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A DNA damage repair gene-associated signature predicts responses of patients with advanced soft-tissue sarcoma to treatment with trabectedin.

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Abbreviations:

AP: apurinic/ apyrimidinic

AUC: area under the ROC curve

BER: base excision repair

CR: complete response

DDR: DNA damage repair

DSB: double strand break

FDR: false discovery rate

FFPE: formalin-fixed paraffin-embedded

GMI: growth modulation index

HR: homologous recombination

MMR: DNA mismatch repair

NER: nucleotide excision repair

NHEJ: non-homologous end joining

OS: overall survival

PcG: polycomb groups

PD: progression disease

PR: partial response

SD: stable disease

TMM: trimmed mean of M-values

TPM: transcripts per million

PFS: progression-free survival

STS: soft-tissue sarcoma

TTP: time-to-progression

ABSTRACT

Predictive biomarkers of trabectedin represents an unmet need in advanced soft-tissue sarcomas (STS). DNA damage repair (DDR) genes, involved in homologous recombination or nucleotide excision repair, had been previously described as biomarkers of trabectedin resistance or sensitivity, respectively. The majority of these studies only focused on specific factors (*ERCC1*, *ERCC5* and *BRCA1*) and did not evaluate several other DDR-related genes that could have a

relevant role for trabectedin efficacy. In this retrospective translational study, 118 genes involved in DDR were evaluated to determine, by transcriptomics, a predictive gene signature of trabectedin efficacy. A six-gene predictive signature of trabectedin efficacy was built in a series of 139 tumor samples from patients with advanced soft-tissue sarcoma. Patients in the high-risk gene signature group showed a significantly worse progression-free survival (PFS) compared to patients in the low-risk group (2.1 vs 6.0 months, respectively). Differential gene expression analysis defined new potential predictive biomarkers of trabectedin sensitivity (*PARP3* and *CCNH*) or resistance (*DNAJB11* and *PARP1*). Our study identified a new gene signature that significantly predicts patients with higher probability to respond to treatment with trabectedin. Targeting some genes of this signature emerges as a potential strategy to enhance trabectedin efficacy.

1. INTRODUCTION

Sarcomas are a heterogeneous group of rare malignant tumors with mesenchymal origin that can affect soft, bone and visceral tissue. Sarcoma incidence is around 50 to 60 new cases per year for every million people, 20% being metastatic at diagnosis. Around 30-40% of localized sarcomas will eventually develop metastasis. In other words, a half of sarcoma population will suffer, sooner or later, metastatic spread. For locally advanced or metastatic soft-tissue sarcoma (STS), the therapeutic options are scarce and the Response Evaluation Criteria in Solid Tumors (RECIST) responses to chemotherapy are infrequent, while the median of survival ranges between 18 and 22 months.[1] While doxorubicin is the backbone of first-line therapy for advanced disease, second-line treatments includes pazopanib and chemotherapeutic agents such as trabectedin, eribulin and gemcitabine combinations. The median progression-free survival (PFS) in first-line therapy for STS ranges between 4.5 and 6.0 months, whereas the

median PFS for most of the second-line drugs is below 5 months. Around 50% of the patients obtain a clinical benefit from these drugs.[2, 3]

Trabectedin is an anti-tumor drug approved for the treatment of adult patients with advanced STS after failure of anthracyclines and ifosfamide, or for whom are unsuited to receive these agents.[4, 5] This compound is a DNA-binding agent that interferes with oncogenic gene transcription, which has been described as its main mechanism of action. Besides, the transcription-coupled nucleotide excision repair (NER) DNA repair machinery, in charge of repairing bulky adducts in DNA, is tricked by the special conformation of trabectedin adducts, ultimately inducing double strand breaks (DSBs) in the DNA. This process generates cell cycle perturbations, with a delayed S phase progression and accumulation of cells in G2 phase.[6] Additionally, trabectedin seems to affect also transcription of cytokines and growth factors in tumor-associated macrophages and circulating monocytes [7, 8], and it sensitizes cancer cells to FAS receptor programmed cell death.[9] It has been also suggested that trabectedin could be a good radiosensitizer, since it causes G2/M arrest, which is the most sensitive cell cycle phase to radiation.[10] The high efficacy of this combination was observed in phase I/II trials in patients with metastatic STS of diverse histologies and patients with localized myxoid liposarcoma.[11, 12]

Trabectedin treatment triggers DNA damage repair (DDR). Therefore, DDR-related factors might be potential predictive biomarkers for this drug. Few retrospective studies supported this observation, and reported that overexpression of genes involved in NER machinery (e.g. *ERCC1* and *ERCC5*) behaved as predictive biomarkers for trabectedin efficacy, whereas the overexpression of homologous-recombination (HR)-associated factors (e.g. *BRCA1*) were associated with trabectedin resistance.[13-15] In line with this, the potential predictive value of *ERCC1* and *ERCC5* was also validated by our group, in a cohort of cases prospectively collected from the randomized phase II trial that compared trabectedin plus doxorubicin versus doxorubicin alone as first line of advanced STS. [16] Nevertheless, the majority of these studies only focused on specific DDR-related proteins/ genes, such as *ERCC1*, *ERCC5* and *BRCA1* and did not analyze several other DDR-related genes that could also have predictive relevance for trabectedin efficacy.

Accordingly, the aim of this retrospective translational study was to evaluate the expression of 118 genes involved in DDR, as potential predictive biomarkers of trabectedin activity, and to determine if any of these genes or a gene signature could predict trabectedin efficacy, in a more robust way than the already described expression of certain limited genes (i.e. *ERCC1*, *ERCC5* and *BRCA1*). New biomarkers or novel insights on the mechanisms underlying drug activity may

allow the development of new therapeutic options for STS and a better selection of patients for trabectedin treatment.

2. METHODS

2.1 Patients and samples

A series of 140 formalin-fixed paraffin-embedded (FFPE) tumor samples, from STS patients treated with trabectedin in real-life setting at any line of advanced disease, was selected from a Spanish Group for Research on Sarcomas (GEIS) registry and was the basis of this translational research. All the samples were collected with the informed consent signed from the patients, according to national regulations, and the study protocol was approved by the Ethics Committees and the institutional review board of each participant center. Study procedures were performed in accordance with the Declaration of Helsinki

2.2 Oncology Biomarker Panel

The HTG EdgeSeq Oncology Biomarker Panel (OBP) direct-transcriptomic assay quantitatively measures the expression of 2549 human RNA transcripts (<https://www.htgmolecular.com/assays/obp>) associated with tumor biology. Among these RNA transcripts, 118 are associated with DDR mechanisms and only these genes were considered for the purpose of this study. For direct-transcriptomics, all the samples used in the assay had to have at least 70% of tumour area; samples with less than 70% tumour or with more than 20% of necrotic tissue underwent macro-dissection. All the samples and controls were quantified in triplicate and no template control was included in each run.

The RNA-Seq libraries were synthesized through HTG EdgeSeq chemistry. In summary, this method consists on a first step that lysed and permeabilized the sample, which allows the release of the RNA. Then, nuclease protection probes were added to the lysed samples to hybridize with the exposed mRNA and target-captured the transcripts. The S1 nuclease was added to the mix, allowing the production of a stoichiometric amount of target mRNA/ nuclease protection probes duplexes. The reaction was blocked by denaturing S1 enzyme by heat. In order to reduce potential biases in the run, the samples were randomized before the inclusion in the HTG EdgeSeq system.

PCR reactions were set up using each hybridized sample as template and specially designed tags that shared common sequences complementary to 5'- end and 3'- sequences of the probes,

as well as with common adaptors that are required for cluster generation on an Illumina sequencing platform. Additionally, each tag contains a unique barcode useful for sample identification and multiplexing. Agencourt AMPure XP (Beckman Coulter, Beverly, MA, USA) was used to clean the PCR amplification product.

Moreover, the libraries were quantified by quantitative PCR, using the KAPA Library Quantification (Roche) kit, according to the manufacturer's instructions. All samples and controls were quantified in triplicate and no template control was included in every run. Library denaturation was performed by adding first 2N NaOH to the library, followed by the addition of 2N HCl. The PhiX was spiked in at a 5% (concentration of 12.5 pM).

One demultiplexed FASTQ file per sample was retrieved from the sequencer for data processing. The HTG EdgeSeq host software performed the alignment of the FASTQ files with the probe list, the results were parsed and the output was obtained as a read counts matrix. The HTG EdgeSeq was ran in the VERIP service lab of HTG in Tucson (HTG Molecular Diagnostics, Tucson, Arizona, USA).

2.3 Data filtering and normalization

Negative control probes were used as quality control to evaluate baseline performance, as previously described by Chandly and colleagues.[17] Briefly, the mean of negative probes for each sample was calculated, and the difference between negative control average and the mean of all negative control probes was obtained (Δ^{mean}). Those samples with a Δ^{mean} outside the bounds of +/-2 standard deviation were excluded from the study. Of 140 sample initially included in the study, 7 were removed from the analysis. Data normalization was performed by applying trimmed mean of M-values method (TMM) using EdgeR package from R/Bioconductor, adjusting for the total reads within a sample.[18]

2.4 Differential gene expression analysis

For differential gene expression analysis, samples were grouped according to PFS, growth modulation index (GMI) or clinical benefit/ response of trabectedin. PFS was measured from the date of initial treatment with trabectedin (PFS) to the event of progression or death, whichever occurred first. Clinical benefit was defined as the percentage of patients achieving complete response (CR) or partial response (PR) and/or stable disease (SD). Response was evaluated according to RECIST v.1.1. GMI was expressed as a ratio of intra-patient successive time-to-progression (TTP): $\text{GMI} = \text{TTP under trabectedin} / \text{TTP for the treatment prior to trabectedin}$.

For PFS analysis, the low-risk group (better prognosis) was made up of the cases with a PFS value higher than the median (3.2 months) and the high-risk group (worse prognosis) comprised of the samples with PFS lower than the median. For GMI analysis, the low-risk group comprised of samples with a GMI ≥ 1.33 and the high-risk group the cases with a GMI < 1.33 . For RECIST response or clinical benefit analysis, samples were grouped as both: cases with response versus cases with stable or progressive disease or cases with response or stable disease versus cases with progression disease.

The differential gene expression between groups was evaluated by a negative binomial generalized log-linear model, using the EdgeR package and implementing the procedure proposed by Robinson and Smyth.[19] The correction for multiple comparisons of Benjamini & Hochberg was applied and a p-value threshold of 0.05 was set.[20] Fold change values were obtained, along with p-values and adjusted p-values (false discovery rate; FDR) for the 118 genes evaluated. The batch effect was taken into account by adding batch information to the model as a co-variable. For data visualization and later analyses, normalized log-TMM values were obtained and the variability due to batch effect was removed using the removeBatchEffect method implemented in limma R package.

All analyses were performed with R/Bioconductor (3.10) running on R version 3.6.0.

2.5 Statistical analysis

The variables that followed binomial distributions (e.g. patient demographics) were expressed as frequencies and percentages. Categorical variables were expressed as absolute and relative frequencies or as continuous variables as median, range (minimum-maximum). Gene expression levels were indicated as mean \pm standard deviation. The comparisons between quantitative and qualitative variables were performed through U of Mann-Whitney non-parametric test. FDR was used to adjust for multiple comparisons. PFS and overall survival (OS) from trabectedin initiation were estimated according to the Kaplan–Meier method. The log-rank test was used to determine the associations between the variables of interest (i.e., gene expression and clinical outcomes). The p-values reported were 2-sided, and the statistical significance was defined at $p \leq 0.05$. Statistical analysis was performed with SPSS 22.0 software (IBM, Armonk, NY, USA).

2.6 Construction of a predictive signature based on gene expression

Raw counts were normalized to transcripts per million (TPM), as it is a more appropriate unit to compare expression across samples than normalized counts from other methods such as

DESeq2 and edgeR. Of 140 cases included, only one patient with PFS=0 was removed from the predictive gene signature analysis.

Univariate Cox regression analysis using the survival package was performed to find individual genes whose expression was significantly associated ($p < 0.05$) with PFS. Additionally, patients were divided, for each gene, into two groups, using an optimal cut-off from the survminer package which maximized the separation of Kaplan-Meier curves of every group, and only those genes with significantly different curves ($p < 0.05$) were further retained.

The remaining genes were used as input to build a gene expression signature using a multivariate Cox regression applying a Lasso penalty to minimize the risk of overfitting with the glmnet package. The penalty parameter was calculated using a 4-fold cross validation (at the minimum partial likelihood deviance) of the complete cohort, randomly dividing 75% and 25% of patients into training and testing sets, respectively, 10000 times.

Risk scores were calculated by multiplying the expression of every gene with its corresponding Cox regression coefficient, for each patient:

Risk score = \sum Cox regression coefficient of Gene i * expression of gene Gene i

All analysis were performed in R (version 4.0.3).

3. RESULTS

3.1 Patient demographics

A subset of 140 STS patients treated with trabectedin at line of advance disease with FFPE tumor sample available for RNA expression analysis was included in this translational study. The median age was 51 (17-79) years with 54% (n=75) being female. Among the 140 STS included, 81% (n=114) were localized at diagnosis, with 66% (n=93) and 34% (n=47) having a somatic or visceral primary tumor location, respectively. Of 140 cases, 59% (n=83) were grade 3, 24% (n=33) were grade 2 and 13% (n=18) were grade 1. The most frequent subtypes were leiomyosarcoma (n=44; 31%), liposarcoma (n=32; 23%), synovial sarcoma (n=14; 10%) and undifferentiated pleomorphic sarcoma (n=14; 10%), while 26% (n=36) were diagnosed with other sarcoma subtypes. Patient demographics are depicted in Table 1.

3.2 Trabectedin outcome and univariate analysis

With a median follow up of 45 months, the median PFS of trabectedin line was 3.5 months, and the median of OS measured from the trabectedin initiation was 12 months. There were 124 out of 140 patients evaluable for response by RECIST with the following distribution: 6 (4.8%) CR, 14 (11.3%) PR, 42 (33.9%) SD and 62 (50.0%) PD as best response. The GMI was ≥ 1.33 in 40 (29%) cases, < 1.33 in 97 (69%) patients, whereas in 3 (2%) cases, this value was not calculable.

In the univariate analysis, grade 3 tumors were associated with a worse PFS [3.0 months (95% CI 2.2-3.8) vs 6.5 months (95% CI 2.0-11.0); $p=0.003$] and worse OS [10.2 months (95% CI 5.7-14.7) vs 17.5 months (95% CI 13.9-21.1); $p=0.041$], and diagnosis of L-sarcoma subtypes was correlated with a better PFS [6.1 months (95% CI 3.6-8.5) vs 3.0 months (95% CI 2.0-4.0); $p=0.001$] and better OS [18.2 months (95% CI 13.2-23.1) vs 7.2 months (95% CI 3.7-10.8); $p=0.001$]. Somatic location was associated with a better PFS in our series [5.6 months (95% CI 3.2-7.9) vs 2.7 months (95% CI 2.1-3.2); $p=0.006$]. Patients receiving trabectedin in first or second line had better PFS [6.3 months (95% CI 1.0-11.5) vs 3.2 months (IC95%: 2.4-4.0); $p=0.001$] (Table 2). Visceral location [HR=1.9 (95% CI 1.3-2.8), $p=0.002$] and non-L-sarcoma [HR=1.9 (95% CI 1.3-2.9), $p=0.001$] were independent factors associated with a worse PFS of trabectedin in the multivariate analysis.

3.3 Expression of DNA damage repair genes in STS

The expression of DDR-related genes was heterogeneous, being *DNAJB8* ($\log_2 2.80 \pm 1.45$) and *APEX1* ($\log_2 9.97 \pm 0.66$) the most underexpressed and overexpressed genes, respectively, among the transcripts analyzed (Supplementary Table S1).

Considering the expression of DDR-related transcripts in relation to the clinical variables grouped as: L-sarcomas vs non-L-sarcomas and grade 3 vs grade 1-2, the outcome was as follows: of 118 genes analyzed, only 2 genes were significantly overexpressed (i.e. *BRIP1*, *DNAJB5*) in L-sarcomas, compared to non-L-Sarcomas, whereas 16 genes were significantly underexpressed (i.e. *DDB2*, *DNAJA1*, *DNAJA2*, *DNAJB2*, *DNAJC10*, *DNAJC14*, *DNAJC16*, *ERCC3*, *ERCC6*, *MMS19*, *MSH2*, *PMS2*, *SMUG1*, *TDG* and *XRCC5*) in L-sarcomas, compared to other STS subtypes. Grade 3 tumours showed a significant overexpression, compared to grade 1-2 cases, of *APEX2*, *BRIP1*, *DNAJC1*, *DNAJC11*, *EXO1*, *FEN1*, *MSH2*, *MSH6*, *NEIL2*, *NEIL3*, *PRKDC*, *RAD21*, *RAD51*, *RAD54L*, *RPA3*, *TREX1* and *XRCC3*, whereas *ATM* and *XPC* were overexpressed in this latter group, compared with grade 3 tumors (Supplementary Table S2).

3.4 Differential gene expression and univariate analysis

Differential gene expression analysis, was carried out in 133 cases after data normalization.

Twenty genes were significantly and differently expressed, by multiple comparisons (FDR < 0.05), for the PFS of trabectedin (Table 3 and Supplementary Figure S1). High expression of *PARP3* (logFC=1.738; FDR<0.001), *POLL* (logFC=1.767; FDR=0.002), *PMS1* (logFC=0.764; FDR=0.003), *RAD52* (logFC=0.875; FDR=0.003), *ATM* (logFC=0.444; FDR=0.014), *NEIL1* (logFC=0.773; FDR=0.021), *TOP3B* (logFC=1.374; FDR=0.030), *CCNH* (logFC=0.620; FDR=0.030), *XRCC5* (logFC=0.862; FDR=0.030), *DDB2* (logFC=0.314; FDR=0.031), *MUTYH* (logFC=0.369; FDR=0.034), *DNAJB14* (logFC=0.420; FDR=0.035) and *XPC* (logFC=0.512; FDR=0.037) were associated with longer PFS, whereas the overexpression of *DNAJB11* (logFC=-0.378; FDR=0.003), *PARP1* (logFC=-0.442; FDR=0.007), *TDG* (logFC=-0.312; FDR=0.030), *TOP3A* (logFC=-0.451; FDR=0.030), *PRKDC* (logFC=-0.392; FDR=0.030), *DNAJC11* (logFC=-0.309; FDR=0.030) and *RAD23B* (logFC=-0.311; FDR=0.035) were associated with worse PFS.

The univariate analysis revealed that *PARP3* and *CCNH* overexpression were markedly related to a longer PFS of trabectedin: 7.7 months (95% CI 4.6-10.9) vs 2.8 months (95% CI 2.1-3.6), $p<0.001$ and 7.9 months (95% CI 4.8-11.0) vs 2.5 months (95% CI 1.9-3.0), $p<0.001$, respectively. On the other extreme, *DNAJB11* and *PARP1* overexpression were notably related to a shorter PFS of trabectedin: 2.5 months (95% CI 2.3-3.7) vs 8.2 months (95% CI 5.2-11.1), $p<0.001$ and 2.5 months (95% CI 1.8-3.1) vs 6.4 months (95% CI 4.0-8.8), $p<0.001$, respectively (Figure 1 and Supplementary Table S3). The remaining genes with statistical significance for the PFS of trabectedin, as well as their impact in OS from trabectedin initiation are represented in Supplementary Table S3.

The overexpression of *BRCA1* gene was significantly associated with worse PFS of trabectedin [2.5 months (95% CI 1.6-3.3) vs 6.1 months (95% CI 2.5-9.7), $p=0.001$]; however, neither the expression of *ERCC1* [5.2 months (95% CI 2.5-8.0) vs 3.0 months (95% CI 2.2-3.9), $p=0.084$] nor of *ERCC5* [3.2 months (95% CI 2.3-4.1) vs 4.4 months (95% CI 1.4-7.4), $p=0.182$] showed a significant association with PFS of trabectedin (Supplementary Table S3).

Considering GMI values, grouped as $GMI \geq 1.33$ vs $GMI < 1.33$, only 6 genes were differentially expressed with statistical significance between both groups (FDR < 0.05). Patients with a $GMI \geq 1.33$ showed significantly higher expression of *DNAJC16* (logFC=1.163; FDR=0.001), *XPC* (logFC=0.799; FDR=0.004), *DNAJB14* (logFC=0.611; FDR=0.006), *ATM* (logFC=0.523; FDR=0.008), *DNAJC7* (logFC=0.684; FDR=0.008) and *NEIL2* (logFC=0.564; FDR=0.039), compared with those with a $GMI < 1.33$ (Table 4).

Differential gene expression reached statistical significance in 20 genes when it was considered obtaining or not clinical benefit (CR+PR+SD vs PD) (Table 5). High expression of *PARP3* (logFC=1.538; FDR=0.007), *POLL* (logFC=1.876; FDR=0.007), *RAD52* (logFC=0.849; FDR=0.009), *TOP3B* (logFC=1.818; FDR=0.009), *XRCC5* (logFC=1.073; FDR=0.009), *DNAJB14* (logFC=0.463; FDR=0.032), *XPC* (logFC=0.559; FDR=0.036), *ATM* (logFC=0.383; FDR=0.036), *MSH5* (logFC=0.843; FDR=0.043) and *NEIL2* (logFC=0.467; FDR=0.043) were associated with clinical benefit of trabectedin, whereas overexpression of *RAD21* (logFC=-0.522; FDR=0.007), *MSH6* (logFC=-0.369; FDR=0.009), *DNAJB11* (logFC=-0.336; FDR=0.011), *TDG* (logFC=-0.332; FDR=0.024), *XRCC4* (logFC=-0.394; FDR=0.024), *PARP1* (logFC=-0.373; FDR=0.036), *ERCC3* (logFC=-0.242; FDR=0.036), *PRKDC* (logFC=-0.380; FDR=0.043), *RAD23B* (logFC=-0.315; FDR=0.043) and *TOP3A* (logFC=-0.423; FDR=0.043) were associated with progressive disease after trabectedin. By contrast, no different gene expression was significantly detected, by FDR, when the grouping was responders (CR+PR) vs non-responders patients (Supplementary Table S4).

Regarding the different analysis performed, only 3 genes were significantly associated with a longer PFS (Table 3), GMI ≥ 1.33 (Table 4) and clinical benefit of trabectedin (Table 5): *ATM*, *DNAJB14* and *XPC* (Supplementary Figure S2). Further, 14 significantly expressed genes were common for PFS and clinical benefit endpoints: *ATM*, *DNAJB11*, *DNAJB14*, *PARP1*, *PARP3*, *POLL*, *PRKDC*, *RAD23B*, *RAD52*, *TDG*, *TOP3A*, *TOP3B*, *XPC* and *XRCC5* (Supplementary Figure S2).

Differential gene expression was also determined according to clinical variables with an independent value for PFS in the multivariate analysis: Supplementary Table S5 (L-sarcomas vs other subtypes) and Supplementary Table S6 (visceral vs somatic location). *PARP3* and *PMS1*, which were genes associated with a better PFS of trabectedin, were overexpressed in L-sarcomas and in tumors with a somatic location, whereas *PARP1*, a gene that correlated with worse PFS of trabectedin, was highly expressed in non-L-sarcomas and tumors with visceral location.

3.5 Predictive gene signature of trabectedin activity

Univariate Cox regression model on a cohort of 139 patients identified 8 genes whose expression was significantly associated with PFS of trabectedin. Further selection of genes whose Kaplan-Meier curves displayed significant differences (patients were divided into two groups based on gene expression median value) yielded 6 final genes. Lasso-penalized Cox regression analysis was performed to construct a predictive gene-signature for trabectedin. The six genes identified

were: Exonuclease 1 (*EXO1*), Cyclin H (*CCNH*), Xeroderma Pigmentosum Group A-Complementing Protein (*XPA*), Poly(ADP-Ribose) Polymerase 1 (*PARP1*), *BRCA1* and Apurinic/Apyrimidinic Endodeoxyribonuclease 2 (*APEX2*).

$$\text{Risk score} = 0.1866930 * \text{Expression}_{\text{EXO1}} + (-0.1966279) * \text{Expression}_{\text{CCNH}} + (-0.2043687) * \text{Expression}_{\text{XPA}} + 0.2293783 * \text{Expression}_{\text{PARP1}} + 0.0129766 * \text{Expression}_{\text{BRCA1}} + 0.2278331 * \text{Expression}_{\text{APEX2}}$$

The six-gene based risk score was calculated for each patient and the optimal cut-off for the risk score (2.146) was determined. An additional univariate Cox regression analysis was performed using risk scores, resulting in a significant association with PFS (Supplementary Table S7). Time-dependent ROC and Kaplan–Meier curves were used to assess the predictive value of the gene signature. The AUCs (area under the ROC curve) for 3-month, 12-month, and 24-month PFS were 0.67, 0.65 and 0.65, respectively (Supplementary Figure S3). Patients in the high-risk group (higher expression of *EXO1*, *PARP1*, *BRCA1* and *APEX2*, and lower expression of *CCNH* and *XPA*) showed a significantly worse PFS compared to the patients in the low-risk group (lower expression of *EXO1*, *PARP1*, *BRCA1* and *APEX2*, and higher expression of *CCNH* and *XPA*), [2.1 months (95% CI 1.8-2.4) vs 6.0 months (95% CI 4.5-7.5), $p < 0.001$ (Figure 2)]. PFS rate at 6 months and at 12 months was 16% and 5%, respectively, for the high-risk group and 48% and 25%, respectively, for the low-risk group. In the multivariate analysis, the high-risk group was an independent factor associated with PFS [HR 2.1 (95% CI 1.4-3.2), $p < 0.001$]; visceral location and non-L-sarcomas maintained the independent value (Supplementary Table S8).

In the patients treated with either doxorubicin or gemcitabine, the DDR-related gene signature was also correlated with the PFS of these drugs. The six-gene based signature failed to significantly predict gemcitabine or doxorubicin activity, in terms of PFS, in our series (Supplementary Table S9).

4. DISCUSSION

A 6-gene predictive signature of trabectedin efficacy was built based on the transcriptomic analysis of 118 DDR-related genes and PFS, in a retrospective series of 139 advanced STS patients. Previously described predictive genes for trabectedin efficacy, as those related to NER machinery, such as *ERCC1* and *ERCC5*, did not show any relevant prognostic role in our series. Rather, other DDR-related genes showed more consistent prediction of trabectedin activity, either in terms of PFS, GMI and/or clinical benefit. For this study, we decided to focus only in the relevance of DDR-related genes, since the DNA DSBs induced by trabectedin along with the involvement of NER machinery and HR repair genes was previously reported as a potential

relevant mechanism of trabectedin activity in STS patients.[13, 21, 15] However, the majority of these studies were limited to few DDR-related genes/ proteins as potential predictive biomarkers of trabectedin, in consequence many other potential predictive factors from the family of DNA repair genes have been unexplored in this regard. Hence, we aimed to evaluate, in a broader way, the predictive role of a large number of DDR-related genes using HTG EdgeSeq next-generation sequencing (NGS)-adapted molecular profiling. Subsequently, an established 6-gene signature was able to markedly separate two significant different populations (favorable or unfavorable signature) for trabectedin efficacy in terms of PFS.

Even when *BRCA1* (expression levels or haplotype status) had been previously associated with the clinical benefit of trabectedin [21, 15], its predictive role was weaker than the gene signature. Overexpression of *XPA* gene, another component of the signature and a NER-associated gene, was in line with previous reports that showed a direct correlation between higher expression of NER-machinery related genes and better trabectedin efficacy.[22, 23] Besides, and to the best of our knowledge, this is the first time that the expression of *EXO1*, *CCNH* and *APEX2* had been associated with trabectedin efficacy. *EXO1* is an XPG family nuclease that participates in several DDR pathways, such as DNA mismatch repair (MMR), HR and non-homologous end joining (NHEJ).[22] Of note, the expression of *EXO1* was associated with resistance to cisplatin and doxorubicin via NHEJ in ovarian cancer, a tumor also sensitive to trabectedin. Nevertheless, the prognostic/ predictive value of *EXO1* in STS is still unknown.[24, 25] Cyclin H, coded by the *CCNH* gene, is a protein that forms a cyclin activating kinase-subcomplex with *CDK7* and *MAT1*. This subcomplex is part of the transcription factor II human (TFIIH) protein complex and it is involved in NER, by regulating cell cycling progression during DNA damage.[26] As expected in accordance to the role of Cyclin H in NER pathway, a high expression of this gene, was associated with trabectedin sensitivity in our signature. *APEX2* is a weak apurinic/ apyrimidinic (AP) endonuclease in the DNA base excision repair (BER) pathway and functions in the context of DNA damage by oxidative and alkylating agents. *APEX2* also exhibits strong activities of 3'-5' exonuclease and 3'-phosphodiesterase, and that has been described to be necessary for the recovery of B-cell lymphocyte progenitors after chemotherapy.[27, 28] Moreover, *APEX2* has been identified as a synthetic lethal gene, in *BRCA1* and *BRCA2* deficient cells.[29] *APEX2* processes AP sites at replication forks, avoiding the blocking of fork progression and the induction of DSBs, which required *BRCA1/2* for repair and DNA synthesis.[29] Of note, trabectedin has been described to cause replication impairment and genome instability, via RNA–DNA hybrid-dependent DNA damage, which is important for drug activity.[30] Accordingly, it is possible that *APEX2* may have a role in resolving replication impairment and genome instability

induced by trabectedin, which may justify its association with lack of drug activity in our gene signature. This could also open the option to target APEX2 as a potential therapeutic strategy in combination with trabectedin if this gene is overexpressed. Remarkably, our 6-gene signature is in line with previous reports [13, 16, 15], wherein the high expression of NER genes (e.g. *XPA* and *CCNH*) and the low expression of HR genes (e.g. *EXO1* and *BRCA1*) would help to identify the STS patients that benefit from trabectedin treatment.

Furthermore, several DDR-related genes were significantly associated with trabectedin activity, in terms of PFS, GMI and/ or clinical benefit. Among these genes, *PARP3* overexpression significantly associated with a better PFS and clinical benefit following trabectedin. *PARP3*, initially described in 1999 [31], is a poly(ADP ribosyl)transferase closely related with *PARP1* and *PARP2* and it is highly expressed in skeletal muscle. [32] *PARP3* is involved in transcriptional silencing and in the maintenance of genomic integrity[33], being part of the Polycomb group (PcG) complexes, which are relevant to maintain the silencing of specific critical genes related to development among others. More precisely, *PARP3* was shown to interact with core proteins of the Polycomb repressive complexes 2 and 3, such as the methyltransferases *EZH2* and *Suz12*, which are relevant in the maintenance of repression through post-translational methylation processes.[33] In turn, *PARP1* expression, a gene also included in our potential predictive molecular signature, was significantly associated with shorter PFS and lack of clinical benefit after trabectedin treatment. The distinct behavior of *PARP1* and *PARP3* genes in our study, may indicate that these proteins may participate differently in some cellular mechanisms, which could affect the response to trabectedin. Both proteins are relevant for DNA genomic integrity[34]; however, it seems that *PARP1* overexpression promotes cell cycle entry[35-37], whereas *PARP3* overexpression interferes with G1/S cell cycle progression[38]. Of note, G1 seems to be the cell cycle phase in which cells are more sensitive to trabectedin.[39] Accordingly, the effect of the overexpression of each one of this proteins in cell cycle progression may justify, at least in part, the different results for both *PARP1* and *PARP3* in our study. Noteworthy, *PARP3* seems to be overexpressed in tumors that benefit from trabectedin treatment (i.e. L-sarcomas and tumors with somatic location), while *PARP1* expression is higher in sarcomas with lower activity of trabectedin (non-L-sarcomas and tumors with visceral location). Therefore, the expression of these genes may be important biomarkers of trabectedin efficacy or resistance, in specific clinical contexts that depend on the histologic subtype or the location of the primary tumor. Besides, the association of *PARP1* high expression with worse trabectedin outcome, support the combination of *PARP1* inhibitors with this chemotherapeutic agent. In line with this observations, the combination of olaparib with trabectedin has been tested in a phase 1b clinical trial in patients with advanced and

non-resectable bone and STS (ClinicalTrials.gov, number NCT02398058). Of 50 patients recruited, 7 had a PR, suggesting that this combination has antitumor activity[40]; however, patient selection based on PARP1 and/ or PARP3 expression levels should be considered for further clinical trials with this combination.

In the study presented here, only *BRCA1* high expression was significantly associated with shorter PFS and OS, while *ERCC1* and *ERCC5* expression, which were previously associated with good prognosis to trabectedin [13, 16, 15], did not had a significant impact in survival. The different methodology used for gene expression analysis could justify the distinct results obtained in terms of survival observed for these two genes; however, it is important to mention that HTG quantitative nuclease protection assays for gene detections had been reported to have similar sensitivity as qPCR.[41, 42] Accordingly, our results may suggest that NER-associated genes *ERCC1* and *ERCC5* are not robust and reliable biomarkers of trabectedin activity. Their predictive value should accordingly be considered with caution; however, a further study with a larger cohort of cases, should validate our data.

Among the study limitations, it is important to mention that the tumor samples used for this retrospective analysis were collected at the diagnostic time, and gene expression levels could had been altered due to the several lines of previous treatment or during the natural progression of the disease. Moreover, another important limitation is related with its initial hypothesis; for this analysis only genes related with DDR were selected and studied. This selection excluded, from the molecular signature, genes involved in other cellular processes, which may also predict trabectedin activity. Since we cannot exclude the relevance of other genes beyond DDR, further analysis, considering all the genes included in the OBP assay are currently ongoing. The absence of an independent validation cohort, or a control cohort treated with any other drug approved for advanced STS, are other limitations of this study. The validation of the predictive value of the gene-based signature in a larger series of cases could also help improve the performance of ROC curves.

5. CONCLUSIONS

In summary, this translational study identified a new molecular signature that could significantly predicts patients with higher probability for trabectedin efficacy. This signature was based on wide transcriptomic analysis of DDR-related genes and provides new insights for potential targets that could enhance the trabectedin efficacy. Validation of this signature is in process with an independent series of advanced STS treated with trabectedin. Finally, the potential value of

specific biomarkers (e.g. PARP3) is currently being tested in the preclinical setting, in order to describe the mechanisms in which they participate under drug treatment.

Data availability statement: The data that support the findings of this study are available from the corresponding [jmartin@mustbesevilla.org] upon reasonable request.

Author contributions: DSM and JMB designed the experiments and analysis. All the authors participated in data acquisition. DSM, MPC, JACV and JMB performed data analysis and interpretation. DSM, MPC, JACV and JMB drafted the manuscript. All the authors reviewed the manuscript and approved its final version.

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from PharmaMar, Lilly, Bayer and Eisai; Travel Grants from PharmaMar; Research Grants from PharmaMar, Eisai, Immix BioPharma and Novartis outside the submitted work. Research Funding for his Institution (for clinical studies) from PharmaMar, Eli Lilly and Company, AROG, Bayer, Eisai, Lixte, Karyopharm, Deciphera, GSK, Novartis, Blueprint, Nektar, Forma, Amgen, Adaptimmune and Daichii-Sankyo.

Table 1. Patient demographics

	N (%)
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Gender	
Male	65 (46)
Female	75 (54)
Stage at diagnosis	
Localized	114 (81)
Metastatic	26 (19)
Sarcoma subtype	
Leiomyosarcoma	44 (31)
Liposarcoma	32 (23)
Synovial sarcoma	14 (10)
Undifferentiated pleomorphic sarcoma	14 (10)
Other	36 (26)
Grade	
1	18 (13)
2	33 (24)
3	83 (59)
Not available	6 (4)
Location	
Somatic	93 (66)
Visceral	47 (34)
Median follow up from diagnostic (months)	45
Median follow up from trabectedin line (months)	12
Median Age, years (range)	51 (17-79)

Table 2. Univariate analysis of clinical factors

Factor	PFS (95% CI)	p	OS (95% CI)	p
Sex:		0.276		0.613
- Female	3.2 (2.5-4.0)		13.1 (8.0-18.2)	
- Male	4.4 (2.5-6.3)		13.1 (4.8-21.5)	
Age:		0.178		0.674
- < 51	5.1 (2.2-8.0)		12.5 (5.8-19.2)	
- > 51	3.0 (2.1-3.9)		13.1 (6.2-20.1)	
Subtype:		0.001		0.001
- L-Sarcoma	6.1 (3.6-8.5)		18.2 (13.2-23.1)	
- Non-L-Sarcoma	3.0 (2.0-4.0)		7.2 (3.7-10.8)	
Grade:		0.003		0.041
- 1 and 2	6.5 (2.0-11.0)		17.5 (13.9-21.1)	
- 3	3.0 (2.2-3.8)		10.2 (5.7-14.7)	
Location:		0.006		0.051
- Somatic	5.6 (3.2-7.9)		17.0 (10.2-23.9)	
- Visceral	2.7 (2.1-3.2)		11.3 (7.6-15.0)	
Stage at diagnosis:		0.057		0.523
- Localized	4.4 (2.1-6.7)		13.8 (8.6-19.0)	
- Metastatic	3.0 (2.2-3.8)		5.4 (0.0-16.7)	
Trabectedin line:		0.001		0.218
- 1/2	6.3 (1.0-11.5)		17.0 (9.5-24.6)	
- >2	3.2 (2.4-4.0)		12.0 (5.8-18.1)	

Table 3. Differential gene expression attending to median PFS (3.2 months)

Gene	logFC	P-Value	FDR
<i>PARP3</i>	1.738	<0.001	<0.001
<i>POLL</i>	1.767	<0.001	0.002
<i>DNAJB11</i>	-0.378	<0.001	0.003
<i>PMS1</i>	0.764	<0.001	0.003
<i>RAD52</i>	0.875	<0.001	0.003
<i>PARP1</i>	-0.442	<0.001	0.007
<i>ATM</i>	0.444	0.001	0.014
<i>NEIL1</i>	0.773	0.001	0.021
<i>TDG</i>	-0.312	0.003	0.030
<i>TOP3A</i>	-0.451	0.003	0.030
<i>TOP3B</i>	1.374	0.003	0.030
<i>CCNH</i>	0.620	0.003	0.030
<i>XRCC5</i>	0.862	0.003	0.030
<i>PRKDC</i>	-0.392	0.004	0.030
<i>DNAJC11</i>	-0.309	0.004	0.030
<i>DDB2</i>	0.314	0.004	0.031
<i>MUTYH</i>	0.369	0.005	0.034
<i>RAD23B</i>	-0.311	0.006	0.035
<i>DNAJB14</i>	0.420	0.006	0.035
<i>XPC</i>	0.512	0.006	0.037
<i>DNAJC21</i>	-0.420	0.009	0.050
<i>NEIL2</i>	0.420	0.012	0.066
<i>EXO1</i>	-0.522	0.017	0.087
<i>DNAJB8</i>	0.700	0.022	0.100
<i>PMS2</i>	0.326	0.023	0.100
<i>DNAJC16</i>	0.541	0.024	0.100
<i>MSH6</i>	-0.230	0.024	0.100
<i>ERCC5</i>	0.242	0.025	0.100

<i>ERCC3</i>	-0.184	0.026	0.100
<i>APEX2</i>	-0.266	0.027	0.100
<i>DNAJB6</i>	-0.177	0.027	0.100
<i>BRCA1</i>	-0.333	0.027	0.100
<i>XRCC4</i>	-0.271	0.029	0.104
<i>RAD21</i>	-0.295	0.030	0.104
<i>MSH4</i>	0.512	0.034	0.114
<i>DNAJB5</i>	0.454	0.035	0.114
<i>OGG1</i>	0.230	0.036	0.116
<i>DNAJC10</i>	-0.241	0.038	0.119
<i>DNAJA1</i>	-0.219	0.041	0.125
<i>MLH3</i>	0.209	0.046	0.135
<i>DNAJC7</i>	0.348	0.048	0.137

FC: fold change; FDR: false discovery rate. A negative fold change means that the gene is overexpressed in cases with PFS below the median.

Table 4. Differential gene expression attending to growth modulation index.

Gene	logFC	P-Value	FDR
<i>DNAJC16</i>	1.163	< 0.001	0.001
<i>XPC</i>	0.799	< 0.001	0.004
<i>DNAJB14</i>	0.611	< 0.001	0.006
<i>ATM</i>	0.523	< 0.001	0.008
<i>DNAJC7</i>	0.684	< 0.001	0.008
<i>NEIL2</i>	0.564	0.002	0.039
<i>DNAJC8</i>	0.402	0.004	0.068
<i>MSH4</i>	0.737	0.006	0.088
<i>XRCC6BP1</i>	0.912	0.009	0.121
<i>TOP3A</i>	-0.446	0.011	0.130
<i>PARP1</i>	-0.358	0.013	0.136
<i>RAD52</i>	0.606	0.016	0.158
<i>BRCA2</i>	0.349	0.022	0.200
<i>PRKDC</i>	-0.343	0.028	0.232
<i>DNAJB5</i>	0.505	0.029	0.232
<i>XRCC2</i>	0.297	0.032	0.233
<i>POLL</i>	0.876	0.037	0.258
<i>ATR</i>	0.215	0.039	0.258

FC: fold change; FDR: false discovery rate. A negative fold change means that the gene is overexpressed in cases with < 1.33.

Table 5. Differential gene expression according to clinical benefit

	logFC	P-Value	FDR
<i>PARP3</i>	1.538	<0.001	0.007
<i>POLL</i>	1.876	<0.001	0.007
<i>RAD21</i>	-0.522	<0.001	0.007
<i>MSH6</i>	-0.369	<0.001	0.009
<i>RAD52</i>	0.849	<0.001	0.009
<i>TOP3B</i>	1.818	<0.001	0.009
<i>XRCC5</i>	1.073	0.001	0.009
<i>DNAJB11</i>	-0.336	0.001	0.011
<i>TDG</i>	-0.332	0.002	0.024
<i>XRCC4</i>	-0.394	0.002	0.024
<i>DNAJB14</i>	0.463	0.003	0.032
<i>PARP1</i>	-0.373	0.004	0.036

<i>XPC</i>	0.559	0.004	0.036
<i>ERCC3</i>	-0.242	0.004	0.036
<i>ATM</i>	0.383	0.005	0.036
<i>MSH5</i>	0.843	0.006	0.043
<i>PRKDC</i>	-0.380	0.006	0.043
<i>RAD23B</i>	-0.315	0.007	0.043
<i>NEIL2</i>	0.467	0.007	0.043
<i>TOP3A</i>	-0.423	0.007	0.043

<i>DNAJC16</i>	0.649	0.009	0.052
<i>DNAJC21</i>	-0.406	0.014	0.070
<i>DNAJC11</i>	-0.271	0.014	0.070
<i>SLK</i>	-0.267	0.018	0.089
<i>DNAJC10</i>	-0.279	0.020	0.094
<i>DNAJA1</i>	-0.247	0.025	0.115
<i>XRCC1</i>	-0.252	0.031	0.133
<i>DNAJB6</i>	-0.178	0.031	0.133
<i>DNAJC7</i>	0.387	0.034	0.136
<i>DNAJC1</i>	-0.244	0.043	0.160
<i>DDB2</i>	0.226	0.044	0.160
<i>BRCA1</i>	-0.315	0.044	0.160
<i>MRE11A</i>	0.249	0.045	0.160

FC: fold change; FDR: false discovery rate. A negative fold change means that the gene is overexpressed in cases progression disease.

Figure 1

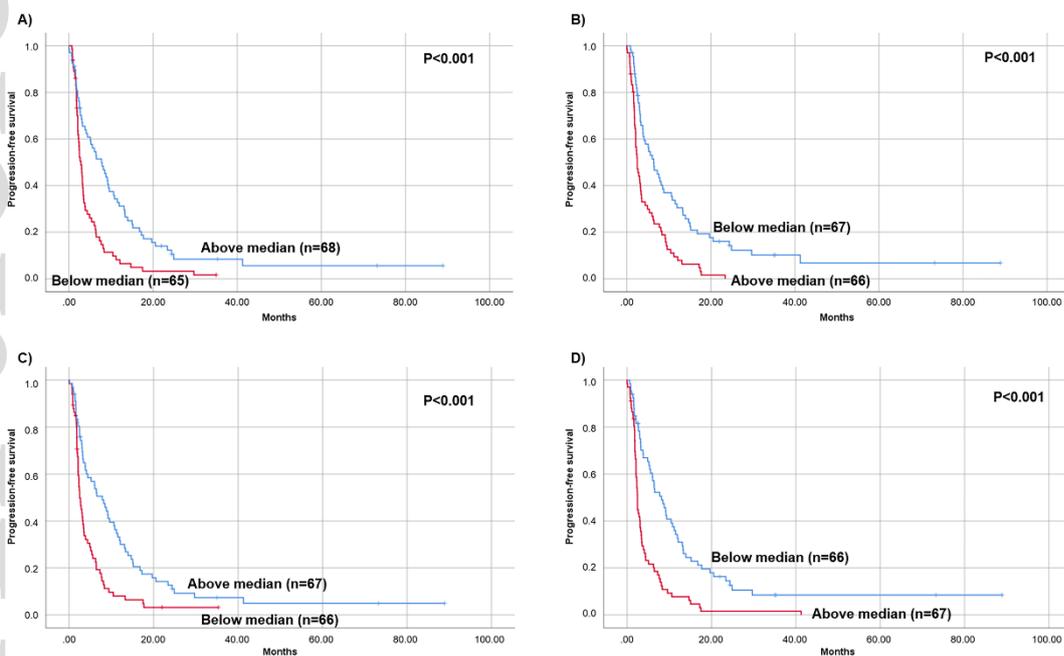


Figure 2

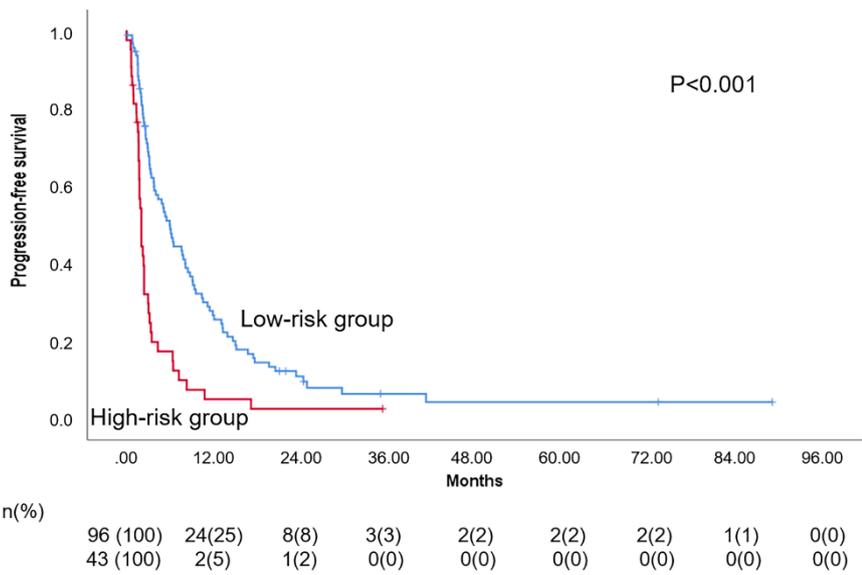


Figure legends

Figure 1. Univariate analysis. A) Progression-free survival according to *PARP3* gene expression; B) Progression-free survival according to *PARP1* gene expression; C) Progression-free survival according to *CCNH* gene expression and D) Progression-free survival according to

DNAJB11 gene expression. Groups were defined according to the median of gene expression; above and below median. Log-rank test statistical significance was defined at $p \leq 0.05$.

Figure 2. Risk-groups Kaplan-Meier curve. Patients were grouped according to the risk scores cut-off value (2.146). Number at risk were represented as n(%). Log-rank test statistical significance was defined at $p \leq 0.05$.

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SUPPORTING INFORMATION

Supplementary Table S1. Expression values of DNA damage repair-related genes

Supplementary Table S2. Gene significantly expressed between L-sarcoma and non-L-sarcomas and between grade 3 tumors vs grade 1+2 tumors

Supplementary Table S3. Univariate analysis of DNA damage repair-related genes with impact in progression-free survival to trabectedin-line

Supplementary Table S4. Differential gene expression according to objective response

Supplementary Table S5. Differential gene expression according to histologic subtype grouping

Supplementary Table S6. Differential gene expression according to tumor location

Supplementary Table S7. Univariate Cox regression analysis for the values of the gene signature (risk scores)

Supplementary Table S8 – Multivariate analysis

Supplementary Table S9 – Correlation between risk groups and progression-free survival of doxorubicin or gemcitabine

Supplementary Figure S1. Volcano plot of genes with prognostic relevance in progression free survival (PFS) of trabectedin. Cases were grouped according to median PFS (3.2 months).

Supplementary Figure S2. Venn diagram comparing the unique and shared genes between progression-free survival, growth modulation index and clinical benefit.

Supplementary Figure S3. Time-dependent ROC curve to assess the predictive value of the gene signature. Area under the ROC curve (AUC) at 3 months (A), 12 months (B) and 24 months (C).