Exploring the role of homologous recombination deficiency and BRCA1/2 mutations in endometrial cancer

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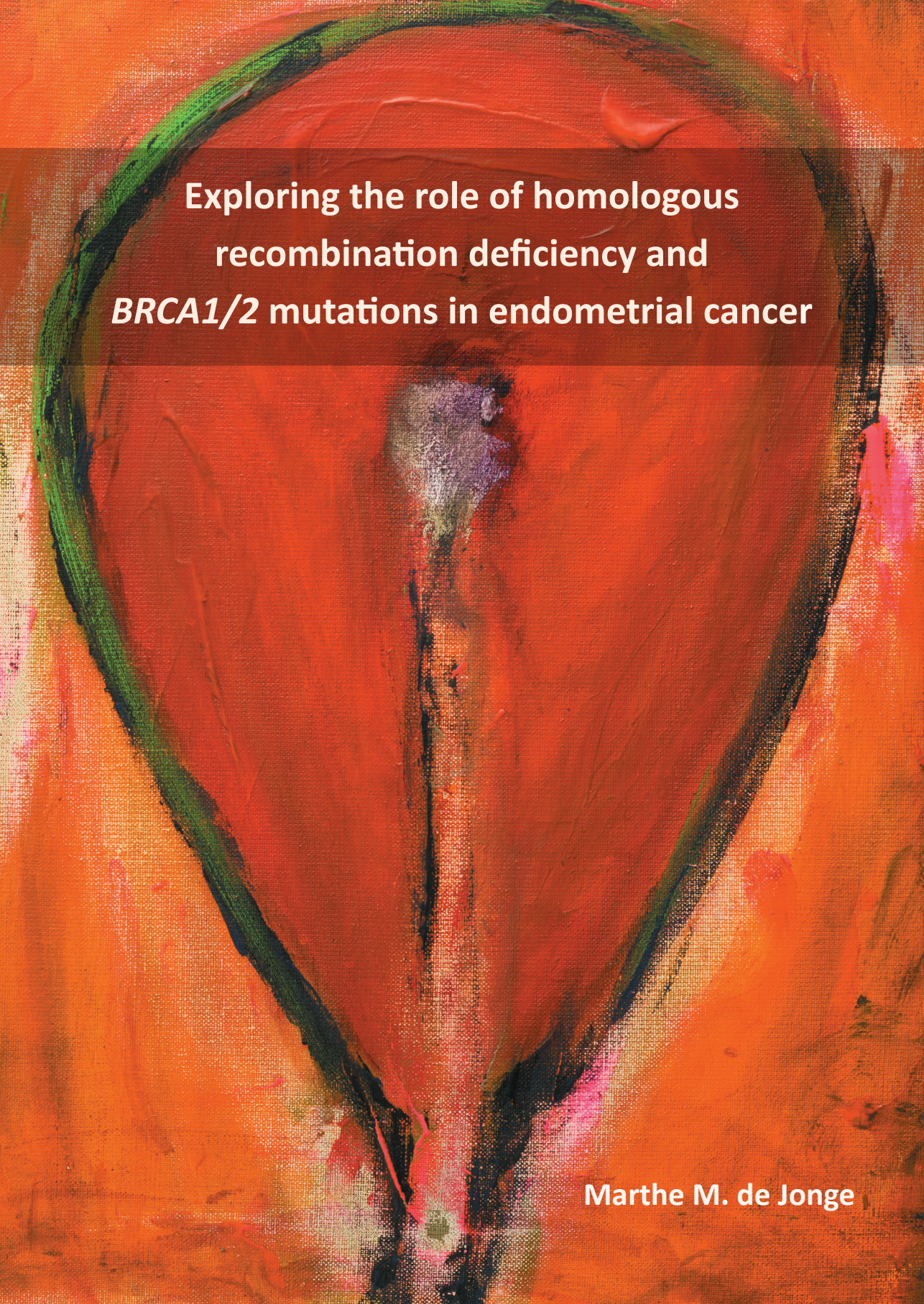
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Marthe M. de Jonge

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Exploring the role of homologous recombination deficiency and *BRCA1/2* mutations in endometrial cancer

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Chapter 1

General introduction

1. General introduction

1.1. Case presentation and introduction outline

A 50 year old woman without a personal cancer history visits the clinical geneticist because her sister recently got diagnosed with a hereditary mutation in the BReast CAncer gene 1 (*BRCA1)*. After genetic testing, it turns out she carries the same germline mutation in *BRCA1 (gBRCA1)*. *gBRCA1* mutations are associated with the hereditary breast and ovarian cancer (HBOC)-syndrome, a syndrome characterized by severely increased life-time risks of developing breast cancer (BC) and tubo-ovarian cancer (OC).[1] To reduce the BC and OC risk, she decides to undergo risk-reducing surgery, including both a bilateral mastectomy and a bilateral risk-reducing salpingo-oophorectomy (RRSO). Three years later, postmenopausal vaginal bleeding occurs. She gets diagnosed with (postsurgical) stage IV uterine serous carcinoma (USC) based on a supraclavicular lymph node metastasis. USC is a rare histologic subtype of endometrial cancer (EC) associated with poor clinical outcome.[2] She undergoes a total abdominal hysterectomy and dissection of the iliac and para-aortal lymph nodes, followed by six cycles of adjuvant carboplatin and paclitaxel chemotherapy. She is still disease free three and a half years later. The occurrence of an EC after RRSO raised the question whether EC is a *BRCA*-associated disease and whether this could have been prevented.

In this introduction, first, a general overview of the main DNA damage response pathways and tumor development is given, with emphasis on homologous recombination repair, the DNA repair pathway for which BRCA1 and BRCA2 are crucial. Second, the molecular alterations in EC will be discussed, with emphasis on similarities between EC and the histologic subtypes of BC and OC that frequently occur in *gBRCA1/2*-mutation carriers. Also, hereditary cancer syndromes associated with EC will be described. Third, the clinical implications for patients of having the *gBRCA1/2*-associatied HBOC-syndrome will be discussed. Finally, the aims and thesis outline will be described.

1.2. DNA repair pathways and tumor development

During life, the DNA of every living organism is continuously being exposed to both endogenous (e.g. reactive oxygen species, deamination) and exogenous (e.g. UV-radiation, chemicals, ionizing radiation, cigarette smoke) genotoxic agents, causing different forms of DNA damage. Adequate recognition and repair of this DNA damage is essential for the maintenance of genomic integrity. If DNA damage persists through replication, this can lead to mutations. Mutations in key regulatory genes might lead to the accumulation of additional mutations, with subsequent uncontrolled cell growth and loss of protective apoptotic and cell cycle control checks, facilitating cancer development.[3, 4] Not surprisingly, genomic instability is an important hallmark of tumor cells.

To prevent the induction of DNA mutations due to the presence of DNA damage, cells have many (interwoven) DNA damage response (DDR) pathways. These DDR-pathways are responsible for the maintenance of genomic integrity, involving the detection of DNA damage, recruitment of DNA repair factors to the site of damage and the actual repair of the lesion.[4, 5] Cells have different DNA damage repair pathways to repair different kinds of DNA damage, Table 1.[4, 5]

Mutations that are acquired during life, are called "somatic mutations". These mutations are only present in cells derived from the mutated cell. Mutations can also be present in gametes. These are the only mutations that can be passed on to the offspring. These mutations will be present in every cell of the offspring and are called "germline mutations".

A subset of familial cancer syndromes have been associated with germline mutations in genes involved in the different DDR-pathways, making family members more prone to cancer development. Table 1 shows the main DNA-damage repair pathways and associated hereditary syndromes.[4-6]

Table 1. DNA-damage repair pathways essential for maintenance of genomic stability

DNA lesion	Repair mechanism	(subset of) genes involved	Associated hereditary syndromes	Associated tumor types
Single strand breaks	Base excision repair	PARP1, XRCC1, Ligase 3, MUTYH		Colorectal
Double strand breaks	Homologous recombination	BRCA1, BRCA2, PALB2, ATM, CHEK1, CHEK2, RAD51	Hereditary breast and ovarian cancer (HBOC)-syndrome, Fanconi anemia	Breast, ovarian, pancreatic leukemia
	Non-homologous end joining	KU70/80, DNA-PK, ligase IV, XRCC4	Severe Combined ImmunoDeficiency (SCID)	
	microhomology-mediated end joining	polymerase theta		
Bulky adducts	Nucleotide excision repair	ERCC4, ERCC1, ERCC2	Xeroderma pigmentosum	Skin
Base mismatches, insertions, deletions	Mismatch repair	MLH1, PMS2, MSH2, MSH6	Lynch syndrome	Colorectal, endometrial
Base alkylation	Direct reversal repair	MGMT		Glioma

Adapted from Lord 2012[4] and Cold Spring Harb Perspect Biol 2012;4:a012773.[6]

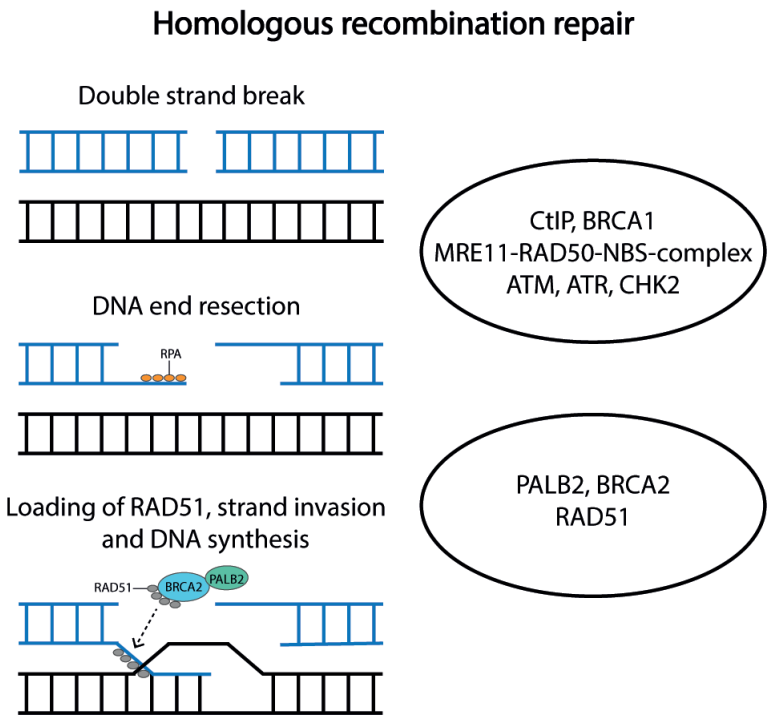


Figure 1: Simplified overview of homologous recombination repair (HR). HR is important for the high fidelity repair of DNA double strand breaks and consists of a series of sequential steps. It is active in the S/G2-phases of the cell cycle, in which a sister chromatid can be used as a template for the DNA repair. In the different steps multiple proteins are involved, some of which are shown in Figure 1. BRCA1, ATM, ATR, CHK2 and the MRE11-RAD50-NBS1 complex are important for the detection of the DNA double strand breaks and the resection the 5' DNA sides to create single strand DNA (ssDNA) ends at the break sites. This ssDNA is subsequently coated with RPA. PALB2/BRCA2 is responsible for the replacement of RPA and subsequent loading of RAD51 to the now exposed ssDNA region. RAD51 forms a nucleoprotein filament with the ssDNA region allowing the DNA to invade the homologous DNA helix, so that it can be used as a template for DNA synthesis to restore the double strand break. Adapted from Roy et al, 2012, Lord and Ashworth, 2016 and Vanderstichele, 2017.[7, 8, 13]

1.2.1. *BRCA1, BRCA2* and homologous recombination repair

BRCA1 and *BRCA2* are tumor suppressor genes that are essential for the maintenance of genomic integrity.[7, 8] They both play a crucial role in homologous recombination repair (HR), a DNA repair pathway that is active during the S and G2 phases of cell cycle, and which is important for the high-fidelity repair of DNA double strand breaks (DSBs) and restoration of lesions that stall the DNA replication fork.[7, 8] *BRCA1/2*-deficient tumors are not capable of performing HR, and are therefore considered to be homologous recombination deficient (HRD).

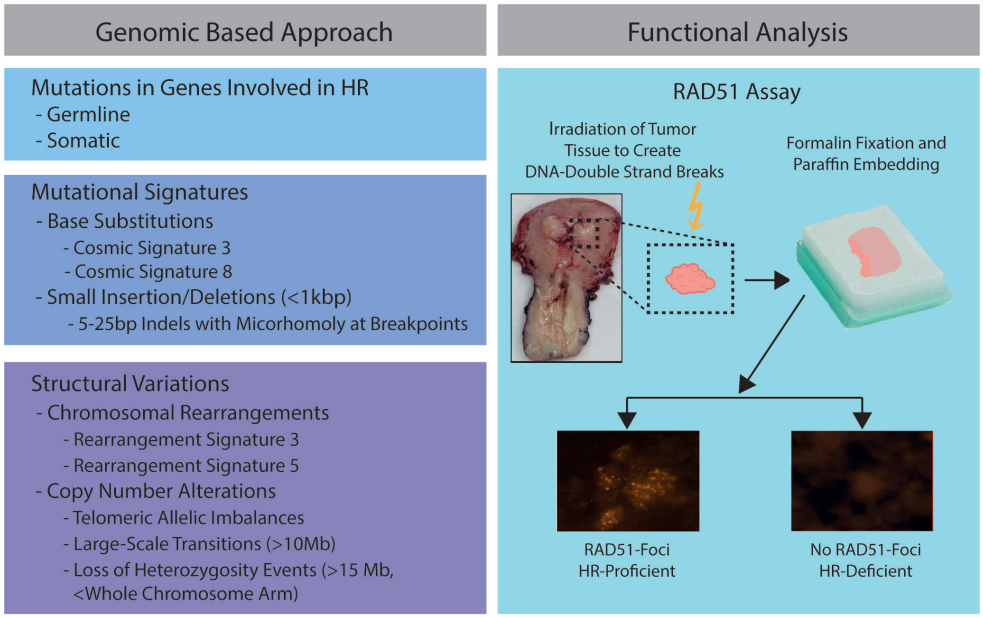


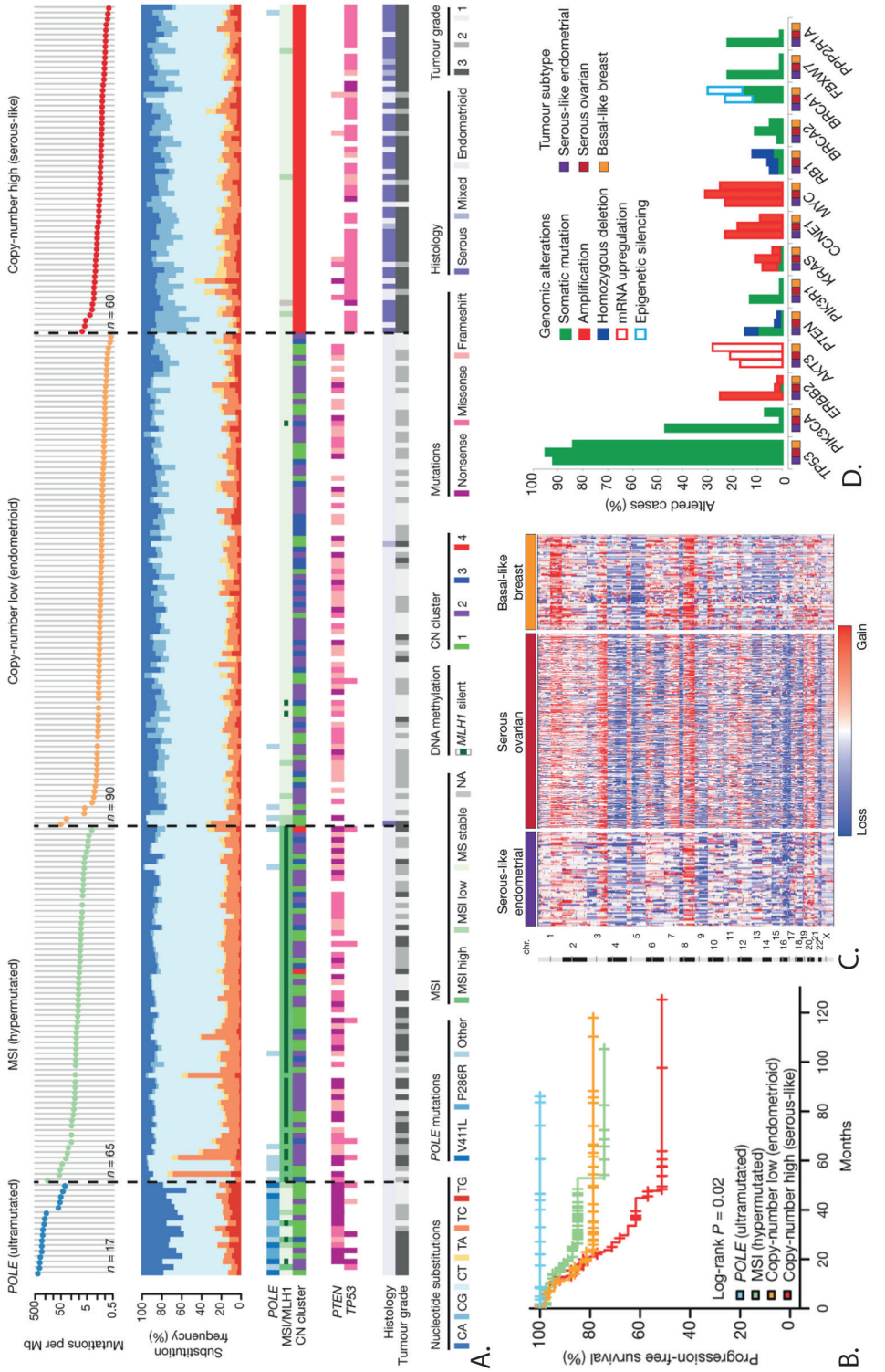
Figure 2: Examples of biomarkers used to indirectly (**A**) and directly (**B**) assess homologous recombination capacity tumor samples.

Besides BRCA1 and BRCA2, multiple other proteins are involved in HR.[7-9] Germline mutations in some of these HR genes also give rise to increased breast- and/or ovarian cancer risk (*PALB2, ATM, CHEK2, RAD51C, RAD51D, BRIP1)*, with risks varying dePending on the gene involved.[10-12] Figure 1 gives an overview of HR and a subset of proteins involved.

1.2.2. *BRCA1/2*-deficient tumors and genomic scars

Mutations in *BRCA1* and *BRCA2* can both be of somatic and germline origin. BCs and OCs with *BRCA1/2* mutations (both germline and somatic) generally show loss of heterozygosity (LOH) of the wild-type allele, resulting in complete loss of *BRCA1* or *BRCA2* function. LOH is an important step in *BRCA1/2*-associated carcinogenesis, as studies showed that BCs and OCs that occurred in *gBRCA1/2* mutation carriers but in which no LOH was present did not show the typical genomic alterations observed in *gBRCA1/2*-associated carcinomas. These tumors instead showed genomic alterations more similar to sporadically occurring, non-*BRCA1/2*-associated carcinomas.[14]

The combination of genomic alterations, called “genomic scars” or “mutational signatures”, that are observed in tumors that are *BRCA*-deficient (“*BRCA*-null tumors”) can be attributed to the accumulation of DNA-DSBs and the use of alternative, error-prone DSB repair pathways like non-homologous end-joining and alternative non-homologous end-joining,


POLE (ultramutated)
MSI (hypermutated)
Copy-number low (endometrioid)
Copy-number high (serous-like)
n = 17
n = 65
n = 90
n = 60
Mutations per Mb
Substitution frequency (%)
POLE
MSI/MLH1
CN cluster
PTEN
TP53
Histology
Tumour grade
A.
Nucleotide substitutions
CA CG CT TA TC TG
POLE mutations
V411L P286R Other
MSI
MSI high MSI low MS stable NA
DNA methylation
MLH1 silent
CN cluster
1 2 3 4
Mutations
Nonsense Missense Frameshift
Histology
Serous Mixed Endometrioid
Tumour grade
3 2 1
B.
Progression-free survival (%)
Months
Log-rank P = 0.02
POLE (ultramutated)
MSI (hypermutated)
Copy-number low (endometrioid)
Copy-number high (serous-like)
C.
Serous-like endometrial
Serous ovarian
Basal-like breast
chr.
Loss
Gain
D.
Altered cases (%)
TP53
PIK3CA
ERBB2
AKT3
PTEN
PIK3R1
KRAS
CCNE1
MYC
RB1
BRCA2
BRCA1
FBXW7
PPP2R1A
Genomic alterations
Somatic mutation
Amplification
Homozygous deletion
mRNA upregulation
Epigenetic silencing
Tumour subtype
Serous-like endometrial
Serous ovarian
Basal-like breast

< Figure 3: A. Molecular characterization of endometrial cancer in four molecular subgroups as proposed by The Cancer Genome Atlas Group (TCGA). **B.** Prognostic significance of the different molecular subgroup proposed by the TCGA. **C.** Somatic copy number alterations in the copy-number high/serous-like endometrial cancer subgroup, serous ovarian cancer and basal-like breast cancer. **D.** Genomic alterations frequently present in serous-like EC, serous ovarian cancer and basal-like breast cancer. Figures A, B, C, D are adapted from the TCGA, Nature 2013 (reprinted under the Creative Commons License).[28]

also called microhomology-mediated end joining. By using techniques like next-generation sequencing (NGS), array-based comparative genomic hybridization (aCGH) and single nucleotide polymorphisms (SNP)-based assays, these genomic scars can be assessed.[7, 13, 15-22] Mutational signatures shown to be overrepresented in *BRCA1/2*-null tumors are for example base-substitutions signature 3 and 8, deletions of >3 base pairs with microhomology at breakpoints, certain genomic rearrangement like rearrangement signatures 3 (small tandem duplications <10 kb) and 5 (deletions <100 kb), and an increased number of somatic copy-number alterations (sCNA) including widespread loss of heterozygosity of areas larger than 15Mb but shorter than the whole chromosome (HRD-LOH), increased number of telomeric allelic imbalances (NtAI) and large-scale state transitions (LST), see Figure 2.[7, 13, 15-22]

There are multiple ways to determine whether a tumor is HRD, see Figure 2. An indirect way is to determine the presence of mutations (either germline or somatic) in key genes involved in HR, like *BRCA1* and *BRCA2,* which will likely result in an HRD-phenotype in the presence of LOH of the wild-type allele. Another indirect way is to assess the presence of beforementioned "genomic scars"[13, 23] that have occurred as a result of the HRD-phenotype. However, assessing the presence of these genomic scars is still costly and not easily implementable in routine diagnostics.

A more direct way to determine HR capacity of tumor cells is by directly measuring the ability of these cells to perform HR. This can be assessed by functional analysis, in which the capacity of tumors cells to recruit RAD51 to ionizing radiation induced DNA DSBs can be measured.[24, 25] As shown in Figure 1, RAD51 is being recruited to the site of the DSB during HR.[8] HRD tumor cells will not be able to recruit RAD51 to the DNA DSBs, and this can thereby be used as a readout for HR capacity, see Figure 2B. The RAD51-assay has already shown to be able to reliably identify cell lines, xenografts and fresh human tumor tissue with defective HR without the necessity of performing expensive genomic analyses.[24-27]

1.3. Molecular alterations in endometrial cancer and similarities with HBOC-associated breast- and ovarian cancer

In 2013, the Cancer Genome Atlas Research Network (TCGA) divided EC in four distinct molecular subgroups based on the comprehensive genomic analyses of 373 endometrioid endometrial carcinomas (EECs), uterine serous carcinomas (USCs), and mixed carcinomas. By integrating tumor mutation burden (TMB), somatic copy number alterations (SCNAs) and

microsatellite instability (MSI) status, the following molecular subgroups with prognostic relevance were identified; (1) the *POLE*/ultramutated group, (2) the microsatellite instability-high (MSI-high)/hypermutated group, (3) the SCNA low/no specific molecular profile (NSMP) group and (4) the SCNA-high/serous-like group, see Figure 3, A-B.[28] Subsequent studies could reproduce these molecular subgroups with similar prognostic relevance using more clinically applicable surrogate markers,[29, 30] and showed that these molecular subgroups were also applicable to other histologic subtypes; uterine carcinosarcomas (UCS), clear cell carcinomas (CCC), undifferentiated carcinomas and dedifferentiated carcinomas.[31-34]

When looking more closely to the "SCNA-hi/serous-like" molecular subgroup, these tumors show striking similarities with high-grade serous tubo-ovarian cancer (HGSOC) and basal-like BC, both being tumors frequently associated with *BRCA1/2* mutations. Molecularly, these tumors for example all harbor a high number of SCNAs and frequent *TP53* mutations, see Figure 3, C-D.[28, 35-39] Furthermore, USC, the most common histologic subtype in the SCNA-hi/serous-like group, and HGSOC are morphologically indistinguishable, see Figure 4. Clinically, both USC and HGSOC generally are at advanced stage of disease at presentation, show frequent intraperitoneal spread and are associated with poor clinical outcome.[2, 40-42] These similarities suggest that a subset of ECs of the SCNA-high/serous-like group could be *BRCA1/2*-associated, and/or harbor other genomic defects causing HRD.

1.4. Endometrial cancer and hereditary cancer syndromes

As previously mentioned, inheritance of genetic alterations can predispose individuals to hereditary cancer syndromes. Hereditary cancer syndromes are characterized by;

- multiple family members at the same side of the family being affected by cancer
- affected family members having increased cancer risks
- affected family members having early age of cancer onset
- affected family members having multiple and/or bilateral primary cancers.[43]

1.4.1. Lynch syndrome

The most well-known hereditary cancer syndrome associated with EC is Lynch syndrome, or hereditary nonpolyposis colorectal cancer (HNPCC) syndrome. Lynch syndrome is an autosomal dominant syndrome, caused by a germline mutation in one of the DNA mismatch repair genes; *MLH1, PMS2, MSH2, MSH6* or the epithelial cell adhesion molecule gene*, EPCAM.*[44] Patients with Lynch syndrome are at highest increased risk to develop colorectal cancer (life-time risk to 70 years; 25-75%) and, for women, EC (life-time risk to 70 years; 12-71%), with risks varying dependent on the mutated gene.[44, 45]

Tumors arising in women with Lynch syndrome are mismatch repair deficient and are characterized by a high tumor mutation burden caused by the inability to recognize and

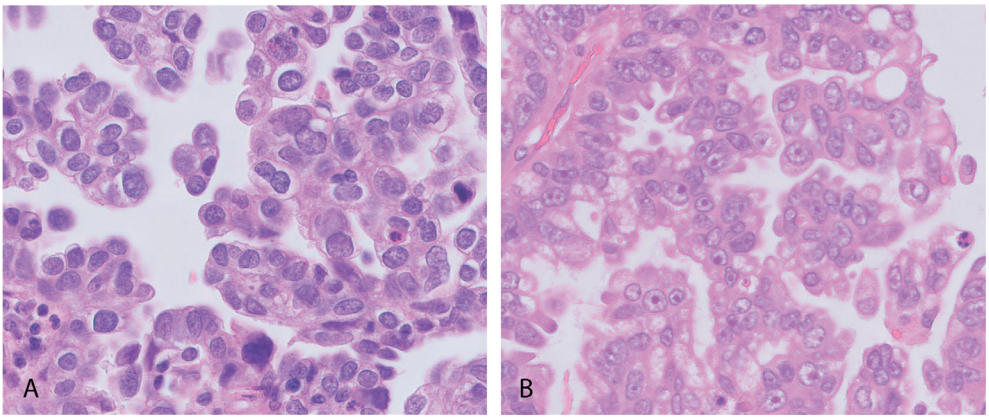


Figure 4: A. Example of an H&E slide of uterine serous carcinoma. **B**. Example of an H&E slide of a high-grade serous tubo-ovarian carcinoma.

repair DNA mismatches, giving rise to hypermutated tumors defined by >10 mutations per megabase.[28, 46] ECs that arise in the context of Lynch syndrome are of the MSI-high/ hypermutated molecular subgroup.

1.4.2 Cowden syndrome

Another hereditary cancer syndrome associated with EC is the Cowden syndrome. This syndrome is caused by a germline mutation in the phosphatase and tensin homolog (*PTEN)* tumor suppressor gene. It is a rare autosomal dominant syndrome in which patients develop hamartomatous tumors in multiple organ systems. The estimated life-time risk for EC is between 10-28%.[47]

1.4.3 *BRCA1/2*-associated HBOC-syndrome

As previously mentioned, women with the g*BRCA1/2*-associated HBOC-syndrome have severely increased life-time risks to develop BC and OC, with reported cumulative risks at age of 70 years for g*BRCA1*-mutation carriers of 50-59% and 34-45%, and for g*BRCA2*-mutation carriers of 42-51% and 13-21% respectively.[1] Other cancer types reported to be increased in *BRCA2*-mutation carriers are pancreatic cancer (both men and women), and prostate cancer (men). Furthermore, there might be an increased risk for stomach and oesophageal cancer (*BRCA1/BRCA2),* uveal melanoma (*BRCA2),* bone (*BRCA2)* and pharyngeal cancer (*BRCA2).*[48-50]

EC is currently not being considered to be part of the HBOC-syndrome. However, beforementioned similarities between a subset of ECs, HGSOCs and basal-like BCs suggest that there might be a role for *BRCA1/2* mutations and HRD in the development of ECs.

Although some studies have reported that women with a *gBRCA* mutation are at increased risk to develop EC, especially USCs[51-55], others were not able to find this association.[56, 57] If a subset of EC is part of the *gBRCA1/2*-associated HBOC, this might have important clinical consequences with regard to the availability of screening for *gBRCA1/2* mutations in EC patients, the extent of risk-reducing surgery (RRSO with/without hysterectomy) and, it might impact treatment strategy choices.

1.5. Clinical consequences of *gBRCA1/2* mutations, HRD and genetic testing

1.5.1 Early cancer detection and prevention

Because of the severely increased life-time risks of developing BC and OC, women with a *gBRCA1/2* mutation can opt for intensified screening programs to identify BC at an early stage.[58, 59] Additionally, women can opt for risk-reducing bilateral mastectomy, which has shown to be highly effective in reducing BC risk, with reported rates by some studies even up to 100%.[60-62]

Screening for early detection of OC has failed to result in survival advantage.[58, 63] The only available effective strategy to prevent OC is by performing a RRSO, which is recommended for *gBRCA1*-mutation carriers at an age of 35-40 years, and for g*BRCA2*-mutation carriers at an age of 40-45 years.[58, 63] Studies showed RRSO to be highly effective in preventing OC/fallopian tube cancer, with cancer reduction rates varying between 71% and 96%.[64] Additionally, studies showed a BC reduction rate after RRSO of approximately 50%, though this protective effect might have been an overestimation because of bias in studies analyzing this effect.[65]

Since patients with *gBRCA1/2* mutations are not considered to be at increased risk to develop EC, it is currently not recommended to routinely perform a risk-reducing hysterectomy concurrently with the RRSO. However, since this is based on small studies, this might change if larger future studies show that *gBRCA1/*2-mutation carriers are at increased risk to develop (a subset of) EC.

1.5.2 Treatment in *gBRCA1/2*-associated carcinomas

Studies showed that *gBRCA1/2*-associated BC and OC are particularly sensitive to drugs that cause DNA damage that is normally repaired via HR, leading to massive genomic instability that is inconsistent with cell viability.[4, 8] Platinum salts (cisplatin and carboplatin) for example are chemotherapeutic agents that cause inter- and intrastrand crosslinks that stall the progression of the replication fork. DNA damage caused by these agents is normally repaired via HR and nucleotide excision repair.[4, 8] Studies showed that patients with carcinomas that harbor mutations in HR genes (including *BRCA1/2*), or which harbored genomic patterns associated with HRD, showed increased platinum-sensitivity and improved progression-free survival, and overall survival.[20, 66-68]

A more recently approved class of drugs are the poly(ADP-ribose) polymerase (PARP)-inhibitors. The PARP enzyme is involved in the repair of singe-strand DNA breaks (SSBs) through the base excision repair. PARP-inhibitors cause SSBs to persist and PARP to be "trapped" to the damaged chromatin site, generating secondary DSBs during the S-phase, which require HR for repair.[4, 8, 69] PARP-inhibitors already showed promising results as maintenance treatment for relapsed platinum-sensitive HGSOC, with most benefit being observed for *BRCA1/2* mutated tumors (both somatic and germline) or tumors with genomic alterations associated with HRD, and for *gBRCA*-mutated BC.[70-73] More recently, PARP-inhibitors have also shown to be highly effective as first-line maintenance treatment for platinum-sensitive *BRCA1/2*-mutated (both germline and somatic) HSGOC.[74]

Currently, PARP-inhibitors are not indicated for treatment of EC. If a subset of EC, especially the SCNA-high/serous-like EC, which have poorest clinical outcome, are indeed frequently *gBRCA1/2*-associated or HRD, PARP-inhibitors might be a new treatment strategy for these women.

1.5.3. Referral and genetic testing for *gBRCA1/2* mutations

Given the major clinical consequences for both patients and family members, it is important to identify patients with the HBOC-syndrome. Table 2 describes the indications for referral to the clinical geneticist for, amongst other, g*BRCA1/2* mutation screening. As can be seen, the main indications are early onset of BC, epithelial OC or a family history of, amongst others, early BC.[59, 63] EC is currently not included as an indication for *gBRCA1/2* mutation testing.

The gold-standard for germline mutation testing is analysis performed on high-quality blood-derived leukocyte-DNA. Depending on the gene analyzed and the mutation sought for, a combination of different techniques is used (next generation sequencing (NGS), sanger sequencing, copy number multiplex ligation probe amplification (CN-MLPA).[75-78] Since 2015, all women with epithelial OC are eligible for *gBRCA1/2* testing,[63, 79] which has significantly increased the referral rates of patients to clinical geneticist.

A more efficient way to select women for referral to the clinical geneticist, would be by preselecting women via mutation analysis performed on tumor-derived DNA. Since *BRCA1/2* mutations are only present in approximately 20% of HGSOC (+/- 14% germline, +/- 6% somatic mutations),[42] such a "tumor-first approach" could possibly prevent referral of around 80% of OC patients to the clinical geneticist and prevent unnecessary patient distress.

Another advantage of this "tumor-first approach" would be the simultaneous detection of both somatic and germline mutations. Though the presence of a germline/somatic *BRCA1/2* mutations is not a prerequisite for PARP-inhibitor maintenance treatment of recurrent platinum-sensitive high-grade OC anymore, the presence of such a mutation is a prerequisite

Table 2. Indications for referral of women to clinical geneticist for (amongst others) *BRCA1/2* mutation testing

Women with history of breast cancer
BC and a family member with a pathogenic g*BRCA1/2* mutation
BC diagnosis <40 years
Bilateral BC, of which the first BC was diagnosed <50 years
Multiple primary BC on one side, of which the first BC was diagnosed <50 years
Triple negative BC <60 years
BC < 50 years and one or more first[a] degree relatives with BC <50 years
BC <50 years and first degree family member with prostate cancer <60 years
BC and two or more first or second[b] degree family members with BC, of which one was diagnosed <50 years (same side of family)
Women with a history of epithelial ovarian/tubal cancer
All patients, irrespective of age at diagnosis (not including borderline tumors)
Women without a cancer history
First or second degree family member with *BRCA1/2* mutation (man or women)
First degree family member with;
BC<40 years
bilateral BC, of which the first BC was diagnosed <50 years
multiple primary BC on one side, of which the first BC was diagnosed <50 years
triple negative BC <60 years
First degree male relative with BC
First degree family member with BC <50 years and first degree family member with prostate cancer <60 years (same side of family)
Two or more first degree family members with BC <50 years
Three or more first or second degree family members with BC, of which one was diagnosed <50 years (same side of family)
First degree family member with OC, irrespective of age

[a]First degree family member: Parents, Children, Siblings. [b]Second degree family member: grandparents, grandchildren, uncles, aunts, half-siblings, children of siblings from same side of the family Abbreviations: BC; breast cancer

for first-line maintenance treatment of platinum-sensitive high-grade OC. Thereby, a "tumor-first approach" would prevent additional mutation analysis for women that tested negative for a *gBRCA1/2* mutation, but who are eligible for first-line maintenance treatment with a PARP-inhibitor treatment in case a somatic *BRCA1/2* mutation would be present.

However, before such a tumor-first approach can implemented as pre-selection tool for referral to the clinical geneticist, the reliability of this method needs to be proven. Tumor tissue used

for pathology diagnoses and DNA-isolation is formalin-fixed and paraffin embedded (FFPE). FFPE-derived tumor DNA is of lower quality than blood-derived DNA, as formalin causes the DNA to be high-fragmented, making mutation analyses more technically challenging.

1.6. Subjects, aims and thesis outline

Whether women with g*BRCA1* and *gBRCA2* mutations are at increased risk to develop EC remains topic of debate, mainly because literature shows contradictory results.[51-57] These contradictory findings are likely attributable to small cohort sizes, limited follow-up and subsequent limited events, and the lack of pathology review. Accurate risk predictions are not only essential for genetic counselling and risk-reducing strategies, but may also provide evidence that a subgroup of EC is HRD, which subsequently provides new treatment options, like PARP-inhibitors.[70-72, 74] Furthermore, now all patients with epithelial OC are being offered genetic testing and having a germline and somatic *BRCA1/2* mutations is a prerequisite for first-line PARP-inhibitor maintenance therapy, more efficient *BRCA1/2* (pre-) screening pathways are desirable.

Therefore, the aims of this thesis were

- to determine whether (a subset of) ECs harbor deficits in the homologous recombination repair pathway.
- to determine whether (a subset of) EC should be considered part of the *gBRCA1/2*-associated HBOC-syndrome.
- to determine whether *BRCA1/2* analyses can reliably be performed on FFPE-derived tumor DNA.

Chapter 2 reports on the occurrence of HRD in EC using a functional *ex vivo* assay. In **chapter 3,** a systematic review and meta-analyses is performed to determine the frequency of *gBRCA1/2* mutations in USCs compared with what would be expected based on population frequencies. **Chapter 4** describes an in depth molecular and morphological characterization of ECs that occurred in women with *gBRCA1/2* mutations. **Chapter 5** reports on the uterine cancer risk in a large cohort of women with a proven *gBRCA1/2* mutation compared with both non-*gBRCA1/2*-mutation carriers and population incidence rates. In **chapter 6,** we validated *BRCA1/2* tumor testing performed on DNA isolated from FFPE-tissue and compared the performance with *BRCA1/2* analyses on leukocyte DNA, which is the gold standard. **Chapter 7** provides a general discussion on the results obtained in this thesis, focusing on potential clinical implications and future perspectives.

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Chapter 2

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 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.

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.

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, USCs 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)/hypermutated, (iii) the SCNA

low/no specific molecular profile (NSMP), and (iv) the SCNA high (SCNA-hi)/serous-like endometrial cancers (4).[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);[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*),[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).[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 cancer 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, cat. 316-030-CL) supplemented with 10% FBS and 1% penicillin-streptomycine (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, 4mA, YXLON Y.TU 225-D02) to induce DNA DSBs. Samples were then incubated on a rotating device (60 rpm) for two hours at 37°C in the OSE culture medium supplemented with 5-Ethynyl-2'-deoxyuridine (EdU) to a final concentration of 20 µmol/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) based on the sum of the tissue vitality (1=poor, 2=moderate, 3=good) and tumor percentage (0=<5%, 1=5%-20% 2=20%-49% 3=≥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_2-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 ≤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-µm 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 µm) and the Wizard Genomic DNA purification KIT (Promega) according to manufacturer's protocol. An H&E cryoslide (5 µm) was made to determine tumor percentage.

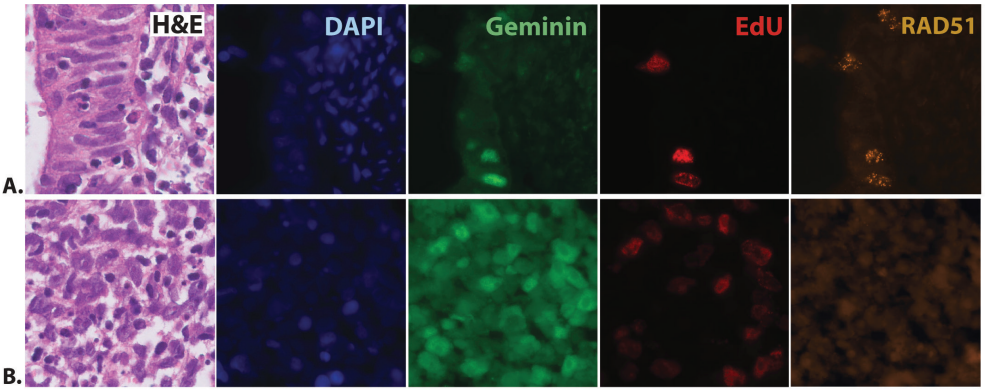


Figure 1: Functional Ex Vivo RAD51 assay to determine homologous recombination repair capacity in endometrial cancer. A, Example of a homologous recombination repair proficient endometrioid endometrial carcinoma (case 26). In the H&E, the presence of tumor tissue is confirmed. Cell nuclei are stained with DAPI. Geminin-staining marks cells in S- and G_2-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.

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 x 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, 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 ≤-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 collegues; HR-genes were categorized as either "core" HR genes (involved in the core HR machinery) or "related" HR genes (involved in closely related processes).[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$^{XT\ HS}$ Custom panel made in SureDesign (Agilent technologies) was used for variant detection with the following HR-genes 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, *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, class 5=pathogenic.[32] Only class 3, 4 and 5 variants are reported in the manuscript. Variants were annotated based 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, *RAD51D;* NM_002878.3.

BRCA1 hypermethylation using MS-MLPA. The presence of *BRCA1* promotor 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 Signalling Technology) and BAP1 (clone C4, 1:100, Santa Cruz Biotechnology) were performed on whole slides (4 µm) as described in the Supplementary Material 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'- tctggcgttctctcctcag-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 ≥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's kappa coefficient (κ) was used to measure interobserver and intertest agreement. IBM SPSS version 23.0 (SPSS, Inc., Chicago, USA) 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$^+$/RAD51$^+$ cells scored after *ex vivo* exposure to ionizing radiation by the 2 independent observers was comparable, with a median score difference

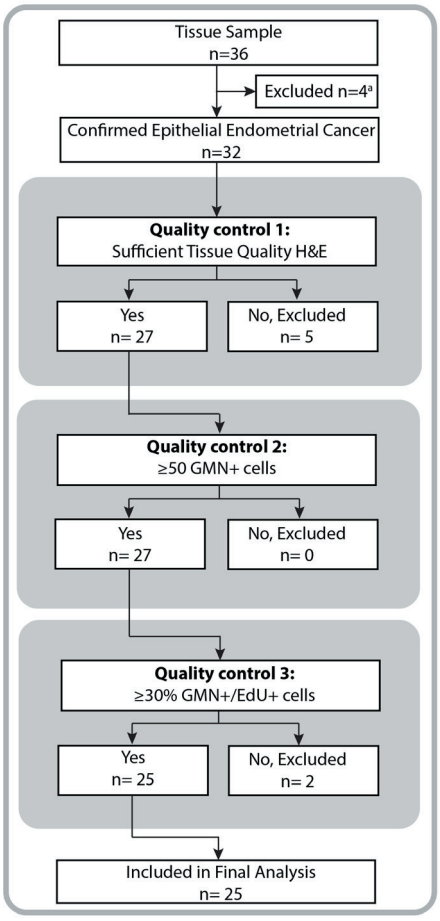


Figure 2: Flowchart illustrating the selection of cases for analysis. Of 36 samples, four[a] cases were excluded because histological evaluation demonstrated no epithelial endometrial malignancy (2x cervical carcinoma, 1x leiomyosarcoma, 1x benign). Tissue was thawed and reanalysed for 10 cases because 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.

within cases of 6% (range: 0%-41%). Interrogator reliability for final HR category assignment was high (κ=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 to 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 to 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$^+$/RAD51$^+$; 23%). The other case was a UCS with an endometrioid epithelial component (case 18; Geminin$^+$/RAD51$^+$; 44%, Fig. 3A and 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 one (6%) MMRd/hypermutated.

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); 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 and Table 1). An association was observed between the SCNA status and the presence of a *TP53* variant, with *TP53* variants being significantly

Table 1. Clinicopathological characteristics stratified for homologous recombination capacity

	HR deficient ***n*** **(%)**	**HR proficient** ***n*** **(%)**	***P*** **value**
Total	6 (100)	17 (100)	
Age, years			
Mean ±SD	70 ±9.3	66 ±10.6	0.431
Tumor			
Primary	6 (100)	17 (100)	
Recurrent	0 (0)	0 (0)	
Histologic subtype			
Endometrioid	0 (0)	11 (65)	**0.014**[a]
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)	**0.019**
3	6 (100)	7 (41)	
FIGO 2009			
I	2 (33)	15 (88)	**0.021**
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)	**0.045**
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)	**0.019**[b]
Copy number high	2 (33)	6 (35)	
Copy number low	0 (0)	10 (60)	
TP53			
Mutation	6 (100)	7 (41)	**0.019**
No mutation	0 (0)	10 (59)	
TCGA subgroups			
TP53	6 (100)	6 (35)	**0.014**
NSMP/POLE/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.
[a]Endometrioid versus non-endometrioid histology was compared.
[b]Copy number extremely high + copy number high versus copy number low was compared.

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

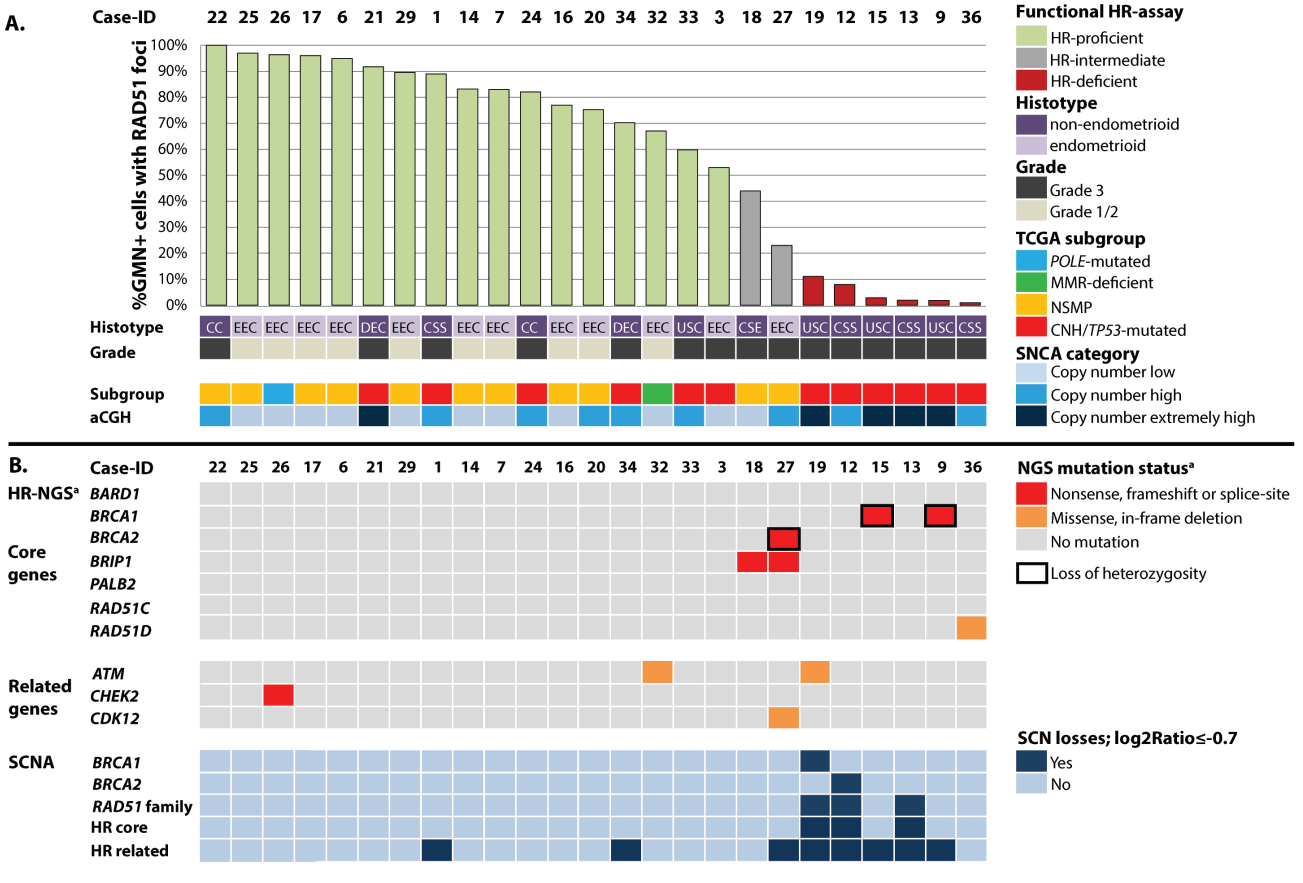
We performed (epi)genetic analysis to identify possible loss-of-function alterations that could explain the HR deficiency. This included NGS (variants HR genes), aCGH/SNP array (high SCN losses of HR genes; $\log_2$Ratio≤-0.7), MS-MLPA (*BRCA1* promoter hypermethylation) and immunohistochemistry (MRE11,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

In 2 out 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, see Fig. 3B and 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 4). Case 36, in which a *RAD51D* VUS was identified, did not show SCN losses in HR genes with a $\log_2$ratio of ≤-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 cancer, harboured 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 in-frame 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,


A.
Case-ID
22 25 26 17 6 21 29 1 14 7 24 16 20 34 32 33 3 18 27 19 12 15 13 9 36
%GMN+ cells with RAD51 foci
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Histotype
CC EEC EEC EEC EEC DEC EEC CSS EEC EEC CC EEC EEC DEC EEC USC EEC CSE EEC USC CSS USC CSS USC CSS
Grade
Subgroup
aCGH
Functional HR-assay
HR-proficient
HR-intermediate
HR-deficient
Histotype
non-endometrioid
endometrioid
Grade
Grade 3
Grade 1/2
TCGA subgroup
POLE-mutated
MMR-deficient
NSMP
CNH/TP53-mutated
SNCA category
Copy number low
Copy number high
Copy number extremely high
B.
Case-ID
22 25 26 17 6 21 29 1 14 7 24 16 20 34 32 33 3 18 27 19 12 15 13 9 36
HR-NGS[a]
Core genes
BARD1
BRCA1
BRCA2
BRIP1
PALB2
RAD51C
RAD51D
Related genes
ATM
CHEK2
CDK12
SCNA
BRCA1
BRCA2
RAD51 family
HR core
HR related
NGS mutation status[a]
Nonsense, frameshift or splice-site
Missense, in-frame deletion
No mutation
Loss of heterozygosity
SCN losses; log2Ratio≤-0.7
Yes
No

< Figure 3: Tumor characteristics (A) and genetic changes (B) stratified for homologous recombination capacity. Each column represents one case. **A**, Cases were classified in TCGA subgroups using surrogate markers as described in the Supplementary Material 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 collegues.[31] Abbreviations: CC: Clear Cell Carcinoma, CSE: CarcinoSarcoma with Endometrioid epithelial component, CSS: CarcinoSarcoma with Serous epithelial component, DEC: Dedifferentiated Endometrial Carcinoma, EEC: Endometrioid Endometrial Carcinoma, HR: Homologous Recombination, USC: Uterine Serous Carcinoma. [a]Only variants with a variant allele frequency of ≥25% are shown. When multiple variants were present in the same gene, the most pathogenic variant is shown.

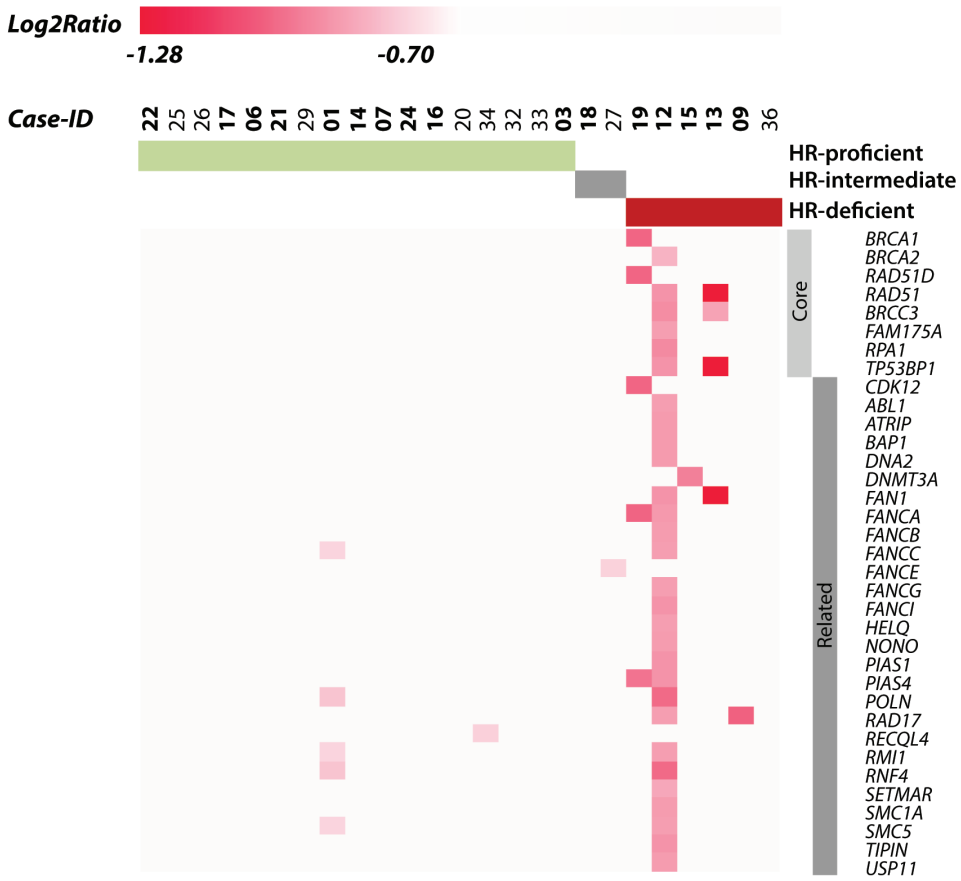


Figure 4: Somatic copy-number losses stratified for homologous recombination (HR) capacity. HR genes were selected and divided in HR-core or HR-related genes as described by Riaz and colleagues.[31] Only those genes with SCN losses of $\log_2$ratio≤-0.7 in at least 1 of the included cases are visualised. Data were extracted from the aCGH data as described in the Supplementary Materials. Bolded cases were analysed using the CGH Agilent platform, others were analysed using the Oncoscan platform.

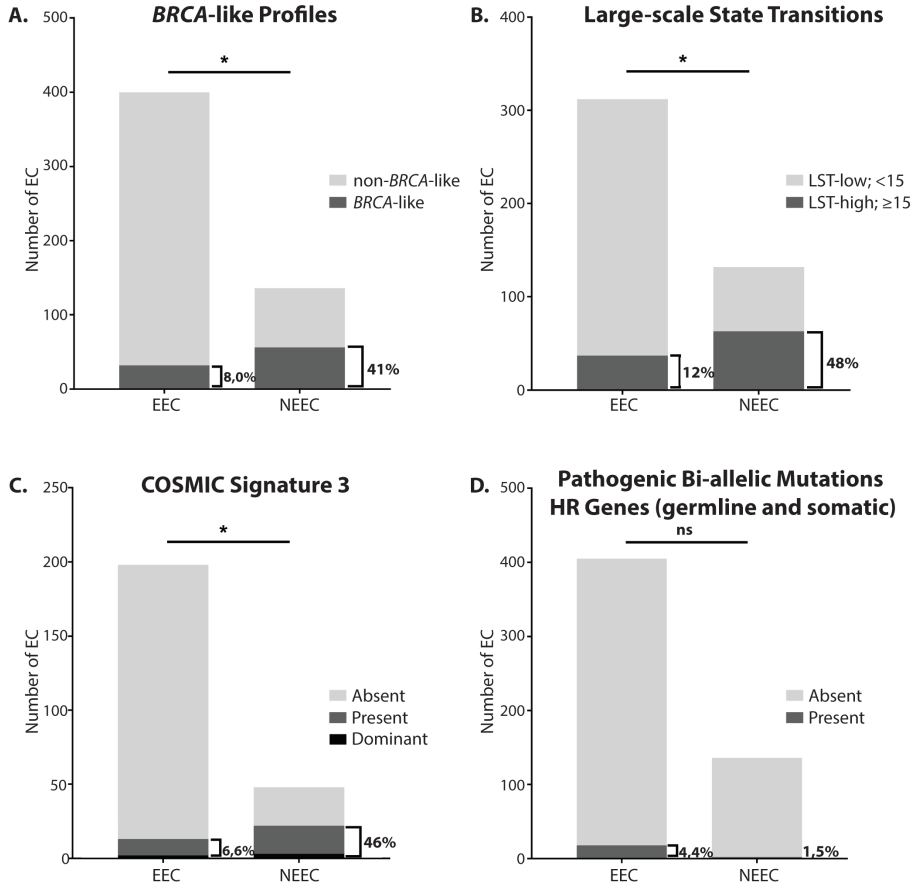


Figure 5: *BRCA*-associated genomic scars as surrogate marker for HR deficiency in the TCGA-endometrial cancer cohort. A, A *BRCA*-like profile was present in 32/400 of EECs and 56/136 of NEECs. **B,** A high LST score (≥15) was present in 37/312 of EECs and 63/132 of NEECs. **C,** COSMIC signature 3 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 biallelic 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).

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%) 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-endometrial cancer cohort supports that HR deficiency occurs in endometrial cancers, 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 HGSOC. 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 cancer 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).[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 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 cancers 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.

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Disclosure of potential conflicts of interest

PCS's spouse is employed by AstraZeneca. No other potential conflicts of interest were disclosed.

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Supplementary materials and methods

Immunofluorescence staining

Sections of 5 µm were used. After deparaffinization in xylene and rehydration in ethanol, target antigen retrieval was performed using DAKO Antigen Retrieval buffer (pH 9.0) at 97°C for 12 minutes using a TissueWave™ 2 Microwave Processor (Thermo Scientific). Cells were permeabilized with a mixture of 0,2% Triton X-100 in phosphate buffered saline (PBS) for 20 minutes, followed by 1 hour incubation at 37°C with DNase (10.000 U/ml, dilution 1/10 in PBS, Roche diagnostics). Blocking was achieved using PBS with 2% FBS and 1% bovine serum albumin (BSA) for 30 minutes at room temperature. Sections were co-stained for RAD51 and Geminin with the primary antibodies (anti-RAD51 (GTX70230, 1/400, GeneTex) and anti-geminin (10802-1-AP, 1/400, Protein Tech group), diluted in blocking buffer) and incubated for 1 hour at room temperature. The secondary antibodies for visualizing the primary antibodies were Alexa Fluor 555 (A21424, 1/1000, Life Technologies) and Alexa Fluor 488 (A11034, 1/1000, Life Technologies), both diluted in blocking buffer. For EdU detection, the Click-iT® reaction cocktail was prepared according to manufacturer's instructions, with Alexa Fluor 647 (cat. C10340, 1/1000, life technologies) for visualization. The tissue sections were incubated for 30 minutes at room temperature with the EdU cocktail mix. Lastly, the tissue sections were mounted with Vectashield ProLong Antifade Mountant with DAPI (Thermofisher Scientific, cat. p36934).

aCGH

For each sample in the Agilent aCGH analysis cohort, DNA was restriction digested and controlled by Agilent Bioanalyzer on DNA 7500 chips (Agilent Technologies, Santa Clara, CA, USA) and labelled with Cy3-dUTP or Cy5-dUTP using Agilent Genomic DNA Labelling Kit PLUS. Hybridization was carried out on Agilent 4x180kb arrays for 24 hours at 65°C in a rotating oven (Robbins Scientific, Mountain View, CA) at 20 rpm, according to the manufacturer's instructions. A commercial DNA was used for control (Promega). Scanning was performed with an Agilent G2505C DNA Microarray scanner using default parameters. Quantification of Cy5 and Cy3 signals from scans was performed with Feature Extraction v10.5.1.1 (Agilent Technologies) using default parameters. Resulting raw signals and log2 (ratio) profiles were normalized and centered according to their dye composition (Cy5/Cy3) and local GC content. These profiles were segmented with the Circular Binary Segmentation algorithm through its implementation in the DNA copy package for R v(v2.6 to v3.1) using default parameters. DNA copy number imbalances were detected considering a minimum of 3 consecutive probes and a minimal absolute amplitude threshold that was specific for each profile. Profiles were centered using the most centered out of the three most populated peaks of the smoothed log2(Test/Ref) distribution. Aberration levels were called by setting a log2(Test/Ref) threshold automatically adapted to the internal noise for each profile, considered as one-fourth of the median value of the absolute differences between consecutive log2(Test/Ref) measures

along the genome. All genomics coordinates were established using the human genome as defined by the UCSC build hg19 (GRCh37). The copy number alterations detected with aCGH were transformed into log2 ratio.

SNP array

The experimental procedure of the OncoScan™ FFPE Assay Kit (335k probes, Thermo Fisher) includes several steps. Probes were added to the sample DNA (80ng as previously stated), and allowed to anneal at 58°C overnight (16–18 h) subsequent to an initial denaturation (95°C for 5 min). Samples were then split into two separate reactions, and proceeded as follows: dATP (A) and dTTP (T) (A/T) were added to one reaction, and dGTP (G) and dCTP (C) (G/C) were added to the second in order to conduct gap fill.

Unincorporated and non-circularized Molecular Inversion Probes (MIP), as well as the remains of the genomic template, were removed by treatment with exonucleases (Affymetrix, Inc.). The circular MIPs that were gap-filled by the A/T or G/C nucleotides were cleaved and their linear form was amplified by PCR. Subsequently, the 120-bp PCR product was cut and the smaller (44-bp) fragment containing the specific SNP genotype was subjected for hybridization onto array. Prior to this, samples were mixed with hybridization buffer and injected into the cartridges for 16–18 h at 49°C and 0.013 × g. Following hybridization, cartridges were removed from the oven, and stained using the GeneChip® Fluidics Station 450 (Affymetrix, Inc.), according to the manufacturer's protocol. Subsequent to staining and washing, arrays were scanned in GeneChip Scanner and the fluorescence of clusters was measured in order to generate a DAT file. Cluster intensities values were automatically calculated using built-in algorithm from DAT files by the Affymetrix GeneChip Command Console software, version 4.0 (Affymetrix, Inc.), and output in a CEL file format was created.

Raw probes intensities were aggregated to probesets and normalized using Affymetrix Power Tools v1.18.2. Normalized Log2Ratio, B-Allele Frequency (BAF) and metadata were extracted from the resulting OSCHP files. The Log2Ratio obtained then went through an additional renormalization step according to local GC-content, and centered as described in the aCGH paragraph. The R ASCAT package (v2.4.2) was then used in order to co-segment Log2Ratio and BAF values and to derive Allele-Specific Copy Number (ASCN) and Total Copy Number (TCN), using the maximal "goodness of fit" criterion to select the optimal ASCN model. This process has been developed in Gustave Roussy and is referred to as "EaCoN" (will be further published).

Genomic instabilisty score (GIS)

We created a software called getStability.py that takes as input a .CBS file and uses a cytoband file from the UCSC in order to classify events in the following classes based on segment lengths (with a 5% tolerance):

1. Whole Chromosome Event
2. Arm + event (which is defined as an event that is greater than one chromosomal arm, but lower than a chromosomal event)
3. Arm event
4. Greater than 30Mb and lower than a whole chromosome arm
5. Between 30Mb and 15Mb event
6. Between 15Mb and 1Mb event
7. Lower than 1Mb event

Based on these results, we summed up the events from classes 4 and 5 and called this result the genomic instability score. Using this instability score, we were able to classify samples in three classes using k-means (python package sklearn, 3 clusters, 2000 iterations, all other parameters set to default).

```
#K-Means analysis
import sklearn.cluster
import numpy as np
import pandas as pd
# Text file containing sample names & Scores
#1) Simple data transformation
a = open('AUAU_cohort', 'r').readlines()
b = [x.rstrip('\n').split('\t') for x in a]
c = [(x[0], int(x[1]), int(x[1])) for x in b]
f = np.array([(x[1], x[1]) for x in c])
#2) Put data in an pandas dataframe so we can keep sample names in (cannot do that with numpy)
g = pd.DataFrame(f, index=[x[0] for x in c])
#3) Prepare the classifier
classifier = sklearn.cluster.KMeans(n_clusters=3, max_iter=2000)
#4) Fit on the classifier
p = classifier.fit(g)
#5) Fill the pandas dataframe with the results from the classifier.
g.assign(rank=p.labels_)
```

Next generation sequencing

Library preparation and target enrichment was performed using the Sureselect$^{XT\ HS}$ Target Enrichment System for Illumina Paired-End Multiplexed Sequencing library reagent kit (Agilent technologies, Santa Clara, California, United States) according to manufacturer's instructions, with manual preparation of 16 sample sets. The captured DNA libraries were sequenced (16 samples per run) using the Illumina MiSeq (Illumina, San Diego, California, United States). The average depth of sequencing was 300X to assure a limit of detection of at least around 5% and a coverage of at least 90% at 200X and 100% at 100X.

The data analysis pipeline included the following algorithms: BWA-MEM v-0.7.12 for read alignment to the hg19 human reference genome and Samtools v-1.2 and Picard-tools v-1.139 for PCR duplicate quantification and removal. GATK Haplotype v-3.4-46, snpEff v-4.0 and MutaCaller-1.7 (home pileup internally developed) were used for variant calling and classification. Variants were called with a minimum allelic frequency threshold of 1% for already classified variants (those known in the internal database) and 5% for non-classified variants, and a read depth threshold of 30X for the total reads at the variant location and at least 10X for the variant.

Several filters were applied to further select for potential relevant variants among the called variants. The population databases Exac and gnomAd were used to automatically filter out polymorphism as soon as the population frequency was higher than 0.5%. Non-classified variants (not known in the internal database) were excluded if the intrarun recurrency within the 16 analyzed samples per illumina run was superior to 4/16 (25%), as this may be an indicator for an artefact or polymorphism.

Variants were categorized using the five-tier pathogenicity classification according to Plon et al., 2008; class 1=benign, class 2=likely benign, class 3=variant of unknown significance (VUS), class 4=likely pathogenic, class 5=pathogenic.[1] An internal database for germline *BRCA1/2* mutations comprising 15 years of sequencing experience in the French population, combined with the data of the UMD-BRCAshare *BRCA1/2* database (www.umd.be/BRCA1/ and www.umd.be/BRCA2/) was used to assign pathogenicity to detected variants. For non-BRCA1/2 alterations, all variants leading to a premature stop codon were considered as deleterious. Additionally, public databases as ClinVar (selection for the three stars curated data only) were applied for variant classification. All missense variants for which no functional data was present were considered as unclassified (class 3).

The presence of all but one pathogenic variant in HR genes, and the *RAD51D* VUS in Case 36 (Supplementary Table S3), were confirmed by bi-directional Sanger sequencing (details of PCR primers and reaction conditions available upon request). The *CHEK2* c.1510G>T variant located in exon 14 could not be confirmed. This region is known to share high homology with several pseudogenes which reduces the sensitivity to identify the variant at low VAF.[2]

The presence of loss of heterozygosity of the wildtype allele (LOH) was assessed based on the variant allele frequency of both the HR gene variants and single nucleotide polymorphisms (SNPs), in combination with the tumor percentage. LOH was considered to be present when the tumor cell percentage was >20%, the HR variant allele frequency was >60%, and/or at least two informative SNPs showed a variant allele frequency of <0.4 or >0.6. Absence of LOH could not be assessed with certainty for the variants with an allelic frequency of <50% due to the possibility of subclonality.

BRCA1-promoter hypermethylation

Approximately 75 ng of FFPE-isolated DNA was denatured for 5 minutes at 98°C and subsequently cooled down to 25°C. After addition of the SALSA probe-mix and MLPA-buffer, samples were incubated for 1 minute at 98°C, followed by hybridization at 60°C for 16-20 hours. Next, ligasebuffer A was added and samples were heated to 48°C. Samples were then split and ligated (ligasebuffer B and Ligase-65 enzyme) with or without the addition of HhaI-enzyme for 30 minutes at 48°C followed by heating for 5 minutes at 98°C. Then, the PCR-mastermix (including SALSA primermix and SALSA polymerase) was added and the following PCR reaction was performed for 35 cycles; 30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°C, followed by an incubation period for 20 minutes at 72°C. The amplified PCR products were separated by electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystems) and analyzed using Coffalyser.Net software (MRC-Holland).

IHC staining

For manually stained sections, following deparaffinization in xylene and rehydration via a graded ethanol series, blockage of the endogenic peroxidase activity (0,3% Methanol/H_2O_2) was performed. Antigen retrieval was achieved using a microwave oven procedure for 10 minutes in 10 mmol/L Tris-EDTA buffer, pH9.0 (MSH6 and PMS2) or in a 10mmol/L citrate buffer, pH 6.0 (PTEN and MRE11). Sections were incubated overnight with antibodies at room temperature (PTEN, MSH6 and MRE11) or at 4°C (PMS2). The sections were then incubated for 30 minutes using a secondary antibody (Poly-HRP-GAM/R/R; DPV0110HRP; ImmunoLogic). For PMS2 and MSH6, before incubation with the secondary antibody, an additional 15 minute incubation step with a linker (EnVision FLEX+ rabbit, SM805, DAKO) was performed. As chromogen, DAB+ (K3468, DAKO) was used. The slides were counterstained with haematoxylin. For the automated stainer standard protocols and the same IHC-clones were used with some slight differences. An additional linker was used for PMS2- and BAP1-stainings and not for MSH6. All BAP1 slides were stained using an automated stainer. As secondary antibody and chromogen we respectively used EnVision™ FLEX /HRP (Dako SM802) and EnVision™ FLEX DAB+ Chromogen (Dako DM827) diluted in EnVision™ FLEX Substrate Buffer (Dako SM803).

IHC scoring

All slides were evaluated by two observers, blinded for patient characteristics and outcome of the functional RAD51 assay.

To assign MMR status, PMS2- and MSH6-IHC expression were scored in three categories as described previously by Stelloo and colleagues; retained, loss and subclonal/regional loss of protein expression.[3] Subclonal/regional loss was defined as a tumor with retained nuclear expression showing focal loss of nuclear expression in a discrete tumour area of at least 10% of the total tumor volume, with positive staining of stromal cells/infiltrate as

an internal control. Tumors in which at least one of the mismatch repair proteins showed loss of expression were considered MMR-deficient (MMRd). Subclonal loss was considered partial MMRd.[3] PTEN staining was evaluated in three categories as described before; negative, positive and heterogeneous.[4] Heterogeneous cases were further subdivided in diffuse patchy staining (considered positive) and subclonal/regional; negative except for a well demarcated area (considered negative). MRE11 expression was scored as negative (no nuclear staining) or positive (weak, moderate or strong nuclear staining). BAP1 expression was scored as positive (nuclear staining) or negative (no nuclear staining).

TCGA classification based on surrogate markers

The EC were classified in previously described molecular subclasses using the following surrogate markers; pathogenic *TP53* variants for SCNA-hi/*TP53*-mutated, *POLE* mutations for *POLE/*ultramutated and mismatch repair(MMR)-IHC (PMS2, MSH6) for MMR-deficient (MMRd)/hypermutated. All ECs without classifying features were classified as SCNA-low/no specific molecular profile (NSMP). Cases with more than one classifying feature were assigned as follows; *POLE/TP53*-mutation to *POLE/*ultramutated, MMRd/*TP53* and MMRd/*POLE* to MMRd/hypermuted, unless the MMR expression loss was subclonal.

Genomic signatures TCGA cohort

- ***BRCA-like* profiles**

Shrunken centroids classifiers were previously trained on a training cohort of 73 ovarian cancer and 110 breast cancer patients to distinguish aCGH copy number profiles of *BRCA1* mutated breast and ovarian cancer cases from control cases and *BRCA2* mutated breast and ovarian cancer cases from control cases.[5] Area under the receiver/operator curves were respectively 0.72 and 0.67. These were subsequently independently validated in the validation cohort consisting of TCGA breast and ovarian cancers. Analyses were combined since the classifier was trained on both tumor types. 86% of the *BRCA1* mutated and methylated breast and ovarian cancers were correctly identified, and 61% of the *BRCA2* mutated breast and ovarian cancers.[5] Although slightly better performance was obtained when analysing tumor types separately, a combined breast and ovarian classifier was hypothesised to perform better in endometrial cancer as tumor type specific aberrations might be smoothed out. We processed the EC TCGA data to have a similar mean and range of values and subsequently applied before mentioned classifiers to this dataset. The classifier assigns a probability of having similar gains and losses as *BRCA* mutated cases ranging between 0 (similar to non-mutated cases) and 1 (similar to mutated cases). A cutoff of 0.5 of the posterior probability was used, as this is a two-class problem in which errors for both classes were considered equally important.

- ***Large-scale state transitions (LST), COSMIC Signature 3 and bi-allelic alterations in HR genes***

Somatic mutation annotation files (MAF), relevant as of 28th January 2016, for individual cancers as part of the TCGA were obtained from Firebrowse (http://firebrowse.org/).[6] EC (UCEC) tumors were classified according to the presence of bi-allelic pathogenic mutation in the 102 homologous recombination genes defined by Riaz and colleagues.[7] Affymetrix SNP Array 6.0 (SNP6) array data was obtained from Firebrowse (http://firebrowse.org/), dated 28th January 2016. To asses for genomic features of genomic instability, the number of large-scale state transitions (LST) was determined for each cancer following methods detailed by Riaz and colleagues,[7] and using the same cut-off to define HR deficiency status (≥15). The proportion of mutations in each endometrial cancer case that were similar to the signatures described by Alexandrov and colleagues,[8] were determined by non-negative least squares regression and had been provided in the supplementary data from Riaz and colleagues.[7] The signature responsible for the majority of the mutations was defined as the dominant signature in that specific cancer. Signature 3 previously has been identified as being associated with *BRCA1* and *BRCA2* mutations.[8] These data were compiled in a matrix for the 541 EC in the TCGA data set.

References

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Supplementary tables and figures

Supplementary Table S1. Clinicopathological characteristics of the cases included in final analysis

	No	%
Total	25	100
Age, years		
Mean (SD)	67,4 ± 9,8	
Tumor		
Primary	24	96
Recurrent	1	4
Histologic subtype		
Endometrioid	12	48
Non-endometrioid	13	52
Serous	*4*	*16*
Carcinosarcoma, serous	*4*	*16*
Carcinosarcoma, endometrioid	*1*	*4*
Clear cell	*2*	*8*
Dedifferentiated	*2*	*8*
Histologic grade		
1	9	36
2	1	4
3	15	60
FIGO 2009		
I	18	72
II	0	0
III	3	12
IV	4	16
Ovarian/tubal involvement		
yes	4	16
no	21	84
Neoadjuvant treatment		
Yes	1	4
No	24	96

Supplementary Table S2. Clinicopathological characteristics of the homologous recombination intermediate cases

	HR intermediate
	n
Total	2
Age, years	
Mean ± SD	69 ±2.1
Tumor	
Primary	1
Recurrent	1
Histologic subtype	
Endometrioid	1
Non-endometrioid	1
Serous	*0*
Carcinosarcoma	*1*
Clear cell	*0*
Dedifferentiated	*0*
Histologic grade	
1+2	0
3	2
FIGO 2009	
I	1
III/IV	1
Adnexal involvement	
yes	1
no	1
PTEN-IHC	
loss of expression	2
normal expression	0
aCGH	
Copy Number extremely high	0
Copy number High	1
Copy number Low	1
TP53	
Mutation	0
No mutation	2
TCGA subgroup	
TP53	0
NSMP/*POLE*/MMRd	2

Abbreviations: HR, homologous recombination; MMRd, mismatch repair deficient; NSMP, no specific molecular profile.

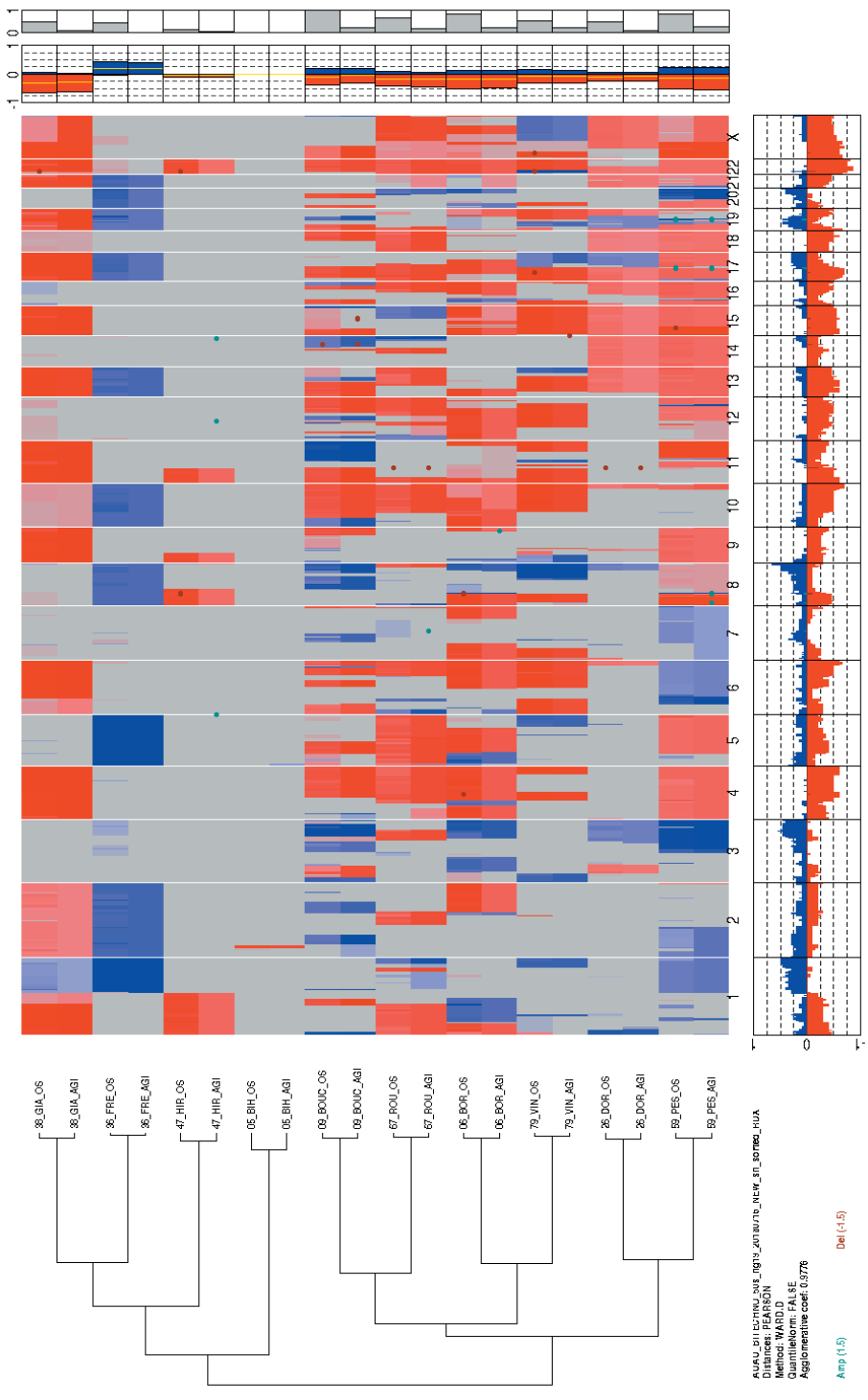
Supplementary Table S3. **Overview of the detected class 3, 4 and 5 variants in the final cohort**

Case-ID	HR status	HR core	HR related	Other	c.DNA change	Amino Acid change	T%	VAF	variant class
01	**proficient**			*TP53*	c.742C>T	p.Arg248Trp	70	78%	class 5
03	**proficient**			*TP53*	c.638G>A	p.Arg213Glu	80	25%	class 5
				TP53	c.742C>T	p.Arg248Trp		28%	class 5
06	**proficient**						50		
07	**proficient**						60		
09	**deficient**	*BRCA1*			c.4327C>T	p.Arg1443*	70	94%	class 5
				TP53	c.659A>G	p.Tyr220Cys		38%	class 5
				TP53	c.742C>T	p.Arg248Trp		41%	class 5
12	**deficient**			*TP53*	c.396G>T	p.Lys132Asn	80	94%	Class 5
13	**deficient**			*TP53*	c.838A>G	p.Arg280Gly	80	95%	class 4
14	**proficient**						50		
15	**deficient**	*BRCA1*			c.3013delG	p.Glu1005fs	70	60%	class 5
				TP53	c.1009C>T	p.Arg337Cys		61%	class 5
16	**proficient**						60		
17	**proficient**						30		
18	**intermediate**	*BRIP1*			c.632delC	p.Pro211fs	70	28%	class 5
19	**deficient**		*ATM*		c.6543G>T	p.Glu2181Asp	70	34%	class 3
				TP53	c.581T>G	p.Leu194Arg		98%	class 4
20	**proficient**						70		
21	**proficient**			*TP53*	c.844C>T	p.Arg282Trp	80	49%	class 5
				TP53	c.919+1G>A	p.?		21%	class 5
22	**proficient**						70		
24	**proficient**			*TP53*	c.993+3A>T	p.?	80	34%	class 4
25	**proficient**						70		
26	**proficient**		*CHEK2*		c.1510G>T	p.Glu504*	60	28%	class 5
				TP53	c.523C>T	p.Arg175Cys		34%	class 4
				POLE	c.857C>G	p.Pro286Arg			class 4
27	**intermediate**	*BRCA2*			c.6373dupA	p.Thr2125fs	80	64%	class 5
		BRCA2			c.6306_6413del	p.Ser2103_Val2138del		28%	reverse
		BRIP1			c.2728G>T	p.Glu910*		39%	class 5
			CDK12		c.2813C>A	p.Ala938Asp		36%	class 3
29	**proficient**						50		
32	**proficient**		*ATM*		c.640delT	p.Ser214fs	75	5,5%	class 5
			ATM		c.7282A>G	p.Arg2428Gly		12%	class 3
			ATM		c.6583C>T	p.His2195Tyr		5,3%	class 3
			ATM		c.5846C>T	p.Ala1949Val		26%	class 3
				MMRd[a]					
33	**proficient**			*TP53*	c.491_520del	p.Lys164_Val173del	70	24%	class 5
34	**proficient**			*TP53*	c.659A>G	p.Tyr220Cys	90	93%	class 5
36	**deficient**	*RAD51D*			c.433C>T	p.Arg145Cys	75	32%	class 3
				TP53	c.818G>A	p.Arg273His		55%	class 5

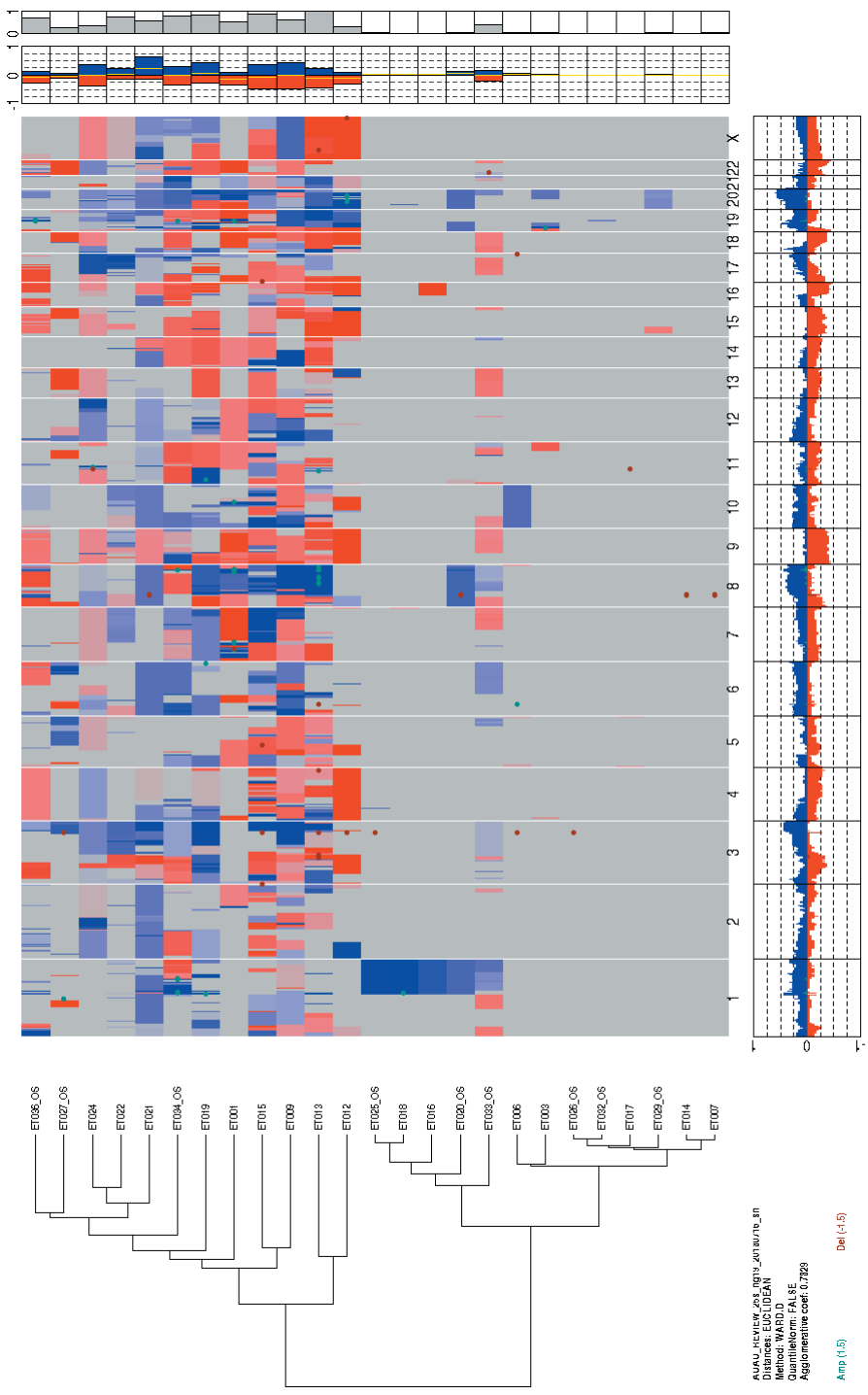
Variants were annotated using build: hg19/GRCh37 and the following transcripts: *ATM,* NM_000051.3*; BRCA1,* NM_007294.3; *BRCA2,* NM_000059.3*; BRIP1,* NM_032043.2; *CDK12*, NM_016507.3; *CHEK2,* NM_007194.3*; POLE,* NM_006231.3*; RAD51D,* NM_002878.3; *TP53,* NM_000546.5.

[a]MMRd based on loss of PMS2 expression in immunohistochemistry.

Abbreviations: HR: Homologous recombination, MMRd: Mismatch repair-deficient, T%: tumor percentage, VAF: variant allele frequency



Supplementary Figure S1: Unsupervised Euclidean hierarchical clustering on ten paired FFPE and frozen ovarian cancer samples analysed respectively by Oncoscan (OS) or CGH Agilent (AGI) platforms. The figure shows perfect match between paired samples independently from their processing (OS or AGI).



Supplementary Figure S2: Unsupervised Pearson hierarchical clustering on the endometrial cancer samples included in our paper. Samples labelled with "_OS" are those analysed by ONCOSCAN platform, others have been analysed using CGH Agilent technology. As can be seen, there is an unbiased natural division between samples.

Chapter 3

Linking uterine serous carcinoma to *BRCA1/2*-associated cancer syndrome; a meta-analysis and case report

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Abstract

Background

Uterine serous carcinoma (USC) shows greater morphological, clinical and molecular similarities to high-grade ovarian tubal serous carcinoma than to other types of endometrial cancer. As high-grade ovarian tubal serous carcinoma is known to be associated with *BRCA1/2* pathogenic germline mutations (PMs), we aimed to explore whether USC is also a constituent of hereditary breast and ovarian cancer syndrome.

Methods

Pubmed, EMBASE and Web of Science were searched in July-2016 for articles assessing the association between USC and germline *BRCA1/2*-PMs. Pooled analysis and comparisons were performed using a random effects logistic model, stratifying for ethnicity (Ashkenazi versus non-Ashkenazi). In addition, tumour tissue from an USC case with a hereditary *BRCA1*-PM was analysed for loss of heterozygosity at the *BRCA1* locus and was functionally analysed for homologous recombination proficiency.

Results

The search yielded 1893 citations, 10 studies were included describing 345 USC patients. For Ashkenazi Jews, the pooled odds ratio of having a germline *BRCA1/2*-PM was increased in USC patients compared with the general Ashkenazi population: odds ratio: 5.4 (95%-confidence interval: 2.2-13.1). In the patient with USC, we identified the known germline *BRCA1*-PM in the tumour DNA. Furthermore, we showed both loss of heterozygosity of the wild-type allele and a deficiency of homologous recombination.

Conclusion

This study suggests that USC may be an overlooked component of *BRCA1/2*-associated hereditary breast and ovarian cancer syndrome. Screening for germline *BRCA1/2*-PMs should be considered in patients diagnosed with USC, especially in cases with a positive first-degree family history for breast and/or ovarian cancer.

Introduction

Uterine serous carcinoma (USC) is an aggressive subtype of endometrial cancer (EC) which constitutes 5-10% of all uterine carcinomas,[1] accounting for almost 40% of EC-related deaths.[2, 3] Treatment options for USC are limited and consist of complete surgical staging or debulking either after or followed by (neo)adjuvant platinum-based chemotherapy and/or adjuvant radiotherapy depending on tumour stage.[4, 5] Despite aggressive treatment approaches, little progress in survival benefit has been achieved in the last decade.

Next-generation sequencing has improved the understanding of the molecular alterations that underlie USC, showing that USC is different from the more common endometrioid endometrial carcinoma at the molecular level while showing striking similarities with the molecular landscape of high-grade ovarian tubal serous carcinomas (HGOTSC). Both USC and HGOTSC show frequent *TP53* mutations (91% and 96%) and a high degree of somatic copy number alterations (SCNA) with similar focal SCNA patterns.[5-10] These similar SCNAs may be related to homologous recombination deficiency (HRD), known to be present in almost 50% of HGOTSC and often caused by *BRCA1/2* defects.[11, 12]

Moreover, USC and HGOTSC show similar histomorphologic and clinical features, as both have the tendency to spread over peritoneal surfaces, are associated with poor survival rates and show good responsiveness to platinum-based chemotherapy, although the latter could not be confirmed by all studies.[5, 11, 13, 14]

HGOTSC is associated with hereditary breast and ovarian cancer syndrome (HBOCS) caused by hereditary pathogenic mutations (PMs) in the *BRCA1* or *BRCA2* genes, which are present in approximately 15% of all HGOTSC.[11] Currently, USC is not considered as a manifestation of HBOCS. Given the many similarities between these two entities, it has been suggested by some that USC is indeed a feature of *BRCA1/2*-associated HBOCS,[15, 16] which might influence genetic counselling and treatment strategies. However, literature on this association has not yet been systematically reviewed.[15, 17]

The aim of this study was to assess whether USC is a component of *BRCA1/2*-associated HBOCS. To address this question, we present a systematic review and meta-analysis and also describe a case report as proof of concept. Furthermore, we determined whether USC patients with a germline *BRCA1/2*-PM showed a higher frequency of either a positive family history and/or personal history for *BRCA1/*2-associated malignancies.

Methods

Systematic review and meta-analysis

Eligibility criteria, literature search and data collection

We searched for studies investigating *BRCA1* and/or *BRCA2* germline mutations in association with USC. We aimed to include case-control studies and cohort-studies/trial-designs. However, examination of the literature failed to identify any case-control study (comparing germline *BRCA1/2*-PM status in USC patients and controls without USC). Also, no cohort study formally compared USC incidence in germline *BRCA1/2*-PM carriers versus non-carriers. We therefore adapted our inclusion criteria so that single-arm case-only studies (studying *BRCA1/2*-PM prevalence in USC patients) and single-arm cohort studies (studying USC frequency in carriers) were also eligible. To allow a comparison, a control group to establish population frequencies of germline *BRCA1/2*-PMs in women with the same ethnic background was extracted from the literature.

For the purposes of this systematic review all studies which investigated at least one mutation in one of the *BRCA*-genes in relation to USC were considered eligible. In these studies, USC was defined by having at least 10% serous histology, with the uterus as primary site of origin. Studies on carcinosarcomas and studies in which no distinction was made between histologic subtypes of EC were excluded. Single-arm cohort studies were only included when patients had a proven germline *BRCA1/2*-PM and the cohort was not enriched for a particular malignancy (thereby preventing selection bias).

Relevant studies were identified by literature search in the PubMed, EMBASE and Web of Science databases using a search strategy which was devised in collaboration with a trained librarian. The search strategy consisted of a combination of Medical Subject Heading (MeSH) and free text words with the following combined keywords: 'BRCA' and 'uterine neoplasms', including all relevant keyword variations (Appendix A). The search was performed in July 2016. No limits or filters were placed on the searches. Reference lists of papers were checked for additional citations to ensure that no references were omitted. Two authors (MJ and AM) independently reviewed the titles and abstracts of the citations to identify studies eligible for inclusion. Articles published in languages other than English, German or Dutch were excluded. Data were reported using the PRISMA checklist (Appendix B).

Data extraction

Data were extracted by two authors (MJ and AM) independently. For the single-arm case-only studies, data on germline *BRCA1/2*-prevalence were extracted. Germline *BRCA1/2*-PM prevalence rates vary among populations and are especially high in Ashkenazi Jews.[15, 18-22]. To avoid bias, in studies describing an ethnic Jewish population, only the data on Ashkenazi Jews were extracted if possible. To reduce the probability of false positive results due to

population stratification, the literature was searched for a control group of the same ethnicity. Data on personal history of breast cancer and first-degree family history of breast and/or ovarian cancer were extracted. For the single-arm cohort studies, USC incidence risk during follow-up was assessed.

Risk of bias assessment

Adequateness of USC diagnosis (revised by expert pathologist, indicated whether mixed-USC cases were also included), risk for population stratification (did studies define the ethnic groups included and were these groups extractable) and potential for selection bias (tamoxifen use) were determined for every study included. For case-only studies, *BRCA1/2*-PM testing (full coverage of the genes or just founder mutations) was assessed. For cohort studies, follow-up was considered sufficient if the mean or median age of the study participants plus the mean or median follow-up together equalled the average age of USC development (age 70 years).

Data synthesis and statistical analysis

To determine whether germline *BRCA1/*2-PMs are more common in women with USC compared with women without USC, first the pooled proportion with 95% confidence interval (CI) of a germline *BRCA1/2*-PM was estimated for patients with USC and for population controls without USC. These pooled proportions were subsequently compared and a pooled odds ratio (OR) with 95% CI was estimated to compare presence of a germline *BRCA1/2*-PM. These estimates were obtained from a logistic regression with a random effect at the study level.

To determine whether germline *BRCA1/2*-PMs are more common in women with USC who have a positive first-degree family history and/or personal history for *BRCA1/*2-associated malignancies compared with the women with no such history, data on personal and family history were extracted from single-arm case studies and a pooled risk ratio (RR) was estimated.

For the single-arm cohort studies, no suitable control group was found, therefore meta-analysis could not be performed.

Statistical analyses were performed in STATA (StataCorp. 2015. *Stata Statistical Software: Release 14*. College Station, TX: StataCorp LP).

Case report

Molecular and functional assays

After obtaining informed consent, normal and tumour DNA was isolated from formalin-fixed paraffin-embedded tissue blocks. Three 0.6-mm cores were taken from normal and tumour tissue. Fully automated DNA isolation was performed as described previously[23] followed by

'next generation sequencing' using a modified two pool version of the Ion AmpliSeq *BRCA1* and *BRCA2* community panel. The Ion Proton (Thermo Fisher Scientific Inc.) system was used for sequencing according to manufacturers' recommendations. Loss of heterozygosity (LOH) was determined by frequency analysis of the pathogenic germline variants and single nucleotide variants in the *BRCA* genes.

The functional RAD51 assay was performed on fresh USC tissue which was obtained from the Pathology department at the Leiden University Medical Centre directly after resection. The research sample was prepared for analysis of RAD51 focus formation by immunofluorescence microscopy as previously described.[24] In brief, cancer tissue was irradiated *ex vivo* with 5 Gy ionising radiation to induce DNA double strand breaks. After 2 h of incubation at 37°C, the tissue was fixed in formalin and embedded in paraffin. As a functional read out for homologous recombination (HR) proficiency, the ability of the cells to recruit RAD51 protein to sites of DNA damage was measured. Tumour samples are considered HRD if less than 20% of the replicating tumour cells form RAD51 foci.[24]

Results

Systematic review and meta-analysis

Search results

The literature search yielded a total of 1893 citations (Pubmed; 778, EMBASE; 700, Web of Science; 415), of which 1365 were unique. Forty-two articles were retrieved for full-text review (See flow-chart, Fig. 1). Of these, thirty-two publications were excluded for reasons described in the flow-chart. Finally, ten publications were included for analysis, of which seven were case-only studies (Table 1) and three were single-arm cohort studies (Table 2). All included studies were identified via the initial database search. Included studies were published between 2000 and 2016.

Risk of bias assessment

Risk of bias assessment is provided in Table 3. Regarding USC diagnosis, in 40% of the studies, USC cases were revised by an expert pathologist and 40% stated whether included USC cases were of pure serous histology or contained mixed-histologic elements. Only one single-arm case study fully covered *BRCA1/2*, whereas five of seven studies only tested for the most common founder mutations in the Ashkenazi Jewish population. For two studies that contained a predominantly ethnic Jewish population,[21, 25] specific data were not extractable for Ashkenazi Jews alone. Data on previous tamoxifen use were given for four of ten studies. Follow-up was inadequate for all studies according to our formulated definition for adequacy (mean or median age study participants plus mean or median follow-up equalled the average age of USC development (age 70 years).

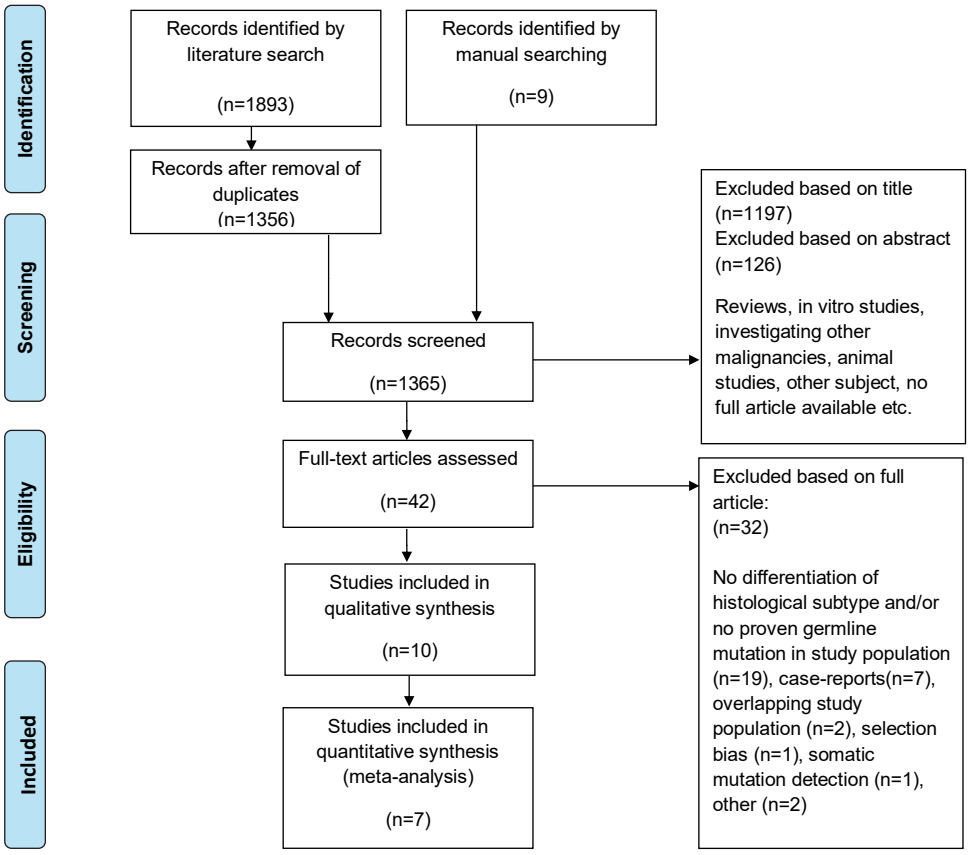

Figure 1: Flowchart illustrating the selection of studies.

Germline BRCA1/2-PM prevalence in USC patients compared to population cohorts

Seven single-arm case studies are summarised in Table 1. Five of the seven reports studied Jewish patients, mainly of Ashkenazi origin, and 2 reports considered an admixed, western population.

Sixteen germline *BRCA1/2*-PMs were identified in 134 Jewish women (mainly of Ashkenazi origin) with USC (Fig. 2). Reported prevalence of germline *BRCA1/*2-PM ranged from 0% to 26.1%, with the pooled proportion being 11.9% (95% CI: 5.1-25.6). Three cases with germline *BRCA1/2*-PMs were found in a total of 207 women from Western origin (mainly Caucasian) with USC. In this group, the pooled proportion was 1.5 (95% CI 0.5-4.1). Of the *BRCA1/2*-PMs found, most were *BRCA1*-PMs (14/19, 74%).

Literature was searched for control groups for both Ashkenazi Jewish and Western women to determine the prevalence of germline *BRCA1/2*-PM. Based on three studies, the

Table 1. Reported prevalence rates of pathogenic germline *BRCA1/2* mutations in uterine serous carcinoma in single-arm case studies

Study	Cases carrier/ Total (%)	*BRCA1/2*-PM detected	LOH[a]	Personal history	Family history case carrier[b]	Mutations tested	Population	Country
Barak et al, 2010	0/34 (0.0)	-	n.a.	-	-	*BRCA1* (185delAG, 5382InsC, Tyr978X) *BRCA2* (6174delT)	75% Ashkenazi Jew, 25% non-Ashkenazi Jews	Israel
Bruchim et al, 2010	6/23 (26.1)[c]	*BRCA2*-6174delT	n.a.	No	Breast	*BRCA1* (185delAG and 5382insC), *BRCA2* (6174delT)	100% Ashkenazi Jew	Israel
		BRCA1-185delAG		No	Breast			
		BRCA2-6174delT		Breast	No			
		BRCA1-5382insC		No	Breast			
		BRCA2-6174delT		No	No			
		BRCA2-6174delT		No	No			
Goshen et al, 2000	0/56 (0.0)	-	-	-	-	*BRCA1* (exon 11, 185delAG, 5382insC, and dup(ex13)) and *BRCA2* (exons 10, 11, 6174delT)[d]	Admixed, not specified, probably admixed Canadian	Canada
Lavie et al, 2000	2/9 (22.2)[e]	*BRCA1*-5382insC	yes	No	Ovarian	*BRCA1* (185delAG and 5382insC), *BRCA2* (6174delT)	100% Ashkenazi Jew	Israel
		BRCA1-185delAG	yes	Breast	Ovarian, Prostate			
Lavie et al, 2010	8/51 (15.7)[f]	*BRCA1*-185delAG	3/4[g]	3/8 (37,5) breast	Breast and/or Ovarian[h]	*BRCA1* (185delAG and 5382insC), *BRCA2* (6174delT)	100% Ashkenazi Jew	Israel
		BRCA1-185delAG			Breast and/or Ovarian[h]			
		BRCA1-185delAG			Breast and/or Ovarian[h]			
		BRCA1-185delAG			Breast and/or Ovarian[h]			
		BRCA1-185delAG			Breast and/or Ovarian[h]			
		BRCA1-5382insC			Breast and/or Ovarian[h]			
		BRCA1-5382insC			Breast and/or Ovarian[h]			
		BRCA2-6174delT			Breast and/or Ovarian[h]			
Levine et al, 2001	0/17 (0.0)	-	-	-	-	*BRCA1* (185delAG and 5382insC), *BRCA2* (6174delT)	>90% Ashkenazi Jew	United States
Pennington et al, 2013	3/151 (2.0)	*BRCA1*-p.E1535X	n.a.	Breast				
		BRCA1-c.2594delC		Ovarian (synchronous), Breast	Breast, Ovarian	*BRCA1*, chr 17:41191313-41282500 *BRCA2*, chr13:32884617-32978809	Admixed; 66% Caucasian, 26% African American, 1% Asian, 2% Other, 5% Unknown	United States
		BRCA1-c.713-2A>C		Esophageal	Cervix			

Abbreviations: PM: pathogenic mutation, LOH: loss of heterozygosity, n.a.: Not available/not extractable. [a] LOH: Partial or complete loss of heterozygosity of the wild-type *BRCA* allele. [b] Family history: first-degree relatives; parents, children, full siblings. [c] Non-Ashkenazi Jews excluded (n=8). [d] According to the authors, the protein truncation assay (*BRCA1* (exon 11) and *BRCA2* (exons 10 and 11) detects approximately 70% of pathogenic mutations. [e] Non-Ashkenazi Jews were excluded (n=3). [f] Non-Ashkenazi Jews were excluded (n=8). [g] Data extracted from study of Lavie *et al*, 2004. [h] Only mentioned that at least 1 first-degree relative showed a history of breast or ovarian cancer.

Table 2. Single-arm cohort studies: incidence of uterine serous carcinoma in women with a pathogenic germline *BRCA1/2* mutation

Study	*BRCA*-PM in USC cases	USC/Total (%)	Expected number of EC[a]	Mean/Median age at enrolment, years (range)	Mean/Median Follow-up, years (range)	Short description
Beiner et al, 2006	n.a.	0/857 (0)	1.13[b]	54.4* (45–70)	3.3* (0.01–9.6)	Women from 11 countries (North America, Europe and Israel) with a germline *BRCA1* (n=619) or *BRCA2 (*n=236) mutation or both (n=2) and an intact uterus were followed until diagnosis of endometrial cancer, ovarian cancer, hysterectomy, death, age of 70 or the date of completion of the last questionnaire.
Reitsma et al, 2013	n.a.	0/315 (0)	0.94[b]	43# (30–71)	6 # (0-27)	Women with a *BRCA1* (n=201) or *BRCA2* (n=144) mutation in the Netherlands who had undergone a risk-reducing salpingo-oophorectomy without hysterectomy from January 1996 until March 2012 at the University Medical Center Groningen were analysed for endometrial cancer using the Dutch nationwide pathology database, PALGA.
Shu et al, 2016	3x *BRCA1* 1x *BRCA2*	4/1083 (0.4[d])	4.3[b] 0.34[c]	45.6# (40.9-52.5)	5.1# (3.0-8.4)	Women with a deleterious *BRCA1* (n=627) or *BRCA2* (n=453) mutation or both (n=3) who had undergone a risk-reducing salpingo-oophorectomy without hysterectomy from January 1995 to December 2011 at 9 academic medical centers in the United states. Censoring occurred at uterine cancer diagnosis, hysterectomy, last follow-up, or death.

Abbreviations: EC; endometrial carcinoma, PM; pathogenic mutation, USC; Uterine serous carcinoma, n.a.: not applicable, * Mean, # Median. [a] Data extracted from article. [b] Expected number of EC. [c] Expected number of serous/serous-like EC. [d] Carcinosarcoma with serous epithelial component (n=1) was excluded.

Table 3. Risk of bias assessment single-arm case studies and single-arm cohort studies

Study	Diagnosis of uterine serous carcinoma		*BRCA1/2* pathogenic mutation detection		Risk population stratification	Selection bias	Follow-up
	All cases revised by expert pathologist	Stated number of pure serous and/ or serous-like/ mixed with serous carcinoma	Full coverage *BRCA1/2*	Only founder mutations tested (*BRCA1* (185delAG, 5382InsC) *BRCA2* (6174delT))	Different ethnic group(s) extractable	Data on previous tamoxifen use available	Adequate follow-up for developing uterine serous carcinoma[a]
Single-arm case studies							
Barak et al, 2010	N	N	N	Y	N	N	n.a.
Bruchim et al, 2010	Y	Y	N	Y	Y	Y	n.a.
Goshen et al, 2000	Y	U	N	N	N	N	n.a.
Lavie et al, 2000	N	U	N	Y	Y	N	n.a.
Lavie et al, 2010	N	U	N	Y	Y	N	n.a.
Levine et al, 2001	Y	Y	N	Y	N	N	n.a.
Pennington et al, 2013	Y	Y	Y	N	N	N	n.a.
Single-arm cohort studies							
Beiner et al, 2006	N	N	n.a.	n.a.	Y	Y	N
Reitsma et al, 2013	N	N	n.a.	n.a.	N	Y	N
Shu et al, 2016	N	Y	n.a.	n.a.	N	Y	N

Abbreviations: Y = yes, N = no, U = unclear, n.a.= not applicable..[a] Follow-up was considered adequate if the mean or median age of the study plus the mean or median follow-up comprised the average age of uterine serous carcinoma development (age 70 years).

reported prevalence of the three most common (founder) germline *BRCA1/2*-PMs [*BRCA1* (185delAG: NM_007294.3:c.68_69delAG, 5382insC: NM_007294.3:c.5266dupC), *BRCA2* (6174delT:NM_000059.3:c.5946delT)] in the general Ashkenazi Jewish population is estimated to be between 1.9 and 2.7%.[18-20] Based on the pooled germline *BRCA1/2*-PM prevalence in the Ashkenazi Jewish population and the pooled germline *BRCA1/*2-PM prevalence in USC cases, the OR for a germline *BRCA1/2*-PM was increased for women with USC: 5.4 (95% CI 2.2-13.1).

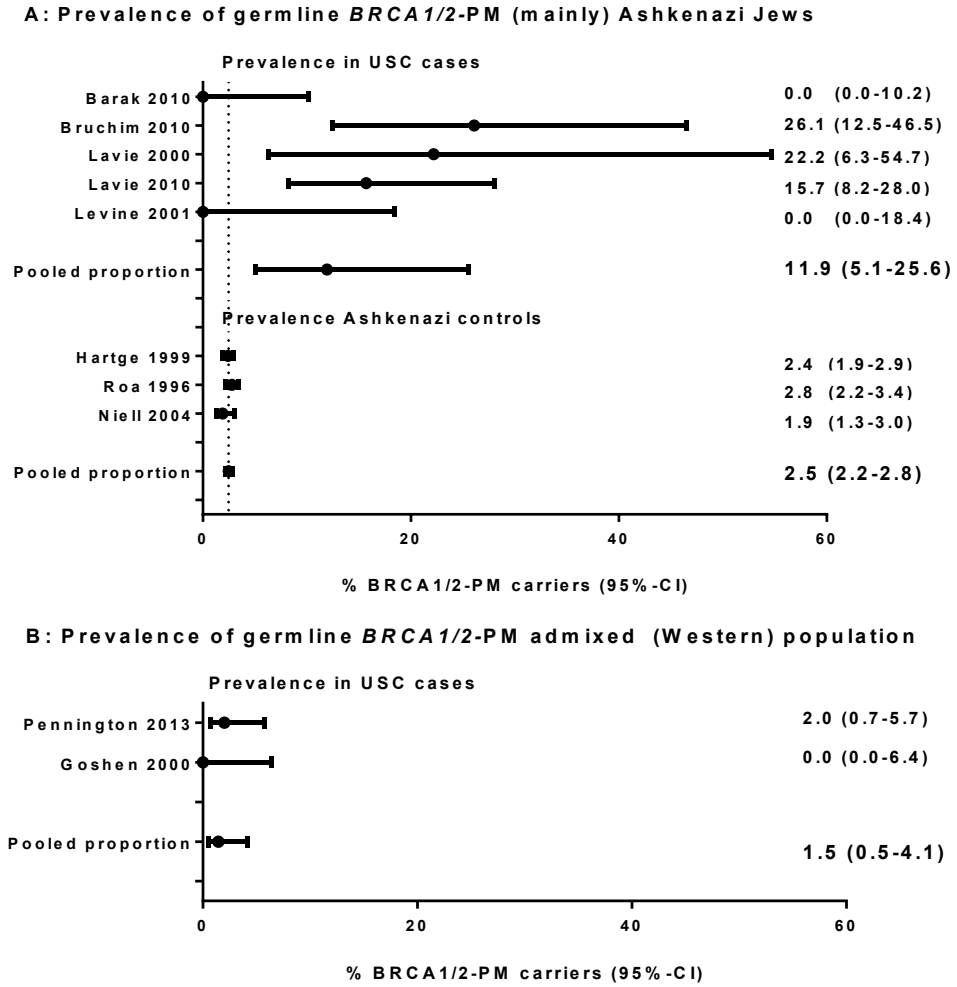

Figure 2. Meta-analysis of data extracted from single-arm case studies: germline *BRCA1/*2-pathogenic mutation prevalence in USC stratified for ethnicity. A: Pooled proportion of germline *BRCA1/2*-PM prevalence in (mainly) Ashkenazi Jewish women with USC compared to the pooled proportion of general Ashkenazi Jewish population. The germline *BRCA1/2*-PM prevalence is significantly higher in Ashkenazi Jews with USC compared to the general population. B: Pooled proportion of germline *BRCA1/2*-PM prevalence in an admixed population of women with USC from Canada and the United States of America. Abbreviations: PM; pathogenic mutation, USC: uterine serous carcinoma

For the general admixed Western population, reported germline *BRCA1/*2-PM prevalence varied between 0.23% and 0.32% based on estimates in women from the UK.[26-28] Because no measures of uncertainty were provided, no formal OR could be estimated. However, since these germline *BRCA1/2*-PM prevalence estimations do not lie within the 95% CI (0.5-4.1) of the pooled proportion of USC patients in the admixed population, this is suggestive for an increased prevalence of germline *BRCA1/2*-PMs in USC women.

Data from three single-arm cohort studies in *BRCA1/2*-PM carriers are summarised in Table 2.[16, 29, 30] The mean follow-up periods in the single-arm cohort studies ranged from 3.3 to 6 years. The median/mean ages at enrolment varied from 43 to 54.4 years.[16, 29, 30] No USCs occurred in two of the single-arm cohort studies. In one study,[16] 4 USCs/mixed USCs occurred in a population of 1083 women in which the expected number of serous/serous-like EC (e.g., serous, undifferentiated, carcinosarcoma) was 0.3.

Personal history and family history in USC patients with germline BRCA1/2-PM

Data on personal and first-degree family history of USC patients correlated to germline *BRCA1/2*-PM status were available in seven studies (Supplementary Table 1). The pooled RR for carriage of a germline *BRCA1/2*-PM in women with USC and a positive first-degree family history for breast and/or ovarian cancer, 4.0 (95% CI: 2.1-7.5), increased compared to women with no such family history (Fig. 3a). In terms of a personal history of breast cancer, the pooled RR for having a germline *BRCA1/2*-PM was 2.1 (95% CI: 0.9-4.9) (Fig. 3B).

Case report

A 53-year-old Caucasian women, with a first-degree family history positive for *BRCA1*-associated breast cancer, was found to be a carrier of this germline *BRCA1*-PM in exon 13 (NM_007294.3:c.4327C>T (p.[Arg1443*]). She presented with postmenopausal bleeding and endometrial curettage showed EC suggestive for USC.

There was no personal history of cancer. A RRSO followed by a prophylactic bilateral mastectomy was performed approximately three years before the onset of symptoms. No (pre)malignancy was diagnosed in either sample.

The patient underwent a total abdominal hysterectomy and dissection of the iliac and para-aortal lymph nodes to achieve maximum cytoreduction.

Pathological examination by a gynaecopathologist revealed USC (<50% of the myometrial thickness) with substantial lymph-vascular space invasion and involvement of 16 of 22 removed iliac and para-aortal lymph nodes. Wilm's tumour 1-IHC was negative in the tumour cells, supporting the primary endometrial origin.[31, 32] One month after surgery, positron emission tomography demonstrated multiple remaining FDG-avid lymph nodes from the

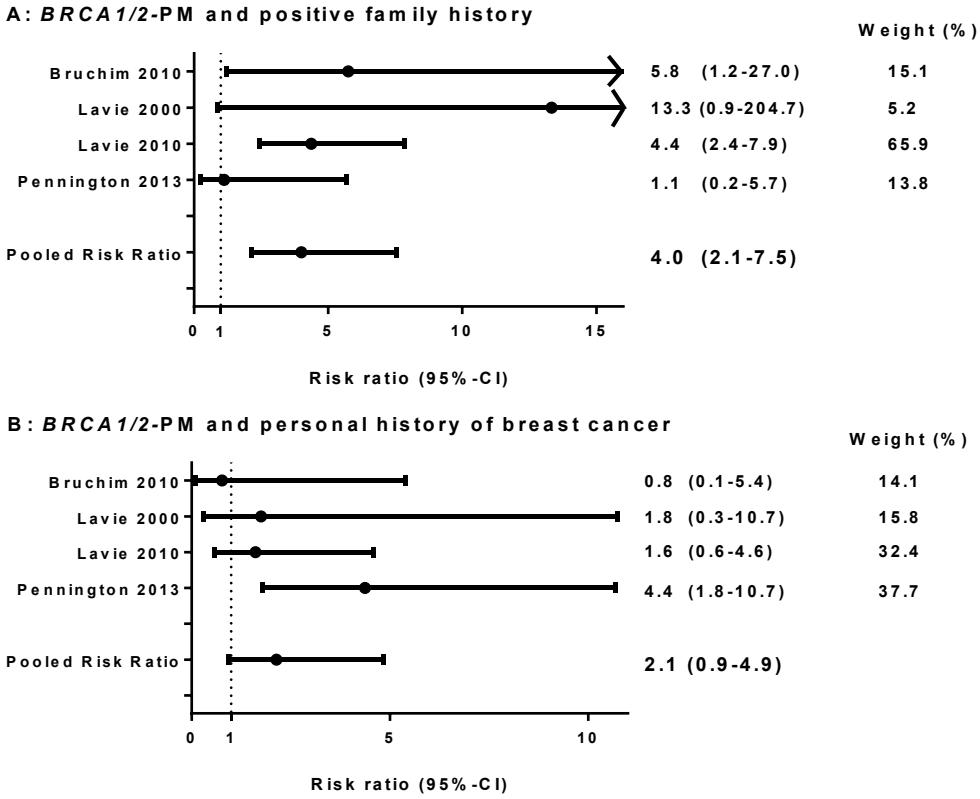

Figure 3. The association of family and personal history with germline *BRCA1/2*-pathogenic mutations in women with uterine serous carcinoma. A: The relative risk for having a germline *BRCA1/2*-PM in women with USC and a positive first-degree family history (parents, children, full-siblings) for breast and/or ovarian cancer was significantly increased compared to women with no such history. B: The relative risk for having a germline *BRCA1/2*-PM in women with USC and a positive personal history for breast cancer is increased, although not significantly. Abbreviations: PM; pathogenic mutation, USC: uterine serous carcinoma

renal vein until the bifurcation of the internal and external iliac artery at both sides. Also, a positive lymph node at the left supraclavicular fossa was detected (FIGO stage IV). After completion of six cycles of adjuvant carboplatin and paclitaxel chemotherapy, computed tomography demonstrated complete radiological remission of residual disease.

The known germline *BRCA1*-PM was detected in the tumour DNA. The tumour showed complete LOH of the *BRCA1* wild-type allele.

In addition, the functional *ex vivo* RAD51 assay showed complete absence of RAD51 ionising radiation induced foci formation in replicating tumour cells, supporting homologous recombination deficiency due to the absence of functional BRCA1 protein (Fig. 4).

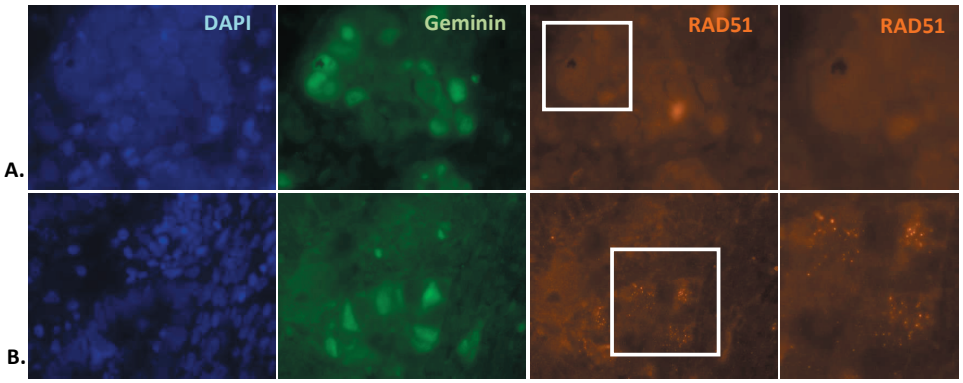

Figure 4: Homologous recombination capacity determined by *ex vivo* RAD51 assay. A: Absence of RAD51 accumulation after ionising radiation in replicating tumor cells stained by Geminin (a marker that is expressed during S and G2 phases of the cell cycle), indicating a homologous recombination deficiency in the tumor cells. DAPI stains cell nuclei. B: RAD51 accumulation was present in replicating tumor cells from an endometroid endometrial carcinoma. In the last column a magnification the RAD51 staining of a subset of the Geminin positive cells is shown.

Discussion

The morphological, molecular and clinical similarities between USC and HGOTSC suggest common etiologies and raise the question of whether USC is a component of *BRCA1/2*-associated HBOCS. In this study, combining a systematic review and a case report, we provide epidemiological, molecular and functional support for the concept of USC as a *BRCA1/2*-associated HBOCS disease.

The main limitation of this study was the absence of case-control studies comparing germline *BRCA1/2*-PM frequencies in USC patients and healthy controls. In addition, no cohort studies formally comparing USC incidence in germline *BRCA1/2*-PM carriers versus non-carriers were available. Control groups were therefore borrowed from the literature. For Ashkenazi Jews, the prevalence of germline *BRCA1/2*-PMs could be estimated based on three large series of Ashkenazi Jews.[18-20] However, data on the Western admixed population were less solid[26-28] and therefore only comparisons of proportions could be performed. Another potential limitation is that, especially in advanced disease, HGOTSC can mimic USC[31, 32] making the ascertainment of the primary site of origin sometimes problematic. Of all USC cases with a germline *BRCA1/2*-PM, only one study reported an USC-case with synchronous ovarian cancer.[33] A Wilm's tumour 1-IHC staining to assist with determination of the primary site of origin,[31, 32] was not routinely performed in these studies. Misclassification of HGOTSC as USC can potentially give bias as it is known that germline *BRCA1/2*-PMs are prevalent in HGOTSC.[11]

Furthermore, substantial heterogeneity was found in the germline *BRCA1/2*-PM prevalence between studies, which can possibly be explained by incomplete analysis of the *BRCA1/*2-genes. *BRCA1* and *BRCA2* are very large genes which, with the exception of population-specific founder mutations, lack mutational hotspots.[34-36] Two of five studies with a Jewish population did not make the distinction between Ashkenazi Jews and other Jews. Since only specific founder mutations characteristic for Ashkenazi Jews were analysed, this may have led to an underestimation of the prevalence of germline *BRCA1/2*-PMs. In addition, one of the two single-arm case studies considering the admixed population used a test only capable of detecting approximately 70% of deleterious *BRCA1/*2-PMs.[17] This approach may have missed 2 of the 3 *BRCA1* mutations identified in the study by Pennington *et al.* [33]. Finally, data on the use of previous tamoxifen-treatment were only available for a subset of studies.[16, 22, 37, 38] Although some studies demonstrate a potential relationship between previous tamoxifen use and the development of USC, which might give bias,[39-41] this relationship remains controversial. Future studies need to be performed to clarify whether tamoxifen indeed increases the risk for non-endometrioid ECs or that this effect has been biased by unknown germline *BRCA1/2*-PMs in the included study-participants with previous breast cancer.

Despite these limitations, this meta-analysis supports a relationship between *BRCA1/2*-PM and USC, especially focussing on studies investigating the prevalence of *BRCA1/2*-PMs in USC patients and comparing this to the prevalence of *BRCA1/2*-PMs in the ethnicity-specific population control groups. In the follow-up studies, this relationship could not be established by two of three studies.[16, 29, 30] However, given that USC develops at a median age of 70 years,[3] all follow-up studies had insufficient follow-up (mean/median 3.3-6 years) relative to the age at enrolment (median/mean age 43-54.4 years) to meaningfully address the question of whether germline *BRCA1/2*-PM carriers have an increased risk of developing USC. Despite this shortcoming, one of the three single-arm cohort studies[16] reported a positive association between germline *BRCA1*-PMs and serous/serous-like carcinoma.

In addition, we described a case of a woman carrying a germline *BRCA1*-PM who developed an USC three years after RRSO. Molecular and functional analysis of tumour DNA demonstrated complete LOH of the *BRCA1* wild-type allele, causing a functional defect in HR, supporting a causal relationship. Of the included studies that additionally analysed LOH, the majority of cases (7/9, 77.8%) demonstrated LOH[15, 16, 38] of the *BRCA1* wild-type allele, further stressing a potential causal relationship between germline *BRCA1/2*-PMs and USC.

Aforementioned relationship has potentially important clinical implications. First, a clinical genetic consultation should be considered for USC patients who have not yet undergone germline *BRCA1/2*-PM testing, especially in the context of a positive first-degree family history, as shown in our meta-analysis.

Second, a prophylactic hysterectomy has to be considered. Since USC is a rare disease, even the 5-fold increased USC risk found in this study does not necessarily carry clinical consequences as the absolute USC risk remains low. However, due to the poor prognosis associated with USC, clinicians should be aware of this relationship and they should inform patients.

Third, it might open up ways for new systemic treatment options, such as use of PARP inhibitors, currently only registered for recurrent platinum-sensitive HGOTSC with germline or somatic *BRCA1/2*-PMs.[42-44] Although platinum derivates might have less effect on USC than HGOTSC,[11, 14, 45] there seems to be a subgroup of USC that is platinum-sensitive.[13] The smaller effect of platinum-based chemotherapy on USC compared with HGOTSC might be explained by the fact that USC more often present with mixed histology. Furthermore, (germline) *BRCA1/2*-PMs are less common in USC compared with HGOTSC. To the best of our knowledge, this is the first study to demonstrate HRD in USC using a functional RAD51 assay. The absence of HR in the tumour cells suggest that USC patients with germline *BRCA1/2*-PM may benefit from PARP inhibitor treatment. Women with a *BRCA1/2*-associated USC should be considered as potential candidates for future trials of PARP inhibitors.

In conclusion, data from our systematic review and meta-analysis support the view that USC is a component of *BRCA1/*2-associated HBOCS. This, together with our case report documenting LOH and HRD in USC, suggests a causal relationship between germline *BRCA1/2*-PMs and the development of USC. As germline *BRCA1/2*-PMs in USC may have therapeutic consequences in terms of use of PARP inhibitors and potentially risk-reducing surgery for patients and family members, clinicians should be aware of this association. Most importantly, this study supports the notion that women with USC should be offered screening for germline *BRCA1/2*-PMs when there is a positive family history for malignancies associated with HBOCS.

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The authors did not receive any funding for this study.

Conflicts of interest statement

None declared.

Appendix A: Search string

PubMed

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Web of science

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Appendix B: PRISMA-checklist

Available online; doi: 10.1016/j.ejca.2016.11.028

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Supplementary table

Supplementary Table S1. Personal and family histories of germline *BRCA1/2*-PM carriers with USC compared to non-*BRCA1/2*-PM carriers in single-arm case studies

	USC and germline *BRCA1/2*-PM			USC without germline *BRCA1/2*-PM		
	Total	Personal history breast cancer (%)	Family history breast/ovarian cancer[a] (%)	Total	Personal history breast cancer (%)	Family history breast/ovarian cancer[a] (%)
Barak et al, 2010	0	-	-	56	n.a.	n.a.
Bruchim et al, 2010	6	1 (16.7)	3 (50.0)	23[b]	5 (21.7)	2 (8.7)
Goshen et al, 2000	0	-	-	56	6 (10.7)	16 (28.6)
Lavie et al, 2000	2	1 (50)	2 (100)	7	2 (28.6)	0 (0.0)
Lavie et al, 2010	8	3 (37.5)	8 (100)	43	10 (23.3)	9 (20.9)
Levine et al, 2001	0	-	-	17	n.a.	n.a.
Pennington et al, 2013	3[c]	2 (66.7)	1 (33.3)	131[c]	20 (13.2)	39 (29.8)
Total	19	7 (36.8)	14 (73.7)	260	38 (14.6)	66 (25.4)

Abbreviations: PM: pathogenic mutation, USC: Uterine serous carcinoma, n.a.: not available/not extractable. [a]: Family history only includes first degree-relatives; parents, children, full-siblings. [b]: Considers Ashkenazi Jews and non-Ashkenazi Jews, data not extractable for Ashkenazi Jews alone. [c]: Personal and family history data available for 134/151 women (including women with germline *BRCA1/2*-PM).

Chapter 4

Germline *BRCA*-associated endometrial carcinoma is a distinct clinicopathologic entity

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Abstract

Purpose

Whether endometrial carcinoma (EC) should be considered part of the *gBRCA1/2*-associated hereditary breast and ovarian cancer (HBOC)-syndrome is topic of debate. We sought to assess whether ECs occurring in *gBRCA carriers are enriched for clinicopathologic and molecular characteristics, thereby supporting a causal relationship.*

Experimental Design

Thirty-eight *gBRCA* carriers that developed EC were selected from the nationwide cohort study on hereditary breast and ovarian cancer in the Netherlands (HEBON), and these were supplemented with four institutional cases. Tumor tissue was retrieved via PALGA (Dutch Pathology Registry). Nineteen morphologic features were scored and histotype was determined by three expert gynecologic pathologists, blinded for molecular analyses (UCM-OncoPlus Assay including 1213 genes). ECs with LOH of the *gBRCA*-wild-type allele (*gBRCA*/LOHpos) were defined "*gBRCA*-associated", those without LOH (*gBRCA*/LOHneg) were defined "sporadic".

Results

LOH could be assessed for 40 ECs (30 *gBRCA1,* 10 *gBRCA2*), of which 60% were *gBRCA/*LOHpos. *gBRCA/*LOHpos ECs were more frequently of nonendometrioid (58%, P=0.001) and grade 3 histology (79%, P<0.001). All but two were in the *TP53-mutated TCGA*-subgroup (91.7%, P<0.001). In contrast, *gBRCA/*LOHneg ECs were mainly grade 1 endometrioid EC (94%) and showed a more heterogeneous distribution of TCGA-molecular subgroups: *POLE-mutated* (6.3%), MSI-high (25%), NSMP (62.5%) and *TP53-mutated* (6.3%).

Conclusions

We provide novel evidence in favor of EC being part of the *gBRCA*-associated HBOC-syndrome. *gBRCA*-associated ECs are enriched for EC subtypes associated with unfavorable clinical outcome. These findings have profound therapeutic consequences as these patients may benefit from treatment strategies such as PARP-inhibitors. In addition, it should influence counseling and surveillance of *gBRCA* carriers.

Translational relevance

We provide novel evidence in favor of endometrial carcinoma (EC) being part of the *gBRCA*-associated HBOC-syndrome. By stratifying ECs that occurred in *gBRCA*-mutation carriers by LOH of the *gBRCA* wild-type allele, we were able to identify ECs associated with the *gBRCA*-mutation (*gBRCA/*LOHpos) and those that occurred sporadically (*gBRCA/*LOHneg). *gBRCA*-associated ECs are distinctly different from sporadic ECs by histology (high grade) and by molecular subtype (*TP53* mutant), both of which are associated with worst clinical outcome. These findings support the concept that EC is part of HBOC-syndrome, which impacts genetic counseling and surveillance programs of *gBRCA* carriers. In addition, our work shows that LOH-status should be considered when assessing PARP-inhibitor sensitivity.

Introduction

Inheritance of a pathogenic mutation in one allele of the breast cancer susceptibility genes, *BRCA1* or *BRCA2*, results in the hereditary breast and ovarian cancer (HBOC) syndrome, characterized by severely increased lifetime risk to develop breast cancer and tubo-ovarian cancer (OC).[1, 2] Other cancer types reported to be increased in patients with germline *BRCA2* mutations (*gBRCA*) are pancreatic and prostate cancer.[3, 4] Whether endometrial carcinoma (EC) should be considered part of *gBRCA*-associated HBOC-syndrome is still under debate due to conflicting data.[5-9] A number of studies have shown an increased risk to develop EC especially for *gBRCA1* carriers, with highest risks observed for an aggressive subtype of EC; the serous-like ECs.[5-7, 9-11] However, others did not observe this increased risk, or attributed it to previous tamoxifen treatment rather than to the *gBRCA* mutation.[8, 9, 11]

LOH of the wild-type *BRCA1* or *BRCA2* allele (g*BRCA*/LOHpos) is an important step in the carcinogenesis of *gBRCA*-associated tumors. This is supported by the observation that *gBRCA/*LOHpos breast cancers and OCs show significantly higher homologous recombination deficiency (HRD)-scores compared to their *gBRCA/*LOHneg counterparts.[12] The HRD-score is based on the presence and quantification of "genomic scars" associated with *BRCA*-deficiency, including the number of regions with LOH,[13] large-scale state transitions (LST),[14] and telomeric allelic imbalances (TAI).[15] Breast cancers and OCs arising in *gBRCA* carriers show variable LOH frequencies, with reported rates of 93% (*gBRCA1*) and 84% (*gBRCA2*) for OCs, and 90% (*gBRCA1*) and 54% (*gBRCA2*) for breast cancers.[12] This signifies the relevance of LOH as a marker of causality and implies that *gBRCA/*LOHneg cancers are in fact sporadic tumors that develop independently of the *gBRCA* mutation and are not HRD.

The finding of recurrent clinicopathologic and molecular features in *gBRCA*-associated breast cancers and OCs has supported the concept that these cancers are distinct entities belonging

to the *gBRCA*-associated HBOC-syndrome. These features can also help identify tumors more likely to harbor *BRCA1/2* mutations. For example, breast cancers arising in *gBRCA1* carriers prototypically are of high-grade and of the basal-like subtype with more frequent necrosis and lymphocytic infiltration.[16, 17] *BRCA1*-associated high-grade serous tubo-ovarian carcinoma (HGSOC) shows more frequent (partial) Solid, pseudoEndometrioid and/or Transitional morphology (SET-morphology), which is distinctly different from the prototypical papillary and infiltrative growth generally encountered in sporadic HGSOC. Other features more frequently observed in *BRCA1*-associated HGSOC are necrosis, a higher mitotic index and an increased number of tumor infiltrating lymphocytes.[18-20] On a molecular level, *gBRCA*-associated breast cancers and OCs share similar somatic copy-number profiles [somatic copy-number alteration (SCNA)-high] and frequent *TP53* mutations.[16-18, 20-22]

The Cancer Genome Atlas (TCGA) Research Network previously defined four distinct molecular subclasses with prognostic relevance in ECs.[22] The "serous-like/SCNA-high" molecular subclass has poorest clinical outcome and interestingly displays molecular similarities to both basal-like breast cancer and HGSOC, including a high number of SCNAs and frequent *TP53* mutations. Moreover, recent studies demonstrated that serous-like/SCNA-high ECs also frequently are HRD.[22-24] This raises the question whether ECs occurring in *gBRCA1/2* carriers might be enriched for certain features, but studies comprehensively evaluating this have not been performed to date.

We aimed to, for the first time, comprehensively describe the clinicopathologic and molecular features, stratified by LOH-status, of a large series of ECs that occurred in *gBRCA* carriers.

Materials and methods

Patient selection

Patients with a history of EC and a pathogenic *gBRCA1/2* mutation were identified from the "*He*reditary *B*reast and *O*varian cancer study, the *N*etherlands (HEBON-cohort study)".[25] The HEBON study is an ongoing nationwide study on families with HBOC for which all patients who undergo genetic testing for *BRCA1/2* and *CHEK2* mutations in one of the participating centers are eligible for inclusion [all eight university medical hospitals in the Netherlands and the Netherlands Cancer Institute (NKI)]. For participants, data on, among others, personal cancer history and therapeutic treatments are collected both retrospectively and prospectively through regular linkages with the Netherlands Cancer Registry (NCR). Data on prophylactic surgery are collected via the Dutch Pathology Registry (PALGA)[26]. All data is centrally collected and managed by trained data managers only. Women were eligible for inclusion when they had (i) a proven pathogenic germline *BRCA1/2* (*gBRCA1/2)* mutation (PLON class 4 or 5)[27], (ii) provided written informed consent for the HEBON-study, and (iii) had a history of epithelial EC

or developed an EC during follow-up, defined as a tumor with an International Classification of Diseases Oncology, Third Edition, First Revision (ICD-O-3.*1;* http://codes.iarc.fr/) topographical code of either C54 (Corpus Uteri) or C55 (Uterus, NOS).

In total, 3,726 *gBRCA* carriers provided informed consent between 1999 and 2014, of which the majority was provided in 2012 and 2013 (62.5%). Of these women, 41 (1.1%) developed an EC. We were able to retrieve 39 of 41 tumors from pathology laboratories across the Netherlands. One tumor was a sarcoma and was therefore excluded. Of these 38 HEBON-ECs, 21 ECs occurred preceding to study enrollment (mean 4.7 years, SD: 2.79) and 16 ECs occurred after study enrollment (mean 4.5 years, SD 3.52). For one case, the date of study enrollment was not available. The HEBON-ECs were supplemented with four ECs from known *gBRCA1/2* carriers previously diagnosed in the Leiden University Medical Center (LUMC).

For all ECs, haematoxylin and eosin (H&E)-stained slides, anonymized pathology reports, and at least one representative formalin-fixed, paraffin embedded (FFPE)-tumor block were collected through PALGA[26] from pathology laboratories across the Netherlands. If applicable, material from the (salpingo-)oophorectomy or OC specimen was also requested. The HEBON study is approved by the medical ethical committees of all participating centers, and all participants gave written informed consent to participate in the study. The HEBON study is performed in accordance with the Declaration of Helsinki. Our study was performed after the study protocol was approved by the HEBON steering committee (date; November 30, 2017) and by the Institutional Review Board of the Netherlands Cancer Institute; IRBd18086. All specimens were handled in compliance with the Code of Conduct for dealing responsibly with human tissue in the context of health research (2011) drawn up by the Federation of Dutch Medical Scientific Societies.

Clinicopathologic characterization

All cases were independently reviewed by three expert gynecologic pathologist (V.T.H.B.M. Smit, T. Bosse, and B.E. Howitt). They were aware that the ECs occurred in *gBRCA* carriers, however they were blinded for LOH status. The World Health Organization (2014) criteria were used for histologic subtype diagnosis. Reviewers were not allowed to use immunostains to aid classification and diagnoses were solely based on H&E stains. Cases were classified ambiguous when overlapping features of both high-grade endometrioid and serous carcinomas were present in the tumor and when tumors failed to show prototypic features of a certain subtype. Discordant cases were discussed during a consensus meeting to assign final histological subtype. ECs with ambiguous morphology were considered non-endometrioid for statistical analyses. After final histologic subtype was assigned, histologic subgroups were made. For ambiguous cases, *TP53* mutation status was used to assign histologic subgroup. Cases were categorized as follows: “Endometrioid” for Endometrioid, mucinous and *TP53*-wildtype ambiguous carcinomas, “serous-like” for uterine serous carcinomas (USCs), uterine

carcinosarcomas (UCSs) and *TP53*-mutant ambiguous carcinomas, or "clear cell" for clear cell carcinomas. Review of adnexa, depth of myometrial invasion, cervical involvement, lymph node status and presence of lymphovascular space invasion was performed by one expert gynaecologic pathologist (T. Bosse) on which FIGO-2009 stage was based upon. When slides were not available, these data were retrieved from the original pathology reports.

Nineteen morphological characteristics were assessed by one expert gynecologic pathologist (B.E. Howitt) on all available tumor slides, blinded for LOH status. For additional details on this, see Supplementary Materials and Methods.

IHC

Cases were stained for p53 (clone DO-7, 1:2,000, DAKO), Wilms tumor 1 (WT1, clone 6F-H1, 1:3,200, Invitrogen), estrogen receptor (ER, Clone EP1, 1:200, DAKO), progesterone receptor (PR, Clone Pgr636, 1:400, DAKO), and CD8 (Clone 4B11, 1:2,000, Novocastra). Procedures and scoring methods are described in the Supplementary Materials and Methods.

Molecular analysis

DNA isolation

Tumor DNA was isolated from FFPE-tissue blocks either by using three 0.6 mm tumor cores (*n*=16) or by using microdissected tissue from 5 to 10 tissue sections (10 µm; *n*=26). DNA isolation was performed fully automated using the Tissue Preparation System (Siemens Healthcare Diagnostics) as described previously.[28] The median tumor cell percentage of the isolated areas was 80% (range: 25%-90%).

Next-generation sequencing

Following extraction, DNA was quantified using the Qubit fluorometric assay (Thermo Fisher Scientific) and further assessed for quantity and quality using a quantitative PCR assay (hgDNA Quantitation and QC kit, KAPA Biosystems). Library preparation and sequencing were performed as previously described for the UCM-OncoPlus Assay.[29] Briefly, approximately 100 ng DNA was fragmented using the Covaris S2 (Covaris). The fragmented DNA was amplified using the KAPA HTP Library Preparation Kit (Kapa Biosystems) along with a set of patient-specific indexes (Roche). The pooled library was captured using a custom SeqCap EZ capture panel (Roche) featuring a collection xGen LockdownProbes (IDT) for 1,213 genes. The pooled captured library was sequenced on the Illumina HiSeq 2500 system (Illumina) in rapid run mode (2 x 101 bp paired end sequencing). Somatic mutation and copy number calling was performed across all 1,213 genes using a custom in-house bioinformatics pipeline previously described.[29] The five-tier pathogenicity classification described by Plon and colleagues, 2008 was used to categorize variants.[27] Only class 4 (likely pathogenic) and 5 (pathogenic) mutations are reported in the manuscript.

LOH of gBRCA1/2 mutations

Known *gBRCA1/2* mutations were assessed for LOH of the wild-type allele by evaluating the following parameters: estimated tumor cell purity, *BRCA1/2* mutation variant allele frequency (VAF), local copy number status, and adjacent SNP-VAF, using a similar approach to what has been described by Khiabanian and colleagues, 2018.[30] For LOH analyses, we applied the following model, taking into account the chromosomal copy number at the *BRCA* locus; VAF=[(1-p)+cmut x p] / [2 x (1-p) + Y x p], with p being the tumor purity, cMut being the mutation's chromosomal copy number and Y being the ploidy of the tumor cells. LOH events occur when cMut = 1 and Y = 1 or cMut > 1 and Y > 1. Because all *BRCA1/2* mutations were germline mutations, the expected VAF in the absence of LOH was 1/2 (50%) for all cases. LOH of the *gBRCA1/2* wild-type allele was considered to be present if (i) cMut = 1 and Y = 1 or cMut > 1 and Y > 1 (ii) the observed *gBRCA1/2* mutation VAF was similar to the expected VAF according to the formula, (iii) adjacent observed SNP VAF (if present) supported the findings and (iv) sequencing quality was sufficient. Mutations that were considered to have an LOH event were classified as either copy-neutral (no evidence of local copy number change) or copy number loss. *gBRCA/*LOHpos ECs were defined as *gBRCA*-associated, *gBRCA/*LOHneg ECs as "sporadic".

Copy-number calling

For the copy-number calling we used a clinically validated bioinformatic tool that has previously been detailed and published.[29] Briefly, copy-number analysis involved evaluation of average exon interval depths recorded via the Genome Analysis Tool Kit DepthofCoverage module. A historical normalized baseline for each interval in the panel was generated using 24 nonmalignant clinical samples. Test sample data were subjected to a normalization algorithm to control for individual gene profile run-specific variability. To detect the potential copy-number regions, fold change and Z-scores were calculated for each interval, and thresholds were set at >200% (gain) or <66% (loss) with Z-score >3 or <-2, respectively. Genes with more than half the intervals showing copy-number changes in the same direction were then identified. Overall copy-number status was assessed manually by assessing the copy-number plots across the entire territory and determining how many large-scale (arm or subarm-level changes) copy-number alterations were present in each case. Cases considered to be "low" copy-number had 0 large-scale copy-number alterations, "borderline" had 1-2 large-scale copy number alterations, and those considered "high" had >2 large-scale copy-number changes.

Microsatellite instability status

For MSI testing, a metric similar to that proposed by Kautto and colleagues, 2017[31] was employed to quantify the stability of a homopolymer locus. For each locus, distribution over different homopolymer lengths (normalized to a fraction of total depth at the locus) was generated. Then, absolute value of the stepwise difference between that sample distribution and normal distribution was calculated as a distance score (*d*). The baseline distribution

was generated using average values across 23 non-malignant spleen samples. Thresholds for assignment of "stable" or "instable" status for a locus involved using training sets of MSI-stable and MSI-high samples, tested previously by PCR assay or IHC staining. Samples with unstable loci <9% were classified as microsatellite stable, 9 to 15% were classified as indeterminate, and >15% were classified as microsatellite instable (MSI).

Tumor mutational burden

Tumor mutational burden (TMB) was quantified as mutations/Mb using a 1,132 gene territory from the UCM-OncoPlus assay. Variants that met any of the following criteria were excluded from the calculation: <10% VAF, synonymous, variants present in either 1,000 Genomes or ExAC population databases. In addition, variants were rescued if there were >10 entries in COSMIC database with an ExAC frequency of <0.001.

Molecular subgroups

The following surrogate markers were used to classify ECs in the four molecular subgroups defined by the TCGA;[22, 32, 33] *POLE* exonuclease domain mutations for the *POLE/*ultramutated group, MSI-high profile for MSI-high/hypermutated group, *TP53* mutations for SCNA-high/serous-like group, and the absence of surrogate markers for no surrogate marker profile (NSMP)/SCNA-low group.[22, 32, 33] When two molecular classifiers were present, subgroups were assigned in line with what has previously been published by the TCGA;[22] *POLE*&MSI-high or *POLE*&*TP53* as *POLE* and MSI-high&*TP53* as MSI-high.

Statistical analysis

Associations between categorical variables were tested using a two-sided Fisher exact test or Chi-square statistics when more than two variables were compared. Associations between continuous variables were tested using the Mann-Whitney *U* test. Overall survival was calculated using the Kaplan-Meier Method with log-rank test, and was calculated from the date of EC diagnosis to the date of death while patients who were alive were censored at the date of last follow-up. For HEBON cases, the date of last linkage with the Dutch Municipal Personal Record Database was used as last date of follow-up (April 11, 2019 for all except for case 2; December 23, 2016). *P* values <0.05 were considered significant. Statistical analysis was performed using IBM SPSS version 23.0 (SPSS, Inc.) and GraphPad Prism (GraphPad Software Inc.).

Results

In total, 42 ECs that occurred in *gBRCA1/2* carriers were analyzed (32 *gBRCA1,* 10 *gBRCA2*). Clinicopathologic characteristics of the complete cohort are described in Supplementary Table S1. The cohort comprised 26 endometrioid ECs (61.9%), of which 17 (40.5%) were

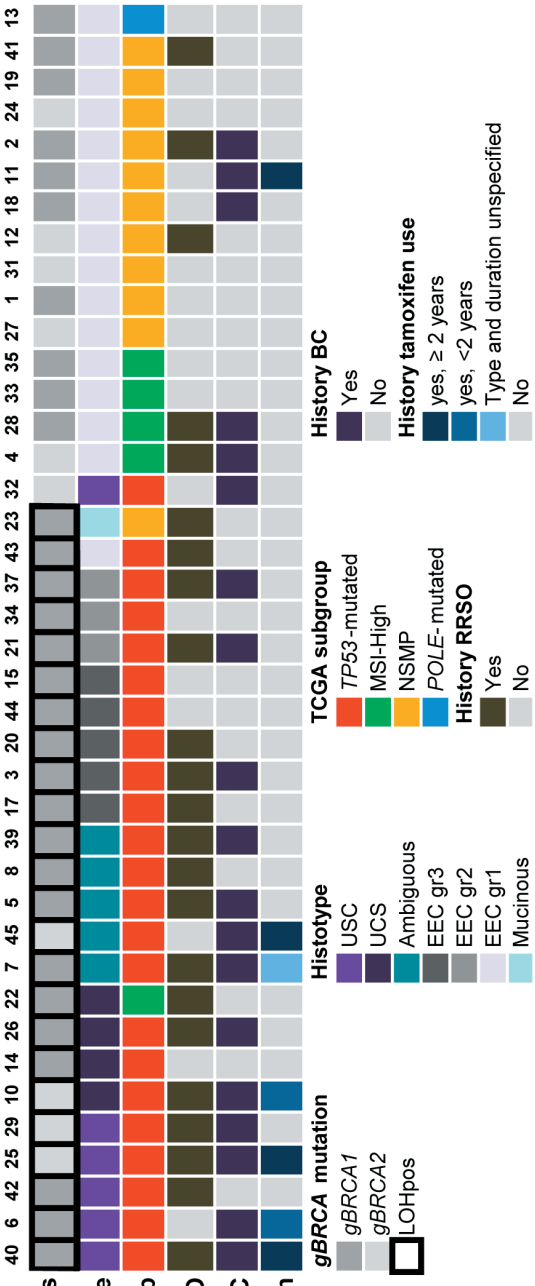

Figure 1: Clinicopathologic and molecular characteristics stratified by LOH status. Case 22 and case 4 were MSI-high and had a *TP53* mutation; they were classified in the MSI-high subgroup in accordance to what is described in the Supplementary Material and Methods. Abbreviations: EEC, endometrioid endometrial carcinoma grade; gr, grade; LOH, Loss of heterozygosity of the *gBRCA1/2* wild-type allele; UCS, Uterine Carcinosarcoma; USC, Uterine serous carcinoma.

Table 1. Clinicopathologic characteristics stratified by LOH status

	LOHpos (n=24)	**LOHneg (n=16)**	***P***
Germline *BRCA1/2* mutation, *n* (%)			
g*BRCA1*	20 (83.3)	10 (62.5)	0.159
g*BRCA2*	4 (16.7)	6 (37.5)	
Age at Diagnosis, median (range), years	60.5 (33-74)	57 (44-67)	0.267
FIGO 2009, No. (%)			
I, II	19 (79.2)	14 (87.5)	0.681
III, IV	5 (20.8)	2 (12.5)	
Salpingo-oophorectomy, *n* (%)[a]			
History of RRSO	18(75)[b]	5 (31.3)	**0.009**[c]
RRSO at time of EC diagnoses	0 (0)	2 (12.5)	
At time of hysterectomy	5 (20.8)	8 (50)	
Therapeutic	0 (0)	1 (6.3)	
History of, *n* (%)			
OC	0 (0)	0 (0)	
BC	13 (54.2)	6 (37.5)	0.349
Tamoxifen use	6[d](25)	1 (6.3)	0.21
STIC or adnexal involvement, *n* (%)	0 (0)	0 (0)	
LVSI present, *n* (%)	10 (41.7)	0 (0)	**0.003**
Not assessable	*1 (2.4)*	*1 (6.3)*	
Histologic subtype, *n* (%)			
Endometrioid	10 (41.7)	15 (93.8)	**0.001**[e]
Mucinous	*1 (4.2)*	*0 (0)*	1.00
Non-endometrioid	14 (58.3)	1 (6.3)	
Serous	*5 (20.8)*	*1* (6.3)	0.373
Carcinosarcoma, serous	*2 (8.3)*	*0 (0)*	0.136[g]
Carcinosarcoma, ambiguous	*2 (8.3)*	*0 (0)*	
Ambiguous	*5 (20.8)*	*0 (0)*	0.071
Histologic subgroups, *n* (%)			
Endometrioid	10 (41.7)	15 (93.8)	**0.001**
Serous-like	14 (58.3)	1 (6.3)	
Histologic grade, *n* (%)			
1&2	5 (20.8)	15 (93.8)	**<0.001**
3	19 (79.2)	1 (6.3)	

NOTE: *P* values in boldface are considered significant ($P < 0.05$). Abbreviations: CN, Copy Number; LOH, Loss of heterozygosity of the *gBRCA1/2* wild-type allele; LVSI, Lymphovascular space invasion. [a]For one case (case 15), no history of salpingo-oophorectomy was reported and they were not removed during hysterectomy. [b]For one case, only an ovariectomy (without salpingectomy) was performed, this was not considered as RRSO. [c]*P* value was calculated over history of RRSO or not. [d]Includes one patient for which the specific hormone treatment was unknown. [e]P value was calculated over endometrioid and nonendometrioid ECs. [g]P value was calculated over carsinosarcoma versus other histotype (independent of epithelial component).

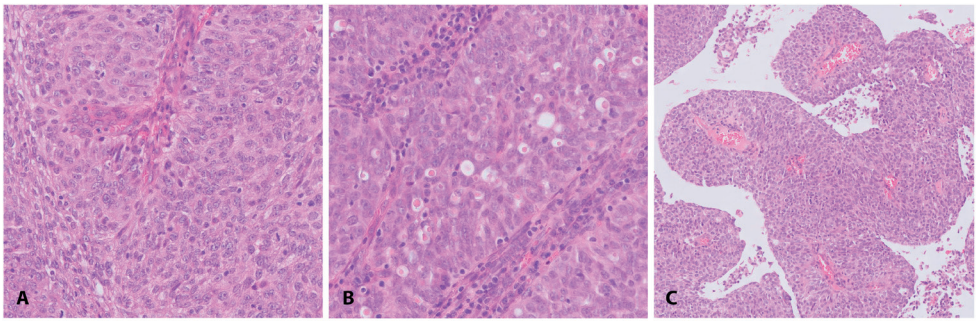

Figure 2: Growth pattern associated with LOH. Hematoxylin and Eosin (H&E) slide of a *gBRCA/*LOHpos endometrial carcinoma classified as ambiguous showing Solid (**A**), pseudoEndometrioid (**B**) and Transitional (**C;** SET)-features.

grade 1, three (7.1%) were grade 2, five (11.9%) were grade 3 and one (2.4%) was a mucinous carcinoma. Sixteen ECs were classified as non-endometrioid (38.1%), of which seven (16.7%) were USC, four (9.5%) were UCS and five (11.9%) were classified as high-grade ambiguous.

Molecular analysis was conducted to stratify for LOH of the *gBRCA1/2* wild-type allele, which succeeded for all but two cases (*n*=40, 95.2%), which were excluded from final analyses (one USC and one EEC grade 1, Supplementary Table S2). The known *gBRCA1/2* mutation was confirmed in all 40 cases included in final analyses. Overall, 60% (24/40) of ECs were *gBRCA/*LOHpos. When stratified for *gBRCA1* and *gBRCA2* mutations, 66.7% (*n*=20/30*)* and 40% (*n*=4/10) showed LOH, respectively (*P*=0.159; Fig. 1; Supplementary Table S2). Plotting the position of the *gBRCA* mutations across the coding DNA sequence for *BRCA1* and *BRCA2* did not show enrichment of mutations in a specific region of the gene [www.cbioportal.org/visualize;[34, 35] Supplementary Fig. S1].

Clinicopathologic, morphologic, and molecular characteristics of g*BRCA* ECs stratified by LOH status

Clinicopathologic characteristics stratified by LOH status are summarized in Table 1 and Fig. 1. Compared with *gBRCA/*LOHneg ECs, *gBRCA/*LOHpos ECs were significantly more often FIGO grade 3 (6.3% vs. 79.2%, *P*<0.001) with non-endometrioid and serous-like histology (both 6.3% vs. 58.3%, *P*=0.001) and more often presented with lymphovascular space invasion (41.7% vs. 0%, *P*=0.003). The 5-year overall survival rate of *gBRCA/*LOHpos ECs was lower (81.3%) compared with *gBRCA/*LOHneg ECs (93.3%, *P*=0.084; Supplementary Fig. S2).

In total, morphologic characteristics were informative for 39 cases (one case was excluded because of neoadjuvant therapy). A higher frequency of “SET features” in *gBRCA/*LOHpos ECs was observed compared with *gBRCA/*LOHneg ECs (52.2% vs. 0%, *P*<0.001; Fig. 2). Other histologic features that were significantly more often observed in *gBRCA/*LOHpos ECs were:

Table 2. Morphologic characteristics stratified by LOH status

	LOHpos (n=23)	LOHneg (n=16)	*P*
Tumor slides assessed/case, median (range)	7 (1-21)	4.5 (1-18)	0.074
Invasion type, *n* (%)			
Destructive	17 (73.9)	4 (25)	**0.004**[a]
Pushing/broad front	2 (8.7)	3 (18.8)	
MELF-type	0 (0)	1 (6.3)	
Adenomyosis-like	0 (0)	3 (18.8)	
No invasion	2 (8.7)	3 (18.8)	
Not analyzable	2 (8.7)	2 (12.5)	
Desmoplastic stromal reaction, *n* (%)[b]	16 (69.6)	5 (31.3)	**0.042**
Predominant growth pattern, *n* (%)			
Glandular	7 (30.4)	16 (100)	**0.001**
"SET-like"	8 (34.8)	0 (0)	
Papillary	4 (17.4)	0 (0)	
Solid	3 (13)	0 (0)	
Mucinous	1 (4.3)	0 (0)	
SET-features (any percentage), *n* (%)			
Solid	15 (65.2)	0 (0)	**<0.001**
Cribriform/pseudoEndometrioid	9 (39.1)	0 (0)	**0.005**
Transitional cell carcinoma-like	5 (21.7)	0 (0)	0.066
SET-features present ≥25%, *n* (%)	12 (52.2)	0 (0)	**<0.001**
Comedo necrosis, *n* (%)	10 (43.5)	2 (12.5)	0.076
Geographic necrosis, *n* (%)[c]	6 (26.1)	0 (0)	**0.03**
Squamous differentiation, *n* (%)	4 (17.4)	6 (37.5)	0.264
Papillary growth, *n* (%)	15 (65.2)	13 (81.3)	0.471

Continue

destructive type of invasion, desmoplastic stromal reaction, non-glandular dominant growth pattern, geographic necrosis, trabecular growth pattern, slit-like spaces, high nuclear grade, tumor giant cells and a higher median mitotic index (Table 2; Supplementary Fig. S3). We did not find a significant difference for intraepithelial TILs or peritumoral lymphocytes assessed on H&E, nor for CD8-positive T-cells (Supplementary Fig. S4). *gBRCA/*LOHpos ECs were more often estrogen receptor negative (45.5% vs. 6.8%, *P*=0.012) and progesterone receptor negative (79.2% vs. 12.5%, *P*<0.001) compared with *gBRCA/*LOHneg ECs.

All ECs were classified into one of the four molecular subgroups previously defined by the TCGA (Fig. 1). All but two *gBRCA/*LOHpos ECs were classified in the *TP53*-mutated subgroup, compared with only one of the *gBRCA/*LOHneg ECs (91.7% vs. 6.3%, *P*<0.001*)*. In line with

Continued

	LOHpos (n=23)	**LOHneg (n=16)**	***P***
Trabecular growth, *n* (%)[d]	8 (34.8)	0 (0)	**0.006**
Jagged lumina, *n* (%)	8 (34.8)	1 (6.3)	0.056
Slit-like spaces, *n* (%)[c]	10 (43.5)	2 (12.5)	**0.04**
Hobnailing, *n* (%)[c]	1 (4.3)	1 (6.3)	1
Nuclear atypia, *n* (%)			
grade 1/2	4 (17.4)	15 (93.8)	**<0.001**
grade 3	19 (82.6)	1 (6.3)	
Tumor giant cells, *n* (%)	11 (47.8)	1 (6.3)	**0.012**
Mitotic index/10 HPF, median (range)	48 (1-197)	12 (1-28)	**<0.001**
Intra-epithelial TILs, *n* (%)	9 (39.1)	6 (37.5)	1
Peritumoral lymphocytes, *n* (%)[c]	16 (69.6)	9 (56.3)	0.323
<10% ER, *n* (%)	11 (45.8)	1 (6.3)	**0.012**
<10% PR, *n* (%)	19 (79.2)	2 (12.5)	**<0.001**
WT-1, *n* (%)			
Negative; ≤1%	17 (70.8)	16 (100)	**0.029**[e]
Heterogeneous; 2-75%	3 (12.5)	0 (0)	
Diffuse positive >75%	4 (16.7)	0 (0)	

NOTE: *P* values in boldface are considerd significant ($P < 0.05$)
Abbreviations: HPF, High power field (0,2 mm^2); LOH, Loss of heterozygosity of the g*BRCA1/2* wild-type allele; MELF, microcystic, elongated and fragmented; SET, **S**olid, psuedo**E**ndometrioid, **T**ransitional; TILs, Tumor infiltrating lymphocytes.
[a]*P* value was calculated over Destructive type of invasion versus other. [b]Not applicable for nine cases which were left out from statistical analysis [five times absence of invasion, four times invasion not analyzable (curettage)], [c]Not evaluable for one case, which was left out from statistical analysis, [d]Not evaluable for two cases, which were left out from statistical analysis. [e]*P* value was calculated over negative nuclear WT-1 expression or positive nuclear WT-1 expression (encompassing both heterogeneous and diffuse positive staining).

this, *gBRCA/*LOHpos ECs more often had a CN-high profile compared to *gBRCA/*LOHneg ECs (95.5% vs.0%, *P*<0.001; Fig. 3). Compared with *gBRCA/*LOHneg ECs, *gBRCA/*LOHpos ECs had significantly more mutations in *TP53* (95.8% vs. 12.5%, *P*<0.001), and fewer mutations in *PTEN* (16.7% vs. 93.8%, *P*<0.001)*, PIK3CA* (16.7% vs. 56.3%, *P*=0.015)*, PIK3R1* (4.2% vs. 43.8%, *P*=0.004)*, ARID1A* (4.2% vs. 43.8%, *P*=0.004) and *CTNNB1* (0% vs. 37.5%, *P*=0.002; Fig. 3). In total, *gBRCA/*LOHpos ECs harbored significantly fewer class 4 or 5 mutations (other than the *gBRCA* mutation) compared with *gBRCA/*LOHneg ECs, no statistically significant difference was observed for TMB (Supplementary Fig. S5A and S5B).

*gBRCA/*LOHpos ECs are not misclassified ovarian cancers

To ensure that the ECs did not represent misclassified OCs, salpingo-oophorectomy specimens were rereviewed to detect (pre)malignant lesions. Of the 40 cases included in our final cohort, 39 (97.5%) cases underwent salpingo-oophorectomy either prior to or at the time of hysterectomy. Women who developed *gBRCA/*LOHpos ECs more often previously underwent a risk-reducing salpingo-oophorectomy (RRSO) compared with women with *gBRCA/*LOHneg ECs (75% vs. 31.3%, *P*=0.009), and the time-interval between the RRSO and EC diagnosis was significantly longer; 73.2 months (range: 35.7-187) versus 12.2 months (range, 4.9-82.9, *P*=0.037). Because this is a historical cohort, sectioning and extensively examining the fimbriated end (SEE-FIM) was not routinely performed. In total, 36 of 39 (92%) adnexal specimens were available for rereview, of which the fimbriae could be (partially) examined for 16 of 22 (72.7%) of *gBRCA/*LOHpos ECs and seven of 14 (50%) of *gBRCA/*LOHneg ECs. None of the ECs showed adnexal involvement and none of the RRSO-specimens showed a serous tubal intraepithelial carcinoma (STIC). In two cases, tubal lesions were detected at the time of hysterectomy; one *TP53* signature (case 6, USC) and one serous tubal intraepithelial lesion (STIL, case 35, EEC grade 1). In addition, according the pathology report of case 31 (EEC grade 1, adnexa not available for review), the tubal lining showed focal epithelial “atypia and p53 positivity”, which could indicate the presence of a p53 signature, STIL, or STIC. Case 31 presented with a simultaneous EEC and endometrioid ovarian cancer, which were considered to be synchronous primary tumors and not to be secondary adnexal involvement of the EC.

A minority of cases displayed WT-1 positivity (*n*=7, 17.5%), of which three (7.5%) displayed heterogenous staining; two USCs, one UCS, and four (10%) displayed diffuse staining; one USC, one UCS, and two ambiguous cases (Table 2). Six out of seven women with a WT-1 positive EC had a history of RRSO, none of which showed a (pre)malignant lesion upon rereview. For all but one (case 5), slides available for rereview included sections through the fimbriae. For case 5 (EC diagnosis 2015), the fimbriae could not be examined because of scarring of the fimbriae as a result of a previous bilateral oophorectomy (1995) performed prior to the salpingectomy (2005), as the complete tubes were submitted for histology review. For the one WT-1 positive EC that did not have a history of RRSO (case 6), both adnexa were removed during therapeutic hysterectomy and a p53 signature was detected in one fallopian tube. When excluding all ECs that displayed WT-1 staining, non-endometroid and serous-like histology remained significantly more common in *gBRCA/*LOHpos ECs compared to *gBRCA/*LOHneg ECs (both *n*=7/17, 41.2% vs. *n*=1/16, 6.3%, *P*=0.039).

gBRCA/LOHpos ECs are not exclusively the result of previous tamoxifen treatment

In total, 19 women had a history of breast cancer, which was not significantly different for women with *gBRCA/*LOHpos ECs compared with *gBRCA/*LOHneg ECs (54.2% vs. 37.5%, *P*=0.349). Although women with *gBRCA/*LOHpos ECs more frequently had a history of

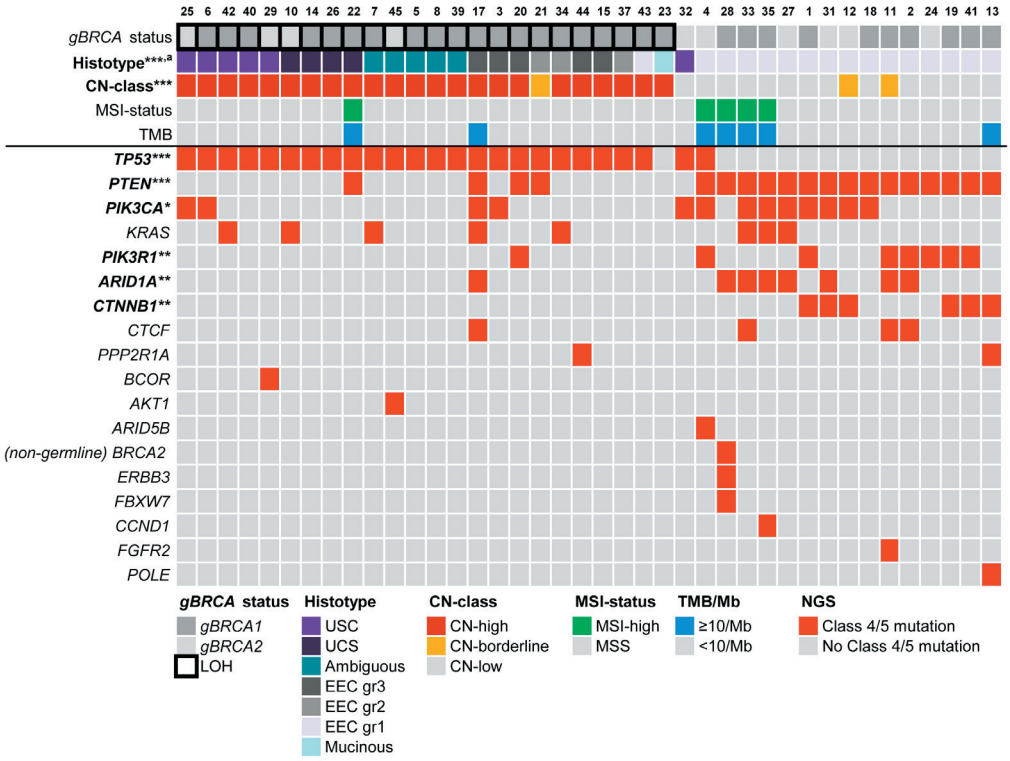

Figure 3: Molecular characteristics of *gBRCA1/2* ECs grouped by LOH status. Case 29 contains a *TP53* mutation NM_000546.5:c.375+5G>T that was considered as likely pathogenic given the predicted effect on splicing in combination with abnormal p53 expression ("null-pattern") in IHC. Bolded cases were considered significant; **P*<0.05, ***P*<0.01, ****P*<0.001. [a]*P* value was calculated over endometrioid and nonendometrioid EC. Abbreviations: CN, Copy number; EEC, endometrioid endometrial carcinoma; gr, grade; LOH, Loss of heterozygosity of the g*BRCA1/2* wild-type allele; UCS, Uterine Carcinosarcoma; USC, Uterine serous carcinoma; MSI-high, Microsatellite instability high; MSS, Microsatellite stable; TMB/Mb, Tumor Mutational Burden / Megabase;

tamoxifen use (including one case for which the type of hormone treatment was not specified), this difference was not significant (*n*=6, 25% vs. *n*=1, 6.3%, *P*=0.210; Table 1;Fig. 1). When excluding all tamoxifen treated individuals, non-endometroid and serous-like histology remained significantly more common in *gBRCA/*LOHpos ECs than in *gBRCA/*LOHneg ECs (both *n*=8/18, 44.4% vs. *n*=1/15, 6.7%, *P*=0.021). Across the entire cohort (both *gBRCA/*LOHpos and *gBRCA/*LOHneg), a history of tamoxifen use was significantly associated with serous-like histology (*n*=6/15, 40% vs. *n*=1/25, 4.0%, *P*=0.007). When only including women who received tamoxifen for 2 or more years (excluding the patient for which hormone treatment duration was unknown), this association was not observed anymore (*n*=3/14, 21.4% vs. *n*=1/25, 4%, *P*=0.123).

Discussion

This is the first study to describe *gBRCA*-associated EC as a distinct entity enriched for high-grade, non-endometrioid tumors with frequent *TP53* mutations and recurring morphologic features. LOH of the wild-type *gBRCA* allele was present in 60% of ECs diagnosed in *gBRCA* carriers**,** and therefore these should be regarded as "*gBRCA*-associated ECs". Importantly, the remaining 40% did not show LOH and therefore are "sporadic ECs" despite the presence of a *gBRCA* mutation. *gBRCA*-associated ECs were histologically high-grade in 79%, which is much more frequent than the 21 to 28% of ECs that would be expected based on population frequencies.[36, 37] We have shown that these tumors are not misclassified OCs, nor exclusively the result of previous tamoxifen treatment. In summary, our findings strongly support that EC is part of the *gBRCA*-associated HBOC syndrome.

There are no strict criteria to which a tumortype should adhere to be considered part of a hereditary cancer syndrome. It is generally accepted, however, that tumors part of a cancer syndrome should occur more frequently and develop at a younger age compared with what would be expected in the general population. A distinct phenotype of tumors in a cancer syndrome is considered to be in support of a causal relationship. Although previous studies show contradictory results about excess risk of EC (all histotypes) for *gBRCA*-carriers,[6-11, 38] most recent studies did find increased risks to develop serous-like ECs, with reported standardized incidence ratios (SIR) ranging from 14.29 to 32.2.[6, 7, 10] These SIRs are comparable to the reported relative risk increase for prostate cancer (up to 20-fold) and pancreatic cancer (up to 10 fold) for *gBRCA2* carriers.[1] The *gBRCA*-associated ECs in our study were diagnosed at a median age of 60.5 years (range 33-74 years). Because these tumors were enriched for EC histotypes that generally occur at an older age (e.g. USC, UCS, EEC grade 3),[36, 37] our data are suggestive that *gBRCA*-associated ECs indeed occur at a younger age compared with their sporadic counterparts, although no definitive conclusions can be drawn without a proper control group. The combination of the excess risk reported in literature and the phenotype of *gBRCA*-associated EC described here strongly support adding (*TP53*-mutated/serous-like) EC to the HBOC syndrome.

Our observation that *gBRCA*-associated (*gBRCA/*LOHpos) EC and sporadic (*gBRCA/*LOHneg) EC show marked histologic and molecular differences supports previous findings that tumors arising in *gBRCA* carriers are not necessarily causally related to the *gBRCA1/*2 mutation.[12] ECs arising in g*BRCA* carriers showed LOH relatively infrequently (67.7% of *gBRCA1* and 40% *gBRCA2*) compared with OCs and breast cancers in *gBRCA1* carriers (93% and 90%) and OCs in *gBRCA2* carriers (84%), but with similar rates to what has been found for breast cancers in *gBRCA2* carriers (54%).[12] This is an important finding, as it emphasizes that tumors that develop in *gBRCA* carriers are not HRD per default, and thereby may not respond to treatments targeting this DNA repair defect. This concept impacts the interpretation of

clinical trials assessing efficacy of PARP inhibitors in tumors with *BRCA1/2* mutations that show LOH relatively infrequently, and suggests that LOH should be included in stratification algorithms for studies assessing therapy efficacy in tumors from *gBRCA*-carriers.[39-42] In fact, LOH status may explain the less pronounced efficacy of olaparib (PARP-inhibitor) for *gBRCA2* carriers with HER2-negative metastatic BC compared to g*BRCA1* carriers as observed in the OlympiAD-trial.[42]

Our observation should increase awareness of the association between *gBRCA* and high-grade EC and may have clinical implications in selecting patients with EC and their families for *gBRCA* testing. Previous studies testing *gBRCA* mutations in unselected EC cohorts resulted in relatively low incidences (0.5% and 0.6%), with only minor increase (1.1% and 3%) when limited to USC and UCS.[43, 44] The morphologic clues described in our study, however, may serve to enrich for *gBRCA* carriers and therefore facilitate cost-effective *gBRCA* testing in patients with EC and their families, a concept that merits further study. Currently, one might consider *gBRCA* testing in patients with high-grade EC with a previous history of breast cancer or a positive family history for *gBRCA*-associated malignancies. Although our study was not aimed to determine the excess risk in women with *gBRCA1/2* mutations to develop EC compared with the general population, our study supports to at least inform *gBRCA* carriers about the association with EC, as the ECs arising in this background are of an unfavorable subtype.

In this study, it was relevant to ascertain that all included carcinomas were of endometrioid and not of tubo-ovarian origin. To exclude misclassification of secondary involvement of the endometrium by HGSOC as EC, we rereviewed all available salpingo-oophorectomy slides with emphasis on putative precursor lesions in the distal fallopian tube. None of the serous-like ECs showed adnexal involvement, supporting the endometrium as primary origin. In addition, we stained all ECs for WT-1, a marker that assists in distinguishing between USC and HGSOC, with reported nuclear positivity rates ranging from 0 to 44% for USCs and 95 to 100% for serous OCs.[45-48] Although cutoff values for WT-1 positivity are unclear, “diffuse WT-1” is generally accepted to be uncommon in EC. WT-1 positivity was observed in seven of 40 ECs (17.5%), of which four (10%) showed diffuse WT-1 positivity. There was no macro- and microscopic indication for a tubo-ovarian carcinoma in the WT-1 positive ECs; nevertheless we cannot completely rule out the theoretical possibility of a “drop-metastasis” from the fallopian tube. The large time interval between the RRSO and EC diagnosis (median 5.7 years, range, 4.0-9.4 years) that was previously performed in six out of seven cases, in combination with the absence of any tubal involvement upon rereview favors primary endometrial origin. For the remainder WT-1-positive EC (case 6), both adnexa were removed during therapeutic hysterectomy, in which a p53 signature was detected unrelated to the EC. We therefore conclude that all cancers in this study, including those that showed WT-1 positivity, are most likely of primary endometrial origin.

Another relevant aspect is a history tamoxifen treatment, as 2 or more years of tamoxifen treatment has been associated with a two- to sevenfold increased risk to develop ECs.[49-52] ECs of tamoxifen-treated individuals are enriched for less favorable histologic subtypes compared to nontreated individuals, especially carcinosarcomas and sarcomas (10.6%-13.8% vs. 2.9%-8.7% respectively), and for ECs with abnormal p53 expression.[49, 53, 54] Tamoxifen is thought to have a stimulatory effect on the endometrium and uterine body while having an anti-estrogenic effect in breast tissue.[49, 55] This stimulatory effect on the endometrium is unlikely the responsible mechanism for the observed association with serous-like ECs as these ECs are mostly hormone independent.[49] A more plausible, alternative hypothesis for this association may be the DNA damaging effect of tamoxifen. It has been suggested that tamoxifen induces the generation of reactive oxygen species (ROS).[56] ROS can cause DNA damage resulting in replicative stress and DNA double-stranded break formation.[1, 57] Previous literature showing the association between tamoxifen use and EC risk did not take *gBRCA* status into account. In our study cohort of *gBRCA* carriers, we found an enrichment for serous-like histology in women previously treated with tamoxifen. We recently showed that BRCA1/2 mediated HR is commonly abrogated in *TP53*-mutated serous-like ECs.[24] Cells that are HRD are more prone to DNA damage due to the error-prone repair of the DNA double-strand breaks caused by ROS and estrogen metabolites.[58] Thereby, we hypothesize that tamoxifen might facilitate (but not initiate) early carcinogenesis of serous-like precursors in *gBRCA1/2* carriers, as these women are already more prone to develop these tumors. This hypothesis should be further studied, as it may alter the balance between advantages and disadvantages of tamoxifen treatment in *gBRCA* carriers.

This study has some limitations. First, we did not include a matched control group of ECs from non-*gBRCA1/2* carriers. Therefore, we are unable to assign sensitivity and specificity of the morphologic features described. Second, we have defined *gBRCA*-associated EC based on LOH status alone and did not interrogate the presence of *BRCA*-related genomic scars to support our definition of *gBRCA*-associated EC. Third, the study design, in which women were included only after providing informed consent and in which ECs were collected both retrospectively (period before providing informed consent) and prospectively (period after providing informed consent), may have led our study cohort to be enriched for ECs with more favorable histotype and survival.

In conclusion, we provide novel evidence that EC is part of the *gBRCA*-related tumor spectrum, with enrichment for EC subtypes associated with unfavorable clinical outcome and distinct histopathologic and molecular features. We also show that tumors with and without LOH of the *gBRCA1/2* wild-type allele are clearly different, thereby providing evidence that establishing LOH status is critical when assessing treatment efficacy of drugs targeting HRD in *BRCA1/2*-mutated tumors.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Supplementary material and methods

H&E slides, pathology reports and FFPE-tumor blocks

Eligible patients from the HEBON-study were linked to PALGA by a HEBON-datamanager. Hysterectomy specimens (with/without curetting) were retrieved from throughout the Netherlands, including the ovarian cancer (OC) and (salpingo-)oophorectomy specimens when applicable. The original pathology reports (pseudonomizyed), haematoxylin and eosin (H&E) slides and a representative formalin fixed paraffin embedded (FFPE)-tumor block were requested via PALGA from pathology laboratories across the Netherlands.

Cancer history and other variables

Data on the following variables were retrieved from the central HEBON-database; *gBRCA1/2* mutation, date of birth, date of death, date of diagnosis of EC, history of breast cancer (BC), history of OC and if applicable, the date of diagnosis, date of risk reducing salpingo-oophorectomy (RRSO). Additionally, we retrieved data on whether a patient retrieved hormone-treatment for BC. As the type of hormone treatment was not specified, we collected more specific information on this from pathology reports and regular questionnaires performed by the HEBON-study (not available for all patients). All data was pseudonymised.

Morphological characterization

Cases were only included for morphological characterization if they did not receive neoadjuvant chemotherapy, as this might affect morphological characteristics. Morphological characteristics were assessed either on the curettage sample (*n*=4) or on the hysterectomy specimen with/without curettage (*n*=35) Slides were first examined at scanning magnification (x40) to determine the dominant pattern of invasion which was categorized as either no invasion (endometrial involvement only), pushing/broad front, destructive, “microcystic, elongated and fragmented”-type of invasion (MELF) or adenomyosis-like.[1] The presence or absence of readily identifiable desmoplastic stromal reaction surrounding the infiltrative glands, squamous differentiation, papillary growth, trabecular growth, jagged lumina, hobnailing, slit-like spaces, tumor giants cells,[2] comedo-necrosis, geographic necrosis,[3] intra-epithelial tumor infiltrating lymphocytes (TILs) and peritumoral lymphocytes were evaluated. The total percentage of **S**olid, cribriform/pseudo**E**ndometrioid and **T**ransitional growth pattern (SET-features) was estimated over all available tumor slides, applying the same criteria as described by Soslow et al.[3] If SET-features were present in >25% of the tumor, it was considered as SET-features being present. Additionally, the predominant growth pattern (glandular, papillary, solid, SET-like or mucinous) was assessed. Nuclear atypia was scored on higher magnification (200x) and was graded as low/grade 1, intermediate/grade 2 or marked/grade 3. Mitotic index was determined per 10 high power files (x400) in enriched areas found upon scanning magnification.

Procedures IHC

One representative formalin-fixed paraffin-embedded (FFPE) tissue block was selected for immunohistochemistry and stains were manually performed on 4 µm whole slides.

Slides were first deparaffinized and rehydrated via graded ethanol series. After blocking the endogenic peroxidase activity (0.3% Methanol/H_2O_2), antigen retrieval was achieved using a microwave oven procedure either in 10 mmol/L Tris-EDTA buffer, pH9.0 (P53, ER, PR, WT-1, CD8) for 10 minutes. Tissue sections were incubated overnight with primary antibodies against P53, ER, PR, WT1 and CD8 at room temperature, followed by incubation with a secondary antibody (Poly-HRP-GAM/R/R; DPV0110HRP; ImmunoLogic) for 30 minutes. DAB+ (K3468, DAKO) was used as chromogen and sections were counterstained with haematoxylin.

Scoring methods

Slides were evaluated by two independent observers and discrepancies were discussed during a consensus meeting.

p53 was categorized as “wild-type” when only focal, weak and heterogeneous staining was present. p53 was considered “abnormal” when either diffuse and strong nuclear staining was observed in >90% of the tumor cell nuclei (“mutant pattern”) or when nuclear staining was completely absent in the presence of “wild-type” staining of stromal cell nuclei/infiltrate as an internal control (“null pattern”).[4]

ER and PR were considered positive when >10% of the tumor cell nuclei showed positive expression. WT-1 was considered positive when nuclear expression was present and samples were divided in the following categories; negative (≤1% nuclear staining), heterogeneous (2-75% nuclear staining) and diffusely positive (>75% nuclear staining).

Besides the assessment of readily identifiable intra-epithelial TILs and peritumoral lymphocytes on H&E slides, we also manually quantified intraepithelial and intrastromal CD8-positive T-lymphocytes, using CD8 immunostained slides. After digitalization, two high power fields (HPF; 0,2 mm^2) were selected in areas enriched for T-lymphocytes in the CD8 immunostain, detected upon scanning magnifications (x20). One HPF was selected in the center of the tumor and one HPF was selected in the invasive margin of the tumor when present. Estimated tumor percentage was at least 70% if possible.

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Supplementary tables and figures

Supplementary Table S1. Clinicopathologic characteristics of the complete cohort

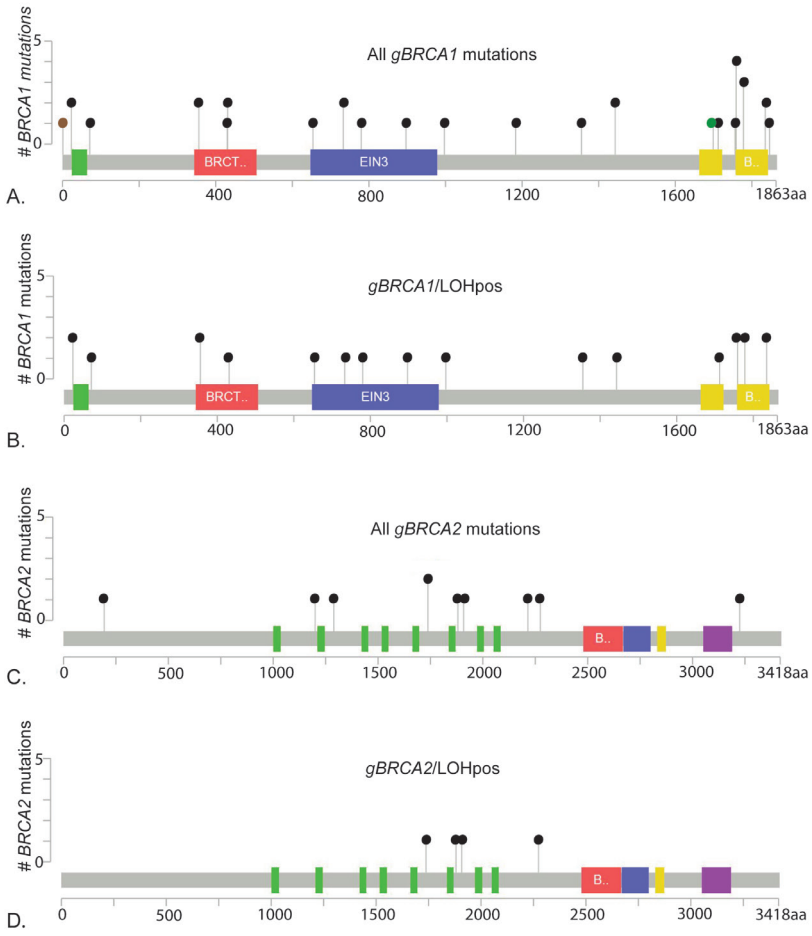
	Cases (n=42)
Germline *BRCA1/2* mutation, No. (%)	
gBRCA1	32 (76.2)
gBRCA2	10 (23.8)
Age at diagnoses, median range, years	58.5 (33-74)
Histologic subtype, No. (%)	
Endometrioid	26 (61.9)
Mucinous	*1 (2.4)*
Non-endometrioid	16 (38.1)
Serous	*7 (16.7)*
Carcinosarcoma, serous	*2 (4.8)*
Carcinosarcoma, ambiguous	*2 (4.8)*
Ambiguous	*5 (11.9)*
FIGO 2009, No. (%)	
I/II[a]	35 (83.3)
III/IV	7 (16.7)
LVSI, No. (%)	10 (23.8)
Not assessable	*2 (4.8)*
Salpingo-oophorectomy, No. (%)	
History of RRSO	23 (54.8)
During hysterectomy	14 (33.3)
RRSO at time of EC diagnoses	2 (4.8)
Therapeutic	2 (4.8)
In situ	1 (2.4)
Ovarian/tubal involvement EC, No. (%)	0 (0)[a]
Precursor lesions fallopian tube, No. (%)	3 (7.1)
"Atypia"[b]	1 (2.4)
P53 signature	1 (2.4)
STIL	1 (2.4)
STIC	0 (0)
History of ovarian Cancer, No. (%)	1 (2.4)[a]
History of Breast Cancer, No. (%)	20 (47.6)
Neoadjuvant treatment, No. (%)	1 (2.4)

[a]One case (case 31) presented with a bilateral endometrioid OC, shortly after which a endometrioid EC was diagnosed in a subsequent curettage. The tumors were considered as two primary tumors (EC FIGO 2009 stage I). The OC was not considered as a history of OC nor as adnexal involvement of the EC. [b]The fallopion tube showing "atypia" was not available for revision. Bolded P values are considered significant (P<0.05). Abbreviations: EC, Endometrial Carcinoma; STIC, Serous Tubal Intraepithelial Carcinoma; STIL, Serous Tubal Intraepithelial Lesion

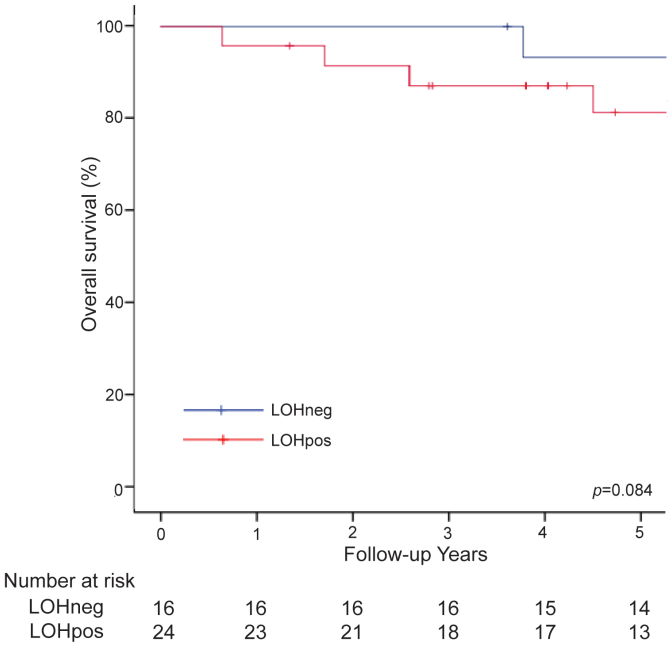
Supplementary Table S2. Germline *BRCA1/2* mutations and variant allele frequencies detected in the FFPE-isolated tumor DNA

Case ID	Tumor (%)	Germline mutation	c.DNA change[a]	Amino Acid change[b]	VAF (%)	LOH	LOH type
44	80	*BRCA1*	c.66dup	p.(Glu23Argfs*18)	72	yes	copy loss
15	90	*BRCA1*	c.68_69del	p.(Glu23Valfs*17)	87	yes	copy neutral
43	40	*BRCA1*	c.213-12A>G	p.?	74	yes	copy neutral
23	80	*BRCA1*	c.1066C>T	p.(Gln356*)	86	yes	copy neutral
8	90	*BRCA1*	c.1066C>T	p.(Gln356*)	73	yes	copy loss
35	70	*BRCA1*	c.1287dup	p.(Asp430Argfs*6)	49	no	none
20	80	*BRCA1*	c.1292dup	p.(Leu431Phefs*5)	77	yes	copy loss
19	70	*BRCA1*	c.1292dup	p.(Leu431Phefs*5)	47	no	none
22	90	*BRCA1*	c.1961del	p.(Lys654Serfs*47)	82	yes	copy neutral
21	70	*BRCA1*	c.2197_2201del	p.(Glu733Thrfs*5)	62	yes	copy neutral
11	80	*BRCA1*	c.2197_2201del	p.(Glu733Thrfs*5)	47	no	none
34[c]	80	*BRCA1*	c.2338C>T	p.(Gln780*)	75	yes	copy neutral
6	90	*BRCA1*	c.2685_2686del	p.(Pro897Lysfs*5)	89	yes	copy loss
26	90	*BRCA1*	c.2989_2990dup	p.(Asn997Lysfs*4)	85	yes	copy neutral
1	80	*BRCA1*	c.3549_3550delinsT	p.(Lys1183Asnfs*27)	40	no	none
14	80	*BRCA1*	c.4065_4068del	p.(Asn1355Lysfs*10)	86	yes	copy neutral
2	60	*BRCA1*	c.4327C>T	p.(Arg1443*)	44	no	none
42	90	*BRCA1*	c.4327C>T	p. (Arg1443Ter)	98	yes	copy neutral
13	80	*BRCA1*	c.5095C>T	p.(Arg1699Trp)	44	no	none
37	30	*BRCA1*	c.5136G>A	p.(Trp1712*)	59	yes	copy neutral
17	70	*BRCA1*	c.5277+1G>A	p.?	71	yes	copy neutral
28	80	*BRCA1*	c.5277+1G>A	p.?	48	no	none
5	80	*BRCA1*	c.5277+1G>A	p.?	84	yes	copy neutral
33	80	*BRCA1*	c.5277+1G>A	p.?	26	no	none
40	80	*BRCA1*	c.5503_5564del	p.(Arg1835Thrfs*24)	77	yes	copy loss
7	90	*BRCA1*	c.5503_5564del	p.(Arg1835Thrfs*24)	89	yes	copy neutral
18	50	*BRCA1*	c.5536C>T	p.(Gln1846*)	48	no	none
39	80	*BRCA1*	c.5333-36_5406+400del	p.?	n/a	yes	two copy loss
41	80	*BRCA1*	c.5333-36_5406+400del	p.?	n/a	no	none
3	90	*BRCA1*	c.5333-36_5406+400del	p.?	n/a	yes	two copy loss
4	80	*BRCA2*	c.582G>A	p.(Trp194*)	50	no	none
32	30	*BRCA2*	c.3599_3600del	p.(Cys1200*)	46	no	none
27	70	*BRCA2*	c.3865_3868del	p.(Lys1289Alafs*3)	47	no	none
12	85	*BRCA2*	c.5213_5216del	p.(Thr1738Ilefs*2)	39	no	none
10	90	*BRCA2*	c.5213_5216del	p.(Thr1738Ilefs*2)	85	yes	copy neutral
45	90	*BRCA2*	c.5637_5640delGAAT	p.Lys1881Glnfs*27	88	yes	copy loss
29	70	*BRCA2*	c.5722_5723del	p.(Leu1908Argfs*2)	62	yes	copy loss
31	80	*BRCA2*	c.6644_6647del	p.(Tyr2215Serfs*13)	46	no	none
25	60	*BRCA2*	c.6816_6817del	p.(Gly2274Argfs*18)	63	yes	copy loss
24	70	*BRCA2*	c.9672dup	p.(Tyr3225Ilefs*30)	46	no	none
38[d]	80	*BRCA1*	c.(?_-232)_(80+1_81-1)del	p.?	n/a	n/a	n/a
16[e]	25	*BRCA1*	c.5266dup	p.(Gln1756Profs*74)	n/a	n/a	n/a

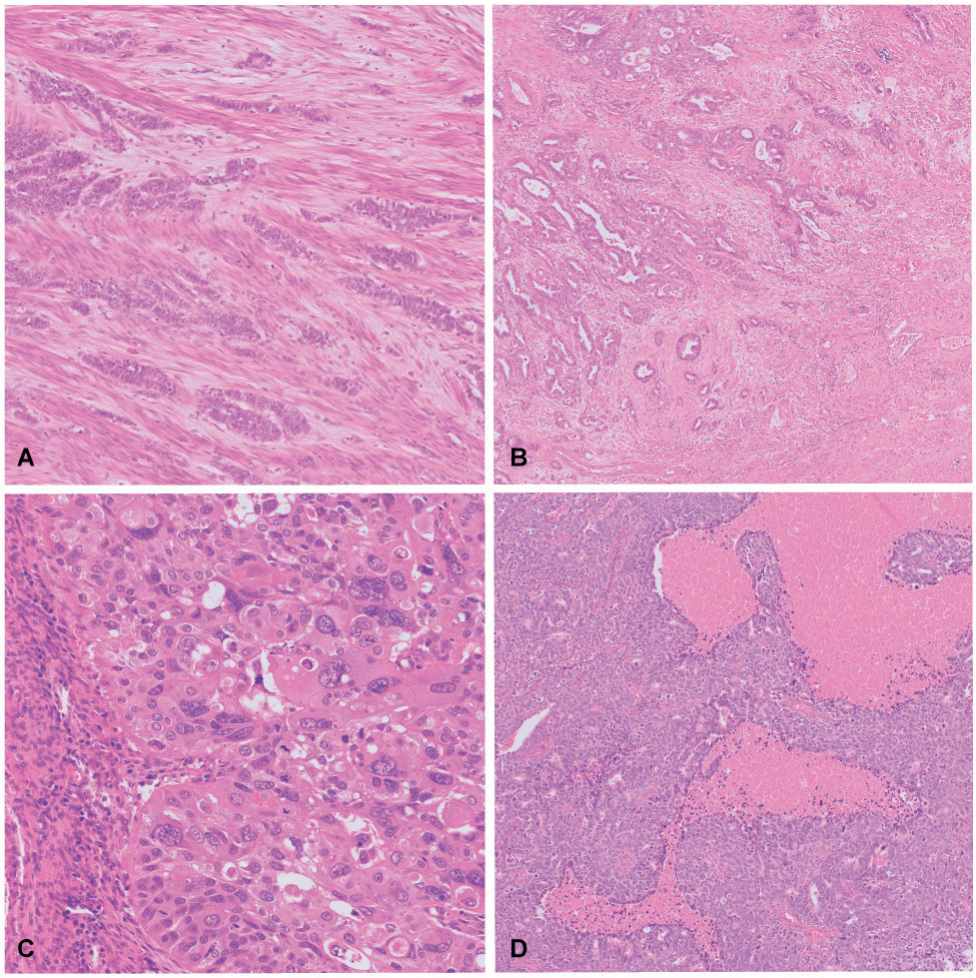
58.3% of the observed LOH (n=14) was copy neutral and 41.7% of LOH (n=10) was associated with copy loss. No somatic loss of function mutations were detected as "second hit". [a]Reference sequences used for mutation annotation; NM_007294.3 for *BRCA1*, NM_000059.3 for *BRCA2*. [b]Reference sequence used for protein annotation; NP_009225.1 for *BRCA1*, NP_000059.3 for *BRCA2*. [c]The patient was a known g*BRCA1* mutation carrier, however, the exact inherited mutation was not specified in the database. [d]The inherited *gBRCA1* exon1/2 deletion could not be confirmed in the tumor DNA and can likely be explained by the limited sensitivity of NGS to detect large exon deletions. Since LOH status could not be assessed, the case was left out from final analyses. [e]Molecular analyses failed due to poor DNA quality. Abbreviations: LOH, Loss of heterozygosity of the *gBRCA1/2* wild-type allele; VAF, Variant allele frequency



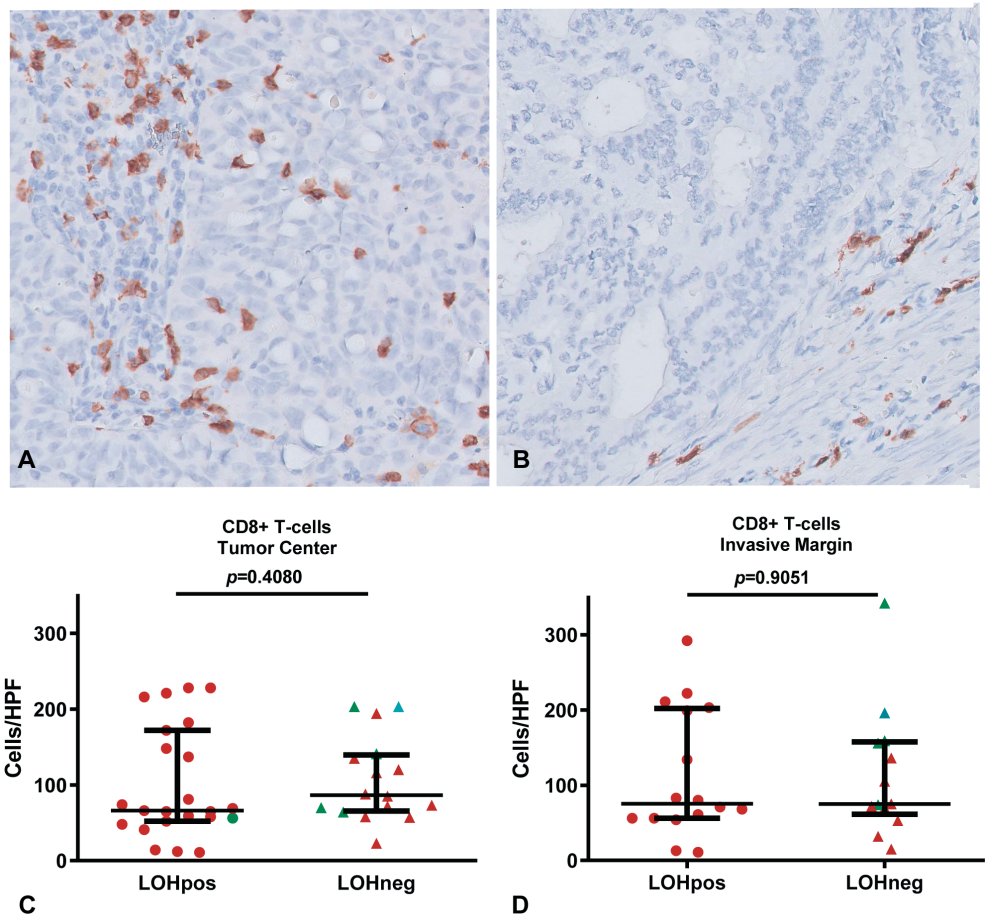
Supplementary Fig. S1: Position and frequency of *gBRCA1/2* mutations across the coding DNA sequence. Distribution and frequency of *gBRCA1* mutations in the study cohort **(A)** and for *gBRCA1/* LOHpos cases only **(B)**. Distribution and frequency of *gBRCA2* mutations in the study cohort **(C)** and for *gBRCA2/*LOHpos cases only **(D)**. Black dot, Truncating variant; Green dot, Missense variant; Brown dot, no protein.



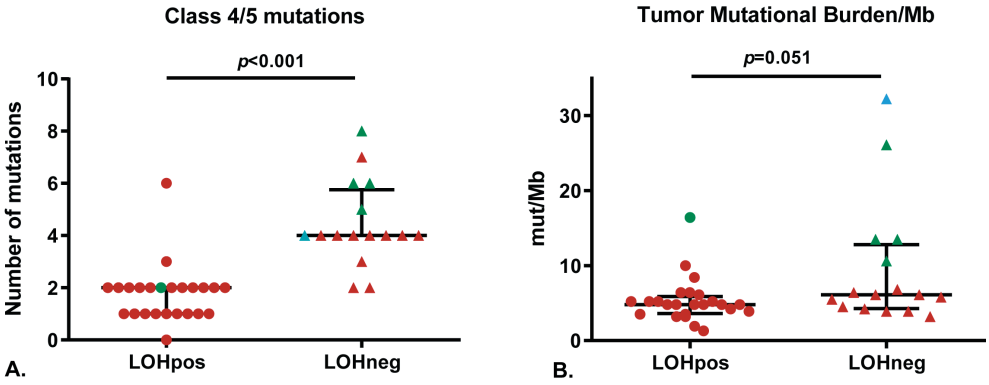
Supplementary Fig. S2: Overall survival of women with *gBRCA*/LOHpos ECs (*n*=24) and *gBRCA*/LOHneg ECs (*n*=16). The cross indicates a censoring event. Of the *gBRCA*/LOHpos ECs, five (20.8%) were diagnosed preceding to the date of enrolment (mean 4.8 years, SD: 4.3) and 19 (79.2%) were diagnosed after the date of enrolment or on the date of enrolment (LUMC cases) (mean 3.7 years, SD: 3.7). Of the g*BRCA*/LOHneg ECs, 14 (87.5%) were diagnosed preceding to the date of enrolment (mean 4.8 years, SD: 2.4) and one (6.3%) was diagnosed after the date of enrolment (mean 2.6 years). For one (6.3%) case, the date of enrolment was missing. Abbreviations: EC, endometrial carcinoma; LOH, loss of heterozygosity of the *gBRCA1/2* wild-type allele.



Supplementary Fig. S3: Examples of morphological characteristics associated with g*BRCA*/LOHpos ECs. A: Trabecular growth and desmoplastic stromal reaction, **B**: Destructive type of invasion, **C:** Tumor giant cells and high nuclear grade, **D**: Geographic necrosis.



Supplementary Fig. S4: CD8-positive T-cell infiltrate stratified by loss of heterozygosity status. Example of CD8-IHC in the center of the tumor (**A**) and at the invasive margin (**B**). The total number of CD8+ T-cells per High Power Field (0,2 mm^2) per case (both intra-epithelial and stromal) compared between *gBRCA*/LOHpos ECs and *gBRCA*/LOHneg ECs are shown for the center of the tumor (*n*=39) (**C**) and at the invasive margin (*n*=29) (**D**). Whiskers represent the interquartile range and the median values are indicated by the horizontal line. Blue dots/triangles indicate *POLE*-mutated ECs, green dots/triangles indicate MSI-high ECs. No significant difference was observed for the number of CD8+ T-cells between *gBRCA*/LOHpos and *gBRCA*/LOHneg ECs, neither was a difference observed when comparing only intra-epithelial CD8+ T-cells or only intrastromal CD8+ T-cells. When excluding the MSI-high (*n*=5) and *POLE*-mutated tumors (*n*=1) from analyses, neither a significant difference in CD8+ T-cell infiltration was detected. Abbreviations: IHC, Immunohistochemistry; LOH, Loss of Heterozygosity of the *gBRCA* wild-type allele.



Supplementary Fig. S5: Class 4/5 mutations and tumor mutational burden stratified by loss of heterozygosity status. A. *gBRCA/*LOHpos ECs harbored significantly fewer class 4/5 mutations (other than the *gBRCA* mutation) compared to *gBRCA/*LOHneg ECs (median 2, range: 0-6 versus 4, range: 2-8, $p<0.001$). The difference remained significant when excluding all *POLE*-mutated ECs and MSI-high ECs. **B.** No statistically significant difference was observed for Tumor Mutational Burden (TMB) when comparing *gBRCA*/LOHpos ECs with *gBRCA/*LOHneg ECs (median 4.8 mut/MB: range 1.3-16.4, versus 6.1 mut/Mb; range: 3.2-32.2, $p=051$). The TMB remained non-significant when excluding all *POLE*-mutated ECs and MSI-high ECs ($p=0.4943$). The whiskers represent the interquartile range and the median values are indicated by the horizontal line. Blue dots/triangles indicate *POLE*-mutated ECs. Green dots/triangles indicate MSI-high ECs. Abbreviations: LOH, Loss of heterozygosity of the *gBRCA* wild-type allele; Mb, Megabase.

Chapter 5

Endometrial cancer risk in women with germline *BRCA1* or *BRCA2* mutations: multicenter cohort study

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Abstract

Background

Endometrial cancer (EC) risk in BReast CAncer gene 1/2 (*BRCA1/2)* mutation carriers is uncertain, therefore we assessed this in a large Dutch nationwide cohort study.

Methods

5,980 *BRCA1/2* (3,788 *BRCA1,* 2,151 *BRCA2,* 41 both *BRCA1/BRCA2)* and 8,451 non-*BRCA1/2* mutation carriers were selected from the HEBON-cohort. Follow-up started at date of nationwide PALGA coverage (January 1, 1989) or at the age of 25 years (whichever came last), and ended at date of EC diagnosis, last follow-up or death (whichever came first). EC risk in *BRCA1/2* mutation carriers was compared to: 1) general population, estimating standardized incidence ratios (SIRs) based on Dutch population-based incidence rates; and 2) non-*BRCA1/2* mutation carriers, using Cox-regression analyses, expressed as hazard ratio (HR). Statistical tests were two-sided.

Results

Fifty-eight *BRCA1/2* and 33 non-*BRCA1/2* mutation carriers developed EC over 119,296 and 160,841 person-years, respectively (SIR = 2.83, 95% confidence interval (CI) = 2.18-3.65; and HR = 2.37, 95% CI = 1.53-3.69, respectively). *BRCA1* mutation carriers showed increased risks for EC overall (SIR = 3.51, 95% CI = 2.61-4.72; HR = 2.91, 95% CI = 1.83-4.66), serous-like EC (SIR: 12.64, 95% CI = 7.62-20.96; HR = 10.48, 95% CI = 2.95-37.20), endometrioid EC (SIR = 2.63, 95% CI = 1.80-3.83; HR = 2.01, 95% CI = 1.18-3.45) and *TP53*-mutated EC (HR = 15.71, 95% CI = 4.62-53.40). For *BRCA2* mutation carriers, overall (SIR = 1.70, 95% CI = 1.01-2.87), and serous-like EC risks (SIR = 5.11, 95% CI = 1.92-13.63) were increased when compared to the general population. Absolute risks by 75 years remained low (overall EC = 3.0%; serous-like EC = 1.1%).

Conclusions

BRCA1/2 mutation carriers have a 2- to 3-fold increased risk for EC, with highest risk observed for the rare subgroups of serous-like and p53-abnormal EC in *BRCA1* mutation carriers.

Introduction

Women with a pathogenic germline mutation in the BReast CAncer genes (*BRCA1* and *BRCA2*) have strongly increased breast carcinoma (BC) and tubo-ovarian carcinoma (OC) risks. Penetrance studies of *BRCA1/*2 mutations report cumulative BC risks at age of 70 years of 50-59% for female *BRCA1* mutation carriers and 42-51% for female *BRCA2* mutation carriers, together with OC risks of 34-45% and 13-21%, respectively.[1]

Whether *BRCA1/2* mutations also confer elevated life-time risk for endometrial cancer (including uterine sarcomas; EC) is unclear. Studies have reported an increased EC risk in *BRCA1/2* mutation carriers compared to country-specific incidence rates (standardized incidence ratios (SIRs), range = 1.9 to 5.3),[2-4] but others found no clearly increased EC risk,[5-7] or found that increased risk was restricted to a rare but aggressive subgroup of EC, ECs with serous-like histology (e.g. uterine serous carcinomas, carcinosarcomas; SIR range = 14.8 to 32.2; **Supplementary Table 1**).[8-12] Furthermore, it has been suggested that the apparent increase in EC risk is not related to the *BRCA1/2* mutation, but to previous BC related tamoxifen-treatment.[2, 3] These conflicting data in previous cohort studies can be attributed to a limited number of ECs (*n*=2-17) as a result of small cohort sizes (315-4,456), low mean/median age at enrolment with limited follow-up periods, or absence of outcome validation (*n*=5*)*.[2-9, 13]

More recently, studies have suggested that in addition to EC of serous-like histology, a larger group of p53-abnormal ECs (one of the four molecularly-defined subgroups),[10, 14, 15] are more common in *BRCA1/2* mutation carriers. EC risks for this molecular subgroup have not yet been determined.

Accurate estimation of EC risk in *BRCA1/2* mutation carriers is important to counselling and clinical management. Therefore, the aim of this study was to confirm and quantify the risk of EC in a large cohort of *BRCA1/2* mutation carriers compared to both the general Dutch population and to non-*BRCA1/2* mutation carriers.

Methods

Study population

BRCA1/2 mutation carriers (*n*=6,072) were selected from the "HEreditary Breast and Ovarian cancer study, the Netherlands (HEBON cohort study)", an ongoing nationwide cohort study of hereditary breast and ovarian cancer (HBOC) families in the Netherlands (for details see[16, 17] and **Supplementary methods**). The HEBON cohort study has been approved by medical

ethical committees of all participating centers. The current study was approved by the Institutional Review Board of the Netherlands Cancer Institute.

BRCA1/2 mutation carriers

Women with a class 5/pathogenic or class 4/likely pathogenic *BRCA1* or *BRCA2* mutation were eligible.[18] The initial cohort consisted of 6,072 *BRCA1/2* mutation carriers, of whom 3,716 provided written informed consent allowing connection to disease registries, 876 who died before they could be invited to join the HEBON cohort, and 1,480 whose connection to disease registries (see below) was approved by the medical ethical committee because they did not respond to a request to participate and did not actively deny the request after three invitations to do so (**Figure 1**).

Dutch population-based cancer incidence rates (comparison group 1)

Age, calendar year- and country-specific EC incidence rates (crude rates/100,000 person-years, stratified by age and calendar time) were obtained from the Netherlands Cancer Registry (NCR) for the calendar years 1989-2015 (May 2020). All tumors with an *International Classification of Diseases for Oncology, Third edition, First revision (ICD-O-3.1;* http://codes.iarc.fr/*)* topographical code of either C54 (Corpus Uteri) and C55 (Uterus, NOS) were included.

In addition, age, calendar year and country-specific EC incidence rates were obtained from the NCR for the following five histologic subgroups based on the morphological ICD-0-3.1 codes: 1) Endometrioid (including mucinous), 2) Serous-like (e.g. uterine serous carcinoma, carcinosarcoma, mixed carcinomas), 3) Clear Cell Carcinoma, 4) Sarcoma and 5) Other (e.g. neuroendocrine carcinoma), see **Supplementary Table 2**.

Non-*BRCA1/2* mutation carriers (comparison group 2)

Non-*BRCA1/2* mutation carriers (*n*=8,575, within-cohort comparison group) were also selected from the HEBON cohort (**Figure 1**). Women were eligible if they: 1) were a member of a family with a proven likely pathogenic or pathogenic *BRCA1* or *BRCA2* mutation (not including variants of unknown significance), and 2) tested negative for this likely pathogenic or pathogenic *BRCA1/2* mutation.

Pathology review and assessment of histologic- and molecular subgroup

To confirm endometrial origin and define histologic and molecular subgroups, pathology reports, H&E-slides and FFPE tumor tissue blocks of ECs of both *BRCA1/2* and non-*BRCA1/2*-mutation carriers were collected via PALGA and centrally revised by at least one expert gynaecopathologist. If pathology review was not possible, histologic subtype and grade were extracted from pathology reports or based on the morphological ICD-0-3.1 code. Although some cases of rare uterine

sarcoma's were included in the study, for simplicity the term ' endometrial cancer' (EC) is used throughout the manuscript.

After review, ECs were classified into the same five histologic subgroups as described for comparison group 1, and were molecular classified similarly to as what has been previously described; p53-abnormal or "other" (including *POLE*-mutant, mismatch repair (MMR)-deficient and no surrogate marker profile group (NSMP)).[10, 14] For cases that were not available for review, assignment to molecular groups was based on histology (see the **Supplementary Methods**).

Data collection and data handling

Pseudonymized data were retrieved for *BRCA1/2* and non-*BRCA1/2* mutation carriers from the central HEBON database. With regular input from the NCR, the Dutch Pathology Registry (PALGA)[19] and the municipal administration (BRP), the HEBON cohort study gathers data centrally, including cancer incidence, date of cancer diagnosis, RRSO, and date of death. In the case of BC, these data also include hormone treatment (HT, type and duration not specified). PALGA is a nationwide archive containing excerpts of all histo- and cytopathology reports in the Netherlands since 1991.[19] For details see the **Supplementary Methods**.

Statistical analysis

Period at risk for EC. Both *BRCA1/2* and non-*BRCA1/2* mutation carriers were assigned a starting date for follow-up based on either nationwide PALGA coverage (Jan 1, 1989) or the date at which women are considered to be at risk for EC (≥25 years of age), whichever was later. Follow-up ended on the date of EC diagnosis (ICD-0-3 topographical code C54/C55), date of death, or date of end of follow-up (January 1, 2016 for *BRCA1/2* mutation carriers who provided informed consent; January 1, 2012 for all others), whichever was earlier. Women were excluded from analyses if an EC occurred before Jan 1, 1989 or before the age of 25 (**Supplementary Figure 1**). We were not informed about the extent of OC surgery and RRSO (whether or not this included a hysterectomy), and therefore the date of OC/RRSO was not used as censoring event.

Comparison 1: BRCA1/2 mutation carriers versus Dutch country-specific incidence rates. For the *BRCA1/2* mutation carrier cohort, expected EC incidence was estimated based on calculated person-time at risk, stratified by age, and calendar-time. SIRs were calculated by dividing observed ECs by expected ECs, and 95% confidence intervals (CIs) and 2-sided *p* values were estimated assuming a Poisson distribution. SIRs were also stratified for histologic subgroup after pathology review, mutation type (*BRCA1/BRCA2),* and attained age.

Comparison 2: BRCA1/2 mutation carriers versus non-BRCA1/2 mutation carriers. Differences in EC occurrence between *BRCA1/2* and non-*BRCA1/2* mutation carriers were analysed using

Cox regression and expressed as Hazard Ratio (HR), with accompanying 95% CI adjusted for age. HRs were also calculated after stratification for mutation type and for histologic and molecular subgroup following pathology review. Women carrying both a *BRCA1*- and *BRCA2* mutation (*n*=41, no ECs) were analysed in both the *BRCA1*- and *BRCA2*-mutation carrier group.

The following sensitivity analyses were performed. First, to exclude potential confounding by tamoxifen use for BC, two separate sensitivity analyses were performed; for the first, patients were censored at date of (first) BC diagnosis that led to HT (type and duration not specified), and for the second, patients were censored at date of (first) BC diagnosis (both analyses included cases with DCIS). Second, to exclude testing bias (testing *BRCA1/2* mutation because of EC diagnosis), person-years at risk began on the date of the *BRCA1/2* DNA test. Third, to minimize potential bias due to unequal observation periods, the end date for follow-up was set to January 1, 2012 for all *BRCA1/2* and non-*BRCA1/2* mutation carriers.

Baseline characteristics between *BRCA1/2* and non-*BRCA1/2* mutation carriers were compared using the Chi-square test (categorical variables) and the Mann-Whitney U-test (numerical variables). Median follow-up time was estimated using the Reverse Kaplan-Meier Method. The cumulative risk of developing EC, and EC of serous-like and endometrioid histology up to age of 75 years was estimated using competing risk analyses.

A *p* value of <0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS version 23.0 and STATA Statistical Software version 14.1 (College Station, TX: StataCorp LP).

Results

Cohort characteristics

A total of 5,980 *BRCA1/2* and 8,451 non-*BRCA1/2* mutation carriers were included (**Figure 1**). Cohort characteristics and follow-up details are described in **Table 1**. The total number of person-years at risk and events (overall and stratified by histologic subgroup) per 5-year age category are shown in **Supplementary Table 3**. Details on EC characteristics and pathology review are described in **Supplementary Table 4-6**.

EC Risk in *BRCA1/2* mutation carriers compared to the Dutch country-specific incidence rates

Overall EC risk in *BRCA1/2* mutation carriers was increased 2.83-fold (95% CI = 2.18-3.65) compared to Dutch EC incidence rates (*BRCA1,* SIR = 3.51, 95% CI = 2.61-4.72; *BRCA2,* SIR = 1.70, 95% CI =1.01-2.87), **Table 2**.

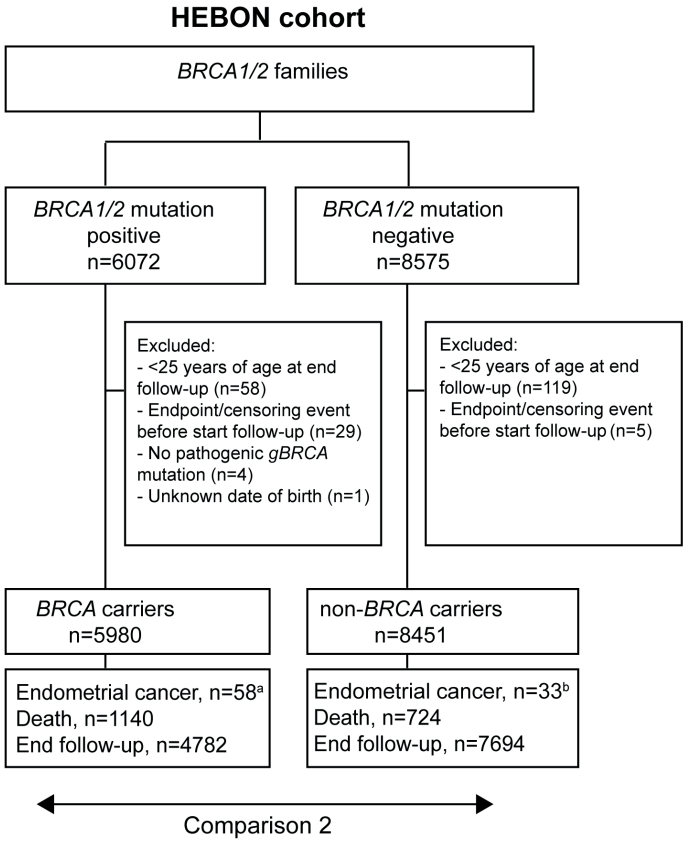

Figure 1. Schematic overview of the *BRCA1/2* mutation carrier cohort and the non-*BRCA1/2* mutation carrier cohort. a. Four events were excluded as they occurred outside of the observation period: two before the start of follow-up (January 1, 1989) and two after the end of follow-up (once on January 1, 2012 and once on January 1, 2016). **b.** Seven events were excluded: five events occurred after the observation period ended (January 1, 2012) and two events were excluded because the tumors were considered of non-endometrial origin after pathology review.

When ECs were stratified by histologic subgroup, *BRCA1/2* mutation carriers were at increased risk for endometrioid EC (SIR = 2.08, 95% CI = 1.49-2.89) and for EC of serous-like histology (SIR = 9.77, 95% CI = 6.23-15.31), **Table 2**. *BRCA1* mutation carriers displayed greater risk for endometrioid EC (SIR = 2.63, 95% CI = 1.80-3.83), and especially for EC of serous-like histology (SIR = 12.64, 95% CI = 7.62-20.96). Risk for EC of serous-like histology in *BRCA2* mutation carriers was lower (SIR = 5.11, 95% CI 1.92-13.63).

Overall EC risks were highest in the youngest age category of 25-40 years (SIR = 9.84, 95% CI = 2.68-25.20), although confidence intervals were broad and the majority of events occurred in older age categories, **Table 3**. For EC of serous-like histology, the highest risks were observed in the age category 60-80 years (SIR = 11.27, 95% CI = 5.99-19.27).

Table 1. Demographic characteristics of *BRCA1/2* mutation carriers and non-*BRCA1/2* mutation carriers

Demographic characteristics	*BRCA1/2* carriers	non-*BRCA1/2* carriers
Total, No. (%)	5980 (100)	8451 (100)[a]
BRCA1 mutation, No. (%)	3788 (63.3)	0 (0)
BRCA2 mutation, No. (%)	2151 (36.0)	0 (0)
BRCA1 and *BRCA2* mutation, No. (%)	41 (0.7)	0 (0)
Median age at start of follow-up (years, IQR)	27.4 (25.0-37.8)	28.0 (25.0-38.2)
<40 years, No. (%)	4737 (79.2)	6657 (78.8)
40-49 years, No. (%)	775 (13.0)	1197 (14.2)
50-59 years, No. (%)	321 (5.4)	395 (4.7)
≥60 years, No. (%)	147 (2.5)	202 (2.4)
Median age at end of follow-up (years, IQR)	51.9 (42.5-61.6)	50.7 (42.1-60.7)
Median observation period (years, IQR)	22.5 (15.2-27.0)	23.0 (16.4-23.0)
Total person-years at Risk (SD)	119296 (7.1)	160841 (5.8)
Of which post *BRCA* DNA test (SD)[b]	56579 (6.3)	48044 (5.1)
Ovarian Cancer History, No. (%)[c]	716 (12.0)	267 (3.2)
Before start observation period, No. (%)	34 (0.6)	19 (0.2)
During observation period, No. (%)	682 (11.4)	248 (2.9)
Endometrial Cancer and simultaneous/history of Ovarian Cancer[d]	5 (0.08)	5 (0.06)
Breast Cancer History, No. (%)[e,f]	2762 (46.2)	2788 (33.0)
Before start observation period, No. (%)	291 (4.9)	140 (1.7)
During observation period, No. (%)	2471 (41.3)	2648 (31.3)
Hormone Treatment		
HT-BC, No. (%)	755 (12.6)	1155 (13.7)
Before start follow-up, No. (%)	14 (0.2)	4 (0.0)
During follow-up, No. (%)	741 (12.4)	1151 (13.6)
HT-BC unknown, No. (%)[f]	209 (3.5)	127 (1.5)
Before start follow-up, No. (%)	72 (1.2)	39 (0.5)
During follow-up, No. (%)	137 (2.3)	88 (1.0)
RRSO History, No. (%)[g]	3619 (60.5)	695 (8.2)
Before start follow-up, No. (%)	19 (0.3)	25 (0.3)
During follow-up, No. (%)	3600 (60.2)	670 (7.9)
History RRSO unknown, No. (%)	119 (2.0)	4324 (51.2)

[a]Includes 96 women with a *BRCA* variant of unknown significance, of whom two developed a endometrial carcinoma (none carried the (likely) pathogenic familial variant). Abbreviations: BC: Breast Cancer, HT: Hormone Treatment, RRSO: Risk-Reducing Salpingo-oophorectomy

[b]Post *BRCA* DNA test; Person-years from date *BRCA1/2*-DNA test until end of follow-up. The date of *BRCA1/2*-mutation test was missing for 1,682 (28.1%) carriers and 1,214 (14.4%) non-carriers. For these women, the date of *BRCA1/2* DNA test was considered to be January 1, 1995. *BRCA1/2* DNA tests were performed from 1995 until 2012 (median year 2007).

[c]Date of OC diagnosis unknown for two non-*BRCA* mutation carriers.

[d]For details, see **Supplementary Table 5 and 6**.

[e]DCIS was considered as BC. Considered the first BC if women had a history of more than one BC.

[f]Date of diagnosis unknown for one BC in the *BRCA* mutation carrier group.

[g]Includes adnexextirpation for reasons other than RRSO, e.g. during hysterectomy or for OC.

Table 2. Observed and expected endometrial cancer rates in *BRCA1/2* mutation carriers, compared to the Dutch country-specific incidence rates

	***BRCA1/2* carriers**	**Dutch population**		
EC subgroups	**Observed**	**Expected**	**SIR (95% CI)**	***P*[a]**
All endometrial cancers	58	20.53	2.83 (2.18-3.65)	<0.001
BRCA1	44	12.53	3.51 (2.61-4.72)	<0.001
BRCA2	14	8.23	1.70 (1.01-2.87)	0.04
Endometrioid	35	16.85	2.08 (1.49-2.89)	<0.001
BRCA1	27	10.27	2.63 (1.80-3.83)	<0.001
BRCA2	8	6.77	1.18 (0.59-2.36)	0.37
Serous-like	19	1.95	9.77 (6.23-15.31)	<0.001
BRCA1	15	1.19	12.64 (7.62-20.96)	<0.001
BRCA2	4	0.78	5.11 (1.92-13.63)	0.01
Sarcoma	3	1.3	2.30 (0.74-7.14)	0.14
BRCA1	1	0.81	1.24 (0.17-8.78)	0.55
BRCA2	2	0.51	3.95 (0.99-15.81)	0.09
Clear cell	1	0.29	3.40 (0.48-24.11)	0.25
BRCA1	1	0.18	5.58 (0.79-39.65)	0.16
BRCA2	0	0.12	NA	NA

[a]*p* values were estimated assuming a Poisson distribution. Abbreviations: SIR: Standardized Incidence Ratio, CI: Confidence Interval NA: not applicable

EC Risk *BRCA1/2* mutation carriers compared to non-*BRCA1/2* mutation carriers

In total, 58 *BRCA1/2* mutation carriers developed ECs compared to 33 non-*BRCA1/2* mutation carriers, over 119,296 and 160,841 at risk person-years, respectively (HR = 2.37, 95% CI = 1.53-3.69), **Table 4**. *BRCA1* mutation carriers displayed higher relative EC risk (HR = 2.91, 95% CI = 1.83-4.66) compared to *BRCA2* mutation carriers (HR = 1.45, 95% CI = 0.75-2.81)**.**

Combined *BRCA1/2* histologic subgroup analysis showed strongly increased risks for EC with serous-like histology (HR = 8.08, 95% CI = 2.34-27.94), with *BRCA1* showing higher relative risk (HR = 10.48, 95% CI = 2.95-37.20) than *BRCA2* mutation carriers (HR = 4.13, 95% CI = 0.83-20.50), **Table 4**. The highest HR was observed for p53-abnormal EC in *BRCA1* mutation carriers (HR = 15.71, 95% CI = 4.62-53.40). The risk for endometrioid EC in *BRCA1* mutation carriers was increased two-fold (HR = 2.01, 95% CI = 1.18-3.45), unlike *BRCA2* (HR = 0.93, 95% CI = 0.41-2.11).

Table 3. Observed and expected endometrial cancer rates in *BRCA1/2* mutation carriers compared to the Dutch country-specific incidence rates, according to attained age

	***BRCA1/2* carriers**	**Dutch population**	
EC subgroup, age categories	**Observed**	**Expected**	**SIR (95% CI)**
All endometrial cancers	58[a]	20.53	2.83 (2.18-3.65)
25-40 years	4	0.41	9.84 (2.68-25.20)
40-60 years	25	10.0	2.50 (1.62-3.69)
60-80 years	28	9.56	2.93 (1.95-4.24)
Serous-like	19	1.95	9.77 (6.23-15.31)
25-40 years	0	0.02	0.00 (0.00-149.82)
40-60 years	6	0.69	8.68 (3.19-18.90)
60-80 years	13	1.15	11.27 (5.99-19.27)

[a]One endometrial cancer occurred after 80 years of age. Given the low number of person-years after 80 years of age, this age category is not presented in the table. Abbreviations: SIR: Standardized Incidence Ratio, CI: Confidence Interval.

When only follow-up after the date of *BRCA1/2* DNA test is considered, EC risk among mutation carriers remained increased, with higher HRs compared to the main analyses, though with broader confidence intervals, **Table 4**. When excluding cases for which the *BRCA1/2* DNA test date was unknown, HRs remained roughly similar, **Supplementary Table 7**.

To eliminate potential confounding by tamoxifen, a sensitivity analyses was performed by additionally censoring at the time of (first) HT-treated BC. This yielded HRs that were similar to the main analyses, both regarding overall EC risk and stratified for mutation-type/histologic/molecular subgroup, **Table 4**. For additional sensitivity analyses, see **Supplementary Table 7**.

When overall EC risk and EC risk stratified by histologic subgroup were compared between non-*BRCA1/2* carriers and Dutch country-specific incidence rates, no statistically significant differences were observed (**Supplementary Table 8**).

At the age of 75 years, the estimated cumulative risk ('life-time risk') for *BRCA1/2* mutation carriers to develop EC was 3.0% (95% CI = 2.20%-3.91%; *BRCA1*: 3.4%, 95% CI = 2.46%-4.81%; *BRCA2*: 2.0%, 95% CI = 1.09%-3.30%), for the subgroup of EC with serous-like histology, this was 1.1% (95% CI = 0.69%-1.80%; *BRCA1*: 1.4%, 95% CI = 0.79%-2.37%; *BRCA2:* 0.6%, 95% CI = 0.21%-1.60%), see **Supplementary Table 9**.

Table 4. Endometrial cancer risks *BRCA1/2* mutation carriers versus non-*BRCA1/2* mutation carriers

	BRCA1/2 carriers			non-*BRCA1/2* carriers				
Subgroup	**Total, No.**	**Event, No.**	**Person-years at risk**	**Total, No.**	**Events, No.**	**Person-years at risk**	**Hazard Ratio (95% CI)[a]**	***P*[b]**
Main analysis								
All	5980	58	119296	8451	33	160841	2.37 (1.53-3.69)	<0.001
BRCA1[c]	3829	44	75366	8451	33	160841	2.91 (1.83-4.66)	<0.001
BRCA2[c]	2192	14	44809	8451	33	160841	1.45 (0.75-2.81)	0.27
Histologic groups								
Endometrioid	5980	35	119296	8451	30	160841	1.61 (0.97-2.66)	0.06
BRCA1[c]	3829	27	75366	8451	30	160841	2.01 (1.18-3.45)	0.01
BRCA2[c]	2192	8	44809	8451	30	160841	0.93 (0.41-2.11)	0.86
Serous-like	5980	19	119296	8451	3	160841	8.08 (2.34-27.94)	0.001
BRCA1[c]	3829	15	75366	8451	3	160841	10.48 (2.95-37.20)	<0.001
BRCA2[c]	2192	4	44809	8451	3	160841	4.13 (0.83-20.50)	0.08
Molecular group								
p53-abnormal[d]	5980	27	119296	8451	3	160841	11.31 (3.37-37.95)	<0.001
BRCA1[c]	3829	23	75366	8451	3	160841	15.71 (4.62-53.40)	<0.001
BRCA2[c]	2192	4	44809	8451	3	160841	4.11 (0.83-20.39)	0.08
Sensitivity analyses								
Start follow-up from date of *BRCA1/2* DNA test[d]								
All histotypes	5771	37	56579	8098	11	48044	3.26 (1.65-6.44)	0.001
Endometrioid	5771	22	56579	8098	10	48044	2.76 (1.26-6.02)	0.01
Serous-like	5771	14	56579	8098	1	48044	18.28 (2.33-143.34)	0.01

Continue

Continued

Subgroup	*BRCA1/2* carriers			non-*BRCA1/2* carriers				
	Total, No.	Event, No.	Person-years at risk	Total, No.	Events, No.	Person-years at risk	Hazard Ratio (95% CI)[a]	*P*[b]
p53-abnormal[d]	5771	21	56579	8098	1	48044	26.64 (3.51-202.32)	0.01
BRCA1, all histotypes[c]	3700	29	37984	8098	11	48044	5.57 (2.69-11.54)	<0.001
BRCA2, all histotypes[c]	2108	8	18971	8098	11	48044	2.18 (0.80-5.91)	0.13
Additional censoring HT-BC[f]								
All	5966	50	113033	8447	30	155002	2.30 (1.44-3.66)	<0.001
Endometrioid	5966	32	113033	8447	28	155002	1.56 (0.93-2.64)	0.09
Serous-like	5966	14	113033	8447	2	155002	8.78 (1.94-39.65)	0.01
p53-abnormal[d]	5966	22	113033	8447	2	155002	13.62 (3.15-59.00)	<0.001
BRCA1, all histotypes[c]	3821	37	72423	8447	30	155002	2.61 (1.58-4.31)	<0.001
BRCA2, all histotypes[c]	2186	13	41461	8447	30	155002	1.60 (0.82-3.12)	0.17

Abbreviations and explanations: BC: Breast Cancer, HT: Hormone Treatment

[a]All hazard ratios were adjusted for age.

[b] The P values assessing the null hypothesis of HR=1.00.

[c]Women with both a *BRCA1* and a *BRCA2* mutation were included in both analyses stratified for *BRCA1/2* mutation status.

[d]Includes cases for which p53-status was unknown (no FFPE tumor block available) and for whom p53-status was based on most common p53-status for the histotype as described in the material and methods. When excluding cases for which p53-status was based on histotype, the number of events remained the same for *BRCA1/2* carriers, but only two events occurred in the non-*BRCA1/2* mutation carriers (HR: 17.07, 95%-CI: 4.0-72.8, p<0.001).

[e]If the date of *BRCA1/2*-DNA test was unknown, this date was considered to be January 1, 1995.

[f]DCIS was considered as BC. If a woman developed a BC/DCIS for which hormone treatment status was unknown, the date of diagnoses was not considered as censoring event.

Discussion

We presented data from a large cohort study that assessed EC risk among *BRCA1/2* mutation carriers (n=5,980). Strengths of the study compared to earlier studies are, high number of events (n=58), long follow-up (median = 22.5 years) and pathology review to validate the outcome. We found that *BRCA1* and *BRCA2* mutation carriers show a 2-3 fold increased EC risk, with highest increased risks found for the subgroups EC of serous-like histology (8-10 fold) and p53-abnormal EC (11-12 fold). We also showed that increased risk cannot be fully explained by previous HT use and is therefore most likely causally associated with *BRCA1/2* mutations.

Conflicting data from earlier cohort studies, most likely due to lack of power, has resulted in uncertainty regarding increased EC risk in *BRCA1/2* mutation carriers (**Supplementary Table 1**),[2-9] as only three of eight reported statistically significantly increased overall EC risk (SIR range = 1.9-5.3). Those figures broadly agree with results from this study (2-3 fold increase).[2-4] A striking observation reported in three of the seven studies that stratified for histotype[2, 4-9] was the statistically significantly increased risk (SIR range = 14.3-32.2) for EC of serous-like histology, which seemed to be restricted to *BRCA1* mutation carriers.[4, 8, 9] Our study confirms that finding, with the highest risk indeed observed for *BRCA1* mutation carriers (10-13 fold), but with *BRCA2* mutation carriers also showing 5-fold increased risk compared to the general population. By contrast, endometrioid EC risk was only increased for *BRCA1*-mutation carriers (2-3 fold). That *BRCA* mutations contribute to the development of EC is further supported by the recent study of Hughley and colleagues,[20] in which they present the 'etiological index': a case-only measure of *BRCA1/2* mutation associated cancer risks based on the fraction of tumors harboring biallelic *BRCA1/2* inactivation. While the *BRCA1/2* etiological index for nonestablished *BRCA1/2*-associated cancers was 1.6, the respective *BRCA1* etiological index of endometrial cancer was 4.0, supporting an etiological role in cancer causation.

A history of tamoxifen use is considered an important confounder when assessing EC risk.[21, 22] These patients also seem to develop less favourable histologic subtypes such as carcinosarcomas, sarcomas and p53-abnormal tumors.[21, 23] As we were not informed about the type of HT (tamoxifen, aromatase inhibitor) women received for their BC, a potential effect was eliminated by censoring for all HT-BC in a sensitivity analysis. We nonetheless found persistent increased risk for EC overall, EC of serous-like histology and p53-abnormal EC, and can therefore conclude that increased EC risk in *BRCA1/2* mutation carriers can, at best, be only partly explained by previous HT/tamoxifen use.

Highest increased EC risks were found for EC with serous-like histology, and more specifically p53-abnormal EC. We have previously shown that ECs in *BRCA1/2* mutation carriers are noticeably enriched for tumors of the p53-abnormal molecular subgroup, that these tumors

demonstrate LOH of *BRCA* wildtype allele,[16] and that ECs of this subgroup are frequently homologous recombination deficient (HRD) or show genomic scars associated with HRD.[15, 24] The molecular alterations in these tumors are similar to those found in high-grade serous OC and basal-like BC, tumor subtypes particularly associated with the *BRCA1/2*-associated HBOC syndrome.[10, 25-27] Due to the above observations, we would argue that ECs with serous-like histology and especially ECs of the p53-abnormal molecular subgroup should be regarded as part of the *BRCA1/2*-associated HBOC syndrome.

A limitation of this study was the possibility of a cancer-related testing bias. EC is not an indication for *BRCA1/2* DNA testing, therefore, although person-time before *BRCA* DNA testing was included in the main analysis, it is unlikely that this influenced the results. Only including person-time after *BRCA1/2* DNA testing resulted in higher HRs (though with broader confidence intervals) compared to the main analysis. This might be due to the older age of the post-*BRCA1/2* DNA testing cohort, as higher SIRs were observed for older age categories (**Table 3**). Another potential limitation is the presence of left censoring, as the possible occurrence of EC in the period before the NCR and PALGA databases achieved nationwide coverage has naturally not been recorded but cannot be entirely excluded. However, since the majority of women were young at start of follow-up and the majority of ECs are recorded >40 years of age (54 of 58 *BRCA1/2* and 31 of 33 non-*BRCA1/2* mutation carriers) any influence is likely minor. Data on previous hysterectomies was unavailable, but as a *BRCA1/2* mutation is not an indication for hysterectomy in the Netherlands this is unlikely to have affected our results. Pathology review could not be performed for all ECs, nor for the Dutch population controls, therefore, a subset of ECs might have been misclassified. This is especially relevant for high-grade EC (review resulted in histologic subgroup changes for 22% of EC in *BRCA1/2* mutation carriers) which are more difficult to classify and more common in *BRCA1/2* mutation carriers.[13, 16] We were not informed about body weight and the use of hormone replacement therapy (HRT) for the majority of cases. Especially obesity, but not modern combined HRT, is a well-known risk factor for endometrial cancer (both endometrioid and non-endometrioid subtypes).[28-31] However, there is no reason to believe that *BRCA1/2* mutation carriers are more frequently obese.

Our results provide important additional information with regard to EC risks, that is essential for adequate genetic counselling of *BRCA1/*2 mutation carriers. Despite the observed increased overall EC risks in *BRCA1/2* mutation carriers, the cumulative overall EC risk (3.0%) and risk for EC of serous-like histology (1.1%) by 75 years remains low (**Supplementary Table 9**), as the life-time risk of developing EC is low in the general population (approximately 1%-1.4%, with ECs of serous-like histology being even less common: 10% of all ECs).[8, 32, 33] Therefore we should not recommend a concurrent risk-reducing hysterectomy at the time of RRSO routinely, especially since this will increase the complication risk of the procedure. Nevertheless, risk-reducing hysterectomy should be considered especially in the presence

of other EC risk factors or when a hysterectomy is considered for other (benign) uterine pathology. Taken together, given the observed relative and absolute risks, the potential hazards and possible benefits of risk-reducing hysterectomy need to be carefully weighed, and shared decision making is crucial in order to conclude about an individually-tailored treatment advice with regard to risk-reducing surgery *BRCA1/2* mutation carriers.

Secondly, ECs that harbor *BRCA1/2* mutations (germline and somatic) will likely benefit from PARP-inhibitor treatment. PARP inhibitors are proven effective maintenance treatment for *BRCA*-associated platinum-sensitive OC,[34] and trials are currently testing efficacy in EC.

Thirdly, although previous studies have reported low incidences of *BRCA1/2* mutations when screening EC patients with a history of BC (3.8%, not selected for histotype)[35] or an unselected cohort of patients with uterine serous carcinomas (2%)[36], *BRCA1/2* mutation incidences in women with p53-abnormal EC, especially with a history of BC, should be studied to determine the potential value of *BRCA1/2* screening in this patient population.

In summary, *BRCA1/2* mutation carriers do have an important increased risk of EC. This is especially the case for the EC subgroups with unfavourable clinical outcome: serous-like EC and p53-abnormal EC. The observed increase in risk cannot be explained by previous BC-related hormone treatment. Importantly, life-time EC risk through 75 years remains low. This report adds critical evidence to the ongoing discussion whether or not EC is a *BRCA1/2*-associated disease, and further supports the mounting evidence that at least serous-like and p53-abnormal EC should be considered to be an integral part of the *BRCA1/2*-associated HBOC syndrome.

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Data availability

The data underlying this article were collected with informed consent in the national collaborative HEBON cohort study. The HEBON steering group provided permission to share the data for this purpose with the study team, including the corresponding author (HOP2016006).

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Supplementary methods

HEBON cohort study

Women are eligible for inclusion in the "HEreditary Breast and Ovarian cancer study, the Netherlands (HEBON cohort study)[1, 2] if they have undergone genetic testing for *BRCA1/2* and *CHEK2* in one of the participating centres (all Dutch academic medical centres and the Netherlands Cancer Institute). The HEBON cohort study collects data from participants via questionnaires and input from the Netherlands Cancer Registry (NCR), the Dutch Pathology Registry (PALGA)[3] and the municipal administration (BRP). The HEBON cohort study is performed in accordance with the declaration of Helsinki.

Data collection

Data entry into the central HEBON database is exclusively performed by trained data managers. Data on the type of mutation (*BRCA1, BRCA2*) and the date of the *BRCA1/2* DNA test were retrieved from the hospitals where screening took place. Data on personal cancer history (based on topographical and morphological codes; ICD-0-3 codes; http://codes.iarc.fr/), date/age/year of cancer diagnosis and treatment history (latter only available for breast cancer (BC) and tubo-ovarian cancer (OC)) were retrieved from the NCR, which registers all cancer diagnoses in the Netherlands and includes other variables such as treatment history. If additional data were found in pathology reports obtained via PALGA (e.g. on treatment history), these were added to the previously mentioned variables. Data on risk-reducing salpingo-oophorectomies (RRSO) and date of RRSO was obtained via PALGA. Data on deaths and date of deaths were obtained via the BRP, supplemented with data from the NCR, pathology reports and information obtained from family members.

For *BRCA1/2* mutation carriers who provided informed consent the most recent data input from the NCR and PALGA was in June and December 2017 (tumour registration/data complete up to January 2016). For all other women (*BRCA1/2* and non-*BRCA1/2* mutation carriers), the most recent NCR and PALGA input dates to April and June 2015, respectively (tumour registration/data complete up to January 2012). The most recent data from the BRP were received in December 2016 for *BRCA1/2* mutation carriers with informed consent, and June 2012 for all other women.

Data handling and missing variables

Four women (*BRCA1/2* mutation carriers, *n*=3/5,980 (0.05%); non-*BRCA1/2* mutation carriers, *n*=1/8,451 (0.1%)) were registered as deceased in the HEBON database while the date/age/year of death was unknown. In these cases, the date of death was considered to be between the last live contact and the end of follow-up.

For 2,896 women (*BRCA1/2* mutation carriers, *n*=1,682/5,980 (28.1%); non-*BRCA1/2* mutation carriers, *n*=1,214/5,980 (14.4%)), the date of the *BRCA1/2* DNA test was unknown. In these cases the date of a *BRCA1/2* DNA test was considered to be 01-01-1995 (date from which *BRCA1/2* DNA testing became regularly available).

The date/age/year of BC diagnosis was unknown for one *BRCA2* mutation carrier. In the subanalyses where BC was added as censoring event, the first occurring censoring event other than date of BC was used for censoring. For the description of baseline characteristics, BC was considered to have occurred during the observation period.

The date/age/year of OC diagnoses was unknown for two non-*BRCA1/2* mutation carriers. For the description of baseline characteristics, OC diagnoses were considered to have occurred during the observation period.

Data on a history of RRSO were manually curated for all women who developed endometrial cancer (including uterine sarcomas; EC) during follow-up, using the pathology reports (if available) retrieved from PALGA.

Data on whether or not women received hormone treatment (HT) for a specific BC was retrieved by the NCR from medical files, and centrally collected by HEBON. This variable was available for the majority of BCs, but the type and duration of HT was not specified. If a women had both a history of HT-treated BC and a tumour with an unknown HT status (*BRCA1/2* mutation carriers *n*=17, non-*BRCA1/2* mutation carriers *n*=8), the date of HT-treated BC was used for all analyses that included HT-status.

Pathology review and histologic and molecular subgrouping

Pathology reports, hematoxylin and eosin (H&E) stained slides and formalin-fixed paraffin-embedded (FFPE) tumour tissue blocks for ECs of HEBON cohort *BRCA1/2* and non-*BRCA1/2* mutation carriers were collected via PALGA and centrally reviewed by at least one expert gynaecopathologist to confirm histotype and endometrial origin. All specimens were handled in compliance with the Code of Conduct for dealing responsibly with human tissue in the context of health research (2011) drawn up by the Federation of Dutch Medical Scientific Societies.

Assignment of histologic subgroups

Histologic subtype diagnosis for cases that were available for pathology review were based on The World Health Organization (2014) criteria. Pathology review was primarily based on morphology (H&E slides without immunohistochemical stains), with the exception of high-grade EC without defining features (ambiguous EC). All cases with high-grade histology without defining features/for which histotype was difficult to establish (“ambiguous”) were reviewed

by at least two gynaecopathologists. When both agreed that the case was "ambiguous", *TP53*-mutation status/P53-IHC expression was used for further differentiation. *TP53*-wildtype/p53-wildtype ambiguous carcinomas were considered to be of the "endometrioid" histologic subgroup, and *TP53*-mutant/p53-abnormal ambiguous carcinomas were considered to be of the "serous-like" histologic subgroup. For cases that were not available for revision, histologic subtype and grade were extracted from pathology reports or, if unavailable, from the morphological ICD-0-3.1 code.

After pathology review, ECs were divided in the same histologic subgroups as the comparison group 1: (1) endometrioid (including *TP53*-wildtype/p53-wildtype ambiguous carcinomas), (2) serous-like (including *TP53*-mutant/p53-abnormal ambiguous carcinomas), (3) clear cell carcinoma, (4) sarcoma and (5) other.

Histologic, molecular and clinical characteristics of a subset of ECs in *BRCA1/2* mutation carriers were comprehensively described previously (case-ID; 1-41).[2]

Assignment of molecular subgroups

In the case of the *BRCA1/2* mutation carriers included in the study by de Jonge and colleagues,[2] the UCM-OncoPlus Assay[4] on FFPE-isolated tumour DNA was used for *TP53* mutation analyses. For the ECs in *BRCA1/2* mutation carriers included in this study, but for which mutation analysis failed/was not available (*n=3),* and for the ECs of both *BRCA1/2* mutation carriers (CaseID 42-62) and non-*BRCA1/2* mutation carriers (CaseID 101-140) that were not included in the study by de Jonge and collegues,[2] p53 immunohistochemistry was used as a surrogate marker to determine *TP53* mutation status. This was either performed manually (clone DO-7, 1:2000, DAKO) as described previously[2] or using the Dako Omnis autostainer (Agilent, Santa Clara, CA). For the Dako Omnis autostainer, slides were deparaffinized and antigen retrieval was achieved on board using EnVision FLEX High pH Target Retrieval Solution for 30 minutes at 97 °C. Slides were then incubated on board at 32 °C with the following primary antibodies: p53, clone DO-7, Ready-To-Use (Dako) for 25 minutes; PMS2, clone EP51.2, ready-to-use for (Dako) for 25 minutes and MSH6 1:400; clone EPR3945 (Abcam) for 20 minutes. For PMS2, this was followed by incubation with a secondary antibody (EnVision FLEX+ rabbit LINKER) for 10 minutes. EnVision FLEX DAB+ was used as chromogen for 5 minutes, followed by counterstaining of the slides for 6 minutes using Mayer's hematoxylin.

Focal, weak and heterogeneous (not subclonal) nuclear p53 staining was considered as p53 "wild-type". Diffuse and strong nuclear staining >90% or completely absent nuclear staining "null pattern" (with positive internal control) was considered as p53-abnormal/mutant. In cases where p53 IHC was inconclusive, molecular analysis using next-generation sequencing was performed to determine final *TP53* mutation status (*n*=1). If a EC showed abnormal p53 expression or a *TP53* mutation, additional staining for MMR proteins (PMS2, MSH6) was

performed. Expression of MMR proteins was scored in three categories (retained, loss and subclonal/regional loss of protein expression) as described previously reported by Stelloo and colleagues.[5] Tumours in which at least one of the mismatch repair proteins showed loss of expression were considered MMR-deficient (MMRd).

Using these surrogate markers, tumours were subsequently classified in one of the molecular subgroups as previously described:[6-8] (1) p53-abnormal or (2) other (including *POLE*-mutant: only analysed for cases included in the study by de Jonge and colleagues;[2] mismatch repair (MMR)-deficient and no surrogate marker profile group (NSMP)). In case both a *TP53* mutation/abnormal p53 expression and a MMRd phenotype were present (not considering subclonal/regional loss of MMRd), cases were assigned to the "other" group.[6, 9]

For cases in which no FFPE block was available for p53 analysis, p53 status was based upon histologic subtype and grade. These were used for classification in the molecular subgroups and subsequent analyses: EEC grade1/2, adenocarcinoma NOS grade 1/2, EEC/adenocarcinoma NOS grade not specified and clear cell carcinomas were assigned to the *TP53*-wildtype group.[6, 10, 11] EEC grade 3 and adenocarcinoma NOS grade 3 were considered 50% *TP53* wildtype, 50% *TP53* mutant.[6, 12] Uterine serous carcinomas and carcinosarcomas were considered *TP53* mutant.[6, 13]

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Supplementary Table 1. Previously published cohort studies on endometrial cancer risk in *BRCA1/2* mutation carriers

Study	Cohort size	Total years at risk	Mean/median age at enrolment	Mean/ median follow-up years	Path review	Observed (of which tamoxifen exposed)	Expected	SIR (95% CI, *p*-value)	EC distribution by *BRCA*-mutation	Countrie(s) from which the study population was retrieved
Beiner et al., 2007	All: 857 *BRCA1;* 619 *BRCA2;* 236 both; 2	2787	54.4 (range: 45-70)	3.3 (range: 0.01-9.6)	No	6 (4) EC *6 (4) endometrioid*	1.13 n.a	5.3 (n.a, *p*=0.0011)[a]	4x *BRCA1* 2x *BRCA2*	North America, Europe, and Israel
Segev et al., 2013	All: 4456 *BRCA1;* 3536 *BRCA2;* 920	25322	42.7 (range: n.a.)	5.7 (range: n.a.)	No	17 (8) EC	9.06	1.87 (1.13-2.94, *p*=0.01)[b]	13x *BRCA1* 4x *BRCA2*	Canada, Italy, USA, Austria, Poland, Norway
Reitsma et al., 2013	All: 315 *BRCA1;* 201 *BRCA2;* 114	2062	43 (range: 30–71)	6 (range: 0-27)	No	2 (0) EC *2 (0) endometrioid*	0.94 *n.a*	2.13 (0.24-7.69, *p*=0.27)	1x *BRCA1* 1x *BRCA2*	The Netherlands
Shu et al., 2016	All: 1083 *BRCA1;* 627 *BRCA2;* 453 both; 3	6377	45.6 (IQR: 40.9-52.5)	5.1 (IQR: 3.0-8.4)	Partly	8 (5) EC *2 (2) endometrioid* *5 (3) serous(-like)* *1 (0) sarcoma*	4.30 3.62 0.34 0.14	1.9 (0.8-3.7, *p*=0.09) *0.6 (0.1-2.0, p=0.88)* *14.8 (4.8-34.6, p<0.001)*[c] *7.1 (0.2-39.4, p=0.13)*	5x *BRCA1* 3x *BRCA2*	USA and United Kingdom
Lee et al., 2017	All: 828 *BRCA1;* 438 *BRCA2;* 390	*n.a.*	43 (IQR: 34-52)	9 (IQR: n.a.)	No	5 (3) EC *5 (3) endometrioid*	2.04 *n.a*	2.45 (0.80-5.72, *p*=0.11)	3x *BRCA1* 2x *BRCA2*	Australia and New Zealand
Saule et al., 2018	All: 369 *BRCA1;* 238 *BRCA2;* 131	1779	*BRCA1:*47.22 (IQ: 1.29) *BRCA2:* 52.75 (IQ: 6.83)	*n.a.*[d] *n.a.*[d]	Yes	2 (0) EC *2 (0) serous*	0.62 0.062	"not increased", *p*=0.26 *32.2 (11.5-116.4, p<0.001)*	2x *BRCA1* 0x *BRCA2*	France

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Study	Cohort size	Total years at risk	Mean/median age at enrolment	Mean/ median follow-up years	Path review	Observed (of which tamoxifen exposed)	Expected	SIR (95% CI, *p*-value)	EC distribution by *BRCA*-mutation	Countrie(s) from which the study population was retrieved
Laitman et al., 2019	All: 2627	32774	*n.a.*	*n.a.*	No	14 (2[e]) EC	3.52	3.98 (2.174-6.673, *p<0.001*)	10x *BRCA1*	Israel, mainly includes founder mutations; (*BRCA1*; 185delAG & 5382insC, *BRCA2*; 6174delT)
	BRCA1; 1463					*5 ([e]) endometrioid*	*n.a*	*n.a.*	*4x BRCA2*	
	BRCA2; 1154					*5 (1) serous(-like)*	0.35	*14.29 (4.639-33.34, p<0.001)*		
	both; 10					*4 (1) sarcoma*	0.106	*37.74 (10.28-96.62, p<0.001)*		
Kitson et al., 2020	All: 2609	59199	20.0 (IQR: 20.0-31.6)	23.8 (IQR: n.a.)	Partly	14 (3[f]) EC	8.22	1.70 (0.74-3.33, *p=n.a.*)	7x *BRCA1*	United Kingdom
	BRCA1; 1350					*7 (2[f]) endometrioid*	*n.a.*	*n.a.*	7x *BRCA2*	
	BRCA2; 1259					*3 (1[f]) serous-like*	0.82	*3.66 (0.01-23.41, p=n.a.)*		
						4 (0[f]) unknown	*n.a.*	*n.a.*		
de Jonge et al., current study	**All: 5980**	119296	27.4 (IQR: 25.0-37.8)	22.5 (IQR: 15.2-27.0)	Partly	58 (8[g]) EC	20.53	2.83 (2.2-3.7), p<0.001[h]	44x *BRCA1*	The Netherlands
	BRCA1; 3788					*35 (3[g]) endometrioid*	16.85	*2.07 (1.5-2.9), p<0.001*[h]	14x *BRCA2*	
	BRCA2; 2151					*19 (5[g]) serous(-like)*	1.95	*9.77 (6.2-15.3), p<0.001*[h]		
	both; 41					*3 (0) sarcoma*	1.3	*2.30 (0.7-7.1), p=0.14*		
						1 (0) clear cell	0.29	*3.40 (0.5-24.1), p=0.25*		

[a]SIR only including non-tamoxifen exposed women; 2.7 (95% CI: n.a., *p*=0.17). [b]SIR only including non-tamoxifen exposed women; 1.67 (95% CI: 0.81-3.07, p=0.1). [c]SIR (serous-like) only including non-tamoxifen exposed women; 11.3 (95% CI: 1.4-40.8, *p*=0.01). [d]Age at end follow-up was given instead of follow-up years; *BRCA1*: 52.75 (IQR: 6.83), *BRCA2*: 56.51 (IQR: 0.8). [e]For 2/5 endometrioid EC, tamoxifen-exposure status was unknown, [f]History of tamoxifen-use was unknown for 4 cases; 1 with endometrioid histology, 2 with serous-like histology and 1 with unknown histology [g]Type and duration of hormone treatment not available. [h]For hazard ratios when additionally censoring for hormone-treated breast cancer, see Table 4.

Supplementary Table 2. Description of morphological ICD-0-3.1 codes assigned to the different histologic subgroups

Endometrioid (includes mucinous)	ICD-0 code	Serous/serous-like	ICD-0 code	Clear cell	ICD-0 code	Sarcoma	ICD-0 code	Other	ICD-0 code
Endometrioid adenocarcinoma, NOS	8380	Serous cystadenocarcinoma, NOS	8441	Clear cell adenocarcinoma, NOS	8310	Endometrial stromal sarcoma, NOS	8930	Neoplasm, malignant	8000
Endometrioid adenocarcinoma, secretory variant	8382	Papillary adenocarcinoma, NOS	8260			Endometrial stromal sarcoma, low grade	8931	Tumour cells, malignant	8001
Endometrioid adenocarcinoma, ciliated cell variant	8383	Serous surface papillary carcinoma	8461			Leiomyosarcoma, NOS	8890	Carcinoma, undifferentiated, NOS	8020
Adenocarcinoma with squamous metaplasia	8570	Papillary serous cystadenocarcinoma	8460			Epithelioid leiomyosarcoma	8891	Large cell carcinoma, NOS	8012
Adenosquamous carcinoma	8560	Cystadenocarcinoma	8440			Myxoid leiomyosarcoma	8896	Large cell neuroendocrine carcinoma	8013
Adenocarcinoma with spindle cell metaplasia	8572	Mesodermal mixed tumour	8951			Rhabdomyosarcoma, NOS	8900	Large cell carcinoma with rhabdoid phenotype	8014
Adenocarcinoma with apocrine metaplasia	8573	Carcinosarcoma, NOS	8980			Myosarcoma	8895	Glassy cell carcinoma	8015
Mucin-producing adenocarcinoma	8481	Mullerian mixed tumour	8950			Pleomorphic rhabdomyosarcoma, adult type	8901	Giant cell carcinoma	8031
Villous adenocarcinoma	8262	Metaplastic carcinoma, NOS	8575			Mixed type rhabdomyosarcoma	8902	Pseudosarcomatous carcinoma	8033
Adenocarcinoma, NOS	8140	Carcinofibroma	8934			Embryonal rhabdomyosarcoma, NOS	8910	Small cell carcinoma, NOS	8041
Carcinoma, NOS	8010	Adenocarcinoma with mixed subtypes	8255			Alveolar rhabdomyosarcoma	8920	Combined small cell carcinoma	8045
Solid carcinoma, NOS	8230	Mixed cell adenocarcinoma	8323			Sarcoma, NOS	8800	Non-small cell carcinoma	8046
Adenocarcinoma in adenomatous polyp	8210					Spindle cell sarcoma	8801	Squamous cell carcinoma, NOS	8070
Adenocarcinoma in villous adenoma	8261					Giant cell sarcoma (except of bone M-9250/3)	8802	Squamous cell carcinoma, keratinizing, NOS	8071
Adenocarcinoma in tubolovillous adenoma	8263					Small cell sarcoma	8803	Squamous cell carcinoma, large cell, nonkeratinizing, NOS	8072
Endometrioid adenofibroma, malignant	8381					Undifferentiated sarcoma	8805	Squamous cell carcinoma, spindle cell	8074

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Endometrioid (includes mucinous)	ICD-0 code	Serous/serous-like	ICD-0 code	Clear cell	ICD-0 code	Sarcoma	ICD-0 code	Other	ICD-0 code
Mucinous adenocarcinoma	8480					Fibrosarcoma, NOS	8810	Basaloid squamous cell carcinoma	8083
						Malignant fibrous histiocytoma	8830	Squamous cell carcinoma, clear cell type	8084
						Malignant perivascular epitheliod cell tumour	8714	Transitional cell carcinoma	8120
						Adenosarcoma	8933	Scirrhous adenocarcinoma	8141
						Stromal sarcoma, NOS	8935	Superficial spreading adenocarcinoma	8143
						Mesenchymoma, malignant	8990	Adenoid cystic carcinoma	8200
						Synovial sarcoma, NOS	9040	Cribriform carcinoma, NOS	8201
						Haemangiosarcoma	9120	Tubular adenocarcinoma	8211
						Chondrosarcoma, NOS	9220	Carcinoid tumour, NOS	8240
						Ewing sarcoma	9260	Mixed adenoneuroendocrine carcinoma	8244
								Neuroendocrine carcinoma, NOS	8246
								Acidophil carcinoma	8280
								Clear cell adenocarcinofibroma	8313
								Granular cell carcinoma	8320
								Follicular adenocarcinoma, NOS (C73.9)	8330
								Adenocarcinoma, endocervical type	8384
								Papillary cystadenocarcinoma, NOS	8450
								Papillary mucinous cystadenocarcinoma	8471
								Mucinous adenocarcinoma, endocervical type	8482

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Endometrioid (includes mucinous)	ICD-0 code	Serous/serous-like	ICD-0 code	Clear cell	ICD-0 code	Sarcoma	ICD-0 code	Other	ICD-0 code
								Signet ring cell carcinoma	8490
								Adenocarcinoma with neuroendocrine differentiation	8574
								Mesonephroma, malignant	9110
								Peripheral neuroectodermal tumour	9364
								Malignant peripheral nerve sheath tumour	9540
								No microscopic confirmation	9990

Supplementary Table 3. Number of person-years at risk and number of events per 5-year age category for *BRCA1/2* mutation carriers and non-*BRCA1/2* mutation carriers

	BRCA1/2 carriers					non-*BRCA1/2* carriers			
Age category	**Person-years at risk, No. (%)**	**All ECs[a], No (%)**	**Endometrioid, No. (%)**	**Serous-like, No. (%)**	**Sarcoma, No. (%)**	**Person-years at risk, No. (%)**	**All ECs[b], No. (%)**	**Endometrioid, No. (%)**	**Serous-like No. (%)**
25-29	14643 (12.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	19663 (12.2)	1 (3.0)	1 (3.3)	0 (0.0)
30-34	17091 (14.3)	2 (3.4)	1 (2.9)	0 (0.0)	1 (33.3)	23376 (14.5)	0 (0.0)	0 (0.0)	0 (0.0)
35-39	17990 (15.1)	2 (3.4)	2 (5.7)	0 (0.0)	0 (0.0)	24984 (15.5)	1 (3.0)	1 (3.3)	0 (0.0)
40-44	17408 (14.6)	2 (3.4)	2 (5.7)	0 (0.0)	0 (0.0)	24303 (15.1)	0 (0.0)	0 (0.0)	0 (0.0)
45-49	15435 (12.9)	7 (12.1)	4 (11.4)	3 (15.8)	0 (0.0)	21314 (13.3)	4 (12.1)	4 (1.3)	0 (0.0)
50-54	12578 (10.5)	7 (12.1)	5 (14.2)	0 (0.0)	2 (66.7)	16704 (10.4)	9 (27.3)	8 (26.7)	1 (33.3)
55-59	9306 (7.8)	9 (15.5)	5 (14.2)	3 (15.8)	0 (0.0)	12133 (7.5)	5 (15.2)	5 (16.7)	0 (0.0)
60-64	6377 (5.3)	11 (19.0)	6 (14.2)	5 (26.3)	0 (0.0)	8124 (5.1)	6 (18.2)	6 (0.2)	0 (0.0)
65-69	4052 (3.4)	14 (24.1)	7 (20.0)	7 (36.8)	0 (0.0)	4580 (2.8)	3 (9.1)	3 (10.0)	0 (0.0)
70-74	2355 (2.0)	3 (5.2)	2 (5.7)	1 (5.3)	0 (0.0)	2709 (1.7)	3 (9.1)	2 (6.7)	1 (33.)
75-79	1225 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1593 (1.0)	1 (3.0)	0 (0.0)	1 (33.3)
80-84	558 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	826 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
85-89	219 (0.2)	1 (1.7)	1 (2.9)	0 (0.0)	0 (0.0)	382 (0.2)	0 (0.0)	0 (0.0)	0 (0.0)
90-94	42 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	121 (0.1)	0 (0.0)	0 (0.0)	0 (0.0)
95+	16 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	27 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a]One clear cell carcinoma occurred at age 59 years, this tumour is not separately mentioned in the columns. [b]None of the non-*BRCA1/2* mutation carriers developed a sarcoma or clear cell carcinoma during follow-up. Abbreviations; EC: Endometrial Cancer

Supplementary Table 4. Details on the included endometrial cancers in the cohort

	***BRCA* carriers**	**Non-*BRCA* carriers**	***p* value**[a]
Endometrial cancer, No. (%)	58 (100)	33 (100)	
Median age at diagnoses, yrs (range)	60.2 (33.1-85.4)	57.4 (29.7-79.8)	0.49
Histotype (after review)			
Endometrioid			
grade 1, No. (%)	18 (31.0)	14 (42.4)	
grade 2, No. (%)	3 (5.2)	2 (6.1)	
grade 3, No. (%)	6 (10.3)	1 (3.0)	
Mucinous, No. (%)	1 (1.7)	0 (0)	
Serous, No. (%)	9 (15.5)	1 (3.0)	
Carcinosarcoma, No. (%)	4 (6.9)	2 (6.1)	
Ambiguous, No. (%)	6 (10.3)	0 (0)	
Low grade endometrial stromal sarcoma, No. (%)	1 (1.7)	0 (0)	
Leiomyosarcoma, No. (%)	1 (1.7)	0 (0)	
Adenosarcoma, No. (%)	1 (1.7)	0 (0)	
Not reviewed, No. (%)	8 (13.8)	13 (39.4)	
Histologic groups			0.02
Endometrioid, No. (%)	35 (60.3)	30 (90.9)	
Serous/Serous-like[b], No. (%)	19 (32.8)	3 (9.1)	
Sarcoma, No. (%)	3 (5.2)	0 (0)	
Clear cell, No. (%)	1 (1.7)	0 (0)	
Other, No. (%)	0 (0)	0 (0)	
Occurrence EC			
After *BRCA1/2* DNA test, No. (%)	28 (48.3)	7 (21.2)	
Before *BRCA1/2* DNA test, No. (%)	20 (34.5)	22 (66.7)	
Date *BRCA1/2* DNA test unknown, No. (%)	10 (17.2)	4 (12.1)	
Pathology review			0.01
Available, No. (%)	50 (86.2)	20 (60.6)	
Not available, No. (%)	8 (13.8)	13 (39.4)	
Histologic group change after review			0.0102
Yes, No. (%)	11 (19.0)	0 (0.0)	
No, No. (%)	39 (67.2)	20 (60.6)	
Not reviewed, No. (%)	8 (13.8)	13 (39.4)	
p53-abnormal, including cases not available for review			<0.001
Yes, No. (%)	27 (46.6)	3 (9.1)	
No, No. (%)	31 (53.4)	30 (90.9)	
P53-abnormal, excluding cases not available for review			<0.001
Yes, No. (%)	27 (46.6)	2 (6.1)	
No, No. (%)	23 (39.7)	17 (51.5)	
Not available, No. (%)	8 (13.8)	14 (42.4)	
P53-status based on;			<0.001
Mutation analyses, No. (%)	37 (63.8)	0 (0.0)	
IHC, No. (%)	13 (22.4)	19 (57.6)	
Histotype, No. (%)	8 (13.8)	14 (42.4)	

Abbreviations: EC: Endometrial cancer, IHC: immunohistochemistry

[a]*p* values were calculated using the Chi-square test (categorical variables) and the Mann-Whitney U-test (numerical variables). [b]Includes six carcinomas of ambiguous morphology that were classified as serous-like based on p53-status.

Supplementary Table 5. Characteristics of endometrial cancers that occurred in the *BRCA1/2* mutation carrier cohort

Case ID[a]	Final histological subgroup[b]	BRCA mutation	Year	Age	Original diagnoses	Diagnoses after pathology review	P53-status[c]	Category change histologic subgroup after review	Molecular subgroup	RRSO, years in between RRSO-EC	Adnexal involvement/ (history of) ovarian malignancy	Adnexal specimens available for revision	Hysterectomy with/ without adnexae
Included as endometrial cancer in main analyses													
1	endometrioid	BRCA1	2011	63	EEC gr1	EEC gr1	wildtype[d]	No	Other	No	No	Yes	Hysterectomy, adnexae removed shortly before (RRSO with EC diagnoses in concurrently performed curettage)
2	endometrioid	BRCA1	2005	55	EEC gr2	EEC gr1	wildtype[d]	No	Other	Yes, 5.1	No	No	Hysterectomy without adnexae (history RRSO)
3	endometrioid	BRCA1	2013	70	EEC gr3	EEC gr3	mutant[d]	No	p53-abnormal	Yes, 15.6	No	Yes	Hysterectomy without adnexae (history RRSO)
11	endometrioid	BRCA1	2004	63	adenocarcinoma NOS, gr1	EEC gr1	wildtype[d]	No	Other	No	No	Yes	Hysterectomy with adnexae
13	endometrioid	BRCA1	2004	50	EEC gr1	EEC gr1	wildtype[d]	No	Other	No	No	Yes	Hysterectomy with adnexae
15	endometrioid	BRCA1	2009	74	EEC gr3	EEC gr3	mutant[d]	No	p53-abnormal	No	No	No, not removed	Hysterectomy without adnexae
17	endometrioid	BRCA1	2013	65	EEC gr3	EEC gr3	mutant[d]	No	p53-abnormal	Yes, 9.0	No	Yes	Hysterectomy without adnexae (history RRSO)
18	endometrioid	BRCA1	2005	59	EEC gr1	EEC gr1	wildtype[d]	No	Other	No	No	Yes	Hysterectomy with adnexae
19	endometrioid	BRCA1	1997	46	EEC gr2	EEC gr1	wildtype[d]	No	Other	No	No	Yes	Hysterectomy, adnexae removed shortly before
20	endometrioid	BRCA1	2011	49	EEC gr2	EEC gr3	mutant[d]	No	p53-abnormal	Yes, 9.5	No	Yes	Hysterectomy without adnexae (history RRSO)
21	endometrioid	BRCA1	2013	65	EEC gr2	EEC gr2	mutant[d]	No	p53-abnormal	Yes, 11.6	No	Yes	Hysterectomy without adnexae (history RRSO)
23	endometrioid	BRCA1	2011	53	mucinous	mucinous	wildtype[d]	No	Other	Yes, 6.1	No	Yes	Hysterectomy without adnexae (history RRSO)
28	endometrioid	BRCA1	2002	44	EEC gr1	EEC gr1	wildtype[d]	No	Other	Yes, 0.4	No	Yes	Hysterectomy without adnexae (history RRSO)
30	endometrioid	BRCA1	2012	39	EEC gr2	n.a.	n.a., considered *TP53* wildtype	n.a.	Other[e]	No	No	No, unknown if removed	Unknown
33	endometrioid	BRCA1	2000	49	EEC gr2	EEC gr1	wildtype[d]	No	Other	No	No	Yes	Hysterectomy with adnexae
34	endometrioid	BRCA1	2005	54	adenocarcinoma NOS, gr2	EEC gr2	mutant[d]	No	p53-abnormal	No[g]	No	Yes	Hysterectomy with fallopian tubes
35	endometrioid	BRCA1	2000	49	EEC gr1	EEC gr1	wildtype[d]	No	Other	No	No	Yes	Hysterectomy with adnexae

Continue

Continued

Case ID[a]	Final histological subgroup[b]	BRCA mutation	Year	Age	Original diagnoses	Diagnoses after pathology review	P53-status[c]	Category change histologic subgroup after review	Molecular subgroup	RRSO, years in between RRSO-EC	Adnexal involvement/ (history of) ovarian malignancy	Adnexal specimens available for revision	Hysterectomy with/ without adnexae
37	endometrioid	BRCA1	2014	33	EEC gr2-3	EEC gr2	mutant[d]	No	p53-abnormal	Yes, 5.1	No	Yes	Hysterectomy without adnexae (history RRSO)
38	endometrioid	BRCA1	2010	56	EEC gr1	EEC gr1	wildtype[d]	No	Other	Yes[h], 4.8	History ovarian clear cell carcinoma	Yes	Hysterectomy without adnexae (history ovariectomy)
41	endometrioid	BRCA1	2007	50	EEC gr2	EEC gr1	wildtype[d]	No	Other	Yes, 0.6	No	Yes	Hysterectomy without adnexae (history RRSO)
47	endometrioid	BRCA1	1999	58	adenocarci-noma NOS, gr1-2	EEC gr1	wildtype	No	Other	No	Simultaneous bilateral HGSOC	Yes	Hysterectomy with adnexae
50	endometrioid	BRCA1	2006	85	EEC gr3	EEC gr3	mutant[d]	No	p53-abnormal	No	No	No	Hysterectomy with adnexae
51	endometrioid	BRCA1	1996	69	EEC gr3	EEC gr3	mutant	No	p53-abnormal	No	No	Yes	Hysterectomy with adnexae
56	endometrioid	BRCA1	2007	63	EEC gr1	n.a.	n.a., considered *TP53* wildtype	n.a.	Other[e]	No	Simultaneous HGSOC left side, right side not reported	No	Hysterectomy with adnexae
60	endometrioid	BRCA1	1994	57	adenocarcino-ma NOS gr1	n.a.	n.a., considered *TP53* wildtype	n.a.	Other[e]	No	No	No, unknown if removed	Unknown
61	endometrioid	BRCA1	2006	68	EEC, gr not specified	n.a.	n.a., considered *TP53* wildtype	n.a.	Other[e]	No	Simultaneous HGSOC right side, left side not involved	No	Hysterectomy with adnexae
62	endometrioid	BRCA1	2008	40	EEC gr1	n.a.	n.a., considered *TP53* wildtype	n.a.	Other[e]	No	No	No	Hysterectomy with adnexae
4	endometrioid	BRCA2	2015	64	EEC gr1	EEC gr1	mutant[d]	No	Other[f]	Yes, 6.9	No	Yes	Hysterectomy without adnexae (history RRSO)
12	endometrioid	BRCA2	2004	62	EEC gr2	EEC gr1	wildtype[d]	No	Other	Yes, 1.0	No	Yes	Hysterectomy without adnexae (history RRSO)
24	endometrioid	BRCA2	2011	67	EEC gr2	EEC gr1	wildtype[d]	No	Other	No	No	Yes	Hysterectomy with adnexae
27	endometrioid	BRCA2	2009	61	EEC gr1	EEC gr1	wildtype[d]	No	Other	No	No	Yes	Hysterectomy with adnexae
31	endometrioid	BRCA2	2011	50	EEC gr2	EEC gr1	wildtype[d]	No	Other	No	Simultaneous bilateral EOC	Yes	Hysterectomy, adnexae removed shortly before
45	endometrioid	BRCA2	1989	65	adenocarcino-ma NOS, gr1	n.a.	n.a., considered *TP53* wildtype	n.a.	Other[e]	No	No	No	Hysterectomy with adnexae
52	endometrioid	BRCA2	2001	67	EEC gr2	EEC gr1	wildtype	No	Other	No	No	Yes	Hysterectomy with adnexae

Continue

Continued

Case ID[a]	Final histological subgroup[b]	BRCA mutation	Year	Age	Original diagnoses	Diagnoses after pathology review	P53-status[c]	Category change histologic subgroup after review	Molecular subgroup	RRSO, years in between RRSO-EC	Adnexal involvement/ (history of) ovarian malignancy	Adnexal specimens available for revision	Hysterectomy with/ without adnexae
58	endometrioid	BRCA2	2009	38	EEC gr1	n.a.	n.a., considered *TP53* wildtype	n.a.	Other[e]	yes, 5.0	No	No, unknown if removed	Unknown
5	serous-like	BRCA1	2015	68	serous	ambiguous	mutant[d]	No	p53-abnormal	Yes, 9.4	No	Yes	Hysterectomy without adnexae (history RRSO)
6	serous-like	BRCA1	2008	64	EEC gr3	serous	mutant[d]	Yes	p53-abnormal	No	No	Yes	Hysterectomy with adnexae
7	serous-like	BRCA1	2010	65	EEC gr1	ambiguous	mutant[d]	Yes	p53-abnormal	Yes, 11.7	No	Yes	Hysterectomy without adnexae (history RRSO)
8	serous-like	BRCA1	2009	65	EEC gr3	ambiguous	mutant[d]	Yes	p53-abnormal	Yes, 7.4	No	Yes	Hysterectomy without adnexae (history RRSO)
14	serous-like	BRCA1	2000	49	carcinosarcoma	carcinosarcoma, serous	mutant[d]	No	p53-abnormal	No	No	Yes	Hysterectomy with adnexae
16	serous-like	BRCA1	1996	59	adenocarcinoma NOS, gr2-3	sereus	mutant[d]	Yes	p53-abnormal	No	No	Yes	Hysterectomy with adnexae
22	serous-like	BRCA1	2015	63	carcinosarcoma, serous	carcinosarcoma, ambiguous	mutant[d]	No	Other[f]	Yes, 4.7	No	Yes	Hysterectomy without adnexae (history RRSO)
26	serous-like	BRCA1	2012	49	EEC gr3	carcinosarcoma, ambiguous	mutant[d]	Yes	p53-abnormal	Yes, 4.0	No	Yes	Hysterectomy without adnexae (history RRSO)
39	serous-like	BRCA1	2003	65	EEC gr2	ambiguous	mutant[d]	Yes	p53-abnormal	Yes	No	Yes	Hysterectomy without adnexae (history RRSO)
40	serous-like	BRCA1	2006	58	EEC gr3	serous	mutant[d]	Yes	p53-abnormal	Yes, 6.8	No	Yes	Hysterectomy without adnexae (history RRSO)
43	serous-like	BRCA1	2006	60	serous	serous	mutant	No	p53-abnormal	No	No	Yes	Hysterectomy with adnexae
44	serous-like	BRCA1	2001	73	adenocarcinoma NOS, gr3	serous	mutant	Yes	p53-abnormal	No	Unknown	No, no hysterectomy/ adnexextirpation	No hysterectomy/adnexextirpation performed, diagnosis based on cervical biopsy, vagina wand biopsy and curretage
48	serous-like	BRCA1	1989	47	adenocarcinoma NOS, gr1	serous	mutant	Yes	p53-abnormal	No	No	No, not removed	Hysterectomy without adnexae
49	serous-like	BRCA1	1997	65	EEC gr3	ambiguous	mutant	Yes	p53-abnormal	No	Unknown	No	Hysterectomy with adnexae
53	serous-like	BRCA1	2001	65	carcinosarcoma, unspecified	carcinosacoma, serous	mutant	No	p53-abnormal	No	No	Yes	Hysterectomy with adnexae

Continue

Continued

Case ID[a]	Final histological subgroup[b]	BRCA mutation	Year	Age	Original diagnoses	Diagnoses after pathology review	P53-status[c]	Category change histologic subgroup after review	Molecular subgroup	RRSO, years in between RRSO-EC	Adnexal involvement/ (history of) ovarian malignancy	Adnexal specimens available for revision	Hysterectomy with/ without adnexae
25	serous-like	BRCA2	2015	64	serous	serous	mutant[d]	No	p53-abnormal	Yes, 4.5	No	Yes	Hysterectomy without adnexae (history RRSO)
29	serous-like	BRCA2	2006	57	serous	serous	mutant[d]	No	p53-abnormal	Yes, 6.1	No	Yes	Hysterectomy without adnexae (history RRSO)
32	serous-like	BRCA2	2009	62	serous	serous	mutant[d]	No	p53-abnormal	No	No	Yes	Hysterectomy with adnexae
55	serous-like	BRCA2	2009	68	large cell carcinoma NOS	ambiguous	mutant	Yes	p53-abnormal	No	Unknown	No, no hysterectomy/ adnexextirpation	No hysterectomy/ adnexextirpation performed, diagnoses based on vaginal and cervical biopsy
36	sarcoma	BRCA1	2007	33	low-grade ESS	low-grade ESS	wildtype	No	Other	No	No	Yes	Hysterectomy without adnexae, fallopian tubes were removed shortly prior to hysterectomy during uterine biopsy
42	sarcoma	BRCA2	1995	53	leiomyosarcoma	leiomyosarcoma	wildtype	No	Other	No	No	No, not removed	Hysterectomy without adnexae
46	sarcoma	BRCA2	2001	54	adenosarcoma	adenosarcoma	wildtype	No	Other	No	No	No, not removed	Hysterectomy without adnexae
59	clear cell	BRCA1	2003	59	clear cell carcinoma	n.a.	n.a., considered *TP53* wildtype	n.a.	Other[e]	No	No	No	Unknown
Endometrial cancer outside observation period main analyses													
10	serous-like	BRCA2	2016	49	EEC gr1	carcinosarcoma, serous	mutant[d]	Yes	p53-abnormal	Yes, 4.3	No	Yes	Hysterectomy without adnexae (history RRSO)
54	serous-like	BRCA1	1988	62	adenocarcinoma NOS, gr3	serous	mutant	Yes	p53-abnormal	No	No	Yes, only left side resected	Hysterectomy and left adnex
57	endometrioid	BRCA2	2012	65	EEC gr2	n.a.	n.a., considered *TP53* wildtype	n.a.	Other[e]	yes, 12.1	No	No	Hysterectomy without adnexae (history RRSO)
Not considered endometrial cancer after revision, outside observation period													
9	No EC	BRCA1	1983	45	endocervical adenocarcinoma	n.a.	n.a.	n.a.		Yes, no EC after revision	No	No	Unknown, RRSO one month before resection

Abbreviations: EEC: Endometrioid endometrial carcinoma, n.a.: not available, NOS: Not otherwise specified, RRSO: Risk-reducing salpingo-oophorectomy, EC: Endometrial cancer [a]CaseIDs number 1-41 correspond with CaseIDs from our previous publication; De Jonge and colleagues, CCR 2019. [b]Based on diagnoses after pathology review when available. [c]P53-status based on IHC, molecular analyses or most common pattern based on literature in case no FFPE blocks were available. "Mutant" includes both overexpression pattern and null-pattern. [d]P53-status based on mutational analyses (see de Jonge and colleagues, CCR 2019). [e]Molecular subgroup based on most prevalent p53 status for the histologic subtype, no FFPE-tumour tissue block was available for determining p53-status. [f]Tumour was also mismatch repair deficient and therefore considered as "other" molecular subgroup. [g]Ovaries were previously removed (without fallopian tubes); this was not considered as RRSO. [h]History of therapeutic ovariectomy because of ovarian clear cell carcinoma.

Supplementary Table 6. Characteristics of endometrial cancers that occurred in the non-*BRCA1/2* mutation carrier cohort

Case ID	Final histological subgroup[a]	Year	Age	Original diagnoses	Diagnoses after pathology review	P53 status[b]	Molecular subgroup	Category change histologic subgroup after review	RRSO, years in between RRSO-EC	Adnexal involvement/ (history of) ovarian malignancy	Adnexal specimens available for revision	Hysterectomy with/ without adenxae
Included as endometrial cancer in main analyses												
101	Endometrioid	1990	46	adenocarcinoma NOS, gr3	n.a.	n.a., considered *TP53* mutant	p53-ab-normal[c]	n.a.	No	No	No	Hysterectomy with adnexae
102	Endometrioid	2010	53	EEC gr1	EEC gr1	wildtype	other	No	No	No	No	Hysterectomy with adnexae
104	Endometrioid	2004	54	EEC gr2	EEC gr1	wildtype	other	No	No	Simultaneous EOC gr1 right side, considered as second primary	Yes	Hysterectomy with adnexae
105	Endometrioid	2000	54	EEC, gr not specified	EEC gr1	wildtype	other	No	No	No	Yes	Hysterectomy with adnexae
106	Endometrioid	1998	65	Adenocarcinoma NOS gr2	EEC gr2	wildtype	other	No	No	No	No	Hysterectomy with adnexae
108	Endometrioid	2009	58	EEC gr1	EEC gr1	wildtype	other	No	No	No	Yes	Hysterectomy with adnexae
110	Endometrioid	2005	60	adenocarcinoma NOS gr1	EEC gr1	n.a., considered *TP53* wildtype	other[c]	No	No	No	Yes	Hysterectomy with adnexae
111	Endometrioid	2003	37	adenocarcinoma NOS gr1	EEC gr1	wildtype	other	No	Unknown	No	No, not removed	Hysterectomy without adnexae
112	Endometrioid	2007	49	adenocarcinoma NOS gr1	EEC gr1	wildtype	other	No	No	Simultaneous EOC right side, considered as second primary	Yes, left side not removed	Hysterectomy with right adnex
113	Endometrioid	1997	48	EEC gr1	EEC gr1	wildtype	other	No	No	No	yes	Hysterectomy with adnexae
114	Endometrioid	2008	57	EEC gr 2	EEC gr3	wildtype	other	No	No	No	yes	Hysterectomy with adnexae
115	Endometrioid	2007	67	EEC gr1	EEC gr1	wildtype	other	No	No	No	yes	Hysterectomy with adnexae
116	Endometrioid	2002	66	adenocarcinoma NOS gr2	EEC gr1	wildtype	other	No	No	Simultaneous bilate-ral serous borderline tumour/low-grade serous carcinoma.	yes	Hysterectomy with adnexae

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Continued

Case ID	Final histological subgroup[a]	Year	Age	Original diagnoses	Diagnoses after pathology review	P53 status[b]	Molecular subgroup	Category change histologic subgroup after review	RRSO, years in between RRSO-EC	Adnexal involvement/ (history of) ovarian malignancy	Adnexal specimens available for revision	Hysterectomy with/ without adenxae
117	Endometrioid	1999	53	adenocarcinoma NOS gr2	EEC gr2	wildtype	other	No	No	No	No	Hysterectomy with adnexae
118	Endometrioid	2002	62	EIN/ adenocar-cioma NOS gr1	EEC gr1	wildtype	other	No	No	No	No	Hysterectomy with adnexae
121	Endometrioid	2011	60	EEC gr1	EEC gr1	wildtype	other	No	No	No	Yes	Hysterectomy with adnexae
124	Endometrioid	1999	56	adenocarcinoma NOS, gr1-2	EEC gr1	wildtype	other	No	No	No	Yes	Hysterectomy with adnexae
126	Endometrioid	2004	52	adenocarcinoma NOS gr1	EEC gr1	wiltype	other	No	No	No	No, not removed	Hysterectomy without adnexae
129	Endometrioid	2011	64	EEC gr3	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	No	No	No	Hysterectomy with adnexae
130	Endometrioid	2000	57	EEC gr1	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	Unknown	No	No, unknown if removed	Unknown
131	Endometrioid	2002	63	EEC gr2	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	No	No	No, unknown if removed	Unknown
132	Endometrioid	2010	74	EEC gr2	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	No	No	No	Hysterectomy with adnexae
133	Endometrioid	2010	53	EEC gr2	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	Yes, 2.7[d]	No	No	Unknown
134	Endometrioid	2008	58	EEC gr2	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	No	No	No, unknown if removed	Unknown
135	Endometrioid	2002	49	EEC gr1	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	No	No	No	hysterectomy with right adnex
136	Endometrioid	2007	72	EEC gr1	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	No	No	No	hysterectomy with right adnex
137	Endometrioid	2011	29	EEC gr1	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	No	Simultaneous bilate-ral serous borderline tumour	No	Hysterectomy with adnexae
138	Endometrioid	1999	60	adenocarcinoma NOS gr2	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	No	No	No, unknown if removed	Unknown
139	Endometrioid	1999	51	adenocarcinoma NOS gr unspe-cified	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	No	No	No	Hysterectomy with adnexae

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Continued

Case ID	Final histological subgroup[a]	Year	Age	Original diagnoses	Diagnoses after pathology review	P53 status[b]	Molecular subgroup	Category change histologic subgroup after review	RRSO, years in between RRSO-EC	Adnexal involvement/ (history of) ovarian malignancy	Adnexal specimens available for revision	Hysterectomy with/ without adenxae
140	Endometrioid	2007	53	EEC gr 1	n.a.	n.a., considered *TP53* wildtype	other[c]	n.a.	No	No	No, unknown if removed	Unknown
103	Serous-like	1997	53	carsinocarcoma, unspecified	carcinosarcoma, endometrioid	wildtype	other	No	No	No	Yes	Hysterectomy with adnexae
123	Serous-like	2007	72	carcinosarcoma, endometrioid	carcinosarcoma, serous	mutant	other	No	No	Simultaneous bilateral serous adenocarcinoma	No	Hysterectomy with adnexae
128	Serous-like	2009	79	serous	serous	mutant	p53-abnormal	No	No	No	Yes	Hysterectomy with adnexae
Endometrial cancer outside follow-up window main analyses												
107	Endometrioid	2012	55	EEC gr1	EEC gr1	wildtype	other	No	No	No	Yes	Hysterectomy with adnexae
109	Endometrioid	2013	69	EEC gr1	EEC gr1	wildtype	other	No	No	No	Yes	Hysterectomy with adnexae
119	Endometrioid	2012	56	undifferentiated/ dedifferentiated carcinoma	dedifferentiated carcinoma	wildtype	other	No	No	No	Yes	Hysterectomy with adnexae
120	Endometrioid	2014	71	EEC gr1	EEC gr2	wildtype	other	No	No	No	Yes	Hysterectomy with adnexae
127	Serous-like	2012	65	carcinosarcoma, unspecified	carcinosarcoma, serous	mutant	p53-abnormal	No	No	No	No	No hysterectomy/adnex extirpation performed (diagnosis based on endometrial curretage)
Not considered endometrial cancer after revision												
122	No EC	1990	59	adenosquamous EC	adenosquamous carcinoma	mutant	No EC	No EC	Unknown	No	No, not removed	Hysterectomy without adnexae
125	No EC	2003	60	EEC gr2	HGSOC	mutant	No EC	No EC	No	Bilateral HGSOC with endometrial involvement	Yes	Hysterectomy with adnexae

Abbreviations: EEC: Endometrioid endometrial carcinoma, EIN: Endometrioid intra-epithelial neoplasia, n.a.: not available, NOS: Not otherwise specified, RRSO: Risk-reducing salpingo-oophorectomy, EC: Endometrial cancer [a]Based on diagnoses after pathology review when available. [b]P53-status based on IHC, molecular analyses or most common pattern based on literature in case no FFPE blocks were available. "Mutant" includes both overexpression pattern and null-pattern. [c]Molecular subgroup based on most prevalent p53 status for the histologic subtype, no FFPE-tumour tissue block was available for determining p53 status. [d]One ovary previously removed.

Supplementary Table 7. Additional sensitivity analyses endometrial cancer risks *BRCA1/2* mutation carriers versus non-*BRCA1/2* mutation carriers

	BRCA1/2 carriers			non-*BRCA1/2* carriers				
Subgroups	**Total (No.)**	**Events (No.)**	**Person-years at risk**	**Total (No.)**	**Events (No.)**	**Person-years at risk (No.)**	**Hazard Ratio (95% CI)[a]**	***p* value[b]**
Start follow-up from date *BRCA* DNA test, excluding cases for which date of *BRCA1/2* DNA test was unknown								
All histotypes	4104	28	29821	6885	7	30055	3.79 (1.61-8.91)	0.002
Endometrioid	4104	17	29821	6885	6	30055	3.76 (1.41-9.97)	0.01
Serous-like	4104	10	29821	6885	1	30055	12.39 (1.49-103.29)	0.02
p53-abnormal[c]	4104	16	29821	6885	1	30055	20.06 (2.56-157.22)	0.004
BRCA1, all histotypes[d]	2493	22	18744	6885	7	30055	7.26 (2.96-17.82)	<0.001
BRCA2, all histotypes[d]	1640	6	11298	6885	7	30055	2.46 (0.72-8.44)	0.15
End follow-up 01-01-2012 for all included women								
All	5936	48	105609	8451	33	160841	2.37 (1.52-3.69)	<0.001
Endometrioid	5936	29	105609	8451	30	160841	1.51 (0.91-2.52)	0.11
Serous-like	5936	15	105609	8451	3	160841	8.16 (2.36-28.22)	0.001
p53-abnormal[c]	5936	20	105609	8451	3	160841	10.95 (3.25-36.87)	<0.001
BRCA1, all histotypes[d]	3796	36	66700	8451	33	160841	2.78 (1.73-4.46)	<0.001
BRCA2, all histotypes[d]	2181	12	39704	8451	33	160841	1.45 (0.75-2.81)	0.27
Additional censoring BC[e]								
All	5689	32	88493	8311	21	139488	2.83 (1.62-4.95)	<0.001
Endometrioid	5689	22	88493	8311	20	139488	1.75 (0.95-3.24)	0.07
Serous-like	5689	6	88493	8311	1	139488	8.90 (1.04-76.54)	0.05
p53-abnormal[c]	5689	9	88493	8311	1	139488	14.25 (1.77-114.22)	0.01
BRCA1, all histotypes[d]	3610	22	53898	8311	21	139488	2.61 (1.41-4.84)	0.002
BRCA2, all histotypes[d]	2115	10	35239	8311	21	139488	1.97 (0.93-4.19)	0.08
Additional censoring HT-BC including cases for which HT was unknown[e]								
All histotypes	5895	49	110215	8407	30	153443	2.30 (1.44-3.68)	<0.001

Abbreviations: BC: Breast Cancer, HT: Hormone Treatment
[a]Hazard ratios were adjusted for age.
[b]The P values assessing the null hypothesis of HR=1.00.
[c]Includes cases for which p53-status was unknown (no FFPE tumour block available) and for which p53-status was based on the most common p53-status for the histotype.
[d]Women with both a *BRCA1* and a *BRCA2* mutation were included in both analyses stratified for *BRCA1/2*-mutation status.
[e]DCIS was considered as BC.

Supplementary Table 8. Observed and expected endometrial cancer rates in non*BRCA1/2* mutation carriers, compared to the Dutch country-specific incidence rates

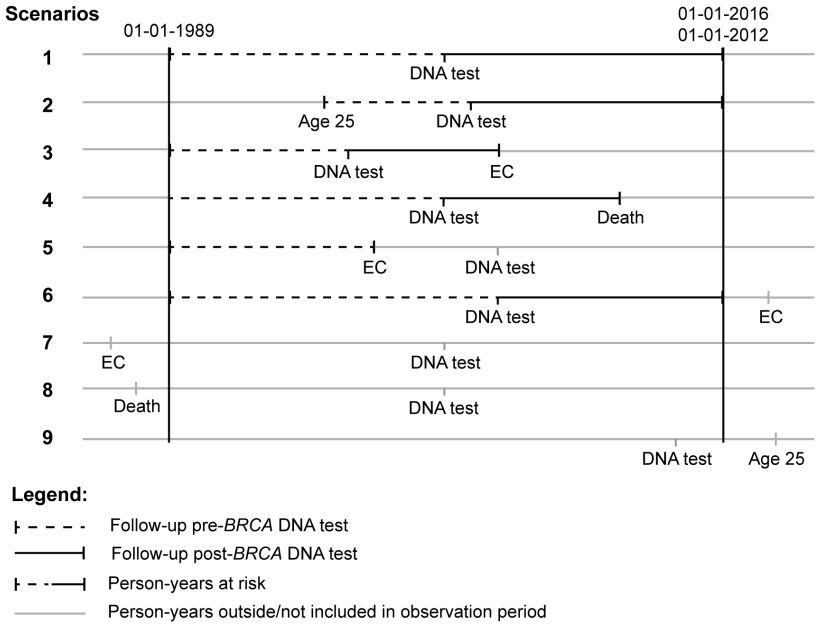
	non-*BRCA1/2* carriers	Dutch population		
EC subtype	**Observed**	**Expected**	**SIR (95% CI)**	***P* value**[a]
All endometrial cancers	33	26.81	1.23 (0.88-1.73)	0.14
Histologic groups				
Endometrioid	30	22.14	1.35 (0.95-1.94)	0.06
Serous-like	3	2.39	1.26 (0.40-3.89)	0.43
Sarcoma	0	1.73	NA	NA
Clear cell	0	0.36	NA	NA

Abbreviations: SIR: Standardized Incidence Ratio, CI: Confidence Interval. NA: not applicable
[a]*p* values were estimated assuming a Poisson distribution.

Supplementary Table 9. Cumulative endometrial cancer risks for *BRCA1/2* mutation carriers by the age of 75 years

EC subtype	**Cumulative risk (%, 95% CI)**
All Endometrial cancers	**2.97 (2.20-3.91)**
BRCA1	3.49 (2.46-4.81)
BRCA2	1.97 (1.09-3.30)
Histologic groups	
Serous-like	**1.14 (0.69-1.80)**
BRCA1	1.42 (0.79-2.37)
BRCA2	0.64 (0.21-1.60)
Endometrioid	**1.70 (1.14-2.44)**
BRCA1	1.97 (1.23-3.01)

Abbreviations, CI: confidence interval



Supplementary Figure 1. Schematic overview of the composition of different observation period scenarios. Follow-up started on the date of nationwide PALGA coverage (01-01-1989) or on the date of attaining 25 years of age (whichever was later). Follow-up ended at date of endometrial cancer diagnosis, date of death, or date of end of follow-up (01-01-2016 for *BRCA1/2* mutation carriers who provided informed consent, 01-01-2012 for all others). The observation period comprised both person-years at risk before *BRCA* DNA testing (dashed line) and person-years at risk after *BRCA* DNA testing (continuous line). Scenario 1 displays the maximum possible observation period. In case endometrial cancer or death occurred before the start of follow-up (scenarios 7 and 8), or age 25 was reached after end of follow-up (scenario 9), cases were excluded. Endometrial cancers that occurred after end of follow-up (scenario 6) were not included as events in the study. Abbreviations; EC: Endometrial cancer

Chapter 6

Validation and implementation of *BRCA1/2* variant screening in ovarian tumor tissue

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Abstract

BRCA1/2 variant analysis in tumor tissue could streamline the referral of patients with epithelial ovarian, fallopian tube, or primary peritoneal cancer to genetic counselors and select patients who benefit most from targeted treatment.

We investigated the sensitivity of *BRCA1/2* variant analysis in formalin-fixed, paraffin-embedded tumor tissue using a combination of next generation sequencing and copy number variant multiplex ligation-dependent probe amplification. After optimization using a training cohort of known *BRCA1/2* mutation carriers, validation was performed in a prospective cohort in which screening of *BRCA1/2* tumor DNA and leukocyte germline DNA was performed in parallel. *BRCA1* promoter hypermethylation and pedigree analysis were also performed.

In the training cohort, 45 of 46 germline *BRCA1/*2 variants were detected (sensitivity 98%). In the prospective cohort (*n*=62), all six germline variants were identified (sensitivity 100%), together with five somatic *BRCA1/2* variants and eight cases with *BRCA1* promoter hypermethylation. In four *BRCA1/2* variant-negative patients, surveillance or prophylactic management options were offered on the basis of positive family histories.

We conclude that *BRCA1/2* formalin-fixed, paraffin-embedded tumor tissue analysis reliably detects *BRCA1/*2 variants. When taking family history of *BRCA1/2* variant-negative patients into account, tumor *BRCA1/2* variant screening allows more efficient selection of epithelial ovarian cancer patients for genetic counseling and simultaneously selects patients who benefit most from targeted treatment.

Introduction

Germline *BRCA1/*2 pathogenic variants confer elevated lifetime risks for epithelial ovarian cancer (EOC), and especially for high-grade serous ovarian, fallopian tube and primary peritoneal cancers (HGSCs).[1-3] Analysis of 489 HGSCs by The Cancer Genome Atlas Research Network demonstrated that germline *BRCA1/2* variants, somatic *BRCA1/2* variants, and epigenetic silencing of *BRCA1* via promoter hypermethylation are frequent events, found in approximately 16%, 7% and 11% of cases, respectively.[4] Other studies reported comparable rates of *BRCA1/2* defects.[1, 3, 5-8]

The high prevalence of pathogenic germline *BRCA1/*2 variants in EOC patients led to the generally accepted recommendation that all women diagnosed with EOC should receive genetic counseling and be offered genetic testing, with some slight differences observed between countries.[9, 10] In the Netherlands, *BRCA1/2* variant screening is recommended for every EOC patient, irrespective of family history, age, and histologic subtype.[10]

BRCA1 and BRCA2 have multiple roles in maintaining genome integrity and are crucial for high-fidelity repair of DNA double strand breaks via homologous recombination-mediated repair.[11, 12] *BRCA1/2*-deficient tumors show specific genomic aberrations associated with this homologous recombination repair deficiency.[13-15] The platinum sensitivity frequently observed in HGSC is thought to be related to the underlying homologous recombination repair deficiency, because homologous recombination repair is involved in the repair of DNA damage induced by these agents.[13, 16, 17] Another group of drugs that exploit the presence of homologous recombination repair deficiency in tumor cells are the poly (ADPribose) polymerase (PARP) inhibitors. By increasing the burden on homologous recombination repair, these drugs induce synthetic lethality in tumor cells with acquired homologous recombination repair deficiency.[11, 18]

Multiple studies have shown that PARP inhibitors improve progression-free survival (PFS) in platinum-sensitive, recurrent EOC.[19-23] Although recent studies also reported a significantly longer PFS of patients with relapsed platinum-sensitive *BRCA1/2* wild-type HGSC receiving niraparib[20] or olaparib[19] compared with placebo treatment, most of the PFS benefit was observed for patients with pathogenic *BRCA1/2* variants. Therefore, identification of patients with either a somatic or a germline *BRCA1/2* variant would significantly improve the selection of patients who benefit most from PARP inhibition.[19, 20, 23]

Although pathogenic germline *BRCA1/2* variants are relatively common in EOC patients, most (approximately 85%) do not have a *BRCA1/2* variant. Referring all women with EOC for genetic counseling is therefore inefficient and causes unnecessary distress. This problem could be overcome by the integration of a reliable tumor screening test in the care pathway of

ovarian cancer patients. A test for genetic variants in *BRCA1/2* should be capable of detecting both germline and somatic variants using tumor DNA derived from formalin-fixed paraffin-embedded (FFPE) tissue. Initial use of a tumor DNA test, followed by referral of only those patients with a *BRCA1/2* variant (somatic or germline) for genetic counseling would avoid an estimated 80% of referrals.

The analysis of *BRCA1/2* in low-quality, highly-fragmented FFPE-derived tumor DNA is technically challenging because *BRCA1/2* are both large genes with a wide mutation spectrum.[24-28] Several studies, mainly using high-quality blood-derived DNA, have shown that next generation sequencing (NGS) can reliably detect *BRCA1/*2 variants.[25, 29-31] Studies analyzing the performance of NGS in FFPE-derived DNA have shown promising results[25, 32-34] but none of the studies simultaneously analyzed high-quality blood-derived DNA in a prospective setting.

The aim of this study was to investigate the performance of *BRCA1/2* variant analysis in DNA isolated from FFPE tumor tissue in comparison with sequencing of leukocyte DNA (currently the gold standard in *BRCA1/2* variant screening). On the basis of the results, we recommend integrating tumor screening within the care pathway of ovarian cancer patients.

Material and methods

Tissue sample and patient selection

Training cohort

The 50 patients in the retrospective training cohort were collected as follows. First, 67 patients were randomly selected who fulfilled the following selection criteria: previously identified germline *BRCA1/2* pathogenic variants at the Laboratory for Diagnostic Genome Analysis of the Leiden University Medical Center, and breast or gynecologic malignancy. From this cohort, 33 samples were selected by expert clinical molecular geneticists (J.T.W., N.v.d.S.) for pathogenic variants that were potentially challenging to detect, including deletions, insertions, and variants in flanking introns and homopolymer regions. An additional 17 cases with pathogenic germline variants were randomly selected (not based on type of variant) to reach a total of 50 cases (Figure 1A).

COBRA cohort

For the prospective clinical implementation of *BRCA1/2* screening in ovarian tumor tissue (COBRA) cohort, women were recruited in seven participating hospitals in the southwestern region of the Netherlands from February 2016 to June 2017. Women with (a history of) EOC and not previously screened for germline *BRCA1/2* variants, were eligible for inclusion. The cohort was enriched for HGSCs. After inclusion, leukocyte DNA was used for routine

germline analysis at the Department of Clinical Genetics. Simultaneously, FFPE tumor tissue blocks were collected for parallel tumor *BRCA1/2* screening at the Department of Pathology, thus allowing detection of both somatic and germline variants (Figure 1B). The study was approved by the medical ethics committee of the Leiden University Medical Center (reference number: P16.009). Sixty-six women gave signed informed consent and were included. Routine germline *BRCA1/2* screening and tumor *BRCA1/2* screening were requested simultaneously, either directly by the treating physician (gynecologist or medical oncologist) or by the clinical geneticist.

Histopathology slides from all cases were revised by an expert gynecopathologist (T.B.) in line with the most recent (2014) World Health Organization classification system.

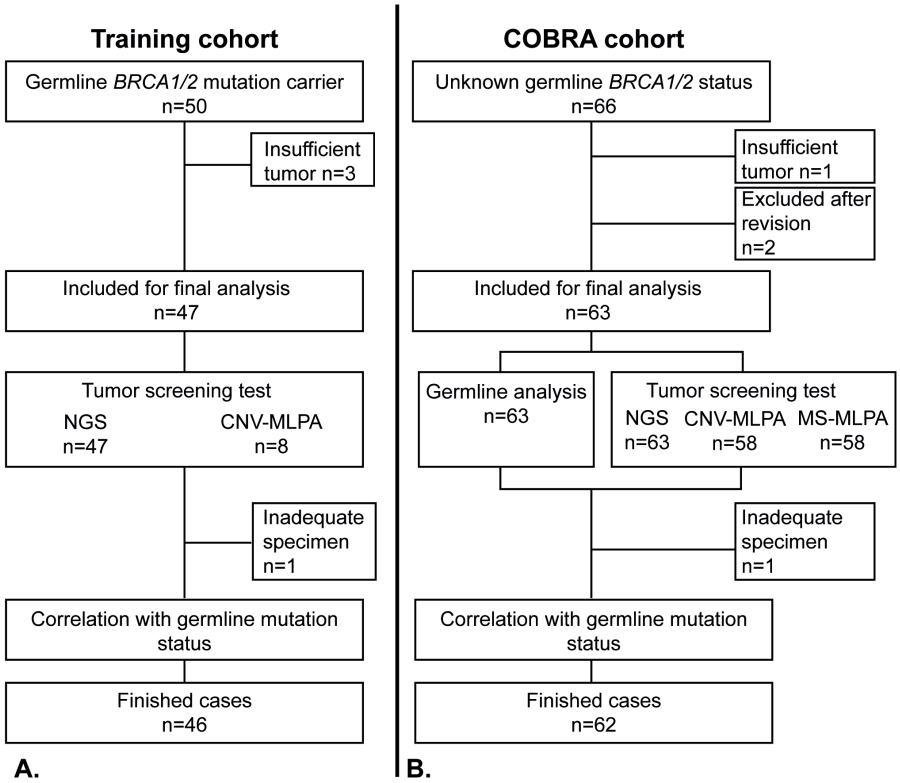

Figure 1. Schematic overview of cohort selection. A: Training cohort. Copy number variant-multiplex ligation-dependent probe amplication (CNV-MLPA) was performed only for cases in which no variant was automatically identified via the software. Of the 33 cases selected for variants that were potentially more challenging to detect, two had insufficient tumor tissue for analysis. **B:** Clinical implementation of *BRCA1/2* screening on ovarian tumor tissue (COBRA) cohort. MS, methylation specific; NGS, next-generation sequencing.

Family history

Pedigrees including first-, second- and third-degree relatives were constructed on the basis of questionnaires. The pedigrees were evaluated by expert clinical geneticists (C.J.v.A., M.N.) for tumor types and age of onset. All family histories of *BRCA1/2*-negative cases were classified on the basis of the presence or absence of an indication for extra surveillance or management options for first-degree relatives, according to current national guidelines.

DNA isolation

Tumor DNA was isolated from FFPE blocks from routine diagnostics. In most cases the tumor tissue underwent at least overnight fixation in formalin. For isolation, either three 0.6-mm tissue cores or the microdissected tumor areas from five 10-µm tissue sections was used. For the purposes of optimization, DNA from paired normal FFPE tissue was isolated and analyzed for a subset of cases in both the training cohort and COBRA cohort. The mean tumor percentage was 61% (range, 30%-90%) for the training cohort, and 65% (range, 10%-95%) for the COBRA cohort. For NGS and methylation-specific multiplex ligation-dependent probe amplification (MLPA), DNA was isolated using the automated Tissue Preparation System (Siemens Healthcare Diagnostics, Erlangen, Germany) as described previously.[35] For copy number variant (CNV) MLPA, crude DNA was manually isolated using overnight proteinase K digestion. FFPE tissue cores did not undergo deparaffinization. For microdissected samples, deparaffinization in xylene was performed, followed by rehydration through a graded ethanol series and staining with haematoxylin. Also, 20 µl of 20% chelex was added during overnight proteinase-K digestion. After overnight incubation in a heat block at 56°C, samples were heated for 10 minutes at 99°C and centrifuged at 13,000 x *g* at 4°C, after which the chelex was removed from the microdissected samples. DNA was quantified using the Qubit dsDNA HS Assay Kit, according to manufacturer's instructions (Qubit 2.0 Fluorometer; Life Technologies, Carlsbad, CA).

Next generation sequencing

BRCA1 and *BRCA2* AmpliSeq NGS libraries were prepared using the Oncomine BRCA Research panel (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. The panel contains 265 amplicons and covers 100% of the coding sequences of *BRCA1* and *BRCA2*, and it also includes flanking intronic sequences (average, 64 bases in 5' and 3' direction). Insert sizes (ie, the amplicon minus the primers) range from 65 to 138 bp. NGS libraries were equimolary pooled to 60 pMol/L, and the final library pool was loaded on an Ion PI chip (ThermoFisher Scientific) using an Ion Chef instrument (ThermoFisher Scientific). Sequencing was performed in an Ion Proton system (ThermoFisher Scientific).

Multiplex ligation-dependent probe amplification

CNV-MLPA was performed using the SALSA MLPA probemix P002 BRCA1 (MRC-Holland, Amsterdam, the Netherlands) on approximately 37.5 ng of DNA in a 20-µl reaction,

according to manufacturer's protocol, with small adaptations. Briefly, the SALSA probe mix and MLPA buffer were added to a solution containing approximately 37.5 ng of DNA and the mix was denatured for 10 minutes at 95°C, followed by hybridization at 60°C for 16 to 20 hours. Next, for ligation the Master mix (ligase buffer A, ligase buffer B and Ligase-65 enzyme) was added at 54°C and samples were heated for 20 minutes at 54°C, followed by 5 minutes at 98°C. The PCR master mix (including SALSA primer mix and SALSA polymerase) was then added, and the following PCR reaction was performed for 35 cycles: 30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°C, followed by incubation for 20 minutes at 72°C. For the training cohort, CNV-MLPA was only performed for cases in which no variant was identified via NGS data analysis. In the COBRA cohort, CNV-MLPA was performed in all cases for which sufficient tumor tissue was available.

Methylation-specific MLPA using the SALSA MLPA ME001 tumor suppressor mix (MRC-Holland) was performed, according to the manufacturer's protocol, with some adaptations. After denaturation of approximately 75 ng of DNA for 5 minutes at 98°C, the SALSA probe mix and MLPA buffer were added and samples were incubated for 1 minute at 98°C, followed by hybridization at 60°C for 16 to 20 hours. Then, ligase buffer A was added at room temperature, and the samples were heated for 2 minutes at 48°C. Samples were then split and ligated for 30 minutes at 48°C (ligase buffer B and Ligase-65 enzyme, with or without the addition of HhaI enzyme), followed by heating for 5 minutes at 98°C. After the master mix was added (SALSA primer mix and SALSA polymerase), a PCR reaction was performed for 35 cycles (30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°C), followed by incubation for 20 minutes at 72°C. Methylation-specific MLPA was performed for all cases from the COBRA-cohort with a [DNA] >7 ng/µl. MLPA data were analyzed using Coffalyser.Net software (MRC-Holland).

For both tests, the ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA) was used for separation of the products by electrophoresis.

Data analysis

The unaligned bam files generated by the proton sequencer were mapped against the human reference genome (GRCh37/hg19) using the TMAP 5.0.7 software with default parameters (https://github.com/iontorrent/TS, last accessed March 6, 2018). Subsequent variant calling was done using the Ion Torrent specific caller, Torrent Variant Caller 5.0.2 (Thermo Fisher Scientific), using the recommended somatic variant caller parameter for the BRCA Oncomine Panel. Briefly, variants were called with a minimum allele frequency threshold of 3.5% and a read depth of at least 100. Strand bias and proximity to a homopolymer region were also used to minimize false positives.

Integrative Genomics Viewer was used for visual inspection of the detected variants. Variants were imported into a local Genetic Assistant database (Geneticist Assistant, Version: 1.4.5;

SoftGenetics, State Collega, PA) which assigns functional prediction, conservation scores, and disease-associated information to each variant. This information is then used to assign pathogenicity to a variant, and the next time the variant is observed, the same pathogenicity is automatically attributed to the observed variant. Variant annotation was based on the NM_007294.3 and the NM_000059.3 transcripts to *BRCA1* and *BRCA2,* respectively.

Data interpretation

Variants were categorized by five-tier pathogenicity status [class 1, benign; class 2, likely benign; class 3, variant of unknown significance (VUS); class 4, likely pathogenic; class 5, pathogenic).[36]

For the training cohort, FFPE-isolated DNA was analyzed at the pathology department (Leiden University Medical Center). Although all cases were known to carry a class 4 or 5 *BRCA1* or *BRCA2* variant, it was not known which germline variant was present in the samples at the time of analysis. All variants identified were later compared with the previously identified germline variant (Figure 1A). For the COBRA cohort, the *BRCA1/2* tumor screening (at the Pathology Department of the Leiden University Medical Center) was performed concurrently with, but independently of, routine leukocyte germline screening (at the Department of Clinical Genetics, Leiden University Medical Center). On completion, the class 3, 4, and 5 variants identified in tumor DNA were compared with the results of the germline analysis (Figure 1B).

Loss of heterozygosity (LOH) of *BRCA1/2* was determined by comparing the variant allele frequency (VAF) of heterozygous SNPs and, when present, the VAF of the *BRCA1/*2 variant in tumor and normal tissue. LOH was considered present when the tumor cell percentage was >20%, the germline *BRCA1/2* variant allele frequency was >60% and/or at least two informative (heterozygous) single nucleotide variants (SNVs) showed a VAF ≤ 0.4 or ≥ 0.6. LOH was considered inconclusive when the tumor cell percentage was <20% or when only one informative SNV was present. LOH was considered absent when the germline *BRCA1/2* variant VAF was ≤ 0.6 and/or at least two informative (heterozygous) SNVs showed a VAF between 0.4 and 0.6, unless a clear difference in VAF of the SNV and/or variant could be observed between the normal DNA sample and the tumor DNA sample. LOH results were manually curated (T.v.W., R.v.E.), taking the tumor cell percentage and the VAF of the SNV or variant into account. SNVs were annotated in an in-house database (geneticist assistant).

Quality control

Sample quality was evaluated by an experienced molecular biologist (T.v.W., R.v.E.). Samples with a low coverage, a high number of low-frequency variants, or a high proportion of C:G>T:A transitions (ie, artifacts caused by formalin fixation)[26, 37] were excluded from further analysis. However, an unequivocal class 3, 4 or 5 variant identified in a poor quality sample

was considered sufficient for analysis. For the training cohort, a patient was only excluded from the final analysis if both the tumor DNA sample and the normal DNA sample failed the quality control.

Statistics

IBM SPSS software version 23.0 (IBM Corp., Armonk, NY) was used for statistical analysis. A one-way ANOVA analysis of variance was used to compare age distributions, and the Mann-Whitney U-test was used for comparison of the age of the tissue blocks. The association between histotype and *BRCA1/2* defects was tested using a two-sided Fisher's exact test. $P \leq 0.05$ was considered significant.

Results

Training cohort

Of the 50 cases in the training cohort, three were excluded because no tumor tissue was available in the archives. For the remaining 47 patients, matching normal tissue DNA was analyzed in 42 cases. Forty-six patients could be included in the final analysis because either the tumor (42/47) or the paired normal (40/42) tissue sample was sequenced with sufficient quality (Figure 1A), hence mutation status was determined on normal FFPE tissue only for four cases. One case was excluded from the analysis because sequencing results for both the tumor and the normal DNA were of insufficient quality. Tissue blocks used for DNA isolation were significantly older for the samples that failed the quality control (n=7; median, 2003; range, 1994-2014) compared to the samples that passed quality control (n=82; median, 2008; range, 1986-2015; $P < 0.05$). The median coverage per amplicon of the samples included in the final analysis is visualized in supplemental Figure S1. All 265 amplicons had a median coverage of at least 100 reads. Per sample, 98% of the amplicons (range 51.3% to 100%) were covered with a sequencing depth of at least 100 reads. Sample R27 (normal FFPE DNA) was an outlier, with only 51.3% of amplicons covered by >100 reads and 10 amplicons that completely failed. Nevertheless, a *BRCA1* variant was clearly detected, and the sample was, therefore, considered to be of sufficient quality for analysis (supplemental Table S1).

Variant analysis

The germline variants found in the 46 cases included in the final analysis are listed in Table 1. In 38 of the 46 cases (83%), a variant (SNV, small insertion or deletion) was detected during initial analysis. The *BRCA1/2* variants could be identified in both the normal and tumor DNA for all samples in which both were analyzed. All germline variants were covered by at least 100 reads and 76% of the variants had a coverage of >1000 reads.

Table 1. Germline variants in the training cohort

ID	Gene	c.DNA Change*	Amino acid change†	T%	VAF tumor	VAF normal	LOH	Histology
R31‡	BRCA1	c.68dupA	p.Cys24fs	40	0.83	0.48	Yes	EEC
R12	BRCA1	c.34C>T	p.Gln12*	70	0.96	0.43	Yes	HGSC
R35‡	BRCA1	c.81-6T>A	p.?	80	0.92	0.53	Yes	HGSC
R49	BRCA1	c.181T>G	p.Cys61Gly	70	0.81	n.a.	Yes	EEC
R11	BRCA1	c.181T>G	p.Cys61Gly	70	0.89	0.51	Yes	HGSC
R19	BRCA1	c.213-12A>G	p.?	40	0.70	0.51	Yes	EEC
R28‡	BRCA1	c.213-12A>G	p.?	65	0.74	0.56	Yes	Breast-NST
R20	BRCA1	c.[594-2A>C;c.641A>G]§	p.?	35	0,57 & 0,61	0,46 & 0,47	n.a.	LGSC
R3‡	BRCA1	c.1292dupT	p.Leu431fs	70	0.77	n.a.	Yes	HGSC
R39‡	BRCA1	c.2019delA	p.Glu673fs	60	0.73	0.45	Yes	Breast-Metaplastic
R34‡	BRCA1	c.2197_2201delGAGAA	p.Glu733fs	60	n.a. ||	0.51	n.a.	Breast-NST
R2‡	BRCA1	c.3436_3439delTGTT	p.Cys1146fs	55	0.73	0.55	Yes	Breast-ILC
R32‡	BRCA1	c.3481_3491delGAAGATACTAG	p.Glu1161fs	80	0.70¶	n.a.	Yes	HGSC
R25‡	BRCA1	c.3485delA	p.Asp1162fs	40	0.61	0.47	Yes	HGSC
R47‡	BRCA1	c.3820dupG	p.Val1274fs	80	0.97	0.47	Yes	Breast-NST
R44‡	BRCA1	c.4035delA	p.Glu1346fs	40	0.58	0.48	Not detected	HGSC
R7	BRCA1	c.4327C>T	p.Arg1443*	80	0.94	0.48	Yes	USC
R14	BRCA1	c.4327C>T	p.Arg1443	50	0.73	0.52	Yes	HGSC
R17	BRCA1	c.4327C>T	p.Arg1443	70	0.84	0.46	Yes	HGSC
R4‡	BRCA1	c.4483delA	p.Arg1495fs	60	0.53	0.51	Not detected	Breast-NST
R9	BRCA1	c.5177_5180delGAAA	p.Arg1726fs	90	n.a.¶	0.48	Yes**	HGSC
R27‡	BRCA1	c.5177_5180delGAAA	p.Arg1726fs	70	0.92	0.54	Yes	Ovarian-Mixed††
R18	BRCA1	c.5266dupC	p.Gln1756fs	75	0.99	0.49	Yes	EOC
R37‡	BRCA1	c.5266dupC	p.Gln1756fs	50	0.80	0.50	Yes	Breast-NST
R5‡	BRCA2	c.658_659delGT	p.Val220fs	60	0.47	0.43	Not detected	Breast-NST
R48	BRCA2	c.771_775delTCAAA	p.Asn257fs	80	0.81	0.56	Yes	Breast-NST
R43‡	BRCA2	c.1147delA	p.Ile383fs	50	0.84	n.a.	Yes	Breast-NST
R46‡	BRCA2	c.1147delA	p.Ile383fs	60	0.71	0.52	Yes	HGSC
R38‡	BRCA2	c.1899_1900insTT	p.Ala634fs	60	0.60	0.49	Yes	Breast-Mixed‡‡
R22‡	BRCA2	c.3599_3600delGT	p.Cys1200*	30	0.63	0.41	n.a.	Breast-NST
R24‡	BRCA2	c.4284dupT	p.Gln1429fs	70	§§	§§	Yes	Breast-NST

Continue

Continued

ID	Gene	c.DNA Change*	Amino acid change†	T%	VAF tumor	VAF normal	LOH	Histology
R33‡	BRCA2	c.5213_5216delCTTA	p.Thr1738fs	80	0.90	n.a.||	Yes	OCS
R10	BRCA2	c.5286T>A	p.Tyr1762*	80	0.71	0.62	Yes	HGSC
R8	BRCA2	c.5682C>G	p.Tyr1894*	60	0.91	0.56	Yes	HGSC
R29‡	BRCA2	c.5946delT	p.Ser1982fs	60	0.86	0.52	Yes	Breast-NST
R21‡	BRCA2	c.6270_6271delTA	p.His2090fs	40	0.75	0.51	Yes	OSC||||
R45‡	BRCA2	c.6275_6276delTT	p.Leu2092fs	70	0.79	0.51	Yes	Breast-NST
R42‡	BRCA2	c.6361_6362delGA	p.Glu2121fs	55	0.88	0.47	Yes	HGSC
R23‡	BRCA2	c.6816_6817delAA	p.Gly2274fs	70	n.a.||	0.38	n.a.	HGSC
R1‡	BRCA2	c.9099_9100delTC¶¶	p.Gln3034fs	50	0.69	0.30	Yes	Breast-NST
R36‡	BRCA2	c.9295_9301delAATTTAC	p.Asn3099fs	60	0.69¶|***	0.48¶|***	Yes	HGSC
CNV-MLPA								
R50	BRCA1	Deletion exon 8 and 9		85	n.a.||	NAP	n.a.	OCS
R15	BRCA1	Deletion exon 22		60	NAP	NAP	n.a.	HGSC
R40‡	BRCA1	Deletion exon 22		30	NAP	n.a.	Yes	HGSC
R26‡	BRCA1	c.5503_5564del	p.Arg1835fs	30	NAP	NAP	Yes	Breast-NST
R41‡	BRCA1	c.5503_5564del	p.Arg1835fs	35	NAP	NAP	Yes	Breast-NST

*Reference sequences: NM_007294.3 for *BRCA1* and NM_000059.3 for *BRCA2.* †NP_009225.1 for BRCA1 and NP_000059.3 for BRCA2. ‡Selected by expert clinical molecular geneticists for variants potentially more challenging to detect, including deletions, insertions, variants in flanking introns and in homopolymer regions. §Reclassified as a variant of uncertain significance. ||quality control failed. ¶Automatically identified after adjustment of the alignment settings. **Amplification of one of the primer pools failed; LOH based on SNVs identified in the succeeded primer pool. ††Clear cell carcinoma-endometrioid carcinoma. ‡‡NST-mucinous. §§Not detected; duplication in homopolymeric region. ||||Grading not reliable because of previous treatment. ¶¶Because of noise at the border of an 8-base pair adenine stretch, the deletion was automatically classified as delACT, but was later manually curated. ***Detected with prior knowledge of the position of the deletion.

Abbreviations; CNV-MLPA, copy number variant-multiplex ligation-dependent probe amplification; EEC, endometrioid endometrial carcinoma; EOC, endometrioid ovarian carcinoma; HGSC, high-grade ovarian, fallopian tube and primary peritoneal cancer; ID, identification; ILC, invasive lobular carcinoma; LGSC, low-grade serous carcinoma; LOH, loss of heterozygosity; NST, invasive carcinoma of no special type; n.a.,not analyzed/not analysable; NAP, not applicable, Nucl., nucleotide; OCS, ovarian carcinosarcoma; OSC, ovarian serous carcinoma; T%, tumor percentage; USC, uterine serous carcinoma; VAF, variant allele frequency.

Deletions and duplications

To detect exon deletions and duplications in *BRCA1*, CNV-MLPA was performed for the eight samples in the training cohort in which no variant was initially detected by the pipeline [either using tumor DNA (n=4), normal DNA (n=2) or both (n=2)]. This resulted in the detection of two germline deletions of exon 22 (R15 and R40), one germline deletion of exons 8 and 9 (R50) and two 62-basepair deletions in exon 24 [c.5503_5564del62, p.Arg1835Thrfs*24 (R26, R41)].

Visual inspection of the sequencing reads in Integrative Genomics Viewer for the remaining three samples revealed an 11-bp deletion (*BRCA1***;** c.3481_3491delGAAGATACTAG) and a 7-bp deletion (*BRCA2*; c.9295_9301delAATTTAC) in samples R32 and R36, respectively. Both deletions were situated at the end of a PCR amplicon, with only a few base pairs left on the short side, resulting in misalignment of the reads. Adjustment of the alignment settings improved the alignment of the reads resulting in automatic identification of both deletions (Supplemental Figure S2).

In sample R24, a known *BRCA2* variant could not be identified. The patient carried a germline duplication (c.4284dupT) in a homopolymer stretch of six thymidines. The duplication could not be identified because of sequencing artifacts present at homopolymer regions (Supplemental Figure S3).

Loss of Heterozygosity

LOH of the wild-type allele was observed in 37 cases (Table 1), whereas three cases did not show LOH. In the remaining six cases, the presence of LOH could not be determined with certainty because of a lack of informative SNPs and/or failure of sequencing of tumor DNA. Of the 16 HGSCs in which LOH could be determined, all but one showed LOH [15/16 (94%)].

Prospective COBRA cohort

In total, 66 women were recruited to participate in the prospective phase of the study (Figure 1B). Four cases (6%) were excluded from the final analysis for the following reasons:

Table 2. COBRA cohort characteristics

	Total cohort	No *BRCA1/2* defect	*BRCA1/2* variant	*BRCA1* promoter hypermethylation	*P* value
Total, *n* (%)	62 (100)	43 (100)	11 (100)	8 (100)	
Age in years, mean (range)	64 (47-89)	66 (47-89)	62 (50-69)	62 (56-71)	0.3
Tumor Type					
HGSC, *n* (%)	54 (87)	35 (81)	11 (100)	8 (100)	0.093*
Non-HGSC, n (%)†	8 (13)	8 (19)	0 (0)	0 (0)	

*The prevalence of HGSC and non-HGSC was compared between women with and without *BRCA1/2* defects.
†The non-HGSC consisted of two low-grade serous carcinomas, two endometrioid ovarian carcinomas, three ovarian clear cell carcinomas, and one ovarian carcinosarcoma.
Abbreviations; COBRA, clinical implementation of *BRCA1/2* screening on ovarian tumor tissue; HGSC, high-grade ovarian, fallopian tube, and primary peritoneal carcinoma.

insufficient tumor tissue available (n=1), quality control of tumor failed (n=1) or no ovarian malignancy after histologic revision (n=2, one metastatic endometrial cancer and one ovarian serous borderline tumor). The characteristics of the COBRA cohort are summarized in Table 2. Fifty-four patients (87%) were diagnosed with HGSC and eight patients (13%) were diagnosed with other histologic subtypes of EOC.

Of the 62 cases included in the final analysis, matched normal FFPE-derived DNA was analyzed for 37 (60%), of which four failed quality control (Supplementary Table S1).

Variant analysis was performed on FFPE cytology material for three samples, two obtained from cytocentrifuged effusions [pleural fluid (P10) and ascites (P60)], and one obtained from a lymph node puncture (P64). All produced data of sufficient quality.

Variant analysis

In total, 11 class 3, 4, or 5 *BRCA1/2* variants were identified in the tumors of 62 EOC patients (Table 3). The 10 detected variants by NGS comprised seven *BRCA1* variants, including three VUSs, and three *BRCA2* variants, including one VUS. One genomic deletion of *BRCA1* exon 22 was detected by CNV-MLPA. For six of the mutated cases in which a variant was detected by NGS, matching normal FFPE-derived DNA was analyzed, five of which produced good quality data. In one case (P30), the variant was also detected in normal FFPE material, suggesting a germline origin. The variants in P11, P14, P52 and P39 were likely somatic, given their absence in the matched normal DNA samples.

Results were compared with leukocyte germline DNA, with findings summarized in Table 3. In the leukocyte DNA, four germline *BRCA1* variants and two germline *BRCA2* variants were detected, all of which were also detected in tumor DNA, resulting in a 100% concordance in the detection of germline variants between the tumor DNA and leukocyte DNA. The remaining four *BRCA1* variants (including two VUSs) and one *BRCA2* variant were somatic variants because they were not detected in the germline DNA. No germline variants were detected in the remaining 51 samples without a *BRCA1/2* variant in tumor DNA.

BRCA1 promoter hypermethylation

With possible future clinical relevance in mind, *BRCA1* promoter hypermethylation was also analyzed in the tumors. *BRCA1* promoter hypermethylation was found in 8 of 57 (14%) cases that had sufficient tumor DNA available for methylation-specific MLPA. None of these cases had a concurrent pathogenic *BRCA1/2* variant.

All 19 *BRCA1/2* defects (germline variants, somatic variants and hypermethylated cases) were detected in patients with HGSC. There was no significant difference in age distribution between women with a *BRCA1/2* variant, with *BRCA1* promoter hypermethylation or lacking a

Table 3. *BRCA1/2* defects in the COBRA cohort

ID	Histology	Gene	cDNA change*,†	Amino acid change‡	T%	VAF tumor	VAF normal	LOH wild-type allele
Germline variants								
p18	HGSC	BRCA1	c.1881C>G§	p.Val627=	70	0,80	n.a.	yes
p32	HGSC	BRCA1	c.2685_2686delAA	p.Pro897fs	85	0,98	n.a.	yes
p56	HGSC	BRCA1	c.5277+1G>A	p.?	80	0.74	n.a.	yes
p30	HGSC	BRCA2	c.4576dupA	p.Thr1526fs	80	0,97	0,48	yes
p62	HGSC	BRCA2	c.5117A>C§	p.Asn1706Thr	80	0.54	n.a.	No
CNV-MLPA, germline								
p41	HGSC	BRCA1	Deletion exon 22	p.?	30	NAP	NAP||	yes
Somatic variants								
p24	HGSC	BRCA1	c.3718C>T	p.Gln1240*	80	0,76	Not present	yes
p11	HGSC	BRCA1	c.3858_3861delTGAG	p.Ser1286fs	70	0,56	Not present	yes
p52¶,**	HGSC	BRCA1	c.4868C>G§	p.Ala1623Gly	40	0.37	Not present	Yes††
p39	HGSC	BRCA1	c.5366C>T§	p.Ala1789Val	95	0.65	Not present	uncertain
p12	HGSC	BRCA2	c.209_210delCT	p.Ser70fs	70	0,82	n.a.‡‡	yes
MS-MLPA								
p7	HGSC	BRCA1	promoter hypermethylation	p.?	80	NAP	n.a.	uncertain
p15	HGSC	BRCA1	promoter hypermethylation	p.?	35	NAP	n.a.	yes
p17	HGSC	BRCA1	promoter hypermethylation	p.?	80	NAP	n.a.	yes
p23	HGSC	BRCA1	promoter hypermethylation	p.?	85	NAP	NAP	yes
p25	HGSC	BRCA1	promoter hypermethylation	p.?	70	NAP	NAP	yes
p36	HGSC	BRCA1	promoter hypermethylation	p.?	95	NAP	NAP	yes
p58	HGSC	BRCA1	promoter hypermethylation	p.?	70	NAP	n.a.	uncertain
p59	HGSC	BRCA1	promoter hypermethylation	p.?	70	NAP	n.a.	uncertain

All variants had a coverage well above 100 reads, reaching >1000 reads in 10 of 11 cases (91%). *Only class 3 (variant of unknown significance), class-4 (likely pathogenic), and class 5 (pathogenic) variants are reported. †Reference sequences: NM_007294.3 for *BRCA1* and NM_000059.3 for *BRCA2*. ‡Reference sequences: NP_009225.1 for BRCA1 and NP_000059.3 for BRCA2. §Variant of unknown significance. ||CNV-MLPA not performed on normal DNA sample. ¶DNA concentration too low to perform MS-MLPA. **Not enough tumor to perform CNV-MLPA. ††LOH of the mutant allele. ‡‡Quality control failed.

Abbreviations; CNV-MLPA, copy number variant-multiplex ligation-dependent probe amplification; COBRA, clinical implementation of *BRCA1/2* screening on ovarian tumor tissue; HGSC, high-grade ovarian, fallopian tube and primary peritoneal cancer; ID, identification, LOH, loss of heterozygosity; MS-MLPA, methylation specific-multiplex ligation-dependent probe amplification; n.a: not analyzed/not analyzable; NAP, not applicable; QCF, quality control failed; T%, tumor percentage; VAF, variant allele frequency.

BRCA1/2 defect (*P*=0.3) (Table 2). In cases with a *BRCA1/2* defect, LOH of the wild-type allele could be determined for 15 of 19 cases (79%). All but one case (93%) showed LOH, one of which was of the mutated allele (P52). The tumor in which no LOH was demonstrated and the one with LOH of the mutant allele both carried a VUS. No informative SNVs were present on the *BRCA1* alleles for the remaining four cases, precluding the analysis of LOH (three with

BRCA1 promoter hypermethylation and one with *BRCA1* variant). None of the six patients with a germline *BRCA1/2* variant had other malignancies in their personal history.

Comparing the frequencies of *BRCA1/2* defects in HGSC with The Cancer Genome Atlas Research Network, fewer germline mutated cases (11% versus 16%), more somatic mutated cases (9% versus 7%) and more cases with *BRCA1* promoter hypermethylation (16% versus 11%) (Supplementary Figure 4).[4]

Family history

Of the 62 patients included in the final analyses, 57 questionnaires regarding family histories were returned, which were then studied by clinical geneticists for suggestions that there was an indication for extra surveillance or management options. Regarding patients without germline *BRCA1/*2 variants, family history would have resulted in policy changes for four patients. Three patients had a positive first-degree family history for OC (P12, P52 and P59), and one patient was suspect for Lynch syndrome (i.e. fulfilled the Bethesda criteria; P55). In families with two cases of EOC but no germline variant, the ovarian cancer risk for first-degree female family members is >10%, a level at which prophylactic surgery should be considered.[38] The patient with a positive first-degree family history for colon cancer <50 years of age had a prior clear cell renal cell carcinoma but no personal history for colon cancer or endometrial cancer. Immunohistochemical staining for mismatch repair proteins did not show abnormalities, making Lynch syndrome unlikely. Nevertheless, because the family fulfilled the familial colorectal cancer criteria, advice for 5-yearly screening of the colon was given.[39]

Discussion

The aim of this study was to evaluate the reliability of *BRCA1/2* variant analysis on FFPE-derived tumor DNA, using a tumor test consisting of semiconductor sequencing with an amplicon-based *BRCA1/*2 panel combined with CNV-MLPA for *BRCA1.* During optimization of the tumor test on the training cohort, 45 of 46 variants were detected, representing a sensitivity of 98% despite enrichment for challenging variants. During prospective validation in the COBRA cohort, all six germline *BRCA1/2* variants in tumor DNA were identified (sensitivity of 100%), together with the identification of an additional five somatic *BRCA1/2* variants and eight cases with *BRCA1* promoter hypermethylation. These results show that *BRCA1/2* variants can be reliably detected in FFPE-derived DNA. In the COBRA cohort, referral based on a positive tumor *BRCA1/*2 variant screening test result may have reduced the referral rate of EOC patients to a clinical geneticist by approximately 80%.

The recent approval of the PARP inhibitors niraparib (US Food and Drug Administration, March 2017; European Medicines Agency, November 2017) and olaparib (US Food and Drug

Administration, August 2017) as maintenance treatment for platinum-sensitive relapsed HGSC regardless of *BRCA1/2* mutation status may undermine the necessity for tumor testing to detect somatic *BRCA1/2* variants. However, these approvals were based on studies showing treatment benefit (ie PFS) of PARP inhibitors in a highly-selected patient population (namely, those patients with platinum-sensitive recurrent HGSC who received at least two lines of platinum-based chemotherapy).[19, 20] *BRCA1/2* loss is known to confer sensitivity to platinum-based chemotherapy, and tumors with similar genomic scars without apparent *BRCA1/2* loss also show increased sensitivity to these agents.[13] Therefore, platinum sensitivity already selects tumors that probably carry DNA repair defects conferring sensitivity to PARP inhibitors. When platinum-based chemotherapy cannot be given or in the event that PARP inhibitors become indicated for adjuvant treatment in the future, this surrogate marker will not serve for patient selection and additional biomarkers will be needed. For the time being, known somatic and germline *BRCA1/2* mutation status helps in the selection of those patients who will derive the greatest treatment benefit from PARP inhibitors.[19, 20, 23] For example, in the study by Ledermann et al.,[19] compared with placebo, women carrying *BRCA1/2* variants showed longer PFS (11.2 versus 4.3 months) than women without *BRCA1/2* variants (7.4 versus 5.5 months).

Although patients with EOC have the highest *a priori* probability for germline variants in *BRCA1/2*, other germline predisposing variants such as *BRIP1, RAD51D* or *RAD51C* have been described.[3, 9] It is, therefore important that patients with a positive family history should still be referred to the clinical genetic services, independent of the result of a *BRCA1/2* tumor test. For example, in the COBRA cohort four patients without a germline *BRCA1/2* variant had a positive family history for either ovarian cancer or colon cancer, which can be an indication to screen for variants in additional genes or for relatives to consider prophylactic surgery. A more comprehensive tumor test incorporating additional genes seems feasible, so this limitation will likely be overcome in the future.

In the COBRA cohort, *BRCA1* promoter hypermethylation was observed in 14% of EOCs. Although hypermethylation is a well-known and common event in HGSC, its clinical relevance remains unclear. The presence of LOH in tumors with *BRCA1* promoter hypermethylation, in combination with the observed homologous recombination deficiency via functional analysis,[15] suggests that hypermethylation is an important driver of tumorigenesis. PARP inhibitor sensitivity is observed in breast cancer cell lines and xenograft tumors with epigenetic *BRCA1* silencing.[40, 41] However, it remains unclear whether this increased sensitivity also applies to patients with *BRCA1* hypermethylated EOC. In a recent study, *BRCA1* hypermethylation was not associated with an increased PARP inhibitor response,[42] whereas in the ARIEL2 trial a subset of *BRCA1*-methylated EOC showed a longer PFS.[23] In the absence of clear data on clinical consequences, testing for *BRCA1* promoter hypermethylation in routine diagnostics may be unnecessary at this time.

It is noteworthy that different populations show different common *BRCA1/2* variants.[43] For example, *BRCA1* genomic deletions are common founder variants in the Dutch population,[44, 45] whereas large deletions in *BRCA2* are rare. CNV-MLPA for *BRCA2* is therefore not routinely performed. In countries in which *BRCA2* exon deletions are more common (eg. Australia and Italy),[45] additional *BRCA2* CNV-MLPA might be necessary.

The wide mutation spectrum seen in *BRCA1/2* and the presence of variants for which the clinical significance is unclear make interpretation of results challenging.[24] Of the six germline *BRCA1/2* variants identified in the COBRA cohort, two were VUSs. Because this category of variants has unclear pathogenicity, it is important that they are discussed in a multidisciplinary team that includes an expert clinical molecular geneticist.[46]

In the training cohort, we showed the importance of optimizing the bioinformatics process for data analysis to prevent variants present in the sequencing data from not being reported automatically. This was also shown by others.[47]

Because *BRCA1/2* screening of ovarian tumor tissue has proved to be a reliable test both in this study and in previous studies,[25, 32] we propose that screening of tumor tissue for *BRCA1/2* variants should be implemented in routine diagnostics as illustrated in Figure 2. Using the tumor screening test to identify women with *BRCA1/2* variants (either germline or somatic in origin) provides an efficient selection method for referral to clinical genetic services. This scheme resembles the previously adopted Lynch syndrome tumor screening program for colorectal and endometrial cancer.[9, 48] When a *BRCA1/2* variant is identified in the tumor screening test, women can be referred for genetic counseling and may subsequently decide whether they want to know if the variant has a germline origin. This scheme is particularly beneficial to those patients (and their relatives) without a *BRCA1/2* variant, as tumor screening will prevent unnecessary distress because of a possible hereditary origin of the EOC. An additional advantage of tumor screening is that subsequent germline analysis only requires verification of a specific variant, avoiding the need (and associated costs) for whole-gene scanning. On the basis of these considerations, implementation of *BRCA1/2* tumor screening in the care pathway of EOC patients may be an efficient and patient-friendly approach.

Although *BRCA1/2* tumor screening proved to be highly sensitive, some technical limitations were observed. Sequencing artifacts present in homopolymer regions prevented the detection of one *BRCA2* variant in the training cohort (Supplemental Figure S3). Previous studies have already highlighted the high rates of error in insertion/deletion calling associated with homopolymer regions.[25, 29, 30, 49, 50] On the basis of data extracted from the Leiden Open Variant Database (http://www.lovd.nl/3.0/home, last accessed October 13, 2017),[28] in combination with our institutional data, we estimate that approximately one homopolymer germline *BRCA1/2* variant in every 250 patients screened could be missed (Supplemental Table S2). Use

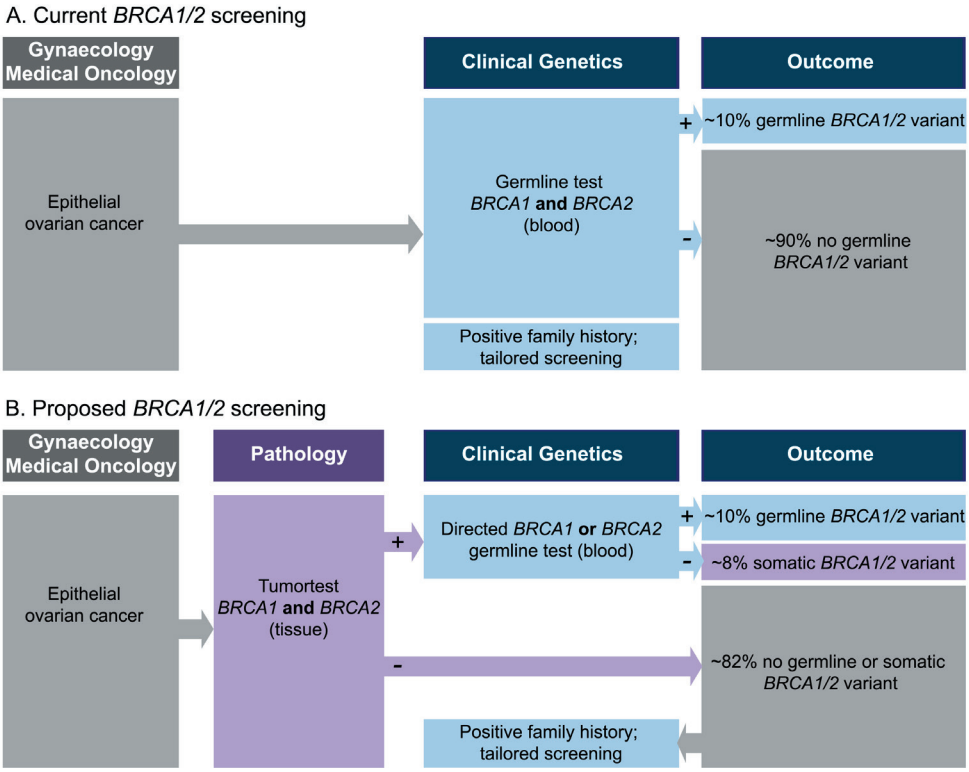

Figure 2. Flowchart illustrating the current epithelial ovarian cancer (EOC) *BRCA1/2* screening pathway (A) and the proposed EOC *BRCA1/2* tumor screening pathway (B). The integration of tumor tissue analysis for *BRCA1/2* variants as part of the ovarian cancer patient pathway is more efficient because it avoids referral of most patients when only those women carrying a *BRCA1/2* mutation or having a suspected family history are being referred for genetic counseling. Percentages are based on the Clinical implementation of *BRCA1/2* screening on ovarian tumor tissue cohort.

of improved sequencing chemistry or sequencing platforms that show better performance with homopolymer regions will mitigate this problem.[50]

A technical limitation, which applies to all amplicon-based sequencing techniques, is the possibility of variants being located at amplicon ends or primer binding sites. Because FFPE-derived DNA is highly fragmented, shorter amplicons are needed, thus increasing the chance of variants being present in amplicon edges or primer locations.

In this study, we optimized and clinically validated a *BRCA1/2* variant tumor screening test of FFPE material. It was demonstrated that the test has adequate sensitivity to detect *BRCA1/2* variants. Therefore, a workflow in which *BRCA1/2* tumor screening is requested by the treating physician and is integrated in routine care for all EOC patients is recommended. This will allow more efficient patient selection for precision medicine, genetic counseling and

preventive options. Awareness of family history remains important and referral to genetic services should be based on both the detection of variants in the tumor test and the presence of affected cases in family histories.

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Supplemental tables

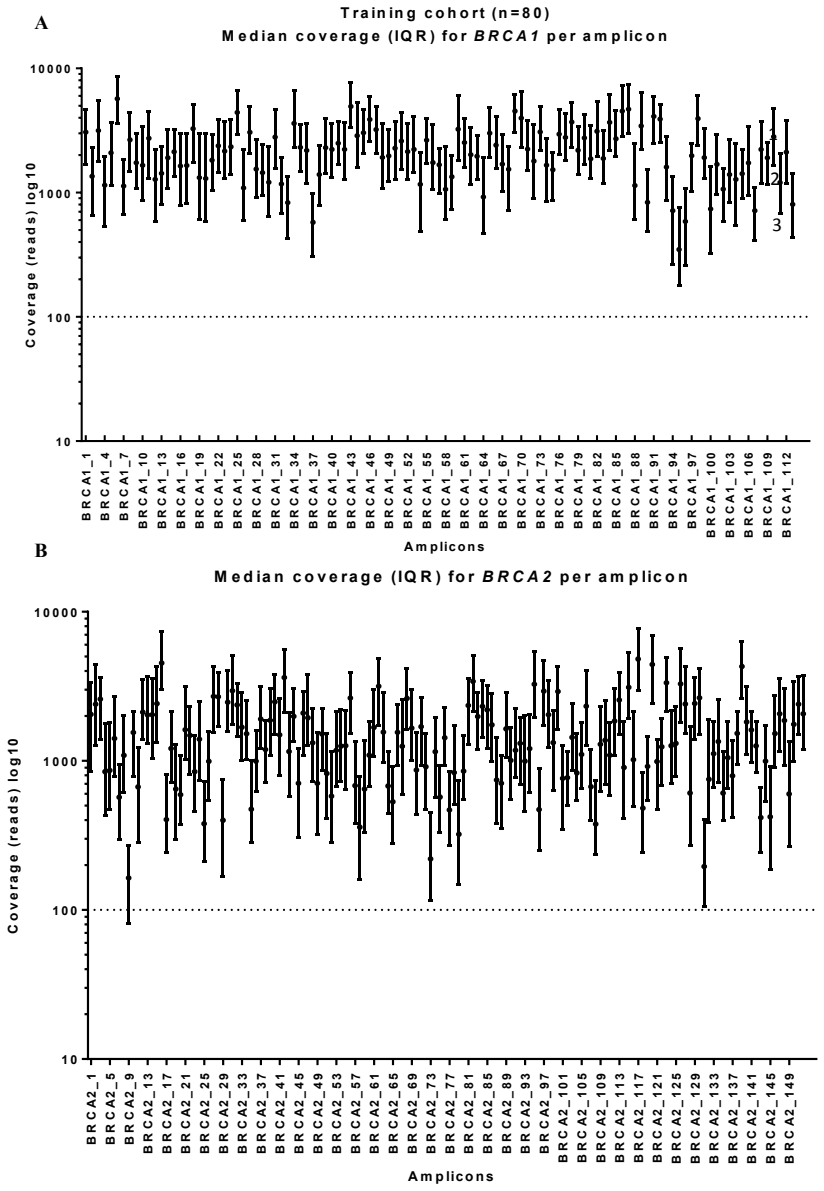
Supplemental Table S1. Samples of insufficient quality for analysis

SampleID	Amplicons covered with 0 reads		Amplicons covered with <100 reads		Reason QC-failed
	Number	%	Number	%	
Training cohort					
R9-tumor	73	27,5	148	55,8	Amplification failure of the majority of amplicons
R16-tumor	18	6,8	237	89,4	Amplification failure of the majority of amplicons and high number of transitions
R16-normal	24	9,1	239	90,2	Amplification failure of the majority of amplicons and high number of transitions
R23-tumor	7	2,6	101	38,1	Amplification failure of the majority of amplicons and high number of transitions
R33-normal	82	30,9	265	100	Amplification failure of the majority of amplicons
R34-tumor	6	2,3	265	100	Amplification failure of the majority of amplicons
R50-tumor	34	12,8	265	100	Amplification failure of the majority of amplicons
COBRA cohort					
p2-normal	36	13,6	129	48,7	Amplification failure of the majority of amplicons
p12-normal	17	6,4	20	7,5	Amplification failure of multiple amplicons
p13-normal	4	1,5	165	62,3	Amplification failure of multiple amplicons
P21-tumor	14	5,3	158	59,6	Amplification failure of the majority of amplicons and high number of transitions
P21-normal	10	3,8	143	54,0	Amplification failure of the majority of amplicons and high number of transitions
P53-normal	118	44,5	265	100	Amplification failure of the majority of amplicons

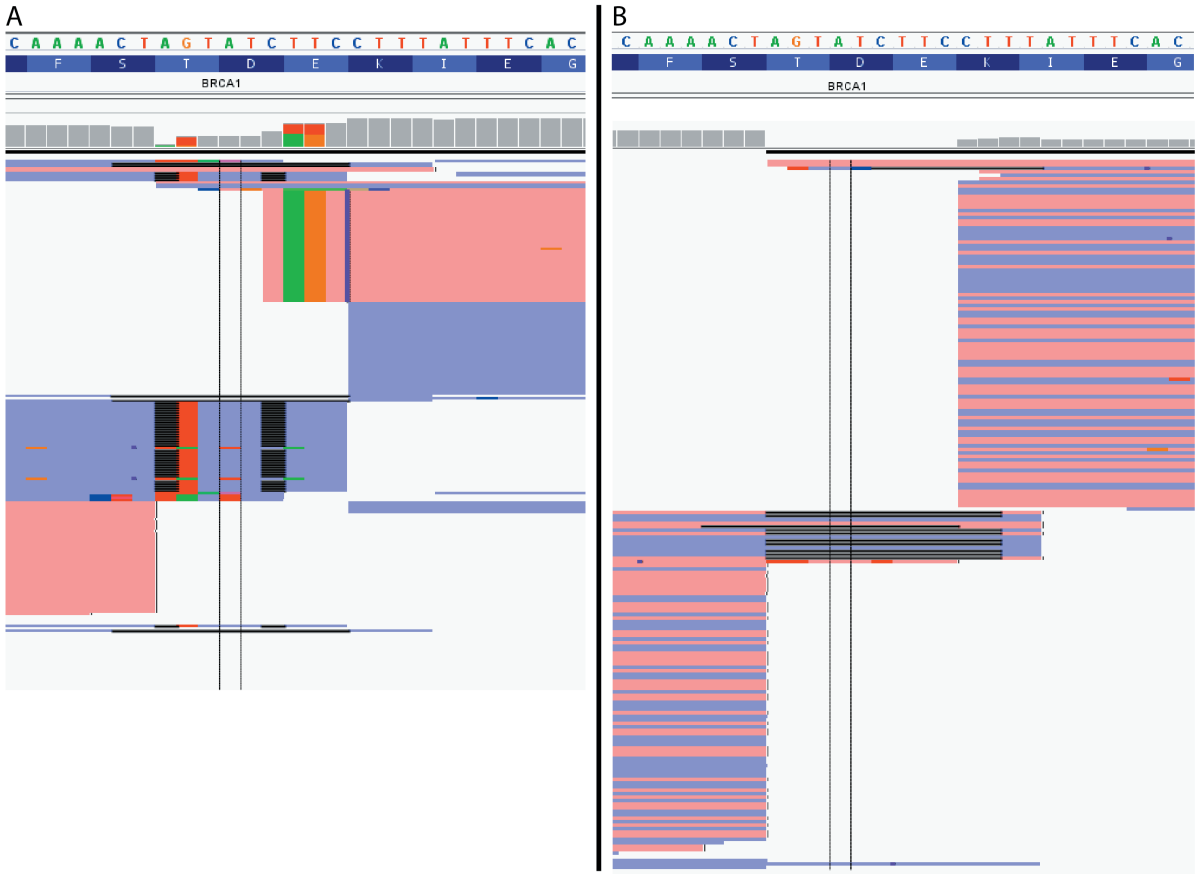
Supplemental Table S2. The 32 homopolymer regions of ≥6 base pairs in the coding sequences of *BRCA1* and *BRCA2*

Genomic Position* *BRCA1*		Stretch	variants †,‡	Frequencies§
41256251	41256256	6A	c.329dup	
41247865	41247870	6A		
41246532	41246538	7A	c.1016del c.1016dup c.1010del	1
41245587	41245594	8A	c.1958_1961del c.1961del c.1961dup	
41244219	41244224	6A	c.3326_3329del c.3329dup	
Genomic position* *BRCA2*		**Stretch**	**Variants†**	**Frequencies‡**
32889779	32889784	6A		
32890628	32890633	6T	c.36del	
32906566	32906571	6A	c.952A>T	
32906603	32906609	7A	c.994del	
32907203	32907208	6A		
32907421	32907428	8A	c.1813dup c.1813del	3 2
32910662	32910667	6A	c.2175dup	
32911074	32911080	7A	c.2588dup	
32911322	32911327	6A	c.2830A>T c.2830del	
32911443	32911449	7A	c.2957del c.2957dup	
32912346	32912352	7A	c.3860del c.3860dup	
32912656	32912661	6T	c.4169del	
32912771	32912776	6T	c.4284dup	1
32913080	32913085	6A		
32913559	32913565	7A	c.5071A>T c.5073del c.5073dup	
32913784	32913789	6A		
32913837	32913843	7A	c.5350_5351del c.5351dup	 1
32914070	32914075	6A		
32914860	32914865	6A		
32929162	32929167	6A		
32937355	32937360	6A		
32953633	32953639	7A		
32954023	32954030	8A	c.9097del c.9097dup	 1
32954273	32954279	7A	c.9253dup	
32972590	32972595	6A		
32972626	32972631	6A		
32972893	32972898	6A		

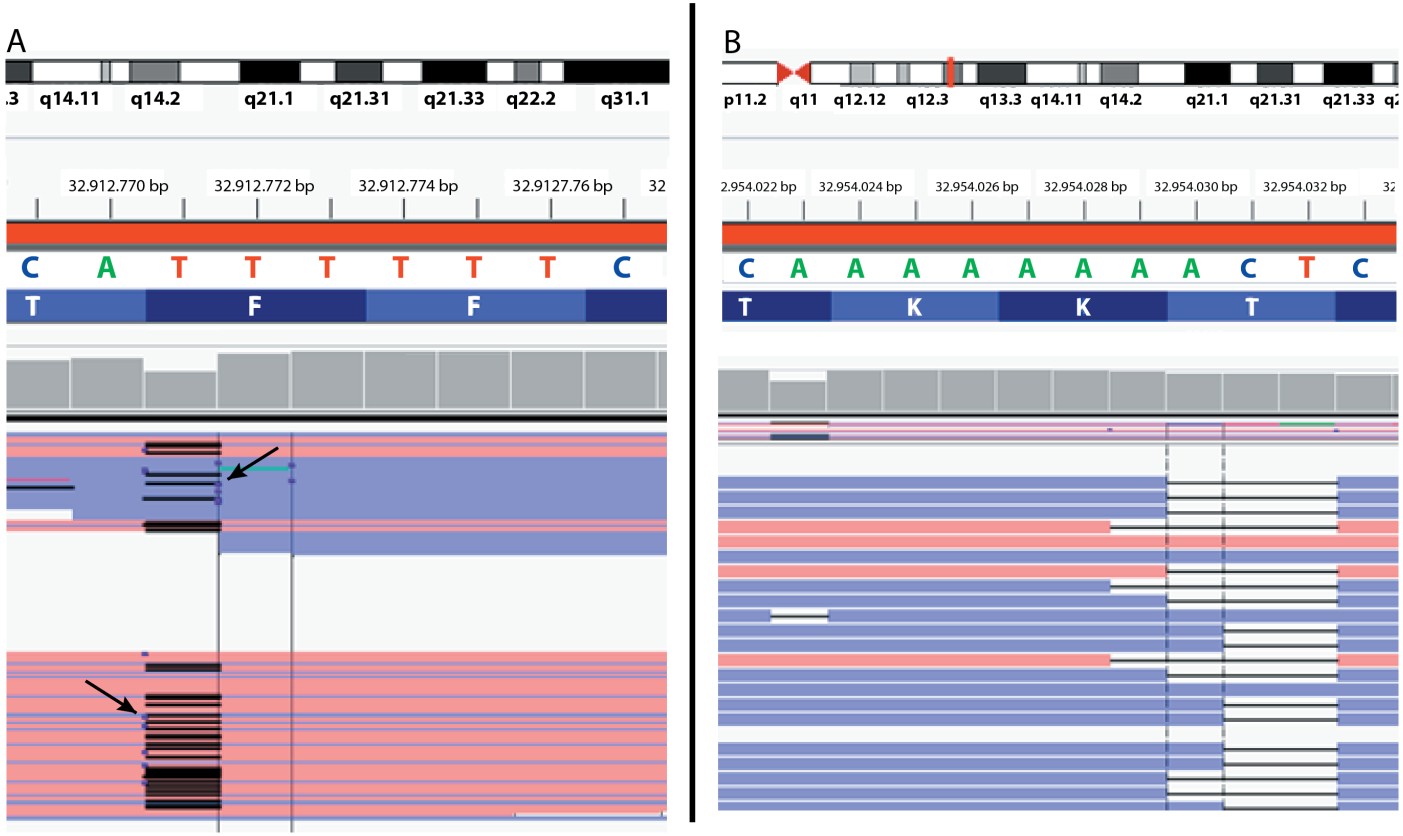
In the LUMC, a class 4 or 5 variant was reported in 236 patients between 2007 and 2016. Of these, nine were variants in homopolymer regions (9/236, 3.8%). Based on the frequency of germline *BRCA1/2* variants observed in the COBRA cohort (9.7%), less than one pathogenic germline variant is estimated to be missed in every 250 patients screened. *Human Reference Consortium 37 (GRCh37). †Reference sequences: *BRCA1:*NM_007294.3, *BRCA2:* NM_ 000059.3 ‡Previously submitted (likely) pathogenic variants extracted from the Leiden Open Variant Database at the genomic positions that are indicated in the table (LOVD; access date: 13-10-2017). §Times detected by LDGA between 2007 and 2016.



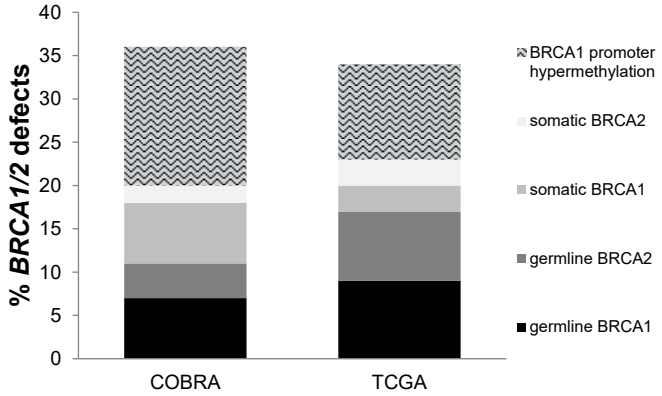
Supplemental Figure S1. Median coverage of the amplicons used to sequence *BRCA1* and *BRCA2* in the training cohort. Error wbars indicate the interquartile range (IQR). The figure only includes the samples that passed quality control. Two samples were excluded from coverage analysis because they were analyzed with an older version of the panel (version 3 instead of version 4). **A:** *BRCA1*; all 113 amplicons have a median coverage >100 reads, with amplicon 95 showing the lowest median coverage, with 348 reads. The median and mean coverage values of all amplicons are 2010 and 2774 reads, respectively. **B:** *BRCA2*; all 152 amplicons have a median coverage >100 reads, with amplicons 9, 73, and 131 showing the lowest median coverage, with 164, 220, and 196 reads, respectively. The median and mean coverage values of all amplicons are 1312 and 1904 reads, respectively. The **dotted line** represents the median coverage >100 reads. *n* = 82 (**A** and **B**, 42 tumor and 40 normal).



Supplemental Figure S2. Adjustments in the alignment improved automatic detection of two variants (R32 and R36). A: Initially, the *BRCA1* deletion (c.3481_3491delGAAGATACTAG) in sample R32 was not automatically detected by the pipeline, and visual inspection of the reads in Integrative Genomics Viewer was needed for identification. **B:** Adjustment of the alignment settings resulted in improved alignment of the reads and automatic detection of the deletion.



Supplemental Figure S3. Sequencing artifacts at the border of homopolymer regions of ≥6 bp interfere with variant detection. A: Sample R24 contains a T duplication (*BRCA2*: NM_000059.3: c.4284dupT) in a stretch of six thymidines. The thymidine duplication is visible in a subset of reads (purple dots; **arrows**) in the Integrative Genomics Viewer but could not be identified because homopolymer instability caused by the T stretch in the form of a bp deletion (black stripes). **B:** Sequencing artifacts present at the border of an 8-bp adenine stretch led the software (Ion Torrent specific caller, Torrent Variant Caller 5.0.2) to call a 2-bp deletion (*BRCA2*: NM_000059.3: c.9099_9100delCT) present in sample R1 as a 3-bp deletion (NM_000059.3: c.9097_9099delACT), which was manually curated.



Supplemental Figure S4. Percentages of *BRCA1/2* defects detected in the high-grade ovarian, fallopian tube, and primary peritoneal cancers (HGSCs) of the clinical implementation of *BRCA1/2* screening on ovarian tumor tissue (COBRA) cohort compared with The Cancer Genome Atlas (TCGA) cohort. Seven percent (4/54) of g*BRCA1* variants, 4% (2/54) of g*BRCA2* variants, 7% (4/54) of s*BRCA1* variants, 2% (1/54) of s*BRCA2* variants, and 16% (8/50) of *BRCA1* promoter hypermethylated cases were detected in HGSCs of COBRA cases included in the final analysis. TCGA found 9% (27/316) of g*BRCA1* variants, 8% (24/316) of g*BRCA2* variants, 3% (10/316) of s*BRCA1* variants, 3% (9/316) of s*BRCA2* variants, and 11% (56/489) of *BRCA1* promoter hypermethylation in HGSCs. Considering the TCGA cohort: One case with both a germline *BRCA1* and *BRCA2* variant was only counted in the germline *BRCA1* mutated group, one case with a simultaneous somatic *BRCA1* variant and germline *BRCA2* variant was only counted in the germline *BRCA2* mutated group, and one case with a simultaneous somatic *BRCA2* variant and germline *BRCA2* variant was only counted in the germline *BRCA2* mutated group.

Chapter 7

Discussion and future perspectives

7. Discussion and future perspectives

BRCA1 and *BRCA2* are tumor suppressor genes that are essential for the maintenance of genomic integrity.[1, 2] They play, together with other genes (e.g. *PALB2, ATM, CHEK2, RAD51C, RAD51D, BRIP1),*[3-5] a crucial role in homologous recombination repair (HR). HR is important for the high-fidelity repair of DNA double strand breaks (DSBs) and restoration of lesions that stall the DNA replication fork.[1, 2] *BRCA1/2*-deficient tumors are not capable of performing HR and are homologous recombination deficient (HRD), resulting in genomic alterations, called "genomic scars" or "mutational signatures (**chapter 1)**.[1, 6-14]

As discussed in **chapter 1**, women with the *gBRCA1/2*-associated hereditary breast and ovarian cancer (HBOC)-syndrome are especially at increased life-time risk to develop basal-like breast cancer (BC) and high-grade serous tubo-ovarian cancer (HGSOC). These tumor types frequently harbour *BRCA1/2* mutations (both somatic and germline) and/or are HRD, and are further characterized by frequent *TP53* mutations and a high number of somatic copy number alterations (SCNA).[15-20] Interestingly, the p53-abnormal/SCNA-high molecular subgroup of endometrial cancer (EC) resembles HGSOC and basal-like BC both molecularly and clinically, suggesting a similar origin and having potential clinical consequences with regard to adjuvant treatment choices and genetic testing.[15-20]

In the first part of this thesis, we aimed to assess whether HRD occurs in EC. Evidence that this DNA repair pathway is abrogated in a subset of EC would support a potential role for *BRCA1/2* (and/or other HR) gene defects in the carcinogenesis of these tumors. In the second part of this thesis, by performing an in depth molecular and morphological characterization of EC that occurred in *gBRCA1/2* mutation carriers, we sought for recurring characteristics further supporting a causal relationship. In addition, we performed a systematic literature search and meta-analysis, and assessed the EC risk stratified by histologic a molecular subgroup in a large nationwide cohort of *gBRCA1/2* mutation carriers with most EC events reported to date. The ultimate goal was to elucidate whether EC is part of the *gBRCA1/2* mutation associated HBOC-syndrome, and to provide further risk estimates that can be used for genetic counselling. In the third and final part of this thesis, we sought for a more efficient way to screen for somatic and germline *BRCA1/2* mutations in tumor specimens, now that this analysis is being routinely requested for women with epithelial ovarian cancer.

7.1. Homologous recombination deficiency in endometrial cancer

In **chapter 2**, we performed a pilot study in which functional assessment of HR was performed in a prospectively collected series of EC. By assessing the ability of proliferating tumor cells to accumulate RAD51 protein at DNA double-strand breaks after *ex vivo* irradiation, we provided evidence that HRD is a frequent event in the p53-abnormal/SCNA-high molecular subgroup of ECs, with 50% of these tumors being HRD. In our series, all HRD-ECs were of non-endometrioid

histology (either uterine serous carcinomas (USC) or uterine carcinosarcomas (UCS)). These results provide evidence that HRD is an important mechanism in tumor development of *TP53*-mutated EC, and provides a rational for treating these patients with therapies exploiting this defect (e.g. platinum compounds, Poly (ADP Ribose) Polymerase (PARP) inhibitors).[12, 21-28]

In our cohort, HRD was only observed in USC and UCS, which was likely the consequence of the small cohort size. Analysis of ECs from the TCGA-cohort presented in the same study showed that *BRCA*-associated genomic scars were present in endometrioid EC as well, though with lower frequencies (50% versus 4%-12% respectively). Also, in **chapter 4,** we showed that a large proportion of *gBRCA1/2*-associated EC were of endometrioid histology (all being p53-abnormal/SCNA-high).[29] That HRD occurs in EC is further supported by Ashley and colleagues,[30] who found mutational signature 3 (associated with HRD) to be the dominant signature in 15%, and second dominant signature in an additional 20% of p53-abnormal/SCNA-high EC, including both USC, endometrioid EC and mixed carcinomas. Furthermore, Jönsson and colleagues[31] found 53% of USC to be HRD (HRD score >42).

7.1.1. Clinical implications

Although most EC have good prognosis, p53-abnormal/SCNA-high EC are still associated with poor clinical outcome.[19, 32-34] The observation that HRD frequently occurs in p53-abnormal/SCNA-high EC provides mechanistic rational for treating these patients with both existing and new treatment strategies.

The best biomarker beyond *BRCA1/2* mutations for predicting efficacy of HRD-directed precision medicine is currently not known.[22, 23, 35-37] Since the presence of HRD can be assessed in multiple ways (e.g. presence of mutations in key HR genes, functional RAD51 assay, presence of "genomic scars" associated with HRD, **chapter 1**; Fig. 2), ideally, a study should be performed in which predictive value of the different available HRD biomarkers is examined side by side. This could for example be performed retrospectively in large already available (combined) cohorts of (recurrent) HGSOC patients treated with PARP inhibitors (e.g. Study 19/NCT00753545, ENGOT-OV16/NOVA trial/NCT01847274, ARIEL2/NCT01891344), and for which formalin-fixed paraffin embedded (FFPE)-tumor blocks are available. The most clinically applicable and predictive biomarker could then be used in future studies.

An advantage of RAD51-based tests above "genomic scar" assays is that it displays the current HR status of the tumor, and that it is rapid and cheap. A disadvantage of RAD51-based tests is the need for fresh tumor specimens/effusions for *ex vivo* irradiation to induce DNA double strand breaks, limiting clinical applicability.[38-42] Interestingly, recent studies suggested that the RAD51 assay could reliably be performed on diagnostic FFPE-tumor tissue without the need for prior induction of DNA damage via *ex vivo* irradiation. This test showed to be predictive for PARP inhibitor sensitivity and discriminative for defects in HR-genes,[43, 44] indicating that

endogenous DNA damage might be sufficient for reliable analyses of HR status. Furthermore, a pilot study presented at the ESGO 2019 (EP1230; http://dx.doi.org/10.1136/ijgc-2019-ESGO.64) in which the results of the RAD51 assay performed on fresh tumor tissue after *ex vivo* irradiation were compared with the RAD51 assay directly performed on diagnostic FFPE-tumor specimens using endogenously present DNA damage (presence of DNA double-strand breaks confirmed with gamma-H2AX staining) showed 100% concordance between both tests. If these findings are confirmed in larger series, the FFPE-RAD51 assay would be an ideal marker to retrospectively investigate the prevalence of HRD in archival diagnostic FFPE tumor specimens, and could be used on larger study cohorts to simultaneously investigate the prevalence of HRD, prognostic and predictive value.

7.1.2. Platinum-based chemotherapy

Our data suggest that p53-abnormal/SCNA-high EC will benefit from platinum-based chemotherapy. The presence of mutations in HR genes, genomic scars associated with HRD, and functional HRD already have shown to be predictive for platinum-based chemotherapy response and to correlate with improved progression free survival and overall survival (OS) in women with ovarian cancer (OC) or breast cancer (BC).[12, 25, 26, 41, 45] Up to recently, prognostic risk group allocation and adjuvant treatment recommendations for EC patients (radiotherapy, chemotherapy) were solely based on clinicopathological risk factors (FIGO stage, grade, histologic subtype, age, lymphovascular space invasion),[46, 47] thereby selecting a histologically and molecularly heterogenous group of tumors. This likely contributed to the heterogenous results of previous clinical trials with regard to the presence of an OS and recurrence free survival (RFS) benefit when adding (platinum-based) chemotherapy to pelvic radiotherapy (CTRT) compared with pelvic radiotherapy (RT) alone, with the absolute benefit being limited for studies that found a positive effect.[48-50] Recently, Leon-Castillo and colleagues[34] investigated the predictive value of the four previously defined molecular subgroups for CTRT benefit in patients with high-risk EC from the randomized PORTEC-3 trial. They found a highly significant absolute benefit (5-year RFS: 22.4%; 5-year OS: 23.1%) when women with p53-abnormal/SCNA-high EC were treated with CTRT compared to RT alone, whereas no (clear) benefit was observed for the remainder molecular subgroups (*POLE*-mutated, mismatch repair deficient (MMRd) and no specific molecular profile (NSMP) group). These findings are in line with our expectations, and it would be interesting to further subdivide the p53-abnormal/SCNA-high EC group of the PORTEC-3 trial by HRD status. By doing this, both the prevalence of HRD in this molecular subgroup could be determined, as well as whether HRD status might be of additional predictive value in selecting patients that benefit most from CTRT. Also, HRD prevalence could be assessed in the other molecular subgroups to determine whether HRD is indeed restricted to the p53-abnormal/SCNA-high molecular subgroup. The FFPE-RAD51 assay would be a promising candidate biomarker for HRD as it is fast, cheap and it can easily be performed on the available FFPE-tissue blocks.

7.1.3. PARP inhibitors

The high prevalence of HRD in p53-abnormal/SCNA-high EC provides rational for treating these women with PARP inhibitors.[21-24, 28] Trials assessing the efficacy PARP inhibitor monotherapy in recurrent or metastatic EC are on their way[51] (Table 1) and results have to be awaited.

Based on our studies, PARP inhibitor effect is to be expected in the p53-abnormal/SCNA-high molecular subgroup, and more specifically, the HRD-group within this subgroup. Since only an estimated 18-26% of unselected EC is expected to be p53-abnormal/SCNA-high,[19, 33] the majority of beforementioned studies might not be able to show an effect, and therefore, might not be able to answer the question whether (a subset of) EC patients benefit from PARP inhibitors. Furthermore, three of four studies exclude carcinosarcomas (NCT03016338, NCT03745950, NCT03745950, NCT04080284), a histotype likely benefitting from PARP inhibitors as studies showed carcinosarcomas to be associated with the p53-abnormal/SCNA-high molecular subgroup, an HRD phenotype, and to be enriched in *gBRCA1/2* mutation carriers.[29, 42]

Ideally, studies assessing the effect of PARP inhibitors in EC should include EC of the p53-abnormal/SCNA-high molecular subgroup, and should randomize this group for either platinum-based CTRT with parp inhibitors (intervention arm) or platinum-based CTRT alone (control arm). Primary outcomes should include OS and RFS. Furthermore, differences in toxicity between the treatment-arms should be registered and evaluated. Finally, diagnostic FFPE-tumor tissue of all included EC should be centrally collected to assess the predictive value of HRD in predicting PARP inhibitor response. A promising trial that is currently in the

Table 1. Trials investigating monotherapy with PARP inhibitors in endometrial cancer

Trial	Patient population	Intervention-arm	control-arm
NCT03016338-Phase 2 (*n=44)*	recurrent/advanced endometrial cancer after at least one line of prior platinum based chemotherapy.	Cohort 1; Niraparib (n=22) Cohort 2; Niraparib and TSR-042[a] (n=22)	n.a.
NCT03617679–Phase 2 (*n=138)*	recurrent/metastastic endometrial cancer after 1-2 prior lines of (chemo) therapy	Rucaparib	Placebo
NCT03745950–Phase 2[b] (*n=147)*	Advanced/metastatic endometrial cancer after 1 line of platinum based chemotherapy	Olaparib	Placebo
NCT04080284 Phase II	Advanced, platinum-sensitive recurrent USC	Niraparib	

[a]anti-PD1 inihibitor, [b]Secondary outcome includes to determine time from response rate according to IHC P53, MMR, NGS BRCA/HRD, MSI

developmental phase and which is planning to assess PARP inhibitor efficacy in the p53-abnormal/SCNA-high molecular subgroup is the RED-trial (p53-abnormal EC) of the Refining Adjuvant treatment IN endometrial cancer Based On molecular features (RAINBO)-program.

7.1.4. Trastuzumab

Another potential therapeutic target for p53-abnormal/SCNA-high EC is the presence of *ERBB2* amplifications, which encodes for the human epidermal growth receptor 2 (HER2) and which is amplified in 25% of P53-abnormal/SCNA-high EC.[19, 52] In our *gBRCA1/2*-carrier cohort described in **chapter 4,** none of the EC displayed *ERBB2* amplifications. In BC, the *ERBB2*-overexpressing subgroup and the basal-like subgroup are two biologically distinct groups[53], the latter being associated with, amongst others, *BRCA1* defects and HRD.[54] This might indicate that the p53-abnormal/SCNA-high EC could possibly be further divided in an HRD-group and an *ERBB2* amplified group, which would be an interesting topic for future studies.

7.2. Endometrial cancer and the *gBRCA1/2*-associated HBOC-syndrome.

By demonstrating that HRD occurs in EC, we provided mechanistic support that a subset of EC might be a *gBRCA1/2*-associated disease, something that has long been topic of debate. Studies that assessed EC risk in *gBRCA1/2* mutation carriers were either small with limited number of events and follow-up, and/or did not stratify the EC for histologic subtype (**chapter 5,** supplementary Table S1).[55-62] This has resulted in conflicting data with regard to EC risk in *gBRCA1/2* mutation carriers, resulting in divided opinions between clinicians and uncertainty whether these risk should be integrated in counselling and clinical management of these women. In **chapter 3, chapter 4** and **chapter 5** we focused on answering the question whether EC is part of the *gBRCA1/*2-associated HBOC syndrome.

By performing a systematic review and meta-analysis (**chapter 3**), we found that the odds ratio for having a *gBRCA1/2* mutation was increased for women with USC compared to what would be expected based on population frequencies. In addition, we described a case of a *gBRCA1* mutation carrier who developed an USC three years after risk-reducing salpingo-oophorectomy (RRSO). The USC showed loss of heterozygosity of the *BRCA1* wild-type allele and showed an HRD phenotype in the functional RAD51 assay, thereby providing evidence that *BRCA1* was involved in the carcinogenesis of this tumor. In **chapter 4**, we comprehensively histologically and molecularly characterized a unique series of 40 EC that developed in *gBRCA1/2* mutation carriers, and found recurring characteristics, further supporting a causal relationship. Since previous studies demonstrated LOH to be an essential event in carcinogenesis of *BRCA1/2*-associated carcinomas,[63] EC with LOH were considered *gBRCA1/2*-associated, whereas EC without LOH were considered “sporadic” (non-*gBRCA1/2*-associated). Sixty percent of EC in *gBRCA1/2* mutation carriers were *gBRCA1/2*-associated, with the remainder being sporadic tumors that likely developed independently

of the *gBRCA1/2* mutation. *gBRCA1/2*-associated EC were clearly enriched for histotypes associated with unfavourable clinical outcome (79.2% USC, UCS, high-grade endometrioid or ambiguous EC)[64], the p53-abnormal/SCNA-high subgroup molecular subgroup (91.7%), and for Solid, pseudoEndometrioid, and/or Transitional morphology (SET morphology) , a growth pattern already shown to be enriched in HGSOC with *BRCA1*- and HR-gene mutations.[17, 18, 65] Now that we learned that ECs not just occur sporadically in *gBRCA1/2* mutation carriers, **chapter 5** focussed on quantifying the EC risk of *gBRCA1/2* mutation carriers using a large nationwide multicenter cohort. With 58 EC events, this was the largest study to date,[55-62] and analyses were stratified for histologic- (endometrioid, serous-like, clear cell, sarcoma, other) and molecular subgroups (p53-abnormal/SCNA-high versus other) after pathology review. We showed that gBRCA1/2 mutation carriers have a 2 to 3-fold increased risk for developing EC, with highest increased risks being observed for the serous-like histological and p53-abnormal/SCNA-high molecular subgroups (approximately 10-fold). When stratified for mutation type, risks were highest for gBRCA1 carriers. Despite these highly increased risks, absolute risks by 75 years remained low because of the rarity of the disease; overall EC, 3.0%; serous-like EC, 1.1%.

Together, by showing that *gBRCA1/*2-associated ECs have a specific phenotype, and providing mechanistic and epidemiologic support for an association between EC and g*BRCA1/2* mutations, we can conclude that ECs, and more specifically ECs of serous-like histology and the p53-abnormal/SCNA-high molecular subgroup, are an integral part of the *gBRCA1/2*-associated HBOC syndrome.

Although our study included most EC events reported to date (**chapter 5,** supplementary table 1), it would be interesting to redo the analysis in 10 years. Despite long follow-up, our cohort was still relatively young, with limited person-years at risk in the age categories above 75-80 years. As can be seen in Figure 1, EC, and especially EC of serous-like histology, is a disease of older age for which incidences remain relatively high, even after the age of 80 years.[66] Therefore, having limited follow-up years and events in these age categories might have influenced the observed increase in risk, especially since we observed a higher EC risk increase for older age categories (table 3, **chapter 5**).[11, 12, 24]

7.2.1 Clinical implications

Now that we provided additional evidence that EC, and more specifically, the rare but aggressive serous-like and p53-abnormal/SCNA-high subgroup of EC, is part of the *gBRCA1/2*-associated HBOC syndrome, the question arises how this should impact current clinical practice.

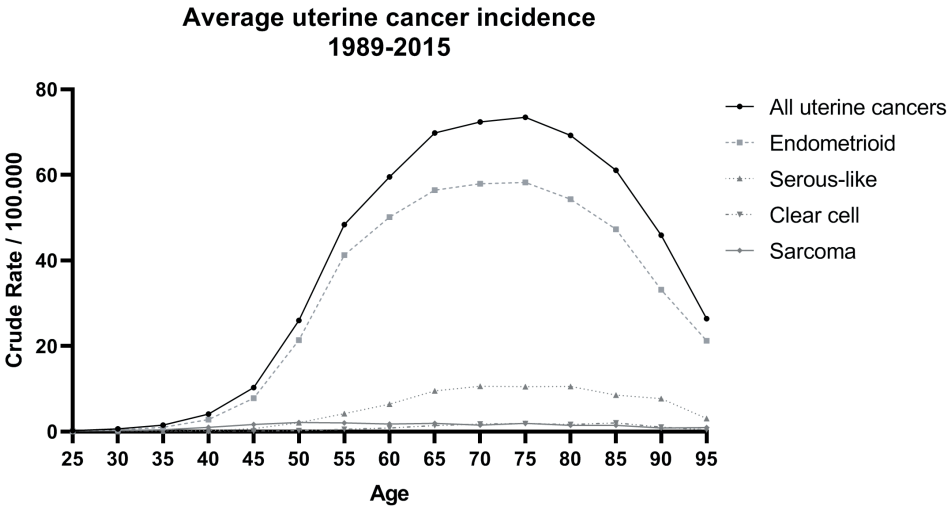


Figure 1. Dutch population uterine cancer incidence, both overall and stratified by histologic subgroup. Data was retrieved from the Dutch Cancer Registry, and was stratified according to histologic subgroups as described in **chapter 5**.

7.2.2. Risk-reducing surgery

Because of the highly increased life-time risks to develop BC and OC (BC: *gBRCA1,* 50- 59% and *gBRCA2,* 42-51%; OC: *gBRCA1,* 34-45% and *gBRCA2,* 13-21%),[67] *gBRCA1/2* mutation carriers can opt for risk-reducing bilateral mastectomy and risk-reducing salpingo-oophorectomy (RRSO).[68] In the Netherlands, it is currently not recommended to perform a concurrent risk-reducing hysterectomy at the time of RRSO since, up to now, EC was not considered to be part of the *gBRCA1/2*-associated tumor spectrum.[68] Although we now showed that EC is part of the *gBRCA1/2*-associated HBOC-syndrome, the low absolute EC risks (overall: *gBRCA1*: 3.4%; *gBRCA2*: 2.0%; serous-like: *gBRCA1*: 1.4%; *gBRCA2*: 0.6%), especially when compared to beforementioned OC and BC risks, support current clinical practice in which routine risk-reducing hysterectomy at the time of RRSO is not routinely recommended. Nevertheless, understanding EC risks is essential for informed decision-making during counselling, and the potential benefits need of performing a hysterectomy should be balanced against the potential hazards.

The main disadvantage of performing an additional risk-reducing hysterectomy at the time of RRSO is the expected increase in surgery-related morbidity. Studies that assessed surgery-related morbidity for total laparoscopic hysterectomy (TLH) that were conducted for benign indications or low-grade malignancy[69], and RRSO[70] reported the following major and minor complication rates (as formulated by the Dutch Society of Obstetrics and Gynecology); major: 4.0% versus 0.6%, and minor: 4.0% versus 3.7% respectively.[69, 70] In addition, de mean length of hospital stay was longer for women that underwent a TLH (4 days, range:2-7)[71, 72] compared

to women that underwent a RRSO (1 day, range: 0-13).[70] To our knowledge, there are no studies that compared complication rates between RRSO with and without risk-reducing hysterectomy in our population of interest, and future studies need to elucidate the true additional morbidity of this procedure.

Reasons to consider a risk-reducing hysterectomy in this population could be the presence of (benign) uterine disease that give symptoms/that will likely give to symptoms in the future, the presence of other risk factors that increase EC risk, anxiety that patients may experience from being at increased EC risk, and, that it is unknown whether there are effective screening modalities to detect early-stage EC in this patient population.

7.2.3. Patient preferences

Although it does not seems rational to routinely perform a risk-reducing hysterectomy at the time of RRSO from a clinical perspective, it would be interesting to conduct a patient preference study to determine patients choice of surgical extent.

This could for example be performed by interviewing patients using a treatment tradeoff method, to assess how patients weigh risk benefits against potential additional complications from extended surgery, and to determine the minimally desired risk benefit from an additional risk-reducing hysterectomy. Figure 2 illustrates an example of a flow-chart that could be used.

7.2.4. PARP inhibitor

The finding that a subset of ECs is *gBRCA1/2*-associated provides additional rationale for treating these women with PARP inhibitors, which was already discussed in paragraph 7.1.3.

7.2.5. Screening for *gBRCA* mutations in uterine cancer patients

DNA testing for hereditary mutations is generally recommended if the expected detection rate is sufficiently high (>5%).[73] Studies that assessed *gBRCA1/2* mutation frequency in an unselected cohort of patients with USC, or EC patients (not selected for histotype) with a history of BC, reported mutation frequencies of 2%74 and 3.8%75 respectively. These data do not support screening for *gBRCA1/2* mutations in EC patients. However, based on our data, highest *gBRCA1/2* mutation frequencies are to be expected in EC with TP53 mutations. Therefore, it would be interesting to perform a future study that determines the *gBRCA1/2* mutation frequency in women that developed TP53-mutated EC, ideally including a subanalyses taking into account mutation incidence when additionally including BC history, family history and/or morphological features enriched in *gBRCA1/2* associated ECs (chapter 4).

7.3. Tumor-based screening for *BRCA1/2* (and other HR gene) mutations

Given the high prevalence of *gBRCA1/2* mutations in HGSOC (16%)[16] and triple-negative BC (14%),[15] germline analysis is routinely being offered to all women with OC, and women

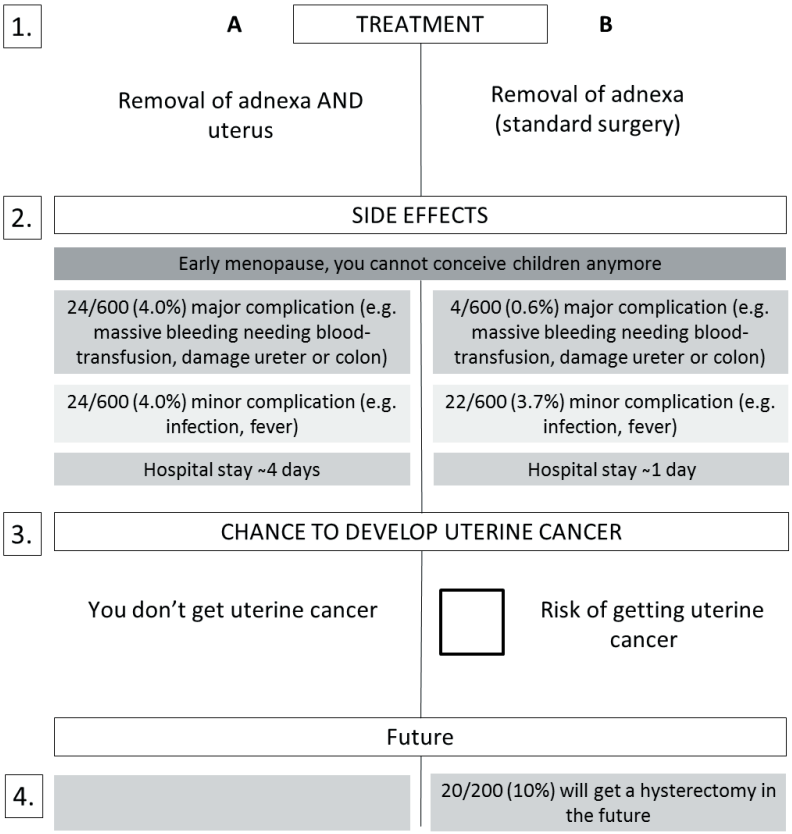


Figure 2: **Example of information on uterine cancer risk and complication rates that could be presented during an interview according to the treatment tradeoff method.**

with triple-negative BC <60 years of age. Furthermore, with the additional registration of PARP inhibitors by the European Medicines Agency as maintenance treatment for first-line platinum-sensitive high-grade epithelial OC in patients with proven *BRCA1/2* mutations (somatic/germline), additional somatic tumor testing will be more regularly required. In **Chapter 6**, we first optimized *BRCA1/2* mutation analysis performed on diagnostic FFPE-tumor tissue in a training cohort of known *gBRCA1/2* mutation carriers, and subsequently validated the tumor test in a prospective cohort of women that developed epithelial OC. We showed that, when using a combination of next-generation sequencing and copy number variant (CNV)-multiplex ligation-dependent probe amplification (MLPA), *BRCA1/2* mutations (both somatic and germline) can reliably be detected. Using this tumor-first approach as pre-screening tool to detect and select patients with *BRCA1/2* mutations for referral to the clinical geneticist could prevent approximately 80% of referrals. Another study in the Netherlands that was simultaneously performed (*BRCA* testing in **O**varian cancer by **Pa**thologist (OPA)-study)

using a different sequencing technique (combination of single-molecule molecular inversion probe-based NGS and CNV-MLPA) also showed the tumor-first approach to be reliable, rapid, feasible in daily practice, and to be appreciated by patients and gynaecologist.[76, 77]

7.3.1. Clinical implications

The tumor-first approach is currently being implemented in different regions of the Netherlands, and is already part of routine diagnostic work-up for all epithelial OC patients (except for women with borderline OC) in other regions (e.g. Leiden, Nijmegen).[68] Because of the consequences of detecting hereditary variants in other HR genes besides *BRCA1/2*, the sequencing panels should also include additional genes (e.g. *ATM, PALB2, CHEK2, RAD51C, RAD51D, BRIP1*).

Sequencing of *BRCA1/2* is challenging. *BRCA1/2* are large genes with a wide mutation spectrum. Because FFPE-derived tumor DNA is highly fragmented, created amplicons are shorter compared to when leucocyte-derived DNA is used, thereby increasing the chance of mutations being located at amplicon ends or primer binding sites, increasing the chance for detection errors. Furthermore, large genomic *BRCA1* deletions (e.g. exon 22 deletion) are common founder variants in the Netherlands.[78] These large deletions are easily overlooked when only using next generation sequencing, making it necessary to perform additional copy number analysis.[79] Furthermore, once variants are detected, interpretation can be difficult, especially when it considers variants of uncertain significance.[80] Because of these challenges, we think *BRCA1/2* analysis should be restricted to academic hospitals with sufficient sequencing experience and in which there is a close collaboration between the pathology department and the clinical genetics department. Also, despite this tumor-first approach having many advantages, it is important that clinicians are aware that because of technical limitations (depending on the technique(s) that is/are used), some variants will not be detected unless additional analyses are being performed (**Chapter 6).** Therefore, if there is a high suspicion for a hereditary variant, patients should always be referred to the clinical geneticist, even if there is no variant detected in the tumor test.

Whether it is necessary to screen all women with epithelial OC (currently recommended by the Dutch guideline)[68] remains topic of debate. Table 2 summarizes the *gBRCA1/2* mutation frequency among different histologic OC subtypes found by two studies. Both studies included pathology review by gynaecologic pathologists and reported highest *gBRCA1/2* mutation incidences in women with HGSOC.[81, 82] Although Alsop and colleagues almost exclusively found *gBRCA1/2* mutations to be associated with high-grade serous histologic subtype after pathology review, Norquist and colleagues also found high incidences in other histologic subtypes, especially carcinosarcomas and high-grade endometrioid carcinomas. Based on these findings, it seems reasonable to exclude women with mucinous OC and grade 1 endometrioid OC from screening for *gBRCA1/2* mutations.

Table 2. *gBRCA1/2* mutation frequency stratified by ovarian cancer histotype

Norquist and colleagues[81]	***gBRCA1/2* mutation frequency (%)**
High-grade serous	16.1
Low-grade serous	5.7
High-grade endometrioid (gr 2/3)	10.9
low-grade endometrioid (gr 1)	0
Carcinosarcoma	13.9
Clear cell	6.9
Alsop and colleagues[82]	
High-grade serous	22.6
Endometrioid (grade not specificied)	1.7[a]
Carcinosarcoma	0
Clear cell	1.6[b]

[a]Originally 8.4%. Eight out of ten (80%) cases were reclassified as serous or unspecified adenocarcinoma after pathology review.
[b]Originally 6.3%. Three out of four (75%) reclassified as high-grade serous carcinoma with focal clear cell alteration after pathology review.

7.3.2. Tumor-based screening in other cancer types

Another major advantage of the tumor-first approach is that it could easily be implemented for other tumor types for which germline mutations have been described in a subgroup of cases (e.g. prostate cancer, pancreatic cancer, (*TP53*-mutated) EC, BC), because referral to the clinical geneticist will only be necessary if a mutation is detected.

7.4. Conclusion

In this thesis, we provided mechanistic, morphologic and epidemiologic evidence that serous-like or p53-abnormal/SCNA-high ECs belong to the *gBRCA1/2*-associated HBOC syndrome. In addition, we demonstrated that HR is frequently abrogated in this molecular subgroup, also in the absence of *BRCA1/2* mutations, thereby providing a strong rationale for future clinical trials assessing the efficacy of treatment strategies exploiting this repair defect in these tumors. Finally, by showing that *BRCA1/2* mutations can reliably be detected in diagnostic FFPE-material, we provided a basis for a more efficient genetic work-up pathway for OC patients, which can also be extended to other tumor types.

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7

Appendices

Nederlandse samenvatting

List of publications

Curriculum Vitae

Dankwoord

Nederlandse samenvatting

Gedurende het leven wordt het DNA van organismen voortdurend blootgesteld aan factoren die schade aan het DNA veroorzaken. Adequate herkenning en reparatie van deze DNA schade is belangrijk. Indien dit niet gebeurt, kunnen namelijk blijvende veranderingen in het DNA van cellen ontstaan, ook wel mutaties genoemd. Deze mutaties kunnen vervolgens leiden tot celdood, ontregelde celgroei en kanker. Om het ontstaan mutaties te voorkomen, hebben cellen meerdere herstelmechanismen beschikbaar. Deze herstelmechanismen worden “DNA damage response pathways” genoemd.

Mutaties die gedurende het leven ontstaan worden *somatische mutaties* genoemd. Deze verworven mutaties zijn maar in een deel van de cellen aanwezig. Erfelijke mutaties zijn daarentegen al aanwezig in de zaadcel of eicel en deze bevinden zich in alle cellen van de nakomeling. Deze mutaties worden ook wel *kiembaanmutaties* genoemd.

Kiembaanmutaties kunnen leiden tot erfelijke tumorsyndromen. Familieleden waarbij de kiembaanmutatie aanwezig is hebben een vergroot risico op het ontwikkelen van kanker. Een bekend erfelijk tumorsyndroom is het erfelijke borst- en eierstokkanker syndroom (HBOC syndroom) waarbij vrouwen een verhoogd risico hebben op het ontwikkelen van borst- en eierstokkanker. Dit syndroom wordt meestal veroorzaakt door kiembaanmutaties in het *BRCA1* of *BRCA2* gen.

Het dragen van een kiembaanmutatie in *BRCA1/2* heeft belangrijke klinische consequenties. Zo kunnen familieleden ervoor kiezen om te laten onderzoeken of zij ook drager zijn van de *BRCA1/2* kiembaanmutatie. Vrouwen met een kiembaanmutatie kunnen vanwege het hoge absolute risico op borst- en eierstokkanker (borstkanker: *BRCA1*, 50-59% en *BRCA2*, 42-51%; eierstokkanker: *BRCA1*, 34-45% en *BRCA2*, 13-21%) kiezen om uit voorzorg hun borsten of eierstokken te laten verwijderen, respectievelijk risico-verlagende mastectomie en risico-verlagende salpingo-ovariëctomie genoemd. Ook is gebleken dat tumoren met een *BRCA1/2* mutatie (zowel somatisch als kiembaan) extra gevoelig zijn voor bepaalde soorten chemotherapie en Poly (ADP Ribose) Polymerase (PARP)-remmers. Deze laatste betreft een vrij nieuwe therapie.

BRCA1 en *BRCA2* zijn tumor suppressor genen die essentieel zijn voor het behoud van het DNA en haar functies. Ze spelen, samen met andere genen (o.a. *PALB2, ATM, CHEK2, RAD51C, RAD51D, BRIP1*), een cruciale rol bij de correcte reparatie van DNA dubbelstrengsbreuken. Dit proces wordt homologe recombinatie reparatie genoemd. *BRCA1/2*-deficiënte tumoren,

waar het BRCA gen bijvoorbeeld niet goed werkt als gevolg van een mutatie, zijn niet in staat om homologe recombinatie uit te voeren en worden daarom homologe recombinatie deficiënt (HRD) genoemd.

Tumoren die HRD zijn hebben bepaalde "genomische littekens" waaraan ze herkend kunnen worden. Daarnaast hebben borsttumoren en eierstoktumoren die ontstaan bij vrouwen met kiembaanmutaties in het *BRCA1* of *BRCA2* gen ook andere karakteristieke kenmerken. Zo worden deze tumoren gekenmerkt door de frequente aanwezigheid van somatische *TP53*-mutaties en een groot aantal somatische copy-number veranderingen (SCNA). Ook tonen deze tumoren vaker een bepaalde kenmerkende groeiwijze. Borsttumoren die ontstaat bij *BRCA1* kiembaanmutatie draagsters zijn bijvoorbeeld vaak van hoge graad, zijn van het basal-like subtype en bevatten veel tumor infiltrerende lymfocyten. Hooggradig sereuze eierstokkanker, de vorm van eierstokkanker die meestal optreedt bij vrouwen met een kiembaanmutatie in *BRCA1* of *BRCA2,* laat vaker een solide, pseudo-endometrioid of overgangstype groeipatroon zien, terwijl de klassieke groeiwijze van deze tumoren juist papillair is.

Baarmoederkanker (endometriumcarcinoom) wordt vooralsnog niet beschouwd als onderdeel van het *BRCA1/2*-geassocieerde HBOC syndroom. Dit komt met name doordat eerdere epidemiologische studies die onderzoek hebben gedaan naar het endometriumcarcinoomrisico bij *BRCA1/2* kiembaanmutatie draagsters tegenstrijdige resultaten lieten zien (**hoofdstuk 5,** aanvullende tabel S1). Deze tegenstrijdige resultaten zijn vermoedelijk het resultaat van een (te) kleine studie omvang, een jonge inclusieleeftijd met korte follow-up met dientengevolge een laag aantal endometriumcarcinoom events.

Wanneer naar tumorkenmerken wordt gekeken zijn er wel degelijk aanwijzingen dat een deel van de endometriumcarcinomen *BRCA1/2*-geassocieerd is. Endometriumcarcinoom kan door de patholoog op twee manieren ingedeeld worden: op basis van hoe deze er onder de microscoop uitziet, ook wel "histologische" typering genoemd, of op basis van de genetische afwijkingen die in de tumor aanwezig zijn, ook wel "moleculaire" typering genoemd. De meest voorkomende histologie van endometriumcarcinoom is het endometrioide-type (80%). Het sereuze subtype komt hierna het meest voor (5-10%). Het sereuze endometriumcarcinoom behoort tot de 'p53-abnormale/SCNA-hoge' moleculaire groep, de groep endometriumcarcinomen met de slechtste overlevingskansen. Opvallend is dat grote overeenkomsten worden gezien tussen borst- en eierstokkanker die voorkomt bij vrouwen met kiembaanmutatie in *BRCA1/2* en endometriumcarcinomen van de p53-abnormale/SCNA-hoge moleculaire groep. Zo hebben deze tumoren net als *BRCA1/2* geassocieerde borst- en eierstokkanker vaak een somatische *TP53* mutatie en tonen ze een hoog aantal SCNA. Ook tonen deze endometriumcarcinomen morfologisch overeenkomsten met *BRCA1/2* geassocieerde eierstokkanker.

Deze overeenkomsten suggereren dat *BRCA1/2* wel degelijk een rol zou kunnen spelen bij de ontwikkeling van endometriumcarcinomen. Indien dit inderdaad het geval is, kan dit gevolgen hebben voor de behandeling van deze endometriumcarcinomen en voor de omvang van risico-verlagende operaties. Daarnaast zou het hebben van bepaalde vormen van endometriumcarcinoom een reden kunnen zijn voor erfelijkheidsonderzoek naar kiembaanmutaties.

Er zijn meerdere redenen om bij vrouwen erfelijkheidsonderzoek naar *BRCA1/2* mutaties te verrichten. Voorbeelden hiervan zijn het optreden van borstkanker bij een vrouw jonger dan 40 jaar, het ontwikkelen van triple negatieve (basal-like) borstkanker onder de leeftijd van 60 jaar (dit betreft het subtype dat vaak gezien wordt bij *BRCA1* mutatie draagsters), of het hebben van een eerste of tweedegraads familielid met een kiembaanmutatie in het *BRCA1* of *BRCA2* gen. Sinds enkele jaren wordt ook geadviseerd om alle vrouwen met een epitheliale vorm van eierstokkanker op *BRCA1/2* mutaties te testen vanwege de hoge kans op het vinden van een *BRCA1/2* kiembaanmutatie (20% in vrouwen met hooggradig sereuze eierstokkanker, waarvan 14% kiembaan en 6% somatisch).

De klassieke werkwijze voor het opsporen van kiembaanmutaties is door middel van bloedonderzoek dat via een afdeling Klinische Genetica wordt uitgevoerd na genetische counseling. Een nadeel van deze werkwijze is dat een groot deel van de vrouwen met eierstokkanker naar de klinisch geneticus wordt verwezen voor erfelijkheidsonderzoek, terwijl circa 85% uiteindelijk geen kiembaanmutatie zal hebben. Ook worden somatische mutaties, dus de mutaties die alleen in de tumor aanwezig zijn, op deze manier niet opgespoord. Dit terwijl het hebben van zo'n mutatie wèl een vereiste kan zijn alvorens behandeling met PARP-inhibitors gegeven kan worden. Omdat zowel kiembaanmutaties als somatische mutaties opgespoord kunnen worden in het tumor-DNA, zou een werkwijze waar eerst het tumor-DNA wordt onderzocht efficiënter zijn. Alleen bij het vinden van een mutatie (die kan zowel somatisch als kiembaan van oorsprong zijn) is dan verwijzing naar de klinisch geneticus nodig. Alvorens dit kan worden gedaan moet echter wel blijken dat deze werkwijze betrouwbaar is.

Inhoud van dit proefschrift

In het eerste deel van dit proefschrift (**hoofdstuk 2, hoofdstuk 3, hoofdstuk 4** en **hoofdstuk 5**) hebben we onderzocht of endometriumcarcinoom onderdeel is van het *BRCA1/2* geassocieerde HBOC syndroom. Dit hebben we gedaan door eerst te onderzoeken of HRD voorkomt in endometriumcarcinomen. Indien dit het geval is, zou dit ondersteunen dat *BRCA1/2* mogelijk een rol heeft bij het ontstaan van endometriumcarcinoom. Hierna hebben we onderzocht of endometriumcarcinomen die ontstaan bij vrouwen met een kiembaanmutatie in het *BRCA1/2* gen bepaalde karakteristieke moleculaire en morfologische kenmerken vertonen. Voorts hebben we onderzocht of vrouwen met een *BRCA1/2* mutatie een verhoogd risico hebben op het ontwikkelen van endometriumcarcinoom ten opzichte van de algemene populatie.

Dit hebben we gedaan door middel van een analyse van eerder gepubliceerde studies (meta-analyse). Ook hebben we gekeken naar het aantal endometriumcarcinomen dat was ontstaan bij vrouwen met een bewezen kiembaan *BRCA1/2* mutatie uit een groot nationaal cohort, het Hereditair Borst- en Eierstokkanker Onderzoek Nederland (HEBON) studie cohort. Deze hebben we vergeleken met het aantal endometriumcarcinomen die waren ontstaan bij vrouwen zonder *BRCA1/2* kiembaanmutatie uit hetzelfde cohort, en met het verwachte aantal endometriumcarcinomen op basis van cijfers van de Nederlandse bevolking.

Tot slot hebben we in **hoofdstuk 6** gekeken of *BRCA1/2* mutatie analyse betrouwbaar uitgevoerd kan worden op tumor DNA. Indien dit het geval is zou deze "tumor-first" benadering toegepast kunnen worden als (pre)screening om te bepalen welke vrouwen naar de klinisch geneticus verwezen moeten worden.

Homologe recombinatie deficiëntie in endometriumcarcinomen

In **hoofdstuk 2** hebben we een pilotstudie verricht naar het voorkomen van HRD in een prospectief verzamelde serie endometriumcarcinomen. Middels een functionele analyse (RAD51-test) hebben we gekeken of tumorcellen in staat waren homologe recombinatie reparatie uit te voeren. RAD51 is een eiwit dat aan het einde van de homologe recombinatie reparatie een functie heeft. In de normale situatie accumuleert RAD51 ter plaatse van de DNA dubbelstrengsbreuk en zorgt die ervoor dat de kapotte DNA streng naast de intacte, homologe DNA streng kan worden gelegd. De intacte DNA streng kan vervolgens als sjabloon worden gebruikt om het gat (de DNA dubbelstrengsbreuk) te repareren. Bij de RAD51-test worden eerst DNA dubbelstrengsbreuken gemaakt in de tumorcellen door de tumor na de operatie, dus buiten het lichaam van de patiënt, te bestralen. Vervolgens wordt gekeken of de tumorcellen in staat zijn om RAD51 naar deze DNA dubbelstrengsbreuken te brengen. Indien geen RAD51-foci gevormd worden betekent dit dat de tumorcellen HRD zijn.

In deze pilotstudie bleek 50% van de endometriumcarcinomen uit de p53-abnormale/SCNA-hoge moleculaire subgroep HRD te zijn. Hoewel endometrioid-type endometriumcarcinoom het meest voorkomt, waren alle HRD tumoren van niet-endometrioïde histologie (sereus endometriumcarcinoom of carcinosarcoom). In de andere moleculaire subgroepen kwam geen HRD voor. Deze resultaten suggereren dat HRD een belangrijk mechanisme is bij de ontwikkeling van p53-abnormale/SCNA-hoge endometriumcarcinomen.

De observatie dat HRD vaak voorkomt in p53-abnormaal/SCNA-hoge endometriumcarcinomen biedt mechanistische ondersteuning om deze patiëntengroep te behandelen met therapieën die dit reparatiedefect uitbuiten, namelijk platinum-bevattende chemotherapie en PARP-remmers. PARP-remmers zijn vooralsnog niet geregistreerd als behandeling voor endometriumcarcinoom en onderzoek naar de effectiviteit van deze geneesmiddelen in de p53-abnormal/SCNA-hoge subgroep van endometriumcarcinoom is nodig.

Endometriumcarcinoom en het *BRCA1/2*-geassocieerde HBOC syndroom

Het feit dat HRD voorkomt in endometriumcarcinomen biedt mechanistische ondersteuning voor de hypothese dat een deel van de endometriumcarcinomen een *BRCA1/2*-geassocieerde ziekte is.

In **hoofdstuk 3**, **hoofdstuk 4** en **hoofdstuk 5** hebben we ons gericht op het beantwoorden van de vraag of het endometriumcarcinoom deel uitmaakt van het *BRCA1/2*-geassocieerde HBOC syndroom. Middels een systematische literatuur review en meta-analyse (**hoofdstuk 3**) bleek de odds ratio voor het hebben van een *BRCA1/2* kiembaanmutatie verhoogd bij vrouwen met een endometriumcarcinoom van het sereuze subtype in vergelijking met wat zou worden verwacht op basis van de populatiefrequenties. Daarnaast beschreven we een casus van een vrouw met een *BRCA1* kiembaanmutatie die drie jaar na de risico-verlagende salpingo-ovariëctomie toch nog endometriumcarcinoom van het sereuze subtype ontwikkelde. Dit sereuze endometriumcarcinoom toonde een HRD fenotype in de functionele RAD51-test. Hiermee bevestigden we dat de *BRCA1* mutatie betrokken was bij de carcinogenese van deze tumor.

In **hoofdstuk 4** hebben we een unieke serie van 40 endometriumcarcinomen die waren ontstaan bij *BRCA1/2* kiembaanmutatie draagsters histologisch en moleculair getypeerd. Vrouwen met een erfelijke *BRCA1* of *BRCA2* mutatie hebben één “kapot” ofwel gemuteerd allel en één normaal allel. Eerdere studies hebben aangetoond dat verlies van dit normale allel, ook wel “verlies van heterozygositeit” (LOH) genoemd, een essentiële gebeurtenis is bij de ontwikkeling van *BRCA1/2*-geassocieerde tumoren. Doordat het normale allel verloren gaat kan er namelijk geen functioneel eiwit meer gevormd worden, wat zorgt voor HRD. Daarom hebben we in deze studie endometriumcarcinomen met LOH als *BRCA1/2*-geassocieerd beschouwd, en endometriumcarcinomen zonder LOH als ‘sporadisch’ (niet-*BRCA1/2*-geassocieerd).

Zestig procent van de endometriumcarcinomen bij *BRCA1/2* kiembaanmutatie draagsters was *BRCA1/2*-geassocieerd, terwijl de rest sporadische tumoren betrof. Het bleek dat deze *BRCA1/2*-geassocieerde tumoren karakteristieke kenmerken toonden. Zo waren deze tumoren duidelijk verrijkt voor histotypen geassocieerd met een ongunstige klinische uitkomst (79,2% sereus, carcinosarcoom, hooggradige endometrioïd of ambigu), de p53-abnormale/SCNA-hoge moleculaire subgroep (91,7%), en voor een groeipatroon dat vaker voorkomt bij eierstokkanker met *BRCA1* of homologe recombinatie genmutaties, namelijk een solide, pseudo-endometrioïde en/of overgangstype groeiwijze (SET-morfologie). Al deze bevindingen ondersteunen een causale relatie tussen het ontstaan van deze tumoren en de aanwezigheid van de *BRCA1/2* kiembaanmutatie.

Nadat we hadden aangetoond dat een deel van de endometriumcarcinomen gerelateerd is aan *BRCA1/2* kiembaanmutaties, hebben we ons er in **hoofdstuk 5** op gericht om het endometriumcarcinoomrisico bij *BRCA1/2* kiembaanmutatie draagsters te kwantificeren waarbij we gebruik maakten van de data van een groot landelijk multicenter cohort studie, de HEBON cohort studie. Met 58 endometriumcarcinoom events is dit tot op heden de grootste studie naar dit onderwerp. In deze studie hebben we de risico analyses gestratificeerd voor histologie (endometrioïd, sereus-achtig, clear cell, sarcoom, andere) en moleculaire subgroep (p53-abnormaal/SCNA-hoog) nadat alle beschikbare endometriumcarcinomen opnieuw waren beoordeeld door de patholoog. We toonden aan dat draagsters van een *BRCA1/2* kiembaanmutatie een 2- tot 3-voudig verhoogd risico hebben op het ontwikkelen van een endometriumcarcinoom. Hierbij werd de grootste risicotoename gevonden voor endometriumcarcinomen met sereus-achtige histologie en endometriumcarcinomen van de p53-abnormale/SCNA-hoog moleculaire subgroep (ongeveer 10-voudig verhoogd). Wanneer werd gestratificeerd voor mutatietype (*BRCA1* of *BRCA2*), waren de risico's het hoogst voor *BRCA1* kiemmutatie draagsters. Ondanks deze sterk verhoogde risico's bleven de absolute risico's voor het ontwikkelen van een endometriumcarcinoom (tot 75 jaar) laag met 3,0% voor alle endometriumcarcinomen, en 1,1% voor sereus-achtige endometriumcarcinomen. Dit komt doordat endometriumcarcinoom een relatief zeldzame ziekte is met 2000 nieuwe gevallen per jaar, waarvan 200-300 van het sereuze subtype zijn.

Samengevat leverden we zowel mechanistisch als epidemiologisch bewijs dat er een associatie bestaat tussen endometriumcarcinoom en *BRCA1/2* mutaties. Daarom kunnen we concluderen dat endometriumcarcinoom, en meer specifiek endometriumcarcinoom met sereuze/sereus-achtige histologie en van de p53-abnormale/SCNA-hoge moleculaire subgroep, een onderdeel vormen van het *BRCA1/2*-geassocieerde HBOC syndroom.

Deze bevindingen hebben mogelijke klinische consequenties. Zoals eerder aangegeven kunnen vrouwen met een *BRCA1* of *BRCA2* kiembaanmutatie vanwege het sterk verhoogde risico op borstkanker en eierstokkanker kiezen voor een risico-verlagende bilaterale mastectomie en risico-verlagende salpingo-ovariëctomie. In Nederland wordt het op dit moment niet aanbevolen om een risico-verlagende baarmoederverwijdering uit te voeren ten tijde van de risico-verlagende salpingo-ovariëctomie. Hoewel we in dit proefschrift hebben aangetoond dat endometriumcarcinoom deel uitmaakt van het *BRCA1/2*-geassocieerd HBOC syndroom, ondersteunt het lage absolute risico om endometriumcarcinoom te ontwikkelen (overall: *BRCA1*: 3.4%; *BRCA2*: 2,0%; sereus-achtig: *BRCA1*: 1,4%; *BRCA2*: 0,6%) het huidige klinische beleid. Desalniettemin is het essentieel voor clinici om deze risico's te kennen, zodat adequate counseling kan plaatsvinden. Hierbij dienen de mogelijke voordelen van het uitvoeren van een risico-verlagende baarmoederverwijdering ten tijde van een risico-verlagende salpingo-ovariëctomie bij de individuele patiënt te worden afgewogen tegen de mogelijke nadelen van deze ingreep.

DNA-testen om kiembaanmutaties op te sporen worden over het algemeen aanbevolen als het verwachte detectiepercentage voldoende hoog is (>5%). Onderzoeken die de *BRCA1/2* kiembaanmutatiefrequentie beoordeelden in een niet-geselecteerd cohort van patiënten met endometriumcarcinoom van het sereuze subtype of endometriumcarcinoom patiënten (niet geselecteerd voor histotype) met een voorgeschiedenis van borstkanker, rapporteerden mutatiefrequenties van respectievelijk 2% en 3,8%. Deze gegevens ondersteunen daarom vooralsnog niet het routinematige screening voor *BRCA1/2* kiembaanmutaties bij patiënten met endometriumcarcinoom.

Op basis van onze gegevens worden de hoogste *BRCA1/2* mutatiefrequenties verwacht bij vrouwen die een p53-abnormaal endometriumcarcinoom hebben ontwikkeld. Het zou daarom interessant zijn om in een toekomstige studie te onderzoeken of de *BRCA1/2* kiembaanmutatie frequentie in deze groep hoger ligt. Zo'n studie zou idealiter uitgevoerd worden inclusief een subanalyse waarbij ook de borstkanker voorgeschiedenis, familiegeschiedenis en morfologische kenmerken die vaker voorkomen bij *BRCA1/2*-geassocieerd endometriumcarcinoom worden meegenomen (**hoofdstuk 4**).

Tumor-first screening naar *BRCA1/2* (en andere HR-gen) mutaties

Gezien de hoge prevalentie van erfelijke *BRCA1/2* mutaties bij vrouwen met hooggradige sereuze eierstokkanker (16%) of triple-negatieve borstkanker (14%), wordt routinematig kiembaananalyse aangeboden aan alle vrouwen met epitheliale eierstokkanker, of triple-negatieve borstkanker jonger dan 60 jaar. Nu PARP-remmers ook zijn geregistreerd door de European Medicines Agency als eerstelijns onderhoudsbehandeling bij patiënten met chemotherapie gevoelig hooggradig epitheliale eierstokkanker met bewezen *BRCA1/2*-mutatie (zowel somatisch als kiembaan), zullen aanvullende somatische tumortests regelmatiger gewenst zijn. In **hoofdstuk 6** hebben we *BRCA1/2* mutatieanalyse verricht op diagnostisch formaline gefixeerd, paraffine ingebed tumorweefsel geoptimaliseerd in een training cohort van bekende kiembaan *BRCA1/2* mutatiedraagsters. Vervolgens hebben we de tumortest gevalideerd in een prospectief cohort van vrouwen met epitheliale eierstokkanker. We toonden aan dat *BRCA1/2* mutaties (zowel somatisch als kiembaan) betrouwbaar kunnen worden opgespoord indien een combinatie van next-generation sequencing en copy number variant-multiplex ligation-dependent probe amplification wordt gebruikt. Door deze tumor-first benadering te gebruiken als pre-screeningstool om patiënten met *BRCA1/2* mutaties in de eierstokkanker op te sporen en te selecteren voor verwijzing naar de klinisch geneticus, zou ongeveer 80% van de verwijzingen naar de klinische genetica kunnen worden voorkomen. Een andere studie in Nederland die gelijktijdig werd uitgevoerd (*BRCA* testing in **O**varian cancer by **Pa**thologist (OPA)-studie) met behulp van een andere sequencing techniek (combinatie van op single-molecule molecular inversion probe-gebaseerde NGS en copy number variant-multiplex ligation-dependent probe amplification), toonde ook aan dat de

tumor-first benadering betrouwbaar en snel uitvoerbaar is in de dagelijkse praktijk. Ook werd deze methode gewaardeerd door zowel de patiënt als de gynaecoloog.

Een groot voordeel van de tumor-first-benadering is dat deze ook gemakkelijk kan worden geïmplementeerd voor andere tumortypen waarin kiembaanmutaties zijn beschreven (bijv. prostaatkanker, alvleesklierkanker, (p53-abnormaal) endometriumcarcinoom, borstkanker).

Ondanks dat deze tumor-first benadering veel voordelen biedt, is het belangrijk dat clinici zich ervan bewust zijn dat vanwege technische beperkingen (afhankelijk van de techniek die wordt gebruikt), sommige mutaties niet zullen worden gedetecteerd tenzij aanvullende analyses worden uitgevoerd (**hoofdstuk 6**). Daarom moeten patiënten met een hoge verdenking op een kiembaan mutatie (bijvoorbeeld sterk belaste familie anamnese, jonge leeftijd bij het ontstaan van de tumor, meerdere verschillende primaire tumoren) altijd worden doorverwezen naar de klinisch geneticus, ook als er geen mutatie is gedetecteerd in de tumortest.

De tumor-first benadering wordt momenteel in Nederland geïmplementeerd met hulp van een door het KWF gefinancierde implementatie studie. Op dit moment maakt het al deel uit van het routine diagnostisch onderzoek voor vrouwen met epitheliale eierstokkanker in verschillende regio's (bijvoorbeeld regio Leiden, Nijmegen). Omdat naast *BRCA1* en *BRCA2* ook andere kiembaanmutaties in meer zeldzame gevallen een oorzaak kunnen zijn voor het ontstaan van erfelijk eierstokkanker, is het genpanel inmiddels uitgebreid (o.a. *ATM, PALB2, CHEK2, RAD51C, RAD51D, BRIP1).*

Conclusie

In dit proefschrift hebben we mechanistisch, morfologisch en epidemiologisch bewijs geleverd dat sereuze of p53-abnormale/SCNA-hoge endometriumcarcinomen tot het *BRCA1/2*-geassocieerde HBOC syndroom behoren. Bovendien hebben we aangetoond dat een groot deel van de tumoren van de p53-abnormale/SCNA-hoge moleculaire subgroep homologe recombinatie deficiënt is, ook in afwezigheid van *BRCA1/2* mutaties. Tot slot, door aan te tonen dat *BRCA1/2* mutaties betrouwbaar kunnen worden gedetecteerd in diagnostisch tumorweefsel, hebben we een basis gelegd voor een efficiëntere genetische work-up van eierstokkanker patiënten die ook kan worden uitgebreid naar andere tumortypen.

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Curriculum Vitae

Marthe Mirande de Jonge werd geboren op 9 januari 1989 in Gouda en groeide op in Oldenzaal. In 2007 behaalde zij haar VWO diploma aan het Twents Carmel College de Thij te Oldenzaal. In 2007 begon zij haar studie Geneeskunde aan de Radboud Universiteit te Nijmegen die zij in 2014 cum laude afrondde. Haar interesse voor onderzoek en pathologie werd gewekt tijdens haar wetenschappelijke stage naar de HPV-type distributie in pre-maligne cervicale afwijkingen. In 2014 startte zij met haar opleiding tot patholoog in het Leids Universitair Medisch Centrum (LUMC) te Leiden, met prof. dr. V.T.H.B.M. Smit als opleider. Naar aanleiding van een casus in de diagnostiek deed zij een literatuurstudie naar het verband tussen sereus endometriumcarcinoom en erfelijke *BRCA1/2* mutaties. Dit onderzoek leidde uiteindelijk tot de start van het promotieonderzoek waarvan het resultaat beschreven staat in dit proefschrift. Haar promotieonderzoek deed zij op de afdeling pathologie van het LUMC en combineerde zij met haar opleiding tot patholoog middels een AIOSKO constructie onder directe begeleiding van prof. dr. V.T.H.B.M. Smit, prof. dr. C.J. van Asperen en dr. T. Bosse. Voor haar onderzoek naar morfologische en moleculaire kenmerken van endometriumcarcinomen ontstaan bij vrouwen met een erfelijke *BRCA1/2* mutatie is zij in 2018 “visiting student researcher” geweest aan de Stanford University Medical Center te Stanford, USA, onder supervisie van B.E. Howitt, M.D.. Hiervoor ontving zij de René Vogels reisbeurs en een reisbeurs van het Leids Universitair Fonds (LUF). In het kader van de studies beschreven in dit proefschrift bezocht zij diverse congressen in het binnen- en buitenland waar ze meerdere presentaties en posterpresentaties gaf. Voor haar voordracht op het United States and Canadian Academy of Pathology (USCAP) congres in 2019 te National Harbor, Maryland, ontving zij een prijs voor beste presentatie door een arts in opleiding. Zij is getrouwd met Klaas Vegter en samen hebben zij zoon Tieme (2020).

Dankwoord

"No one can whistle a symphony. It takes a whole orchestra to play it."

H.E. Luccock, 1885-1960

Met dank aan alle mensen die hebben bijgedragen aan de totstandkoming van dit proefschrift.