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ORIGINAL ARTICLE

Unraveling the complexity of HRD assessment in ovarian cancer by combining genomic and functional approaches: translational analyses of MITO16-MaNGO-OV-2 trial

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Background: Ovarian cancer (OvC) constitutes significant management challenges primarily due to its late-stage diagnosis and the development of resistance to chemotherapy. The standard treatment regimen typically includes carboplatin and paclitaxel, with the addition of poly (ADP-ribose) polymerase inhibitors for patients with high-grade serous ovarian cancer (HGSOC) harboring *BRCA1/2* mutations. However, the variability in treatment responses suggests the need to investigate factors beyond *BRCA1/2* mutations, such as DNA repair mechanisms and epigenetic alterations. Notably, homologous recombination repair deficiency (HRD) is observed in an additional 20% of HGSOC cases, indicating a broader spectrum of DNA repair defects. Existing commercial HRD assays have certain limitations, prompting a global effort to develop new genomic and functional tests through academic research.

Materials and methods: This study investigates, in the 187 high-grade serous and endometrioid tumors from the MITO16/MaNGO-OV-2 trial, academic HRD genomic tests in conjunction with a RAD51 immunofluorescence assay to assess functional activation of HRD. Additionally, the study incorporates analysis of microRNA-506 (miR-506) expression as a putative epigenetic effector.

Results: The RAD51 test identified HRD in 73% of the samples and genomic HRD testing in 57%, with HRD identified in 45% of samples by both tests. The significant discrepancy between the two assays emphasizes the need to refine tumor classification for HRD. A three-group genomic classification unveiled superior progression-free survival (PFS) in high- and mild-HRD tumors compared with negative-HRD tumors. High concordance between RAD51 and genomic testing in high-HRD tumors suggests a subset of 'super-HRD' tumors exhibiting superior PFS. High expression of miR-506 may be used to further refine HRD status.

Conclusions: The study underscores the complexities of HRD assessment and advocates for a combined genomic and functional approach to enhance predictive accuracy in OvC treatment responses.

Key words: ovarian cancer, HRD, RAD51, miR-506

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INTRODUCTION

Ovarian cancer (OvC) is the primary cause of death among gynecological malignancies, characterized by a lack of early diagnosis and the development of chemo-resistance, resulting in poor long-term disease control.[1] The standard first-line chemotherapy treatment involves the combination

of carboplatin and paclitaxel. However, the heterogeneity of treatment response among patients prompts the exploration of additional predictive biomarkers of response/resistance such as impairment in DNA repair mechanisms and epigenetic changes.[2] Data coming from The Cancer Genome Atlas analysis have clearly shown that ∼30% of the patients with high-grade serous ovarian cancer (HGSOC) carry a germline or somatic mutation in *BRCA1* or *BRCA2* (*BRCA1/2*) genes, involved in the homologous recombination repair (HRR) pathway. These patients present better prognosis, higher response to platinum and better outcome after maintenance treatment with poly (ADP-ribose) polymerase inhibitors (PARPi). It has been commonly recognized that HRR deficiency (HRD) is not restricted to patients with *BRCA1/2* mutations. Other mutations and epigenetic changes in HRR genes account for a further 20% of HRD in HGSOC.[3]

The efficacy of niraparib and rucaparib has been documented in first line also for 'all-comers' patients, although better response is observed for *BRCA1/2*-mutated and HRD patients.[4,5] Nonetheless, several HRR-proficient (HRP) patients do respond to platinum and PARPi raising the question of a better understanding of the biology and better testing to predict the response to treatment.[4,6]

The myChoice CDx assay by Myriad Genetics was the first commercially available HRD assay, assessing mutations in *BRCA1/2* genes and genomic instability patterns topically developed by HRD tumors.[7] The assay combines the results of three molecular parameters, expressed as the sum of loss of heterozygosity (LOH), telomeric allelic imbalance and large-scale state transitions.[7] The definitions of HRD positivity varied between the trials.[7] All trials used an HRD score cut-off of $\geq$42 to define HRD status, but as the methodology for cut-off selection has not been shown, the test is considered poorly reproducible, and the definition of an unequivocal score value is far to be achieved.[4,6,7] The Foundation Medicine CDxBRCA is another test detecting *BRCA1/2* mutations and assessing the percentage of the genome affected by LOH.[8] Tumors are considered to be HRD if the LOH score is $\geq$16.[8]

Many other genomic tests have been recently developed and proved to be able to predict response to PARPi with similar efficacy than the myChoice CDx assay by Myriad Genetics.[2] Despite their efficacy, these commercial tests have limitations, including 'unknown' status (i.e. tumors for which the test was unable to provide a definitive determination of the HRD status), false negatives and high costs.[2] Furthermore, these tests are static and do not catch the dynamic evolution of the sensitivity to treatment.[2,9] New genomic and functional academic tests are being developed globally to overcome these limitations.

In the preliminary experience from our group, we investigated two academic genomic tests to assess the HRD status and combined these with the RAD51 assay to evaluate the functional activation of the HRR pathway.[10] The performance of our academic HRD test has been recently confirmed in an independent cohort of patients using two different pipelines for shallow whole-genome sequencing (WGS) compared with the myChoice CDx assay.[11]

In this paper, we further explore this concept increasing the sample size and combining also with other putative epigenetic effectors such as microRNA-506 (miR-506) whose overexpression in cellular models impairs RAD51 foci formation following induction of DNA damage, increasing cell line sensitivity to platinum salts and PARPi,[12,13] and correlates to better prognosis in OvC patients.[12]

MATERIALS AND METHODS

Study design

This study is part of the translational activities associated with the MITO16A/MaNGO-OV2 (EudraCT number: 2012-003043-29, hereafter indicated as MITO16A) clinical trial, coordinated by the Clinical Trials Unit at Istituto Nazionale Tumori IRCCS "Fondazione G. Pascale" in Naples. MITO16A is a phase IV trial that aims to explore the prognostic role of selected biomarkers in epithelial ovarian cancer patients treated in first line with chemotherapy (paclitaxel + carboplatin × 6) plus bevacizumab (BEV) (15 mg/kg) for 15 months. Overall, 398 patients were prospectively enrolled.[14]

Sample collection and processing

MITO16A samples have been collected and processed in the coordinating center. A 5-μm section was cut from each formalin-fixed paraffin-embedded (FFPE) block, stained with hematoxylin—eosin and reviewed by a trained gynecologic pathologist. For the current analysis, 187 samples of all available high-grade serous and endometrioid samples with available DNA and FFPE blocks were selected for the dedicated analyses.

Extraction of total nucleic acids was carried out as described.[15] Briefly, extraction of genomic DNA from FFPE tissues was carried out from two cores of paraffin-embedded tissues using the GeneRead DNA FFPE Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. Total RNA was extracted from two 1-mm cores of FFPE tissues using the QIAGEN miRNeasy FFPE Kit. RNA concentration was assessed using the NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA) and samples with RNA concentration $<$80 ng/μl were excluded from the study. Sample purity and RNA integrity were evaluated as described.[15] For 140 out of 187 patients, miRNA expression profiles were also available (see following section). The institutional review boards of the involved institutions have approved the study design. All patients provided informed consent to the use of their data for research purposes before enrolment.

Immunofluorescence staining and scoring

The immunofluorescence-based RAD51 test was carried out on FFPE patient tumor samples as described in Castroviejo-Bermejo et al. and Cruz et al.[16,17]

The following primary antibodies were used: rabbit anti-RAD51 (Abcam ab133534, 1 : 1000), mouse anti-geminin (NovoCastra NCL-L, 1 : 100 in patient-derived xenograft samples, 1 : 60 in patient samples), rabbit anti-geminin (ProteinTech 10802-1-AP, 1 : 400), mouse anti-BRCA1 (Abcam ab16780, 1 : 200), mouse anti-phospho-H2AX (Millipore #05-636, 1 : 200). Goat anti-rabbit Alexa fluor 568 (Invitrogen; 1 : 500), goat anti-mouse Alexa fluor 488 (Invitrogen; 1 : 500), donkey anti-mouse Alexa fluor 568 (Invitrogen; 1 : 500) and goat anti-rabbit Alexa fluor 488 (Invitrogen; 1 : 500) were used as secondary antibodies.[16] Biomarker scoring was carried out on to life images using a 60×-immersion oil lens.[16,18] One hundred geminin-positive cells from at least three representative areas of each sample were analyzed and biomarker scores were calculated as the percentage of geminin-positive cells with five or more nuclear foci of any size. Twenty-five (13%) samples with low γH2AX (<25% of geminin-positive cells with γH2AX foci) and 34 (18%) samples with <40 geminin-positive cells were not included in the analyses, due to insufficient endogenous DNA damage or tumor cells in the S/G2 phase of the cell cycle and poor quality of the samples, respectively (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2024.104091). Tumors showing >10% of geminin-positive cells with BRCA1 foci were considered BRCA1 proficient; tumors showing >10% of geminin-positive cells with RAD51 foci were considered HRP.[16,17]

BRCA1/2 status definition

For 144 out of the 187 patients included in this study, the *BRCA1/2* mutational status was available, with 43 patients being germline or somatically mutated and 96 wild type (wt) as recorded from case report forms or determined by targeted hybrid capture sequencing, as previously described.[10]

HRD based on shallow WGS DNA libraries

Shallow WGS DNA libraries for Illumina sequencing were prepared using the Watchmaker DNA Library Prep Kit (Watchmaker Genomics, Boulder, CO), using 100 ng of DNA, following the same procedure already published.[11] The paired-end sequencing reaction was carried out on the Illumina NextSeq550 Dx System (Illumina, San Diego, CA), loading the pool with a concentration of 1.5 pM and 2% Phix 1.5 pM. Through the bioinformatic analysis, six independent models spanning the 5- to 1000-kb genome window, implemented using the large-scale genomic alteration (LGA) method[19] and a neural network module,[20] provided the predicted HRD status, as previously described.[10] Based on the LGAs of myChoice CDx assay by Myriad Genetics carried out on the first 100 samples,[10] we identified the following groups of patients: HRD deficient (LGA > 20), HRD mild (15 < LGA < 19) and HRD negative (LGA < 14) corresponding to a genomic score >50, 42-50 and <42, respectively, as referred to the Myriad scoring.[19]

miR-506 expression

miRNA expression profiling was carried out on 100 ng of total RNA using Agilent SurePrint G3 8 × 60K microarrays designed on miRBase 21.0 and covering the expression of 2549 miRNAs, essentially following Agilent's protocols (see Bagnoli et al.[21] for details). Relative expression of miR-506 was considered after data normalization and filtering.

Statistical methods

Data were described using median and interquartile range for continuous variables and frequencies and percentages for qualitative variables. The characteristics of the studied population were compared with those of the population enrolled in the MITO16A trial (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2024.104091). To evaluate the concordance between the genomic test and functional HRD by RAD51 assay, Cohen's κ statistic with 95% confidence interval (CI) was used. The κ statistic was interpreted as <0, indicating no agreement, 0.00-0.20 as slight, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as substantial and 0.81-1.00 as an almost perfect agreement. The prognostic value for the genomic test, the functional HRD by RAD51 and miR-506 expression assay was investigated in terms of PFS and overall survival (OS). PFS was defined as the time from registration to documented progression according to RECIST criteria, death due to any cause or last follow-up date. OS was defined as the time from randomization to death due to any cause or last information on the vital status. Survival curves were calculated using the Kaplan—Meier method and compared by a log-rank test. Hazard ratios (HRs) were estimated using the Cox regression model. In the multivariable models, the following covariates were added: age (as category <65 versus ≥65 years), Eastern Cooperative Oncology Group performance status (PS; 0 versus 1-2), residual disease (none; ≤1 cm; >1 cm/not operated) and International Federation of Gynecology and Obstetrics stage (III versus IV). All the analyses were carried out with STATA 14 MP (StataCorp 2015, Stata Statistical Software: Release 14, StataCorp LP, College Station, TX).

RESULTS

Out of the 187 samples examined, 181 (97%) and 125 (67%) were evaluable for genomic testing and RAD51 assay, respectively (Supplementary Figure S1A, available at https://doi.org/10.1016/j.esmoop.2024.104091). The analysis of concordance was conducted on 121 samples for which both evaluations were available (Supplementary Figure S1A, available at https://doi.org/10.1016/j.esmoop.2024.104091). Supplementary Figure S1B, available at https://doi.org/10.1016/j.esmoop.2024.104091, details the causes of the failure rate for the RAD51 test: 34 out of 184 samples (18%) did not present enough geminin-positive cells and were considered low-proliferation samples; 25

out of 184 samples (13%) exhibited insufficient DNA damage for evaluation in the functional HRD test. The characteristics of the 183 analyzed patients closely mirrored those of the participants in the MITO16A study (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2024.104091). miRNA expression profiles were available for 140 of 187 samples (Supplementary Figure S1A, available at https://doi.org/10.1016/j.esmoop.2024.104091).

Functional HRD by RAD51 assay

In the assessment of functional HRD using the RAD51 assay, γH2AX emerged as the primary sensor of the HRR pathway. The presence of γH2AX foci indicated double-strand breaks, serving as the internal positive control for the RAD51 assay. In the 125 (69%) evaluable samples (Supplementary Figure S1A, available at https://doi.org/10.1016/j.esmoop.2024.104091), γH2AX showed a median expression of 53% (Figure 1A): these findings suggested that the majority of HGSOC cases displayed high levels of endogenous DNA damage (Supplementary Figure S1B, available at https://doi.org/10.1016/j.esmoop.2024.104091, and Figure 1A). The functionality of the BRCA1 protein, a key mediator in the HRR pathway, was assessed by immunofluorescence. Sixty-one (49%) of the samples exhibited negative BRCA1 immunofluorescence (Figure 1B), indicating a loss of function in *BRCA1* due to somatic or germline mutations or epigenetic modifications. RAD51, the key effector of the HRR pathway, was evaluated by immunofluorescence to determine HRD status, revealing that 91 (73%) tumors were HRD in our cohort (Figure 1C). Fifty-seven out of 61 (93%) *BRCA1*-negative tumors were HRD by RAD51; on the contrary, the main cause of HRD in this series was the loss of function of *BRCA1* which occurred in 57 out of 91 (63%) HRD tumors.

Functional HRD by RAD51 did not predict response to platinum salts in univariate analyses for PFS (Figure 2A) (HR 0.79, 95% CI 0.51-1.23, $P = 0.302$) or OS (Figure 2B) (HR 0.76, 95% CI 0.42-1.39, $P = 0.378$). The association with PFS slightly improved in multivariate analyses when adjusted for PS, age, stage and residual disease (HR 0.65, 95% CI 0.41-1.03, $P = 0.07$).

Evaluation of HRD by academic genomic testing

Analysis by academic genomic testing identified 69 out of 121 tumors (57%) as HRD (Supplementary Table S2A, available at https://doi.org/10.1016/j.esmoop.2024.104091). HRD genomic testing was predictive of response to platinum salts in terms of PFS in both univariate (HR 0.56, 95% CI 0.40-0.78, $P = 0.001$) (Figure 2C) and multivariate analyses (HR 0.52, 95% CI 0.37-0.74, $P < 0.001$). No differences were observed in terms of OS (HR 0.70, 95% CI 0.44-1.13, $P = 0.147$) (Figure 2D).

No correlation was found between HRD status by RAD51 and HRD genomic test (Pearson $r = 0.17$, $P = 0.06$). RAD51 identified up to 33 HGSOC as HRD, which were negative HRD (neg-HRD) for genomic testing (Supplementary Table S2A, available at https://doi.org/10.1016/j.esmoop.2024.104091) (Cohen's κ coefficient = 0.19, 95% CI 0.02-0.36). To investigate the discrepancy, Ki67 expression was examined in discordant cases, revealing positive percentages >10%, suggesting suitability for immunohistochemistry/immunofluorescence assays. Additionally, the expression of γH2AX, as a positive internal control, was rechecked in tumors with a RAD51 score <1, all presenting an adequate percentage of endogenous DNA damage, with a median of 50% (data not shown).

Group re-classification by academic testing

A three-group genomic classification based on HRD genomic testing identified high-HRD, mild-HRD and neg-HRD tumors (Materials and Methods, Supplementary Table S2B, available at https://doi.org/10.1016/j.esmoop.2024.104091 and Capoluongo et al.[10]). High-HRD, mild-HRD and neg-HRD tumors constituted 43% (77/181), 14% (25/181) and 44% (79/181) of samples, respectively (Supplementary Table S2B, available at https://doi.org/10.1016/j.esmoop.

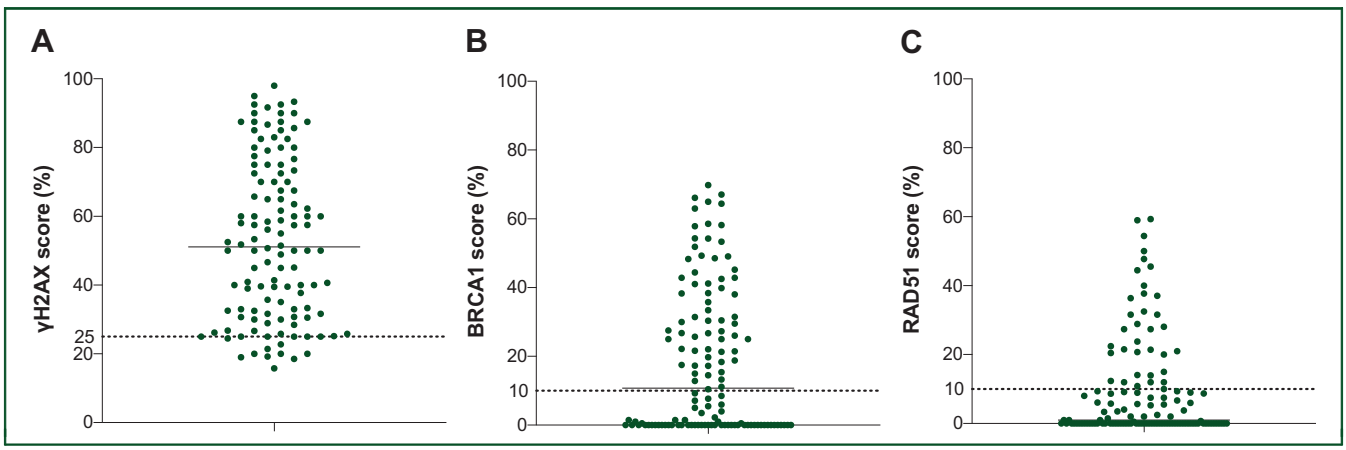

Figure 1. Functional HRD in ovarian cancer according to RAD51 assay. (A) Distribution of γH2AX: γH2AX is the main sensor of the HRR pathway; the presence of γH2AX foci in >25% of geminin-positive cells indicates the presence of double-strand breaks and it is the internal positive control of the RAD51 assay. (B) Distribution of BRCA1: BRCA1 is a mediator of the HRR pathway; the presence of BRCA1 foci in >10% of geminin-positive cells indicates the correct functionality of the BRCA1 protein. (C) Distribution of RAD51: RAD51 is the main effector of the HRR pathway; the presence of RAD51 foci in at least 10% of geminin-positive cells reveals the correct functionality of the HRR pathway. Dotted lines represent each cut-off for positive expression.
HRD, homologous recombination repair deficiency; HRR, homologous recombination repair.

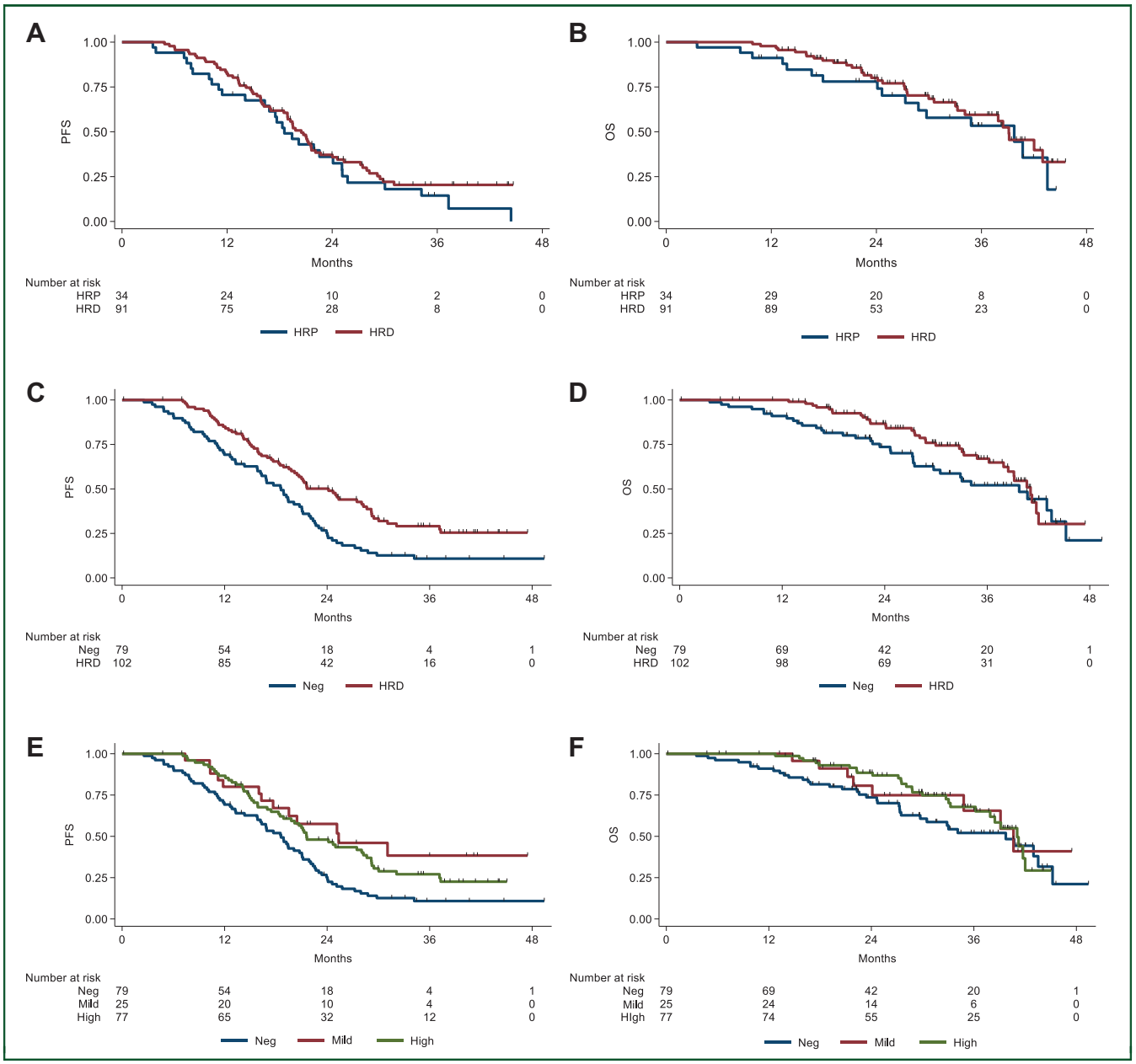

Figure 2. Prognostic role of functional and genomic HRD. (A) PFS in HRD and HRP tumors according to RAD51 assay. (B) OS in HRD and HRP tumors according to RAD51 assay. (C) PFS in HRD and neg-HRD tumors according to genomic HRD assay. (D) OS in HRD and neg-HRD tumors according to genomic HRD assay. (E) PFS in high-HRD, mild-HRD and neg-HRD tumors according to genomic HRD assay. (F) OS in high-HRD, mild-HRD and neg-HRD tumors according to genomic HRD assay. HRD, homologous recombination repair deficiency; HRP, homologous recombination repair proficient; neg, negative; OS, overall survival; PFS, progression-free survival.

2024.104091). Both high- and mild-HRD tumors demonstrated statistically superior PFS in univariate (high versus neg: HR 0.59, 95% CI 0.41-0.85, $P = 0.004$; mild versus neg: HR 0.45, 95% CI 0.25-0.82, $P = 0.009$) (Figure 2E) and multivariate analyses (high versus neg: HR 0.53, 95% CI 0.37-0.77, $P = 0.001$; mild versus neg: HR 0.48, 95% CI 0.27-0.88, $P = 0.017$) compared with neg-HRD tumors. No differences were noted in terms of OS (Figure 2F) (high versus neg: HR 0.70, 95% CI 0.42-1.16, $P = 0.161$; mild versus neg: HR 0.74, 95% CI 0.34-1.59, $P = 0.433$).

Among high-HRD tumors, there was a high concordance with the RAD51 test, with 86% of tumors being HRD for both tests (Supplementary Table S2B, available at https://doi.org/10.1016/j.esmoop.2024.104091). These 'super-HRD' tumors demonstrated statistically superior PFS compared with 'super-HRP' tumors (neg-HRD and HRP for RAD51) (Cox PFS: HR 0.52, 95% CI 0.29-0.95, $P = 0.04$).

Combined genomic and functional HRD test

Combining genomic and functional HRD tests, 102 out of 121 (84%) OvC samples were HRD for RAD51 and/or genomic HRD tests. HRD tumors for at least one test displayed statistically superior PFS compared with HRP tumors for both tests in univariate (HR 0.60, 95% CI 0.41-0.86, $P = 0.005$) and multivariate (Figure 3A) (HR 0.49, 95% CI 0.34-

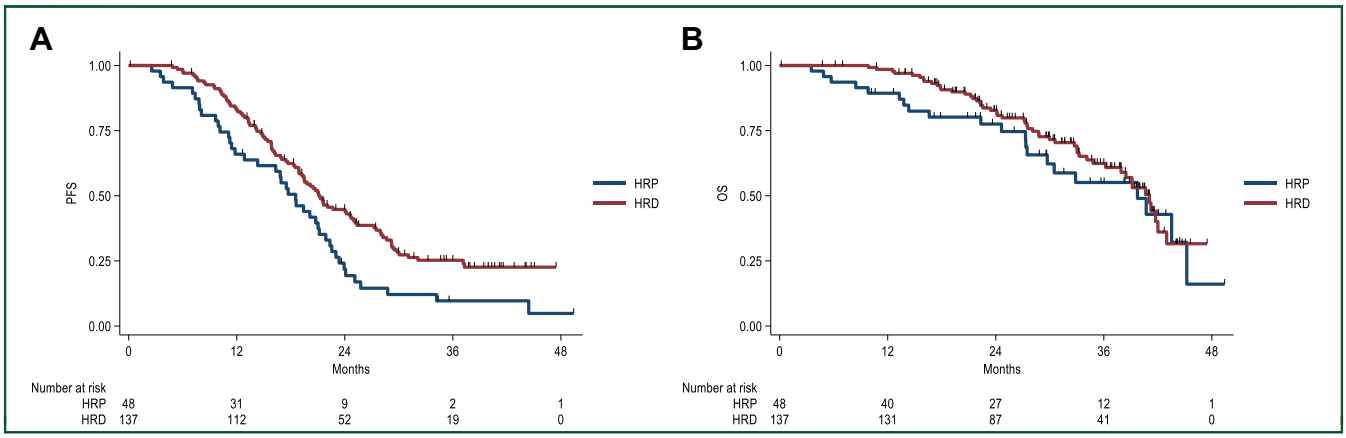

Figure 3. Prognostic role of combined functional and genomic HRD test. (A) PFS in HRD tumors for at least one HRD test compared with HRP tumors for both tests. (B) OS in HRD tumors for at least one HRD test compared with HRP tumors for both tests.
HRD, homologous recombination repair deficiency; HRP, homologous recombination repair proficient; OS, overall survival; PFS, progression-free survival.

0.72, $P < 0.001$) analyses. No differences were observed in terms of OS (Figure 3B) (HR 0.79, 95% CI 0.47-1.32, $P = 0.365$).

miR-506 and HRD status in OvC samples of MITO16 trial

miR-506 exerts a regulatory role on genes related to DNA repair recapitulating a BRCAness phenotype when highly expressed in tumors. In a preliminary analysis on 72 of 100 samples used to set up the academic genomic assay,[10] we observed a significant relative higher expression (as assessed by microarray profiling) of miR-506 in the group of patients defined as HRD by genomic testing (Supplementary Figure S2A, available at https://doi.org/10.1016/j.esmoop.2024.104091). In the present case material relative miR-506 expression was evaluable for 140 samples (Supplementary Figure S1A, available at https://doi.org/10.1016/j.esmoop.2024.104091). In this further analysis, we took into consideration that miR-506, due to its regulatory functions, is a tumor-suppressive miRNA and it is therefore downregulated in various cancers, with its high expression being a clinically favorable prognostic factor.[12,22] The upper quartile of miR-506 relative expression, showing a trend in PFS advantage (Supplementary Figure S2B, available at https://doi.org/10.1016/j.esmoop.2024.104091) but not in OS (not shown), is indeed associated with 68% and 76% of HRD samples by the genomic testing and the functional assay, respectively (Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2024.104091). Since from a biological point of view, what makes the difference is a high expression of miR-506, we correlated the genomic (Supplementary Figure S2C, available at https://doi.org/10.1016/j.esmoop.2024.104091) and functional (Supplementary Figure S2D, available at https://doi.org/10.1016/j.esmoop.2024.104091) HRD status only considering the samples with a relative expression of miR-506 above the median value. Although the sample size did not allow reaching a statistically significant difference between HRP and HRD samples, a clear trend in higher expression of miR-506 in samples defined HRD, particularly by RAD51 assay, was evident (Supplementary Figure S2D, available at https://doi.org/10.1016/j.esmoop.2024.104091).

BRCA1/2 mutations and HRD status in OvC samples of MITO16 trial

Among the 96 *BRCA1/2*-wt patients, 29 (46%) were HRD and 34 (56%) were neg-HRD for the genomic testing; 47 (75%) were HRD and 16 were HRP, according to the RAD51 assay (Supplementary Figure S3A, available at https://doi.org/10.1016/j.esmoop.2024.104091). The concordance of the two HRD methods resembled the results of the overall population: 24 (83%) and 5 (17%) of the HRD samples by genomic testing were HRD and HRP for RAD51 assay, respectively; 23 (68%) and 11 (32%) of the neg-HRD samples by genomic testing were HRD and HRP for RAD51 assay, respectively (Supplementary Figure S3A and B, available at https://doi.org/10.1016/j.esmoop.2024.104091). Supplementary Figure S3C and D, available at https://doi.org/10.1016/j.esmoop.2024.104091, illustrates the peculiar case of the patient with the highest expression of miR-506: she was classified as neg-HRD for genomic testing but HRD for the functional assay, supporting once again the importance of combining genomic and functional assays in order to increase the number of HRD patients who might benefit from PARPi.

DISCUSSION

OvC poses a challenge in clinical management due to its late-stage diagnosis and the emergence of chemo-resistance, particularly in HGSOC. The conventional treatment involving carboplatin and paclitaxel has been augmented with maintenance strategies, including PARPi, especially for *BRCA1/2*-mutated HGSOC. However, the inherent heterogeneity in treatment responses prompts a deeper exploration of biomarkers beyond *BRCA1/2* mutations for expanding/refining the patient population benefiting from PARPi and platinum salts.[9]

Numerous studies have investigated biomarkers of HRD beyond *BRCA1/2* mutations. Next-generation sequencing panels, while dynamic, are constrained by current knowledge and may exclude genes not yet known to be part of HRR. Genomic HRD tests, or genomic scars, are static and unable to identify tumors regaining HRR ability under

treatment pressure. The RAD51 assay, however, is both functional and dynamic, capturing the actual HRR status of tumors and identifying those that may have regained HRR ability during the natural history of the disease.[16,17,23]

Trials like PRIMA and PAOLA demonstrated the association between genomic HRD status and PARPi response in HGSOC.[4,6] While the PAOLA trial indicated that only tumors classified as HRD by myChoice CDx assay benefited from olaparib, in the PRIMA trial, it was found that HRP tumors still exhibited benefits from treatment with niraparib. This discrepancy may arise from several factors, including the sensitivity and false-negative rate of the tests used to determine HRD status. Furthermore, niraparib is known to be more potent in trapping PARP1, which may enhance its efficacy even in 'weaker' HRD tumors.

Academic laboratories have been pivotal in developing novel HRD testing approaches, trying to increase the plethora of patients who may benefit from PARPi. Previous work by our group compared two academic genomic HRD tests and RAD51 assay with Myriad as a reference standard, showing a high level of concordance between genomic HRD tests and Myriad.[10] In this study, a larger cohort from the MITO16a trial was analyzed, revealing complementary insights from genomic testing and RAD51 assay. Despite technical challenges, the RAD51 assay identified heightened endogenous DNA damage in 70% of HGSOC cases. However, functional HRD by RAD51 did not predict response to platinum salts in terms of PFS or OS. This discrepancy with other studies may be attributed to technical challenges which led to a higher failure rate in surgical samples, reducing the sample size of our research.[24-26] Despite its potential, the RAD51 assay faced a 30% failure rate in this study, possibly linked to the distinct quality of paraffin-embedded ovarian surgical samples compared with breast biopsies.[27] Furthermore, the follow-up data of the present study are influenced by the addition of BEV to front-line treatment.[14] Genomic HRD testing classified 57% of tumors as HRD, aligning with existing literature.[4,6] A notable discordance emerged between HRD status identified by RAD51 and genomic testing, emphasizing the need for further investigation. The introduction of a three-group genomic classification based on HRD genomic testing revealed superior PFS not only in high- but also in mild-HRD tumors compared with neg-HRD tumors. Among high-HRD tumors, a high concordance with the RAD51 test was noted and 'super-HRD' tumors showed superior PFS compared with 'super-HRP'[6] tumors. Considering both genomic and functional HRD tests, 84% of OvC samples were HRD for at least one test, demonstrating superior PFS compared with tumors that were neg-HRD and HRP. This study underscores the complexity of HRD assessment and suggests that a combined genomic and functional approach may enhance predictive accuracy for OvC treatment responses.

The clinical significance of HRD testing is further highlighted by recent approvals of PARPi as first- and second-line maintenance therapy in platinum-sensitive HGSOC.[28-31] Recently, the European Medicines Agency approved olaparib in combination with BEV (an anti-vascular endothelial growth factor monoclonal antibody) as first-line therapy in HRD-positive platinum-sensitive HGSOC patients, using an HRD diagnostic test with demonstrated clinical validity, the myChoice CDx assay by Myriad Genetics.[2,6,8,28,32] As the most widely used test, Myriad has limitations, including unknown status, false negatives and high cost.[33,34] Newer approaches, combining genomic testing and functional activity, are crucial to optimizing the management of PARPi-eligible patients. In this context, the combined score could be helpful to: (i) refrain from using platinum-based chemotherapy sensitivity as a selection biomarker for PARPi, thereby minimizing toxicities for patients who may not derive benefits from such treatments; (ii) utilize the RAD51 assay as a pre-screening tool to identify patients with germline *BRCA1/2* mutations, considering the low incidence of these mutations in this specific subset of patients; (iii) expand the pool of patients who could benefit from PARPi beyond those with platinum sensitivity or genomic alterations.

Considering the complexity of HRR pathways, we should take into consideration also the possible epigenetic regulation of this process possibly mediated by miRNAs. miR-506 is the most well-known miRNA in the ChrXq27.3 miRNA cluster, which has been linked to better prognosis in HGSOC patients.[35] Alongside miR-506, miR-508 and miR-509, also found in this cluster, are known to have tumor-suppressive roles.[22] miR-506 responds to genotoxic stress, suggesting its involvement in the DNA damage response.[36] Similar expression patterns are observed for miR-508 and miR-509, hinting at their potential roles in DNA damage response as well, with miR-509 being a further regulator of RAD51.[37] Given their highly correlated expression,[21,35] it is likely that these miRNAs share target genes or regulate similar signaling pathways. Our data suggested a possible role of miR-506 expression as a refinement of HRD status; however, we need to rely on a more sensitive detection method such as digital PCR, enabling detection of rare transcripts (and miRNAs) and small quantitative variations, and to expand the analysis on other miRNAs regulating the same pathways, such as miR-509.

Looking ahead, the study suggests a roadmap for future research: (i) standardizing HRD assessment methodologies should be prioritized to facilitate cross-study comparisons; (ii) combining genomic and functional HRD testing can help identify patients with 'weak HRD' who may benefit from PARPis, as suggested by the findings from the PRIMA study; we believe that this approach may provide a deeper understanding of patient selection for targeted therapies and represents the novelty of our work; (iii) the significant discrepancy noted between the RAD51 test and genomic HRD testing highlights the need for improved tumor classification based on HRD status, which we address in our study; (iv) discrepancies among different assays can be due to some pre-analytical issues[38] related to the cellularity and quality of the sample that can impair the HRD assessment, at both functional and genomic levels.

The present paper confirms the need of a better standardization and harmonization in HRD assessment, particularly

to overcome the pre-analytical limitations through the integration of multiple tests, including both genomic and functional assays, as we have reported in this article. Ongoing analyses on samples from patients enrolled in the MITO35 trial (EudraCT number: 2021-000244-21) will be crucial to confirm the predictive role of the combined score and of miRNA regulation to PARPi response.

In conclusion, this study provides a valuable contribution to the ongoing discourse on HRD assessment in OvC. By highlighting the complexities and nuances of genomic and functional testing, the research prompts a reevaluation of current approaches and underscores the need for a more personalized and dynamic understanding of HRD for improved patient outcomes.

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DATA SHARING

The core dataset from this study is available online (https://doi.org/10.5281/zenodo.14288047). Full data will be shared upon publication after a reasonable request to the corresponding author.

DISCLOSURE

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