to the transformed (*z* score) gene expression data from the other cohorts described in the Results section and the microarray data from CHARTED trial patients [18]. *Low* term was assigned for the lower tertile, and *wt* for the mid and high tertiles of each gene. TSG<sub>low</sub> was considered when two or more out of the three TSGs presented low expression and TSG<sub>wt</sub> for the remaining cases.

CRPC-FS and OS, calculated from the date of start of ADT to the time of developing CRPC, and to the time of death or last follow-up visit, respectively, were analyzed by the Kaplan-Meier method and compared by log-rank test. CRPC-FS definition, treatment response criteria, and progressive disease definitions followed the Prostate Cancer Working Group 2 criteria [19]. Univariate and multivariate analyses of variables of interest were performed by a Cox regression analysis. Analyses were performed with R software (version 3.6.3; R Foundation for Statistical Computing, Vienna, Austria).

## 3. Results

### 3.1. Patients, samples, and TSG expression signature

A total of 226 patients were enrolled in the study: 218 were eligible and eight were excluded due to insufficient tumor sample (*n* = 4) or RNA quantity (*n* = 4). Of the eligible

patients, 125 were treated with ADT + D and 93 with ADT alone (Table 1). Most formalin-fixed paraffin-embedded (FFPE) samples were obtained from the primary tumor (93.1%). The median follow-up was 46.3 (range 6.7–223.5) mo. As shown in Figure 1A, 24.4%, 30.5%, and 23.9% of patients were considered RB1<sub>low</sub>, PTEN<sub>low</sub>, and TP53<sub>low</sub>, respectively. According to our criteria, 19.2% of patients were TSG<sub>low</sub>. Moreover, we explored whether there were differences in *AR* expression between TSG<sub>low</sub> and TSG<sub>wt</sub> groups, observing that *AR* mRNA levels were lower in TSG<sub>low</sub> (*p* = 0.002; Fig. 1B).

Overall, there were no differences in clinical characteristics between TSG<sub>low</sub> and TSG<sub>wt</sub> (Supplementary Tables 1–6). Regarding individual TSG RNA levels, low *PTEN* expression correlated with de novo stage IV disease in the global series (*p* = 0.022) and the ADT + D cohort (*p* = 0.004), and with visceral metastases in the ADT + D cohort (*p* = 0.049). Moreover, *RB1* (*p* = 0.017) and *TP53* (*p* = 0.047) expression correlated with visceral metastases in the ADT cohort (Supplementary Fig. 1–3).

### 3.2. Comparison between TSG determination by nCounter and other techniques

In 60 patients, TSG mRNA was determined by both nCounter and RNA-Seq, observing a high correlation of mRNA levels of each gene by both techniques (Supplementary Fig. 4).

A targeted TSG mutation analysis was performed in 54 patients treated with ADT + D. Mutations in at least one TSG were present in 30 patients (55.6%); *RB1* was mutated in 11 (20.4%), *PTEN* in 18 (33.3%), and *TP53* in 19 (35.2%) patients, whereas 14 (25.9%) presented mutations in more

**Table 1 – Characteristics of patients from the global cohort<sup>a</sup>**

	Global cohort	ADT + D cohort	ADT cohort	<i>p</i> value
Patients, <i>n</i> (%)	218	125 (57.3)	93 (42.7) <sup>b</sup>	
Age (yr)				
Median (range)	66.4 (46.3–84.6)	66.6 (46.3–83.4)	66.1 (51–84.6)	0.467
Tumor origin, <i>n</i> (%)				
Primary	203 (93.1)	117 (93.6)	86 (92.5)	0.791
Metastatic	15 (6.9)	8 (6.4)	7 (7.5)	
Stage at diagnosis, <i>n</i> (%)				
<IV	42 (19.3)	9 (7.2)	33 (35.5)	<b>&lt;0.001</b>
IV	165 (75.7)	116 (92.8)	49 (52.7)	
NA	11 (5)	–	11 (11.8)	
Gleason sum at diagnosis, <i>n</i> (%)				
≤7	53 (24.3)	22 (17.6)	31 (33.3)	<b>0.004</b>
≥8	158 (72.5)	102 (81.6)	56 (60.2)	
NA	7 (3.2)	1 (0.8)	6 (6.5)	
Presence of visceral metastases, <i>n</i> (%)				
Yes	33 (15.1)	25 (20)	8 (8.6)	<b>0.034</b>
No	181 (83)	100 (80)	81 (87.1)	
NA	4 (1.9)	–	4 (4.3)	
Disease volume, <i>n</i> (%)				
High	148 (67.9)	97 (77.6)	51 (54.8)	<b>0.002</b>
Low	65 (29.8)	27 (21.6)	38 (40.9)	
NA	5 (2.3)	1 (0.8)	4 (4.3)	
ECOG performance status score, <i>n</i> (%)				
0	93 (42.7)	54 (43.2)	39 (41.9)	0.777
1 or 2	114 (52.3)	69 (55.2)	45 (48.4)	
NA	11 (5.0)	2 (1.6)	9 (9.7)	

ADT = androgen deprivation therapy; D = docetaxel; ECOG = Eastern Cooperative Oncology Group; *n* = number of cases; NA = not available.

<sup>a</sup> The *p* values are based on Fisher exact test and Wilcoxon Mann-Whitney *U* test for categorical and continuous variables, respectively. Significant *p* values (*p* < 0.05) are bold indicated.

<sup>b</sup> Five patients were excluded from survival analysis due to lack of complete follow-up data.

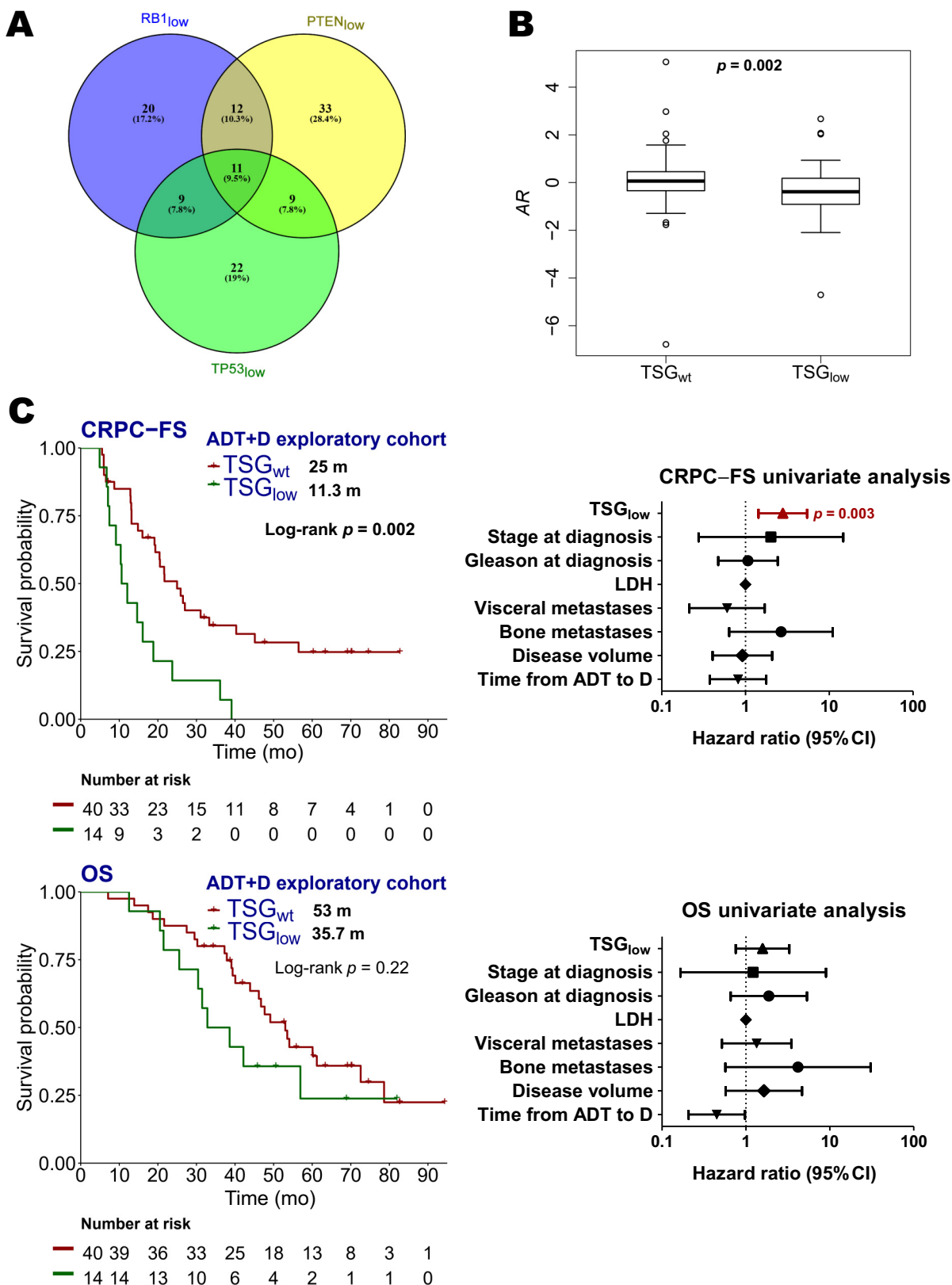


Fig. 1 – (A) Venn diagram of the RB1<sub>low</sub>, PTEN<sub>low</sub>, and TP53<sub>low</sub> patients with complete follow-up in the global (ADT ± D) cohort. (B) Boxplot of androgen receptor (AR) RNA expression levels (z score) according to TSG expression in the global (ADT ± D) cohort (Wilcoxon test; p value). (C) Kaplan-Meier curves representing CRPC-FS and OS according to TSG expression and forest plots representing the univariate analysis in the ADT + D exploratory cohort. ADT = androgen deprivation therapy; CI = confidence interval; CRPC = castration-resistant prostate cancer; CRPC-FS = CRPC-free survival; D = docetaxel; LDH = lactate dehydrogenase; m: median months; OS = overall survival; TSG = tumor suppressor gene. Significant p values ( $p < 0.05$ ) are bold indicated.

than two TSGs and four (7.4%) in all three genes. Most of the pathogenic variants were missense (46.9%) or nonsense mutations (46.7%). Details of pathogenic mutations found in each TSG are shown in [Supplementary Table 7](#).

A significant correlation was observed between the presence of mutations in *PTEN* and low *PTEN* RNA levels ( $p = 0.026$ ; [Supplementary Fig. 5A](#)). The mutational status of none of the individual TSGs was associated with clinical outcomes ([Supplementary Fig. 5B and 5C](#)). The presence of two or more TSG mutations (TSG<sub>mut</sub>) was correlated with shorter CRPC-FS (hazard ratio [HR] 2, 95% confidence interval [CI] 1.1–4,  $p = 0.036$ ; [Supplementary Fig. 5D](#)).

IHC was carried out in tumor samples from 73 patients from the ADT + D cohort with available tumor for IHC. Finally, 48 (65.8%) samples were assessable for RB1, 52 (71.2%) for *PTEN*, and 56 (76.7%) for *TP53*. Thirty-eight (79.2%) samples presented alterations in RB1, 26 (50%) in *PTEN*, and 28 (50%) in *TP53*. Altered IHC for RB1 and *PTEN* correlated significantly with low levels of RNA expression ( $p = 0.007$  and  $p < 0.001$ , respectively; [Supplementary Fig. 6A](#)). The alteration by IHC of none of the individual TSGs correlated with clinical outcomes, nor having two or more altered TSGs (in 34 [66.7%] patients; [Supplementary Fig. 6B–D](#)).

### 3.3. TSG mRNA expression signature in an exploratory cohort

The series of 54 patients from the ADT + D cohort, with both mutational and nCounter expression data, was considered the exploratory cohort ([Supplementary Table 8](#)). In this cohort, TSG<sub>low</sub> correlated with shorter CRPC-FS (HR 2.8, 95% CI 1.4–5.4,  $p = 0.002$ ; [Fig. 1C](#)). Moreover, the model that included the TSG assessed by mRNA expression (Akaike Information Criterion [AIC] score: 275.8) fitted better than the one that included their mutational status (AIC: 279.8).

### 3.4. TSG mRNA expression signature validation in patients treated with ADT + D

Internal validation of the results was performed in the additional 71 patients treated with ADT + D ([Supplementary Table 8](#)), where TSG<sub>low</sub> correlated with lower CRPC-FS (HR 2.4, 95% CI 1.1–5.3,  $p = 0.033$ ) and OS (HR 3.2, 95% CI 1.4–7.3,  $p = 0.006$ ). Moreover, TSG<sub>low</sub> was independently associated with shorter CRPC-FS (HR 4.1, 95% CI 1.6–10.4,  $p = 0.003$ ) and OS (HR 3.7, 95% CI 1.6–8.7,  $p = 0.003$ ; [Fig. 2](#)).

### 3.5. TSG mRNA expression signature in patients treated with ADT alone

The established cutoffs were also analyzed in a cohort of 93 patients treated with ADT alone ([Table 1](#)). In this cohort, TSG<sub>low</sub> was not associated with either CRPC-FS (HR 1.4, 95% CI 0.8–2.3,  $p = 0.23$ ) or OS (HR 1.6, 95% CI 0.9–2.7,  $p = 0.091$ ; [Fig. 3](#)).

### 3.6. Exploring TSG mRNA expression as a predictor of treatment benefit

To explore whether TSG mRNA expression was a predictor of treatment benefit, we analyzed together the patients treated with ADT + D and those treated with ADT (global cohort;

[Table 1](#)). TSG<sub>low</sub> patients had shorter CRPC-FS (HR 1.9, 95% CI 1.3–2.7,  $p = 0.001$ ) and OS (HR 1.8, 95% CI 1.2–2.7,  $p = 0.002$ ) than TSG<sub>wt</sub> patients. Moreover, TSG<sub>low</sub> correlated independently with shorter CRPC-FS (HR 1.8, 95% CI 1.3–2.7,  $p = 0.002$ ) and OS (HR 2, 95% CI 1.3–3.1,  $p = 0.002$ ; [Fig. 4](#)). However, no interaction between TSG expression and treatment was observed regarding CRPC-FS ( $p = 0.11$ ) or OS ( $p = 0.45$ ).

### 3.7. Independent series validation

In the microarray data from the CHARTED trial [18], 27.5% of the patients were classified as TSG<sub>low</sub> patients. In the multivariate analysis, TSG<sub>low</sub> was independently correlated with shorter CRPC-FS (HR 2.2, 95% CI 1.1–4.3,  $p = 0.02$ ; [Fig. 5A](#)).

Analyzing TSG<sub>low</sub> and TSG<sub>wt</sub> populations separately according to treatment, we found that there were no significant differences in CRPC-FS ( $p = 0.3$ ) or OS ( $p = 0.5$ ) between TSG<sub>low</sub> patients treated with ADT + D or ADT alone. Moreover, TSG<sub>wt</sub> patients treated with ADT + D had the longest CRPC-FS (HR 0.4, 95% CI 0.2–0.6,  $p < 0.001$ ) and OS (HR 0.4, 95% CI 0.3–0.7,  $p = 0.001$ ), compared with ADT-treated patients. However, as observed in our series, no interaction between the TSG expression and treatment was observed regarding CRPC-FS ( $p = 0.116$ ) or OS ( $p = 0.051$ ; [Fig. 5B and 5C](#)).

### 3.8. Individual assessment of TSG

In the global cohort, RB1<sub>low</sub> (HR 1.6, 95% CI 1.2–2.2,  $p = 0.006$ ) and *PTEN*<sub>low</sub> (HR 1.8, 95% CI 1.3–2.5,  $p < 0.001$ ) correlated with CRPC-FS. Moreover, RB1<sub>low</sub> (HR 1.6, 95% CI 1.1–2.2,  $p = 0.018$ ), *PTEN*<sub>low</sub> (HR 1.7, 95% CI 1.2–2.3,  $p = 0.003$ ), and *TP53*<sub>low</sub> (HR 1.5, 95% CI 1.1–2.2,  $p = 0.023$ ) correlated with OS ([Supplementary Fig. 7](#)). In the multivariate analysis, RB1<sub>low</sub> correlated with CRPC-FS (HR 1.5, 95% CI 1–2.1,  $p = 0.03$ ), *PTEN*<sub>low</sub> correlated with CRPC-FS (HR 1.6, 95% CI 1.2–2.3,  $p = 0.003$ ) and OS (HR 1.5, 95% CI 1.1–2.2,  $p = 0.018$ ), and *TP53*<sub>low</sub> correlated with OS (HR 1.6, 95% CI 1.1–2.4,  $p = 0.013$ ; [Supplementary Fig. 8](#)).

The multivariate analysis including TSG<sub>low</sub> was the best accurate model for both CRPC-FS and OS compared with those that included the low expression from an individual gene ([Supplementary Table 9](#)).

## 4. Discussion

In this study, we show that the mRNA expression of the TSG<sub>low</sub> signature (low expression of two or more of the TSGs *RB1*, *PTEN*, and *TP53*) is independently associated with lower CRPC-FS and OS in mHSPC patients. The prognostic value of the TSG signature was validated independently in the molecular dataset from patients included in the CHARTED trial [2,18]. Besides, we found that the lower expression of any of the individual genes was also independently associated with an adverse prognosis, although the TSG<sub>low</sub> signature was a better model for CRPC-FS and OS prediction. We also explored whether the TSG signature could be useful to predict treatment benefit. We found that in the CHARTED series, when analyzing TSG<sub>low</sub> and TSG<sub>wt</sub>

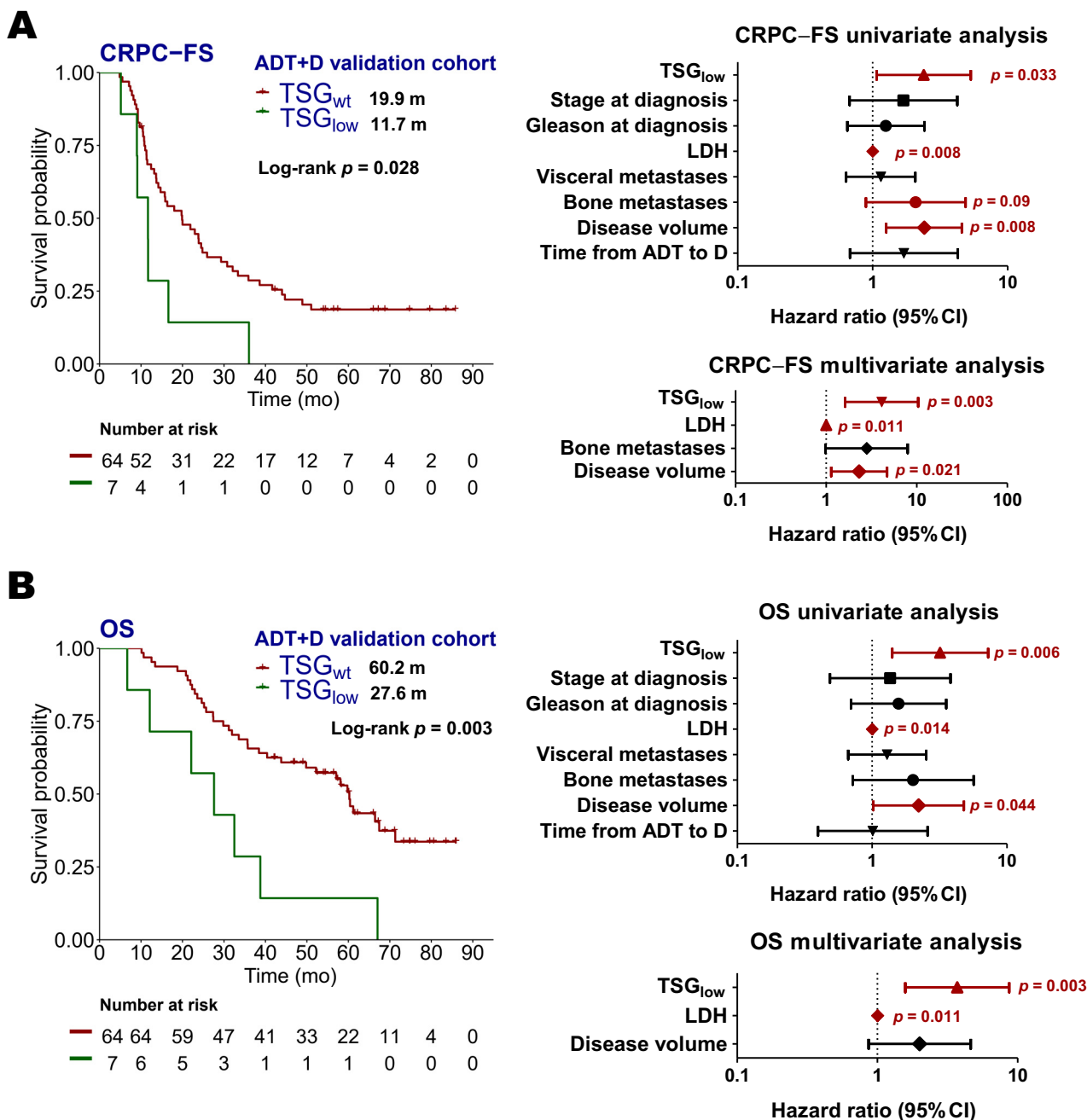


Fig. 2 – Kaplan-Meier curves representing (A) CRPC-FS and (B) OS according to TSG expression and forest plots representing the univariate and multivariate analyses in the ADT + D validation cohort. ADT = androgen deprivation therapy; CI = confidence interval; CRPC = castration-resistant prostate cancer; CRPC-FS = CRPC-free survival; D = docetaxel; LDH = lactate dehydrogenase; m = median months; OS = overall survival; TSG = tumor suppressor gene. Significant  $p$  values ( $p < 0.05$ ) are bold indicated.

populations separately according to treatment, there were no significant differences in CRPC-FS and OS between TSG<sub>low</sub> patients treated with ADT alone or ADT + D. Moreover, TSG<sub>wt</sub> patients treated with ADT + D presented longer CRPC-FS and OS than those treated with ADT alone. However, no interaction was observed between the TSG signature and treatment in either our study or the CHARTED series. Thus, we may not conclude that TSG<sub>low</sub> patients do not benefit from adding D to ADT.

As one of the established standards of care for mHSPC ADT + ART, it will be relevant to test the TSG signature in

patients receiving this treatment strategy, as well as in those treated with ADT + ART + D [9–11], in order to elucidate whether they would benefit from D addition.

While most of the previous studies have focused on studying TSG genomic alterations or IHC protein expression, just a few of them have analyzed TSG RNA expression. Both IHC and next-generation sequencing (NGS) have been proved to be able to correlate TSG alterations with clinical outcomes, but they have not been compared rigorously [12,15]. One study conducted in tumor-derived xenografts, which studied TSG alterations by IHC, RNA expression, and

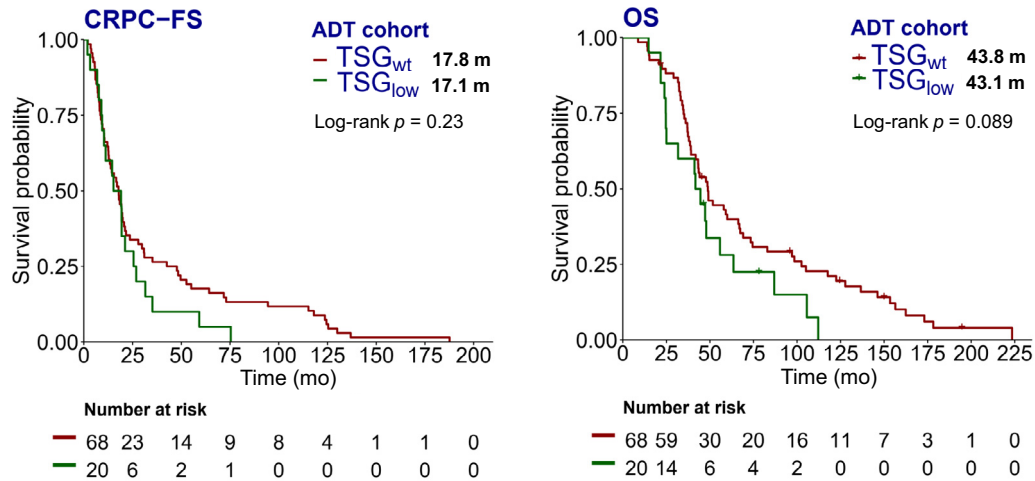


Fig. 3 – Kaplan-Meier curves representing CRPC-FS and OS according to TSG expression in the ADT cohort. ADT = androgen deprivation therapy; CRPC = castration-resistant prostate cancer; CRPC-FS = CRPC-free survival; m = median months; OS = overall survival; TSG = tumor suppressor gene.

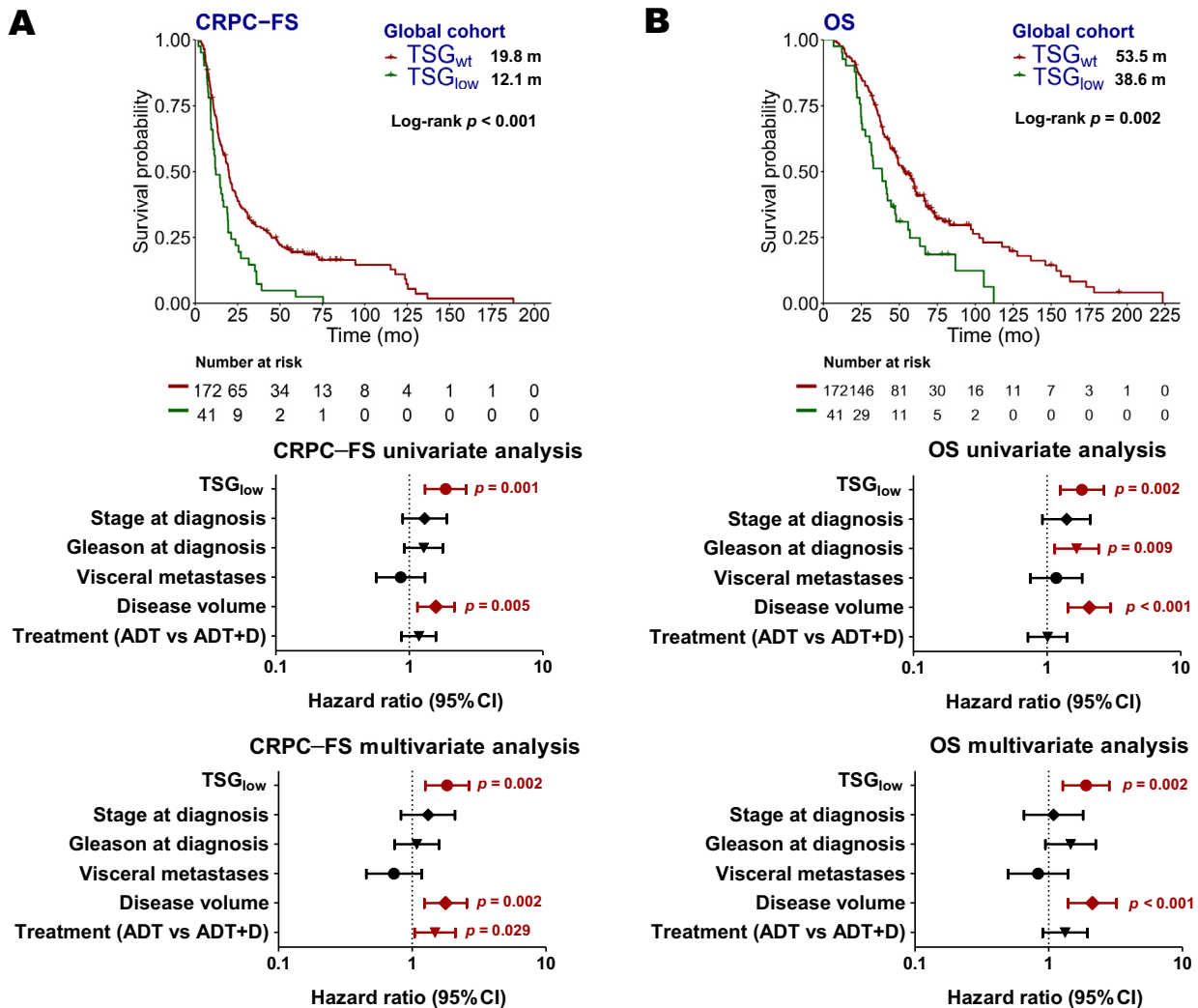
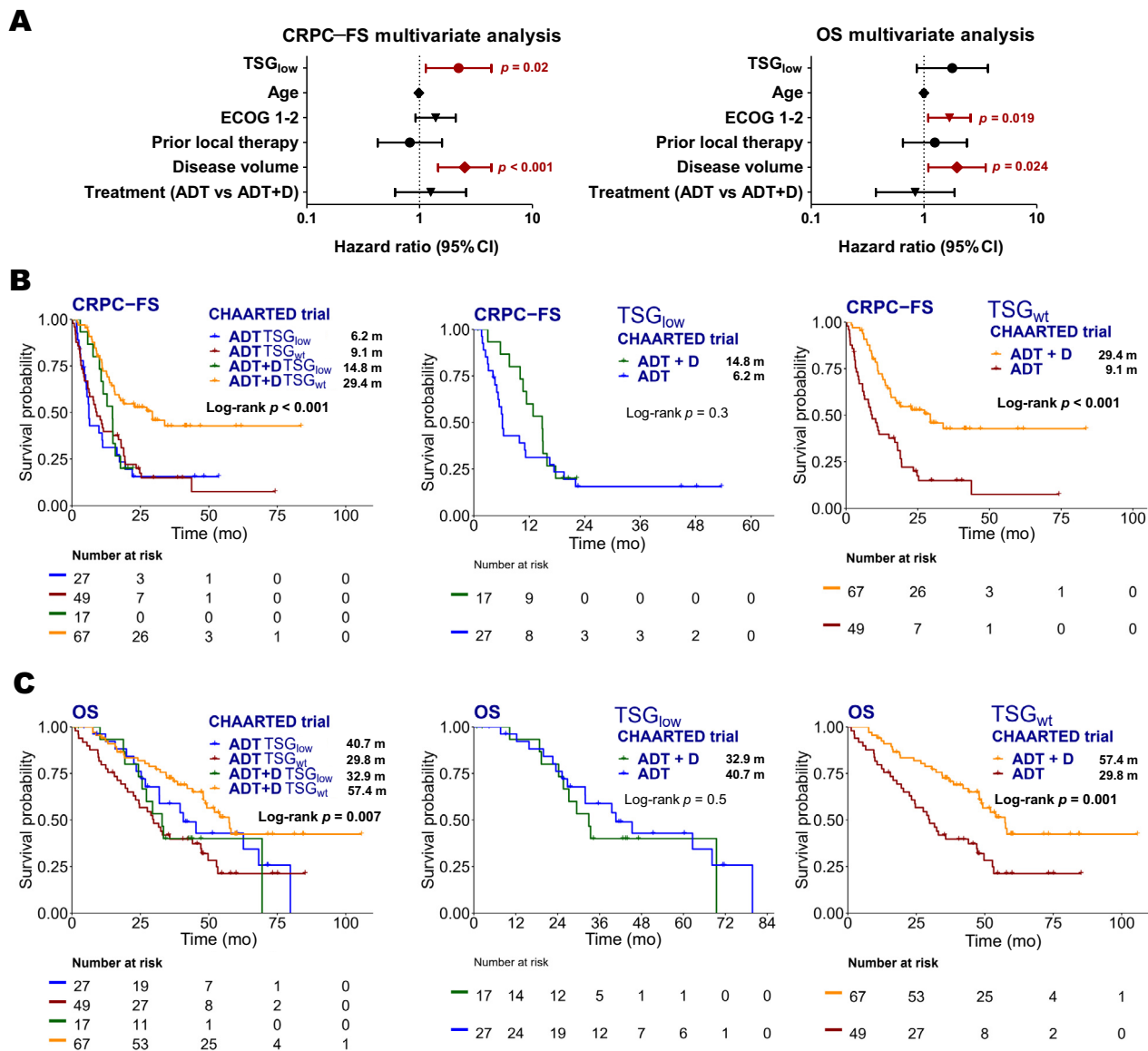


Fig. 4 – Kaplan-Meier curves representing (A) CRPC-free survival (CRPC-FS) and (B) overall survival (OS) according to TSG expression and forest plots representing the univariate and multivariate analyses in the global (ADT ± Docetaxel [D]) cohort. ADT = androgen deprivation therapy; CI = confidence interval; CRPC = castration-resistant prostate cancer; m = median months; TSG = tumor suppressor gene. Significant  $p$  values ( $p < 0.05$ ) are bold indicated.



**Fig. 5 – (A)** Forest plots representing the multivariate analysis of TSG expression of microarray data from the CHAARTED trial for CRPC-free survival (CRPC-FS) and overall survival (OS). Kaplan-Meier curves representing (B) CRPC-FS and (C) OS according to TSG expression in the CHAARTED trial segregated by treatment: ADT + docetaxel (D) arm and ADT arm. ADT = androgen deprivation therapy; CI = confidence interval; CRPC = castration-resistant prostate cancer; ECOG = Eastern Cooperative Oncology Group; m = median months; TSG = tumor suppressor gene. Significant *p* values (*p* < 0.05) are bold indicated.

DNA sequencing, found a good genotype-to-phenotype correlation [20]. Here, we have studied TSG alterations by DNA, RNA, and IHC in a subset of patients. We have observed a correlation between mRNA expression of *PTEN* and *PTEN* mutations, and between mRNA and IHC expression of *PTEN* and *RB1*. For *TP53*, we could not find a strong association between IHC patterns, mutations, and RNA expression. As prostate tumor tissues show low basal expression of *TP53*, IHC cannot detect *TP53* loss that results from nonsense, frameshift, or indel alterations that may also lead to low RNA expression [21,22]. Moreover, there is a lack of standardized criteria for TSG determination by IHC in prostate cancer.

Focusing on DNA-RNA discordance, cases with lower RNA expression without any genetic alteration may be explained by post-transcriptional alterations, changes in methylation

patterns, or interactions with noncoding RNA that can affect mRNA expression without the presence of a mutation [23]. Moreover, some genetic variants such as copy number alterations or large deletions are often not detected by NGS conventional assays. In our study, mRNA expression of TSG was a better outcome predictor than genomic alterations.

*TP53*, *PTEN*, and *RB1* are recurrently altered in CRPC. In this context, the presence of two or more TSG alterations (mainly defined by genomic loss or mutations or altered protein expression by IHC) is associated with aggressive clinical features, resistance to conventional therapies, aggressive evolution, and NE dedifferentiation [12,24–26]. A gene expression signature reflecting *TP53/RB1* loss is associated with diminished responses to AR antagonists and reduced survival [27].

It is known that molecular alterations of primary prostate cancer may differ from those of CRPC. Mateo et al. [15] studied genomic aberrations in matched hormone-naïve and CRPC biopsies from 61 patients who developed mCRPC, and found differences in *TP53*, *RB1*, and *PI3K/AKT* mutational status between same-patient samples. Furthermore, cell plasticity-related changes that occur as a result of ARTs [24,28] may not be present in treatment-naïve primary tumors. Notably, in one study, 40% of *TP53/RB1*-deficient tumors were classified as AR-active adenocarcinomas; therefore, NE differentiation is not a necessary consequence of *TP53/RB1* inactivation [26]. Similarly, in a prior study in mHSPC, we did not find a correlation between TSG and NE markers mRNA expression [17]. Thus, the absence of NE markers expression in mHSPC does not exclude the presence of TSG alterations. Moreover, we found in the present study that lower TSG expression correlated with lower *AR* expression. Thus, TSG alterations in noncastrated tumors may preclude the development of NE dedifferentiation and androgen-independent progression during CRPC progression [29].

Several studies addressed the clinical implications of TSG genomic alterations in mHSPC patients. In a large massively parallel targeted sequencing study, where patients with altered TSG were defined by harboring any copy number loss or deleterious mutation of one or more TSGs, authors found that patients with prostate tumors with compound TSG mutations had poorer outcomes [13]. A meta-analysis and systematic review of 11 studies including 1682 mHSPC patients found that high-volume and de novo mHSPC were enriched with *TP53* alterations [30]. We found in the present study that lower *PTEN* levels correlated with de novo mHSPC in the global and ADT + D series and the presence of visceral metastases in the ADT + D series, and that lower *RB1* and *TP53* expression correlated with visceral metastases in the ADT series. However, analyzing together, we did not find differences in clinical characteristics between TSG<sub>low</sub> and the rest of the patients, and notably, the TSG<sub>low</sub> signature was an independent adverse prognostic factor for CRPC-FS and OS [17]. This may suggest that this molecular signature may be more accurate than clinical characteristics in predicting the outcome in mHSPC patients.

## 5. Conclusions

In conclusion, our study shows the adverse prognostic factor of the TSG<sub>low</sub> signature in mHSPC patients. The investigation of this signature in patients receiving ADT + ART or the triple therapy with ADT, ART, and D may be of interest in order to determine the benefit of D addition according to the TSG status. Overall, the adverse clinical implications of having TSG alterations support the investigation of new therapeutic strategies in metastatic prostate cancer patients with these molecular alterations.

**Author contributions:** Begoña Mellado had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Acquisition of data:** Mellado, Jiménez, Garcia de Herreros, Reig, Marín-Aguilera, Aversa, Ferrer-Mileo, García-Esteve, Rodríguez-Carunchio, Trias, Font, Rodriguez-Vida, Climent, Cros, Chirivella, Domènech, Figols, Carles, Suárez, Herrero Rivera, González-Billalabeitia, Cívico, Sala-González.

**Analysis and interpretation of data:** Mellado, Jiménez, Garcia de Herreros, Reig, Marín-Aguilera, Aversa, Ferrer-Mileo, García-Esteve, Rodríguez-Carunchio, Trias.

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**Obtaining funding:** Mellado.

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**Supervision:** Mellado.

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## Appendix A. Supplementary data

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