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Theranostic biomarkers and PARP-inhibitors effectiveness in patients with non-*BRCA* associated homologous recombination deficient tumors: Still looking through a dirty glass window?

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ABSTRACT

Breast cancer susceptibility gene 1 (*BRCA1*) and breast cancer susceptibility gene 2 (*BRCA2*) deleterious variants were the first and, still today, the main biomarkers of poly(ADP)ribose polymerase (PARP)-inhibitors (PARPis) benefit. The recent, increased, numbers of individuals referred for counseling and multigene panel testing, and the remarkable expansion of approved PARPis, not restricted to *BRCA1/BRCA2*-Pathogenic Variants (PVs), produced a strong clinical need for non-*BRCA* biomarkers.

Significant limitations of the current testing and assays exist. The different approaches that identify the causes of Homologous Recombination Deficiency (HRD), such as the germline and somatic Homologous Recombination Repair (HRR) gene PVs, the testing showing its consequences, such as the genomic scars, or the novel functional assays such as the RAD51 foci testing, are not interchangeable, and should not be considered as substitutes for each other in clinical practice for guiding use of PARPi in non-*BRCA*, HRD-associated tumors. Today, the deeper knowledge on the significant relationship among all proteins involved in the HRR, not limited to *BRCA*, expands the possibility of a successful non-*BRCA*, HRD-PARPi synthetic lethality and, at the same time, reinforces the need for enhanced definition of HRD biomarkers predicting the magnitude of PARPi benefit.

Introduction

In recent years, understanding of the role of germline genetic testing has rapidly increased, moving from preventive paths including screening programs and risk-reducing strategies, to the development of effective drugs for the treatment of tumors associated with Hereditary Cancer Predisposition Syndromes and sporadic cancers harboring the same deleterious, somatic, gene variants [1]. The landscape of germline genetic testing has intersected with the rapidly expanding area of predictive biomarker testing to identify more patients who may be eligible for innovative therapies, first of all the poly(ADP)ribose polymerase (PARP)-inhibitors (PARPis). Breast cancer susceptibility gene 1 (*BRCA1*) and breast cancer susceptibility gene 2 (*BRCA2*) deleterious variants were the first and, still today, the main biomarkers of PARPi benefit [2].

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PARPi have proved to be effective in patients with breast, ovarian, pancreatic and prostate cancers with a germline or sporadic *BRCA* pathogenic or likely pathogenic variants (PVs) [3]. With the implementation, in a few years, of the multidisciplinary team, of novel oncogenic models such as the mainstreaming cancer genetics, and the increased number of individuals referred for counseling and multigene panel testing, novel genetic and genomic biomarkers are being used or are under investigation to determine whether a patient will benefit from the treatment [4]. Importantly, the recent expansion of approved PARPis, not restricted to *BRCA1/BRCA2*-PVs, produced a strong clinical need for non-*BRCA* biomarkers of PARPi benefit. Today, to identify patients who will benefit from PARPi treatment beyond *BRCA1/2* PVs is among the main current clinical challenges. Parallel to novel indications of PARPis irrespective of *BRCA* PVs, our ability to stratify patients should improve the treatment selection through testing optimization in the clinic [5]. However, significant limitations for the current testing and assays exist [6]. The different approaches that identify the causes of Homologous Recombination Deficiency (HRD), such as the germline and somatic Homologous Recombination Repair (HRR) gene PVs, or testing showing its consequences, such as the genomic scars, or novel assays that measure the functional Homologous Recombination (HR) activity itself, such as the RAD51 foci assays, are not interchangeable, and should not be considered as substitutes for each other in clinical practice for guiding use of PARPi [7].

This review aims to outline the current and emerging scenario of non-*BRCA* biomarkers that might predict the effectiveness of PARPi. For such HRD-positive patients, including but not limited to *BRCA1/2* related-tumors, treatment options are expanded by PARPi and other potential emerging strategies in the near future. Therefore, identifying these patients is nowadays crucial to refine the clinical decision-making process.

Genetic and functional assays: Discovering what lies deeper in the clinic

Today, the identification of patients harboring HRR, non-*BRCA* PVs, that could benefit from PARPi is a crucial step widely debated. The evaluation of an HRR-deficient phenotype could be assessed at different assay levels following the cause-effect relation that closely links HR loss (triggering event) and genomic instability (result of HR loss) [8,9]. Although promising, the HRR biomarkers currently available in clinical practice are inadequate predictors of response to PARPi both because they do not allow a dynamic view of the tumor and its heterogeneity, and because they are unable to detect acquired resistance to PARPi due

Table 1
Clinical trials for PARP-inhibitors in patients with HR deficiency or *BRCA1/BRCA2* pathogenic Variants (PVs), using biomarkers or functional assays for patient selection.

Tumor	Treatment	Phase	Biomarkers	Companion Diagnostic	Clinical trial ID
Ovarian cancer	Rucaparib	III	Germline or somatic *BRCA1/2* PVs	(Foundation Medicine, Inc.)	**ARIEL4** R. Kristeleit, 2022 NCT02855944
Ovarian cancer	Veliparib in combination with carboplatin and paclitaxel	III	Germline or somatic *BRCA1/2* PVs or HR deficiency	Myriad BRACAnalysis CDx or Myriad myChoice HRD CDx	**VELIA** E M Swisher, 2022 NCT02470585
Ovarian cancer	Rucaparib	III	*BRCA1/2* negative PVs	Not specified	**MAMOC** - NCT04227522
Breast cancer	Veliparib in combination with carboplatin and paclitaxel	III	Germline *BRCA1/2* PVs	Myriad BRACAnalysis CDx	**ABT-888** S. Stodtmann, 2022 NCT02163694
Pancreatic cancer	Fuzuloparib	III	Germline *BRCA1/2* or *PALB2* PVs	Not specified	NCT04300114
Prostate cancer (mCRPC)	Fuzuloparib monotherapy or in combination with apatinib	II	HRR gene PVs	Central laboratory-based testing	NCT04869488
Prostate cancer (mCRPC)	IMP4297	II	HRR gene PVs	Not specified	NCT04822961
Prostate cancer	Niraparib in combination with abiraterone and prednisone	III	Germline HRR gene PVs	Not specified	**AMPLITUDE** - NCT04497844 ——— **MAGNITUDE** - NCT03748641
Prostate cancer	Rucaparib	III	Germline *BRCA1/2* or *ATM* PVs	(Foundation Medicine, Inc.)	**TRITON3** MC Maia, 2020 NCT02975934
Prostate cancer	Talazoparib in combination with enzalutamide	III	DDR gene PVs	FoundationOne Liquid CDx or FoundationOne CDx	NCT04821622
Advanced solid tumors	Pamiparib (BGB-290) in combination with tislelizumab	I	Germline or somatic *BRCA1/2* PVs or HR deficiency PVs	(Myriad Genetic Laboratories, Inc.)	NCT02660034 M Friedlander, 2019

DDR, DNA damage response; HR, homologous recombination; mCRPC, metastatic castration-resistant prostate cancer. Clinical trials are accessible at https://clinicaltrials.gov/.

to HR reactivation in HRD tumors [9]. In this scenario, the current landscape of diagnostic tools available is various and includes a wide range of genetic and genomic tests, and involves the use of various tumor samples [9] (Table 1). To date, Next-Generation Sequencing (NGS) multigene panels, containing most HR-related genes, allow the identification of genetic variants in about 10 to more than 500 genes using different available platforms and sequencing chemistries to detect common SNVs and small indels as well as large rearrangements in exonic/intronic regions [10]. Several commercially available and in-house multigene panels are worldwide available for the evaluation of HR loss "causes", including mainly pathogenic alterations and large rearrangements responsible for protein loss or inactivation. The two most spread NGS sequencing approaches are multiplex polymerase chain reaction (PCR)-based (amplicon sequencing), and hybrid capture-based target enrichment, and their choice strictly depends on size targets. Amplicon-based sequencing allows the identification of smaller/known targets by using a primers' pool specifically designed to target a specific region of interest following a polymerase chain reaction. The amplification products are termed "amplicons" and represent the DNA fragments containing the genomic region of interest on which sequencing will be then concentrated. Contrariwise, hybrid capture-based target enrichment allows the identification of larger/unknown targets by combining a specific capture method by using DNA or RNA single-stranded oligonucleotides, called probes, to select the regions of interest with deep sequencing coverage metrics [11]. Considering the HR loss "effects", the evaluation of genomic instability (GI) has represented in the last years an unmissable opportunity to properly address patients toward a tailored and optimal targeted treatment. In fact, HRD is a phenotype characterized by the inability of a cell to effectively repair DNA Double Strand Breaks (DSBs) using the HRR pathway: alterations in these genes have been considered "causes" of HRD (e.g., genetic, and epigenetic events) [9]. The recognition of HRD has transformed the therapeutic paradigm in the same tumors, mainly in the high-grade serous ovarian cancer (HGSOC). Currently, the Food and Drug Administration (FDA) has approved several diagnostic tests to select patients suitable for PARPi treatment based on HR status [12]. Two of these tests, Myriad myChoice® CDx and FoundationOne CDx, simultaneously evaluate genetic alterations in several genes along with genomic instability [9]. Myriad myChoice® CDx, is currently the most widely used diagnostic test and is an NGS-based in vitro diagnostic test allowing the identification of single nucleotide variants, insertions, deletions and large rearrangements in the coding regions and intron/exon boundaries of the *BRCA1* and *BRCA2* genes and several other HRR genes, and simultaneously determines the Genomic Instability Score (GIS) as a result of loss of heterozygosity (LOH), telomeric allelic imbalance (TAI), and large-scale state transitions (LST) measurements. A positive GIS is defined for cut-off values $\geq$ 42 or $\geq$ 16, depending on the company ownerships, but overall, a lower score suggests HR proficiency [13]. The score of 42 is the most used and has been adopted in several clinical trials such as PRIMA and PAOLA-1 which have evaluated the therapeutic efficacy of various PARPis [13]. The FoundationOne CDx (F1CDx) is an FDA-cleared comprehensive genomic profiling (CGP) platform that applies hybrid-capture NGS-based in vitro testing to evaluate 324 cancer genes from formalin fixed paraffin embedded (FFPE) tumor tissue samples. F1CDx detects the presence of genetic alterations in various HRR genes such as PVs, copy number alterations (CNA), rearrangements, and complex biomarkers including tumor mutational burden (TMB) and microsatellite instability (MSI), as well as the percentage of tumor tissue samples affected by genomic loss of heterozygosity [14]. This test is employed in the randomized clinical trials with second and third line rucaparib (ARIEL 2 and ARIEL 3), and it has also been approved as a complementary diagnostic assay to determine the genomic LOH percentage obtained by genotyping a large number of PVs throughout the genome. According to the ARIEL2 study, examining ovarian cancer (OC) samples, the optimal cut-off obtained for LOH to identify HRD tumors was 14 % [15], while in the subsequent ARIEL 3 study, the cut-off was revised to 16 % as a threshold [16]. Currently, different companies and scientific communities are developing additional affordable tests to assess HRD. Among them, the kit made by SOPHiA Genetics detects genetic alterations through NGS in 28 genes of the HRR pathway and in combination with whole genome sequencing identifies copy number variations indicative of an HRD scar [17]. Furthermore, the commercially available test distributed by Amoy Scientific Company, evaluates HRD status in OC tissue samples by testing *BRCA* genes and the genomic scar score (GSS). A PV in *BRCA* genes or a positive GSS status (GSS $\geq$ 50.0) is highly indicative of a positive HRD status [17].

However, several technical and clinical issues for known assays are recognized. The standardization of methods for HRD assessment along with the prospective study in clinical trials are urgently needed. Several reports showed that HRD-negative and HR-negative patients could maintain a well responsiveness to PARPi administration, highlighting thus the lack of accuracy in HRD detection assays. Additionally, the timely evaluation of genomic instability should consider the restoration of the HR functions as consequence of reversion mutations and/or epigenetic changes, and a different HRD score thresholds should be taken into account for different tumor types. Furthermore, at a practical level, the evaluation of HRR genes and genomic scars could be worriedly influenced by several pre-analytical and analytical factors, such as an incorrect selection of the sample, the limited percentage of neoplastic cells in the FFPE samples, the intratumor heterogenicity, as well as the tumor evolution due to neoadjuvant chemotherapy (CT). This element could eliminate platinum-sensitive clones selecting, thus, resistant ones while maintaining a high genomic scar score. The scientific community has faced the common goal of overcoming these limitations, attempting to standardize protocols of libraries constructions and bioinformatic pipelines and ensure the turnaround time, reproducibility, and interpretation of molecular test data [18,19].

The clinical urgency of HRD testing in tissue specimens for all patients likely to benefit from PARPi therapies and for the personal and family cancer risk prevention has prompted possible in-house testing solutions due to the significant exclusion rate due to both the difficulty of non-reimbursable testing and stringent testing outsourcing criteria. In this context, the test evaluating the formation of RAD51 foci is a promising tool for the selection of patients who may benefit from PARPis. RAD51 foci assay measures RAD51 protein accumulation. Upon binding at overhangs formed during repair of DNA DSBs, RAD51 forms a filament known as foci which can be identified by immunohistochemistry (IHC) staining or immunofluorescence (IF) [20]. The identification and measure of these foci is a potential powerful tool to identify deficiency of HR pathway [21].Several research evaluated the use of this assay in different tumors as endometrium [22], lung [23] and colon [24], beyond breast [25,26] and ovarian cancers [27,28], which are the most studied. Llop et *al.* [29], in the retrospective biomarker analysis from the GeparSixto randomized clinical trial, reported the validity of quantifying RAD51 nuclear foci in untreated Triple Negative Breast Cancer (TNBC) to establish the concordance between RAD51 score and tumor *BRCA* (t*BRCA*) status or genomic HRD score (Myriad myChoice®). The RAD51 test was highly concordant with genomic test [concordance rate 87 % (95 % CI 79 % to 93 %], and capable of identifying tumors that benefit from addition of carboplatin in terms of pathological complete response (pCR). Interestingly, with the concordance rate of 65 % between RAD51 test and t*BRCA* status, the RAD51 test was able to detect a high proportion of non-t*BRCA*-mutated cases with HRD (45 %), likely associated to PVs in non-*BRCA* HRR genes or showing epigenetic silencing of the pathway [29]. This element is particularly relevant because reflect the partial discordance existing between the HRD status and HRR mutations.

The "signs" of impaired HR DNA repair other than *BRCA*: The basis for the synthetic-lethal interaction between HRD and PARP inhibitors

The best characterized HRR genes are certainly *BRCA1* and *BRCA2*: germline and somatic PVs, as well as epigenetic modifications, have been strongly related to the HRD phenotype and associated with the occurrence of several tumor types, including breast and ovarian cancers, but also prostate and pancreatic cancers [30]. However, deleterious variants in HR-related genes other than *BRCA1/2*, such as *ATM, CHEK2, PALB2, RAD51*, and *BARD1*, also confer an HRD or "BRCAness" phenotype, as they code for multiple protein co-factors that are necessary for functional HRR [31,32]. Today, the significant relationship among all proteins involved in DSB repair, not limited to BRCA, expands the possibility of a successful HRD-PARPi synthetic lethality and, at the same time, reinforces the need for enhanced definition of HRD biomarkers of PARP-inhibitors effectiveness.

Ovarian cancer

HRD is considered an important biomarker with both predictive and prognostic value in HGSOC. While 13–21 % of patients harbor germline *BRCA1/2* PVs [13,33], and an additional 6 % harbor somatic *BRCA1/2* PVs, up to half of HGSOC are predicted to be defective in HRR [34]. Some HRR genes, although at different penetrance levels, have been associated with OC risk [35] and mainly concern *RAD51C*, *RAD51D*, *BRIP1* and *PALB2* genes [36–38]. In addition to point mutations, large rearrangements involving genes other than *BRCA1/2*, such as *RAD50* and *NBS1*, have been reported [39]. Furthermore, HRD can be the consequence of EMSY (a *BRCA2*-interacting transcriptional repressor) amplification, an alteration found in approximately 6 % of cases [40,41]. In addition to BRCAness due to genetic PVs, promoter methylation in other HR genes, such as *RAD51C* and *PALB2*, has been described [42,43] (Fig. 1).

In light of these data, deleterious variants in other non-*BRCA* HRR pathway genes, and the consequent genomic instability have been reported to be relevant events that should be included in the current diagnostic and therapeutic algorithm. However, the optimal strategy to identify HRD and potential PARPi responders in OC remains undefined.

Prostate cancer

Several studies of HRR mutations in men with prostate cancer indicated that, beyond *BRCA2*, the strongest link is for *ATM* and *CHEK2* PVs. Mutation prevalence is heterogeneous among the studies. In a retrospective study of 944 men with metastatic prostate cancer, HRR germline or somatic PVs were found in 16 % of patients, mainly *BRCA2* (11.4 %), followed by *ATM* PVs (5.8 %) [44]. In a second retrospective study of 692 patients, 11.8 % showed HRR PVs, and still *BRCA2* (5.3 %) was the most common mutated gene; *CHEK2* and *ATM* genes were mutated in 1.9 % and 1.6 %, respectively [45]. The prevalence of HRR PVs was higher in metastatic prostate cancers (mPCs), compared to primary tumors, thus germline or somatic *BRCA2* mutations occured in ~ 13 % of metastatic tumors, and 3 % of patients with localized disease [46,47]. Altogether, HRR PVs have been identified in 15–25 % of metastatic castration-resistant prostate cancer (mCRPC) patients [48]. Furthermore, although the main data concern *BRCA2* gene, the presence of HRR PVs has been also associated with aggressive tumors showing higher tumor stage, Gleason grade, and prostate-specific antigen (PSA) levels at diagnosis [49–51], and poor prognosis with higher rates of lymph node involvement, metastases, and prostate cancer-specific death [52,53].

Pancreatic cancer

Germline PVs in non-*BRCA* DNA damage response (DDR) pathway genes are found in up to 16 % of patients with pancreatic ductal

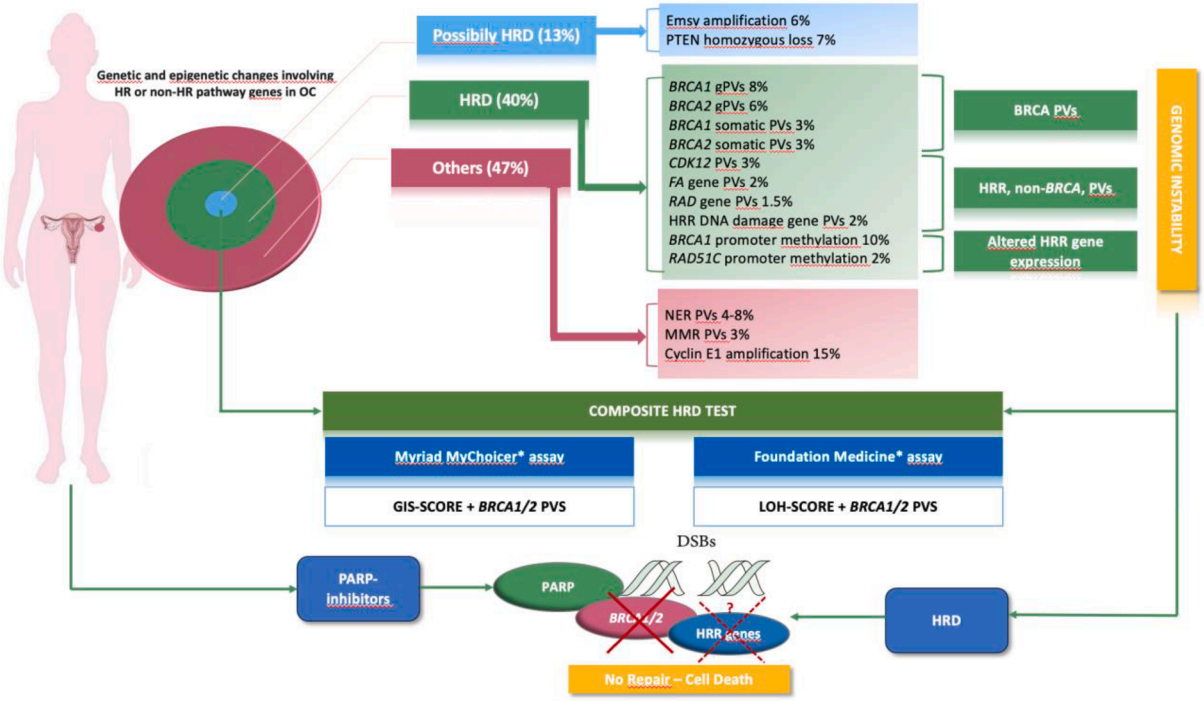

Fig. 1. Frequency of genetic and epigenetic changes involving HR pathway genes or non-HR pathway genes that modulate HR pathway in Ovarian Cancers. Current approved diagnostic tests to select patients suitable for PARPi treatment, simultaneously evaluate genetic alterations in several genes along with genomic instability. While in the first-line maintenance setting HRD testing clearly predicts the magnitude of PARPi benefit, as demonstrated across PAOLA-1 and PRIMA trials, in the PAOLA-1 setting the predictive value of HRR gene panels is debated. GIS, Genomic Instability Score; HR, Homologous Recombination; HRD, Homologous Recombination Deficiency; LOH, Loss Of Heterozygosity; PVs, Pathogenic Variants.

adenocarcinoma (PDAC) [54]. In addition, comprehensive genomic profiling shows that up to 13.7 % of patients have DDR alterations. The most strongly associated with pancreatic cancer are *ATM*, *PALB2*, *CHEK1*, *RAD50*, *BARD1*, *FANCA*, and *ARID1A* genes [55].

After *BRCA2*, PVs of *ATM* gene are the second most frequent germline and somatic alterations in PDAC (germline *ATM* PVs, 2–3.09 %; somatic *ATM* PVs, 2.2–9 %), conferring to PV carriers a lifetime increased risk of PDAC comparable to *BRCA* [55].

Breast cancer

Beyond *BRCA1/2*, PVs in other cancer susceptibility genes have a significant association with breast cancer (BC) risk, including *PALB2, ATM, CHEK2, BRIP1, BARD1, RAD51C* and *RAD51D* [31,56]. PVs in the *PALB2* gene are associated with a high risk of BC (odds ratios 5.0–10.6), and PVs in *ATM* and *CHEK2* with a moderate risk of BC (odds ratios 2.1–2.5) [57,58]. Also in the context of bilateral BC, multigenic panel analysis showed the presence of approximately 15 % PVs in other genes, such as *PTEN, PALB2, CHEK2, ATM* and *RAD51C* [59].

Although the exact magnitude of some of these HRR-associated gene cancer risk has not yet been defined, they are often included in multigene panel testing. This element raises the question of clinical management of these sub-populations, and requires a deeper knowledge of to the potential effectiveness of PARPi in BC patients harboring PVs other genes than *BRCA1/2* (Table 2).

Non-*BRCA* HRD status as potential druggable target: The magnitude of PARPi benefit

Ovarian cancer

PARPi effectiveness in HRD tumors, without *BRCA* PVs, was confirmed in the recurrent and frontline setting, although with a different degree of benefit. In a phase II study by Gelmon *et al.* including recurrent HGSOC, olaparib was found to be effective in platinum-sensitive patients, irrespective of the *BRCA* PVs [60]. These data were confirmed in a phase II randomized trial (Study 19) in which olaparib maintenance treatment significantly improved the PFS compared with placebo (hazard ratio [HR] 0.35 [95 % CI 0.25–0.49]; $p < 0.0001$) in the overall population of platinum-sensitive HGSOC, with the greatest clinical benefit in patients with *BRCA* PVs (HR 0.18 [95 % CI 0.10–0.31]; $p < 0.0001$) [61].

Following these data, other 4 positive randomized phase III trials were completed and granted the full approvals for PARPi as maintenance therapy in the recurrent setting and have further clarified their role and management [16,62–64] (Table 3). All five studies, albeit with some differences, shared some criteria: enrolled patients were affected by platinum-sensitive HGSOC, in complete or partial response (CR/PR) to the last platinum-based therapy. Only the SOLO2 trial included *BRCA*-mutated patients, while the other studies included all comers. Overall, PARPi maintenance was active in all subgroups, regardless of *BRCA* or HRD status, albeit with a different magnitude of benefit [65].

Nonetheless, in 2022, the FDA revisited the regulatory approval of niraparib in the recurrent maintenance setting and limited the indication to women harboring a mutation in the *BRCA* genes. This decision is

Table 2
Current clinical indications of PARP-inhibitors restricted or not restricted to *BRCA1/2*-mutated ovarian, breast, prostate and pancreatic cancer patients.

Cancer type	Drug (Trial)	Approval	Treatment setting	Molecular alterations	Companion diagnostic
PARP inhibitor indications *BRCA1/2*-restricted					
Ovarian cancer	Olaparib (SOLO 2[63])	FDA, EMA	Maintenance in relapsed OC	Germline or somatic *BRCA1/2* PVs	Myriad BRACAnalysis CDx
Ovarian cancer	Olaparib (SOLO 1[66]) and rucaparib (ATHENA-MONO[67])	FDA, EMA (only for olaparib)	Maintenance in newly diagnosed OC	Germline or somatic *BRCA1/2* PVs	Olaparib: Myriad BRACAnalysis CDx or BRCA1/2 genetic testing assay (BGI) Rucaparib: FoundationOne CDx
Breast cancer	Olaparib (OlympiAD [114]) and talazoparib (EMBRACA[115])	FDA, EMA	Monotherapy in advanced or metastatic HER2-neg BC	Germline *BRCA1/2* PVs	Myriad BRACAnalysis CDx
Breast cancer	Olaparib (OlympiA[116])	FDA, EMA	Adjuvant monotherapy in HER2-neg high-risk EBC	Germline *BRCA1/2* PVs	Local or central testing (Myriad BRACAnalysis CDx)
Prostate cancer	Rucaparib (TRITON 2[117])	FDA	Monotherapy in II line (mCRPC)	Germline or somatic *BRCA1/2* PVs	FoundationOne CDx
Pancreatic cancer	Olaparib (POLO[118])	FDA	Maintenance (mPaC)	Germline *BRCA1/2* PVs	Myriad BRACAnalysis CDx
PARP inhibitor indications No-*BRCA1/2*-restricted					
Ovarian cancer	Niraparib (PRIMA[68]) and olaparib (plus bevacizumab) (PAOLA 1[69])	FDA, EMA	Maintenance in newly diagnosed OC	HRD defined by *BRCA1/2* PVs or genomic instability	Myriad myChoice CDx
Ovarian cancer	Olaparib (STUDY19 [61]), rucaparib (ARIEL3[16]), and niraparib (NOVA[62])	FDA, EMA	Maintenance in relapsed OC	Not selected on the basis of *BRCA1/2* PVs or HRD	Olaparib: not selected for BRCA1/2 PVs Rucaparib: Myriad BRACAnalysis CDx, Foundation Medicine's T5 NGS assay
Prostate cancer	Olaparib (PROFOUND[71])	FDA, EMA	mCRPC progressed following anti-androgen therapy	Germline or somatic PVs in HR genes (*BRCA1/2, PALB2, RAD51C, RAD51D, ATM*)	FoundationOne CDx

HR, Homologous Recombination; HRD, Homologous Recombination Deficiency; EBC, Early BC; mCRPC, metastatic castration-resistant prostate cancer; mPaC, metastatic pancreatic cancer patients; PVs, Pathogenic Variants.

Table 3
Randomized phase III trials on PARPi as a maintenance therapy in the ovarian cancer recurrent setting.

STUDY	Phase	No of Pts (Exp/ Con)	Experimental arm	Control Arm	Mean age (years)	No. of *BRCA* m Pts (%)	Discontinuations due to adverse events- no (%)	PFS HR (95 %CI)
Study 19 [61]	Phase II, double-blind	265 (136/ 129)	Olaparib 400 mg twice a day (capsules)	Placebo	58 (exp) 59 (con)	136 (51.3)	Exp: 8 (5.8)Con: 2 (1.5)	***BRCAm***: 0.18 (0.10–0.31) ***BRCAwt***:0.54 (0.34–0.85)
SOLO 2 [65]	Phase III, double-blind	295 (196/ 99)	Olaparib 300 mg twice daily (tablets)	Placebo	56 (exp) 56 (con)	295 (100)	Exp: 21 (10.8) Con: 2 (2)	***BRCAm:*** 0.30 (0.22–0.41)
NOVA [62]	Phase III, double-blind	733 (487/ 246)	Niraparib 300 mg once daily	Placebo	NA	223 (30.4)	Exp: 54(14.7) Con: 4(2.2)	***BRCAm***: 0.27 (0.17–0.41) ***HRDpos BRCAwt***: 0.38 (0.23–0.62) ***HRDneg:*** 0.58 (0.36–0.92)
ARIEL3 [16]	Phase III, double-blind	564 (375/ 189)	Rucaparib 600 mg twice daily	Placebo	61 (exp) 62 (con)	196 (34.8)	Exp: 50(13.4) Con: 3 (1.6)	***BRCAm***: 0.23 (0.16–0.34) ***LOH high BRCAwt***: 0.44 (0.29–0.66) ***LOH low BRCA wt:*** 0.58 (0.40–0.85)
NORA [63]	Phase III, double-blind	265 (177/ 88)	Niraparib 300 mg once daily (16 pts) Niraparib at individualized starting dose^^ (249 pts)	Placebo	53 (exp) 55 (con)	110 (37.7)	Exp: 7 (4)Con: 5 (5.7)	***BRCAm***: 22 (0.12–0.39) ***BRCAwt***:0.40 (0.26–0.613.12.)

Pts, patients; Exp, experimental arm; con, control arm; NA, not assessable; PTS, patients; *BRCA*m, *BRCA* mtated; *BRCA* wt, *BRCA* wild type; HRDpos, homologous recombination deficiency test positive; HRD neg, homologous recombination deficiency test negative; LOH, loss of heterozigosity;^Wild-type *BRCA* included patients with no known *BRCA* PVand those with a *BRCA* PVof unknown significance;^^patients with a baseline body weight < 77 kg or a platelet count < 150,000/μL received 200 mg (N = 235), while other patients received 300 mg (N = 14).

based on an FDA review of the final overall survival (OS) analysis of the phase 3 ENGOT-OV16/NOVA (NCT01847274) trial. While this trial previously served as the basis for the approval of niraparib as second-line maintenance therapy, final OS results from the study showed the secondary end-point of OS to have a hazard ratio (HR) of 1.06 (95 % CI, 0.81–1.37) in patients without germline *BRCA* PVs. These have not only limited options for treatment in the US but also fueled uncertainties at a global level about how and when to use these agents.

Moreover, it is now accepted that PARPi should be used as early as possible and many experts suggest that patients should receive a PARPi maintenance in the frontline setting, to improve OC management and delay the occurrence of PARPi resistance.

The incorporation of PARP inhibitor maintenance in the first-line setting of OC patients harboring a *BRCA1/2* PVs is now definitely established and data from all clinical trials have confirmed the unprecedented benefit related to PARPi administration in these women [66–69].

Nonetheless, in the same trials, an important survival advantage was found also in patients harboring PVs in other HRR genes, identified as HRD patients.

In the phase 3 PRIMA trial [68], comparing the efficacy of niraparib maintenance therapy with placebo, a minority of patients were HRD-positive without *BRCA* PVs (20.5 %); in this group, the median PFS was 19.6 months compared with 8.2 months of the placebo arm, (HR, 0.50; 95 % CI, 0.31–0.83). It was also interesting that overall HRD patients achieved a 24-month OS rate of 91 % vs 85 %, respectively (HR, 0.61; 95 % CI, 0.27–1.39), while HRD-negative patients had a 24-month OS of 81 % vs 59 %, respectively (HR, 0.51; 95 % CI, 0.27–0.97). Niraparib was also investigated in the PRIME study, in which PFS of HRD-positive *BRCA*-Wild-Type (WT) patients was 14 months longer than those receiving placebo (24.8 months vs 11.1, HR, 0.58; 95 % CI, 0.36–0.93).

Similar positive results were found in the phase 3 ATHENA-MONO trial [67], investigating rucaparib instead of niraparib in HGSOC newly diagnosed patients. Similarly, HRD-positive patients without *BRCA* deficiency were roughly 21.4 % and experienced a median PFS of 20.3 months vs 9.2 months of those under placebo (HR, 0.58; 95 % CI, 0.33–1.01).

Finally, the PARPi olaparib, was combined with bevacizumab maintenance in the PAOLA1 trial which included newly diagnosed, advanced, HGOC who responded to first-line chemotherapy; in this study, HRD-positive *BRCA*-WT patients were 18.6 % [69]. Overall, women with HRD-positive disease achieved a median PFS of 37.2 months compared with 17.7 months (HR, 0.33; 95 % CI, 0.25–0.45). Interestingly, those with HRD-positive, *BRCA*-WT ovarian cancer registered a median PFS of 28.1 months vs 16.6 months (HR, 0.43; 95 % CI, 0.28–0.66). Notably, in this trial data of OS were presented and women with HRD-positive (*BRCA*-mutated and *BRCA*-WT) disease experienced a median OS of 75.2 compared with 57.3 months of the control arm (HR, 0.62; 95 % CI, 0.45–0.85). Moreover, in patients with HRD-positive *BRCA*-WT disease the median OS was not reached (NR) vs 52 months of those under placebo (HR, 0.71; 95 % CI, 0.45–1.13).

Taken together, these data suggest that in OC patients with HRD-positive tumors, even in the absence of a *BRCA1/2* PVs, there is a significant and clinically meaningful benefit of adding PARPi maintenance therapy (alone or in combination with bevacizumab) following response to platinum-based chemotherapy. Genomic scar assays provide information on the magnitude of benefits that PARPi could generate depending on HRD status. However, it is important to highlight that beyond *BRCA* PVs, HRR multigene panels and HRD genomic instability tests are not interchangeable. While in the first-line maintenance setting HRD testing clearly predicts the magnitude of PARPi benefit, as demonstrated across PAOLA-1 and PRIMA trials, in the PAOLA-1 setting HRR gene panels are not predictive of maintenance olaparib plus bevacizumab. Indeed, Pujadee-Lauraine *et al.* [70] showed that non-*BRCA* HRR PVs were not associated with improved PFS with olaparib plus bevacizumab.

Of course, molecular tests for the identification of HRD patients are crucial to guide the use of PARPi and should be implemented. In the meantime, multigene panels and HRD genomic testing should not be considered as substitutes for each other in clinical practice.

Prostate cancer

The presence of a *BRCA* PV directs therapeutic management with PARPi. However, whether and to what extent PARPis can be used for prostate cancer patients with non-*BRCA* HRR PVs, remains controversial. The phase III randomized PROfound study evaluated olaparib versus enzalutamide or abiraterone according to HRR gene mutations [71). In patients with *BRCA1*, *BRCA2* or *ATM* PVs, the PARPi olaparib significantly improved in radiologic PFS and increased OS compared with standard of care (19.1 vs 14.7 months, respectively; HR 0.69, 95 % CI 0.50–0.97, p = 0.02). However, an exploratory gene-level analysis showed different HR for *BRCA1/2* and *ATM*-mutated patients. The HR for OS was 0.42 (95 % CI 0.12–1.53) for *BRCA1* PV carriers, 0.59 (95 % CI 0.37–0.95) for *BRCA2* PV carriers, and 0.93 (95 % CI 0.53–1.75) for patients carrying *ATM* PVs [72].

In cohort B, including patients with PV in 12 additional HRR genes (*BARD1, BRIP1, CDK12, CHEK1, CHEK2, FANCL, PALB2, RAD51B, RAD51C, RAD51D or RAD54L),* there was no evidence of olaparib efficacy (HR 0.88; CI 95 % 0.58–1.36) [73]. These findings underline a different outcome to PARPi depending on the HRR involved genes. However, while the FDA approved olaparib in mCRPC patients harboring any HRR genes identified by the FoundationOne CDx test, the EMA approved olaparib only in *BRCA1/2* mutated setting.

The interim analysis of the phase III Propel trial showed that I-line olaparib/abiraterone improved PFS vs placebo/abiraterone (HR 0.66, 95 % CI 0.54–0.81, P < 0.001) in mCRPC patients regardless of HRR status. Despite the effect being more evident in patients with HRR PVs (HR 0.50, 95 % CI 0.34–0.73) than patients without HRR PVs (HR 0.76, 95 % CI 0.60–0.9), the results were nonetheless significant. Therefore, additional data on the PARPi effectiveness in non-*BRCA* mutated prostate cancer patients are needed [74].

Pancreatic cancer

Beyond *BRCA2*, *ATM* gene mutations are the second most frequent alterations in PDAC. Although the *ATM* gene encodes a kinase involved in the DNA double-strand break repair pathway, bi-allelic deleterious variants of *ATM* have not been shown to result in susceptibility to PARPi-like *BRCA* PV carriers [75,76].

Furthermore, recent findings showed benefit from PARPi in PDAC patients with tumors harboring somatic bi-allelic loss of *BRCA1/2* and *PALB2* genes, opening additional scenarios of HRD also in pancreatic cancers [77]. Particularly, in the single-arm phase II study of maintenance rucaparib in patients with platinum-sensitive PDAC and germline or somatic PV in *BRCA1*, *BRCA2*, or *PALB2*, the finding of efficacy in patients with germline *PALB2* and somatic *BRCA2* PVs expands the potential population likely to benefit from PARPi [78].

Breast cancer

Several trials are ongoing to test PARP-inhibitors efficacy beyond germline *BRCA1/2* PVs in BC patients, such as the phase II studies VIOLETTE (NCT03330847), DOLAF (NCT04053322), and NOBROLA (NCT03367689). To date, most BC patients benefiting from PARPi harbored germline *PALB2* PVs [79]. In the phase II olaparib expanded trial TBCRC-048 (NCT03344965), among HER2-negative metastatic BC patients carrying PVs in non-*BRCA1/2* HRR-related genes, confirmed responses were only achieved in patients with germline *PALB2* PVs [Objective Response Rate (ORR), 82 %] and somatic *BRCA1/2* (ORR, 50 %) PVs. Median PFS was 13.3 months (90 % CI, 12 months – not available [NA]) for *PALB2* PVs carriers, and 6.3 months (90 % CI, 4.4 months - NA) for somatic *BRCA1/2* PV carriers. No responses were observed in BC patients harboring *ATM* or *CHEK2* PVs [79]. Instead, anecdotical response to olaparib is reported in metastatic HR-positive, human epidermal growth factor receptor 2 (HER2)-negative breast cancer patients carrying germline PV in the *BRIP1* gene [80].

In the phase II PETREMAC trial (NCT02624973), olaparib efficacy was evaluated in patients with early triple-negative BC. Excluding germline *PALB2* and *BRCA1/2* PV carriers, 12 out of 14 responders showed somatic HR PVs and/or *BRCA1* methylation, revealing olaparib response beyond germline *BRCA1/2* PVs [81].

In the single-arm phase II RUBY trial (NCT02505048), the efficacy of rucaparib was assessed in HER2-metastatic BC patients with either high LOH scores or non-germline *BRCA1/2* PVs. The results suggested that a small subset of these BC patients, without germline *BRCA1/2* PVs, could derive benefit from PARPi, and highlighted the need for additional biomarkers to select the patients [82].

Toward the liquidomics. The future of biomarker discovery in HRD-associated tumors

Emerging DNA-based biomarkers: Circulating tumor (ctDNA)

In the era of precision medicine, liquid biopsy is under investigation as the first or complementary approach to tissue for tailoring the molecular testing of sporadic tumor snapshots [83]. Translational research has been focused on elucidating whether the role of primary or acquired somatic reversion variants was closely related to patient prognosis and/or prediction of response to PARPi and platinum-based chemotherapies [83]. Dealing with diagnostic accuracy, the PROfound, TRITON-2 trials on prostate cancers, and other explorative clinical studies, have shown a 75–80 % concordance rate in HRR gene variants between tumor tissue and cell-free DNA (cfDNA) [84,85]. Certainly, the unfortunate addition of sequence variants, such as substitutions and insertions/deletions (indels), to pre-existing deleterious somatic or germline variants in HRR genes can restore the open reading frame and subsequent protein functionality in tumor cells becoming HRR-proficient [86].

In this fascinating scenario, circulating tumor DNA (ctDNA) mutational profiling of HRR genes (including *BRCA1*, *BRCA2*, *RAD51*, A*TM, CDK12, PALB2*, *ARID1A,* and *MAPK* pathway genes) using customized amplicon and hybridization-based NGS techniques could be useful for diagnostic and therapeutic purposes, mostly considering the multiclonal heterogeneity in the pretreated setting [4,87].

Namely, in the resistant setting of HGSOC patients harboring germline variants in the HRR pathway, the role of ctDNA acquired somatic PVs has been investigated, showing that the MRE11A p.K464R point mutation (as well as a few novel somatic variants in several other genes playing a role in the regulation complex of DDR proteins) appeared to be responsible for resistance to olaparib, leading to shorter PFS and poor prognosis [88]. On the contrary, in the baseline setting, the same p.K464R or p.K373E point mutation within the *CHEK2* gene together with other variants in the *TP53*, *ATM, PMS2* genes have been associated with statistically significant higher PFS, differently from *PIK3CA*, *EGFR* and *ERBB2* genes [88]. Further, the monitoring of ctDNA mutated allelic frequency (MAF) clearance of such PVs during treatment seemed to easily follow CA125 serum levels [88]. Thus, screening cfDNA molecular profiles of HRR genes could provide useful insights into identifying valid biomarkers for monitoring PARPi-based treatment.

The use of different NGS platforms employing specific probes could enable the on-treatment MAF evaluation for the longitudinal tracing of clonal driver variants. From this perspective, as compared to the low-quality nucleic acids of FFPE tumor tissue, ctDNA testing reliably proved to detect clonal reversion mutations in HGSOC patients harboring germline or somatic variants in the *BRCA1/2* genes [89].

However, considering the significant paucity of data, larger studies are still warranted to define the prevalence of reversion mutations, while monitoring sub-clonal HRR PVs and evaluating their relationship with treatment response in the clinical setting [90].

The silence of genes: The DNA methylation

In recent years, a growing body of literature has studied how

epigenetics could influence the onset and progression of differing neoplasms, contributing to treatment resistance [91]. Epigenetics can affect the regulation of gene expression through various mechanisms, with the most notorious and studied pathways being mainly three: DNA methylation, histone modification and non-coding RNAs. Here, we focus on DNA methylation, perhaps the most known epigenetic modification regarding the addition of a methyl group to the cytosine base generating 5-methylcytosine (5mC) [92].

In mammals, DNA methylation occurs through the covalent modification of cytosine residues in CpG dinucleotides, a reaction catalyzed by DNA methyl transferases (DMNTs). CpG dinucleotides are mostly concentrated in extended regions of DNA characterized by the presence of repeated sequences, or in short stretches of CpG-rich DNA ("CpG islands") predominantly found within the promoters of human genes, thereby significantly influencing gene transcription [93].

Chemical and pathological agents together with alterations in methylation machinery or DNMTs could be responsible for the abnormal cell DNA methylation status, inducing cancer proliferation and chemoresistance [94]. It has been found that DNA methylation, arising in about 70 % of CpG islands and approximately 40 % of CpG-rich genes [93], could be linked to the onset of some malignancies including ovarian and breast cancers, as it negatively affects transcription by reducing the levels of proteins involved in DNA damage repair [92].

Aberrant DNA methylation, repressing gene expression by promoting or inhibiting the recruitment of regulatory proteins into DNA [95], is an event that, occurring early in cancer development, could be representative of tumor heterogeneity while being easily detected in a minimally invasive manner in circulating cell-free DNA, thus representing one of the most promising cancer biomarkers in both the diagnostic and clinical scenario [96]. Namely, DNMT could potentially serve as a therapeutic target, especially in the clinical setting resistant to platinum-based agents and PARPis.

DNA methylation and platinum resistance

It had been suggested that chemoresistance to platinum-based chemotherapy, developed during the treatment course, could be due to epigenetic mechanisms impacting the transcription of genes involved in reduced drug influx to the cell, increased extracellular export, increased DDR routes pathway or activation of apoptosis pathways [97]. The identification of promising DNA methylation biomarkers for predicting response to platinum could be useful in the clinical management of cancer patients. Namely, promoter DNA methylation of genes involved in pathways such as MLH1/MSH2 or NRF2/KEAP1 had been associated with platinum resistance owing to the creation of DNA adducts or inhibition of apoptosis, respectively [98]. However, no biomarker has been approved in this field so far, mostly due to the limited sample size of the studies [99]. From this standpoint, another critical issue regards the percentage of neoplastic cells in the tumor sample, with values widely ranging from 30 % [100] to 90 % [101]. Such significant variability among studies sharply influences the determination of methylation levels and the diagnostic accuracy of the tests, affecting the reproducibility and validation of results [102].

DNA methylation and PARPis

Besides being involved in the MMR route [103]. PARP1 controls other cellular processes such as transcription regulation [104] and differential DNA methylation [105]. As a matter of fact, most methylation changes occur globally causing epigenetic silencing of oncosuppressor genes during carcinogenesis. Reale *et al.* [106] showed that auto-poly (ADP-ribosylation) of PARP1 could recruit DNMT, inhibiting its activity and thus preventing the hypermethylation. Pulliam and colleagues showed that treatment with specific DNTM inhibitors could restore sensitivity to PARPis regardless of *BRCA* status [107]. In this context, the addition of DNMT inhibitors to PARPis could synergistically improve the antiproliferative action in cancer cells, even independently of DDR status [108].

Conclusion

Although *BRCA1/2* PVs are effective predictors of sensitivity to PARPi, mainly in OC, current biomarkers of non-*BRCA* HRR PVs are insufficient for guiding the use of PARPi. Genomic scar assays have demonstrated their value in increasing the number of patients likely to benefit from PARPi in several clinical trials, particularly in the in OC first-line setting. However, several limitations for known assays are recognized. They show genome scars that reflect the HRD status but will not disappear if the tumor HRD phenotype changes under the pressure of treatment, leading to HR repair restoration [109,110,111].

In the future, liquid biopsy using blood or other different biological fluids may represent a surrogate of neoplastic tissue and a minimally invasive option to access the longitudinal monitoring of clinically approved molecular biomarkers [112]. Currently, the analysis of ctDNA, isolated from peripheral blood, is the main liquid biopsy approach studied in HRD-associated tumors, despite its use still presenting issues of a technical and biological nature. Maybe, the emerging "liquidomics" [113] will be able to represent the missing, complementary, element integrating the better genetic and genomic knowledge.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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