RAD51 as biomarker for the identification of homologous recombination deficient gynaecological carcinomas

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Chapter 2

RAD51 as a functional biomarker for homologous recombination deficiency in cancer: a promising addition to the HRD toolbox?

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Abstract

Carcinomas with defects in the homologous recombination (HR) pathway are sensitive to PARP inhibitors (PARPi). A robust method to identify HR-deficient (HRD) carcinomas is therefore of utmost clinical importance. Currently available DNA-based HRD tests either scan HR-related genes such as *BRCA1* and *BRCA2* for the presence of pathogenic variants or identify HRD-related genomic scars or mutational signatures by using whole-exome or whole-genome sequencing data. As an alternative to DNA-based tests, functional HRD tests have been developed that assess the actual ability of tumors to form HR intermediates by quantifying for instance their ability to accumulate RAD51 protein at DNA double-strand breaks. Clinical implementation of a RAD51-based HRD test to identify *BRCA1/2*-related and non-*BRCA1/2*-related HRD tumors can assist in patient selection for PARPi treatment. Here, we present an overview of currently available HRD tests and discuss the pros and cons of the different methodologies including their sensitivity for the identification of HRD tumors, their concordance with other HRD tests, and their capacity to predict therapy response.

Keywords: BRCA1; BRCA2; genomic scars; homologous recombination deficiency; mutational signatures; RAD51; RAD51-FFPE test; RECAP test

1. Introduction

The BRCA1 and BRCA2 proteins play crucial roles in homologous recombination (HR), the major DNA damage repair (DDR) pathway for error-free repair of DNA double-strand breaks (DSBs). The presence of germline pathogenic variants (PV) in the breast cancer susceptibility genes *BRCA1* (OMIM: 604370) and *BRCA2* (OMIM: 600185) is associated with an increased risk for breast and ovarian cancer [1]. Emerging evidence indicates that not only defects in *BRCA1/2*, but also in other HR-related genes can lead to HR-deficiency (HRD) [2]. Additionally, HRD is also observed in other cancers including prostate and pancreatic cancer. HRD tumors are particularly sensitive to platinum-based chemotherapy and poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi) [3]. Therefore, there is great clinical interest in tests that can identify HRD tumors. In this review, we provide an overview of the currently available HRD tests, including both DNA-based and RAD51-based functional HRD tests.

1.1 Homologous recombination

A proliferating cell accumulates approximately 50 DNA DSBs per day from endogenous sources (e.g. oxidative damage, replication fork collapse, and telomere erosion) [4-7]. In addition, DNA DSBs can be induced by exogenous sources (e.g. ionizing radiation and chemotherapeutic agents). Various repair systems have evolved to deal with DSBs including non-homologous end joining (NHEJ), theta-mediated end joining (TMEJ), and homology directed repair (HDR) [8,9]. DSB repair via NHEJ, which is active during the whole cell cycle, can lead to the introduction of single nucleotide insertions and/or deletions (indels) [9]. TMEJ is active post-replication in cases where the sister chromatid cannot serve as a template. DSB repair via this pathway can result in deletions with microhomology and templated insertions [8]. During the late S/G2 phase of the cell cycle when a sister chromatid is available that may serve as repair template, DSBs can be error-free repaired by HDR [10]. Repair of DSBs is initiated by the binding of the Mre11-Rad50-Nbs1 (MRN) complex, which activates ataxia telangiectasia mutated (ATM) protein kinase causing the phosphorylation of the histone variant H2AX at Serine 139 in the vicinity of the break [11,12]. In the case of HR, accumulation of phosphorylated H2AX (γH2AX) at the site of a DSB and inhibition of 53BP1 by BRCA1 initiates the co-localization of the MRN complex, BRCA1, and CtBP-interacting protein (CtIP) promoting 5' to 3' DNA end resection and end processing [10]. The resected, single-strand DNA (ssDNA) ends are coated by Replication Protein A (RPA) to protect the strands from degradation. The repair process is continued with the phosphorylation of the BRCA1 protein, which forms a protein complex with PALB2 to recruit BRCA2 to the ssDNA ends. Subsequently, RPA is replaced by the RAD51 DNA recombinase through its interaction with BRCA2 to form RAD51-ssDNA nucleofilaments. These RAD51-ssDNA nucleofilaments interact with dsDNA to scan for

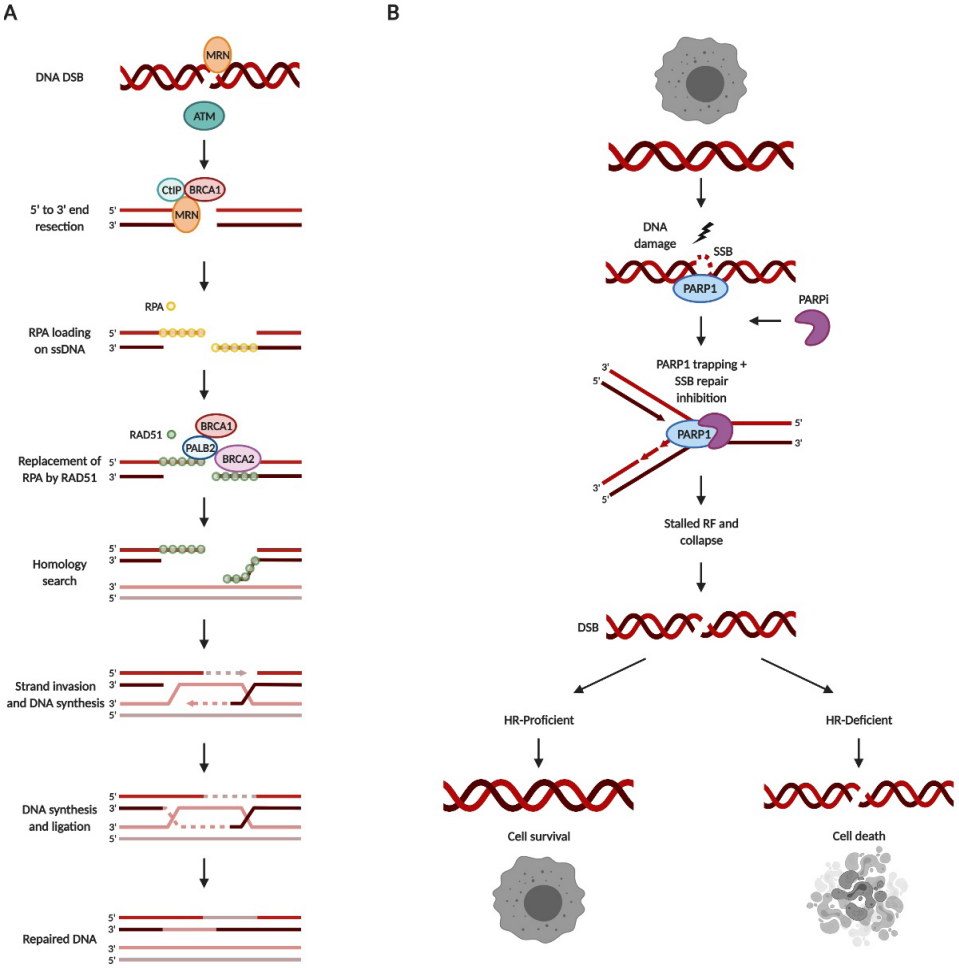

Figure 1. Homologous recombination (HR) and the mechanism of action of PARP inhibitors (PARPi). A) Simplified scheme of the HR pathway [5,9,10]. Double-strand breaks (DSBs) are recognized by the MRN complex, followed by the activation of ATM and the recruitment of BRCA1 and exonucleases to promote 5′ to 3′ end resection. RPA protects the single-strand DNA (ssDNA) overhangs from degradation and is replaced by RAD51 to form a RAD51 nucleoprotein filament, a process supported by BRCA1, BRCA2, and PALB2. The RAD51 filament invades the sister chromatid to search for the homologous sequence. After the formation of a displacement loop, lost genetic information is copied from the homologous template strand to allow error-free repair. B) Simplified scheme of the mechanism of action of PARPi [15,16]. PARPi inhibit PARP1, an enzyme involved in single-strand break (SSB) repair. The inhibition of SSB repair through PARP1 trapping may lead to stalling and collapse of replication forks (RFs), resulting in the generation of relatively high quantities of DSBs. Accumulation of unrepaired DSBs in HR-Deficient cells after treatment with PARPi will subsequently lead to cell death. Created with BioRender.com.

sequence complementarity. In a still poorly understood fashion, strand invasion and the formation of Holliday junctions occurs, a process that is promoted by the BRCA1-BARD1 complex, and DSBs are error-free repaired using the homologous sequence of the sister chromatid as template for DNA synthesis (Figure 1A) [9,13]. The inability of cells to perform HR may lead to the formation of genomic aberrations, genomic instability, and ultimately cancer [14].

1.2 Targeting HRD cells with PARPi

In 2005, the first reports appeared that described the high sensitivity of HRD cells for PARPi [15,16]. PARP1 is essential in the repair of DNA single-strand breaks (SSBs) as it fulfills a crucial function in the base excision repair (BER) pathway. PARPi obstruct the catalytic activity of PARPs and trap PARP on sites of DNA damage [17]. Consequently, replication fork (RF) stalling and collapse may occur, eventually leading to the generation of DSBs from unresolved SSBs [17]. In the absence of HR, the repair of DSBs solely depends on error-prone repair pathways, such as NHEJ and TMEJ, leading to the accumulation of mutations and unrepaired breaks, and eventually cell death (Figure 1B) [18]. The synthetic lethality between *BRCA1/2* deficiency and PARPi has not only been shown *in vitro* [19], but also *in vivo* using *BRCA1/2* deficient mammary tumor-bearing mouse models [20] and *ex vivo* using *BRCA1/2* deficient mouse mammary tumor organoids [21].

1.3 Approved PARP inhibitors

The promising *in vivo* mouse experiments showing effective treatment of *BRCA1/2* deficient tumors with PARPi led in 2012 to the first clinical trial investigating the efficacy of PARPi treatment in relapsed ovarian cancer (OC) patients [22]. This study was soon followed by several other studies investigating treatment response of various PARPi in diverse patient groups, including ovarian [22-33], breast [34-37], pancreatic [38], and prostate cancer patients [39,40], all indicating improved progression-free survival (PFS) of patients treated with PARPi compared to patients treated with placebo. Interestingly, the highest clinical benefit for PARPi was observed among *BRCA1/2* deficient cases, followed by non-*BRCA1/2* deficient HRD cases as determined by the MyChoice® companion diagnostic (CDx) test (Section 2.2) [23,24,41,42]. Based on these clinical studies, the Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved four different PARPi in the last seven years (Figure 2). Olaparib, rucaparib, and niraparib have been approved as maintenance treatment for advanced platinum-sensitive OC patients [23-26,28,29,31,43]. Importantly, olaparib and niraparib are also approved as first-line maintenance treatment in advanced platinum-sensitive OC patients [30,32]. In the meantime, olaparib and talazoparib became available for advanced HER2-negative,

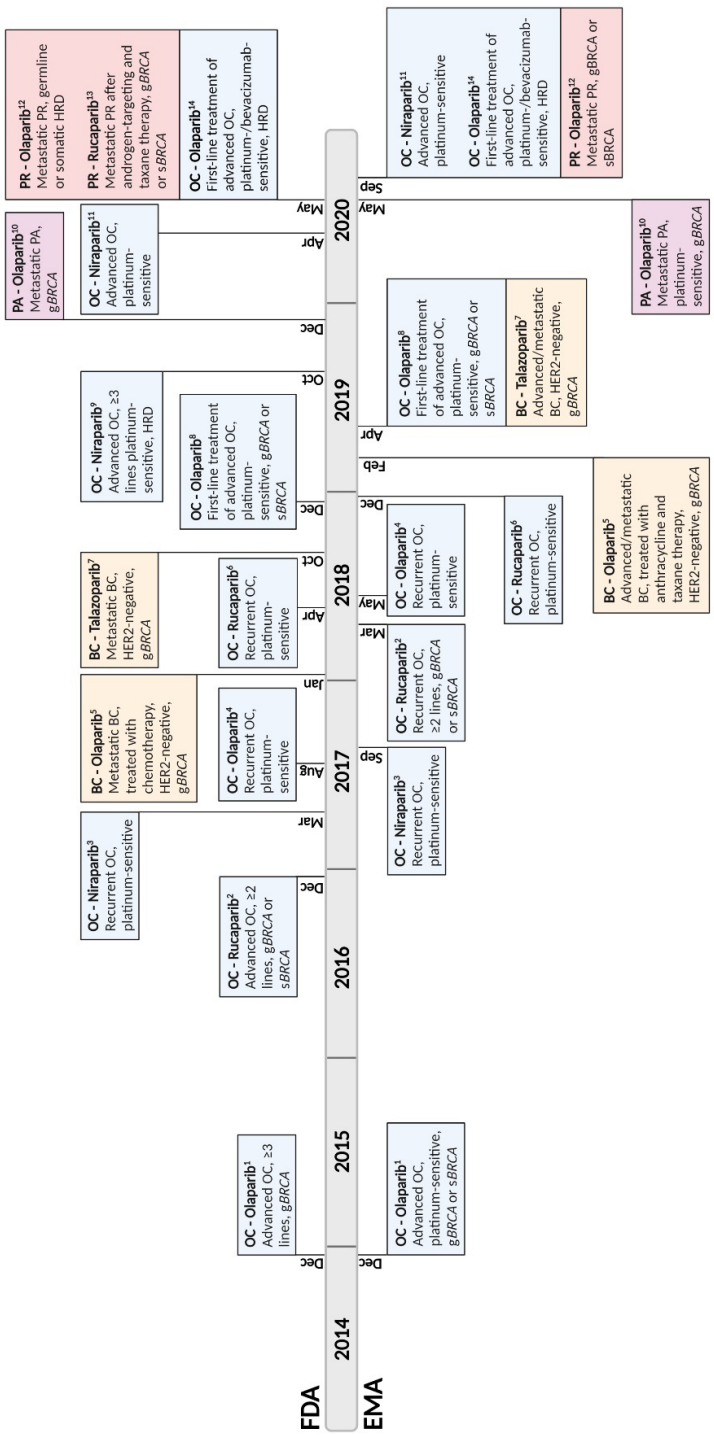

Figure 2. PARP inhibitor approvals by the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) for ovarian, breast, prostate, and pancreatic cancer. FDA approved PARPi are presented above the timeline, EMA approved PARPi are presented below the timeline. Cancer type, approved PARPi and corresponding clinical trials (numbers) on which the approval was based are indicated in bold. Disease characteristics are described for each PARPi approval. Approvals were based on the following clinical trials: 1. NCT01078662 [43]; 2. Study 10 and ARIEL2 [25]; 3. ENGOT-OV16/NOVA [23]; 4. SOLO-2 and Study19 [26,28]; 5. OlympiAD [34]; 6. ARIEL3 [24]; 7. EMBRACA [35]; 8. SOLO-1 [29]; 9. QUADRA [31]; 10. POLO [38]; 11. PRIMA [30]; 12. PROfound [40]; 13. TRITON2 [39]; 14. PAOLA-1 [32]. Abbreviations: OC = ovarian cancer (blue); BC = breast cancer (yellow); PA = pancreatic cancer (purple); PR = prostate cancer (red); X lines = platinum-based chemotherapy treatment; g*BRCA* = germline *BRCA* PV; s*BRCA* = somatic *BRCA* PV. Created with BioRender.com.

germline *BRCA* (*gBRCA*) breast cancer (BC) patients [34,35]. The group of cancer patients eligible for PARPi treatment quickly expanded, now also including g*BRCA* pancreatic cancer patients and patients with advanced prostate cancer harboring a *BRCA* PV (germline or somatic) or showing HRD, as olaparib or rucaparib treatment led to antitumor activity and/or improved PFS [38-40]. Remarkably, in all of the aforementioned studies, PARPi benefit was not only observed among patients with *BRCA* PV or HRD tumors, but also in patients with HR-Proficient (HRP) tumors, as determined with the MyChoice® CDx or FoundationOne® CDx test (section 2.1 and 2.2). These findings raise the question if the current patient selection procedures are sufficiently accurate to identify the true responders to PARPi. The development and clinical validation of informative HRD tests are therefore of utmost importance to improve patient selection for PARPi treatment.

2. DNA-based HRD tests

Next-generation sequencing (NGS) of DNA to identify PV in HR related genes, including *BRCA1* and *BRCA2,* is currently the gold standard for the identification of patients eligible for PARPi treatment. Alternative methods to identify HRD tumors based on the patterns of mutations that arise during tumorigenesis in the absence of HR have been developed as well. In this section, we summarize the methodology and clinical application of DNA-based HRD tests.

2.1 Gene panel sequencing and methylation analysis of HR-related genes

The simplest DNA-based method to identify HRD tumors is to screen for PVs (either germline or somatic) in the *BRCA1/2* genes (Figure 3), as used in the FDA approved CDx tests BRACAnalysis® (germline) and FoundationFocus™ BRCA (germline/somatic) [44]. Several prospective clinical trials showed that platinum-sensitive OC patients with a *BRCA* PV (either germline or somatic) derive the greatest clinical benefit from PARPi treatment in both the first-line and recurrent setting [23,24,26,27,29,30,32,45,46]. Although these studies showed the robustness of *BRCA1/2* deficiency as positive predictor of PARPi response in OC patients, the negative predictive value was generally poor, as some *BRCA* wild-type (wt*BRCA*) patients also benefitted from PARPi treatment. Follow-up studies revealed a more complex genetic landscape of HRD, extending beyond *BRCA1/2* PVs [1,47-49]. Analysis of 102 HR-related genes in 8178 TCGA-registered tumors of different origins, among which ovarian, breast, and prostate cancers, showed that 13% of tumors have at least one PV (with simultaneous loss of heterozygosity (LOH) of the wildtype allele) in one of these genes; of which the *BRCA1/2* genes were most frequently affected, followed by *CHEK2*, *PALB2*, *RAD51C* and *RAD51D* [50]. Several pre-clinical studies indicated that deficiencies in the Fanconi's anemia genes, and also in other DNA repair genes such as

ATM and *CDK12* lead to platinum/PARPi sensitivity *in vitro* [47,50-52]. Clinical validation of these findings is challenging as the occurrence of PVs in non-*BRCA* HR-related genes is relatively rare. However, the GOG-0218 trial showed an improved PFS and overall survival (OS) to PARPi treatment for OC patients with PVs in non-*BRCA* HR-related genes compared to patients who did not harbor PVs in *BRCA* or HR-related genes [53]. Similarly, patients with prostate cancer harboring PVs in *ATM* or *PALB2* also had an improved PFS with PARPi treatment over conventional therapy. The clinical benefit was, however, lower than for

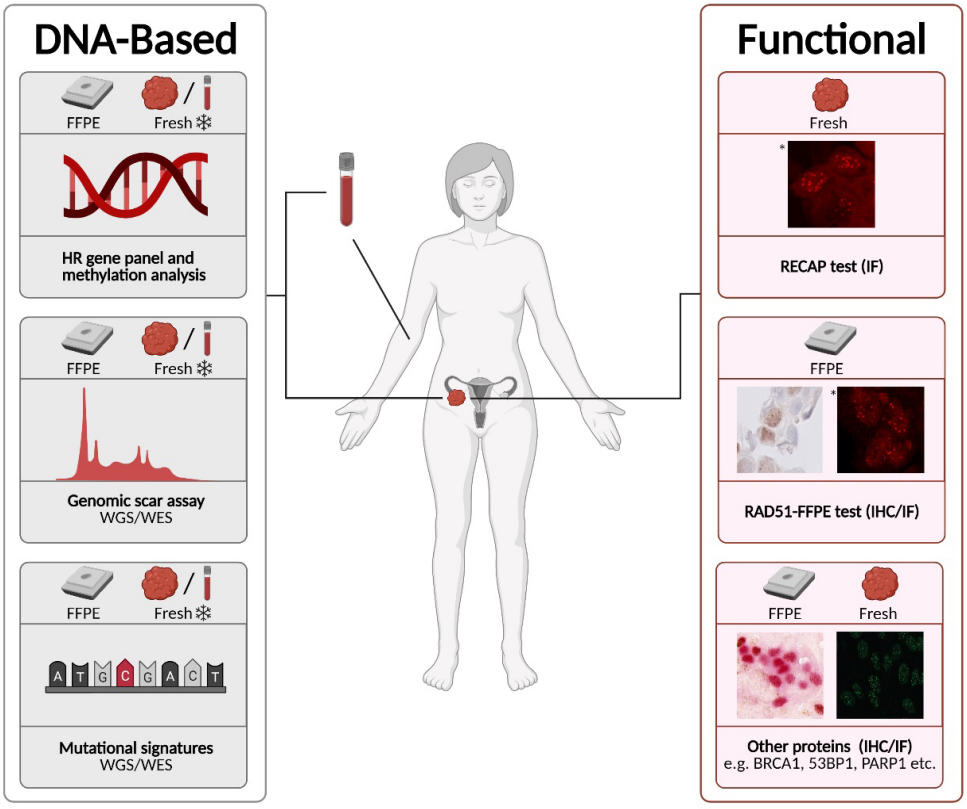

Figure 3. DNA-based and functional HRD tests. DNA-based HRD tests (left) include HR gene panel and methylation analysis, genomic scar analysis, and mutational signature analysis. DNA is isolated from FFPE tissue, fresh frozen tumor tissue, and liquid biopsies (whole blood samples). Functional HRD tests (right) include the RECAP test, the RAD51-FFPE test, and the quantification of other proteins via IF/IHC analysis. FFPE tissue or fresh (cryopreserved) tumor tissue is required for the various tests. * Adapted from van Wijk *et al.* [62]. Abbreviations: HR = homologous recombination; FFPE = formalin-fixed paraffin-embedded; WGS = whole-genome sequencing; WES = whole-exome sequencing; RECAP =REcombination CAPacity; IHC = immunohistochemistry; IF = immunofluorescence. Created with BioRender. com.

patients harboring a *BRCA1/2* PV [40,54,55]. Currently, two CDx tests (FoundationOne® and FoundationOne® Liquid) are available that screen for PVs in over 300 HR-related genes (germline/somatic) [44]. They do not, however, capture HRD tumors that may arise from epigenetic alterations, such as *BRCA1* and *RAD51C* promoter hypermethylation (Figure 3) [48,56,57]. *BRCA1* and *RAD51C* promoter hypermethylation has been associated with rucaparib response in OC patients [27,58], although other studies did not find a correlation between platinum/PARPi response and the presence of *BRCA1* or *RAD51C* promoter hypermethylation [57,59,60]. A possible explanation for these conflicting results is of technical nature, as it became clear that both copies of *BRCA1* must be methylated to affect BRCA1 function, which is not taken into account in most clinical studies evaluating epigenetic alterations as biomarker for HRD [61].

2.2 Genomic scars

Due to the absence of HR and the dependency on error-prone repair pathways, HRD tumors show characteristic patterns of chromosomal rearrangements also known as 'genomic scars' [49]. These HRD specific genomic scars can be used to identify HRD tumors (Figure 3).

2.2.1 Array comparative genomic hybridization analysis

As a consequence of HRD, copy number variants (CNVs) may be induced that can be detected by array comparative genomic hybridization (aCGH). Based on genomic data of BC samples harboring PVs in *BRCA1/2*, classifiers were developed allowing identification of *BRCA1*- and *BRCA2*-like (*BRCA*-like^{CGH}) BC tumors of patients who were diagnosed as non-*BRCA1/2* carriers [63,64]. Recently, an alternative classifier has been developed that requires less formalin-fixed paraffin-embedded (FFPE)-derived DNA and uses multiple-ligation-dependent probe amplification (MPLA) data to identify CNVs. This 'digitalMLPA' assay was able to accurately detect *BRCA1*- and *BRCA2*-like patterns in BC samples [65].

2.2.2 SNP-array analysis

Single nucleotide polymorphism (SNP)-array analysis represents an alternative approach to identify and characterize chromosomal rearrangements. Three distinct types of genomic scars can be distinguished, i.e. telomeric allelic imbalance (TAI) [66], loss of heterozygosity (LOH) [67], and large-scale transition (LST) [68]. Whereas each of these genomic scars has been validated using tumor cohorts of patients harboring *BRCA1/2* PVs, only genomic LOH was prospectively evaluated for PARPi benefit in OC patients in phase II and III ARIEL trials (ARIEL2 and ARIEL3) [24,27]. Both the ARIEL2 and ARIEL3 trials showed that patients in the LOHhigh group (>14% genomic LOH) displayed an increased PFS after

treatment with rucaparib compared to patients in the LOHlow group [24,27]. Importantly, rucaparib treatment benefit was also observed in some patients with LOHlow scores, indicating that the predictive value of genomic LOH was insufficiently discriminative [24,27]. A combination of TAI, LOH, and LST scores generated a higher association with *BRCA1/2* deficiency compared to the individual scores [69,70]. The MyChoice® CDx test, which is based on such a combination score, was used in several clinical trials to evaluate the predictive value of genomic scar HRD testing regarding PARPi or platinum treatment benefit in OC patients [42]. Most of these trials, including the NOVA, ARIEL3, and Study19 trials, indicated that the highest clinical benefits for PARPi and platinum-based chemotherapy was observed among *BRCA1/2* deficient HRD cases, followed by non-*BRCA1/2* deficient HRD and *BRCA1/2*-wildtype non-HRD cases [23,24,41,42]. In two studies that evaluated the association between HR status and platinum-based therapy response in BC patients, the MyChoice® CDx test failed to discriminate between responders and non-responders [36,71]. In the above mentioned studies, PARPi and platinum benefit were frequently observed among patients without *BRCA1/2* deficiency and with a MyChoice® HRD-negative score, indicating that patient selection is still suboptimal.

2.3 Mutational signatures

HRD tumors rely for the repair of DSBs solely on error-prone pathways, which results in the relatively frequent introduction of specific types of mutations, such as specific base substitutions or deletions with microhomology [8,72]. The presence of these specific types of passenger mutations or so called 'mutational signatures' can serve as a biomarker for HRD (Figure 3). Whole-genome sequencing (WGS) is used to obtain the mutational spectrum of a given tumor which can be categorized into specific signatures using bioinformatic analysis [72]. Base substitution "COSMIC signature 3" for example, is strongly associated with the presence of large insertions and deletions and is the main signature found in somatic and germline *BRCA1/2* deficient OC and BC [72]. *SignatureAnalyzer* [73], HRDetect [74], and Signature Multivariate Analysis (SigMA) [75] are algorithms that can be used to quantify the presence of various mutational signatures in tumor sequence data in order to determine the likelihood of a tumor to be HRD. *SignatureAnalyzer* was applied on a cohort of 995 invasive BC samples and successfully identified 88% (88/100) of the *BRCA1/2* BC cases based on COSMIC signature 3. In a recent study, *SignatureAnalyzer* was validated on a cohort of 981 BC from the TCGA cohort with known MyChoice® HRD scores [76]. Of 92 samples with a MyChoice® HRD score, nine did not have COSMIC signature 3, including three with a *BRCA1/2* PV, indicating that *SignatureAnalyzer* was not 100% sensitive for the identification of tumors with a *BRCA1/2* PV and MyChoice® HRD score [76]. In addition to COSMIC signature 3, COSMIC signature 8 is also frequently observed among tumors with *BRCA1/BRCA2* PVs [77]. HRDetect incorporates COSMIC signature 3 and

8, but also the number of deletions with microhomology, rearrangement signatures (RS3, RS5), and an HRD index (calculated as the sum of TAI, LOH, and LST) to generate a weighted HRD score [74]. The test identified *BRCA1/2* deficiency (germline and/or somatic) with a sensitivity of 99% in a cohort of 560 triple-negative BC (TNBC) samples [74]. Additionally, it allowed identification of HRD TNBC tumors beyond *BRCA1/2* deficiency [74,78]. A recently developed computational tool called Signature Multivariate Analysis (SigMA) can identify the presence of COSMIC signature 3 on targeted gene panel data and does not, in contrast to SignatureAnalyzer and HRDetect, require WGS or whole-exome sequencing (WES) data [75]. Although using targeted gene panel data over WGS or WES data would be beneficial, the sensitivity of SigMA for the identification of COSMIC signature 3-positive tumors is only 74% [75]. It is still unclear if mutational signature-based assays can improve patient selection for the treatment with PARPi and/or platinum-based chemotherapy.

3. RAD51-based functional HRD tests

As an alternative to DNA-based HRD tests, functional tests that assess the ability of replicating tumor cells to perform HR have been developed for use in breast, ovarian and endometrial cancer [79-83]. These so called RAD51-based HRD tests measure RAD51 protein accumulation at single-strand overhangs formed during repair of DSBs via HR (Fig 1A), which can be visualized as foci by immunofluorescence (IF) or immunohistochemistry (IHC) staining (Figure 3). The quantification of RAD51 foci is a powerful tool to identify HRD tumors (absence of RAD51 foci in proliferating cells) and retrospective clinical validation studies show promising results for RAD51 as biomarker for PARPi and/or platinum response. The detection of additional proteins like e.g. 53BP1 might be informative as well (Figure 3), as 53BP1 deficiency with RAD51 proficiency was an indicator for a poor PFS and OS in platinum-treated OC patients [84].

The use of RAD51 foci as a functional readout for HR in tumor tissues was first described in 2009 [85] (Table 1). Not long after, three studies described a method to evaluate RAD51 foci in fresh tumor biopsies from BC patients [85-87], with the main message that tumors with a low fraction of RAD51-positive ($RAD51^+$) cells responded better to platinum-based chemotherapy than tumors with a high fraction of $RAD51^+$ cells (Table 1). This first proof of concept initiated several studies that evaluated the use of a RAD51-based functional HRD test in a variety of tumors including breast [79,80,85-96], ovary [81,83,88,89,91,97-101], pancreas [88,89,102], endometrium [82], prostate [89], lung [91,103], head and neck [104], mesothelioma [91], colon [89] and osteosarcoma [89] (Table 1). In addition, different specimen types have been used for the analysis of RAD51 foci: cell lines, organoids, needle biopsies, ascites and solid tumor tissue samples (Table 1). Several RAD51-based functional HRD tests have been developed that differ in the applied DNA damaging agent (ionizing radiation, chemotherapy, PARPi, or none), the proliferation marker used (geminin, cyclin A2 or Ki67), and the number of cells counted to calculate a RAD51 score (Table 1).

Table 1. Overview RAD51-based functional HRD tests. Abbreviations: IF = immunofluorescence; IHC = immunohistochemistry; IR = irradiation; HR = homologous recombination; HRD = homologous recombination deficiency; PARPi = PARP inhibitor; % = percentage; PDX = patient-derived xenograft; NA = not applicable.

Publication	Tumor type	Specimen type	IF/ IHC	DNA damage induction	Time to fixation	RAD51 foci cutoff	HRD threshold	Prolifera-tion marker	Minimal number of cells scored	HRD score calculation	HRD score correlation to PARPi/Platinum sensitivity	Treat-ment model
Sakai *et al.*, 2008 [102]	Pancreas	Cell lines (*n* = 14)	IF	15 Gy IR	12 h	5	None	None	100	Increase in $RAD51^+$ /Total cells	Yes (platinum sensitivity)	Cell line
Willers *et al.*, 2009 [85]	Breast	Fresh pre-treatment biopsies (*n* = 7)	IF	8 Gy IR	4 h	2	None	None	20-100	Increase in $RAD51^+$ /Total cells	NA	NA
Drew *et al.*, 2010 [88]	Breast, ovarian, pancreas	Cell lines (*n* = 9)	IF	10µM Rucaparib	24 h	Unknown	None	None	30	Increase in $RAD51^+$ /Total cells	Yes (rucaparib sensitivity)	PDX
Asakawa *et al.*, 2010 [86]	Breast	Fresh pre- and post-treatment biopsies (*n* = 60)	IHC	Neoadjuvant epirubicin plus cyclophosphamid e	18-24 h	Unknown	1	None	Unknown	0 for no $RAD51^+$ cells, 1 for <10% $RAD51^+$ cells, 2 for 10-79% $RAD51^+$ cells and 3 for ≥80% $RAD51^+$ cells	Yes (tumor volume reduction/better tumor response rate)	Patients
Graeser *et al.*, 2010 [87]	Breast	Biopsies (*n* = 68)	IF	Anthracycline based chemotherapy	24 h	1	10%	Geminin	100-500	% of $RAD51^+$ /$geminin^+$ cells	Yes (complete response to chemotherapy)	Patients
Mukhopadhyay *et al.*, 2010 [97]	Ovarian	Ascites (*n* = 25)	IF	10µM Rucaparib	24 h	None	2-fold increase	None	30	Increase in RAD51 foci over untreated controls	Yes (rucaparib sensitivity)	Primary culture
Nowsheen *et al.*, 2011 [104]	Head and neck squamous carcinoma	Cell lines (*n* = 4)	IF	3 Gy IR	4, 8, 24 h	10	Unknown	None	500	% of $RAD51^+$ /total tumor cells	No (veliparib sensitivity)	Cell line
Mukhopadhyay *et al.*, 2012 [98]	Ovarian	Ascites (*n* = 50)	IF	10µM Rucaparib	24 h	None	2-fold increase	None	30	Increase in RAD51 foci over untreated controls	Yes (rucaparib sensitivity/ platinum sensitivity, lower tumor progression and higher median survival)	Primary culture and patients

Table 1 continued

Publication	Tumor type	Specimen type	IF/ IHC	DNA damage induction	Time to fixation	RAD51 foci cutoff	HRD threshold	Prolifera-tion marker	Minial number of cells scored	HRD score calculation	HRD score correlation to PARPi/Platinum sensitivity	Treat-ment model
Oplustilova *et al.*, 2012 [89]	Breast, ovary, prostate, colon, pancreas, osteosarcoma	Cell lines (*n* = 6)	IF	1μM KU 58948 (PARP1 inhibitor)	24 h	8	None	Cyclin A2	Unknown	% of RAD51$^+$ /cyclin A2$^+$	No (KU 58948 sensitivity)	Cell line
Birkelbach *et al.*, 2013 [103]	Non-small cell lung cancer	Cell lines (*n* = 16) tissue samples (*n* = 13)	IF	16μM Cisplatin/ 10μM Olaparib (cell lines); 8 μM Cisplatin/10Gy IR (tissue samples)	5 h	2	10%	None	200-400	% of RAD51$^+$ /total tumor cells	Yes (cisplatin/olaparib sensitivity)	Cell line/Tiss ue sample
Naipal *et al.*, 2014 [79]	Breast	Biopsies (*n* = 45)	IF	5 Gy IR	2 h	5	20%	Geminin	30	% of RAD51$^+$ /geminin$^+$ cells	Yes (olaparib sensitivity)	Tissue sample
Patterson *et al.*, 2014 [91]	Non-small cell lung cancer, Mesothelioma , Breast and Ovarian	Pleural effusion (*n* = 12)	IF	10μM Rucaparib	24 h	None	2-fold increase	None	50	Increase in RAD51 foci over untreated controls	No (improved OS)	Patients
Shah *et al.*, 2014 [99]	Ovarian	Cell lines (n=7) and PDX tissues (*n* = 8)	IF	4 Gy IR	8 h	10	10%	None	500	% of RAD51$^+$ /total tumor cells	Yes (veliparib sensitivity)	Cell line and PDX
Mutter *et al.*, 2017 [90]	Breast	Tissue samples (*n* = 49)	IF	10 Gy IR	4 h	5	None	Ki67	200	Increase in RAD51$^+$ /Total cells	NA	NA
Tumiati *et al.*, 2018 [81]	Ovarian	Ascites and tissue samples (*n* = 32)	IF	10 Gy IR	4, 8 and 24 h	Unknow n	20%	Cyclin A2	Unknown	% of RAD51$^+$ /CyclinA2$^+$ cells	Yes (platinum sensitivity/ longer time-to-progression, improved OS)	Patients
Castroviejo-Bermejo *et al.*, 2018 [92]	Breast	Human tissue samples (*n* = 23) and PDX (n=46)	IF	None	NA	5	10%	Geminin	40	% of RAD51$^+$ /geminin$^+$ cells	Yes (olaparib sensitivity)	PDX

Publication	Tumor type	Specimen type	IF/ IHC	DNA damage induction	Time to fixation	RAD51 foci cutoff	HRD threshold	Prolifera-tion marker	Minial number of cells scored	HRD score calculation	HRD score correlation to PARPi/Platinum sensitivity	Treat-ment model
Cruz *et al.*, 2018 [93]	Breast	Human tissue samples (*n* = 20) and PDX (*n* = 13)	IF	None	NA	5	10%	Geminin	100	% of $RAD51^{-}$ /$geminin^{+}$ cells	Yes (olaparib sensitivity)	PDX
Meijer *et al.*, 2018 [80]	Breast	Tissue samples (*n* = 125)	IF	5 Gy IR	2 h	5	20%	Geminin	30	% of $RAD51^{+}$ /$geminin^{+}$ cells	NA	NA
Hill *et al.*, 2019 [100]	Ovarian	Organoids (*n* = 33)	IHC	10 Gy IR	4 h	Unknown	Unknown	Geminin	Unknown	Detection of $RAD51^{+}$ /$geminin^{+}$ cells over multiple higher power fields	Yes (olaparib sensitivity)	Organoi ds
Jonge *et al.*, 2019 [82]	Endometrial	Tissue samples (*n* = 25)	IF	5 Gy IR	2 h	5	20%	Geminin	50	% of $RAD51^{+}$ /$geminin^{+}$ cells	NA	NA
Kopper *et al.*, 2019 [101]	Ovarian	Organoids (*n* = 6)	IF	5 Gy IR	2 h	5	20%	Geminin	50	% of $RAD51^{+}$ /$geminin^{+}$ cells	Yes (niraparib sensitivity)	Organoi ds
Meijer *et al.*, 2019 [105]	Breast	Biopsies (*n* = 41)	IF	5 Gy IR	2 h	5	20%	Geminin	30	% of $RAD51^{+}$ /$geminin^{+}$ cells	Yes (platinum sensitivity)	Patients
Parmar *et al.*, 2019 [106]	Ovarian	ascites from PDX (*n* = 14)	IHC	10 Gy IR	4 h	Unknown	Unknown	None	Unknown	Presence/absence of RAD51 foci	Yes (olaparib sensitivity)	PDX
Chopra *et al.*, 2020 [94]	Breast	Biopsies (*n* = 25)	IHC	Rucaparib	24-48h	5	20%	Geminin	30	% of $RAD51^{+}$ /$geminin^{+}$ cells	NA	NA
Eikesdal *et al.*, 2020 [95]	Breast	Biopsies (*n* = 32)	IF	None	NA	5	10%	Geminin	40	% of $RAD51^{+}$ /$geminin^{+}$ cells	Yes (olaparib sensitivity)	Patients

Table 1 continued

Publication	Tumor type	Specimen type	IF/ IHC	DNA damage induction	Time to fixation	RAD51 foci cutoff	HRD threshold	Prolifera-tion marker	Minial number of cells scored	HRD score calculation	HRD score correlation to PARPi/Platinum sensitivity	Treat-ment model
van Wijk *et al.*, 2020 [83]	Ovarian	Tissue samples (*n* = 49)	IF	5 Gy IR	2 h	5	20%	Geminin	40	% of RAD51$^+$ /geminin$^+$ cells	Yes (better OS)	Patients
Waks *et al.*, 2020 [96]	Breast	Biopsies (*n* = 8)	IHC	PARPi and platinum chemotherapy	Unknown	3	One RAD51$^+$ cell	Geminin	Unknown	Detection of RAD51$^+$ /geminin$^+$ cells in three 40x fields	Yes (PARPi/platinum response)	Patients
Carreira *et al.*, 2021 [107]	Prostate	Biopsies (*n* = 52)	IF	None	NA	5	10%	Geminin	40	% of RAD51$^+$ /geminin$^+$ cells	Yes (olaparib sensitivity, improved PFS and OS)	Patients
van Wijk *et al.*, 2021 [62]	Endometrial and Ovarian	Tissue samples (*n* = 70)	IF	None	NA	2	15%	Geminin	40	% of RAD51$^+$ /geminin$^+$ cells	NA	NA
Llop-Guevara *et al.*, 2021 [108]	Breast	Tissue samples (*n* = 133)	IF	None	NA	5	10%	Geminin	40	% of RAD51$^+$ /geminin$^+$ cells	Yes (carboplatin sensitivity)	Patients

3.1 The REcombination CAPacity (RECAP) test

One of the most frequently used RAD51-based functional HRD tests that has been validated on different tumor and specimen types is the REcombination CAPacity (originally termed REpair CAPacity) or RECAP test [79,80,82,83] (Table 1). An important characteristic of this test is the inclusion of geminin (GMN) as an S/G2 phase cell proliferation marker [9], as HR is only active during these phases of the cell cycle. DSBs are induced by irradiation of viable tumor specimens with 5 Gy ionizing radiation (IR) followed by a two hours recovery time. A GMN-positive (GMN^+) cell with at least five RAD51 foci is considered $RAD51^+$ and the RAD51 score is defined as the percentage of (GMN^+/$RAD51^+$) cells within a population of GMN^+ cells. Tumors are classified into three HR groups depending on their RAD51 score: HR-Deficient (HRD; 0-20%), HR-Intermediate (HRI; 21-50%), and HR-Proficient (HRP; 51-100%). Evaluation of 125 BC samples resulted in the classification of twenty-four (19%) cases as HRD of which 16 (67%) could be explained by *BRCA1/2* deficiencies [80]. In a cohort of 25 endometrial cancer (EC) samples that was enriched for non-endometrioid tumors, six out of 25 (24%) cases were classified as HRD based on the RECAP test and only two of these (33%) could be explained by the presence of PVs in *BRCA1/2* [82]. The RECAP test was also applied on OC samples where HRD cases were only identified among HGSOC samples. Ten out of 39 (26%) HGSOC samples were identified as HRD, and among the nine HRD cases that were sequenced, eight (89%) could be explained by *BRCA1/2* deficiencies [83]. No *BRCA1/2* deficiencies were observed in HRP cases in any of the above mentioned studies, indicating that the RECAP test identified *BRCA1/2* deficient cases with a sensitivity of 100% in OC, BC and EC [80,82,83]. Although sample sizes were small, two studies showed a trend towards improved OS in HGSOC patients with tumors with low RECAP scores treated with platinum-based chemotherapy [81,83]. While these studies show promising results, the need for fresh, tumor tissue and the *ex vivo* induction of DNA damage by IR limits the clinical implementation of the RECAP test.

3.2 Analysis of RAD51 in FFPE tumor tissue

Recently, a method was developed to analyze RAD51 foci in diagnostic FFPE tumor tissue [62,92,93]. To prevent tumor misclassification as being HRD, the presence of endogenous DNA damage is a prerequisite. This can be determined through quantification of the fraction of tumor cells with γH2AX foci. In the first two studies evaluating BC FFPE tumor tissue for the analysis of RAD51 foci, >25% tumor cells of a sample had to contain γH2AX foci to be eligible for inclusion [92,93]. In a subsequent study, this inclusion criterium became more strict, and >25% of GMN^+ tumor cells had to contain γH2AX foci since only these cells were analyzed for RAD51 foci analysis [95]. When this quality criterium was applied to OC and EC samples, almost all samples were eligible for the quantification of RAD51 foci in FFPE tumor tissue, indicating that the inclusion of a γH2AX quality control

might only be necessary to confirm that cases with a low RAD51 score contained sufficient levels of endogenous DNA damage [62].

The RAD51 score is dependent on the combination of two parameters: the percentage of GMN^+ cells with RAD51 foci and the number of RAD51 foci per nucleus. For BC samples, an HRD threshold of 10% GMN^+ cells with RAD51 foci and a cut-off of five foci/nucleus showed the best correlation with PARPi response in g*BRCA1* PDX and *gBRCA1/2* patient samples [93]. This outcome was confirmed by a second study that identified all *BRCA1/2* deficient tumors as HRD [92]. In the RIO trial, where FFPE tumor samples of rucaparib-treated patients were analyzed for RAD51 foci formation, an HRD threshold of 20% with a RAD51 foci number cut-off of five captured all BC samples with *gBRCA*, *gPALB2*, *RAD51C* and *BRCA1* promoter methylation [94]. Since not only PVs in HR-related genes and HR gene promoter methylation can lead to HRD, one study calibrated the HRD threshold and foci cut-off for the analysis of RAD51 foci in FFPE tissues from OC and EC based on matching RECAP scores and *BRCA* status [62]. An HRD threshold of 15% in combination with a RAD51 foci number cut-off of two yielded the highest sensitivity, identifying 90% of *BRCA1/2* deficient and 87% of RECAP-HRD cases [62]. Clinical validation will be required to confirm if these HRD thresholds and RAD51 foci number cut-offs result in the highest sensitivity and specificity for the identification of HRD and HRP tumors.

4. Strengths and limitations of HRD tests

The presence of PVs in the *BRCA1/2* genes is currently the best biomarker to predict platinum-based chemotherapy and PARPi response [109,110]. Since PVs in other HR-related genes can also lead to HRD, targeted gene panel sequencing (at relatively low costs and short turnaround time), represents an attractive tool for large-scale clinical implementation [83] (Table 2). On the other hand, the presence of PVs in other, non-*BRCA,* HR-related genes is rare and their impact on patients' sensitivity to PARPi/platinum-based chemotherapy is still largely unknown [110]. Additionally, many tumors with a variant of uncertain significance (VUS) in one of these genes are identified, for which the impact on gene function is uncertain [111]. Alternative biomarkers which are able to identify HRD tumors irrespective of the molecular basis might therefore be preferred. Patients with HRD BC identified by applying *BRCA1/2*-like^{CGH} classifiers displayed an improved OS and therapy outcome after treatment with high-dose platinum-based chemotherapy [65,112,113]. It is unknown if this classifier can be used for other tumor types as well. Clinical studies using the MyChoice® CDx test have shown promising results in the identification of OC patients who are PARPi sensitive. The test is, however, only available commercially, about 15% of the tests are not informative, and PARPi response is also observed among HRD-negative patients [23,24,41,42]. HRDetect has shown to be

highly sensitive in the identification of *BRCA1/2* deficient cancers in different tumor types, including BC, OC, and pancreas cancer [38,65,74]. In a prospective clinical trial on TNBC, HRDetect scores were predictive for rucaparib response [94]. Clinical implementation of HRDetect is limited by the necessity to isolate DNA from frozen tumor tissue to generate high-quality WGS data, the complexity of data analysis, and the high costs associated with WGS (Table 2).

RAD51-based functional HRD tests have been performed on different tumor types demonstrating high sensitivity for the identification of *BRCA*-HRD tumors with competitive performance with regard to reliability, costs and turnaround time (Table 2). The first generation RAD51-based test (i.e. RECAP test) relies on the use of fresh tumor tissue and requires *ex vivo* induction of DNA damage which precludes the application in routine clinical diagnostics. The RAD51-FFPE test, which measures RAD51 foci in diagnostic FFPE samples, does not suffer from these drawbacks facilitating clinical validation and implementation [62,92,93]. However, a small fraction of tumor samples (3-8%) is non-informative as they contain insufficient numbers of proliferating tumor cells to allow calculation of a RAD51 score [62,92]. Other drawbacks of the RAD51-based HRD tests are the labor intensity of manual scoring and bleaching of the immunofluorescence signal during confocal microscopy, limiting the options for digitalization and automatic quantification. Applying immunohistochemical staining in combination with automatic slide scanning and scoring would bypass these limitations. Several studies already showed promising results regarding the predictive value of RAD51-based HRD tests for

Table 2. Strengths and limitations of DNA-based and functional HRD tests. Input 'all' refers to fresh (cryopreserved) and FFPE tumor tissue that is obtained from solid tumors, tumor biopsies or ascites. Abbreviations: FFPE = formalin-fixed paraffin-embedded; PARPi = PARP inhibitor; + = low; ++ = intermediate; +++ = high; NA = not applicable. Estimates based on various studies; i [109,114,115]; ii [74,78,94,116]; iii [80-83]; iv [62,92-95,108].

	HRD test	Input	Costs	Turnaround time	Expertise required	Clinical validation	Ability to identify PARPi resistance
DNA-based	Gene panel and methylation[i]	All	++	+	+	+++	++
	Genomic scars[i]	All	++	+	+++	+++	+
	Mutational signatures[ii]	All	+++	+	+++	+	+
Functional	RECAP[iii]	Fresh/ cryopreserved	++	+	++	+	+++
	RAD51-FFPE[iv]	All	+	++	++	+	+++

PARPi and/or platinum sensitivity using organoids, PDX models, and retrospective clinical study cohorts [92,93,95,96,100,101,105] (Table 1). Most importantly, RAD51-based HRD tests can, unlike DNA-based HRD tests, assess the actual HR status of the tumor and also distinguish between HRD and HRP cells within a heterogeneous tumor. Functional HRD tests are therefore, in contrast to most DNA-based tests, expected to be able to detect acquired resistance to PARPi due to HR restoration in HRD tumors (section 6.2), which will majorly impact clinical decision-making in the recurrent setting (section 6.3).

5. Concluding remarks

In the near future, it is important to evaluate the clinical validity and utility of the various DNA-based and functional HRD tests. Cross-validation between HRD tests will give valuable insights into their reliability and applicability. The main focus of these efforts should be on maximizing the accuracy in the prediction of PARPi/platinum response, while reducing the turnaround time and complexity of HRD tests. As a consequence, robust HRD tests will become available that will positively impact the lives of many cancer patients.

6. Expert opinion

Before DNA-based and functional HRD tests can be implemented in routine clinical practice, there are still some key issues that should be addressed. Calibration of HRD thresholds and clinical validation of both DNA-based and functional HRD tests should become the focus of HRD test development in the next years.

6.1 Calibration of HRD thresholds

Calibration of HRD tests (DNA-based and functional) was initially based on *gBRCA* BC and OC tumors [74,79,117]. For the MyChoice® CDx test, both *gBRCA* tumors and tumors harboring *BRCA1* promoter methylation were used to establish a threshold to define tumors to be HRD or HRP [117]. Importantly, correlation of obtained MyChoice® CDx test results with PARPi and/or platinum response in patients was not absolute and several studies indicated that the applied threshold should be adjusted (either increased or decreased) to improve PARPi/platinum sensitivity prediction [41,76,118,119]. This discrepancy may be due to the HRD threshold being tumor-specific or that solely the *BRCA* status of a tumor is insufficient to reliably calibrate an HRD test to capture all PARPi/platinum-sensitive tumors. It may be important to note that HRDetct was classified using *gBRCA* BC tumor data and its performance on other tumor types is uncertain [74]. The first study evaluating HRDetect as a predictive biomarker for PARPi response showed that HRDetect scores correlated with rucaparib response in TNBC patients [94]. More studies

are, however, required to confirm that the current HRDetect thresholds are suitable for different tumor types and treatment conditions.

The early RAD51-based functional HRD tests were calibrated on *gBRCA* and *wtBRCA* cell lines, using the absence of RAD51 foci induction after platinum/PARPi treatment over untreated controls to define HRD [85,88,97,102]. Later, tumor samples with or without *BRCA1/2* deficiency, derived from patient-derived tumor xenografts, served as the gold standard to establish the HRD threshold for the RECAP test [79]. The threshold for functional HRD combines two parameters, the percentage of cells with RAD51 foci (mostly between 10-20% $RAD51^+/GMN^+$ cells or $RAD51^+$ /total cells) in combination with RAD51 foci number cut-offs from two to five foci per nucleus [62,83,92-95]. In TNBC patients, two clinical studies showed that low RAD51 scores associated with HRDetect high scores and rucaparib/olaparib response [94,95]. A recent study on TNBC showed high concordance of low RAD51 scores with *BRCA1/2* deficiency and MyChoice® HRD scores. Additionally, RAD51 score was predictive for the clinical benefit of carboplatin addition to neoadjuvant chemotherapy (NACT) [108]. Ideally, one would apply various DNA-based and functional HRD tests to large series of tumor samples for which clinical outcome data (preferably PARPi response) are available to establish faithful thresholds for each test. In this way, it should also become clear if tumor type-specific HRD thresholds are required.

6.2 HRD phenotype reversal and prediction of PARPi/platinum resistance

Resistance to PARPi and/or platinum-based chemotherapy has been observed in HRD tumors and might be caused through restoration of HR activity. Multiple mechanisms have been described for reversal of HRD phenotype including acquisition of secondary mutations in e.g. *BRCA1/2, RAD51C/D,* or *PALB2* that restore the reading frame [102,120-123] and loss of HR gene promoter methylation [124]. Additional reversal mechanisms involve loss of expression of 53BP1 [125,126], Shieldin factors [127], CTC/polα [127], or DYNLL1/ATMIN [127], and stabilization of mutant BRCA1 protein by HSP90 [128].

An HRD test that captures restored HR activity might therefore be of clinical relevance, especially in the recurrent setting [96,102,121]. Several studies, using targeted next-generation sequencing, showed that genetic events restoring the reading frame can be detected in recurrent disease, but their clinical relevance is still unclear [96,129,130]. Genomic scar/mutational signature-based assays reflect the historical accumulation of mutations and might therefore not be sensitive enough to identify reversion of HRD. Implementation of RAD51-based functional HRD tests may tackle shortcomings of DNA-based HRD tests as they assess HR status independent of the molecular mechanism. Indeed, RAD51 scores from pre- and post-resistance tumor samples correlated to PARPi/platinum response and resistance [96].

Also non-HR-related mechanisms leading to PARPi and/or platinum resistance have been described using preclinical models, including the upregulation of P-glycoprotein cellular efflux pumps, restoration of poly ADP-ribosylation (PARylation), loss of PARP1 and stabilization of stalled replication forks [96,102,120,122,131,132]. HRD tests might therefore not be sufficient to capture all patients who develop PARPi/platinum resistance.

6.3 Implementation of HRD testing in the clinic

In the last years, the application of HRD tests within (retrospective) clinical studies mainly focused on OC and BC patients, while HRD has been described for a broad range of tumor types, including endometrial, pancreas, prostate, lung, kidney, gastric, biliary tract, bladder, hepatocellular and gastroesophageal cancer [115,133]. Once PARPi has been demonstrated to be effective in these patients inclusion of an HRD test as part of the standard routine diagnostics would be relevant. In the primary setting, a RAD51-based functional HRD test on FFPE tissue material may serve as a pre-screening tool since it has a high sensitivity for the identification of both *BRCA*-related and non-*BRCA*-related HRD cases. Gene panel sequencing (including *BRCA1/2*) can subsequently be restricted to RAD51-HRD tumors to identify patients with germline PVs predisposing to hereditary cancer. As the specificity of the RAD51-based HRD test is suboptimal, additional genomic scar/mutational signature analysis may be considered for RAD51-HRD cases in which no *BRCA1/2* PV is identified. Tumors without an HRD genomic scar/mutational signature or classified as RAD51-HRP should be treated with conventional therapy. In the recurrent setting, the application of a RAD51-based functional HRD test on a biopsy might be most appropriate since this test is expected to also identify tumors that are PARPi resistant due to reversion of the HRD phenotype. In case the recurrent tumor displays an HRD phenotype, the patient may receive PARPi.

Alternative treatment strategies have been applied for patients whose tumors showed reversal of HR activity [131]. Interestingly, *BRCA1/2* deficient PARPi/platinum-resistant tumor cells appear to rely on ATR for survival and a combination treatment of PARPi and ATRi was able to overcome PARPi and platinum-resistance in OC PDX models [134]. Semi-quantitative analysis of ATR expression in FFPE OC tumor tissue might be a useful additional biomarker to explore treatment options in *BRCA1/2* deficient patients with acquired PARPi/platinum resistance [135]. Also, the combination of PARPi/platinum with wee1-like protein kinase (WEE1) inhibitors showed promising results in the treatment of different tumor types, including resistant tumors [131,136,137]. In the years to come, more data will become available on the mechanisms through which patients acquire PARPi/platinum resistance. Depending on this knowledge, additional biomarkers should be developed to discriminate between HR-dependent and HR-independent resistance

mechanisms. Only then, informed choices about subsequent treatment of the patient can be made.

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Declaration of interest

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