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

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Chapter 8

Summary, general discussion and future perspectives

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This thesis illustrates the process of translating a fundamental insight into the development of a (pre-) clinically applicable test, i.e., from bench to bedside. Bringing research findings further to clinically applicable tools or the development of new medication is challenging, and requires patience, creativity, investments, and above all, collaboration.

The patient plays a central role in the field of (bio)medicine, where we are constantly searching for ways to optimize treatment procedures. However, it is only since recently that we are transitioning from a 'one-size-fits-all' to a more personalized therapy for many diseases. This transition is particularly visible in the field of oncology; with the arrival of new targeted therapies such as poly (ADP-ribose) ribose polymerase inhibitors (PARPi) and immune checkpoint inhibitors, the question of 'who should be treated with what?' becomes more complex. In addition, healthcare professionals need to decide when and for how long these targeted therapies should be given, and if it should be in addition or instead of conventional therapies (e.g. surgery, chemotherapy and radiation therapy). Biomarkers play a crucial role in therapy decision-making, particularly in precision medicine. They can be used to characterize the tumor, identify molecular targets for targeted therapy, reveal potential resistance mechanisms, and predict if a patient will respond to a certain therapy. Because of their importance in the clinic, validation of a new biomarker can take, in the best case scenario, several years, going through the typical phases of discovery and early validation, retrospective validation, prospective validation, regulatory approval, and finally clinical implementation.

One of the biomarkers that is currently in the validation phase (both retrospectively and prospectively) is homologous recombination (HR) deficiency (HRD), which is being evaluated as predictive biomarker for platinum and/or PARPi sensitivity of tumors. Between 2017 and 2022 the PARP-inhibitors olaparib, rucaparib and niraparib were FDA (U.S. Food & Drug Administration) approved in patients with platinum-sensitive ovarian cancer (OC), independently of the *BRCA* status and HR status, as first line and second line or subsequent lines of treatment. However, as of December 2022, the FDA withdrew the approval of all three drugs as treatment option for OC patients as single-agent therapy and, for niraparib and rucaparib, the approvals for 2nd line or subsequent line maintenance therapy for OC patients who do not harbor a *BRCA1* and/or *BRCA2* pathogenic variant (PV) based on newly published overall survival data of several clinical trials [1]. PARPi are now FDA approved for platinum-sensitive OC patients (recurrent disease) and for platinum-

sensitive OC patients with either *BRCA1/2* deficient or HRD tumors (advanced disease), HER2-negative breast cancer (BC) patients with *BRCA1/2* deficient tumors, for platinum-sensitive patients with *BRCA1/2* deficient pancreatic cancer, and for patients with *BRCA1/2* deficient prostate cancer (Table 1). The EMA (European Medicines Agency) approved olaparib for platinum-sensitive OC (recurrent disease) and for platinum-sensitive OC with *BRCA1/2* deficiency or HRD (newly diagnosed, advanced disease), for HER2-negative BC patients with *BRCA1/2* deficient tumors, for platinum-sensitive patients with *BRCA1/2* deficient pancreatic cancer and for patients with *BRCA1/2* deficient prostate cancer (Table 1). Rucaparib and niraparib are EMA approved as maintenance treatment for platinum-sensitive OC, independent of *BRCA* and HR status. However, Dutch recommendations from the NVMO-committee BOM in the Netherlands recently stipulate that niraparib should only be used as maintenance treatment for platinum-sensitive OC patients with either *BRCA1/2* deficient or HRD tumors [2]. The debate about the criteria that a patient's tumor must meet for treatment with PARPi is still ongoing.

Table 1. PARP inhibitor (PARPi) approvals by the FDA (U.S. Food & Drug Administration) and EMA (European Medicines Agency).

PARPi approvals	Ovarian Cancer	Breast Cancer	Pancreatic Cancer	Prostate Cancer
FDA	Platinum-sensitive (recurrent disease) Platinum-sensitive, *BRCA1/2* deficient or HRD (maintenance treatment of advanced disease)	HER2-negative, *BRCA1/2* deficient	Platinum-sensitive, *BRCA1/2* deficient	*BRCA1/2* deficient
EMA	Platinum-sensitive (recurrent disease) Platinum-sensitive, *BRCA1/2* deficient or HRD (newly diagnosed, advanced disease)	HER2-negative, *BRCA1/2* deficient	Platinum-sensitive, *BRCA1/2* deficient	*BRCA1/2* deficient

Historically, both for DNA- and functional HRD tests, *BRCA* status served as an important standard; the HRD test should at least identify the samples with *BRCA1/2* deficiency as HRD. However, we now know that also pathogenic variants in other HR-related genes

can lead to HRD, which are tumor type specific. Among OC this mainly concerns point mutations in the *RAD51C*, *RAD51D*, *BRIP1* and *PALB2* genes, but also large rearrangements in the *RAD50* and *NBS1* genes and amplification of *EMSY* can lead to HRD in OC. Promoter hypermethylation of *RAD51C* and *PALB2,* in addition to promoter hypermethylation of *BRCA1,* has also been described as possible cause of HRD in OC. In BC, PVs in the *PTEN*, *PALB2*, *CHEK2*, *ATM* and *RAD51C* genes are commonly described in the context of HRD beyond *BRCA1/2* PVs. For prostate cancer, PVs in *ATM* and *CHEK2* are linked to HRD and alterations in the *ATM, PALB2, CHEK1, RAD50, BARD1, FANCA* and *ARID1A* genes are strongly associated with pancreatic cancer [3]. There is still limited data on the effect of PVs in other HR-related genes on PARPi sensitivity. Studies investigating other underlying causes of HRD are still ongoing and even describe mechanisms other than (epi)genetic defects in HR-related genes that can induce HRD, such as alterations in RNA-binding protein-encoding genes [4]. We probably just see the proverbial tip of the iceberg when we screen tumors for PVs in HR-related genes with the goal to identify all HRD tumors. Two FDA-approved diagnostic HRD tests, the Myriad MyChoice® CDx and FoundationOne® CDx simultaneously evaluate genetic alterations along with genomic instability. Out of the two, the Myriad MyChoice® CDx is the most commonly used HRD test to identify HRD OC patients eligible for PARPi treatment by calculating a genomic scar score consisting of the sum of LOH, TAI and LST. However, several clinical trials in which the Myriad MyChoice® CDx test was used to classify tumors as HRD reported that even patients classified as HR-Proficient (HRP) were responsive to PARPi treatment, highlighting the suboptimal accuracy of the HRD test. Other companies and research groups are developing additional HRD tests to assess HRD from a genetic and genomic perspective, such as a kit developed by SOPHiA genetics that detects genetic alterations in 28 HR-related genes and in combination with WGS identifies HRD-specific copy number variations [5]. Amoy Scientific Company produces a commercially available HRD test that determines the HR status based on the presence of PVs in the *BRCA1/2* genes combined with a LOH-genomic scar score [5]. New and improved HRD testing methodologies offer the opportunity to pinpoint better which patients will benefit the most from PARPi and/or platinum-based treatment. Here, new insights on the development of alternative methods to assess HRD status are discussed.

There are several ways to identify if a tumor is HRD, and **chapter 2** summarized the different methodologies, both DNA-based and functional, which are currently under development. Each of these methods have shown potential, mostly in retrospective studies, as predictive biomarker for platinum and/or PARPi sensitivity. However, each methodology has its own test parameters and HRD-thresholds, and it is still unclear how these HRD tests perform on large and various patient cohorts. In addition, it is also important to understand if an HRD test yields a reliable result in both the primary and

recurrent setting, as tumors evolve over time and under treatment pressure, potentially resulting in a platinum/PARPi resistant tumor. Several reversion mutations in the *BRCA1* and *BRCA2* genes have been identified in *BRCA1/2* deficient tumors, which led to restoration of the genes and consequently to treatment resistance. Our ability to identify such reversion mutations is, however, still in the very early days. DNA-based HRD tests capture all genomic and/or mutational events that have taken place in a tumor, but this does not necessarily reflect the current HR status of the tumor. In addition, it is rather complex and time-consuming to interpretate test results from DNA-based HRD tests.

To fulfill the need of a real-time HRD test, functional HRD tests were developed, which capture the current HR status of a tumor by evaluation of RAD51 accumulation at sites of DNA damage. As RAD51 is a key player in the HR pathway, working downstream of BRCA1 and BRCA2, it is considered a good candidate biomarker for the functional assessment of HR. The visualization of RAD51 foci via immunofluorescence (IF) staining in cell lines has been performed since the late 1990s, just after its discovery, and since the early 2000s researchers started exploring the option of RAD51 IF staining in tumor samples. Staining tumor material comes with additional challenges, due to the presence of a mixture of cell types and extracellular matrix, leading to differences in fluorescence background staining and harder to distinguish tumor cells. Incorporation of additional markers to assist in tumor cell recognition and cell cycle phase, in particular the G2/S phase in which HR takes place, highly increases the reliability of the RAD51 foci assessment. However, many analyses currently performed on tumor material do not incorporate such control markers. The first RAD51-based functional HRD test incorporating both DAPI to assess tumor cell morphology and a geminin (GMN) IF to identify cells in G2/S phase in addition to the RAD51 IF, was described for BC samples in 2014 and later named as the REcombination CAPacity (RECAP) test. In this test, a fresh tumor tissue or biopsy is *ex vivo* irradiated with ionizing radiation and subsequently fixed and embedded into paraffin prior to a co-IF staining with DAPI, GMN and RAD51. **Chapter 3** described the validation of the RECAP test in a cohort of 49 OC samples of different histological subtypes in relation to the identification of *BRCA1/2* deficient OC, reaching a sensitivity of 100%. In addition, the RECAP test identified patients with HRD tumors, who had a more beneficial treatment outcome towards platinum-based chemotherapy. This proof-of-concept was confirmed in other studies evaluating HRD biomarkers in relation to both platinum- and PARPi sensitivity. Although the RECAP test became the 'gold standard' in functional HRD testing, practical limitations such as the availability of fresh tumor material and the need for *ex vivo* irradiation was holding back the development towards clinical implementation of the test.

The necessary steps to undertake were to 'simplify' the RECAP test and to adjust it in such a way that functional HR assessment could take place in the routine diagnostics,

where no *ex vivo* culturing of tumor tissue and irradiation was possible. With this in mind, a RAD51-based functional HRD test was developed that can be performed on formalin-fixed paraffin-embedded (FFPE) tumor material blocks, which are routinely made in the pathology department. The concept remained the same, with a co-IF for DAPI, GMN and RAD51, but in contrast to the RECAP test, no fresh tumor material was required and endogenous DNA damage was utilized instead of exogenous induced DNA damage. We called this new test the RAD51-FFPE test, and in **chapter 4** we described the methodology and validation of this new RAD51-based HRD test on our previously published ovarian and endometrial cancer (EC) cohorts. Importantly, we were able to calibrate test parameters that were suiting both ovarian and endometrial tumor samples of different histological subtypes by using both the *BRCA* status and RECAP test results, leading to a sensitivity of 90% to identify *BRCA1/2* deficient samples and a sensitivity of 87% for the identification of RECAP-HRD samples. However, the specificity of the RAD51-FFPE test could be improved, reaching 73% for the identification of RECAP-HR-Proficient (HRP) samples (OC and EC combined). We identified samples with a RECAP-HRP score, while the RAD51-FFPE test resulted in an HRD score. This 'overcalling' of HRD was present even though a γH2AX control for the presence of endogenous DNA damage was incorporated. Importantly, the nature and quantity of DNA double-strand breaks (DSBs) and the size of the RAD51 foci differ between the endogenous (RAD51-FFPE) and radiation-induced (RECAP) DNA damage. For this reason, a new co-IF protocol with different RAD51 and GMN antibodies was developed for the RAD51-FFPE test. However, it cannot be ruled out that differences in pan-nuclear background staining between co-IF stained slides of the RECAP and RAD51-FFPE tests led to an underscoring of RAD51 foci in RAD51-FFPE slides, as manual scoring is still the standard procedure. Another possible explanation for the overcalling of HRD in the RAD51-FFPE test is the highly variable time-to-fixation of diagnostic samples, which can lead to suboptimal fixation and a decrease in the immune reactivity of proteins. For RECAP samples, the time-to-fixation is always exactly two hours and fixation is performed on tumor samples of equal size. Other technical aspects, such as the thickness of the tissue section, the microscope slide used for the co-IF staining, the microscope used for scoring and the experience level of the observer(s) can affect the quality and visibility of RAD51 foci, which is particularly important considering the fact that RAD51 foci in RAD51-FFPE samples are typically smaller and less pronounced compared to RAD51 foci in RECAP samples.

Genomic studies revealed that HRD signatures cannot only be identified among OC, but also among other carcinomas such as endometrial, bladder, biliary tract, colorectal, osteosarcoma, hepatocellular, and gastroesophageal carcinoma [6,7]. As of today, only a small number of studies have been performed evaluating the prevalence of HRD among a broader range of carcinomas. Our next step was to explore the prevalence of HRD among

gynaecological carcinomas with the RAD51-FFPE test, while continuing the validation of the test with *BRCA1/2* deficiency and RECAP test outcomes. In **chapter 5** we showed that HRD can be commonly identified among EC patients with high-grade serous p53abn tumors, both with and without *BRCA1/2* deficiency. However, no HRD was observed among cervical and vulvar carcinomas. As PARPi are currently not among the available treatment options for patients with EC, it is important to validate our results in other (clinical) studies.

Chapter 6 presents functional analysis of HRD by applying the RAD51-FFPE test in 63 BC samples, which showed a high sensitivity of 88% for the identification of RECAP-HRD samples with a specificity of 76% for the identification of RECAP-HRP samples. Similar to our observations in OC and EC, a possible overcalling of HRD was reported for BC samples, suggesting that the overcalling may not be tumor-specific, but rather of technical origin. Nevertheless, the RAD51-FFPE test was able to identify all *BRCA1/2* deficient BC samples as HRD and showed its compatibility with FFPE samples, indicating that further validation of the test was desirable.

In the development of the RAD51-FFPE test, both *BRCA* status and RECAP status were taken into account as gold standards, but it became clear that additional tests were needed besides the RAD51-FFPE test to be able to filter out the 'true' HRD samples. **Chapter 7** describes the largest validation cohort of over 200 triple-negative breast carcinoma (TNBC) samples for which detailed (epi)genetic data of HR-related genes and whole-genome sequencing (WGS) data were available, and on which both HRDetect (a mutational signature score), a Genomic Scar test (the sum of loss of heterozygosity (LOH), telomeric allelic imbalances (TAI), and large-scale transitions (LST)), and the RAD51-FFPE test were performed. These data showed that the majority of samples were classified in the same HR group based on the RAD51-FFPE, HRDetect and Genomic Scar test results, but several samples were still classified differently between tests. The RAD51-FFPE test reached a sensitivity of over 80% for the identification of tumor samples with a *BRCA1*, *BRCA2* or *PALB2* PV, and for the identification of HRDetect-High and Genomic SCAR-HRD samples. Although the RAD51-FFPE test classified a higher number of samples as HRD compared with the HRDetect and Genomic Scar test, it is, among these three, the only functional HRD test measuring HRD at a point in time of tumor development relevant for treatment decisions, namely in the resected tumor, and it can be performed within a short time span. Considering the fact that one HRD test is not like the other, prospective and retrospective validation of HRD testing in relation to platinum- and PARPi sensitivity should become the focus point.

Future perspectives

There is an urgent need to improve both the sensitivity and the specificity of HRD testing. Clinical trials in which HRD tests are retrospectively or prospectively validated are still limited and large-scale validation is required before clinical implementation is possible. It is important to compare and complement various HRD tests with each other, as HRD can be evaluated from different angles (both from a DNA and a functional perspective), and to validate these results with patients' sensitivity to PARPi and platinum-based therapy.

It may even be possible that HR status should not be measured as a binary outcome, but rather as a gradient. Hypothetically, tumors could be clearly HRP or HRD and easy to identify by an individual HRD test, or somewhere in between and harder to classify as belonging to either group, leading to discrepancies between HRD test results. We may need to introduce an intermediate HR group in the RAD51-FFPE test, similar to the HRDetect-Intermediate group, to identify those samples for which the RAD51-FFPE score should be interpreted with caution. Additional (HRD) testing can be performed to characterize the tumor better for personalized treatment choices. We identified tumors with scores close to the HRD threshold with the RAD51-FFPE test, suggesting that these tumors are less obvious HRD or HRP. In some of these samples, tumor heterogeneity was identified in terms of RAD51 positivity, with tumor areas full of RAD51-positive, but other tumor areas with many RAD51-negative cells. Currently, no standardized method for the interpretation of these heterogenous samples exists. It would be interesting to investigate whether the presence of RAD51-positive subclones in primary tumor material could predict future PARPi/platinum resistance. In addition, RAD51-based HRD tests have the ability to identify real-time changes in HR status of tumor cells at different time points during the treatment course. However, obtaining tumor biopsies to perform RAD51-FFPE testing is more invasive than using liquid biopsies for the analysis of circulating tumor DNA (ctDNA), which has already shown its potential in detecting clonal reversion mutations in high-grade serous OC patients harboring PVs in *BRCA1/2* genes [8].

Given the (at the moment of writing still) high costs of whole-genome sequencing (WGS) and the complexity of data analysis that are required for tests such as HRDetect, it is worth exploring if alternative HRD tests, either as stand-alone or combined with other tests, can perform as well as extensive WGS-based HRD tests. In **chapter 7**, we showed that combining the RAD51-FFPE test with a genomic scar test, similar to the Myriad MyChoice® CDx test, can be a reliable method to capture HRD samples, as we showed that the combination of RAD51-FFPE test results with a genomic scar score led to the identification of 98% of HRDetect-High TNBC samples, including the *BRCA1/2* and *PALB2* deficient samples. Another advantage of combining the RAD51-FFPE test with a DNA-

based HRD test is that the likelihood of obtaining an HR-classification is increased when the tests are complemented, as in some cases the tumor cell percentage is too low to obtain DNA and hence to perform a DNA-based HRD test, while it is sufficient to calculate a RAD51-FFPE score based on the FFPE tumor section. The opposite can also happen, as the number of GMN-positive cells can be too low for an informative RAD51-FFPE score, while DNA is available for analysis.

From a technical perspective, the RAD51-FFPE test ideally becomes a co-immunohistochemistry (co-IHC) staining with GMN and RAD51, thereby reducing the auto-fluorescence background, allowing digital scanning and full automatization of RAD51-FFPE score calculation. Another aspect that can be analyzed further is the incorporation of an additional marker to lower the number of false-positive HRD samples. This can either be a DNA damage marker (e.g. 53BP1) to filter the samples that do not have sufficient endogenous DNA damage, and/or by staining of another protein that is involved in HR, such as Replication Protein A (RPA) that acts upstream of RAD51. From a clinical perspective, large prospective and retrospective studies are required to test if the RAD51-FFPE test can predict PARPi/platinum response. For these analyses, the RAD51-FFPE test as stand-alone, but also in combination with other DNA-based HRD tests should be evaluated. Recent studies showed that deep learning methodologies can be applied to H&E stained tumor tissue sections to predict typical 'HRD' profiles [9,10], which could assist in the classification of tumors for which functional HR classification by evaluation of RAD51 foci or by DNA-based analyses did not result in a clear HR classification.

HRD testing should become an integral part of the routine diagnostics for the tumor characterization of a broad group of patients, not only limited to patients with gynaecological carcinomas. With the recent FDA and local NVMO guideline updates regarding the therapeutic indication of PARPi, now incorporating *BRCA1/2* deficiency or HRD as a prerequisite for many patients, the availability of a reliable, cost-effective HRD test becomes even more urgent. Critical evaluation of the currently available HRD tests in relation to PARPi sensitivity remains required and efforts should be made in the clinical validation of HRD tests in development.

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