

# Frequent Homologous Recombination Deficiency in High-grade Endometrial Carcinomas

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## Abstract

**Purpose:** The elevated levels of somatic copy-number alterations (SCNAs) in a subset of high-risk endometrial cancers are suggestive of defects in pathways governing genome integrity. We sought to assess the prevalence of homologous recombination deficiency (HRD) in endometrial cancers and its association with histopathologic and molecular characteristics.

**Experimental Design:** Fresh tumor tissue was prospectively collected from 36 endometrial cancers, and functional HRD was examined by the ability of replicating tumor cells to accumulate RAD51 protein at DNA double-strand breaks (RAD51 foci) induced by ionizing radiation. Genomic alterations were determined by next-generation sequencing and array comparative genomic hybridization/SNP array. The prevalence of *BRCA*-associated genomic scars, a surrogate marker for HRD, was determined in the The Cancer Genome Atlas (TCGA) endometrial cancer cohort.

**Results:** Most endometrial cancers included in the final analysis ( $n = 25$ ) were of non-endometrioid (52%), grade 3 (60%) histology, and FIGO stage I (72%). HRD was observed in 24% ( $n = 6$ ) of cases and was restricted to non-endometrioid endometrial cancers (NEEC), with 46% of NEECs being HRD compared with none of the endometrioid endometrial cancers (EEC,  $P = 0.014$ ). All but 1 of the HRD cases harbored either a pathogenic *BRCA1* variant or high somatic copy-number (SCN) losses of HR genes. Analysis of TCGA cases supported these results, with *BRCA*-associated genomic scars present in up to 48% (63/132) of NEEC versus 12% (37/312) of EEC ( $P < 0.001$ ).

**Conclusions:** HRD occurs in endometrial cancers and is largely restricted to non-endometrioid, *TP53*-mutant endometrial cancers. Evaluation of HRD may help select patients that could benefit from treatments targeting this defect, including platinum compounds and PARP inhibitors.

## Introduction

Endometrial cancer is the most common gynecologic malignancy in developed countries (1), with surgery as its primary

treatment modality. To guide adjuvant treatment, women with endometrial cancers are stratified according to risk of recurrence using clinicopathologic characteristics (2, 3). A heterogeneous group of 15%–25% of endometrial cancers are currently considered at high-risk of disease recurrence. This group consists of patients with non-endometrioid endometrial carcinomas [NEEC; uterine serous carcinoma (USC), uterine carcinosarcoma (UCS), clear cell carcinoma (CCC), undifferentiated carcinoma (UC), mixed endometrial cancers], endometrioid endometrial cancers (EEC) grade 3 stage IB–IV and EEC grade 1 and 2 stage II–IV (2–6). These patients have the poorest clinical outcome, despite optimum adjuvant treatment, which currently comprises a combination of pelvic radiotherapy with or without (platinum–taxane based) chemotherapy (3–5). In the cohort of Hamilton and colleagues, high-risk EEC grade 3, USC, and CCC represented only 28% of the total endometrial cancer cohort but accounted for 74% of endometrial cancer–related deaths (4), emphasizing the need for better systemic treatments to improve outcomes for these patients.

The Cancer Genome Atlas Research Network (TCGA) analyzed EECs, USC, and mixed carcinomas and identified 4 distinct molecular subclasses based on mutational load and somatic copy-number alterations (SCNAs). These 4 subclasses are respectively (i) the *POLE*/ultramutated, (ii) the microsatellite instability–high (MSI-high)/hypermethylated, (iii) the SCNA-low/

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### Translational Relevance

The prognosis for women with high-grade endometrial cancers is poor, with little improvement in the last 2 decades. The mainstay of treatment is surgery (hysterectomy) with or without lymphadenectomy. Although adjuvant radiotherapy is considered standard for high-risk endometrial cancers, the added value of chemotherapy has been subject of recent trials. The randomized PORTEC-3 trial found a significant 5-year failure-free survival benefit (75.5% vs. 68.6%,  $P = 0.022$ ) for women with high-risk endometrial cancer treated with adjuvant chemotherapy both during and after radiotherapy versus radiotherapy alone. However, biomarkers predicting chemotherapeutic benefit for patients with endometrial cancers have not been defined to date. In this article, we provide functional evidence that homologous recombination is frequently abrogated in a subset of endometrial cancers, in particularly the "serous-like," *TP53*-mutated subclass, which have the worst clinical outcome. Our results suggest that homologous recombination deficiency (HRD) holds promise as a marker to guide treatment decisions in high-risk endometrial cancers, and supports prospective trials investigating agents such as platinum compounds and PARP inhibitors to target this repair defect in these cancers.

no specific molecular profile (NSMP), (iv) and the SCNA-high (SCNA-hi)/serous-like endometrial cancers (7). Each of these has distinct risk of recurrence and clinical outcome, with *POLE*/ultramutated tumors showing excellent outcome and the SCNA-hi/serous-like subgroup showing the worst prognosis. The first 3 of these subclasses consist mainly of EEC with variants in *PTEN* as the most frequent genetic alteration. In contrast, the SCNA-hi subclass almost exclusively comprises of USC and grade 3 EEC and is strongly associated with pathogenic variants in *TP53* (7). Interestingly, recent studies demonstrated that rare non-endometrioid subtypes, such as UCS, CCC, and dedifferentiated carcinomas appear to be composed of the same 4 molecular subclasses, with UCS being mostly SCNA-hi/*TP53*-mutated and CCC, UC, and dedifferentiated endometrial cancers being more heterogeneous (8–11). The clinical relevance of these observations has increased by the recognition that the TCGA molecular subclasses of endometrial cancers can be recapitulated using pragmatic surrogate markers resulting in subgroups with differing prognoses (12, 13).

Another interesting observation of the TCGA study were the similarities between the SCNA spectra of the SCNA-hi/*TP53*-mutated endometrial cancers subclass with those of high-grade serous ovarian tubal carcinomas (HGSOCs) and basal-like breast cancers (7, 8). Both HGSOC and basal-like breast cancer are part of the hereditary *BRCA1/2*-related breast and ovarian cancer syndrome (HBOC syndrome; refs. 14, 15); characterized by failure of high-fidelity homologous recombination (HR) repair of DNA double-strand breaks (DSBs) mediated by *BRCA1* and *BRCA2* proteins (15, 16). Although endometrial cancer is not generally regarded as part of HBOC syndrome, case and cohort studies indicate that serous/serous-like endometrial cancers (including carcinosarcomas) are more prevalent in germline *BRCA1/2* mutation carriers than in the general population (17, 18). Furthermore, germline alterations in other HR-related

genes have been described in patients with endometrial cancer (e.g., *ATM*, *BARD1*, *BRIP1*, *CHEK2*, *NBN*, *RAD51C*; ref. 19), raising the question of whether a subset of endometrial cancer is HR-deficient. Shen and colleagues showed that *PTEN* has a role in the DSB-repair system by regulating the expression of *RAD51*, a key protein in HR-repair (20). Given the frequent somatic *PTEN* alterations in endometrial cancers, particularly in MMRd, *POLE*, and NSMP-EC, it is conceivable that HR deficiency might also occur in these subclasses.

There are several methods to determine HR deficiency in tumors. Besides sequencing of genes involved in HR, one can also assess the presence of specific "genomic scars" caused by the use of alternative, error-prone pathways to repair DSBs in the absence of HR. Examples of such alterations that are overrepresented in *BRCA1/2*-null tumors include COSMIC Signature 3 and SCNA profiles associated with widespread loss of heterozygosity (LOH), large-scale state transitions (LST), and telomeric allelic imbalances (TAI; refs. 16, 21–24). A more direct way of testing HR capacity and one which more closely reflects the current status of the tumor, is to determine the ability of tumor cells to perform HR in a functional assay. For this, fresh viable tumor tissue is exposed *ex vivo* to ionizing radiation to induce DNA DSBs. In HR-proficient tumor cells, *RAD51* protein will be recruited to these breaks leading to the formation of *RAD51*-containing ionizing radiation-induced foci (IRIF). In the case of HR-deficient tumor cells, *RAD51*-IRIF formation will be impaired (16, 25–27). The *RAD51* assay, as a functional read out for HR, has been shown to reliably identify cell lines, xenografts, and fresh human tumor tissue with defective HR (25–28).

The aim of this study was to assess the prevalence of HR deficiency in endometrial cancers using a functional *RAD51*-IRIF assay, evaluate its association with clinicopathologic characteristics, and define the underlying molecular etiology.

## Materials and Methods

### Patient selection

Fresh endometrial cancers tissue was obtained from patients who underwent surgery at the Leiden University Medical Center (LUMC; Leiden, The Netherlands) between August 2015 and January 2017. All patients with epithelial endometrial cancer (including carcinosarcomas) were eligible for inclusion. After transportation of the surgical specimen to the pathology department, fresh tumor tissue was donated for research if sufficient tumor tissue was available. All cases obtained a unique research number and histotype was assigned by an experienced gynecopathologist (T. Bosse). The local medical ethics committee approved the study protocol (B16.019) and specimens were handled according to the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (Dutch Federation of Medical Scientific Societies).

### Functional *ex vivo* *RAD51* assay to determine HR capacity

Fresh endometrial cancer tissue samples were kept at 4°C in OSE Culture Medium (Wisent Bioproducts, catalog No. 316-030-CL) supplemented with 10% FBS and 1% penicillin-streptomycin (100 U/mL). Tissue was manually cut in 5-mm slices and after an incubation period of at least 4 hours at 37°C, the slice was irradiated with 5-Gy ionizing radiation (200 kV, 4 mA, YXLON Y.TU 225-D02) to induce DNA DSBs. Samples were then incubated on a rotating device (60 rpm) for 2 hours at 37°C in the OSE

culture medium supplemented with 5-ethynyl-2'-deoxyuridine (EdU) to a final concentration of 20  $\mu\text{mol/L}$  (component A, catalog No. C10340, Click-iT EdU Imaging Kits, Invitrogen), according to manufacturer's instructions. After incubation, tissue slices were fixed in formalin (4%) and embedded in paraffin. Leftover endometrial cancer tissue was stored in liquid nitrogen in Recovery Cell Culture Freezing Medium (Sigma, catalog No. 12648010) to ensure viability after cryostorage.

**Immunofluorescent staining.** After irradiation and incubation, tumor samples were costained for RAD51, Geminin, and EdU using anti-RAD51 (GTX70230, GeneTex), anti-geminin (10802-1-AP, Protein Tech group), and the Click-iT reaction cocktail for EdU detection. For details, see Supplementary Materials and Methods.

**Quality control and scoring of the RAD51 assay.** To ensure high-quality data, we applied 3 stringent inclusion criteria. First, a semiquantitative analysis of the quality of the tumor tissue was performed on a hematoxylin and eosin (H&E)-stained serial section of the irradiated tumor slice used for the RAD51-IRIF assay. The tissue quality was scored (score 1–2 = poor, 3–4 = moderate, 5–6 = good) on the basis of the sum of the tissue vitality (1 = poor, 2 = moderate, 3 = good) and tumor percentage (0 = <5%, 1 = 5%–20%, 2 = 20%–49%, 3 =  $\geq$ 50%). Samples were excluded when the total tissue quality score was 2 or less, or when the tumor percentage was <5%. Second, we only included samples for which we were able to score RAD51-IRIF in at least 50 geminin-positive cells, defined by complete nuclear staining. Geminin is a cell-cycle marker to identify cells in the S/G<sub>2</sub>-phase, the cell-cycle phases in which HR is active. Third, >30% of the geminin-positive cells had to be EdU-positive. EdU is a nucleoside analogue that is actively incorporated into the DNA during DNA synthesis (29). Absence or low levels of EdU incorporation are indicative for limited DNA replication capacity of the tumor cells. As

nonproliferative cells are not able to perform HR, this criterion avoids incorrect classification of tumors as HR-deficient.

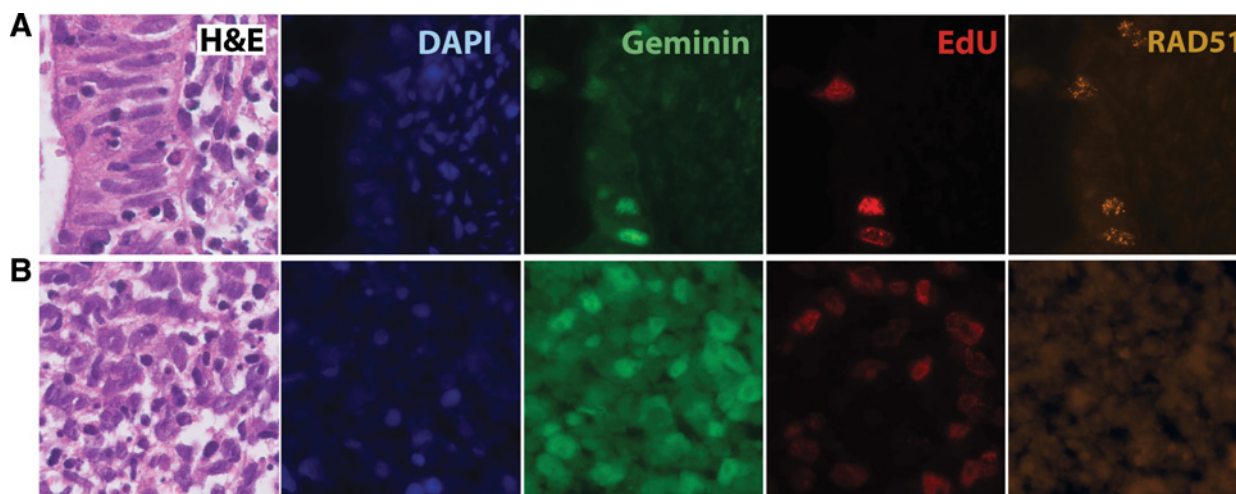
When 1 of these 3 criteria was not met, cryopreserved tissue from the same tumor was thawed, irradiated, and analyzed. If this "back-up" sample also failed to meet all the quality controls, the tumor sample was excluded from further analysis.

For scoring, we used preestablished cut-off values (25). A tumor was considered HR-proficient when more than 5 RAD51-IRIF per nucleus were present in >50% of geminin-positive cells and HR-deficient when  $\leq$ 20% of geminin-positive tumor cells formed RAD51-IRIF after ionizing radiation (Fig. 1). RAD51-IRIF formation in 21%–50% geminin-positive tumor cells was considered HR-intermediate. All cases were scored for Geminin, RAD51, and EdU by 2 independent observers via immunofluorescence microscopy and the average score was used for the category assignment.

#### Genetic and epigenetic analyses

**DNA isolation.** Tumor DNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue blocks either by taking 3 0.6-mm tumor cores or by microdissection of tumor areas (10- $\mu\text{m}$  slides). DNA isolation was performed fully automated using the Tissue Preparation System (Siemens Healthcare Diagnostics) as described previously (30). In addition, for a subset of cases, high-quality tumor DNA was isolated from frozen tumor tissue using 5–10 whole cryosections (20  $\mu\text{m}$ ) and the Wizard Genomic DNA purification KIT (Promega), according to manufacturer's protocol. An H&E cryoslide (5  $\mu\text{m}$ ) was made to determine tumor percentage. The Qubit dsDNA HS Assay Kit was used for DNA quantification, according to manufacturer's instructions (Qubit 2.0 Fluorometer, Life Technologies).

**aCGH/SNP array to determine SCNAs.** SCNAs were determined using either the Agilent SurePrint G3 CGH Microarray (8  $\times$  60k probes, Agilent technologies) on 300-ng DNA derived from frozen tumor tissue ( $n = 16$ , case ID; 1, 3, 6, 7, 9, 12, 13, 14,



**Figure 1.**

Functional *ex vivo* RAD51-assay to determine homologous recombination repair capacity in endometrial cancer. **A**, Example of a homologous recombination repair-proficient endometrioid endometrial carcinoma (case 26). In the H&E, the presence of tumor tissue is confirmed. Cell nuclei are stained with DAPI. Geminin-staining marks cells in S- and G<sub>2</sub>-phase. RAD51 foci can be visualized in geminin-positive tumor cells 2 hours after *ex vivo* exposure to X-rays (5 Gy). **B**, Example of a homologous recombination repair-deficient carcinosarcoma (case 13). After *ex vivo* treatment with ionizing radiation, only 2% of the geminin-positive cells demonstrates accumulation of RAD51-foci.

15, 16, 17, 18, 19, 21, 22, 24) or the OncoScan FFPE Assay Kit (335k probes, Thermo Fisher Scientific) on 80-ng FFPE-isolated DNA ( $n = 9$ , case ID; 20, 25, 26, 27, 29, 32, 33, 34, 36). Prior paired analysis of ten ovarian tumor samples showed that the SCNA were similar independently of the platform used (Supplementary Fig. S1). Furthermore, unsupervised Pearson hierarchical clustering performed on the included tumor samples demonstrated a natural division between samples independent of the platform used (Supplementary Fig. S2). For both platforms, samples were included when the tumor cell percentage was at least 30%. The mean tumor cell percentage of the DNA derived from frozen tumor tissue samples included for the aCGH was 78% (range: 30%–95%). The mean tumor cell percentage of the FFPE tissue-isolated DNA samples for the SNP array was 71% (range: 50%–90%). Analysis was performed according to manufacturer's instructions. Microarray data is available upon request. For details, see Supplementary Materials and Methods.

**Genomic instability score.** The genomic instability score (GIS) was calculated as the number of altered segments superior to 15 Mbp and inferior to chromosome arm, and samples were classified in 3 categories using an unsupervised machine learning (kmeans – python scikit) based on GIS. For details on the analysis, see Supplementary Materials and Methods. The 3 categories were SCNA-low, SCNA-high, and SCNA-extremely high.

**Somatic copy-number losses.** As a marker for potential loss of function of HR genes, the presence of "high somatic copy-number (SCN) losses" was determined for all cases by using a very stringent cut-off value;  $\log_2$  ratio  $\leq -0.7$ . This stringent cut-off value was used to select for SCN losses in genes that are more likely clonal and/or homozygous. The same cutoff was applied for both platforms (CGH Agilent and OncoScan) as both yield similar results. HR genes were defined according to a previously published list by Riaz and colleagues HR genes were categorized as either "core" HR genes (involved in the core HR machinery) or "related" HR genes (involved in closely related processes; ref. 31).

**Next-generation sequencing.** Next-generation sequencing (NGS) was performed using FFPE-isolated tumor DNA with a total input of 500–1,000 ng per sample. The mean tumor cell percentage of the included samples was 68% (range: 30%–90%). An Agilent Sureselect<sup>XT</sup> HS Custom panel made in SureDesign (Agilent technologies) was used for variant detection with the following HR-gene design: *ATM*, exons 2–63; *BARD1*, exons 1–10; *BRCA1*, exons 1–24; *BRCA2*, exons 2–27; *BRIP1*, exons 2–20; *CDK12*, exons 1–14; *CHEK2*, exons 2–15; *PALB2*, exons 1–13; *RAD51C*, exons 1–9; and *RAD51D*, exons 1–14. Additional genes included in the panel were *TP53* (exons 1–12) and *CCNE1* (only for amplification detection). For details on the data analysis, see Supplementary Materials and Methods.

Variants were categorized using the 5-tier pathogenicity classification according to Plon and colleagues, 2008; class 1 = benign, class 2 = likely benign, class 3 = variant of unknown significance (VUS), class 4 = likely pathogenic, and class 5 = pathogenic (32). Only class 3, 4, and 5 variants are reported in the manuscript. Variants were annotated on the basis of build GRCH37 (hg19) using the following transcript numbers: *ATM*, NM\_000051.3; *BRCA1*, NM\_007294.3; *BRCA2*, NM\_000059.3; *BRIP1*, NM\_032043.2; *CHEK2*, NM\_007194.3; *CDK12*, NM\_016507.3; and *RAD51D*, NM\_002878.3.

**BRCA1 hypermethylation using MS-MLPA.** The presence of *BRCA1* promoter hypermethylation was assessed for all cases using tumor DNA isolated from FFPE tissue. For this, the SALSA MLPA ME001 tumor suppressor mix (MRC-Holland) was used as described in the Supplementary Materials and Methods.

**IHC analysis.** If not yet performed in routine diagnostics (Autostainer Link 48, DAKO), additional IHC stainings for PMS2 (Clone EP51, 1:25, DAKO), MSH6 (Clone EPR3945, 1:400, GeneTex), PTEN (Clone 6H2.1, 1:200, DAKO), MRE11 (clone 31H4, 1:400, Cell Signaling Technology), and BAP1 (clone C4, 1:100, Santa Cruz Biotechnology) were performed on whole slides (4  $\mu$ m) as described in the Supplementary Materials and Methods.

**POLE sequencing.** Unidirectional Sanger sequencing was performed to screen exons 9 (forward), 13 (reverse), and 14 (reverse) for somatic *POLE* exonuclease domain mutations as described previously using FFPE tumor DNA (33). To sequence exon 14, the following primers were used; forward: 5'- tctggcgtctctctcag-3', reverse: 5'- cgacaggacagataatgctcac-3'. Mutations were confirmed by Sanger sequencing in the opposite direction. *POLE* transcript NM\_006231.3 was used for variant annotation.

**TCGA classification based on surrogate markers.** All endometrial cancers included in this study were classified according to the previously described molecular subclasses using a surrogate marker approach. For details, see Supplementary Materials and Methods.

#### BRCA-associated genomic scars in the TCGA cohort

To determine the presence of *BRCA*-associated genomic scars in the TCGA-EC cohort, SCNA data, and somatic mutation annotation files (MAF) were obtained from Firebrowse (<http://firebrowse.org/>) using data version 2016\_01\_28; doi:10.7908/C11G0KM9 (34). First, we assessed the presence of specific patterns of somatic copy-number gains and losses that have previously been linked to *BRCA1* or *BRCA2*-mutated breast and ovarian cancer to classify tumors in *BRCA*-like or non-*BRCA*-like (35). Second, we assessed the number of LST (cut-off used to define HR-deficiency  $\geq 15$ ), the presence of COSMIC signature 3 and the presence of biallelic pathogenic mutations in 102 HR genes as defined by Riaz and colleagues (31). For details regarding these analyses, see Supplementary Materials and Methods.

#### Statistical analysis

Comparison of age between groups was performed using the unpaired *t* test. Associations between all categorical variables were tested using a 2-sided Fisher exact test. A *P* value of  $<0.05$  was considered significant. Cohen kappa coefficient ( $\kappa$ ) was used to measure interobserver and intertest agreement. IBM SPSS version 23.0 (SPSS, Inc.) and R (<http://r-project.org>) were used for statistical analysis.

## Results

### Homologous recombination repair deficiency and clinicopathologic characteristics

Fresh tumor tissue was prospectively obtained from 36 patients. Twenty-five samples (12 EEC and 13 NEEC) passed our stringent quality controls and were included for further analyses (Fig. 2). Clinicopathologic characteristics of the total cohort are

described in Supplementary Table S1. The percentage of Geminin<sup>+</sup>/RAD51<sup>+</sup> cells scored after *ex vivo* exposure to ionizing radiation by the 2 independent observers was comparable, with a median score difference within cases of 6% (range: 0%–41%). Interrogator reliability for final HR category assignment was high ( $\kappa = 0.85$ ).

In total, 6 (24%) endometrial cancers were classified as HR-deficient, 17 (68%) as HR-proficient, and 2 (8%) as HR-intermediate. Clinicopathologic characteristics of groups stratified by HR status are shown in Table 1 and Fig. 3A. HR-intermediate cases are described in Supplementary Table S2. HR deficiency was significantly associated with non-endometrioid histology; all 6 (100%) HR-deficient tumors were NEEC, compared with none of 12 EEC tested ( $P = 0.014$ ). The 6 HR-deficient NEEC were either USC ( $n = 3$ , 50%) or UCS with serous epithelial component ( $n = 3$ , 50%). The 17 HR-proficient tumors were histologically more diverse; 11 (65%) EEC, 2 (12%) CCC, 2 (12%) dedifferentiated carcinomas, 1 (6%) USC, and 1 (6%) UCS with serous epithelial component. When only considering USC and UCS (both with serous and endometrioid epithelial component), 6 of 9 tumors (67%) were HR-deficient.

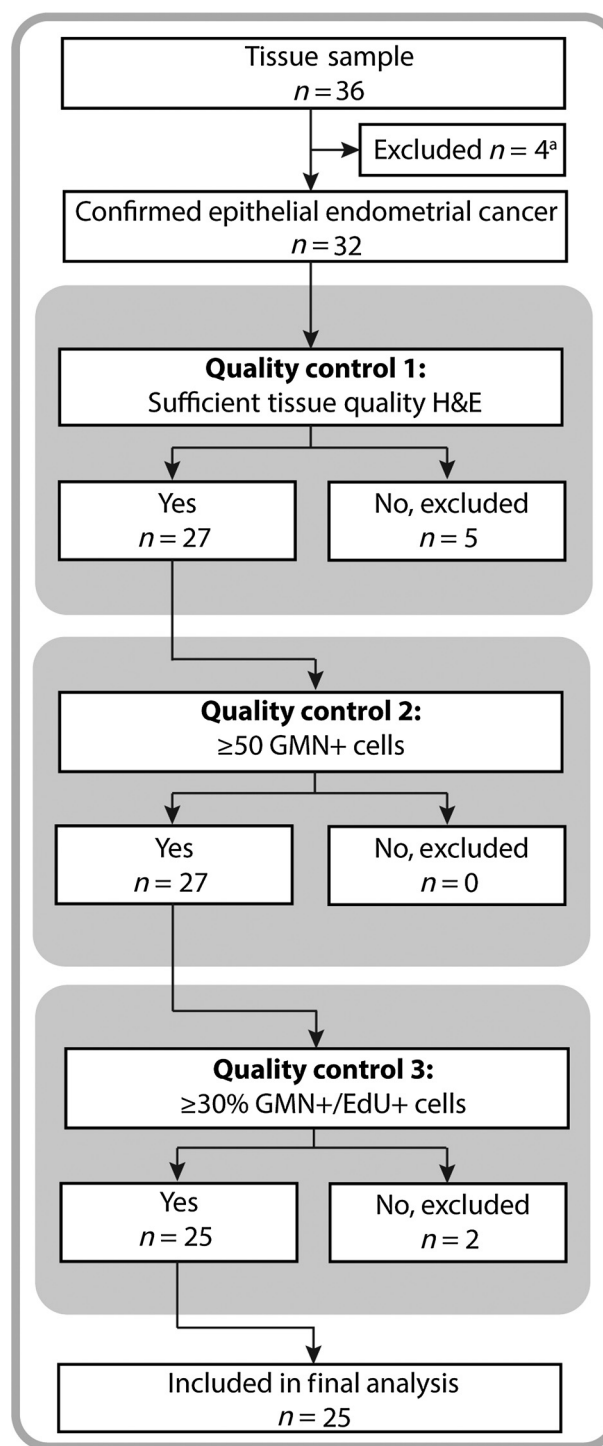
HR-deficient endometrial cancers were more often high grade (grade 3; 100%) compared with HR-proficient endometrial cancers (41%,  $P = 0.019$ ), reflecting the non-endometrioid histology in the HR-deficient group. HR-deficient endometrial cancers presented more often in a high FIGO stage compared with HR-proficient endometrial cancers (I vs III/IV;  $P = 0.021$ ) and had more frequent lymphovascular space involvement ( $P = 0.045$ ). We did not observe an association between HR deficiency and loss of PTEN expression by IHC, with 1 (17%) of the HR-deficient cases showing PTEN loss compared with 47% of HR-proficient cases ( $P = 0.340$ ). There was also no association between HR capacity and age of endometrial cancer diagnosis ( $P = 0.431$ ). *TP53* variants were more often present in HR-deficient tumors (100%) compared with HR-proficient tumors (41%;  $P = 0.019$ ). In total, 46% of the *TP53*-mutated endometrial cancers were HR-deficient.

Two cases were assigned HR-intermediate. One was a grade 3 EEC that was just above the threshold of being HR-deficient (case 27; Geminin<sup>+</sup>/RAD51<sup>+</sup>; 23%). The other case was a UCS with an endometrioid epithelial component (case 18; Geminin<sup>+</sup>/RAD51<sup>+</sup>; 44%, Fig. 3A; Supplementary Table S2).

#### Homologous recombination repair capacity and molecular subgroups

Surrogate markers were used to classify the endometrial cancers into the 4 molecular subgroups as defined by the TCGA study (Table 1; Fig. 3A). HR-deficient endometrial cancers were significantly more often classified as SCNA-hi/*TP53*-mutated compared to HR-proficient endometrial cancers, with all HR-deficient endometrial cancers being SCNA-hi/*TP53*-mutated compared with 6 (35%) of the HR-proficient endometrial cancers ( $P = 0.014$ ). The HR-proficient group was heterogeneous with all molecular subgroups represented; 9 (53%) NSMP, 6 (35%) SCNA-hi/*TP53*-mutated, 1 (6%) *POLE*/ultramutated, and 1 (6%) MMRd/hypermuted.

To further characterize our cohort, we performed SCNA analyses using a genomic instability score (GIS) based on the number of altered segments greater than 15 Mbp and smaller than a whole chromosome arm. For this, samples were classified in 3 categories using unsupervised machine learning (k-means clustering);



**Figure 2.**

Flowchart illustrating the selection of cases for analysis. Of 36 samples, 4<sup>a</sup> cases were excluded because histologic evaluation demonstrated no epithelial endometrial malignancy (2× cervical carcinoma, 1× leiomyosarcoma, 1× benign). Tissue was thawed and reanalyzed for 10 cases because they did not pass 1 of the quality controls (QC1,  $n = 5$ ; QC2,  $n = 0$ ; QC3,  $n = 5$ ). For 3 cases (all initially excluded during QC3), this procedure resulted in sufficient quality improvement to allow inclusion for final analysis. For 1 case, only frozen tissue was available, which was of sufficient quality. In total, 25 cases passed all quality controls.

SCNA-low, SCNA-high, and SCNA-extremely high. All HR-deficient endometrial cancers (100%) were either SCNA-high ( $n = 2$ ) or SCNA-extremely high ( $n = 4$ ), compared with 7 (41%; 6 SCNA-high, 1 SCNA-extremely high) of the HR-proficient endometrial cancers ( $P = 0.019$ , Fig. 3A; Table 1). An association was observed between the SCNA status and the presence of a *TP53* variant, with *TP53* variants being significantly more common in SCNA-high or extremely high endometrial cancers (79%, 11/14) compared with SCNA-low endometrial cancers (18%, 2/11,  $P = 0.005$ ).

#### Genetic alterations in HR genes and relation to HR phenotype

We performed (epi)genetic analysis to identify possible loss-of-function alterations that could explain the HR deficiency. This included NGS (variant HR genes), aCGH/SNP array (high SCN losses of HR genes;  $\log_2$  ratio  $\leq -0.7$ ), MS-MLPA (*BRCA1* promoter hypermethylation), and IHC (MRE11, BAP1).

In 2 of 6 HR-deficient endometrial cancers, the presence of a pathogenic *BRCA1* variant with LOH of the wild-type allele could explain the HR-deficient phenotype (case 9; *BRCA1*, c.4327C>T, p.Arg1443\*, and case 15; *BRCA1*, c.3013delG, p.Glu1005fs; Fig. 3B; Supplementary Table S3). Two other HR-deficient cases harbored a VUS in an HR gene; case 36; *RAD51D*, c.433C>T, p.Arg145Cys and case 19; *ATM*, c.6543G>T, p.Glu2181Asp. As it is uncertain whether these variants will affect protein function and the variant allele frequency (VAF) was low (32%, and 34%, respectively) with tumor percentages of 75% and 70%, respectively, it is unlikely that these variants were causative for the observed HR deficiency.

High SCN losses in HR core and HR-related genes were observed for both cases in which no variants were identified (cases 12 and 13) and for case 19, in which a VUS in *ATM* was detected (Figs. 3B and ). Case 36, in which a *RAD51D* VUS was identified, did not show SCN losses in HR genes with a  $\log_2$  ratio of  $\leq -0.7$ . None of the included cases demonstrated *BRCA1* promoter hypermethylation or IHC BAP1 or MRE11 expression loss.

In the HR-proficient endometrial cancers, variants in HR genes were present in 2 cases (Fig. 3B). Case 26, the *POLE*-mutated tumor, harbored a class 5 *CHEK2* variant c.1510G>T, p.Glu504\* (VAF: 28%) that likely occurred as a consequence of the *POLE* mutation as it is concordant with the known mutational bias it causes (36). Case 23, the MMRd endometrial cancers, harbored 4 *ATM* variants. One of the 4 *ATM* variants was a class 5 variant; c.640delT, p.Ser214fs, VAF: 5.5%, and the remaining 3 were all VUS (Supplementary Table S3). None of the HR-proficient endometrial cancers demonstrated high SCN losses of the HR core genes. Cases 01 and 34 did show high SCN losses in HR-related genes (Figs. 3B and 4).

Two endometrial cancers demonstrated an HR-intermediate phenotype (Fig. 3A and B; Supplementary Table S2). Case 27 harbored 2 *BRCA2*, 1 *BRIP1*, and 1 *CDK12* variant. The *BRCA2* variant with the highest VAF (64%) was a duplication of an adenine; c.6373dupA, p.Thr2125fs. In addition, an inframe deletion (c.6306\_6413del, p.Ser2103\_Val2138del) spanning the frameshift variant was present with a VAF of 28%, likely restoring the *BRCA2* function in a subset of the tumor cells. Case 18 harbored a class 5 *BRIP1* variant; c.632delC, p.Pro211fs with a VAF of 28%. None of the HR-intermediate cases demonstrated

high SCN losses in the HR core genes. Case 27 did show SCN losses in 1 HR-related gene (Figs. 3B and 4).

#### BRCA-associated genomic scars in the TCGA cohort

To validate the occurrence of HR deficiency in an additional endometrial cancer cohort, we used SCNA data and somatic MAFs from the TCGA study to determine the presence of *BRCA*-like profiles (data available for  $n = 536$ ), LSTs (data available for  $n = 444$ ), COSMIC signature 3 (data available for  $n = 246$ ), and pathogenic biallelic alterations in HR genes (data available for  $n = 541$ ). Because our data showed a clear difference in the presence of HR deficiency between EEC and NEEC, we stratified the cohort by histotype (EEC vs. NEEC, the latter including both mixed endometrial cancers and USC). Both a *BRCA*-like profile and a high LST score were significantly more common in NEEC (*BRCA*-like profile, 41.2%; LST, 47.7%)

**Table 1.** Clinicopathologic characteristics stratified for homologous recombination capacity

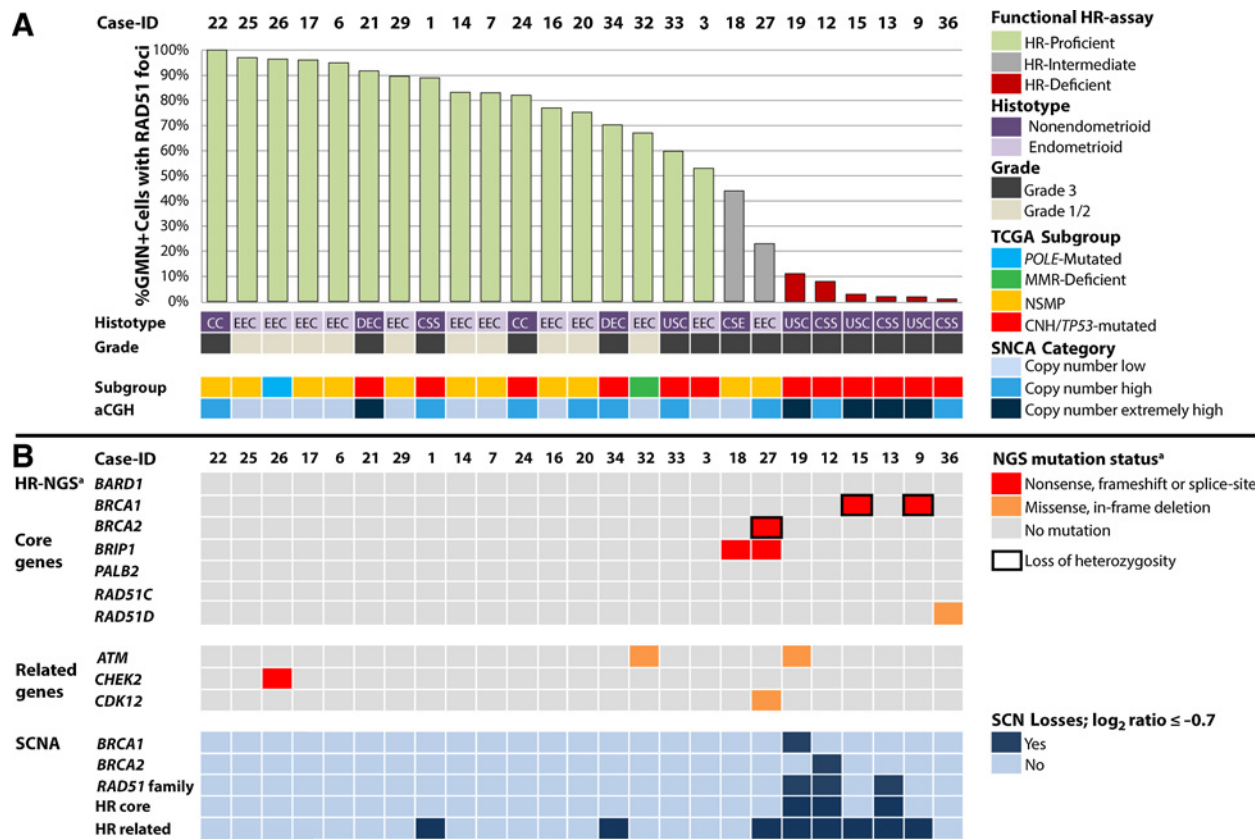
	HR-deficient <i>n</i> (%)	HR-proficient <i>n</i> (%)	<i>P</i>
Total	6 (100)	17 (100)	
Age, years			
Mean $\pm$ SD	70 $\pm$ 9.3	66 $\pm$ 10.6	0.431
Tumor			
Primary	6 (100)	17 (100)	
Recurrent	0 (0)	0 (0)	
Histologic subtype			
Endometrioid	0 (0)	11 (65)	<b>0.014<sup>a</sup></b>
Non-endometrioid	6 (100)	6 (35)	
Serous	3 (50)	1 (6)	
Carcinosarcoma	3 (50)	1 (6)	
Clear cell	0 (0)	2 (12)	
Dedifferentiated	0 (0)	2 (12)	
Histologic grade			
1 + 2	0 (0)	10 (59)	<b>0.019</b>
3	6 (100)	7 (41)	
FIGO 2009			
I	2 (33)	15 (88)	<b>0.021</b>
III/IV	4 (67)	2 (12)	
Adnexal involvement			
Yes	1 (17)	2 (12)	1.00
No	5 (83)	15 (88)	
LVSI			
Yes	4 (67)	3 (18)	<b>0.045</b>
No	2 (33)	14 (82)	
PTEN-IHC			
Loss of expression	1 (17)	8 (47)	0.340
Normal expression	5 (83)	9 (53)	
aCGH			
Copy number extremely high	4 (67)	1 (6)	<b>0.019<sup>b</sup></b>
Copy number high	2 (33)	6 (35)	
Copy number low	0 (0)	10 (60)	
<i>TP53</i>			
Mutation	6 (100)	7 (41)	<b>0.019</b>
No mutation	0 (0)	10 (59)	
TCGA subgroups			
<i>TP53</i>	6 (100)	6 (35)	<b>0.014</b>
NSMP/ <i>POLE</i> /MMRd	0 (0)	11 (65)	

NOTE: Bolded *P* values are considered significant ( $P < 0.05$ ). *P* values were calculated using the 2-sided Fisher exact test for the categorical variables and the unpaired *t* test for the difference in age.

Abbreviations: LVSI, lymphovascular space involvement; MMRd, mismatch repair deficient; NSMP, no specific molecular profile.

<sup>a</sup>Endometrioid versus non-endometrioid histology was compared.

<sup>b</sup>Copy number extremely high + copy number high versus copy number low was compared.



**Figure 3.**

Tumor characteristics (A) and genetic changes (B) stratified for homologous recombination capacity. Each column represents 1 case. A, Cases were classified in TCGA subgroups using surrogate markers as described in the Supplementary Materials and Methods. Case 26 contained a *POLE* variant and a *TP53* variant and was classified in the *POLE*-mutated subgroup. Case 09 demonstrated subclonal loss of *PMS2* with normal expression in the tumor tissue on which the RAD51 assay was performed, together with a *TP53* variant, and was classified as *TP53* mutant. B, HR genes were categorized as either being involved in the core process of HR ("core" genes) or being involved in related processes to HR ("related" genes), as previously described by Riaz and colleagues (31). Abbreviations: CSE, carcinosarcoma with endometrioid epithelial component; CSS, carcinosarcoma with serous epithelial component; DEC, dedifferentiated endometrial carcinoma; HR, homologous recombination. \*Only variants with a VAF of  $\geq 25\%$  are shown. When multiple variants were present in the same gene, the most pathogenic variant is shown.

compared with the EEC (*BRCA*-like profile, 8.0%; LST, 11.9%),  $P < 0.001$  (Fig. 5A and B). COSMIC signature 3 was present in 6.6% of EEC and 45.8% of NEEC ( $P < 0.001$ , Fig. 5C). It was present as dominant signature in 1.0% ( $n = 2$ ) of EEC and 6.3% of NEEC ( $n = 3$ ,  $P = 0.052$ ). Somatic or germline pathogenic biallelic variants in HR pathway genes were present in 4.4% of EEC and in 1.5% NEEC ( $P = 0.19$ , Fig. 5D). The high prevalence of *BRCA*-associated genomic scars in the TCGA-EC cohort supports that HR deficiency occurs in EC, especially in NEEC, as observed in our prospective cohort.

## Discussion

Using a functional assay to assess homologous recombination repair capacity, we found that HR deficiency is common in endometrial cancers, especially in NEEC (46%). The observation that all HR-deficient endometrial cancers were *TP53*-mutated and of USC or UCS histology (comprising 67% of the included USC/UCS) further extends the established parallels between a subset of endometrial cancer and HGSOE. In 5 of 6 HR-deficient tumors, we identified alterations in core HR genes

(2 cases with a pathogenic variant in *BRCA1* and 3 cases with high SCN losses of HR core genes). Independent validation using the TCGA endometrial cancers cases in which we determined the prevalence of *BRCA*-associated genomic scars underscored the high prevalence of HR deficiency in NEEC.

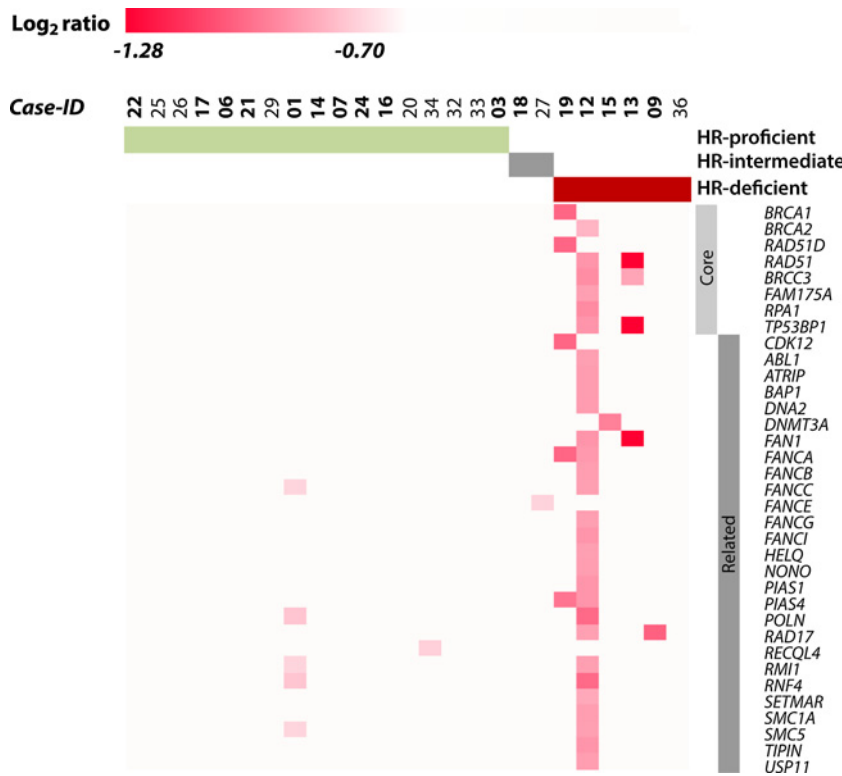
Using established cut-off values to assign endometrial cancers to different HR categories, we were able to assign 23 of 25 endometrial cancers into either the HR-deficient or HR-proficient category, leaving 2 cases in the HR-intermediate category (cases 27 and 18). Case 27 was a second recurrence of a *TP53* wild-type grade 3 EEC after 2 previous lines of platinum-based chemotherapy. At initial treatment, there was a partial response (according to the RECIST criteria) after 3 courses of neoadjuvant carboplatin/paclitaxel. Genomic analysis identified 2 *BRCA2* variants; 1 truncating frameshift variant and 1 in-frame deletion, spanning the region containing the frameshift variant. It is likely that the in-frame deletion is a secondary somatic variant (partially) restoring the *BRCA2* function, a scenario described previously (37). This is a relevant observation as it suggests that *TP53* wild-type endometrial cancers with endometrioid histology may also be HR-deficient.

*PTEN* alterations are frequent in endometrial cancers, particularly in EEC and may modulate DSB-repair capacity by regulating the expression of *RAD51* (20). *In vitro* studies have shown contradictory results, with some reporting no correlation between *PTEN* loss and HR deficiency (38, 39), whereas others did find a correlation (40). In our study, we did not observe a correlation between HR capacity and IHC *PTEN* expression.

On the basis of the high prevalence of HR deficiency in our cohort, one might speculate that a proportion of, especially the serous/serous-like endometrial cancers would be responsive to platinum-based chemotherapy (41, 42). The PORTEC-3 trial suggested that the addition of platinum/taxane-based chemotherapy to radiotherapy in patients with USC resulted in a similar failure-free survival benefit as for the overall cohort of patients with high-risk endometrial cancers, although this benefit was not significant (43). Furthermore, a grouped analysis among 1,203 patients with advanced or recurrent endometrial cancers participating in 4 gynecologic oncology group (GOG) trials found similar overall response rates to chemotherapy for USC as for other histotypes (EEC, CCC; ref. 44). In contrast, the pooled analysis of the NSGO-EC-9501/EORTC-55991 trials showed a significant progression-free survival benefit of the addition of adjuvant (platinum-based) chemotherapy for EEC but not patients with USC and CCC (45). Possible explanations for these different trial outcomes may be the small number of included USC, the different chemotherapy combinations used within trials (apart from PORTEC-3) and finally, the major difficulties pathologist are having with assigning histotype, particularly in high-grade endometrial cancers (46). For these reasons, future endometrial cancer trial designs, in which (platinum-based) chemotherapy is included, should consider HR status as a biomarker for treatment stratification.

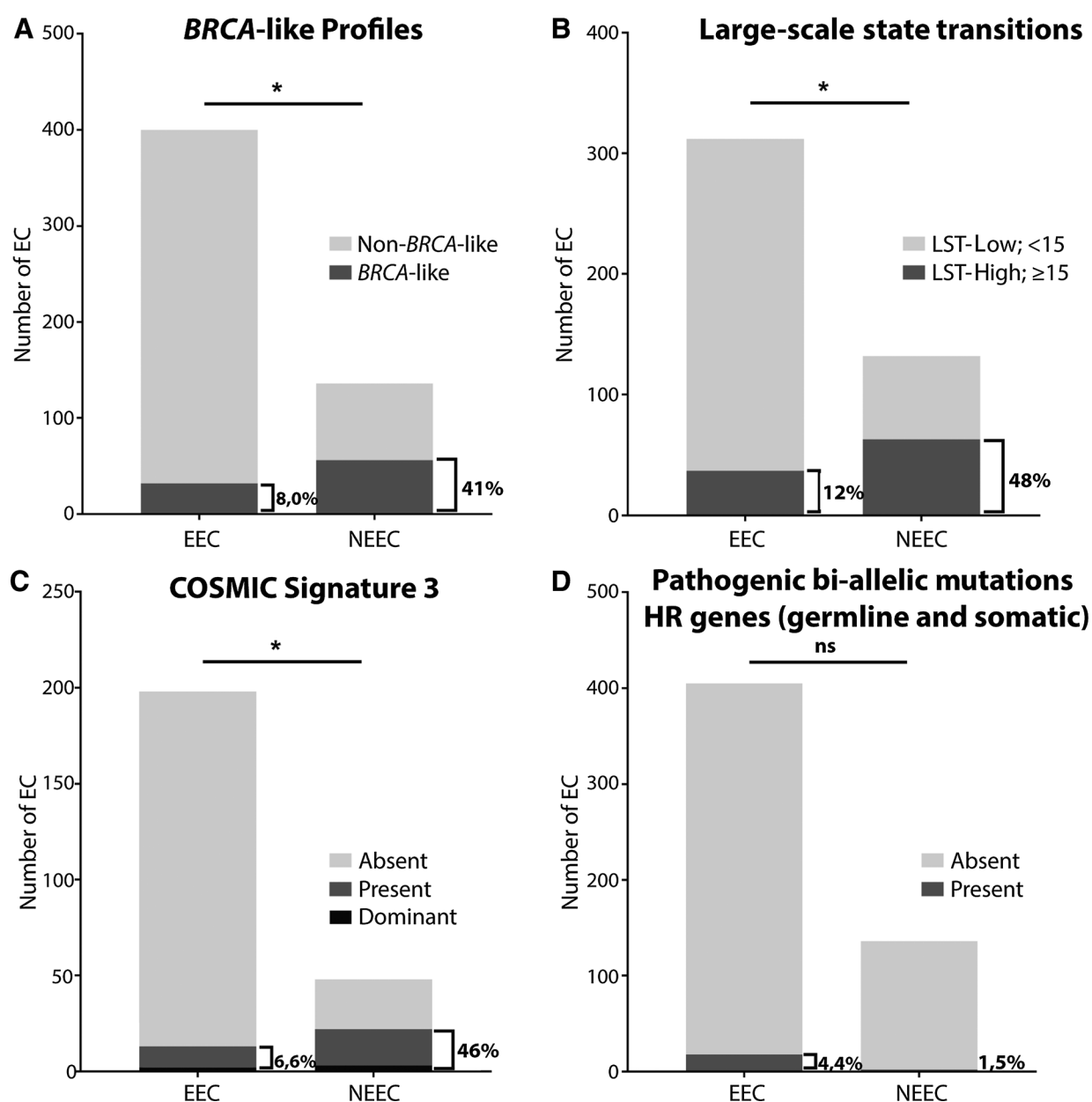
Multiple studies have already shown that PARP inhibitors improve progression-free survival in patients with platinum-sensitive recurrent ovarian cancer (47–49). Although most treatment benefit is observed for *BRCA1/2*-mutated tumors, an increased beneficial effect could also be observed for tumors with genetic alterations that are suggestive for HR deficiency as assessed by "genomic scar" assays (47–49). Our results suggests PARP inhibitors as a potential new treatment modality for the HR-deficient subgroup of endometrial cancer, which is further supported by a recently published case report in which a patient with EEC with a germline *BRCA2* variant (and a somatic hit of the wild-type allele) experienced a durable response to the PARP inhibitor, olaparib (50).

The performance of several candidate "HRD biomarkers" to predict therapy response are currently being studied, among which many that include the analysis of pathogenic variants in HR genes or the presence of *BRCA*-associated "genomic scars" in tumor DNA (16, 21–23, 51). At this moment, it is still unknown which of the available HRD biomarkers is most powerful to predict therapy response. The HR status, as determined by the *RAD51* assay used in this study, has been shown to be strongly associated with achieving a complete pathologic response to neoadjuvant chemotherapy in patients with breast cancer (26), could predict *in vitro* PARP-inhibitor cytotoxicity in primary cell cultures obtained from epithelial ovarian cancers (52, 53) and could predict platinum sensitivity as well as improved survival outcome in patients with EOC and HGSOC (27, 53). Because the *RAD51* assay is performed on fresh, irradiated tumor tissue, it currently has limited potential to be routinely used in clinical diagnostics, whereas methods that can assess "genomic scars" in FFPE-derived DNA are more suitable for this purpose (51). Interestingly, in the recently published study of Cruz and



**Figure 4.** Somatic copy-number losses stratified for homologous recombination (HR) capacity. HR genes were selected and divided in HR-core or HR-related genes as described by Riaz and colleagues. Only those genes with SCN losses of log<sub>2</sub> ratio ≤ -0.7 in at least 1 of the included cases are visualized. Data were extracted from the aCGH data as described in the Supplementary Materials. Bolded cases were analyzed using the CGH Agilent platform, others were analyzed using the Oncoscan platform.



**Figure 5.**

BRCA-associated genomic scars as surrogate marker for HR deficiency in the TCGA-endometrial cancer cohort. **A**, A BRCA-like profile was present in 32/400 of EECs and 56/136 of NEECs. **B**, A high LST score ( $\geq 15$ ) was present in 37/312 of EECs and 63/132 of NEECs. **C**, COSMIC signature 3 was present in 13/198 of EECs and 22/48 of NEECs, and it was present as dominant signature in 2/198 of EECs and 3/48 of NEECs. **D**, Pathogenic bi-allelic mutations in HR genes were present in 18/405 of EECs and 2/136 of NEECs. The intertest agreement (accuracy and Cohen's  $\kappa$  coefficient, respectively) were as follows; 0.82 and 0.46 for LST versus BRCA-like profiles, 0.84 and 0.40 for LST versus signature 3, 0.85 and 0.36 for BRCA-like profiles versus signature 3 (ns, not significant; \*,  $P < 0.001$ ).

colleagues, low levels of RAD51 foci in nonirradiated tumors correlated with PARP-inhibitor sensitivity in xenograft models (54). When this approach can be validated on (archived) human FFPE tumor tissue, the assessment of RAD51 to define HR status would become clinically feasible.

Our study is not without limitations. Our cohort is enriched for high-grade endometrial cancer cases, because we prospectively recruited patients in the LUMC, which is a referral center for endometrial cancer. Therefore, the prevalence of HR deficiency in

our endometrial cancer cohort is likely an overestimate, given the strong association with NEEC. Studies on larger cohorts are necessary to establish a more precise estimate of the prevalence of HR deficiency among the diverse endometrial cancer histotypes. Finally, the molecular analysis we performed was extensive, but not exhaustive. We used a targeted NGS panel and a aCGH/SNP array to identify the molecular cause of HR deficiency. In the future, whole-exome/genome sequencing may be preferred, not only to have the possibility to identify pathogenic variants in

additional genes, but also to explore the relationship between the outcome of the RAD51 assay and established genomic scars.

In conclusion, we are the first to demonstrate that HR is frequently abrogated in a subset of endometrial cancers, in particularly the "serous-like," *TP53*-mutated subclass of endometrial cancers, the group with the worst clinical outcome. This study provides a strong rationale for future clinical trials aiming to target HR-deficient high-grade endometrial cancers with therapies exploiting this defect, such as platinum compounds and PARP inhibitors.

### Disclosure of Potential Conflicts of Interest

Immediate family members of P.C. Schouten are employees of AstraZeneca. E. Rouleau reports receiving speakers bureau honoraria from and is a consultant/advisory board member for AstraZeneca and Bristol-Myers Squibb. A. Leary reports receiving commercial research grants from Merus and Gamamabs, and is a consultant/advisory board member for AstraZeneca, Clovis, Gridstone, Seattle Genetics, Gamamabs, Tesaro, and Biocad. No potential conflicts of interest were disclosed by the other authors.

### Disclaimer

The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

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