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## **Cracking the homologous recombination deficiency code: how to identify responders to PARP inhibitors**

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## Highlights

- Homologous recombination deficiency (HRD) is targetable by PARP inhibitors
- HRD tests are helpful to select patients who may benefit from PARP inhibitors
- Genomic scars assays increased the number of patients eligible for PARP inhibitors
- Functional tests allow a real-time assessment of homologous recombination activity
- Acquired resistances to PARPi have been described and need to be carefully studied

**Abstract:**

DNA double-strand breaks are the most critical DNA damage to cells, and their repair is tightly regulated to maintain cellular integrity. Some cancers exhibit homologous recombination deficiency (HRD), a faithful double-strand break repair system, making them more sensitive to poly (ADP ribose) polymerase inhibitors (PARPi). PARPi have shown substantial efficacy in *BRCA*-mutated ovarian cancer for several years, and their indication has gradually been extended to other tumour locations such as breast, prostate and pancreas. More recently, PARPi were demonstrated to be effective in cancers with an HRD phenotype beyond *BRCA* mutations. Today, a major challenge is developing tests capable of detecting the HRD phenotype of cancers (HRD tests) and predicting sensitivity to PARPi to select patients likely to benefit from this therapy. This review provides a synthesis of the existing HRD tests, divided into three main approaches to detect HRD: the investigation of the HRD causes, the study of its consequences and the evaluation of the HR activity itself.

**Keywords:** Homologous recombination deficiency, PARP inhibitors, *BRCA*, HRD tests, genomic scars, RAD51 foci

## 1 Hacking the homologous recombination pathway with PARP inhibitors

DNA repair pathways are essential for the maintenance of cellular integrity. Among these pathways, homologous recombination (HR) allows the faithful repair of DNA double-strand breaks. Numerous molecular actors are involved, such as the damage sensors *ATM*, *ATR*, *CHEK1*, *CHEK2*, the MRN complex composed of the *MRE11*, *NBS1* and *RAD50* sensors, the mediators *BRCA1*, *BRCA2*, *PALB2* and the effector protein RAD51, an essential protein allowing the invasion of the complementary strand necessary for the damaged sequence re-synthesis [1]. Alterations in each player can lead to HR deficiency (HRD), which causes genomic instability that cancer cells use to gain selective advantages. In addition to well-described alterations in *BRCA1* or *BRCA2*, defects in other HR genes may also be responsible for an HRD phenotype, also called “BRCAness”. According to The Cancer Genome Atlas (TCGA), around 50% of ovarian cancers could have an HRD phenotype [2], followed by prostate, pancreatic and breast cancers [3]. These repair defects increase the sensitivity of tumour cells to DNA damaging agents. This Achilles’ heel has been exploited in therapy with PARP (poly(ADP-ribose) polymerase) inhibitors (PARPi). Through catalytic inhibition of the PARP enzyme and PARP-trapping on damaged DNA, these molecules prevent PARP activity, a protein involved in repairing single-strand breaks (SSB). Simultaneous inhibition of the SSB repair pathway and HR deficiency (HRD) leads to apoptosis of tumour cells by synthetic lethality [4]. The development of PARPi has led to significant progress in the management of advance stage high grade serous ovarian cancers. Currently, these drugs are indicated as first-line maintenance treatment in patients with newly diagnosed advanced ovarian cancers responding to platinum-based chemotherapy, alone or in combination with Bevacizumab in those with HRD-associated diseases [5–7]. They are also used in the maintenance treatment of platinum-sensitive recurrent ovarian cancers. In other tumour types, PARPi are indicated in germline *BRCA1/2* mutated metastatic breast and pancreatic cancers, as well as in *BRCA1/2*-mutated metastatic castration-resistant prostate cancers [8–11]. The actual main challenge is to identify patients that will benefit from PARPi therapy beyond *BRCA1/2* mutations. This prompted the development of tests able to detect HRD in cancers. This review will focus on three different approaches: i) identifying the causes of HRD, ii) identifying its consequences, or iii) measuring the HR activity itself (Figure 1).

## **Glossary:**

**HR** (*Homologous recombination*): cellular mechanism responsible for the faithful repair of double-strand breaks.

**HRD** (*Homologous recombination deficiency*): defects in homologous recombination leading to the inability to repair DNA double-strand breaks properly. These defects can be due to mutations in the genes involved in the pathway, epigenetic modifications or other causes.

**Cancer HRD status:** an HRD-positive cancer is a cancer that has HR defects, an HRD-negative cancer (or HRP for Homologous recombination proficient) is a cancer that has a functional HR pathway.

**HRD tests:** technics to determine the cancer HRD status.

**HRD phenotype:** a set of phenotypic characteristics shared by HRD-positive cancers (such as sensitivity to platinum salts or PARPi).

**HRR genes** (*Homologous recombination repair genes*): genes involved in the HR pathway and whose alterations are responsible for the HRD phenotype.

## **2 Identifying the source code: HRD causes**

### *Germline and somatic BRCA1/2 mutations*

The increased *in vitro* sensitivity of *BRCA*-mutated tumour cells to PARPi [12,13], and the significant frequency of these mutations in several cancers led to the use of *BRCA1/2* mutations as the first biomarker to identify patients who could benefit from PARPi therapy.

Clinical trials in ovarian cancer demonstrated the substantial positive predictive value of *BRCA1/2* mutations. The *BRCA*-mutated patients' subgroup benefited the most from PARPi [14–16]. Clinical benefits are comparable whether the *BRCA1/2* mutations are germline or somatic [17], which has led to the indication of PARPi in *BRCA*-mutated ovarian cancer regardless of the origin of the mutation. However, patients with *BRCA*-wildtype ovarian cancer (*wtBRCA*) also significantly benefited from PARPi in some studies to a lesser extent [18]. Currently, PARPi are indicated as first-line maintenance therapy in newly diagnosed advanced ovarian cancers responding to platinum-based chemotherapy as well as

maintenance treatment for platinum-sensitive recurrent ovarian cancers with or without *BRCA* mutation. These indications could still evolve. First results from the phase III OReO/ENGOT Ov-38 trial point to a clinical benefit in PFS of retreatment with Olaparib compared to placebo in heavily pre-treated ovarian cancers after response to platinum-based chemotherapy, regardless of *BRCA* status (HR 0.57; CI95% 0.37-0.87; P=0.022 in the *BRCA*-mutated cohort and HR 0.43; CI95% 0.26-0.71; P=0.002 in the non-*BRCA*-mutated cohort) [19].

Results from clinical trials in breast (OlympiAD, EMBRACA) [20,21] and pancreatic cancers (POLO) [22] also showed a clinical benefit of PARPi in patients with *BRCA1/2* germline mutations leading to the use of *gBRCA* as a biomarker in these cancers to select metastatic patients for PARPi prescription. Recently, results from the OlympiA trial - comparing adjuvant Olaparib in early-stage breast cancer at high risk of recurrence versus placebo - demonstrated a significant improvement in invasive disease-free survival (HR 0.58 CI99.5% 0.41-0.82; P<0.001) in patients with germline *BRCA1/2* mutation treated with Olaparib [23]. Recommendations are awaited regarding the use of Olaparib and the *gBRCA* biomarker at an earlier stage of breast cancer.

Commercial tests have been developed to screen for *BRCA1/2* mutations. In the United States, FDA approved the BRACAnalysis CDx test (Myriad Genetics<sup>®</sup>), which detects *BRCA1/2* germline mutations, and the FoundationFocus CDx<sub>BRCA</sub> test (Foundation Medicine<sup>®</sup>), which detects *BRCA1/2* tumour mutations, as "companion diagnostic" (CDx) tests to select patients who may be eligible for a PARPi treatment [24]. In Europe, *BRCA* mutation testing is usually performed in accredited laboratories, and a single centralised test is not a prerequisite for PARPi treatment. Some authors suggested that capture-based next-generation sequencing (NGS) should be preferred when exploring mutations, including CNV detection, in formalin-fixed paraffin-embedded (FFPE) tumours [25].

### *Other HRR genes mutations*

Alterations of homologous recombination repair genes (HRR genes) beyond *BRCA1/2* could also generate HR deficiency and sensitise the tumour to PARPi. These HRR genes (from

10 to 30 depending on the panel) are now routinely assessed by commercial or academic NGS panels. However, modifications of non-*BRCA1/2* HRR genes are less frequent, and the study of their association with the response to PARPi remains a challenge today, as suggested by some trial results.

In metastatic prostate cancer (mCRPC), the phase III PROfound trial evaluated Olaparib *versus* Enzalutamide or Abiraterone in patients according to HRR gene alterations. The *BRCA1/2* and *ATM*-mutated patient cohort had a significant improvement in radiologic PFS with Olaparib (HR 0.34; CI95% 0.25-0.47) [26]. However, Olaparib did not appear to be effective in the subgroup of patients with an *ATM* mutation (HR 1.04 CI95% 0.64-1.87). An exploratory analysis of this trial also reported a lower efficacy of Olaparib in cohort B (*BARD1, BRIP1, CDK12, CHEK1, CHEK2, FANCL, PALB2, RAD51B, RAD51C, RAD51D or RAD54L* mutation) with a hazard ratio of 0.88 (CI95% 0.58-1.36) [27]. These results highlight a disparity of response to PARPi depending on the affected genes. While the FDA approved Olaparib in patients with mCRPC mutated in any of these HRR genes identified by the FoundationOne CDx test (Foundation Medicine®), the EMA chose to grant Olaparib marketing authorisation only in *BRCA1/2* mutated mCRPC.

Another study, the phase II Olaparib Expanded trial TBCRC-048, evaluated the response to Olaparib in 54 patients with HER2-negative metastatic breast cancer mutated in HRR genes other than *gBRCA*. Confirmed responses were only achieved in patients with germline *PALB2* mutations (82% (9/11); CI90% 53-96%) and somatic *BRCA1/2* mutations (50% (8/16); CI90% 28-72%). Among the patients with mutations in other HRR genes, such as *ATM* (0/8), *CHEK2* (0/7) or *BRIP1* (0/1) [28], there were no confirmed responses.

In ovarian cancer, an exploratory biomarker analysis of ARIEL2 samples suggested that *RAD51C* and *RAD51D* mutations confer similar sensitivity to Rucaparib as *BRCA1/2* mutations [29]. However, the role of other HRR genes in response to PARPi remains unclear. Recently, Pujade-Lauraine *et al.* investigated the predictive value of different panels of non-*BRCA* HRR genes in the PAOLA1 cohort. The panels allowed the detection of a small proportion of patients carrying a non-*BRCA* HRR mutation (3.7-9.8% depending on the panel). Furthermore, detecting a mutation was not associated with improved PFS with Olaparib + Bevacizumab [30]. Additional clinical trials are ongoing to further explore the various genetic



abnormalities associated with HRD and provide greater insight into their pathogenicity and bi-allelic inactivation.

### *HRR genes epigenetic changes*

Epigenetic modifications can also be responsible for HR deficiency. Hypermethylation of the *BRCA1* and *RAD51C* promoters is observed in 11% and 3% of ovarian cancer cases, respectively [2]. Hypermethylation of the promoter CpG islands prevents transcription and thus leads to decreased gene expression [31]. However, the correlation between *BRCA1* or *RAD51C* hypermethylation and platinum salts or PARPi activity seems challenging to establish, with contrasting results depending on studies [15,32,33].

A recent meta-analysis of 15 ovarian cancer studies shows that although *BRCA1* promoter methylation shares some histopathological features with *BRCA1* mutations (young age at diagnosis, high grade), epigenetic silencing is not associated with improved survival after platinum chemotherapy compared to *wtBRCA* patients [34]. This could be due to the rapid demethylation of the *BRCA1* promoter - epigenetic changes being dynamic mechanisms - under the pressure of chemotherapy, conferring resistance to the treatment. The study also shows substantial heterogeneity in the techniques employed to detect *BRCA1* methylation (methylation-specific PCR, methylation-sensitive restriction enzyme, genome-wide methylation array), which could explain the resulting disparity regarding the predictive/prognostic value of *BRCA1* methylation [34].

Recently, Kondrashova et al. highlighted the importance of *BRCA1* methylation zygosity. Sensitivity to PARPi was achieved in homozygous-methylated tumours (methylation of all *BRCA1* copies), but not with heterozygous methylation [35]. An exploratory analysis of ARIEL2 samples also showed that homozygous *BRCA1* hypermethylation conferred similar sensitivity to Rucaparib as *BRCA1/2* mutations [29]. Prospective studies incorporating this parameter may provide more insight on the impact of *BRCA1* methylation on response to PARPi. Today, *BRCA1* or *RAD51C* methylation is not used as a biomarker for PARPi response in cancer.

### *Genotypic tests limitations*

The main limitation of extended HRR panels is the difficulty to associate the inactivation of non-*BRCA1/2* genes with response to PARPi in clinical trials. Moreover, these NGS-based tests frequently identify variants of uncertain significance (VUS) with unknown functional impact on the HR pathway. As an example, up to 50% of *BRCA* variants are classified as VUS, according to a recent survey [36].

The choice of the tumour material for HRR genes screening is also critical. First, the use of FFPE material at the time of relapse should be preferred since the tumour HRD phenotype may change between the first diagnosis and the relapse. On the other hand, the quantity and the quality of the tissue preserved in FFPE block are sometimes inadequate and responsible for a non-contributive sample. The rate was as high as 31% in available prostate-biopsies in PROfound [26].

Approaches using liquid biopsy, already routinely validated in lung cancers, are emerging in prostate cancer to address these issues. Recent data presented at the ASCO 2021 suggested high concordance between tumour tissue and circulating tumour DNA analysis for *BRCA1*, *BRCA2*, and *ATM* genes from patient samples enrolled in the PROfound trial (81% positive concordance (143/177); CI95% 75-87 and 92% negative concordance (291/315); CI95% 89-95) [37]. Moreover, Quigley et al. reported that liquid biopsy could detect *BRCA2* reversion mutations associated with PARPi resistance [38]. Thus, a liquid biopsy would provide a real-time reflection of the cancer HRD status. In the United States, the FoundationOne® Liquid CDx test (Foundation Medicine®), performed on plasma, has been approved as a companion test for introducing Olaparib and Rucaparib in mCRPC [24].

In summary, the mechanisms responsible for HR deficiency have not been fully identified. The genotypic approach cannot, therefore, be exhaustive. One way to overpass the limitation of HRR genes sequencing is to look at the consequences of HRD on the tumour genome.

### **3 Looking for the program prints: HRD consequences**

HR defects are the source of significant genomic instability. This instability results in somatic mutations (substitutions, short indels) - which are the basis of mutational signature

tests - as well as large chromosomal rearrangements generating structural and copy number abnormalities throughout the genome, referred to as genomic scars.

### *Genomic scars assays*

Three types of genomic scars attributable to HR deficiency were described in 2012 using SNP-array techniques: 1) loss of heterozygosity (LOH) that corresponds to the loss of regions greater than 15Mb but less than the entire chromosome [39], 2) large-scale transitions (LSTs) that represent chromosomal breaks of at least 10 Mb between adjacent genomic regions [40], 3) telomeric allelic imbalance (TAI) that is the unequal contribution between the paternal and maternal alleles in telomeres [41] (Figure 2). Assessment of each of these lesions resulted in 3 scores, all individually correlated with *BRCA1/2* deficiency.

In 2014, Timms et al. demonstrated a better correlation to *BRCA1/2* deficiency by combining the 3 LOH/LST/TAI scores [42]. This combination provided the basis for the commercial myChoice® CDx test (Myriad Genetics®), now approved by the FDA as a companion diagnostic test to guide the initiation of Olaparib in association with Bevacizumab in newly diagnosed advanced ovarian cancer.

The myChoice® CDx test combines the sum of LOH, LST and TAI identified within a tumour to generate a Genomic Instability Score (GIS). The analysis of breast and ovarian tumours cohorts revealed a bimodal distribution of the GIS, with two populations: *BRCA1/2*-wildtype tumours with a low GIS; and *BRCA1/2*-deficient tumours with a high GIS. The threshold was set to 42 from this dichotomy to discriminate HRD tumours (GIS ≥ 42) with a 95% sensitivity [43]. This number means that 5% of *BRCA1/2*-deficient tumours exhibit a GIS < 42, supporting the importance of parallel *BRCA1/2* sequencing in the combined myChoice® CDx test. Importantly a fraction of *wtBRCA1/2* tumours also has a high GIS, presumably due to alteration of non-*BRCA1/2* HRR genes. HRD+ cancers are therefore defined by the presence of a *BRCA1/2* mutation and/or a GIS ≥ 42.

The myChoice® HRD score has been studied in several clinical trials investigating PARPi in ovarian cancer. In relapsed ovarian cancer, evaluation of Niraparib by HRD status in the NOVA [14] and QUADRA [44] trials revealed a continuum of benefit (Table 1). Maximum

benefit was achieved in the subgroup of patients with *BRCA1/2* mutations, followed by the HRD subgroup and finally the HRP subgroup. These results underline the inability of the myChoice® test to discriminate a subset of patients who derive no benefit from PARPi. In first-line maintenance therapy, similar results were reported in the PRIMA [6] trial with a benefit of Niraparib regardless of HRD status. Only the phase III PAOLA [7] trial showed that the subgroup of HRP patients did not benefit from Olaparib combined with Bevacizumab. These findings led to the approval of Olaparib in combination with Bevacizumab as first-line maintenance therapy only in patients with advanced HRD+ ovarian cancer (*BRCA1/2* mutated and/or high GIS) who have responded to first-line platinum-based chemotherapy.

Foundation Medicine® has developed a second FDA-approved commercial test detecting genomic scars has been developed by. The FoundationFocus CDx<sub>BRCA LOH</sub> test determines a LOH score defined by the percentage of LOH regions within the tumour genome combined with the search for *BRCA1/2* alterations.

This test was evaluated in ARIEL2 and ARIEL3 trials studying Rucaparib in platinum-sensitive relapsed ovarian cancer [15,16]. Subgroup analysis by HRD status in ARIEL3 revealed a clear improvement in PFS in the *tBRCA* subgroup (16.6 vs 5.4 months; HR 0.23 CI95% 0.16-0.39) followed by the HRD+ subgroup (*tBRCA* or *wtBRCA*-LOH<sup>high</sup>: 13.6 vs. 5.4 months; HR 0.32 CI95% 0.24-0.42), the *wtBRCA*-LOH<sup>high</sup> subgroup (9.7 vs. 5.4 months; HR 0.44 CI95% 0.29-0.66), and finally the HRP subgroup (*wtBRCA*-LOH<sup>low</sup>: 6.7 vs. 5.4 months; HR 0.58 CI95% 0.4-0.8) [16]. This test identifies patients without *BRCA1/2* mutations who could also benefit from Rucaparib. However, as in PRIMA and NOVA trials with the myChoice® test, this test does not identify a subgroup not benefiting from PARPi.

LOH score analysis in ovarian cancer is now integrated into Foundation Medicine's FoundationOne CDx test, which also includes analysis of an extensive panel of genes, including *BRCA1/2* and many other HRR genes.

### *Genomic scars assays limitations*

Genomic scars assays have demonstrated their value to increase the number of patients likely to benefit from PARPi, particularly in the first-line setting. These tests provide

information on the magnitude of benefits that PARPi could generate depending on HRD status. However, except in the PAOLA-1 trial, genomic scars assays did not identify a subgroup of patients who would not benefit from PARPi. One hypothesis to explain this difference between PAOLA-1 and PRIMA or other trials at relapse, is the use of platinum sensitivity as an inclusion criterion. PRIMA included high-risk patients, some with residual disease after primary debulking surgery (PDS). They were selected based on a documented response to platinum chemotherapy. In contrast, in PAOLA-1, 60% of the patients included did not have residual disease after PDS and sensitivity to platinum could not be documented [45].

Adjustments of the thresholds used to define HRD status could be more discriminating to identify a non-responding population. For example, an exploratory analysis of the Study19 trial investigating the response to Olaparib by HRD status using the myChoice® test with a cut-off set at 33 instead of 42 supports this. In this analysis, the "GIS < 33" subgroup of patients had a lower PFS benefit from Olaparib (HR 0.71, CI95% 0.34-1.46) than the "GIS < 42" subgroup (HR 0.60, CI95% 0.31-1.17) [46]. Prospective studies in new clinical trials might be of interest to define the optimal threshold.

Another significant limitation is that these assays capture genome scars, i.e. "indelible" marks that reflect a deficiency in HR during the tumour process but will not disappear if the tumour HRD phenotype changes. Indeed, tumour cells are constantly adjusting, and acquired resistances, due to the pressure of treatment, can reverse the HRD phenotype leading to HR pathway restoration (Figure 3). Of importance, secondary somatic mutations of *BRCA1/2* can make these genes functional again and restore a proficient HR phenotype. A recent meta-analysis found that 26% of cancers (out of 327 cases studied) that became resistant to platinum salts or PARPi had *BRCA1/2* reversion mutations [47]. This phenomenon has been described in ovarian, breast, prostate and pancreatic cancers. HR restoration can also be achieved by demethylation of the *BRCA1* promoter allowing its re-expression or by decreasing 53BP1 expression, an actor favouring the non-homologous end joining (NHEJ) pathway instead of HR pathway [4]. Other mechanisms of acquired HR-unrelated resistance to PARPi, such as developing alternative systems meant to protect the replication fork destabilised by PARPi [4] or the increase in PARPi efflux via P-glycoproteins [48], cannot be detected with these tests.

### *Mutational signatures*

Focusing on somatic mutations occurring throughout the tumour genome rather than in target gene regions (frequency, type, close environment) is another way to identify HRD patients [49]. This approach is referred to as a "mutational signature".

In 2013, Alexandrov et *al.* described 20 mutational signatures through analysis of whole-genome (WGS) or whole-exome sequencing (WES) data from more than 7000 tumours representing 30 different tumour types [50]. To date, more than fifty mutational signatures based on single nucleotide substitutions (SBS) have been identified [51]. These signatures are based on the analysis of the different nucleotide substitutions along the genome (C>A, C>G, C>T, T>A, T>C, T>G) as well as the adjacent 5' and 3' nucleotides. This results in 96 possible trinucleotide combinations, whose frequency is evaluated to be related to given mutational processes. Signature 3 (SBS3) corresponds to an equivalent distribution of the trinucleotide combinations across the tumour genome and correlates with *BRCA1/2* deficiency. It was observed in 10% of samples examined by Alexandrov et *al.* within three tumour types: breast, ovary and pancreas [50]. Also observed in *BRCA1/2*-intact tumours, this SBS3 signature could be associated with other mechanisms of HRD.

New bioinformatics tools are being developed to exploit signature 3 from routine cancer gene panels used in clinics instead of WGS. As such, the SigMA tool is based on a likelihood measure comparing the mutation profile from the sequencing of a gene panel for a given sample to average profiles from WGS data representing different mutational signatures. Its application to a cohort of platinum-treated ovarian cancers identified a subgroup of "wtBRCA Sig3+" patients (n=76) with an overall survival similar to *BRCA*-mutated patients (n=19; P=0.74) and significantly better than "Sig3-" patients (HR 0.54; IC95% 0.35-0.84; P=0.006) [52].

The HRDetect tool combines six genomic signatures based on substitutions, rearrangements or genomic scars determined by WGS [53]. Evaluated on a cohort of 560 breast cancers, with a threshold set at 70% (HRDetect  $\geq$  0.7) to define tumours associated with *BRCA1/2* deficiency, this test shows excellent performance (sensitivity of 98.7%), better

than for each of the genomic signatures used individually. In addition, the test identified approximately one-third of *wtBRCA* tumours with a score  $\geq 0.7$ , increasing the number of patients with tumours similar to *BRCA1/2*-deficient cancers, which could potentially benefit from PARPi. This promising tool has been validated in breast, ovarian, and pancreatic cancer cohorts to predict *BRCA1/2*-deficiency; the next step will be to evaluate its ability to predict PARPi sensitivity in these tumours. In this way, HRDetect was used in the Phase II RIO trial to determine the HRD status of 43 newly diagnosed TNBC treated with Rucaparib. HRD+ status was observed in 69% of TNBC. The HRDetect signature was associated with greater sensitivity to Rucaparib as measured by the decrease in plasma ctDNA concentration before treatment and after 15 days of Rucaparib (n=15, p=0.027). Initial results were supporting the potential ability of the HRDetect assay to identify PARPi-sensitive tumours [54].

## 4 Testing the program in real-time: dynamic approaches

### *Transcriptome signatures*

Several teams have focused on transcriptomic signatures to detect HRD cancers and predict sensitivity to PARPi. The study of the transcriptome - DNA transcribed into RNA - provides information about which genes are expressed at a given time under a given condition. DNA chips or RNA sequencing can study thousands of genes simultaneously, generating a gene expression profile (GEP). The idea is to see if HRD cells have a different GEP than HRP cells and if this correlates with their sensitivity to PARP inhibitors to make them predictive tools for the response to PARPi in routine, without having to analyse each HR gene individually. In 2014 Peng et al. defined a transcriptomic signature composed of 230 genes capable of identifying HRD in various cell lines independently of the altered HRR gene [55].

Moreover, transcriptomic approaches better assess the HRD phenotype at a given time, as gene expression is dynamic and can vary with acquired resistance. Konstantinopoulos et al. described an HRD-associated BRCAness Profile consisting of 60 genes that predicted response to PARPi in Capan-1 clones with deleterious *BRCA2* mutation or mutation reversion [56]. However, these GEP need to be evaluated on a larger scale and in clinical trials.

### Functional studies

Another way to detect HRD tumours is to measure HR activity using functional assays directly. The objective is to find surrogate markers of the HRD phenotype independent of causal events. The most described functional assay is based on the quantification of RAD51 foci. The RAD51 recombinase is the key effector enzyme of HR, involved in the final steps of the repair pathway. When cells harbour double-strand DNA breaks and HR is functional, nuclear foci of RAD51 are formed. Conversely, *BRCA1/2*-deficient tumours showed no RAD51 foci [57]. This approach allows analysis of HR functionality regardless of the HR deficiency causes upstream of RAD51.

Initial studies have been performed on FFPE samples from breast tumour biopsies obtained 24 hours after neoadjuvant anthracycline chemotherapy. Anthracyclines are cytotoxic DNA intercalators that also can inhibit topoisomerase II, generating double-strand breaks. Graeser et al. [58] quantified RAD51 foci by immunofluorescence in G2/S phase cells through co-labelling with geminin (cell cycle regulatory protein expressed during the G2/S phase) as HR relies on the sister chromatid to repair the damaged sequence and can only take place after DNA replication.. After ensuring the absence of foci in a basal state in 99% of tumour cells from analysis of chemotherapy-naive biopsies, they defined a RAD51 score as the percentage of geminin-positive RAD51-positive cells (i.e., having at least one RAD51 focus per nucleus). They established a threshold of 10% below which tumours are considered RAD51-foci<sup>low</sup> and therefore HRD. Of the breast tumours analysed, 26% had a RAD51 score < 10%, with a significant frequency of a low score in the TNBC subtype (67%) [58].

Other authors have analysed the RAD51 foci formation after induction of *ex-vivo* DNA damage. Naipal et al. studied the RAD51 foci formation in chemotherapy-naive breast tumours, with exogenous induction of DNA damage by irradiation (IRIF for ionising radiation-induced foci) [59]. The irradiated tumours were incubated for two hours before being analysed by immunofluorescence. Tumours were considered HRP if more than 50% of the geminin-positive cells had at least 5 RAD51 foci per nucleus, and HRD if less than 20% of the geminin-positive cells had at least 5 RAD51 foci. This test, known as RECAP (for REpair CAPacity), was validated in a cohort of 170 breast cancers, detecting 19% of HRD cancers, of



which 16/23 were *BRCA1/2* altered, and 7/23 were *wtBRCA*, indicating the ability to detect HRD cancers beyond *BRCA1/2* deficiency [59]. The RECAP was also evaluated in a cohort of 49 ovarian cancer samples (solid tumours and ascites fluids), identifying 20% HRD phenotype cancers, mostly *BRCA1/2*-deficient [60]. A drawback of this test is that it requires fresh tissue, making this technique difficult to apply in a laboratory routine.

More recently, Castroviejo-Bermejo et al. have developed a protocol for detecting RAD51 foci in tumour samples without prior induction of DNA damage [61]. This protocol is based on DNA damage generated endogenously by tumour cells, sufficient to trigger the double-strand break repair pathway if it is functional. Fourteen non-*gBRCA* breast tumours out of 23 tested had a low RAD51 score (defined threshold  $\leq 10\%$ ), among which 11 had germline *PALB2* mutation, showing the ability of this test to detect HRD cancers beyond *BRCA1/2* deficiency on FFPE samples and in the absence of DNA damage induction [61].

Cruz et al. investigated the ability of the RAD51 functional assay to detect tumours with primary or acquired resistance to PARPi. In 13 PDX models from breast and ovarian tumours with germline *BRCA1/2* mutation, the 10 Olaparib-resistant models had detectable RAD51 foci (RAD51 score  $36 \pm 2\%$  for PARPi-resistant vs  $5 \pm 3\%$  for PARPi-sensitive,  $P=0.0017$ ). Different mechanisms of resistance were identified (hypomorphic *BRCA1* isoforms or loss of 53BP1), demonstrating the ability of the assay to detect PARPi-resistant tumours regardless of the mechanism of resistance [62].

Lastly, Blanc-Durand et al. evaluated the RAD51 functional test in a cohort of 155 ovarian cancer patients treated with neoadjuvant platinum-based chemotherapy (CHIVA trial). They identified 55% (76/139) of RAD51-negative patients who were shown to have significantly improved PFS compared with RAD51-positive patients (20.8 vs 14.1 months;  $p=0.02$ ) [63]. Quantification of RAD51 foci could thus predict patient outcome under platinum-based chemotherapy and needs to be evaluated in patients treated with PARPi.

However, some points about RAD51 foci quantification assays need to be clarified, such as the delay to quantify foci after induction of DNA damage (2h in Naipal et al. study versus 24h for Graeser et al.), or the number of foci retained to consider a cell as HR-proficient. On

the other hand, these assays will not detect events responsible for HRD that would take place downstream of RAD51 as it could be the case with a defect of the polymerase  $\eta$  [64], an enzyme involved in the last phase of HR, or in the case of "loss of function" mutations of RAD51 itself. Furthermore, mechanisms of PARPi resistance independent of HR restoration, as mentioned above, will not be detectable either.

Recently, functional assays studying the stability of replication fork, challenged in *BRCA1/2*-deficient tumours, have emerged [65]. Indeed, *BRCA1/2* are involved in the protection of the replication fork. *BRCA1/2*-deficient cells have less replication fork stability during S-phase, associated with greater chemosensitivity. Conversely, these tumour cells may develop alternative mechanisms to limit replication fork degradation, leading to PARPi resistance. DNA fibre assays measure the dynamics of the replication fork by incorporating two labelled thymidine analogues sequentially into a cell culture medium. The labelled DNA is then extracted and observed under a fluorescence microscope. Depending on the labelling of the DNA strands obtained, their length and their position on the replication fork, it provides information on the fork stability or degradation and its speed of movement [66,67]. At present, only a few studies assessing the correlation between the state of replication fork and response to PARPi have been published. The team of Hill *et al.* performed these tests on organoids from patient ovarian tumours. Of 11 organoid cultures with a stable replication fork, all were resistant to Olaparib. However, of the 17 organoids with replication fork instability, only 2 showed sensitivity to Olaparib, arguing against a correlation between fork instability and PARPi sensitivity [68].

## 5 HRD testing: an ongoing process

PARPi have significantly improved the management of ovarian cancers and are becoming increasingly crucial in breast, prostate and pancreatic cancers. Many tests are being developed to predict cancer HRD phenotype and better select patients likely to benefit from PARPi.

Initially based on the detection of *BRCA1/2* mutations, HRD tests have evolved with the development of HRR genes sequencing panels since HR deficiency may be due to defects in other HRR genes. However, as recently illustrated by Pujade-Lauraine *et al.* [30], none of

them was associated with a benefit from PARPi. While somatic and germline mutations in *BRCA1/2* are associated with a real benefit of PARPi in patients, the role of HRR gene panels remains unclear. In Europe, these extensive HRR gene panels are not approved to guide PARPi prescription.

More global approaches have emerged to overcome the difficulties of analysing each gene individually. Genomic scars and mutational signature assays capture genome-wide chromosomal abnormalities and mutations resulting from the genomic instability generated by HRD. Genomic scars have thus allowed the use of PARPi to be extended to a larger number of patients. HRD, as measured by *BRCA* mutation or genomic instability score (GIS), is required to introduce first-line maintenance therapy Olaparib in combination with Bevacizumab in ovarian cancer. However, these tests detect past events, not necessarily reflecting the tumour HRD status at the time of treatment. These “indelible” marks are useful in first-line treatment. However, they could lead to a wrong conclusion about the benefit of treatment if an event restoring HR during tumour progression has occurred under the pressure of several lines of chemotherapy. Therefore, it is imperative to develop tests that best reflect the tumour HRD phenotype in real-time.

Functional tests are on an increasing trend and attempt to address this issue. The detection of RAD51 foci enables the analysis of whether HR occurs in tumour cells at a given time. Progress made in developing these assays, which allow the analysis of FFPE tumour tissue without prior damage induction, makes this approach applicable in routine use and gives rise to real hope. However, the currently reported protocols are time-consuming and sometimes difficult to interpret. They require multiple immunofluorescence slides to identify the HRD status, leading to difficulties in their routine use. Moreover, it is essential to compare these tests to the actual gold standard (*BRCA1/2*, GIS) in clinical trials to better characterise their predictive value and place in the care pathway. Each of these tests thus provides elements to determine the HRD phenotype of a tumour, although no single test is perfect. The combination of several of these tests could provide a better predictive value and needs to be deepened, keeping in mind that the set of results must be compatible with the time to initiate treatment.

Many paths still need to be explored regarding HRD testing, which is becoming essential in a broad range of solid tumours.

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Illustration captions:

### **Figure 1: Detection of HRD through different approaches**

HRD causes: identifying the cause of HRD through the study of HR genes (mutations, epigenetic changes); HRD consequences: identifying the consequences of HRD by measuring genomic instability marks (genomic scars, mutational signatures); HR efficiency: measuring the activity of the HR itself (functional tests such as RAD51 foci assays).

### **Figure 2: Genomic scars associated with HRD**

Three types of genomic scars were described in 2012 as markers of BRCA1/2 deficiency. These three scars result in an allelic imbalance measurable in SNP-array or through whole genome sequencing of SNPs. LOH is defined by a loss of material greater than 15Mb but less than the whole chromosome. TAI corresponds to an allelic imbalance at the telomeric ends. LSTs include large rearrangements greater than 10Mb such as duplications, deletions, transitions or interchromosomal translocations. Created with Biorender.

### **Figure 3: Main mechanisms of resistance to PARPi**

1) Restoration of homologous recombination - a. HRD cells: Mutation (red star) of BRCA1 resulting in loss of function. HRP cells: Secondary deletion (blue line) eliminating the initial mutation and restoring the reading frame, resulting in a functional protein. b. HRD cells: 53BP1 expression inhibits the recruitment of nucleases required for homologous recombination and points towards NHEJ repair. Conversely, in HRP cells, there is a decrease in 53BP1 expression, favouring homologous recombination. c. HRD cells: methylation of the BRCA1 promoter preventing expression of the protein. HRP cells: demethylation of the promoter and BRCA1 re-expressed. 2) Protection of the replication fork - ATR and CHEK1 allow the recruitment of proteins involved in the stabilisation of the replication fork. In parallel, other proteins stabilise the RAD51 nucleofilament and prevent the recruitment of nucleases to limit fork degradation. 3) Other mechanisms of resistance to PARPi have been described such as depletion of the poly (ADP-ribose) glycohydrolase (PARG) enzyme that catabolises PARP, increased efflux by P-glycoproteins or mutations in PARP making PARP-trapping impossible. Created with Biorender.

**Table 1: Clinical trials in which HRD status was assessed by genomic scars assays**

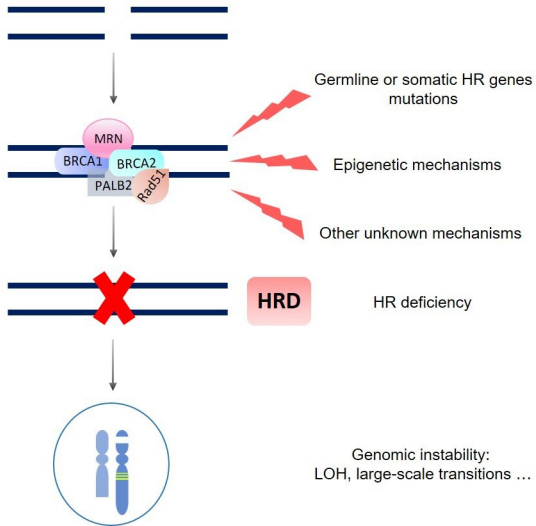
	Clinical trial	PARPi	Patients	HRD test	Results (PFS)
First line maintenance therapy	<b>PRIMA</b>	Niraparib	-Newly diagnosed HGSOC -Complete or partial response to first line platinum chemotherapy	Myriad Genetics myChoice® HRD  HRD+ : tBRCA and/or GIS ≥ 42	- <b>ITT population</b> : 13.8 vs 8.2 months ( <i>HR</i> 0.62 95%CI 0.50-0.76 ; p<0.0001) - <b>HRD+</b> ( <i>n</i> =373): 21.9 vs 10.4 months ( <i>HR</i> 0.43 95%CI 0.31-0.59 ; p<0.0001)  - <b>tBRCA</b> ( <i>n</i> =223): 22.1 vs 10.9 months ( <i>HR</i> 0.40 95%CI 0.27-0.62) - <b>wtBRCA GIS+</b> ( <i>n</i> =150): 19.6 vs 8.2 months ( <i>HR</i> 0.50 95%CI 0.31-0.83) - <b>wtBRCA GIS-</b> ( <i>n</i> =350): 8.1 vs 5.4 months ( <i>HR</i> 0.68 95%CI 0.49-0.94)
	<b>PAOLA-1</b>	Olaparib	-Newly diagnosed HGSOC -Complete or partial response to first line platinum chemotherapy + Bevacizumab	Myriad Genetics myChoice® HRD  HRD+ : tBRCA or GIS ≥ 42	- <b>ITT population</b> : 22.1 vs 16.6 months ( <i>HR</i> 0.59 - 95%CI 0.49-0.72)  - <b>HRD+</b> ( <i>n</i> =387): 37.2 vs 17.7 months ( <i>HR</i> 0.33 - 95%CI 0.25-0.45) - <b>wtBRCA GIS+</b> ( <i>n</i> =150): 28.1 vs 16.6 months ( <i>HR</i> 0.43 95%CI 0.28-0.66) - <b>HRP or unknown</b> ( <i>n</i> =419) : 16.9 vs 16 months ( <i>HR</i> 0.92 95%CI 0.72-1.17) - <b>HRP</b> ( <i>n</i> =277): 16.6 vs 16.2 months ( <i>HR</i> 1.00 95%CI 0.75-1.35)

<b>NOVA</b>	Niraparib	-Recurrent platinum-sensitive HGSOC -After ≥ 2 lines of platinum based chemotherapy	Myriad Genetics myChoice® HRD  GIS+ : ≥ 42	- <b>gBRCA</b> : 21.0 vs 5.5 months ( <i>HR</i> 0.27 95%CI 0.173-0.410 ; p<0.0001) - <b>non-gBRCA</b> : 9.3 vs 3.9 months ( <i>HR</i> 0.45 95%CI 0.338-0.607 ; p<0.0001) - <b>non-gBRCA GIS+</b> : 12.9 vs 3.8 months ( <i>HR</i> 0.38 95%CI 0.24-0.59)  - <b>wtBRCA GIS+</b> ( <i>n</i> =115): 9.3 vs 3.7 months ( <i>HR</i> 0.38 95%CI 0.23-0.63) - <b>wtBRCA GIS-</b> ( <i>n</i> =134): 6.9 vs 3.8 months ( <i>HR</i> 0.58 95%CI 0.36-0.92)
<b>QUADRA</b>	Niraparib	-Recurrent HGSOC -After ≥ 3 lines of platinum based chemotherapy	Myriad Genetics myChoice® HRD	NA
<b>ARIEL2 part 1</b>	Rucaparib	-Recurrent platinum-sensitive HGSOC -After ≥ 1 line of platinum based chemotherapy	Foundation Medicine T5 NGS*  LOH high: ≥ 14%	- <b>tBRCA</b> ( <i>n</i> =40): 12.8 months (95%CI 9.0-14.7) - <b>wtBRCA-LOH high</b> ( <i>n</i> =82): 5.7 months (95%CI 5.3-7.6) - <b>wtBRCA-LOH low</b> ( <i>n</i> =70): 5.2 months (95%CI 3.6-5.5)
<b>ARIEL3</b>	Rucaparib	-Recurrent platinum-sensitive HGSOC -After ≥ 2 lines of platinum based chemotherapy	Foundation Medicine T5 NGS*  HRD+ : tBRCA or LOH ≥ 16%	- <b>ITT population</b> : 10.8 vs 5.4 months ( <i>HR</i> 0.36 95%CI 0.30-0.45 ; p <0.0001) - <b>tBRCA</b> ( <i>n</i> =196): 16.6 vs 5.4 months ( <i>HR</i> 0.23 - 95%CI 0.16-0.34) - <b>HRD+</b> ( <i>n</i> =354): 13.6 vs 5.4 months ( <i>HR</i> 0.32 95%CI 0.24-0.42 ; p<0.0001)  - <b>wtBRCA-LOH high</b> ( <i>n</i> =158): 9.7 vs 5.4 months ( <i>HR</i> 0.44 95%CI 0.29-0.66) - <b>wtBRCA-LOH low</b> ( <i>n</i> =161): 6.7 vs 5.4 months ( <i>HR</i> 0.58 95%CI 0.4-0.8)

In purple: primary endpoint, In black: exploratory analyses in predefined subgroups, In blue: post-hoc analyses

\* Test developed for research use (non commercial)

HGSOC: high-grade serous ovarian cancer, PFS: progression free survival, HR: hazard ratio, *gBRCA*: *BRCA* germline mutation, *tBRCA*: *BRCA* tumour mutation, *wtBRCA*: unmutated *BRCA*, HRD+: having a homologous recombination deficiency, LOH high/low: loss of heterozygosity high/low, NA: not applicable



## HRD causes

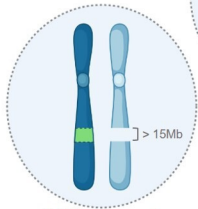
→ individual or panel genes study

## HR efficiency

→ fonctionnal study

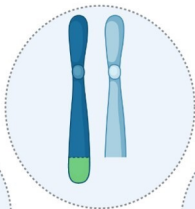
## HRD consequences

→ genomic scars, mutational signatures



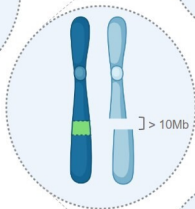
**LOH : Loss of heterozygosity**

Loss of one of the two alleles of a gene

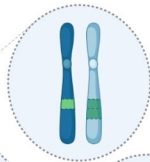


**TAI : Telomeric allelic imbalance**

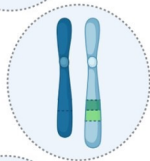
Loss of one of the two alleles in the telomeric region



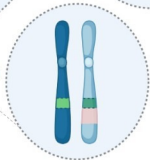
**LST : Large scale transition**



Duplication



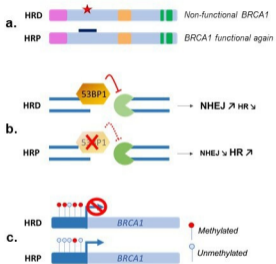
Transition



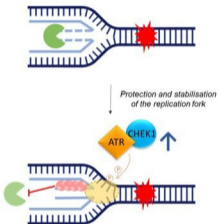
Interchromosomal translocation



### 1) HR restoration



### 2) Protection of the replication fork



### 3) Other mechanisms

