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

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Chapter 5

Exploring homologous recombination deficiency among endometrial, cervical and vulvar carcinomas

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Abstract

Background

Homologous recombination deficiency (HRD) is commonly identified among ovarian cancer and breast cancer patients, who are particularly sensitive to PARP inhibitors (PARPi) and/or platinum-based chemotherapy. Recently it became clear that HRD is also prevalent among endometrial cancers (EC) by the assessment of RAD51 foci at sites of DNA damage after *ex vivo* irradiation of viable tumor tissue (RECAP test). In the current study, we assessed the prevalence of HRD among 94 EC cases, using both the RECAP and RAD51-FFPE test on matching tumor samples. In addition, we assessed if functional HRD could be identified among 28 cervical (CC) and 16 vulvar (VC) carcinomas.

Methodology

EC FFPE samples were subjected to three quality control steps before case data were included for analysis, i.e., general assessment of H&E slides by a pathologist to confirm tumor tissue quality, the calculation of a RAD51-FFPE score with a minimum of 30 geminin-positive (GMN^+) cells, and the calculation of a yH2AX score for samples with a RAD51-FFPE HRD score. To assess the performance of the RAD51-FFPE test, we performed the RECAP test on matching fresh tumor samples. Next, the presence of functional HRD was analysed in the context of the Cancer Genome Atlas Research Network (TCGA) EC molecular subgroups (*POLE* mutant, mismatch repair deficient (MMRd), no specific molecular subtype (NSMP) and p53 abnormal (p53abn)). For CC and VC, the RECAP test was performed on a set of 28 and 16 fresh tumor tissues respectively.

Results

Half of the ECs had a RAD51-FFPE score ≤ 15% and were thus considered HRD. Based on the RECAP test, 16% of ECs were HRD. All RECAP-HRD samples were also RAD51-FFPE HRD. ECs identified as HRD by the RAD51-FFPE test were more likely to be high-grade and non-endometrioid. In addition, patients with RAD51-FFPE HRD tumors were more likely to have advanced-stage disease. HRD was observed in all molecular subgroups, but was most frequently observed in p53abn tumors (83% of p53abn EC). The pilot study with CC and VC revealed no HRD samples.

Conclusion

This study demonstrates that approximately half of EC are HRD based on the RAD51-FFPE test, but a substantially smaller fraction was classified as HRD based on the RECAP test. HRD was commonly observed among p53abn EC samples. HRD testing of EC could

assist in the stratification of patients for treatment with DNA damaging agents, including platinum-based chemotherapy and/or PARP inhibitors. In contrast, HRD was not identified among cervical and vulvar cancer.

1. Introduction

Homologous recombination (HR) is the major DNA damage repair pathway for error-free repair of DNA double-strand breaks (DSBs) [1]. BRCA1 and BRCA2 play key roles in HR, and *BRCA1/2* deficiency is still among the strongest predictive biomarkers for PARPi and platinum-sensitivity [2-4]. However, recent evidence showed that genetic defects in other HR-related genes such as *PALB2, ATM, MRE11, RAD50*, and *RAD51C,* but also epigenetic alterations such as hypermethylation of the *BRCA1* or *RAD51C* gene promoters can lead to HR-Deficiency (HRD) [5-7]. HRD causes genomic instability and accumulation of genetic alterations, due to the fact that HRD cells rely on alternative error-prone DNA repair pathways, consequently leading to the development and progression of cancer [8,9]. At the same time, HRD tumors are particularly sensitive to treatment with PARP inhibitors (PARPi) and/or platinum-based chemotherapy. This led to great efforts to develop reliable HRD tests beyond *BRCA1/2* genetic testing [10]. These HRD tests measure HRD based on the genomic footprint, the so-called 'genomic scar' (loss of heterozygosity (LOH), telomeric allelic imbalances (TAI), large-scale transition (LST)), the presence of HRD mutational signatures, or by functional assessment of HRD based on the presence of RAD51 foci at DSBs [11-15]. The availability of HRD tests led to the identification of HRD among a variety of carcinomas, of which breast cancer (BC) and ovarian cancer (OC) are the most commonly described, followed by prostate and pancreatic cancer [10]. For patients with these carcinomas, PARPi treatment is now available under the prerequisite that the tumor has a *BRCA1/2* pathogenic variant (PV) and/or an HRD score [10]. It became clear, however, that HRD can be identified among other carcinomas for which PARPi treatment is not yet available, including patients with endometrial cancer (EC) [1,16].

EC is the 6th most commonly diagnosed cancer in women worldwide, with 417.000 new cases and 97.000 deaths reported in 2020 [17]. EC is a disease with an heterogenous clinical course and standard treatment of EC consists of surgery and adjuvant pelvic external beam radiotherapy (EBRT) treatment [18]. Approximately 15-20% of EC patients have a high-risk of recurrent disease and disease-related death based on clinicopathological features, including, but not restricted to, advanced stage disease, high-grade tumors and non-endometroid histology (NEEC) [19]. ECs can be classified into four distinct molecular subgroups based on both somatic mutations and copy number alterations: 1) the '*POLE*mut' ECs, characterized by pathogenic mutations in the exonuclease domain of DNA polymerase-ε, resulting in an ultra-high tumor mutational burden, 2) mismatch repair-deficient (MMRd) ECs, which show loss of mismatch repair proteins, resulting in microsatellite instability (MSI-high), 3) p53abn ECs, which have a low tumor mutational burden and high somatic copy number alterations (SCNA-high), and 4) no specific molecular profile (NSMP) ECs, which have no single identifying molecular feature (SCNA-

low) [20]. The four molecular subclasses have been shown to be of prognostic value over the years, with *POLE*mut EC patients having an excellent therapeutic outcome and p53abn EC patients having the poorest prognosis [20-25]. The prognostic performance of these molecular subgroups were reviewed in the context of the international randomized Adjuvant Chemoradiotherapy Versus Radiotherapy Alone in Women with High-Risk Endometrial Cancer (PORTEC-3) trial [22]. The PORTEC-3 trial demonstrated that the addition of adjuvant chemotherapy to EBRT (CTRT) versus EBRT alone had a small but significant benefit in failure-free survival (FFS), especially in NEECs [26]. Another study showed that p53abn ECs benefited the most from the addition of platinum-based chemotherapy to EBRT, independent of histological subtype [22]. Novel trial designs are now directed on further improving the prognosis of patients in the p53abn molecular subgroup by adding targeted adjuvant treatment to CTRT. Following the success of DNA damaging agents, i.e., PARPi and/or platinum- based chemotherapy in the treatment of OC, trial designs, including the Refining Adjuvant treatment IN endometrial cancer Based On molecular features (RAINBO) p53abn-RED trial, are focusing on the role of PARPi in the adjuvant treatment of EC [27].

Although the need for better treatment options is highest among p53abn ECs, it cannot be excluded that EC patients with other molecular subtypes could benefit from PARPi and/or platinum-based therapy. Patients with endometrioid histology who have stage III or IV disease and/or grade 3 histology, and patients with uterine serous carcinoma (USC), a subtype of EC that has similar characteristics to high-grade serous ovarian cancer (HGSOC), are described in literature as candidates for PARPi and/or platinum-based therapy [20,28]. Several studies have confirmed the presence of HRD with DNA-based HRD tests among a variety of EC and it was estimated that 37% of USCs harbored HR-related PVs and 53% of USCs had a genomic scar HRD score (based on the sum of LOH, TAI and LST) [29-33]. In addition, Bustamante *et al.* showed that 68-76% of the CN-high EC were classified as HRD, based on the percentage of genome-wide LOH, a marker of HRD [31]. Nevertheless, these studies were small, mainly performed with DNA-based HRD tests, and still provide limited insight in the true prevalence of HRD in EC.

In the last decade, the development of functional tests to identify HRD tumors accelerated. First, the REcombination CAPacity (RECAP test), which measures the accumulation of RAD51 protein at sites of DNA damage after *ex vivo* irradiation of fresh tumor tissue, was developed and validated for BC and OC [13,34-36]. One study investigated functional HRD in a selected cohort of 25 ECs by using the RECAP test, showing that 24% of EC samples, all NEECs, were functionally HRD [37]. Recently, a functional RAD51-based HRD test that can be performed on formalin-fixed paraffin-embedded (FFPE) tumor tissue, was validated

on EC samples [38]. Avoiding the need for irradiated fresh tumor tissue (as is the case for the RECAP test) greatly facilitates potential implementation in the routine diagnostics [38]. Functional HRD tests have the advantage that they measure the HR status real-time (at surgical removal), while DNA-based HRD tests show all historic mutational or DNA-damaging events that occurred in a tumor, which do not necessarily reflect the latest HR status [10]. For these reasons, the RAD51-FFPE test is an efficient and quick method to determine HR status on a large cohort of tumor samples.

In this study, we investigated the prevalence of functional HRD in an extensive cohort of EC by using both the RAD51-FFPE and RECAP test, and we investigated HR status in the context of the four molecular subgroups. In an explorative manner, we also investigated if functional HRD could be observed among cervical (CC) and vulvar (VC) carcinomas, as previous genetic studies showed that HRD could be observed among these carcinomas [16,39,40].

2. Materials and Methods

2.1 Patient material

FFPE tumor material of 109 EC specimens obtained between June 2010 and January 2020 were collected from the Pathology archives of the Leiden University Medical Center (LUMC). All obtained EC specimens received a pseudo-anonymized study code. The study protocol was approved by the local medical ethics committee of the LUMC on 7 February 2011 and 24 May 2017 (B16.019, P10.226 and G17.041). The EC specimens were handled according to the Dutch 'Code for Proper Secondary Use of Human Tissue', as described by the Dutch Federation of Medical Scientific Societies. For this study, RAD51-FFPE scores and RECAP scores obtained in the studies of de Jonge *et al* [37] and van Wijk *et al* [38] were included (n = 25/109). Furthermore, the study cohort was extended with an additional 84 EC specimens (total: n = 109), for which the RAD51-FFPE test and RECAP test was performed as described below.

2.2 Selection of FFPE EC material

For each of the EC specimens, a representative tumor tissue block was selected by an expert gynaecopathologist (T.B.). As a first quality control (QC) step (QC tissue quality), the hematoxylin and eosin (H&E) slide was reviewed, and a block containing at least 70% vital tumor tissue was selected. Clinicopathological data were obtained from the PA-report, including histological subtype, tumor grade, and (if available) *POLE* status and *BRCA* status.

2.3 Co-Immunofluorescence (co-IF) staining for RAD51/Geminin

EC samples that passed the QC tissue quality were stained with a co-IF staining for RAD51 and geminin (GMN), as described previously [38]. Briefly, tissue sections were incubated for 60 minutes in a 60 °C stove and then incubated overnight in a 37 °C stove prior to the co-IF staining. Next, unstained sections were deparaffinized in xylol, after which sections were rehydrated with 100%, 90% and 70% ethanol and washed with distilled water. Sections were then heated in Antigen Retrieval buffer (DAKO, pH 9.0, Agilent, Santa Clara, CA, USA, cat. S2375) at 97 °C for 12 minutes in the TissueWave™ 2 Microwave Processor (ThermoFisher Scientific, Waltham, MA, USA). After a cooling down period of 30 minutes, sections were washed in distilled water and permeabilized for five minutes in DAKO wash buffer (DAKO Agilent, Santa Clara, CA, USA, cat. S3006). After blocking for 10 minutes (DAKO wash buffer with 1% BSA), sections were incubated for 60 minutes with the primary anti-RAD51 antibody (rabbit, monoclonal, Abcam, Cambridge, UK, cat. ab133534) and anti-GMN antibody (mouse, monoclonal, NovoCastra, Leica Biosystems, Buffalo Grove, IL, USA, cat. NCL-L) with concentrations of 1:1000 and 1:60, respectively. The sections were washed, incubated with blocking buffer for 10 minutes, followed by incubation with secondary antibodies in blocking buffer for 30 minutes (concentration 1:500; goat-anti-mouse IgG Alexa Fluor 488 and goat-anti-rabbit IgG Alexa Fluor 555 (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA, cat. A-11001 and cat. A-21428). Additional washing steps were performed and sections were dehydrated with ethanol (70%-90%-100%). Lastly, sections were mounted with ProLong Gold antifade mountant including DAPI (ThermoFisher Scientific, Waltham, MA, USA, cat. P36935) and stored at -20 °C.

2.4 RAD51-FFPE score calculation

Co-IF stained sections (DAPI, GMN and RAD51) were scored manually using the Leica DM6B microscope (63x/1.40-0.6 oil objective). All samples were scored by two independent observers. First, the 20x and 63x objective were used, respectively, to get a general idea of the tissue quality, location of tumor cells, staining quality and heterogeneity of the tumor with respect to RAD51 staining in all three above described channels. Obvious technical problems or tissue-artefacts were noted. Next, the DAPI channel was used to orientate and select areas with vital tumor cells, based on cell morphology. At least 40 GMN^+ cells were scored in at least three till five distinct areas (QC GMN^+ cells). GMN^+ cells were randomly selected in distinct areas and in selected cells the number of RAD51 foci was determined (0, 1, 2, 3, 4, ≥5). The RAD51-FFPE score of each observer comprised of the percentage of GMN^+ cells with at least 2 RAD51 foci. The average score of two observers was considered the final RAD51-FFPE score.

2.5 Co-Immunohistochemistry (co-IHC) staining for γH2AX/Geminin

For EC specimens with a RAD51 score ≤ 15% (classified as HRD), an additional co-IHC for γH2AX/GMN was performed on sections of FFPE tumor tissue (QC γH2AX score). γH2AX, a marker of DNA damage, in GMN^+ cells served as a proxy for the presence of endogenous DNA damage (≥ 25% of $γH2AX^+/GMN^+$ cells). The protocol as described by van Wijk *et al.* was applied [38]. The primary antibodies that were used were anti-γH2AX-antibody (mouse, monoclonal, MilliporeSigma, St. Louis, MO, USA, cat. 05-636, clone JBW301) and the anti-GMN-antibody (rabbit, polyclonal, Proteintech, Manchester, UK, cat. 10802-1-AP) in the concentrations 1:30.000 and 1:5000, respectively. Slides were incubated with liquid chromogen DAB+ (Abcam, Cambridge, UK, cat. ab7084) and Fast Red (Abcam, Cambridge, UK, cat. ab64254). After the staining, at least 40 GMN^+ cells were reviewed in three to five vital tumor areas on a Zeiss Axio Imager.M2 light microscope (63x oil objective). For each of the GMN^+ cells, the number of γH2AX foci was determined (0, 1, 2, 3, 4, ≥ 5). A γH2AX score was calculated as the percentage of GMN^+ cells that had at least 2 γH2AX foci. Final γH2AX-scores were calculated as the average of γH2AX scores of two observers. All stained sections were evaluated by two independent observers. Samples with insufficient numbers of endogenous DNA damage (γH2AX score <25%) were excluded from the final analysis.

2.6 RECAP test

The RECAP test was performed on viably frozen tumor tissue matching with the 80 EC samples for which an informative RAD51-FFPE score was calculated, as described previously [13,37]. Briefly, thawed EC samples that were obtained during hysterectomy were irradiated with 5Gy ionizing irradiation. After a two-hour incubation time the irradiated samples were fixed and embedded into paraffin. H&Es of (irradiated) tumor blocks were reviewed for tumor cell percentage and tissue vitality by an expert pathologist. Co-IF staining with RAD51 (anti-RAD51 antibody, GeneTex, Alton Pkwy Irvine, CA, USA, cat. GTX70230) and GMN (anti-GMN antibody, ProteinTech, Manchester, UK, cat. 10802-1-AP) was performed on sections of FFPE tumor blocks. In total, 40 GMN^+ cells were reviewed per sample. The RECAP score was calculated as the percentage of GMN^+ cells that had at least five RAD51 foci per nucleus. If the RECAP score was ≤ 20%, a sample was classified as HRD and if the RECAP score was > 50%, a sample was classified as HR-Proficient (HRP). Samples with a RECAP score between 21% and 50% were classified as HR-Intermediate (HRI).

The RECAP test was performed on viably frozen tumor tissue of patients with cervical cancer (CC) and of patients with vulvar cancer (VC) as described above.

2.7 Molecular classification of EC samples

The EC samples were molecularly classified using the surrogate markers (*POLE* sequencing, IHC of MMR proteins, and p53 IHC) . IHC stainings were performed, i.e., p53 IHC and MSH6 (Clone EPR3945, 1:400, GeneTex) and PMS2 (Clone EP51, 1:25, DAKO) IHC, as described by the Jonge *et al.* [37]. Expression of PMS2 and MSH6 was evaluated by an expert pathologist to assign mismatch repair (MMR)-status (MMR-deficient (MMRd) or MMR-proficient (MMRp)). The expression of PMS2 and MSH6 was assigned in one of two categories; retained expression or loss of expression, as described by Stelloo *et al.* [41]. If a tumor had loss of PMS2 or MSH6 expression, an IHC-staining with the other MMR-proteins (MLH1 and MSH2) was performed. If there was subclonality in the loss of expression (≥ 10%) of PMS2/MLH1 or MSH6/MSH2 complex, an EC was classified as MMRd. Similarly, p53 IHC was reviewed by an expert pathologist (T.B.) as described by Castillo-Leon *et al.* [22]. p53 IHC was scored abnormal if >10% of the tumor area showed one of the well-known abnormal/mutant-like staining patterns. These abnormal patterns include: 1) p53 nuclear overexpression in over 80% of tumor cells (overexpression pattern), 2) complete absence of p53 staining with a positive internal control (null/deletion-type pattern), or 3) significant p53 staining in the cytoplasm of >80% of the tumor cells (cytoplasmic pattern) [42] .

2.8 Statistical Analyses

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) and SigmaStat 3.5 (Systat Software Inc, San Jose, CA, USA). GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA), Adobe Illustrator CC 2020 (Adobe Inc, San Jose, CA, USA) and BioRender software (Toronto, ON, Canada) were used to create figures. For the scope of this study, RECAP-HRI were considered HRP. For numerical data, Student's t-test was used to test differences between two groups with normally distributed numerical data and Mann-Whitney Rank Sum test was used with data that was not normally distributed. For categorical data, the Chi-Square test was used to test differences between two groups. Fisher's exact test was performed when ≥ 1 of the values was 0 and/or when ≥ 20% of the values was less than five. We used a Cohen Kappa coefficient (k) to calculate the interobserver variability and intertest agreement. A p-value of <0.05 was considered significant.

3. Results

3.1 HRD in EC as defined by the RAD51-FFPE test

In total, 109 FFPE ECs were reviewed by an expert gynaecopathologist and 94/109 (86%) displayed good tissue vitality and a tumor cell percentage (TCP) > 70% (QC tissue quality, MM section 2.2, Fig. 1). Next, the RAD51-FFPE test was performed on these 94 EC samples (MM section 2.3). For 83 out of 94 (88%) EC samples, a RAD51-FFPE score was successfully calculated (Fig. 1, MM section 2.4). The remaining 11 samples (12%), had an insufficient number (<40) of GMN$^+$ cells (QC GMN$^+$ cells) for a final RAD51-FFPE score. In total, 43 EC samples were classified as HRD by the RAD51-FFPE test and for these samples a γH2AX score was calculated to confirm the presence of endogenous DNA damage (QC γH2AX score, MM section 2.5). Three samples had a γH2AX score <25% and were therefore excluded from analysis (Fig. 1, Fig. S1). The mean γH2AX-score in EC samples included in the final analysis was 71% (range: 27-100%). There was no significant correlation between γH2AX-scores and p53-status (p = 0.706, Fig. S2) nor between γH2AX-scores and tumor grade (p = 0.822, Fig. S2).

In total, RAD51-FFPE scores were obtained for 80 EC samples (Fig. 1). There was substantial agreement in RAD51-FFPE scores between two observers (Cohen's Kappa value = 0.7998, test parameters: RAD51 foci cut-off of 2 and HRD threshold of 15%). Forty out of 80 EC samples (50%) were classified as HRD.

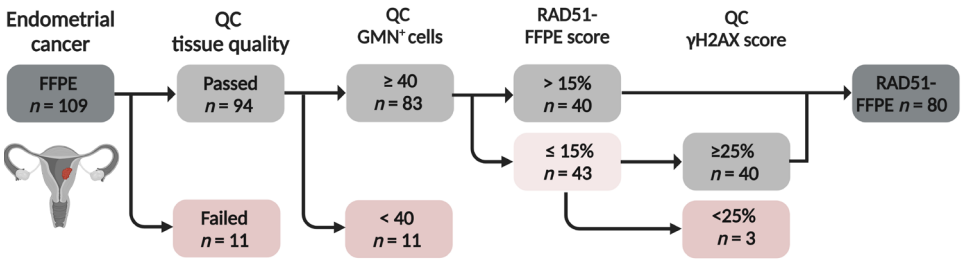

Fig. 1. Flowchart of the RAD51-FFPE test. The RAD51-FFPE test was performed on formalin-fixed paraffin embedded (FFPE) endometrial cancer (EC) samples. A RAD51-FFPE score was obtained if at least 40 geminin-positive (GMN$^+$) cells were scored. EC samples with a RAD51-FFPE score of ≤ 15% were considered HRD. HRD samples were subjected to an additional quality control step by performing a γH2AX/geminin immunohistochemistry staining to confirm the presence of sufficient endogenous DNA damage. If the γH2AXscore was ≤ 25%, the sample was excluded from the final analysis. HRP samples and HRD samples with a γH2AX score above 25% were included in the final analysis.

3.2 Correlation of RAD51-FFPE and RECAP HR-classification in EC

In a previous study, we calibrated the RAD51-FFPE test parameters based on the RECAP test HR-classification outcome on a set of 25 EC samples [38]. To confirm the validity of the previously established RAD51-FFPE test parameters in this extended EC cohort, we performed the RECAP test on matching freshly frozen tumor material (MM section 2.6).

In total, RECAP scores were calculated for 50/80 (63%) of samples with RAD51-FFPE scores (Fig. S3). Out of the 30 excluded samples, 27 (90%) did not have (vital) tumor tissue (Table S1). Eight out of the 50 included samples (16%) were classified as HRD, 9/50 (18%) were classified as HRI and 33/50 (66%) were classified as HRP (Fig. 2).

Importantly, all RECAP-HRD samples were also classified as RAD51-FFPE HRD (Fig. 2, Fig. 3). Seven out of nine (78%) RECAP-HRI samples were classified as RAD51-FFPE-HRD (Fig. 2). Applying the previously established RAD51-FFPE test parameters (HRD threshold 15%, RAD51 foci cutoff of two) for EC [38], a 100% sensitivity was reached for the identification of RECAP-HRD samples with a specificity of 57%.

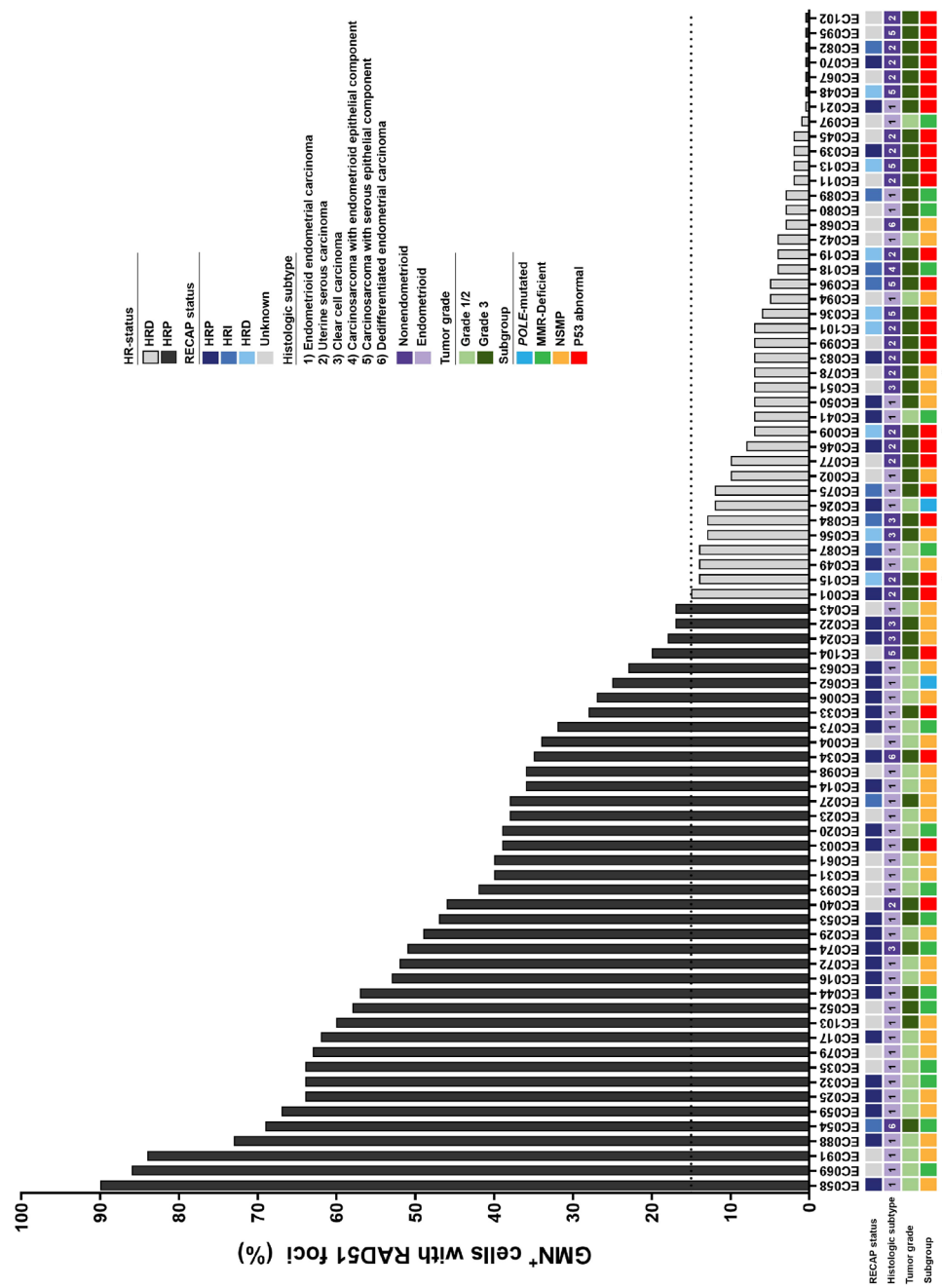

Fig. 2. RAD51-FFPE scores of the total EC cohort *n* = 80. RAD51-FFPE scores were calculated as the percentage of GMN$^+$ cells with at least two RAD51 foci. The dotted line indicates the HRD threshold of 15%. *These samples harbor a pathogenic variant in *BRCA1* with loss of the wildtype allele.

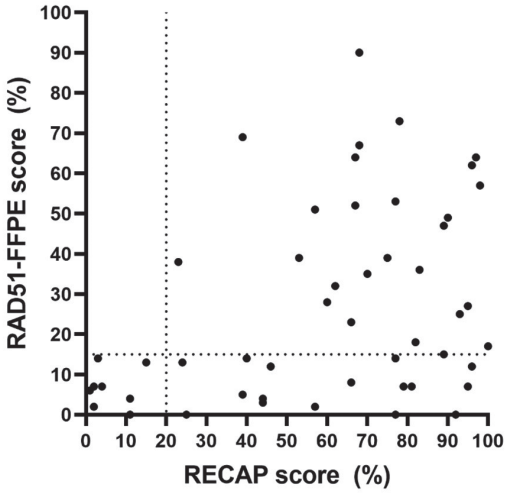

Fig. 3. Correlation RAD51-FFPE and RECAP scores in EC (*n* = 50). The RAD51-FFPE score was calculated as the percentage of GMN$^+$ cells with at least two RAD51 foci. The RECAP score was calculated as the percentage of GMN$^+$ cells with at least five RAD51 foci. Pearson correlation coefficient $p = 0.0069$.

3.3 Functional-HRD in relation to clinicopathologic characteristics

Next, we evaluated the relation between HR status and clinicopathologic characteristics of the EC samples. All but one of the ECs were primary tumors (Table 1). There was no significant association between age at diagnosis and HR-status ($p = 0.083$). There was a significant correlation between FIGO stage and HR-status, with more patients with HRD tumors having advanced-stage disease ($p = 0.020$, Table 1). The ECs that were classified as HRD were more likely to be NEEC ($p < 0.001$, Table 1). Similarly, there was a significant association between grade 3 tumors and HRD ($p < 0.001$, Table 1), which may reflect the enrichment in NEEC in the HRD group. With regard to histological subtypes, the 40 HRD ECs were grade 1/2 EEC ($n = 7$), grade 3 EEC ($n = 6$), uterine serous carcinoma ($n = 16$), clear cell carcinoma ($n = 3$), uterine carcinosarcoma ($n = 6$) or dedifferentiated EC ($n = 1$) (Table 1, Figure 2).

Table 1. Clinicopathologic characteristics stratified for homologous recombination capacity as determined by the RAD51-FFPE test.

	HRD *n* = 40	HRP *n* = 40	*p*-value
Age (years) Mean ±SEM	69 (±1.43)	65 (±1.76)	$p = 0.083$
Tumor			
Primary	40	39	
Recurrent	0	1	
Histologic subtype			***p* <0.001***
Endometrioid	12	32	
Non-endometrioid			
Serous	16	2	
Carcinosarcoma	7	1	
Clear cell	3	3	
Dedifferentiated	2	2	
Tumor grade			***p* <0.001**
1/2	7	26	
3	33	14	
FIGO 2009			***p* = 0.020**
I/II	25	35	
III/IV	15	5	
TP53			***p* <0.001****
Wildtype	15	34	
Loss of expression	24	6	
Ambiguate	1		

Abbreviations: HRD = homologous recombination deficient, HRP = homologous recombination proficient. *Endometrioid was compared with non-endometrioid. **The ambiguate sample was not taken along in the statistical analysis.

3.4 HRD in relation to molecular subgroup classification

Next, we evaluated the relation of HR status with the molecular subgroup classification of the EC samples (Fig. 2). A significant association between p53abn and RAD51-FFPE HRD was observed (Table 1; $p < 0.001$). Among the molecular subgroups, 6/17 (35%) MMRd, 9/32 (28%) NSMP and 24/29 (83%) p53abn EC samples were classified as HRD by the RAD51-FFPE test (Fig. 2). Of the two samples with a *POLE*mut in our cohort, one was classified as HRD by the RAD51-FFPE test (Fig. 2). When HR status as determined by the RECAP test was used, HRD ECs were more likely to be p53abn (Table S2; $p = 0.0028$). In total, 1/17 (6%) NSMP, 7/20 (35%) p53abn EC samples and none of the MMRd samples were classified as HRD by the RECAP test (Fig. 2). Both *POLE*mut samples were classified as HRP by the RECAP test (Fig. 2).

3.5 No HRD in CC and VC as defined by the RECAP test

In an explorative manner, we evaluated if HRD could be identified among patients with CC or VC. A few genetic studies suggested that HRD can be identified among CC and VC [16,40]. However, to our knowledge, no studies have been performed yet that determined functional HR status in these cancer types. For this reason, we decided to perform the RECAP test on a small set of CC (*n* = 28) and VC (*n* = 16). In the CC set, one sample was identified with an HRI score of 48%. The other 27 samples were classified as HRP (96%, Fig. S4). In the VC set, all samples were classified as HRP (100%, Fig. S5). Since no HRD was identified among CC and VC with the RECAP test, we did not pursue the HRD testing with the RAD51-FFPE test.

4. Discussion

To our knowledge, this is the largest EC cohort in which the presence of functional HRD is analyzed. Half of the 80 ECs included in this study were classified as HRD and the RAD51-FFPE test had a 100% sensitivity to identify RECAP-HRD samples with a 57% specificity to identify RECAP-HRP samples.

The high incidence of HRD among OC and BC is well known, but recently, PARPi and/or platinum-based chemotherapy is also being considered as an option for the potential treatment of other gynaecological carcinomas. Among these are EC and CC, and the first clinical trials with PARPi and platinum therapy were established in the last years [27,39,43]. Considering the relation of HRD with PARPi and/or platinum sensitivity, we sought to investigate the prevalence of HRD among EC, CC and VC. A high prevalence of HRD (50%), as determined with the RAD51-FFPE test, was observed among EC. This percentage should, however, be interpreted with caution, as previous studies on OC, BC, and EC showed that the RAD51-FFPE test may overestimate the number of HRD samples [15,38]. Based on the RECAP test results alone, 16% of the ECs were classified as HRD, which was slightly lower than the 24% of HRD that was reported among the first 25 included EC samples [37]. Importantly, the clinicopathologic characteristics of the samples in the HRD and HRP groups between our study and the study that included the first 25 included EC samples were similar (Table S2) [37].

Previous studies have shown that (functional) HRD is a frequent event in USC and/or p53abn EC [31,32,37], and our results support these findings; the HRD ECs that were identified in our cohort, both with the RAD51-FFPE and the RECAP test, were more likely to have aggressive features, as having advanced-stage disease and being NEECs, grade 3 and p53abn tumors. Our results show that based on the RAD51-FFPE test, HRD is present in grade 1/2 EEC (n = 7). However, for the four samples for which a RECAP score was available, a discrepancy in HR-classification between the RAD51-FFPE and RECAP test was observed, as three samples were classified as RECAP-HRP (EC026, EC041, EC049 Fig. 2) and one sample as RECAP-HRI (EC087, Fig. 2). The RECAP test was used as a reference in this study, implying that at least four grade 1/2 EEC, of the seven that were classified as HRD in the RAD51-FFPE test, may have been inaccurately done so. One possible explanation for this may be that the level of proliferation and genetic instability in low-grade p53 wildtype tumors is insufficient for a reliable RAD51-FFPE test result, even though they all passed the γH2AX QC. Importantly, γH2AX foci are frequently thought of as a surrogate for the presence of DNA DSBs, but inconsistencies in the presence of both γH2AX foci and DNA DSBs in terms of quantity and localization have been observed [44-46], suggesting that γH2AX might be a suboptimal marker for DNA DSBs, especially in low-grade ECs. An alternative DNA damage marker might be 53BP1. However, loss of *TP53BP1*, which encodes

for 53BP1, was frequently observed in treatment-naïve ECs, implying that 53BP1 may not be an accurate alternative as DNA damage marker for low-grade ECs [32].

In an exploratory manner, we investigated the presence of HRD in the context of the molecular subgroup classification. Importantly, pathogenic variants (PVs) in *BRCA1/2* and other HR-related genes may occur in the context of a hypermutated (*POLE*mut) and/or ultramutated (MMRd) phenotype. For example, the study of De Jonge *et al.* demonstrated that one *POLE*mut EC harbored a PV in *CHEK2* [37]. Similarly, studies of Hatakayatama *et al.* and Lee *et al.* showed that *BRCA1/2* PVs are present in *POLE*mut and/or MMRd EC and colorectal cancers [47,48]. The functional effect of these potential 'passenger mutations' on protein level has yet to be investigated. In our study, 18% (7/40) of HRD EC were either *POLE*mut (n = 1) or MMRd (n = 6). The RECAP test did not classify these samples as HRD. Three samples (EC009, EC015 and EC078) harbored a PV in *BRCA1* with loss of the wildtype allele, of which two were p53abn and one of the NSMP subtype.

We showed that HRD, as determined with both the RAD51-FFPE and RECAP test, was frequently observed in p53abn EC (83% of p53abn EC), which is the subgroup of patients that also showed the most benefit from the addition of platinum-based chemotherapy to EBRT in a previous published study [22]. But the samples analyzed in our study were not sequenced with an extensive HRD gene panel or screened for HRD genomic scars/mutational signatures. In addition, due to the nature of this retrospective study, the correlation with PARPi/platinum sensitivity could not be determined. Several clinical trials have been initiated to investigate the clinical benefit of both PARPi and platinum-based chemotherapy in EC, but the correlation with HRD is yet to be determined.

Our research included a pilot study investigating the prevalence of HRD among CC and VC. An *in vitro* study showed that CC primary cell lines were responsive to PARPi treatment and a phase I clinical trial (NCT01237067) investigating combined carboplatin and olaparib sensitivity in patients with CC is currently ongoing [39,49]. In addition, genetic studies suggested that HRD can be identified among CC and VC [16,40]. However, we did not identify any functional RECAP-HRD sample among our CC and VC cohorts. Although our sample size was small, it may suggest that HRD is not a common feature among CC and VC.

In conclusion, we demonstrated that HRD is common in EC, especially in high-grade p53abn EC. Nonetheless, the presence of HRD was not restricted to the p53abn subgroup and was identified in other molecular subgroups, albeit at lower frequencies (*POLE*mut, MMRd, NSMP). The RAD51-FFPE test is an efficient tool to identify HRD among ECs, which could assist in the stratification of patients who might benefit from treatment with PARPi and/or platinum-based chemotherapy.

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Supplementary Materials

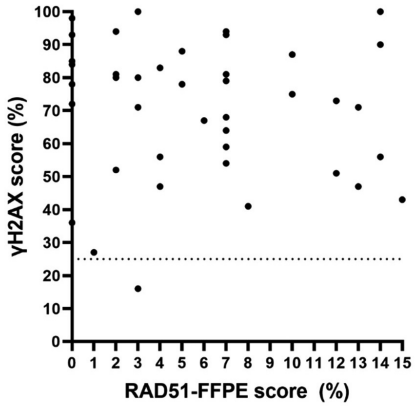

Fig. S1. Scatterplot indicating the relationship between the RAD51-FFPE score (x-axis) and γH2AX-score (y-axis) for EC samples with a RAD51-FFPE score ≤ 15%. The RAD51-FFPE score was calculated as the percentage of GMN^+ cells with at least two RAD51 foci. The γH2AX score was calculated as the percentage of GMN^+ cells with at least two γH2AX foci. Samples with a γH2AX score < 25% were excluded from analysis. One sample had a γH2AX score of 16% and two samples did not have an informative γH2AX score and were subsequently excluded from analysis.

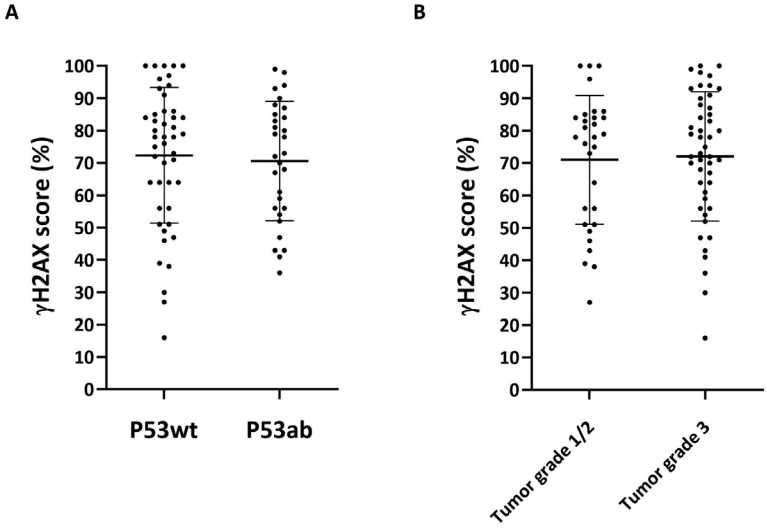

Fig. S2. A) Scatterplot indicating γH2AX-scores for p53 wildtype (p53wt) and p53 abnormal (p53abn) endometrial cancers (EC). There was no significant correlation between γH2AX-scores and p53-status (p = 0.706). B) Scatterplot indicating γH2AX-scores for low-grade (grade 1/2) and high-grade (grade 3) EC. No significant association was observed between γH2AX-scores and tumor grade (p = 0.822). The lines indicate means and standard deviations.

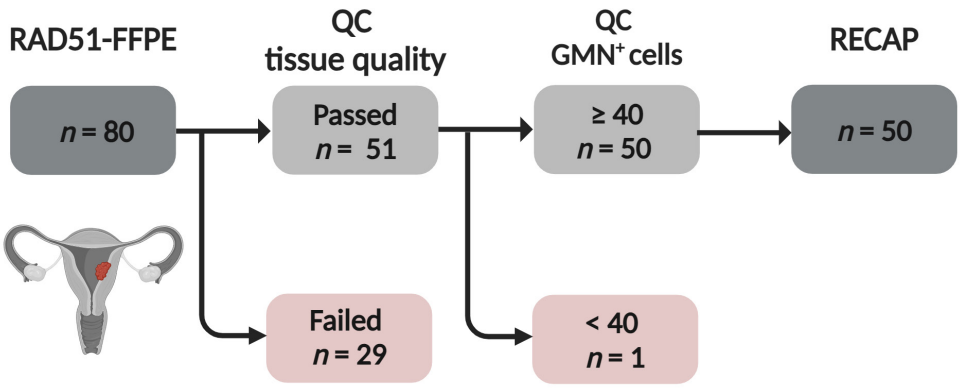

Fig. S3. Flowchart of the RECAP test. The RECAP test was performed on viably frozen tumor samples matching the included RAD51-FFPE samples. Tissue samples were thawed and the RECAP test was performed (MM section 2.6). The tissue quality was assessed by an experienced gynaecopathologist and only samples with vital tumor tissue and sufficient tumor cells passed the tissue quality QC. A RECAP score was obtained if at least 40 geminin-positive (GMN^+) cells were scored (QC GMN^+ cells).

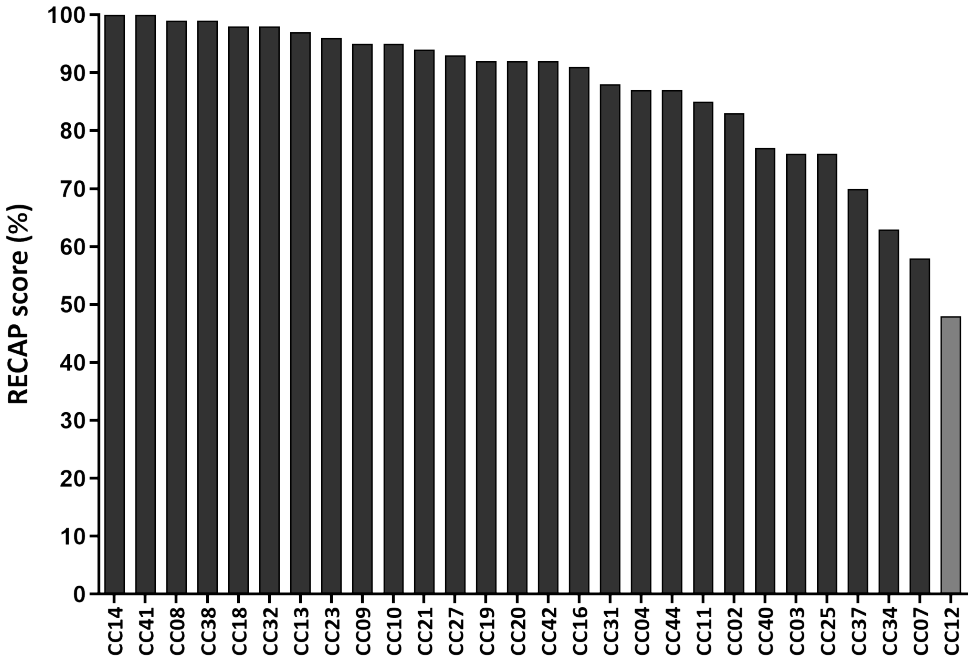

Fig. S4. RECAP scores in a cohort of cervical carcinoma (CC) samples (*n* = 28). The RECAP score was calculated as the percentage of GMN^+ cells with at least five RAD51 foci.

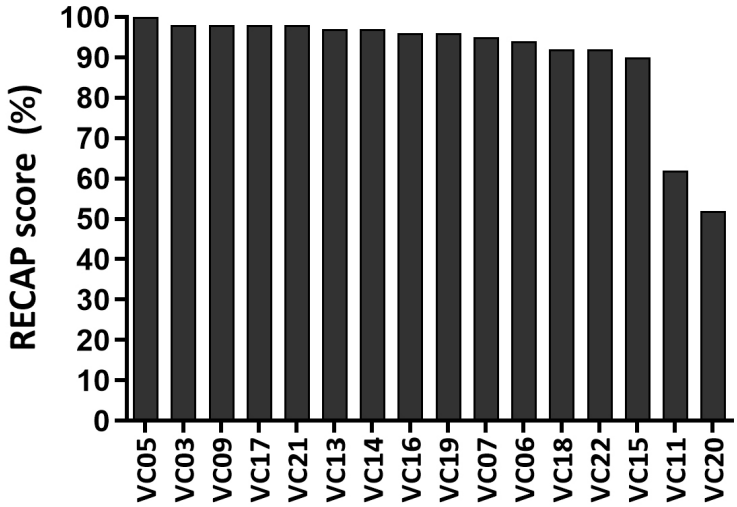

Fig. S5. RECAP scores in a cohort of vulvar carcinoma (VC) samples (*n* = 16). The RECAP score was calculated as the percentage of GMN^+ cells with at least five RAD51 foci.

Table S1. Overview of the reasons for exclusion of RECAP samples.

Reason for exclusion	Number of samples
No (vital) tumor tissue	27
Insufficient GMN^+ cells	1
No viably frozen sample available	2

Table S2. Clinicopathologic characteristics stratified for homologous recombination capacity as determined by the RECAP test.

	HRD ***n* = 8**	**HRP/HRI** ***n* = 42**	***p*-value**
Age (years) Mean ±SEM	66 (±4.09)	68 (±1.32)	$p = 0.541$
Tumor			
Primary	8	41	
Recurrent	0	1	
Histological subtype			***p* <0.001 ***
Endometrioid	0	28	
Non-endometrioid			
Serous	4	6	
Carcinosarcoma	3	2	
Clear cell	1	4	
Dedifferentiated	0	2	
Tumor grade			***p* = 0.018**
1/2	0	19	
3	8	23	
FIGO 2009			$p = 0.082$
I/II	4	34	
III/IV	4	8	
TP53			***p* = 0.0028**
Wildtype	1	29	
Loss of expression	7	13	

Abbreviations: HRD = homologous recombination deficient, HRP = homologous recombination proficient. *Endometrioid was compared with non-endometrioid.