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

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Chapter 6

Performance of a RAD51-based functional HRD test on paraffin-embedded breast cancer tissue

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Abstract

Purpose

BRCA1/2 deficient breast cancers (BC) are highly sensitive to platinum-based chemotherapy and PARP inhibitors due to their deficiency in the homologous recombination (HR) pathway. However, HR-Deficiency (HRD) extends beyond *BRCA*-associated BC, highlighting the need for a sensitive method to enrich for HRD tumors in an alternative way. A promising approach is the use of functional HRD tests which evaluate the HR capability of tumor cells by measuring RAD51 protein accumulation at DNA damage sites. This study aims to evaluate the performance of a functional RAD51-based HRD test for the identification of HRD BC.

Methods

The functional HR status of 63 diagnostic formalin-fixed paraffin-embedded (FFPE) BC samples was determined by applying the RAD51-FFPE test. Samples were screened for the presence of (epi)genetic defects in HR and matching tumor samples were analysed with the RECAP test, which requires *ex vivo* irradiated fresh tumor tissue on the premise that the HRD status as determined by the RECAP test faithfully represented the functional HR status.

Results

The RAD51-FFPE test identified 23 (37%) of the tumors as HRD, including three tumors with pathogenic variants in *BRCA1/2*. The RAD51-FFPE test showed a sensitivity of 88% and a specificity of 76% in determining the HR-class as defined by the RECAP test.

Conclusion

Given its high sensitivity and compatibility with FFPE samples, the RAD51-FFPE test holds great potential to enrich for *BRCA1/2* deficient and HRD tumors in a fast and cost-effective manner, even in scenarios where DNA-based testing may be challenging or not easily accessible in routine clinical practice. This is particularly important considering the potential implications for treatment decisions and patient stratification.

Keywords

Breast cancer, homologous recombination deficiency, RAD51-FFPE test, RECAP test, *BRCA1/2* deficiency and biomarker.

1. Introduction

Breast cancer (BC) accounts for 30% of newly diagnosed female cancers and is the second highest leading cause of cancer death for women [1]. Germline pathogenic variants (PVs) in *BRCA1* or *BRCA2* (*gBRCA1/2*) are observed in 3% of unselected BC and in 10-15% of patients with triple-negative BC (TNBC) [2]. BRCA1 and BRCA2 play an essential role in homologous recombination (HR), the DNA damage repair pathway that allows DNA double-strand breaks (DSBs) to be repaired in an error-free manner [3]. Next to *BRCA1* and *BRCA2,* PVs in other HR-related genes such as *PALB2*, *RAD51C* and *RAD51D* as well as epigenetic silencing of *BRCA1* and *RAD51C* via hypermethylation of the gene promoter have been shown to lead to HR-Deficiency (HRD) [4]. Overall, HRD can be observed in approximately 18% of BC [4], a substantial group of patients who could potentially benefit from treatment with platinum-based chemotherapy or poly (ADP-ribose) polymerase (PARP) inhibitors [5,6]. At this moment however, PARP inhibitors (PARPi) are only approved for the treatment of patients with *gBRCA1/2* or suspected *gBRCA1/2*, human epidermal growth factor receptor type 2 HER2-negative BC with early (olaparib) or recurrent disease (olaparib and talazoparib) [7-10]. Various efforts are currently undertaken to develop DNA-based and functional HRD tests to identify an additional group of BC patients with HRD tumors who might benefit from treatment with PARPi [11].

DNA-based HRD tests comprise those identifying PVs in HR-related genes, mutational signatures, and/or genomic scars by next-generation sequence (NGS) analysis of tumor DNA [12-15]. Currently, several clinical studies are ongoing to determine the accuracy of DNA-based HRD tests to predict platinum and/or PARPi response [12,16,17]. The MyChoice® HRD test, in which a score is calculated based on genomic loss of heterozygosity (LOH), telomeric allelic imbalance (TAI), and large-scale transition (LST), was shown to be a good predictor for PARPi sensitivity in ovarian cancer (OC) patients [18,19]. The MyChoice® HRD test did not, however, predict carboplatin sensitivity in TNBC patients [20]. Alternatively, HRDetect, which applies both mutational signature and genomic scar analysis, was predictive for rucaparib response in a prospective clinical trial with TNBC patients [21]. Importantly, patient selection based on DNA-based HRD tests is still suboptimal, as PARPi and platinum benefit are also observed among patients with non-HRD tumors [20,22].

In RAD51-based functional HRD tests, the ability of tumor cells to accumulate RAD51 protein at sites of DrNA damage in proliferating (geminin-positive, GMN^+) tumor cells is assessed [23-27]. RAD51 scores (i.e. the percentage of $RAD51^+/GMN^+$ cells) are used as a functional HR read-out in tumor samples, where low RAD51 scores indicate HRD. The first developed RAD51-based HRD test, the REcombination CAPacity (RECAP) test, has shown a high sensitivity in identifying breast and ovarian tumors with PVs in *BRCA1/2* or *BRCA1*

promoter hypermethylation [23,26,28-31]. However, the requirement for fresh tumor tissue poses a significant limitation for its clinical implementation. To address this limitation, the RAD51-FFPE test has been introduced as a more practical alternative. Unlike the RECAP test, the RAD51-FFPE test utilizes FFPE diagnostic tumor samples, eliminating the need for fresh tumor tissue [27]. The RAD51-FFPE test parameters were established based on its sensitivity to identify i) tumors with *BRCA1/2* PVs, and ii) tumors identified as HRD using the functional RECAP HRD test on ovarian and endometrial tumors [27].

Although sample sizes were small, two studies have demonstrated a correlation between low RAD51-FFPE scores and sensitivity to platinum or PARPi in metastatic BC and TNBC patients respectively [32,33]. In a recent biomarker analysis from the GeparSixto trial, low RAD51-FFPE scores exhibited a strong association with the presence of *BRCA1/2* PVs and MyChoice® HRD, accurately predicting the clinical benefit of adding carboplatin to neoadjuvant chemotherapy (NACT) treatment in TNBC [34]. Another study, evaluating PARPi sensitivity in over 100 patient-derived xenograft models from BC, showed that low RAD51-FFPE scores displayed a higher accuracy in predicting PARPi response compared to HR gene mutations and genomic HRD analysis, including both MyChoice® HRD and HRD signature assessment by HRDetect [35].

The accuracy of the RAD51-FFPE test to identify 'true' HRD samples in BC remains uncertain. In this study, we evaluate the performance of the RAD51-FFPE test using previously defined test parameters for ovarian and endometrial cancer. Our findings demonstrate that the RAD51-FFPE test can achieve a high sensitivity in identifying *BRCA1/2* deficient and RECAP-HRD samples in BC samples, regardless of the histological subtype.

2. Materials and methods

2.1. Patient material

Archival diagnostic FFPE tumor tissue blocks of BC patients who underwent surgery at the Leiden University Medical Center (LUMC) were collected between May 2013 and August 2019. The selection was made based on the availability of matched, cryopreserved tumor tissue that could be used for the REcombination CAPacity (RECAP) test. All samples were coded with a unique research code. The local medical ethics committee of the LUMC approved the study protocols on 7 February 2011 and 24 May 2017 (P10.226, B16.019, G17.041) and samples were handled according to the "Code for Proper Secondary Use of Human Tissue" in the Netherlands as established by the Dutch Federation of Medical Scientific Societies.

2.2. γH2AX/GMN co-immunohistochemistry staining (co-IHC)

Tissue sections (4µm) were stained for γH2AX (mouse, monoclonal, MilliporeSigma, St. Louis, MO, U.S., cat. 05-636, clone JBW301) and GMN (rabbit, polyclonal, Proteintech, Manchester, U.K., cat. 10802-1-AP) according to a previously published protocol [27].

2.3. Co-immunofluorescence (co-IF) staining for RAD51 and geminin

Tissue sections (4µm) were stained for DAPI, RAD51 (rabbit, monoclonal, Abcam, Cambridge, U.K., cat. ab133534) and GMN (mouse, monoclonal, NovoCastra, Leica Biosystems, Buffalo Grove, IL, U.S., cat. NCL-L) according to a previously published protocol [27].

2.4. Sample selection

Based on the availability of cryopreserved BC tumor samples, representative matching diagnostic FFPE blocks containing >70% vital tumor tissue were selected by a mamma pathologist (D.C.). Samples with ductal carcinoma in situ (DCIS) were not included in this study and pleural-fluid samples were additionally screened for the presence of p53 mutant cells based on IHC staining to confirm the presence of tumor cells.

Next, the presence of sufficient GMN^+ cells was confirmed based on a GMN/RAD51 co-IF for both RECAP and RAD51-FFPE test samples. At least 40 GMN^+ cells, randomly selected in 3-5 vital tumor tissue areas, were considered sufficient. Tumor samples with <40 GMN^+ cells in the co-IF were excluded for analysis.

For RAD51-FFPE test samples, the presence of endogenous DNA damage in tumor cells of FFPE samples with a RAD51-FFPE score of ≤15% (section 2.6) was confirmed by evaluation of a γH2AX/GMN co-IHC. At least 40 GMN+ cells, randomly selected in 3-5 vital tumor tissue areas, were manually counted by two independent observers on a Zeiss Axio Imager.M2 light microscope, 63x oil objective. The number of γH2AX foci were counted per selected GMN^+ cell (0,1,2,3,4 or ≥5 γH2AX foci). The γH2AX score was determined by calculation of the average percentage of $γH2AX^+$/ GMN^+ cells (cut-off ≥2 γH2AX foci) of two observers. Diagnostic FFPE tumor samples with a γH2AX score <25% were excluded for analysis due to the absence of sufficient endogenous DNA damage.

2.5. RAD51-FFPE score calculation

Diagnostic FFPE tumor tissue sections were stained for DAPI, GMN and RAD51 in a co-IF staining and scored manually with a Leica DM6B microscope, 63x/1.40-0.6 oil objective with an EL6000 light source. DAPI was used to get an overall impression of the sample (either whole tumor section or pleural fluids enriched for tumor cells), assess cell morphology

and locate 3-5 areas of the sample enriched with vital tumor cells. Within vital tumor areas, GMN^+ cells were identified and ≥40 GMN^+ cells were selected at random. A cell was considered GMN^+ when the nucleus was completely stained with a granular pattern. The number of RAD51 foci within a GMN^+ cell was determined (0, 1, 2, 3, 4 or ≥5 foci) and cells were categorized accordingly. For each RAD51 foci cut-off, a RAD51-FFPE score was calculated as the percentage of $RAD51^+/GMN^+$ cells by each observer. Final RAD51-FFPE scores were calculated as the average RAD51-FFPE score of two independent observers.

2.6. RECAP test

A detailed description of the methodology of the RECAP test has previously been published [26]. In short, tumor samples (thawed after cryopreservation), were irradiated with ionizing radiation to induce DNA double strand breaks (DSB) and incubated at 37°C for two hours prior to fixation and paraffin embedding. Irradiated tumor samples with high tumor percentage and sufficient tumor vitality (see quality description in section 2.4) were included and stained for geminin (GMN; anti-geminin antibody, ProteinTech, Manchester, U.K., cat. 10802-1-AP) and RAD51 (anti-RAD51 antibody, GeneTex, Alton Pkwy Irvine, CA, U.S., cat. GTX70230) with a co-immunofluorescence (co-IF) staining. Forty GMN^+ cells were evaluated for the presence of ≥5 foci/nucleus ($RAD51^+$). The percentage of $RAD51^+/GMN^+$ cells was represented as the RECAP score. Tumor samples were considered HR-Deficient (HRD) with a RECAP score of ≤20%, HR-Intermediate (HRI) with a RECAP score of 21-50% and HR-Proficient (HRP) with a RECAP score of >50%.

2.7. Genetic and epigenetic analyses

Next-generation sequence (NGS) analysis was performed using maximum 30 ng of tumor DNA per sample isolated from FFPE tissue blocks. The mean tumor cell percentage of included samples was 61% (range: 10–80%). All samples were sequenced with an HRD targeted gene panel. The custom Ampliseq HRDv2 gene panel (SeqStudio Genetic Analyzer, Thermo Fisher Scientific) was used for variant detection in the coding exons of the following genes: *ATM* (exon 2-63), *BARD1* (exon 1-11), *BRCA1* (exon 2-24), *BRCA2* (exon 2- 27), *BRIP1* (exon 2- 20), *CDK12* (exon 1- 14), *CHEK1* (exon 2- 13), *CHEK2* (exon 2- 15), *FANCL* (exon 1- 14), *PALB2* (exon 1- 13), *PPP2R2A* (exon 1- 10), *RAD51B* (exon 2- 11), *RAD51C* (exon 1- 9), *RAD51D* (exon 1 -10), *RAD54L* (exon 1- 18), *TP53* (exon 1- 11), *PIK3CA* (hotspots in exon 2, 5, 7, 8, 10, 14, 19 and 21) and *ERBB2* (hotspots in exon 8 and 17- 21). Details on request (DT). Sequencing was performed in an Ion GeneStudio S5 Series sequencer (ThermoFisher Scientific). The raw, unaligned sequencing reads were mapped against human reference genome (hg19) using TMAP software. Torrent Variant Caller was used for variant calling and variants were categorized using the 5-tier pathogenicity classification according to Plon *et al.*: Class 1 = benign, Class 2 = likely benign, Class 3 = variant of uncertain significance

(VUS), Class 4 = likely pathogenic, and Class 5 = pathogenic [36]. When needed, variants were interpreted using Integrative Genomic Viewer or the Alamut™ Visual Plus software (SOPiA GENETICS ™. Only class 3, 4 and 5 variants are reported in this manuscript. LOH analysis of the NGS data was performed as described previously by de Jonge *et al* [37].

In addition to sequence analysis, promoter hypermethylation of *BRCA1* using MS-MLPA was measured on samples for which sufficient DNA was available, as described previously [37].

2.8. Statistical analysis

Figures were created with Graphpad Prism 8.0 (GraphPad Software, San Diego, CA, U.S.), Adobe Illustrator CC 2020 (Adobe Inc, San Jose, CA, U.S.) and BioRender software (Toronto, ON, Canada). Statistical analysis was performed with Graphpad Prism 8.0, IBM SPSS version 25.0 (SPSS Inc.), and SigmaStat 3.5 (Systat Software Inc, San Jose, CA, U.S.). Student's t-tests were performed to test differences between two groups containing normally distributed numerical data and Mann-Whitney Rank Sum tests when numerical data was not normally distributed. Categorical data of two groups were tested with Chi-square test or Fisher's Exact test. Fisher's Exact test was chosen when at least one of the expected values was less than one and when over 20% of the expected values were less than five. To test if numerical data was correlated between two groups, Pearson's correlation coefficient was calculated. A p-value of <0.05 was considered significant.

3. Results

3.1 RAD51-FFPE test

A cohort of 74 diagnostic FFPE BC samples was selected based on the availability of cryopreserved tumor tissue. The RAD51-FFPE score in these samples was determined by analyzing the accumulation of RAD51 protein at sites of DNA damage in proliferating tumor cells (Fig. 1). In seven samples, of which five had a tumor grade 1 or 2, the number of GMN^+ cells was insufficient to determine a RAD51 score [27].

Out of the 67 tumor samples for which a final RAD51-FFPE score was determined, 27 samples were categorized as HRD. For these samples, an additional GMN/γH2AX staining was performed to confirm that the low RAD51 score was not caused by insufficient levels of endogenous DNA damage (Fig. S1). Four samples (Two no special type grade 3, one lobular grade 2, and one papillary carcinoma grade 1) with an γH2AX score (percentage of GMN^+ cells showing at least two γH2AX foci) of <25% were excluded (Fig. 1, Fig. S2, Materials and Methods section 2.4). In total, the HR status was successfully determined for 63 out of the 74 (85%) BC samples, with 37% (23/63) being classified as HRD.

3.2 (Epi) genetic defects in HR genes in relation to RAD51-FFPE scores

To assess the sensitivity of the RAD51-FFPE test to identify breast tumors with genomic-HRD, i.e., (epi)genetic defects in HR-related genes, we performed NGS analysis applying an HRD gene panel comprising 18 genes and applied MS-MLPA to identify *BRCA1* promoter hypermethylation (Materials and Methods section 2.7).

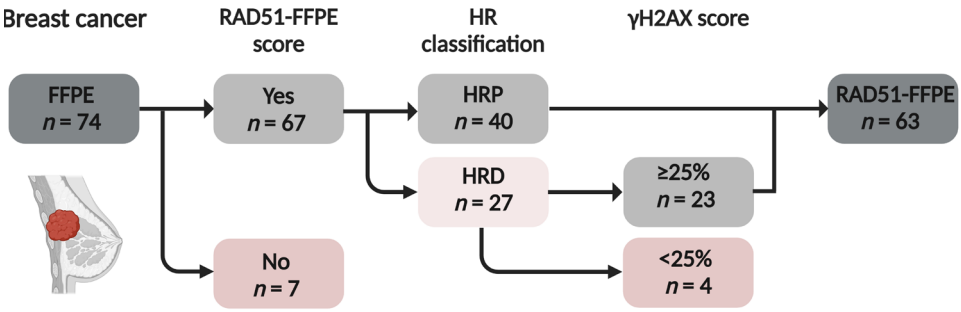
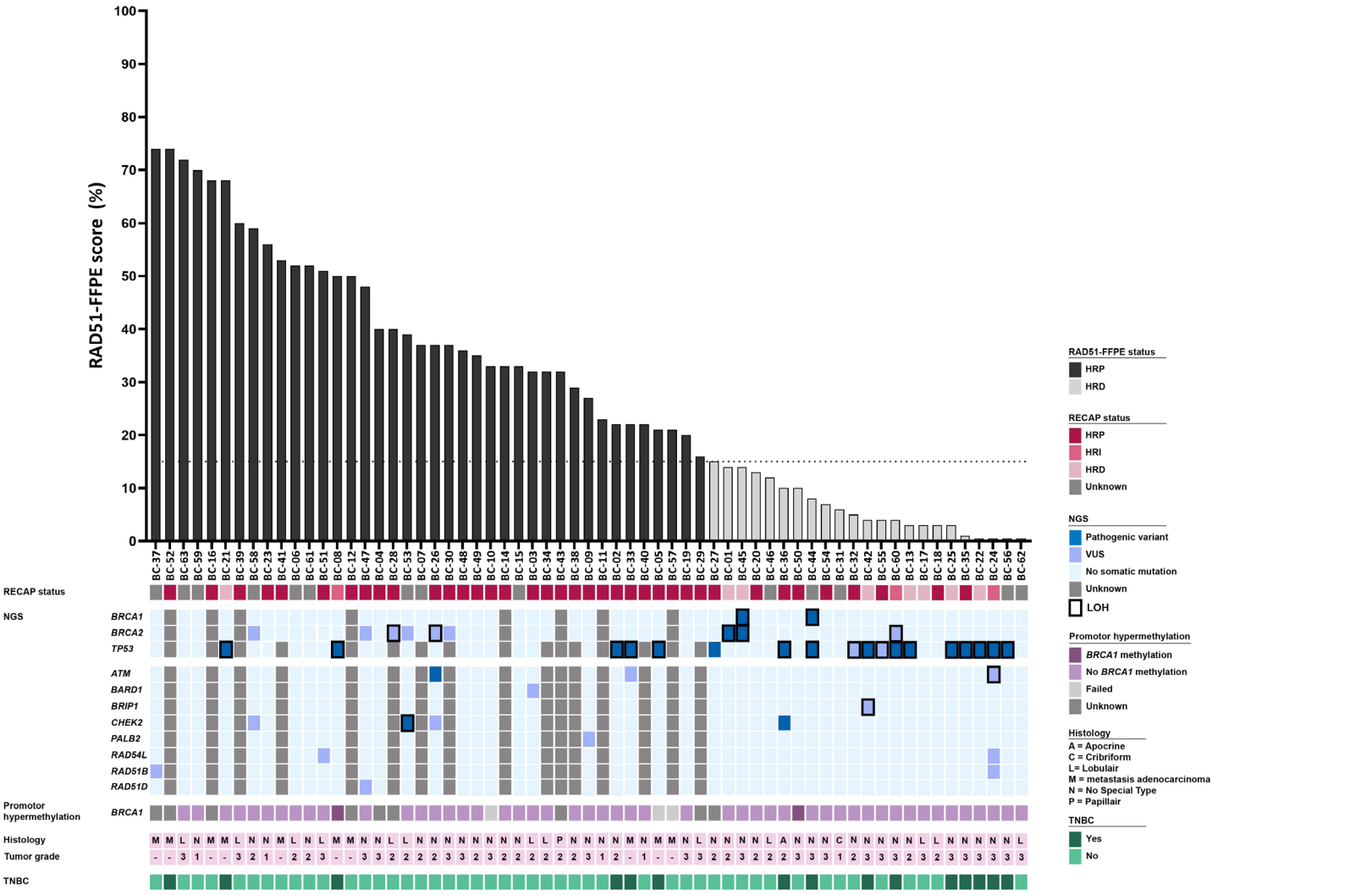


Fig. 1. Flowchart for the inclusion of RAD51-FFPE BC samples. In total, 74 breast cancer samples were analyzed with the RAD51-FFPE test. Sixty-seven samples contained sufficient geminin-positive (GMN^+) tumor cells to allow assessment of a RAD51-FFPE score. Twenty-seven samples were initially classified as HRD, but four samples were excluded from the cohort as they contained insufficient levels of endogenous DNA damage. In total, 63 samples were included in the RAD51-FFPE study cohort. There were no significant differences in patient and tumor characteristics between included and excluded samples (Table S1). Abbreviations: FFPE = formalin-fixed paraffin-embedded, HRP = homologous recombination proficient, HRD = homologous recombination deficient.

A PV in *BRCA1* and/or *BRCA2* with LOH of the wild-type allele was identified in three out of 23 HRD tumors, providing an explanation for the observed HRD phenotype (i.e. BC-01 (*BRCA2* PV), BC-44 (*BRCA1* PV) and BC-45 (*BRCA1* and *BRCA2* PV), Fig. 2, Table S2)). In one HRD sample, a *CHEK2* PV with a VAF of 0.46 was identified (BC-36). One HRD tumor harbored a variant of uncertain significance (VUS) with a variant allele frequency (VAF) ≥0.5 in *BRIP1* with LOH of the wild-type allele (BC-42; Fig. 2, Table S3). Two other HRD tumors harbored a VUS in *ATM* and *BRCA2* with LOH of the wild-type alleles respectively, but both had a VAF <0.5 (BC-24 and BC-60; Fig. 2, Table S3).

Fig. 2. RAD51-FFPE score in relation to RECAP status, HR gene panel results, *BRCA1* promoter hypermethylation status and tumor characteristics. RAD51-FFPE scores were calculated as the percentage of geminin-positive (GMN^+) cells with ≥2 RAD51 foci. Abbreviations: HRP = homologous recombination proficient, HRI = homologous recombination intermediate, HRD = homologous recombination deficient, RECAP = REcombination CAPacity, LOH = loss of heterozygosity, VUS = variant of uncertain significance, NGS = next-generation sequencing, TNBC = triple-negative breast cancer.


RAD51-FFPE score (%)
100
90
80
70
60
50
40
30
20
10
0
BC-37 BC-52 BC-63 BC-59 BC-16 BC-21 BC-39 BC-58 BC-23 BC-41 BC-06 BC-61 BC-51 BC-08 BC-12 BC-47 BC-04 BC-28 BC-53 BC-07 BC-26 BC-30 BC-48 BC-49 BC-10 BC-14 BC-15 BC-03 BC-34 BC-43 BC-38 BC-09 BC-11 BC-02 BC-33 BC-40 BC-05 BC-57 BC-19 BC-29 BC-27 BC-01 BC-45 BC-20 BC-46 BC-36 BC-50 BC-44 BC-54 BC-31 BC-32 BC-42 BC-55 BC-60 BC-13 BC-17 BC-18 BC-25 BC-35 BC-22 BC-24 BC-56 BC-62
RECAP status
NGS
BRCA1
BRCA2
TP53
ATM
BARD1
BRIP1
CHEK2
PALB2
RAD54L
RAD51B
RAD51D
Promotor hypermethylation
BRCA1
Histology
Tumor grade
TNBC
RAD51-FFPE status
HRP
HRD
RECAP status
HRP
HRI
HRD
Unknown
NGS
Pathogenic variant
VUS
No somatic mutation
Unknown
LOH
Promotor hypermethylation
BRCA1 methylation
No BRCA1 methylation
Failed
Unknown
Histology
A = Apocrine
C = Cribriform
L= Lobulair
M = metastasis adenocarcinoma
N = No Special Type
P = Papillair
TNBC
Yes
No

Among the 40 samples that were classified as HRP, no PVs were identified in *BRCA1* or *BRCA2*. In one sample a *CHEK2* PV with a VAF ≥0.50 and LOH of the wild type allele was identified (BC-53). A PV in *ATM* with a VAF <0.5 was identified (BC-26). Various VUSes in *ATM*, *BRCA2*, *CHEK2*, *RAD51B*, and *RAD54L* were identified among the HRP samples, of which five had a VAF ≥0.50 (Fig. 2, Table S3).

All genetic variants identified are listed in Table S3. *BRCA1* promoter hypermethylation was identified in one HRP (BC-08) and in one HRD sample (BC-50).

In conclusion, the sensitivity of the identification of BC samples with a PV in *BRCA1* and/or *BRCA2* was 100% in our cohort.

3.3 The RAD51-FFPE test identifies RECAP-HRD BC samples with high sensitivity

To determine the sensitivity and specificity of the RAD51-FFPE test to identify functional HRD we performed the RECAP test on viable, cryopreserved matching tumor samples on the premise that the HRD status as determined by the RECAP test faithfully represented the functional HR status.

3.3.1 The RECAP test

The RECAP test is a RAD51-based functional HRD test using viable, tumor tissue for the identification of HRD in BC [23,28,30,31]. In contrast to the RAD51-FFPE test, tumor tissue is irradiated with 5 Gy ionizing radiation to induce DNA damage prior to fixation. As we had access to cryopreserved tumor tissue from the 63 BC samples in our cohort with informative RAD51-FFPE scores, we determined RECAP scores on matched tumor samples. Quality control steps involved assessment of tissue quality to determine tissue vitality and the presence of ≥40 GMN^+ tumor cells [26]. Out of 63 BC samples, a total of 14 samples were excluded based on these criteria (Fig. S3). In total, RECAP scores were determined for 49/63 (78%) BC samples, of which eight (16%) were classified as HRD, three (6%) as HR-Intermediate (HRI) and 38 (78%) as HRP (Fig. 2, Fig. S3). Fig. 3 shows representative immunofluorescence (IF) images of HRP and HRD samples from matched RECAP and RAD51-FFPE samples.

3.3.2 The correlation between RECAP and RAD51-FFPE scores

For 49 BC samples both RAD51-FFPE and RECAP scores were obtained (Fig. 2 and 4). The RAD51-FFPE test showed a high sensitivity to identify HRD BC samples as defined by the RECAP test (RECAP-HRD) as seven out of the eight RECAP-HRD BC samples were identified as HRD by the RAD51-FFPE test, including two samples with a *BRCA1/2* PV (Fig.2 and 4, Table S4). One RECAP-HRD sample (BC-21, RECAP score 0%) was scored as HRP (68%) by the RAD51-FFPE test (Fig. 4, Table S1).

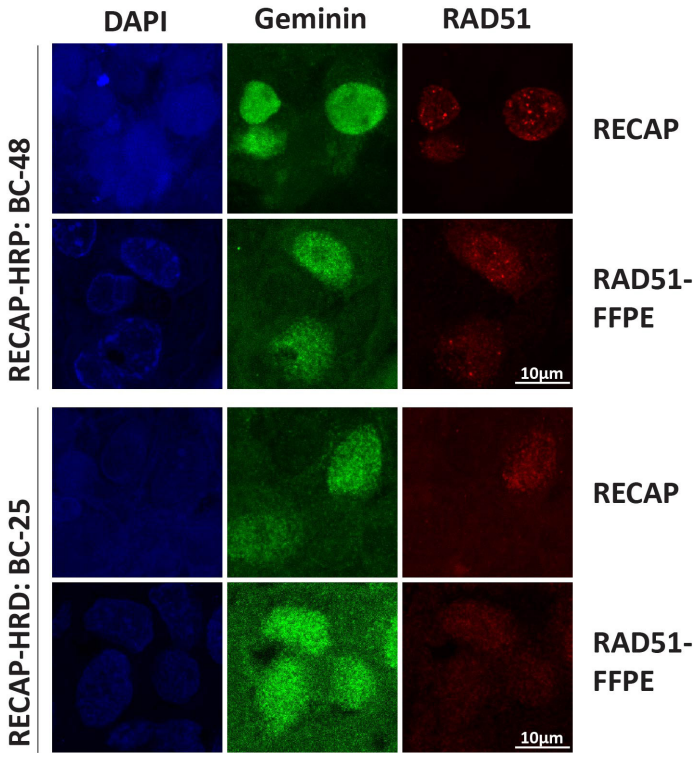


Fig. 3. Microscopy illustration of RECAP and diagnostic FFPE immunofluorescence slides of an homologous recombination proficient (HRP) and homologous recombination deficient (HRD) BC. Abbreviations: BC = breast cancer, RECAP = REcombination CAPacity, FFPE = formalin-fixed paraffin-embedded, HRP = homologous recombination proficient, HRD = homologous recombination deficient.

For the 38 BC samples classified as RECAP-HRP, 29 samples were classified as HRP by both tests. Nine samples were classified as HRD by the RAD51-FFPE test (Fig. 2 and 4, Table S4).

Using the test parameters previously established for OC and EC (HRD threshold of 15% with a RAD51-FFPE foci cut-off of two), the RAD51-FFPE test demonstrated an 88% sensitivity and 76% specificity in identifying the same HR-class as defined by the RECAP test [27]. Adjusting the RAD51 foci cut-off and/or the percentage of $RAD51^{+}/GMN^{+}$ cells to define HRD did not improve the sensitivity and specificity of the RAD51-FFPE test (Fig. S4 and Table S6).

The three RECAP-HRI samples were not taken along for the calculation of the sensitivity and specificity of the RAD51-FFPE test as the RAD51-FFPE test does not have an HRI scoring class.

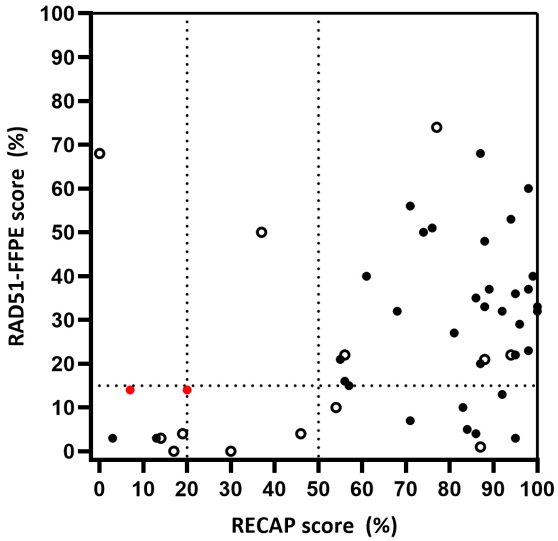


Fig. 4. RECAP versus RAD51-FFPE scores (*n* = 49). RAD51-FFPE scores were determined as the percentage of geminin-positive cells with at least two RAD51 foci. RECAP scores were determined as the percentage of geminin-positive cells with at least five RAD51 foci. HRD thresholds for the RAD51-FFPE test and RECAP test are indicated with dashed lines. Samples with a pathogenic variant in *BRCA1* and/or *BRCA2* are indicated in red. TNBC samples are indicated with open circles. Abbreviations: FFPE = formalin-fixed paraffin-embedded, RECAP = REcombination CAPacity, TNBC = triple-negative breast cancer.

3.4 HRD is commonly identified among TNBC and TP53 mutated breast cancers

Our cohort consisted of a heterogenous set of BC samples with different histological subtypes (Table S1). To investigate if HRD is more prevalent in specific tumor types, we stratified clinicopathologic characteristics by HR-status as determined by the RAD51-FFPE test (Table 1). Although not statistically significant, HRD tumors were more often observed in TNBC, 9/15 (60%) and in *TP53* mutated BC, 12/17 (71%) (Table 1).

No differences were observed in tumor grade and age at diagnosis between the HRD and HRP groups (p = 0.141 and p = 0.080 respectively, Table 1). The tumor samples derived from pleural fluid at recurrent disease (n = 10) were all classified as HRP (p = 0.010, Table 1). No differences in clinicopathologic characteristics between included and excluded RAD51-FFPE samples were observed (Table S2).

Stratification of clinicopathologic characteristics based on HR group classification by the RECAP test displayed only a significant difference between HRD and HRP groups for the presence of *BRCA1/2* PVs (Table S5).

Table 1. Clinicopathologic characteristics stratified for HR status as determined with the RAD51-FFPE test. Differences between the HRD and HRP groups were statistically tested with a Student's t-test for the age at diagnosis and the chi-square or Fisher's Exact test for the other characteristics.

	***HRD (*n* = 23) *n* (%)**	***HRP (*n* = 40) *n* (%)**	***P*-value**
Age at diagnosis (Years ± SEM)	67.9 (±3.09)	61.7 (±1.98)	$p = 0.080$
Tumor			**$p = 0.010$[a]**
Primary	23 (100)	30 (75)	
Recurrent		10 (25)	
Histological subtype			$p = 0.531$[b]
No special type (NST)	17 (74)	20 (67)	
Lobular	4 (17)	9 (30)	
Other			
Papillary		1 (3)	
Apocrine	1 (4)		
Cribriform	1 (4)		
Tumor grade			$p = 0.141$[c]
1	1 (4)	4 (10)	
2	8 (35)	15 (38)	
3	14 (61)	11 (28)	
NA		10 (25)	
Hormone receptor status			$p = 0.063$[d]
TNBC	9 (39)	6 (15)	
Other			
ER+/PR+/Her2Neu-	11 (48)	23 (58)	
ER+/PR+/Her2Neu+	1 (4)	3 (8)	
ER+/PR-/Her2Neu-	2 (8)	4 (10)	
ER+/PR-/Her2Neu+	-	3 (8)	
ER-/PR-/Her2Neu+	-	1 (3)	
***TP53* PV**			$p = 0.053$[e]
Yes	12 (52)	5 (21)	
No	11 (48)	19 (79)	
***BRCA1/2* PV**			$p = 0.068$[e]
Yes	3 (13)		
No	20 (87)	32 (100)	
***BRCA1* promoter hypermethylation**			$p = 1.000$[a]
Yes	1 (5)	1 (3)	
No	21 (95)	28 (97)	

Abbreviations: NA = not applicable, HRD = homologous recombination deficient, HRP = homologous recombination proficient, PV = pathogenic variant. *Due to rounding corrections, the total percentage is not always 100%. a) Fisher's exact. b) Chi-square, 'NST vs 'lobular'. c) Chi-square, 'grade 1-2' vs 'grade 3'. d) Chi-square, 'TNBC' vs 'other'. e) Chi-square.

4. Discussion

Here, we determined the sensitivity and specificity of the RAD51-FFPE test in a diagnostic series of BC using the *ex vivo* RAD51-based HRD test (RECAP test) and *BRCA1/2* deficiency as gold standards for HRD classification.

In this study, RAD51 scores were successfully determined for 63 BC samples. Thirty-seven percent of the BC samples in our cohort was identified as HRD using the RAD51-FFPE test, including three tumors with a *BRCA1/2* PV. The prevalence of HRD was higher among TNBC (60%) and tumors with *TP53* PVs (71%), which is in line with results obtained in other studies evaluating the prevalence of HRD with a RAD51-based HRD test on diagnostic TNBC tumor samples [21,33,38].

Two samples harbored a pathogenic variant in *CHEK2*, one with LOH of the wild type allele, classified as HRP (BC-53) and one with unknown LOH status of the wildtype allele that was classified as HRD (BC-36). These results are in line with findings indicating that loss of CHK2 activity does not lead to HRD [39,40]. Similarly, breast tumors with *ATM* PVs lack HRD-related mutational signatures, in line with our functional classification of sample BC-26 with an *ATM* PV as HRP [41]. In six BC samples VUSes with a VAF≥0.50 were identified in *ATM*, *BRIP1*, *CHEK2*, *BRCA2*, *RAD51B*, and *RAD54L* with LOH of the wild type allele in two samples. The c.2240A>G, p.(Glu747Gly) VUS in *BRCA2* is located outside the known functional domains and has been reported not to affect protein function, in line with the observed HRP phenotype of the tumor [42]. One HRD sample harbored a *BRIP1* VUS c.2768T>G, p.(Leu923Arg) with loss of the wildtype allele. Based on the available information, it is unlikely that this variant can explain the HRD phenotype observed in the sample. Not only is the effect of the missense variant on BRIP1 activity yet unknown, previous research in cell lines showed that *BRIP1* deficiency does not impair RAD51 foci formation [43].

The RAD51-FFPE test correctly identified the three *BRCA1/2* deficient tumors present in our set as HRD. The sensitivity for the identification of functional HRD as defined by the RECAP test was 88% with a specificity of 76% using previously validated thresholds for OC and EC [27]. The observed frequency of 16% HRD in our RECAP analyses is in line with previously reported RECAP results in a cohort of 125 BC [23]. Using the RAD51-FFPE test, we observed a higher frequency of HRD samples compared to the RECAP test (Table S4). It is important to realize that there are differences in the nature and quantity of DNA double strand breaks and the size of the RAD51 foci between the endogenous (RAD51-FFPE) and radiation-induced (RECAP) DNA damage. In addition, it cannot be ruled out that time-to-fixation might play a role as has previously been described for HER2 assessment [44,45]. While the fixation period is exactly two hours for all RECAP samples, it varies considerably

for the RAD51-FFPE samples due to their diagnostic nature. If the detection of RAD51 foci is compromised in samples with suboptimal time-to-fixation (potentially affecting the immune reactivity of the protein), this might explain the false positive HRD samples observed. However, further investigation is required to explore this possibility.

Given the high incidence of BC and the relatively low frequency of PVs in *BRCA1* or *BRCA2* in these carcinomas, there is a clear demand for a sensitive method to enrich for HRD tumors in a fast and cost-effective manner. The availability of a sensitive, non-DNA-based, method like the RAD51-FFPE test addresses this need and fills an important gap in the diagnostic landscape, as in contrast to the RECAP test, no *ex vivo* irradiated fresh tumor tissue is required. Although the RAD51-FFPE test may overestimate the number of 'true' HRD samples, it faithfully captured all *BRCA1/2* deficient samples. It therefore offers a reliable and efficient method with a success rate of more than 90% to identify HRD tumors even in situations where DNA-based testing is challenging or not readily accessible in routine clinical practice. This is particularly important considering the potential implications for treatment decisions and patient stratification.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Author contributions

Conceptualization, L.M.v.W., H.V., and M.P.G.V.; Data curation, L.M.v.W., S.V., N.T.t.H. and D.C.; Formal analysis, L.M.v.W., S.V., C.J.H.K., N.T.t.H., and D.T.; Funding acquisition, H.V., and M.P.G.V.; Investigation, L.M.v.W., S.V. and N.T.t.H.; Methodology, L.M.v.W., H.V. and M.P.G.V.; Project administration, M.P.G.V.; Resources, M.P.G.V.; Supervision, H.V. and M.P.G.V.; Visualization, L.M.v.W.; Writing—original draft, L.M.v.W., H.V. and M.P.G.V.; Writing—review & editing, N.T.t.H., C.J.H.K., and D.C.

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Data availability

All data generated or analysed during this study are included in this published article (and its supplementary information files).

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Supplementary Information

Additional analyses

Adjustments of RAD51 foci cut-off and HRD threshold to define HRD in BC

The HRD threshold and RAD51 foci cut-off for the evaluation of HR status of breast FFPE samples has been previously described at 10% with a RAD51 foci cut-off of five [24,25,33-35], while an HRD threshold of 10% and 20% with a RAD51 foci cut-off of one and five respectively, was applied when biopsies were analyzed from TNBC patients after receiving neoadjuvant chemotherapy or rucaparib treatment [21,46]. Since our BC cohort was heterogeneous, including tumor samples with different histological subtypes and treatment history (Suppl. Table 1), we explored whether changing the RAD51-FFPE test parameters would lead to an increase in sensitivity and/or specificity. A sensitivity and specificity analysis for commonly described HRD thresholds (5,10, 15, 20%) and RAD51 foci cut-offs (1, 2, 3, 4 or 5) showed that the highest sensitivity and specificity of 88% and 76% respectively was reached with an HRD threshold of 5% with a RAD51 foci cut-off of four, and with an HRD threshold of 15% and a RAD51 foci cut-off of two, as applied in our study (Table S6).

To investigate whether alternative, not previously described HRD thresholds, would reach a better sensitivity and specificity using RAD51-FFPE scores to classify tumors with the RECAP outcome as gold standard, Receiver Operating Characteristic (ROC) curves were plotted and HRD thresholds were applied ranging from 0-100% with a 1% step size (Fig. S4). Importantly, RAD51-FFPE foci cut-offs of 2, 3 and 4 led to a very good performance with area under the curve (AUC) scores > 0.8 (0.804, 0.814, and 0.817 respectively). The highest sensitivity and specificity was reached when applying an HRD threshold of 7% with a RAD51 foci cut-off of three, leading to a sensitivity of 88% with a specificity of 84% (Fig. S4C). The second best test parameters were an HRD threshold of 15% with a RAD51 foci cut-off of two, a 5% HRD threshold with a RAD51 foci cut-off of four, and a 4% HRD threshold with a RAD51 foci cut-off of five, all leading to a sensitivity of 88% with a specificity of 79% for the identification RECAP-HRD. All RAD51-FFPE test parameter combinations led to a 100% sensitivity for the identification of samples with a *BRCA1/2* PV.

Supplementary Figures

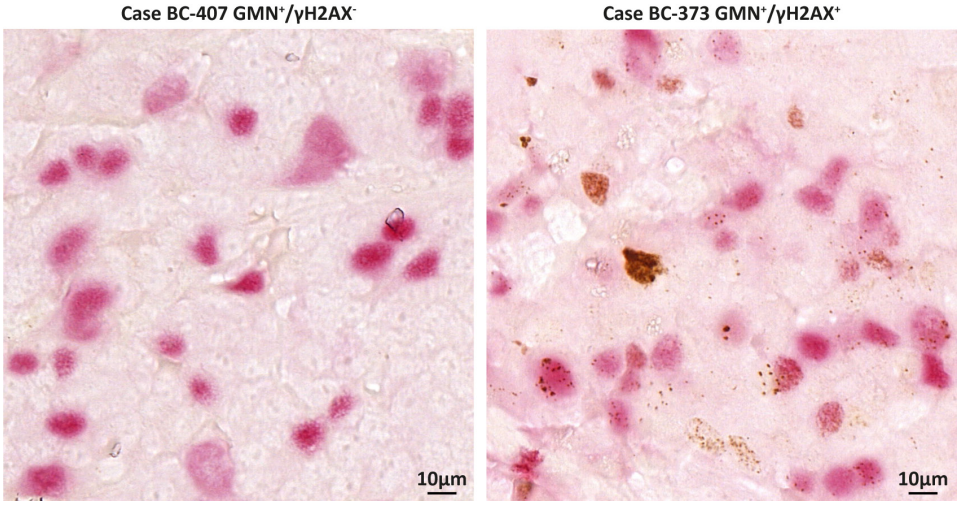


Fig. S1. Immunohistochemical slides of tumors with a low and high γH2AX score. Geminin (GMN, pink)/γH2AX (brown) immunohistochemical slides of a tumor with a γH2AX score <25% (left) and ≥25% (right).

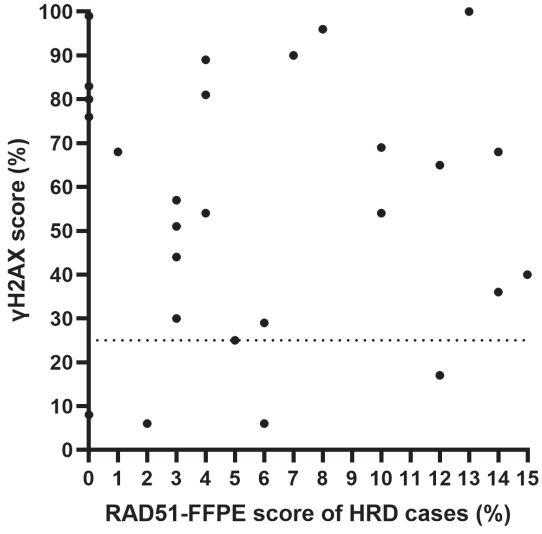


Fig. S2. γH2AX scores of RAD51-FFPE HRD samples. In total, 27 samples had a RAD51-FFPE score ≤15% and were stained for GMN/γH2AX by IHC and γH2AX scores (% GMN+ cells with ≥2 γH2AX foci) were calculated**.** Samples with a γH2AX score <25%, were excluded from analysis. Twenty-three samples had a γH2AX score ≥25%. Abbreviations: FFPE = formalin-fixed paraffin-embedded, HRD = homologous recombination deficient, GMN = geminin.

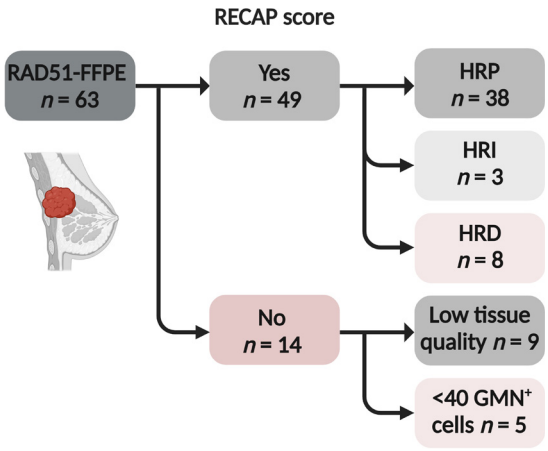
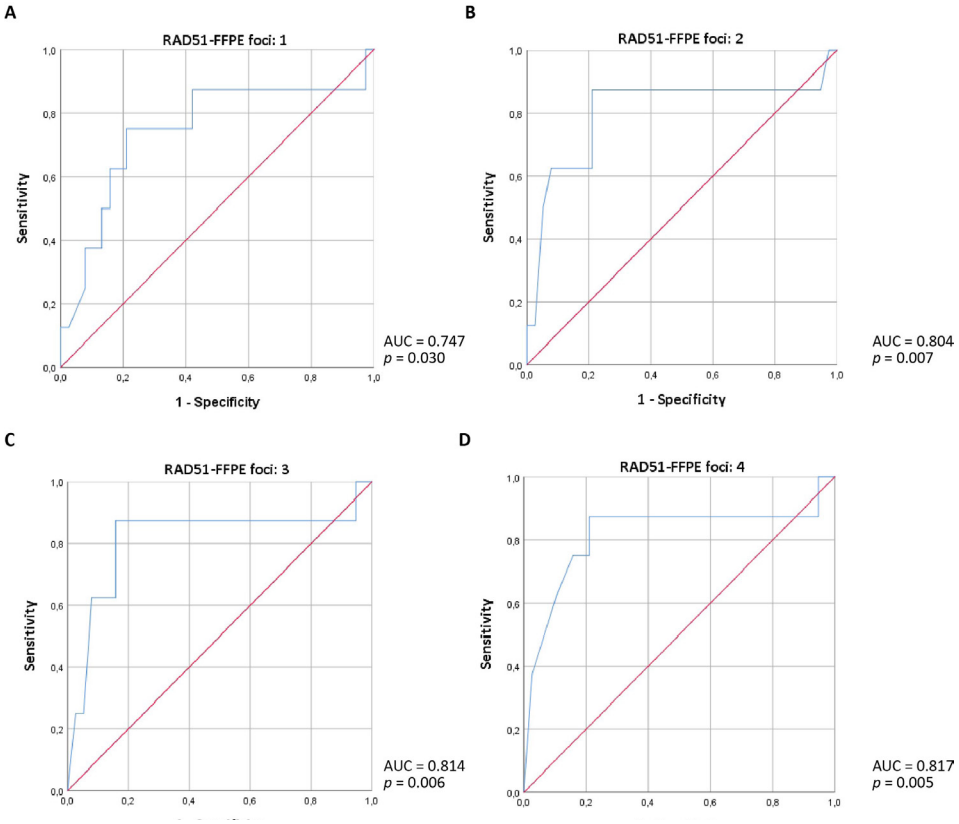


Fig. S3. Flowchart for the inclusion of RECAP samples. For the 63 FFPE samples with an informative RAD51-FFPE score, the RECAP test was performed using the available cryopreserved tumor tissue. RECAP scores were successfully calculated for 49 samples. For 14 samples, no RECAP scores were calculated due to insufficient tissue quality (no vital tissue and/or a low tumor cell percentage) or number of geminin-positive (GMN^+) cells (<40).



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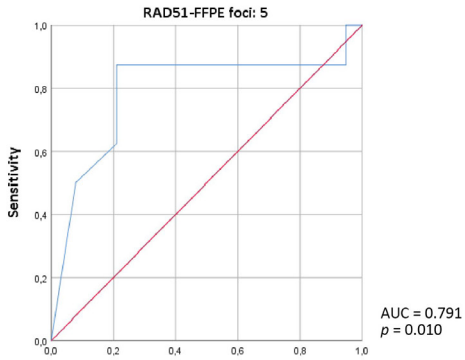

Fig. S4. ROC curves for RAD51-FFPE scores with a foci cut-off ranging from 1 till 5. HR classification based on RECAP scores was used as the gold standard. Tumors with RECAP scores ≤20% were considered HRD and tumors with RECAP scores of >50% were considered HRP. RECAP-HRI samples were excluded from analysis. A) ROC curve for RAD51-FFPE scores with a cut-off of 1 foci. AUC = 0.747, *p* = 0.030 and 95% CI (0.528-0.966). Highest sensitivity with most optimal specificity was reached at an HRD threshold of 32%, with a sensitivity of 88% and a specificity of 58%. B) ROC curve for RAD51-FFPE scores with a cut-off of 2 foci. AUC = 0.804, p = 0.007 and 95% CI (0.589-1.000). Highest sensitivity with most optimal specificity was reached at an HRD threshold of 15%, with a sensitivity of 88% and a specificity of 79%. C) ROC curve for RAD51-FFPE scores with a cut-off of 3 foci. AUC = 0.814, p = 0.006 and 95% CI (0.602-1.000). Highest sensitivity with most optimal specificity was reached at an HRD threshold of 7%, with a sensitivity of 88% and a specificity of 84%. D) ROC curve for RAD51-FFPE scores with a cut-off of 4 foci. AUC = 0.817, *p* = 0.005 and 95% CI (0.605-1.000). Highest sensitivity with most optimal specificity was reached at an HRD threshold of 5%, with a sensitivity of 88% and a specificity of 79%. E) ROC curve for RAD51-FFPE scores with a cut-off of 5 foci. AUC = 0.791, *p* = 0.010 and 95% CI (0.580-1.000). Highest sensitivity with most optimal specificity was reached at an HRD threshold of 4%, with a sensitivity of 88% and a specificity of 79%. Abbreviations: FFPE = formalin-fixed paraffin-embedded, AUC = area under curve, ROC = Receiver Operating Characteristic.

Supplementary Tables

Table S1. Overview tumor characteristics informative study cohort *(n = 63).*

Code	RAD51-FFPE score	RECAP score	Tumor grade (NA for recurrent disease samples)	TNBC Yes/No	ER	PR	HER2 Neu	PV in HR gene(s) (LOH wildtype allele yes/no)	*TP53* PV (LOH *wt* allele yes/no)	*BRCA1* promoter methylation
BC-01#	14%	7%	2	No	+	+	-	*BRCA2* (yes)	No	No
BC-02	22%	94%	2	Yes	-	-	-	No	Yes (yes)	No
BC-03	32%	68%	2	No	+	+	-	No	No	No
BC-04	40%	61%	3	No	+	+	-	No	No	Unknown
BC-05	21%	88%	NA	Yes	-	-	-	No	Yes (yes)	Failed****
BC-06	52%	Failed QC	2	No	+	-	-	No	No	No
BC-07	37%	Failed QC	2	No	+	-	-	No**	Unknown	No
BC-08	50%	37%	NA	Yes	-	-	-	No	Yes (yes)	Yes
BC-09	27%	81%	3	No	+	-	+	No	No	No
BC-10	33%	100%	3	No	+	+	-	No	No	Failed****
BC-11	23%	98%	1	No	+	+	-	Unknown***	No	No
BC-12	50%	74%	NA	No	+	+	+	Unknown***	Unknown	Unknown
BC-13	3%	13%	2	No	+	+	-	No	Yes (yes)	No
BC-14	33%	88%	2	No	+	+	-	Unknown***	No	No
BC-15	33%	Failed QC	2	No	+	+	-	No	No	No
BC-16	68%	87%	NA	No	-	-	+	Unknown***	Unknown	Unknown
BC-17	3%	3%	3	No	+	+	-	No	No	No
BC-18	3%	95%	2	No	+	+	-	No	No	No
BC-19	20%	87%	3	No	+	+	-	No	No	No
BC-20	13%	92%	2	No	+	+	-	No	No	No
BC-21	68%	0%	NA	Yes	-	-	-	No	Yes (yes)	No
BC-22	0%	17%	3	Yes	-	-	-	No	Yes (yes)	No
BC-23	56%	71%	1	No	+	+	-	No	No	No

BC-24	0%	30%	3	Yes	-	-	-	No	Yes (yes)	No
BC-25	3%	14%	3	Yes	-	-	-	No	Yes (yes)	No
BC-26*	37%	98%	2	No	+	-	-	*ATM* (no)	No	No
BC-27	15%	57%	2	No	+	+	-	No	Yes (unknown)	Unknown ***
BC-28	40%	99%	2	No	+	+	-	No**	Unknown***	Unknown ***
BC-29	16%	56%	3	No	+	-	+	No**	No	Unknown ***
BC-30	37%	89%	3	No	+	+	+	No**	No	No
BC-31	6%	Failed QC	1	No	+	+	-	No	No	No
BC-32	5%	84%	2	No	+	+	-	No	No	No
BC-33*	22%	56%	NA	Yes	-	-	-	No	Yes (yes)	No
BC-34	32%	92%	2	No	+	+	-	No**	No	No
BC-35	1%	87%	3	Yes	-	-	-	No	Yes (yes)	No
BC-36	10%	54%	2	Yes	-	-	-	*CHEK2* (no)	Yes (yes)	No
BC-37	74%	Failed QC	NA	No	+	+	-	No	No	Unknown ***
BC-38	29%	96%	2	No	+	+	-	No**	No	No
BC-39	60%	98%	3	No	+	+	-	Unknown***	Unknown***	No
BC-40	22%	95%	1	No	+	+	-	No**	No	No
BC-41	53%	94%	NA	No	+	+	-	No**	Unknown***	No
BC-42	4%	19%	3	Yes	-	-	-	No	Yes (yes)	No
BC-43	32%	100%	2	No	+	-	-	Unknown***	No	Unknown ***
BC-44	8%	Failed QC	3	Yes	-	-	-	*BRCA1* (yes)	Yes (yes)	No
BC-45	14%	20%	3	No	+	+	+	*BRCA1* and *BRCA2* (yes)	No	No
BC-46	12%	Failed QC	2	No	+	+	-	No	No	No
BC-47	48%	88%	3	No	+	+	-	No	No	No
BC-48	36%	95%	3	No	+	+	-	No	No	No

Table S1 continued

Code	RAD51-FFPE score	RECAP score	Tumor grade	TNBC Yes/No	ER	PR	HER2 Neu	PV in HR gene(s) (LOH wildtype allele yes/no)	*TP53* PV (LOH *wt* allele yes/no)	*BRCA1* promoter methylation
BC-49	35%	86%	2	No	+	+	-	No	No	No
BC-50	10%	83%	3	No	+	+	-	No	No	Yes
BC-51	51%	76%	3	No	+	-	+	No	No	No
BC-52	74%	77%	NA	Yes	-	-	-	Unknown***	No	Unknown ***
BC-53	39%	Failed QC	2	No	+	+	-	*CHEK2* (yes)	No	No
BC-54	7%	71%	3	No	+	+	-	No	No	No
BC-55	4%	86%	3	No	+	-	-	No	Yes (yes)	No
BC-56	0%	Failed QC	3	Yes	-	-	-	No	Yes (yes)	No
BC-57	21%	55%	NA	No	+	+	-	Unknown***	No	Failed****
BC-58	59%	Failed QC	2	No	+	+	-	No	No	No
BC-59	70%	Failed QC	1	No	+	+	+	No	No	No
BC-60	4%	46%	3	Yes	-	-	-	No	Yes (yes)	No
BC-61	52%	Failed QC	2	No	+	+	-	No	No	No
BC-62	0%	Failed QC	3	No	+	-	-	No	No	No
BC-63	72%	Failed QC	3	No	+	-	-	No	No	No

Abbreviations: FFPE = formalin-fixed paraffin-embedded, RECAP = REcombination CAPacity, TNBC = triple negative breast cancer, PV = pathogenic variant, QC = quality control, NA = not applicable, NGS = next-generation sequencing. # This sample was obtained from a man. *Tumor samples that were obtained after neoadjuvant chemotherapy (NACT) treatment. **Tumor samples were only sequenced for *BRCA1* and *BRCA2*. ***Tumor samples were not sequenced. ****NGS performed, but failed.

Table S2. Comparison of clinicopathologic characteristics between included and excluded RAD51-FFPE BC samples. Differences between the successful and not successful groups were statistically tested with a Student's t-test for the age at diagnosis and Fisher's Exact test for the other characteristics.

	Included* **(*n* = 63)** ***n* (%)**	**Excluded *** **(*n* = 11)** ***n* (%)**	***p*-value**
Age at diagnosis (Years ±SEM)	63.9 (1.72)	61.7 (4.38)	$p = 0.622$
Tumor			
Primary	53 (88)	11 (100)	$p = 0.341$[a]
Recurrent	10 (12)		
Histological subtype			$p = 0.427$[b]
No special type (NST)	37 (70)	9 (82)	
Lobular	13 (25)	1 (9)	
Other			
Papillary	1 (2)	1 (9)	
Apocrine	1 (2)		
Cribriform	1 (2)		
Tumor grade			$p = 0.741$[c]
1	5 (8)	3 (27)	
2	23 (37)	4 (36)	
3	25 (40)	4 (36)	
N/A	10 (16)		
Hormone receptor status			$p = 0.437$[d]
ER+/PR+/Her2Neu-	34 (54)	6 (55)	
ER+/PR+/Her2Neu+	4 (6)		
ER+/PR-/Her2Neu-	6 (10)	2 (18)	
ER+/PR-/Her2Neu+	3 (5)	1 (9)	
ER-/PR-/Her2Neu+	1 (2)	1 (9)	
TNBC	15 (24)	1 (9)	
****NACT**			$p = 1.000$[a]
Yes	2 (3)		
No	61 (97)	11 (100)	

Abbreviations: N/A = not applicable, HRD = homologous recombination deficient, HRP = homologous recombination proficient, NACT = neoadjuvant chemotherapy. *Due to rounding corrections, the total percentage is not always 100%. **Tumor samples that were obtained after NACT treatment. a) Fisher's exact. b) Fisher's exact, 'NST' vs 'Lobular'. c) Fisher's exact, 'grade 1-2' vs 'grade 3'. d) Fisher's exact, 'TNBC' vs 'other'.

Table S3. Overview genetic variants identified in our BC cohort.

Code	RAD51-FFPE HR status	RECAP HR status	Gene	Variant	Class *	VAF	LOH yes/no	Reported in literature
BC-01	HRD	HRD	*BRCA2*	NM_000059.3:c.9154C>T, p. (Arg3052Trp)	5	0.75	Yes	
BC-02	HRP	HRP	*TP53*	NM_000546.5:c.659A>G, p.(Tyr220Cys)	5	0.69	Yes (1 SNP)	
			PIK3CA	NM_006218.4:c.1624G>A, p.(Glu542Lys)	5	0.65	Yes	
BC-03	HRP	HRP	*BARD1*	NM_000465.4:c.1694G>A, p.(Arg565His)	3	0.35	No	
BC-05	HRP	HRP	*TP53*	NM_000546.5:c.785G>T, p.(Gly262Val)	4	0.71	Yes (1 SNP)	
			PIK3CA	NM_006218.4:c.1035T>A, p.(Asn345Lys)	4	0.49	No	
BC-08	HRD	HRI	*TP53*	NM_000546.5:c.328delC, p. (Arg110ValfsTer13)	4	0.97	Yes	
BC-09	HRP	HRP	*PALB2*	NM_024675.4:c.1099G>A, p.(Glu367Lys)	3	0.48	No	
BC-13	HRD	HRD	*TP53*	NM_000546.5:c.536A>G, p.(His179Arg)	5	0.80	Yes	
				NM_001126114.2:c.1025A>C, p.(Ter342Ser)	3	0.88	Yes	
BC-15	HRP	N/A	*PIK3CA*	NM_006218.4:c.1624G>A, p.(Glu542Lys)	5	0.37	Unknown	
BC-20	HRD	HRP	*PIK3CA*	NM_006218.4:c.1624G>A, p.(Glu542Lys)	5	0.40	Unknown	
BC-21	HRP	HRD	*TP53*	NM_000546.5:c.580_582delCTT, p.(Leu194del)	4	0.85	Yes	
BC-22	HRD	HRD	*TP53*	NM_000546.5:c.916C>T, p.(Arg306*)	5	0.89	Yes	
BC-23	HRP	HRP	*PIK3CA*	NM_006218.4:c.1633G>A, p.(Glu545Lys)	5	0.28	No	
BC-24	HRD	HRI	*TP53*	NM_000546.5:c.517G>C, p.(Val173Leu)	5	0.35	Yes	
			RAD54L	NM_003579.4:c.777G>T, p.(Met259Ile)	3	0.09	No	
			RAD51B	NM_133509.4:c.854-3C>T	3	0.11	No	
			ATM	NM_000051.3:c.2867G>A, p.(Gly956Glu)	3	0.10	Yes	
BC-25	HRD	HRD	*TP53*	NM_000546.5:c.581T>G, p.(Leu194Arg)	4	0.85	Yes	
BC-26	HRP	HRP	*BRCA2*	NM_000059.3:c.1265A>C, p.(Asn422Thr)	3	0.14	Yes	
			CHEK2	NM_007194.4:c.480A>G, p.Ile160Met	3	0.28	No	
			ATM	NM_000051.3:c.6733G>T, p.(Glu2245Ter)	4	0.12	No	
				NM_000051.3:c.8213T>G, p.(Leu2738Ter)	5	0.11	No	

BC-27	HRD	HRP	*PIK3CA*	NM_006218.4:c.3140A>G, p.(His1047Arg)	5	0.04	Yes	
			TP53	NM_000546.5:c.814G>A, p.(Val272Met)	4	0.01	Unknown	
BC-28	HRP	HRP	*BRCA2*	NM_000059.3:c.2240A>G, p.(Glu747Gly)	3	0.63	Yes	Functional, Ikegami *et al,* 2020 [42]
BC-30	HRP	HRP	*BRCA2*	NM_000059.3:c.10123A>C, p.(Ser3375Arg)	3	0.48	No	
BC-32	HRD	HRP	*TP53*	NM_001126114.2:c.1025A>C, p.(Ter342Ser)	3	0.66	Yes	
BC-33	HRP	HRP	*TP53*	NM_000546.5:c.722C>T, p.(Ser241Phe)	5	0.90	Yes	
			PIK3CA	NM_006218.4:c.241G>A, p.(Glu81Lys)	4	0.19	No	
			ATM	NM_000051.3:c.115A>G, p.(Thr39Ala)	3	0.50	No	
BC-35	HRD	HRP	*TP53*	NM_000546.5:c.578A>G, p.(His193Arg)	4	0.81	Yes	
BC-36	HRD	HRP	*CHEK2*	NM_007194.4:c.1100delC, p.(Thr367Metfs*15)	5	0.46	Unknown	
			TP53	NM_000546.5:c.541C>T, p.(Arg181Cys)	5	0.41	Yes	
			PIK3CA	NM_006218.4:c.3140A>G, p.(His1047Arg)	5	0.32	No	
BC-37	HRP	N/A	*RAD51B*	NM_133509.4:c.315+2T>A, p.(?)	3	0.43	No	
				NM_133509.4:c.997T>C, p.(Phe333Leu)	3	0.50	No	
BC-42	HRD	HRD	*TP53*	NM_000546.5:c.785G>T, p.(Gly262Val)	4	0.53	Yes	
			BRIP1	NM_032043.3:c.2768T>G, p.(Leu923Arg)	3	0.75	Yes	
BC-44	HRD	N/A	*TP53*	NM_000546.5:c.637C>T, p.(Arg213*)	5	0.67	Yes	
			BRCA1	NM_007294.4:c.5137delG, p.(Val1713Ter)	4	0.23	Yes	
BC-45	HRD	HRD	*BRCA1*	NM_007294.4:c.2359dupG, p.(Glu787GlyfsTer3)	4	0.7	Yes	
			BRCA2	NM_000059.3:c.3865_3868delAAAT,p.(Lys1289AlafsTer3)	4	0.85	Yes	
			PIK3CA	NM_006218.4:c.1633G>A, p.(Glu545Lys)	5	0.24	Unknown	
BC-47	HRP	HRP	*BRCA2*	NM_000059.3:c.5282G>C, p.(Gly1761Ala)	3	0.11	Unknown	
			RAD51D	NM_002878.3:c.515C>T, p.(Ala172Val)	3	0.21	Unknown	
BC-49	HRP	HRP	*PIK3CA*	NM_006218.4:c.3140A>G, p.(His1047Arg)	5	0.28	Unknown	
BC-51	HRP	HRP	*PIK3CA*	NM_006218.4:c.1637A>G, p.(Gln546Arg)	5	0.34	Unknown	
			RAD54L	NM_003579.4:c.1526G>A, p.(Arg509Gln)	3	0.58	Unknown	
BC-53	HRP	N/A	*CHEK2*	NM_007194.4:c.1100delC, p.(Thr367Metfs*15)	5	0.79	Yes (1 SNP)	
			PIK3CA	NM_006218.4:c.1633G>A, p.(Glu545Lys)	5	0.36	No	
			BRCA2	NM_000059.3:c.4048C>T, p.(His1350Tyr)	3	0.30	No	

Table S3 continued

Code	RAD51-FFPE HR status	RECAP HR status	Gene	Variant	Class *	VAF	LOH yes/no	Reported in literature
BC-55	HRD	HRP	*PIK3CA*	NM_006218.4:c.3140A>T, p.(His1047Leu)	5	0.37	No	
				NM_006218.4:c.1034A>T, p.(Asn345Ile)	4	0.20	No	
			TP53	NM_000546.5:c.660T>G, p.(Tyr220Ter)	4	0.52	Yes	
BC-56	HRD	N/A	*TP53*	NM_000546.5:c.524G>A, p.(Arg175His)	5	0.75	Yes	
BC-58	HRP	N/A	*PIK3CA*	NM_006218.4:c.1624G>A, p.(Glu542Lys)	5	0.51	No	
			BRCA2	NM_000059.3:c.4828G>A, p.(Val1610Met)	3	0.35	No	
			CHEK2	NM_007194.4:c.556A>C, p.(Asn186His)	3	0.56	Unknown	Functional, Delimitsou *et al.*, 2019 and Boonen *et al.*, 2022 [40,47]
BC-60	HRD	HRI	*TP53*	NM_000546.5:c.377A>G, p.(Tyr126Cys)	5	0.51	Yes	
			PPP2R2A	NM_002717.4:c.547dupA, p.(Ile183AsnfsTer4)	4	0.68	Yes	
			BRCA2	NM_000059.3:c.-40+5G>C, p.(?)	3	0.25	Yes	
BC-61	HRP	N/A	*PIK3CA*	NM_006218.4:c.1624G>A, p.(Glu542Lys)	5	0.24	Unknown	
BC-63	HRP	N/A	*ERBB2*	NM_004448.3:c.2329G>T, p.(Val777Leu)	4	0.41	Unknown	

Table S4. HR classification by the RECAP and RAD51-FFPE tests.

	RECAP HRD	RECAP HRP
RAD51-FFPE HRD	7	9
RAD51-FFPE HRP	1	29

Abbreviations: HRD = homologous recombination deficient, HRP = homologous recombination proficient, FFPE = formalin-fixed paraffin-embedded.

Table S5. Clinicopathologic characteristics stratified for HR status as determined with the RECAP test.

	***HRD (*n* = 8) *n* (%)**	***HRP (*n* = 38) *n* (%)**	***p*-value**
Age at diagnosis (Years ± SEM)	68.3 (±5.52)	61.9 (±2.16)	$p = 0.243^{a}$
Tumor			$p = 1.000^{b}$
Primary	7 (88)	31 (82)	
Recurrent	1 (13)	7 (18)	
Histological subtype (primary)			$p = 1.000^{c}$
No special type (NST)	6 (86)	23 (74)	
Lobular	1 (14)	6 (19)	
Other			
Papillary		1 (3)	
Apocrine		1 (3)	
Cribriform			
Tumor grade			$p = 0.405^{d}$
1	-	3 (8)	
2	2 (25)	14 (37)	
3	5 (63)	14 (37)	
N/A	1 (13)	7 (18)	
Hormone receptor status			$p = 0.055^{e}$
TNBC	4 (50)	6 (16)	
Other			
ER+/PR+/Her2Neu-	3 (38)	23 (61)	
ER+/PR+/Her2Neu+	1 (13)	2 (5)	
ER+/PR-/Her2Neu-	-	3 (8)	
ER+/PR-/Her2Neu+	-	3 (8)	
ER-/PR-/Her2Neu+	-	1 (3)	
***TP53* PV**			$p = 0.206^{b}$
Yes	5 (63)	7 (30)	
No	3 (38)	16 (70)	
***BRCA1/2* PV**			**$p = 0.040^{b}$**
Yes	2 (25)	-	
No	6 (75)	30 (100)	
***BRCA1* promoter hypermethylation**			$p = 1.000^{b}$
Yes	-	1 (3)	
No	8 (100)	29 (97)	

Abbreviations: N/A = not applicable, HRD = homologous recombination deficient, HRP = homologous recombination proficient, PV = pathogenic variant. *Due to rounding corrections, the total percentage is not always exactly 100%. a) Student's t-test. b) Fisher's exact c) Fisher's exact, 'NST' vs 'lobular'. d) Fisher's exact, 'grade 1-2' vs 'grade 3'. e) Fisher's exact, 'TNBC' vs 'other'.

Table S6. Sensitivity and specificity analysis for the RAD51-FFPE test with the RECAP test serving as gold standard. Commonly used HRD threshold and RAD51-FFPE foci cut-off parameters were applied to determine the sensitivity and specifitiy of the RAD51-FFPE test. A 5% HRD threshold with a foci cut-off 4 and a 15% HRD threshold with a foci cut-off of two led to the highest sensitivity and specificity for the identification of RECAP-HRD.

HRD threshold	Foci number	RECAP-HRD *n* = 8	RECAP-HRP *n* = 38	All *n* = 46
5%	**1**	25	92	80
	2	63	89	85
	3	75	84	83
	4	88	76	78
	≥5	88	68	72
10%	**1**	50	87	80
	2	63	82	78
	3	88	71	74
	4	88	61	65
	≥5	88	45	52
15%	**1**	63	82	78
	2	88	76	78
	3	88	63	67
	4	88	37	46
	≥5	88	29	39
20%	**1**	75	76	76
	2	88	71	74
	3	88	39	48
	4	88	26	37
	≥5	88	24	35

Abbreviations: RECAP = Recombination CAPacity test, HRD = homologous recombination deficient, HRP = homologous recombination proficient.