Novel **BRCA1** and **BRCA2** Tumor Test as Basis for Treatment Decisions and Referral for Genetic Counselling of Patients with Ovarian Carcinomas

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**ABSTRACT:** With the recent introduction of Poly(ADP-ribose) polymerase inhibitors, a promising novel therapy has become available for ovarian carcinoma (OC) patients with inactivating **BRCA1** or **BRCA2** mutations in their tumor. To select patients who may benefit from these treatments, assessment of the mutation status of **BRCA1** and **BRCA2** in the tumor is required. For reliable evaluation of germline and somatic mutations in these genes in DNA derived from formalin-fixed, paraffin-embedded (FFPE) tissue, we have developed a single-molecule molecular inversion probe (smMIP)-based targeted next-generation sequencing (NGS) approach. Our smMIP-based NGS approach provides analysis of both strands of the open reading frame of **BRCA1** and **BRCA2**, enabling the discrimination between real variants and formalin-induced artefacts. The single molecule tag enables compilation of unique reads leading to a high analytical sensitivity and enabling assessment of the reliability of mutation-negative results. Multiplex ligation-dependent probe amplification (MLPA) and Methylisation-specific multiplex ligation-dependent probe amplification (MS-MLPA) were used to detect exon deletions of **BRCA1** and methylation of the **BRCA1** promoter, respectively. Here, we show that this combined approach allows the rapid and reliable detection of both germline and somatic aberrations affecting **BRCA1** and **BRCA2** in DNA derived from FFPE OCs, enabling improved hereditary cancer risk assessment and clinical treatment of ovarian cancer patients.

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**KEY WORDS:** ovarian cancer; **BRCA1**; **BRCA2**; cancer predisposition; **BRCA** testing; personalized medicine; PARP-inhibitor; single molecule molecular inversion probes

**INTRODUCTION**

Ovarian carcinoma (OC) is one of the most frequently diagnosed types of cancer in females in Europe with an age-standardized incidence rate of 13.1 per 100,000 [Ferlay et al., 2013]. OC is most frequently diagnosed in patients above the age of 65 years [Yancik, 1993; Lowe et al., 2013]. However, the average age of onset is lower in patients who carry an OC predisposing germline aberration [Prat et al., 2005; Weissman, et al., 2012]. During the last decades, a modest improvement in OC survival has been reported [Lowe et al., 2013], but due to the low mean age-standardized 5-year survival (37.6%), the estimated number of OC-related deaths remains high in Europe (7.6 per 100,000; age-standardized rates) [Ferlay et al., 2013; De Angelis et al., 2014].

A promising novel therapy for OC patients is based on the inhibition of poly(ADP-ribose) polymerase (PARP), which is synthetically lethal in cancer cells with acquired inactivation of the homologous recombination-mediated repair pathway [Bryant et al., 2005; Farmer et al., 2005]. Multiple clinical trials with PARP inhibitors, including olaparib and niraparib, have demonstrated tolerability and efficacy of these treatments in OC patients [Audeh et al., 2010; Sandhu et al., 2013]. Moreover, progression-free survival of OC patients is further improved when olaparib is administered in combination with other treatments (e.g., paclitaxel, carboplatin, and cediranib) [Liu et al., 2014; Oza et al., 2015]. Since PARP inhibitors are predominantly lethal for cells that have lost the ability of homologous recombination-mediated repair, patients who have developed tumors with defects in this pathway show the highest response rates to such treatment [Mateo et al., 2015]. The highest response rates to treatments with olaparib were observed in OC patients with mutations affecting the homologous recombination genes **BRCA1** (MIM# 113705) or **BRCA2** (MIM# 600185) [Audeh et al., 2010; Ledermann et al., 2014]. Since genomic aberrations affecting **BRCA1** and **BRCA2** are among the

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most prevalent mutations observed in OCs [Cancer Genome Atlas Research, 2011; Kanchi et al., 2014; Patch et al., 2015], a substantial number of OC patients may benefit from treatments with PARP inhibitors.

Genomic aberrations affecting *BRCA1* and *BRCA2* are frequently encountered in both sporadic and familial OCs [Cancer Genome Atlas Research, 2011; Kanchi et al., 2014] (OMIM #604370 and #612555). Approximately 10%–15% of all OC patients carry a pathogenic germline aberration in *BRCA1* or *BRCA2* [Daly et al., 2010; Hennessy et al., 2010; Kanchi et al., 2014]. Loss of heterozygosity (LOH) of the wild-type allele is the tumor-initiating second hit in the majority of these patients [Foster et al., 1996; Berchuck et al., 1998]. Somatic mutations in *BRCA1* and *BRCA2* are observed in approximately 3.5%–8.5% and 2.5%–4% of OCs without an underlying germline mutation, respectively [Merajver et al., 1995; Foster et al., 1996; Berchuck et al., 1998; Cancer Genome Atlas Research, 2011; Kanchi et al., 2014]. Hypermethylation of the promoter of *BRCA1* is observed in approximately 10%–15% of these carcinomas [Baldwin et al., 2000; Bianco et al., 2000; Esteller et al., 2000; Cancer Genome Atlas Research, 2011]. Importantly, germline mutations, somatic mutations, and promoter hypermethylation appear mutually exclusive in OCs [Cancer Genome Atlas Research, 2011; Dworkin et al., 2009]. In total, *BRCA1* and *BRCA2* are mutated in 19%–22% of OCs and, consequently, these patients may benefit from PARP-inhibitor treatment [Hennessy et al., 2010; Cancer Genome Atlas Research, 2011; Kanchi et al., 2014]. Based on the genetic heterogeneity of the observed mutation spectrum, sequencing of the entire open-reading frame (ORF) of *BRCA1* and *BRCA2* using tumor-derived DNA is required to identify the patients who may benefit from this treatment.

Sequencing of *BRCA1* and *BRCA2* using tumor-derived DNA is hampered by the complexity of these genes, the low quality of the DNA derived from formalin-fixed, paraffin-embedded (FFPE) tumor samples and the low percentage of neoplastic cells in these samples. Several next-generation sequencing (NGS) approaches to determine the mutation status of *BRCA1* and *BRCA2* have been developed, but most approaches were validated using high-quality DNA (i.e., blood-derived DNA) [Feliubadalo et al., 2013; Hirotsu et al., 2015; Strom et al., 2015]. Therefore, these approaches can successfully be implemented in a diagnostic setting to screen for germline defects in *BRCA1* and *BRCA2* using blood-derived DNA [D’Argenio et al., 2015; Trujillano et al., 2015], but cannot be used to sequence low quality and highly fragmented DNA derived from FFPE tumor blocks. Recently, three multiplex PCR-based targeted NGS methods to sequence *BRCA1* and *BRCA2* in DNA derived from FFPE material have been evaluated [Ellison et al., 2015; Mafficini, et al., 2016]. However, these methods have relatively low levels of amplicon tiling, do not allow for strand-specific amplification, and lack single molecule tagging. As a consequence, possible drop-outs of amplicons and PCR jackpotting effects may result in false-negative results [Ellison et al., 2015] or false-positive calls due to deamination artefacts [Lou et al., 2013; Chen et al., 2014; Wong et al., 2014] and detection of low frequency variants is hampered [Jabara et al., 2011; Hiatt et al., 2013].

Here, we show that single-molecule molecular inversion probe (smMIP)-based targeted sequencing [Hiatt et al., 2013] is a reliable method to detect both germline and somatic mutations in *BRCA1* and *BRCA2* in FFPE samples, which can be applied to identify OC patients who may benefit from treatments with PARP inhibitors and are at high risk of carrying a germline *BRCA1* mutation.

### Materials and Methods

#### Patient Selection

A retrospective cohort of OC patients who were tested for germline *BRCA1* and *BRCA2* mutations after genetic counselling at the department of Human Genetics of the Radboudumc or the MaastrichtUMC+ was selected. All patients included signed informed consent for the use of stored material for research purposes (Radboudumc) or did not refuse use of stored material for research purposes (according to local policy, MaastrichtUMC+). OC derived from patients with a pathogenic germline *BRCA1* or *BRCA2* mutation were included in our study regardless of the histological phenotype. In contrast, patients without a germline mutation only those reported as serous OC were selected. Histological review was performed for all tumors by an expert pathologist (MS and JB). FFPE OCs were obtained between 1998 and 2014 at different time points during treatment and were categorized as either diagnostic (biopsy or primary debulking operation) or postchemotherapy (interval or secondary debulking operation). This study was approved by the medical ethics committee/institutional board (CMO, study 2014-1472) of the Radboudumc.

#### Isolation and Quality Assessment of FFPE Ovarian Cancer-Derived DNA

DNA isolation from FFPE OC samples was performed using standard procedures. First, 200 μl of 5% Chelex-100, diluted in TET lyses buffer with GlycoBlue, and 20 μl proteinase K (20 mg/ml) were added to the isolated sections of the FFPE ovarian cancer. This sample was sequentially incubated and mixed (350 rpm) at 56°C for 16 hr, at 37°C for 48 hr, and 95°C for 10 min. Incubation was followed by centrifugation at 16,000 g for 1 min at room temperature and the supernatant was collected. Next, 20 μl of NaAc (3 M, pH 5.2) and 440 μl ice-cold ethanol (EtOH) was added to the supernatant (on ice) and mixed. After centrifugation at 16,000 g for 10 min at 4°C, the supernatant was removed and the remaining pellet was washed with 1 ml ice-cold 70% EtOH. To remove the EtOH, the sample was centrifuged at 16,000 g for 2 min at 4°C and the supernatant was removed. The pellet was air-dried and, subsequently, dissolved in 80 μl of TE and incubated for 4 min at 56°C. The DNA concentration was determined (Qubit Flurometer; Life Technologies, Interleuvenlaan, Belgium) contained extension and ligation arms (40-45bp) and a linker sequence (30bp) and were designed to capture a target region of 112 nucleotides. This study was approved by the medical ethics committee/institutional board (CMO, study 2014-1472) of the Radboudumc.

#### Targeted Sequencing of *BRCA1* and *BRCA2* by smMIPs

A total of 157 and 260 smMIPs were designed, covering all coding regions and intron–exon boundaries of *BRCA1* and *BRCA2*, according to previously published methods [O’Roak et al., 2012; Hiatt et al., 2013; Boyle et al., 2014] with minor modifications (see Neveling et al., in press; and Supp. Table S1). Briefly, these 75–80 bp long oligonucleotides (i.e., smMIPs; ordered from Integrated DNA Technologies, Interleuvenlaan, Belgium) contained extension and ligation arms (40–45bp) and a linker sequence (30bp) and were designed to capture a target region of 112 nucleotides. In addition, all smMIPs contained a stretch of five random nucleotides (molecular tag), enabling the detection of 1,024 unique (tagged) reads per DNA fragment.
smMIP. Both DNA strands (i.e., plus and minus strand) of the target regions were targeted by independent smMIPs, enabling double tiling of these regions of interest. If the extension or ligation arm targeted a common SNP (MAF > 1%), two different smMIPs were designed to recognize and target both alleles. Next, smMIPs were pooled in an equimolar manner and phosphorylated by adding T4 polynucleotide kinase and 10X T4 DNA ligase buffer supplied with 10mM ATP (New England Biolabs, Ipswich, MA). To improve proper and equal coverage of the target regions, the smMIP pool was rebalanced based on initial sequencing results obtained using reference (blood-derived) DNA [Neveling et al., in press]. Targeted sequencing of BRCA1 and BRCA2 using DNA derived from FFPE OCs was performed as previously described [Weren et al., 2015], using a slightly modified smMIP capture protocol [O’Roak et al., 2012; Hiatt et al., 2013]. Briefly, smMIP capture was performed on 10 μl of input DNA (20–500ng) supplied with 15 μl capture mixture (0.01 μl ampiclase DNA ligase [100U/μl]; Illumina, Madison, WI), 2.5 μl 10X ampiclase buffer [Illumina], 0.27 μl smMIP pool dilution [6.6X10^6 μM], 0.32 μl Hemo KlenTaq [10U/μl; New England Biolabs], 0.03 μl dNTPs [0.25mM], and 11.9 μl H2O. The mixture was incubated at 95°C for 10 min, and subsequently at 60°C for 24 hr. Incubation was followed by exonuclease treatment: 0.5 μl exonuclease 1 (New England Biolabs), 0.5 μl exonuclease III (New England Biolabs), 0.2 μl 10X ampiclase buffer (Illumina), and 0.8 μl H2O was added to the (cooled) capture samples (consecutively incubated at 37°C and 95°C for 45 and 2 min, respectively). Subsequently, 10 μl of the sample was used to perform a PCR reaction by adding 1.3 μl of barcoded reverse primer (10 μM; Illumina), 12.5 μl 2X iProof (BioRad Laboratories, Venendaal, the Netherlands), 0.125 μl Hemo KlenTaq forward primer (100 μM; Illumina), and 1.075 μl H2O (final volume: 25 μl; PCR program: 98°C, 30 sec – 24x [98°C, 10 sec - 60°C, 30 sec - 72°C, 30 sec - 72°C, 2 min - 4°C, ∞]). Sequencing of the barcoded samples was performed using the Illumina NextSeq500 system, with 2 × 151-bp paired-end reads; smMIP libraries required spike-in of custom primers as described previously [O’Roak et al., 2012]. On average, 44 OC samples were sequenced per NextSeq500 Mid Output run. Obtained bcl files were converted into fastq files that were separated into barcode files. Double tiling was achieved for up to 99.3% of the ORF of BRCA1 and BRCA2, including the ~20 and ~20 intronic regions.

Fastq files were analyzed using the SeqNext software package (version 4.2.2; JSI Medical Systems GmbH, Kippenheim, Germany). Briefly, based on the single-molecule tag, consensus reads were generated and variants in coding regions were called if present in ≥ 5% of all reads and ≥ 3 unique variant reads. For details, see Supplementary Data.

Sequencing Data Analysis

Fastq files were analyzed using the SeqNext software package (version 4.2.2; JSI Medical Systems GmbH, Kippenheim, Germany). First, sequencing read data (fastq files) were mapped to exonic regions plus adjacent intronic regions of BRCA1 (NM_000924.3) and BRCA2 (NM_000059.3). Reads with undefined nucleotides in their barcodes or of low quality were ignored, and to generate consensus reads, all bases should be sequenced at least once in the consensus reads, at least two tagged reads are required to create a consensus read, and reads with less than 30% consensus were discarded. Reads were excluded from alignment if these contain more than 15% mismatches compared with the reference or if less than 50% of the bases match to the reference. The minimal absolute sequencing depth and expected sequencing depth for variant calling were 20 and 30 unique reads, respectively. In addition, variants were called if the variant was observed in ≥ 5% of all reads and ≥ 3 unique variant reads were present. To exclude (FFPE induced) cytotoxic deamination artefacts, C-G:T-A transition calls were manually discarded if the variant was not present in the smMIP covering the opposite strand (i.e., targeting the guanine nucleotide). Subsequently, all variants in coding regions and the intron–exon boundaries of BRCA1 and BRCA2 were selected for analysis. Variants were considered common SNPs if these were observed in our in-house database of BRCA1 and BRCA2 germine variants (accessed 01/03/2016; our in-house database contains 264 and 413 germine variants (in the close proximity) of the BRCA1 and BRCA2 locus, respectively). We previously applied BRCA sequencing (i.e., exonic- and adjacent intronic regions) on blood-derived DNA from 76 patients (of whom 90 of the 107 OCs were derived) using Sanger- or Iontorrent-based sequencing prior to this study. All germine variants in BRCA1 and BRCA2 that were identified in these screenings were included in our analysis to confirm that these variants were also present in the smMIP-based targeted sequencing data derived from the patient-matched FFPE samples. The other 17 patients had only been evaluated for the mutation segregating in their family. Insertion and deletion calls in regions known to be prone for false-positive calls (i.e., variant calls in homopolymer stretches of eight adenine residues and variant calls in nucleotides at the end of a sequencing read, which were not present in the sequencing reads derived from overlapping smMIPs) were considered false positives.

Remaining variants were validated using Sanger sequencing or by independent resequencing of the corresponding sample using our smMIP-based targeted sequencing approach. Variants were submitted to the locus-specific databases at LOVD: www.lovd.nl/BRCA1, www.lovd.nl/BRCA2.

To determine the accuracy per nucleotide of our method, we determined the number of true positives (n = 996), false positives (n = 14), and false negatives (n = 18) based on the results of 107 samples. The number of true negatives (n = 1,442,122) was determined based on 90 samples of which both BRCA genes were completely sequenced using Sanger- or Iontorrent-based sequencing of blood-derived DNA prior to the smMIP-based NGS analysis.

Multiplex Ligation-Dependent Probe Amplification Methylation-Specific Multiplex Ligation-Dependent Probe Amplification

Multiplex ligation-dependent probe amplification (MLPA) was performed according to the manufacturers protocol to detect intragenic copy-number variations affecting BRCA1 [kit P077; MRC Holland, Amsterdam, The Netherlands]. For data analysis, the GenMarker software (Sofgenetics, State College, PA) was used using the population normalization mode. Using this population normalization mode, possible chromosomal aberrations at other genomic regions (e.g., aneuploidies) would not hamper the MLPA analysis (in contrast to other approaches that only use probes outside the genomic locus of BRCA1 as a reference). In short, MLPA analysis was performed with 38 probes targeting the BRCA1 locus and 10 probes targeting other chromosomes. For data analysis, peak intensities were adjusted based on the average of peak intensities from all probes (i.e., population normalization). Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) was performed and analyzed according to the manufacturers protocol (kit ME001 C2) (MRC Holland).

LOH Analysis

LOH of the BRCA1 and the BRCA2 locus was determined using the variant allele frequency (VAF) of common SNPs (n = 16 for
BRCA1; n = 20 for BRCA2) and confirmed germline variants called in a heterozygous state (5% ≤ VAF ≤ 95%). As 95% of the samples had a neoplastic cell percentage of at least 32% (median 65%, range 15%–90%), an average major VAF of >60% was considered as a marker for LOH of the corresponding locus. Noteworthy, LOH analysis may have been hampered by the low percentage of neoplastic cells in a minor subset of these samples.

Statistics

A two-tailed Fisher exact test was applied to determine whether the frequency of high-grade serous OCs statistically differed between patients with a germline mutation in BRCA1 or BRCA2. The predetermined level of significance was P = 0.05.

Results

Sample Selection and Coverage of smMIP-Based Sequencing of BRCA1 and BRCA2

For the evaluation of our approach, 127 ovarian tumor samples derived from 96 patients were tested: 29 with a BRCA1, 14 with a BRCA2, and 53 without a germline mutation in either gene. For 20 samples, including 16 samples that were poorly amplifiable based on our control PCR, the sequencing depth of the ORF of BRCA1 and BRCA2 and total number of mapped reads was low (Supp. Fig. S1A and B). In the remaining 107 samples, the average number of unique reads per smMIP (after deconvolution of the PCR duplicates) was high (Fig. 1A). The average number of unique reads per coding base pair (including canonical splice sites) was 647 for BRCA1 (146–1,476) and 592 for BRCA2 (47–1,679) (Supp. Fig. S1C) (for the total number of tagged reads, see Supp. Fig. S1D). These unique reads were equally mapped to the plus and minus strands of the ORF, revealing that both strands were successfully targeted by our method (Fig. 1B). On average, 98.8% and 97.4% of the ORFs including the -20 and +20 intronic regions of BRCA1 and BRCA2 were covered with at least 20 and 30 unique reads reflecting a 95% chance of detecting a variant with a VAF of 30% and 20%, respectively (Fig. 1C).

Interpretation of smMIP-Based Mutation Detection

All pathogenic germline mutations in BRCA1 (n = 31) and BRCA2 (n = 16), known prior to smMIP-based targeted sequencing, were called by the NextSeq software in 47 tumor samples derived from 38 patients (Fig. 2A). The average number of unique variant reads and percentage of variant calls was high for these variants in both BRCA1 (524 [28–2,125]; 82.3% [64%–95%]) and BRCA2 (686 [23–2,832]; 71.3% [45%–94%]) (Table 1). Moreover, 745 out of 763 class 1/2 germline variants, known prior to smMIP-based targeted sequencing in 90 samples, were called (Fig. 2B). Based on the detection of 792/810 germline variants, an overall sensitivity of 97.8% (95% CI, 96.8%–98.8%) is estimated. The positions of the 18 variants that were not called using our standard variant calling settings were inspected visually. Five variants were missed due to LOH leading to a low percentage of variant reads in samples with a high tumor cell percentage. Thirteen variants were false negative due to a poor read depth at four SNP positions in five samples with a low number of total mapped reads (< 20,000 unique reads) (Supp. Fig. S1E).

1,135 variants were called in 107 samples. In addition to the 47 pathogenic germline mutations, 937 of these variants were known benign germline variants (class 1 or 2) present in our in-house database of BRCA1 and BRCA2 variants. Of the remaining 151 variants, 125 were C-to-T transitions reflecting FFPE-induced cytosine deamination artefacts (Supp. Table S2). The majority of these (n = 64) were observed in only two samples. In total, 12 variants were somatic mutations as they were confirmed present in the tumor, but absent in germline DNA. Five different somatic mutations, represented in eight carcinomas from five patients, were considered pathogenic, whereas the other four somatic variants were considered benign passenger mutations (Table 2). The remaining 14 variants were false-positive variant calls due to sequencing artefacts. They were called with a low percentage and number of variant reads (Supp. Fig. S2A and B) at either the last nucleotide of the targeted region or in a stretch of eight adenosines. Thus, after exclusion of deamination artefacts, the percentage of false-positive calls is low (1.4%, 14/1,010).

In total, 16,033 nucleotides were analyzed per sample. Therefore, based on these results, the accuracy per nucleotide of our method is 99.998% (996 true positive, 14 false positive, 1,442,122 true negative, and 18 false negative).

Detection of Copy-Number Variants Affecting BRCA1

All 127 DNA samples were screened for copy-number variants (CNVs) affecting the genomic locus of BRCA1 using MLPA. Prior to this screening, it was established that three of these patients carried a germline deletion encompassing exon 22 of BRCA1. These deletions were confirmed in all FFPE OC samples (n = 4) derived from these patients. No CNVs were detected in the other FFPE OC samples. Noteworthy, the relative number of unique reads covering exon 22 was strongly decreased in the smMIP NGS data derived from patients with a germline deletion affecting this exon (Supp. Fig. S3).

Detection of BRCA1 Promoter Methylation

Possible methylation of the BRCA1 promoter was determined using a MS-MLPA assay for all 127 DNA samples. Methylation of the promoter of BRCA1 was not observed in FFPE OC samples derived from patients with a germline or somatic pathogenic mutation affecting BRCA1 (n = 43) or BRCA2 (n = 22). In contrast, methylation of the BRCA1 promoter was observed in 13 samples derived from 17% of the sporadic patients (9/53). In all 24 patients, from whom multiple OC specimen were available, the methylation of the BRCA1 promoter was fully concordant in tumor samples at diagnosis and at interval or secondary debulking (Supp. Table S3).
Figure 1. A: Average number of unique reads per smMIP. X-axis: number of unique smMIPs included in our panel to sequence BRCA1 (n = 157 smMIPs) and BRCA2 (n = 260 smMIPs) (ordered for the genomic position of the target). Y-axis: number of unique reads obtained per smMIP. The average and median number of unique reads per smMIP is 254 and 154, respectively. Numbers are based on sequencing results obtained from 107 FFPE samples (for details; see main text or Materials and Methods). B: Number of unique reads/smMIPs mapping to/targeting the plus or minus strand of the ORF of BRCA1 and BRCA2. smMIPs targeting plus/minus strand: total number of smMIPs targeting the plus/minus strand of the corresponding base-pair position (range 0–3 smMIPs per strand per position). Average reads plus/minus strand: average number of unique reads mapping to the plus and minus strand based on 107 ovarian carcinoma samples. On average, the plus/minus strand were covered 356x/291x (BRCA1) and 334x/258x (BRCA2), respectively. Bars on the X-axis represent the nucleotides located in the exons of BRCA1 and BRCA2, including the canonical splice sites. Red bars indicate regions (4 and 16 bp, respectively) without any smMIPs or mapped reads at the corresponding locus. Note that 100% of the ORF including the canonical splice sites is sequenced and that 99.8% is sequenced on both the plus and minus strand. C: Percentage of the ORF of BRCA1 and BRCA2 covered with at least 20 and 30 unique reads in 107 FFPE ovarian carcinoma samples. On average, 98.8% (median 99.8%, range 79.3%–100%) and 97.4% (median 99.6, range 60.9%–100%) of the ORF of BRCA1 and BRCA2, including the −20 and +20 intronic regions, was covered with a sequencing depth of at least 20x and 30x, respectively.

LOH at the BRCA2 locus was observed in 73% of the FFPE samples derived from carriers of a pathogenic germline mutation in BRCA2 (Supp. Table S4). Based on these data, we consider that LOH occurred in the OC of 12 out of 14 patients (86%) with a BRCA2 germline mutation (Table 1). LOH of the wild-type allele is presumed in all four lesions with a somatic pathogenic BRCA2 mutation (Table 2; Supp. Table S5).

LOH of the respective loci is not an indication for a BRCA1 or BRCA2 mutation. LOH at the BRCA1 locus was observed in 73% of the tumors with a pathogenic germline mutation in BRCA2 and in 80% of the tumors without a somatic pathogenic mutation in BRCA1 or methylation of the BRCA1 promoter. LOH of the BRCA2 locus was observed in 56% of tumors with a pathogenic germline mutation in BRCA1 and in 46% of the sporadic tumors.
Histology Review of the OCs

Histology revision by an expert pathologist revealed that 83% of the carcinomas derived from germline mutation carriers had a high-grade serous histology (n = 35). The other patients with a germline mutation in BRCA1 or BRCA2 presented carcinomas with a mixed (n = 2), high-grade endometrioid (n = 1), clear cell (n = 1), and poorly/undifferentiated (n = 3) histology (Supp. Table S6A). The frequency of high-grade serous OCs did not differ between carcinomas derived from patients with a germline mutation in BRCA1 (22/28) or BRCA2 (13/14) (P = 0.39).

Although OCs of patients without a germline BRCA1 or BRCA2 mutation were selected based on their reported serous histology, revision revealed that 13% of these patients did not develop low- or high-grade serous carcinomas (seven out of 54). These patients developed carcinomas with a mixed (n = 1), low-grade endometrioid (n = 1), high-grade endometrioid (n = 1), or poorly/undifferentiated (n = 4) carcinomas (Supp. Table S6B). All carcinomas that showed methylation of the promoter of BRCA1 had a high-grade serous histology (n = 9 patients). In contrast, high-grade serous histology was observed in only three of the five carcinomas with somatic pathogenic mutations in BRCA1 or BRCA2. Two patients with a somatic pathogenic mutation in BRCA2 developed a carcinoma with either a mixed or poorly/undifferentiated histology (Table 2).

Discussion

Reliable and sensitive analysis of the mutation status of BRCA1 and BRCA2 in FFPE OC samples is important, now that patients with germline and somatic pathogenic mutations in BRCA1 and BRCA2 are eligible for therapy with PARP inhibitors. Analysis of tumor DNA can be used as a prescreen for germline mutation analysis in blood only if germline mutations in tumor DNA can be assessed efficiently. Here, we show that reliable analysis of germline and somatic mutations is possible using a combination of smMIP-based mutation detection and MLPA on DNA isolated from FFPE OCs.

Due to the high a priori risk of 10%–15% to carry a germline BRCA1 or BRCA2 mutation, all new OC patients are now eligible for germline DNA testing in many countries, including The Netherlands [Lancaster et al., 2015; Oncoline, 2015]. Our novel method enables reliable assessment of the tumor DNA mutation status of BRCA1 and BRCA2 in FFPE material derived from OCs. Performing such a BRCA tumor test on all newly diagnosed OC patients may be an efficient way to select all patients who may eventually benefit from treatment with PARP inhibitors and are simultaneously at high risk of carrying a pathogenic germline BRCA mutation, as approximately 75% of those that are tested positive for BRCA mutations in the tumor will have a germline mutation (i.e., hereditary predisposition; germline status of the mutation is confirmed in DNA derived from blood) [Cancer Genome Atlas Research, 2011]. This would limit genetic counselling procedures and concomitant distress to OC patients with a positive BRCA tumor test or a positive family history of ovarian cancer. This procedure would also be...
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<th>Patient ID</th>
<th>Germline mutation</th>
<th>Nucl. change</th>
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<td>756</td>
<td>80</td>
<td>Y</td>
</tr>
<tr>
<td>P057</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>2</td>
<td>80</td>
<td>933</td>
<td>68</td>
<td>Y</td>
</tr>
<tr>
<td>P070</td>
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<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>2</td>
<td>50–60</td>
<td>411</td>
<td>76</td>
<td>Y</td>
</tr>
<tr>
<td>P078</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>2</td>
<td>4</td>
<td>1,215</td>
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<td>Y</td>
</tr>
<tr>
<td>P001</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>Poorly/undifferentiated</td>
<td>1</td>
<td>50</td>
<td>58</td>
<td>87</td>
<td>Y</td>
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<tr>
<td>P007</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
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<td>1</td>
<td>90</td>
<td>57</td>
<td>95</td>
<td>Y</td>
</tr>
<tr>
<td>P022</td>
<td>BRCA1</td>
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<td>p.Glu1250*</td>
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<td>1</td>
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<td>405</td>
<td>90</td>
<td>Y</td>
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<tr>
<td>P025</td>
<td>BRCA1</td>
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<td>p.Glu1250*</td>
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<td>1</td>
<td>90</td>
<td>28</td>
<td>93</td>
<td>Y*</td>
</tr>
<tr>
<td>P029</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>85</td>
<td>150</td>
<td>83</td>
<td>Y</td>
</tr>
<tr>
<td>P033</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>75</td>
<td>358</td>
<td>88</td>
<td>Y</td>
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<tr>
<td>P040</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
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<td>483</td>
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<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>70</td>
<td>322</td>
<td>84</td>
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<tr>
<td>P048</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>90</td>
<td>1,100</td>
<td>91</td>
<td>Y</td>
</tr>
<tr>
<td>P066</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>60–70</td>
<td>150</td>
<td>68</td>
<td>Y</td>
</tr>
<tr>
<td>P076</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>80–90</td>
<td>667</td>
<td>88</td>
<td>Y</td>
</tr>
<tr>
<td>P077</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>Mixed carcinoma</td>
<td>1</td>
<td>80</td>
<td>1,602</td>
<td>88</td>
<td>Y</td>
</tr>
<tr>
<td>P079</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>80</td>
<td>751</td>
<td>92</td>
<td>Y</td>
</tr>
<tr>
<td>P085</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>90</td>
<td>190</td>
<td>86</td>
<td>Y</td>
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<tr>
<td>P086</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>70</td>
<td>1,134</td>
<td>90</td>
<td>Y</td>
</tr>
<tr>
<td>P093</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>Mixed carcinoma</td>
<td>1</td>
<td>65</td>
<td>265</td>
<td>80</td>
<td>Y</td>
</tr>
<tr>
<td>P094</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>85</td>
<td>590</td>
<td>88</td>
<td>Y</td>
</tr>
<tr>
<td>P096</td>
<td>BRCA1</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>60</td>
<td>786</td>
<td>68</td>
<td>Y</td>
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<tr>
<td>P028</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>2</td>
<td>40</td>
<td>23</td>
<td>52</td>
<td>Y</td>
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<tr>
<td>P086</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>2</td>
<td>30</td>
<td>282</td>
<td>63</td>
<td>Y*</td>
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<tr>
<td>P006</td>
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<td>p.Glu1250*</td>
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<td>64</td>
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<td>p.Glu1250*</td>
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<td>Y</td>
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<tr>
<td>P034</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>50</td>
<td>138</td>
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<td>P044</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
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<td>727</td>
<td>92</td>
<td>Y</td>
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<tr>
<td>P067</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>70</td>
<td>409</td>
<td>52</td>
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<tr>
<td>P068</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>45</td>
<td>2,832</td>
<td>84</td>
<td>Y</td>
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<tr>
<td>P069</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>60</td>
<td>89</td>
<td>79</td>
<td>Y</td>
</tr>
<tr>
<td>P071</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>40</td>
<td>515</td>
<td>45</td>
<td>N</td>
</tr>
<tr>
<td>P072</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>80</td>
<td>1,351</td>
<td>89</td>
<td>Y</td>
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<tr>
<td>P073</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>55</td>
<td>1,299</td>
<td>90</td>
<td>Y*</td>
</tr>
<tr>
<td>P074</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>55</td>
<td>1,207</td>
<td>75</td>
<td>Y</td>
</tr>
<tr>
<td>P075</td>
<td>BRCA2</td>
<td>c.3748G&gt;T</td>
<td>p.Glu1250*</td>
<td>High-grade serous</td>
<td>1</td>
<td>55</td>
<td>1,207</td>
<td>75</td>
<td>Y</td>
</tr>
</tbody>
</table>

*The indicated nucleotide change is based on the cDNA sequence.

**Based on pathogenic germline variant call in FFPE material; no additional informative SNPs available.

P*: Possibly LOH, but the analysis is hampered by the low percentage of tumor cells in the corresponding FFPE sample.

Cost-effective as it will reduce the number of germline tests and decreases double testing on both tumor and germline DNA. We are currently evaluating whether this altered diagnostic pathway starting with tumor DNA BRCA testing in newly diagnosed ovarian cancer as a prescreen for treatment and genetic counselling to initiate germline DNA testing on DNA derived from blood is feasible and is adopted as well as appreciated by both patients and professionals.

Most NGS approaches to determine the mutation status of BRCA1 and BRCA2 have been developed to sequence blood-derived, high-quality DNA and, consequently, can only be implemented in a routine diagnostic setting to screen for germline mutations in these genes [Feliu-Badalo et al., 2013; D’Argenio et al., 2015; Hirotsu et al., 2015; Strom et al., 2015; Trujillano et al., 2015]. Our smMIP-based NGS approach provides double tiling of the ORF of BRCA1 and BRCA2 by targeting the plus and minus strand using independent overlapping smMIPs and enables the detection of unique reads by the introduction of a single-molecule tag. The detection of unique reads enables the recognition of biased amplification of only a limited number of template molecules, which is commonly observed when a low amount of amplifiable input DNA is available (e.g., DNA derived from FFPE OCs). Formalin-induced cytosine deamination artefacts, which frequently occur in DNA derived from FFPE samples [Chen et al., 2014], can be recognized due to the targeting of the plus and minus strand using independent overlapping smMIPs (i.e., double tiling). Our approach enables the reliable detection of mutations in BRCA1 and BRCA2 in DNA derived from FFPE material. Furthermore, since smMIP-based NGS is a low-cost and easily scalable method, extending this approach to sequence additional genes in FFPE samples is feasible [O’Roak et al., 2012; Kumar et al., 2014]. Since patients with defects in other homologous recombination genes may also benefit from PARP inhibitors, extending our smMIP design with probes targeting these genes could be considered...
In five of the 51 ovarian cancer patients without a germline \textit{BRCA1} or \textit{BRCA2} mutation included in this study, a somatic, pathogenic mutation affecting \textit{BRCA1} or \textit{BRCA2} was encountered. This confirms that these somatic mutations are relatively common in sporadic ovarian cancer patients [Cancer Genome Atlas Research, 2011; Kanchi et al., 2014]. In comparison with amplicon-based target enrichment, our smMIP-based approach has the advantage that the number of sequenced template molecules can be measured using the single-molecule tag. This allows a proper risk estimation of the probability of false-negative results given a chosen limit of detection [Eijkelenboom et al., 2016]. Our analyses were aimed at a minimal sequencing depth of 30 unique reads, which should be sufficient to detect \textgreater 95% of variants present at a VAF of 20% or higher. On average, 97.4% of the ORFs including 20 exon-flanking nucleotides of \textit{BRCA1} and \textit{BRCA2} reached this sequencing depth, which was mostly much higher reflecting high complexity of our sequence libraries. Given the percentages of neoplastic cells in our samples was at least 40% in 90% of the tumor samples derived from sporadic patients, the chance that a somatic mutation was missed is low.

Acquired hypermethylation of the promoter of \textit{BRCA1} in the OC was observed in 17% of the patients without a germline \textit{BRCA} mutation. As described by others [Cancer Genome Atlas Research, 2011], hypermethylation was mutually exclusive with germline and somatic \textit{BRCA1} and \textit{BRCA2} mutations. In line with previous reports [Esteller et al., 2000], we noticed that LOH of \textit{BRCA1} was observed in all OCs with methylation of the \textit{BRCA1} promoter, suggesting that the hypermethylation is driving the tumorigenesis and will probably lead to homologous recombination-deficient tumors. Therefore, patients who develop OCs with hypermethylation of the \textit{BRCA1} promoter are predicted to benefit from PARP-inhibitor treatments [Stefansson et al., 2012; Veek et al., 2010]. A systematic evaluation of the effect of PARP inhibitors in this patient group seems justified.

$$\frac{\text{HUMAN MUTATION, Vol. 38, No. 2, 226–235, 2017}}{\text{233}}$$

### Table 2. Variant Calling of Somatic Mutations in \textit{BRCA1} and \textit{BRCA2} in FFPE Samples

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Gene</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Carcinoma phenotype</th>
<th>FFPE samples Neoplastic cells (%)</th>
<th>*Var. reads</th>
<th>Var. reads (%)</th>
<th>Pathogenic$^a$ LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>P047</td>
<td>\textit{BRCA1}</td>
<td>c.121del</td>
<td>p.His41fs</td>
<td>High-grade serous</td>
<td>2</td>
<td>60–70</td>
<td>486</td>
<td>41</td>
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<tr>
<td>P061</td>
<td>\textit{BRCA1}</td>
<td>c.929del</td>
<td>p.Gln310fs</td>
<td>High-grade serous</td>
<td>2</td>
<td>60</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>P050</td>
<td>\textit{BRCA2}</td>
<td>c.7971dup</td>
<td>p.Tyr2658fs</td>
<td>Mixed carcinoma</td>
<td>2</td>
<td>50</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>P062</td>
<td>\textit{BRCA2}</td>
<td>c.512del</td>
<td>p.Arg18Leufs</td>
<td>Poorly/undifferentiated</td>
<td>1</td>
<td>90</td>
<td>789</td>
<td>75</td>
</tr>
<tr>
<td>P091</td>
<td>\textit{BRCA2}</td>
<td>c.7878G&gt;C</td>
<td>p.Trp2636Gys</td>
<td>High-grade serous</td>
<td>1</td>
<td>70</td>
<td>339</td>
<td>47</td>
</tr>
<tr>
<td>P048$^a$</td>
<td>\textit{BRCA2}</td>
<td>c.6970C&gt;G</td>
<td>p.His2324Asp</td>
<td>High-grade serous</td>
<td>1</td>
<td>60–70</td>
<td>235</td>
<td>37</td>
</tr>
<tr>
<td>P063</td>
<td>\textit{BRCA2}</td>
<td>c.4154C&gt;T</td>
<td>p.Ser1385Leu</td>
<td>Low-grade endometrioid</td>
<td>1</td>
<td>30</td>
<td>55</td>
<td>8</td>
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<tr>
<td>P063</td>
<td>\textit{BRCA2}</td>
<td>c.4347C&gt;G</td>
<td>p.Phe1449Leu</td>
<td>Low-grade endometrioid</td>
<td>1</td>
<td>30</td>
<td>128</td>
<td>6</td>
</tr>
<tr>
<td>P079$^e$</td>
<td>\textit{BRCA2}</td>
<td>c.8599A&gt;C</td>
<td>p.Trh2867Pro</td>
<td>Poorly/undifferentiated</td>
<td>1</td>
<td>90</td>
<td>59</td>
<td>36</td>
</tr>
</tbody>
</table>

$^a$Variants were considered pathogenic as they were either truncating or a known class 5 missense mutation and considered benign passenger mutations based in silico prediction tools.

$^b$Probably LOH, but the analysis is hampered by the low percentage of tumor cells in the corresponding FFPE sample.

$^c$Patients with a \textit{BRCA1} germline mutation.

$^d$Weakly conserved nucleotide (PhyloP: 1.90), Align GVGD: class C0 (GV: 130.59; GD: 22.66), SIFT: tolerated (score: 0.39).

$^e$Weakly conserved nucleotide (PhyloP: 1.58), Align GVGD: class C0 (GV: 353.86 - GD: 0.00), SIFT: tolerated (score: 0.51).

$^f$Weakly conserved nucleotide (PhyloP: 0.29), Align GVGD: class C0 (GV: 180.03 - GD: 0.00), SIFT: tolerated (score: 1).

$^g$Weakly conserved nucleotide (PhyloP: 0.61), Align GVGD: class C0 (GV: 129.31 - GD: 1.62), SIFT: tolerated (score: 0.07).

For more details regarding these in silico predictions, see Supplementary Data.

[McCabe et al., 2006; Mateo et al., 2015]. This smMIP-based analysis of \textit{BRCA1} and \textit{BRCA2} is a paradigm for reliable mutation analysis of complete ORFs or hotspot regions of other genes [Eijkelenboom et al., 2016].

If settings are used that filter out deamination artefacts, the amount of false-positive calls using standard variant calling settings at a cut-off value of 5% VAF is very low. In our analysis, no false-positive calls were observed in 90% of the samples (96/107), whereas in nine samples only a single false-positive variant was called and in two samples two and three false-variant positives were called, respectively. All these calls could easily be recognized as false-positive calls. Therefore, interpretation of sequencing results is very objective. The sensitivity of our smMIP-based NGS approach was 97.8% based on standard variant calling of 810 \textit{BRCA1} and \textit{BRCA2} germline variants (100% was detected by visual inspection). Variants were missed either due to insufficient sequencing depth at three specific regions in samples with relatively low total coverage or due to loss of the allele containing the SNP, a phenomenon often observed in human cancers [Lengauer et al., 1998]. If germline mutations in \textit{BRCA1} or \textit{BRCA2} underlie the development of an OC, LOH will generally affect the allele without the causative germline mutation leading to an increase in mutant allele frequencies [Cancer Genome Atlas Research, 2011; Kanchi et al., 2014]. However, low frequent variant calls can reliably be detected using smMIP-based NGS; we successfully identified somatic (passenger) variants with a low percentage of variant reads (e.g., 6% VAF). Noteworthy, the detection of low-percentage variant calls can be easily improved by increasing the length of the molecular tag; a stretch of eight random nucleotides would enable the detection of 65,536, instead of 1,024, unique reads per smMIP [Hiatt et al., 2013]. Nevertheless, caution is required regarding the detection of germline variants in cancer samples with a high percentage of neoplastic cells (>90%), especially if mutation analysis is performed on tumor samples that are obtained after chemotherapy, as therapy may lead to reversion of the mutation [Norquist et al., 2011; Patch et al., 2015]. We have not yet optimized the detection of exon deletions and duplications that may inactivate \textit{BRCA1} and \textit{BRCA2} using our smMIP approach. For these aberrations, MLPA methods that are currently used in routine diagnostics of germline mutations can be applied.
germline mutations in BRCA1 and BRCA2 develop (high-grade) serous OCs [Boyd et al., 2000; Hensssey et al., 2010]; however, a significant subset (17%) of OCs derived from patients with germline mutations in BRCA1 and BRCA2 reveal a different histological phenotype. Therefore, we recommend that sequencing of BRCA1 and BRCA2 should be considered in all patients with OCs irrespective of their histological subtype.

In conclusion, a combined approach of smMIP-based NGS, MLPA, and MS-MLPA allows the reliable detection of both germline and somatic alterations affecting BRCA1 and BRCA2 in FFPE OCs (MLPA and MS-MLPA were only performed for BRCA1). This approach enables the identification of (1) patients who may benefit from therapeutic treatments that are based on the mutation status of BRCA1 and BRCA2 (e.g., PARP-inhibitors) and (2) those at high risk of a pathogenic germline mutation in BRCA1 and BRCA2 (i.e., hereditary predisposition). Therefore, our approach will further improve clinical treatment and hereditary cancer risk assessment of ovarian cancer patients.

Acknowledgments
We thank Jay Shendure, Evan Boyle, and Beth Martin for scientific input regarding our smMIP design. We also thank the Radboud Genomics Technology Center for performing the sequencing runs described in this manuscript. We would like to thank all the hospitals (i.e., Amphia Hospital, Canisius-Wilhelmina Hospital, Elkerliek Hospital, Jeroen Bosch Hospital, Laboratory Clinical Pathology Midden Brabant, Maastricht UMC+), Stichting PAMM, Laboratory Pathology East Netherlands, Radboudumc, Rijnstate en Zuyderland Medical Center) who provided FFPE material for this study. M.J.L.L. and N.H. designed the study. R.D.A.W., A.R.M., A.E., H.O., M.v.A., M.S., and J.J.L.L. supervised the work. R.D.A.W. was responsible for patient selection. A.H. and H.O. designed molecular inversion probes. A.S.S., M.S., J.A.d.H., and M.J.L.L. were responsible for interpretation histology. A.S.S., N.H., E.B.G.G., M.J.B., and M.J.L.L. were responsible for patient selection. A.H. and H.O. designed molecular inversion probes. A.R.M., M.R.N., N.H., and M.J.L.L. supervised the work. R.D.A.W. and M.J.L.L. wrote the manuscript, with assistance and final approval from all coauthors.

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