Order out of chaos: decoding chromosomal instability patterns in copy number high female cancers

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CHAPTER 3

Causality and functional relevance of *BRCA1* and *BRCA2* pathogenic variants in non-high-grade serous ovarian carcinomas

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ABSTRACT

The identification of causal *BRCA1/2* pathogenic variants (PVs) in epithelial ovarian carcinoma (EOC) aids selection of patients for genetic counselling and treatment decision making. Current recommendations therefore stress sequencing of all EOCs, regardless of histotype. While it is recognised that *BRCA1/2* PVs cluster in high-grade serous ovarian carcinomas (HGSOC), this view is largely unsubstantiated by detailed analysis. Here, we aimed to analyse the results of *BRCA1/2* tumour sequencing in a centrally-revised, consecutive, prospective series including all EOC histotypes. Sequencing of *n*=946 EOCs revealed *BRCA1/2* PVs in 125 samples (13%), only eight of which were found in non-HGSOC histotypes. Specifically, *BRCA1/2* PVs were identified in high grade endometrioid (3/20; 15%), low-grade endometrioid (1/40; 2.5%), low-grade serous (3/67; 4.5%), and clear cell (1/64; 1.6%) EOCs. No PVs were identified in any mucinous ovarian carcinomas tested. By re-evaluation and using loss of heterozygosity and homologous recombination deficiency analyses, we then assessed whether the eight 'anomalous' cases were (1) potentially histologically misclassified and (2) whether the identified variants were likely causal in carcinogenesis. The first 'anomalous' non-HGSOC with a *BRCA1/2* PV proved to be a misdiagnosed HGSOC. Next, germline *BRCA2* variants, found in two p53-abnormal high-grade endometrioid tumours, showed substantial evidence supporting causality. One additional, likely causal variant, found in a p53-wildtype low-grade serous ovarian carcinoma, was of somatic origin. The remaining cases showed retention of the *BRCA1/2* wildtype allele, suggestive of non-causal secondary passenger variants. We conclude that likely causal *BRCA1/2* variants are present in high-grade endometrioid tumours but are absent from the other EOC histotypes tested. Although the findings require validation, these results seem to justify a transition from universal to histotype-directed sequencing. Furthermore, in-depth functional analysis of tumours harbouring *BRCA1/2* variants combined with detailed revision of cancer histotypes can serve as a model in other *BRCA1/2*-related cancers.

INTRODUCTION

Epithelial ovarian carcinomas (EOC) have an estimated incidence of 5.2 per 100,000[1] and consist predominantly of high-grade serous ovarian carcinoma (HGSOC) histotype. A substantial range of other histotypes are also recognized, constituting over a quarter of all ovarian carcinomas, which include high-grade and low-grade endometrioid ovarian carcinoma (EnOC), low-grade serous ovarian carcinoma (LGSOC), clear cell ovarian carcinoma (CCOC), mucinous ovarian carcinoma (MOC), as well as other very rare EOC histotypes.

A *BRCA1* or *BRCA2* (*BRCA1/2*) pathogenic variant (PV), in combination with loss of heterozygosity (LOH) of the wildtype allele, results in a deficiency in the homologous recombination (HR) DNA repair pathway and an HR-deficient (HRD) tumour. This fundamental tumour vulnerability can be exploited via treatment with platinum-based chemotherapy or poly (ADP-ribose) polymerase inhibitors (PARPi).[2] Over the last decade, use of PARPi in a frontline or relapsed setting has increased the progression-free survival of EOC,[3-10] breast cancer,[11, 12] pancreatic cancer[13] and prostate cancer[14] patients, with the greatest benefit seen in tumours with a *BRCA1/2* PV and LOH.

Consequently, sequencing of tumour DNA to identify *BRCA1/2* PVs has rapidly become an important adjunct to decision-making in PARPi treatment. In light of the pivotal role of *BRCA1/2* sequencing, the American Society of Clinical Oncology (ASCO)[15] and the European Society of Medical Oncology (ESMO)[16] both recommend sequencing of EOCs for germline and somatic *BRCA1/2* PVs. The rationale for these screening recommendations is twofold: (1) to guide genetic counselling and (2) stratify for PARPi eligibility. In recent years, genetic testing strategies for germline *BRCA1/2* PVs have broadly shifted towards variant pre-screening of tumour tissue, an efficient and patient-friendly approach to preselect patients with an indication for genetic counselling.[17, 18]

Although clinical guidelines recommend *BRCA1/2* sequencing in EOC regardless of histological subtype, PVs are commonly thought to be confined to HGSOC. This perception was reinforced by studies that reported occult HGSOC precursors lesions (serous tubal intraepithelial carcinomas) in specimens obtained during prophylactic surgery in a subset of *BRCA1/2* PV carriers,[19, 20] whereas precursor lesions of other non-HGSOC EOC histotypes were not enriched in *BRCA1/2* PV carriers.

Despite these observations, meta-analyses showed that *BRCA1/2* PVs are not exclusive to HGSOC.[21, 22] A further complicating factor is that accurate identification of EOC

histotypes was debatable[23] prior to the improved 2014 World Health Organization (WHO) classification.[24] As a consequence, previously reported non-HGSOC carrying *BRCA1/2* PVs may have been misdiagnosed cases of HGSOC.

The important, unresolved question we address here is the distribution of *BRCA1/2* PVs in a large, prospectively collected series of EOCs revised by expert gynaecopathologists. We therefore analysed the results of *BRCA1/2* tumour sequencing in a centrally-revised, consecutive series including all EOC histotypes. In the case of PVs identified in non-HGSOC, which we subsequently term 'anomalous samples', additional HRD analyses were conducted in order to assess causality and help refine tumour sequencing recommendations.

MATERIALS AND METHODS

Cohort selection, central pathology revision and BRCA1/2 sequencing of EOC

The study was approved by the ethics committees of the Leiden University Medical Center (Leiden, The Netherlands) and University Medical Center Groningen (Groningen, The Netherlands) (nWMO-D4-2022-030). A waiver of informed consent was waived, according to local regulations.

Tumour DNA from EOCs (all histotypes) was prospectively sequenced for *BRCA1/2* variants using next-generation sequencing (NGS) in two large academic medical centres as part of a national implementation project. The consecutive series (centre 1: September 2017 – November 2022; centre 2: July 2018 – December 2021) comprised practically all newly-diagnosed EOCs in the rural regions covered by these centres. Cases were excluded from the final analyses due to (pre-)analytical or other reasons (including a non-evaluable EOC histotype due to insufficient tissue for ancillary immunohistochemistry (IHC)) (supplementary material, Figure S1, final cohort *n*=946). The final centrally-revised EOC cohort comprised both HGSOC (*n*=690/946; 73%) and non-HGSOC histotypes (*n*=256/946; 27%) (Table 1).

Central pathology revision and BRCA1/2 sequencing of epithelial ovarian carcinomas

Prior to *BRCA1/2* sequencing, EOCs were centrally reviewed in a prospective manner by expert gynaecopathologists as part of routine care and classified according to the most recent WHO guidelines.[25, 26] Diagnosis was made using histologic variables (e.g., squamous differentiation was used as a defining feature for endometrioid histotype) in combination

with ancillary immunohistochemistry markers (e.g., PAX8, WT1, p53, NapsinA, PR). The methodology and specifications of sequencing are described in supplementary material, Table S1. PVs (class 4 and 5) in *BRCA1/2* were reported.[27] The pathogenicity of each *BRCA1/2* variant is evaluated in our routine molecular diagnostics by clinical molecular biologists at the department of Pathology and discussed with the Clinical Genetics department. Large deletions and genomic rearrangements were not routinely tested.

Inter-pathologist agreement

A subset of referred EOCs was histologically classified in both a local hospital and a university medical centre, allowing calculation of inter-pathologist agreement. Only referred EOCs that were fully categorized according to the most recent WHO guidelines (i.e., 2014[26] or 2020[25] WHO guideline) were included in the agreement analysis (i.e., exclusion of 'EOC not otherwise specified'; *n*=362/946; 38%)

In-depth analysis of non-HGSOC with BRCA1/2 PVs

Non-HGSOC with a *BRCA1/2* PV were initially re-evaluated by two expert gynaecopathologists using representative H&Es and diagnostic IHC markers (already performed in routine diagnostics) to exclude misclassification. Next, the origin (germline or somatic) of identified PVs was confirmed by routine diagnostic genetic germline testing at clinical genetic services. Cases were then re-sequenced using a comprehensive multi-biomarker NGS panel (Oncomine™ Comprehensive Assay Plus (OCA+)) (Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States of America). The OCA+ results included, but were not restricted to, locus-specific LOH (LOH defined as: copy number total ≥1 with minor allele copy number of zero), tumour mutational burden, *POLE* PVs, a microsatellite instability (MSI-) score, as well as a genomic instability metric (GIM). A GIM score of ≥ 16 is associated with HRD.[28] A variety of HRD tests were then performed. Firstly, the percentage of genome-wide LOH (gwLOH%) was determined using the OCA+ data, followed by shallow whole genome sequencing (Illumina NovaSeq6000 sequencing, single-end, 150 bp, 5 million reads per sample) (Illumina, San Diego, California, United States of America) to obtain copy number (CN-) signatures, as described.[29] Lastly, functional HR status was assessed using the RAD51-FFPE test (co-immunofluorescence staining of RAD51 (rabbit, cat. ab133534, 1:1000, Abcam, Cambridge, United Kingdom) and geminin (mouse, cat. NCL-L, 1:60, NovoCastra, Leica Biosystems, Buffalo Grove, Illinois, United States of America)).[30] Tumours with a RAD51 score of ≤ 15% were considered HRD.[30]

Statistical analyses

Histotype agreement between local and central pathologists was assessed and the 95% confidence interval (CI; Normal Approximation or Wald interval) and Cohen's kappa coefficient calculated. Interpretation of Cohen's kappa coefficient was as previously described,[31] with a coefficient of 0.40–0.59 indicating weak, 0.60–0.79 indicating moderate and 0.80–0.90 indicating a strong level of agreement. Prevalence is reported together with the 95% CI (Normal Approximation or Wald interval). Logistic regression analysis was performed to analyse the relationship between *BRCA1/2* PVs and EOC histotypes. For the modelling, EOC histotypes were dichotomized into HGSOC and non-HGSOC histotypes. The odds ratio (OR) and 95% CI (Normal Approximation or Wald interval) were estimated to express this relationship. *P*-values below 0.05 were considered statistically significant. Statistical analysis was performed using SPSS statistics (IBM, SPSS Inc., version 25).

RESULTS

Pathologist agreement in histotyping

To assess interobserver agreement in histotyping, a comparison of histotypes assessed locally versus centrally was performed. The inter-pathologist agreement revealed agreement of 90% (95% CI, 87% - 93%) between local and central histotype assessment (Cohen's kappa value 0.79; substantial agreement).[31] Nevertheless, some variance in EOC histotyping was observed (Figure 1A), including in EOCs carrying a *BRCA1/2* PV (Figure 1B).

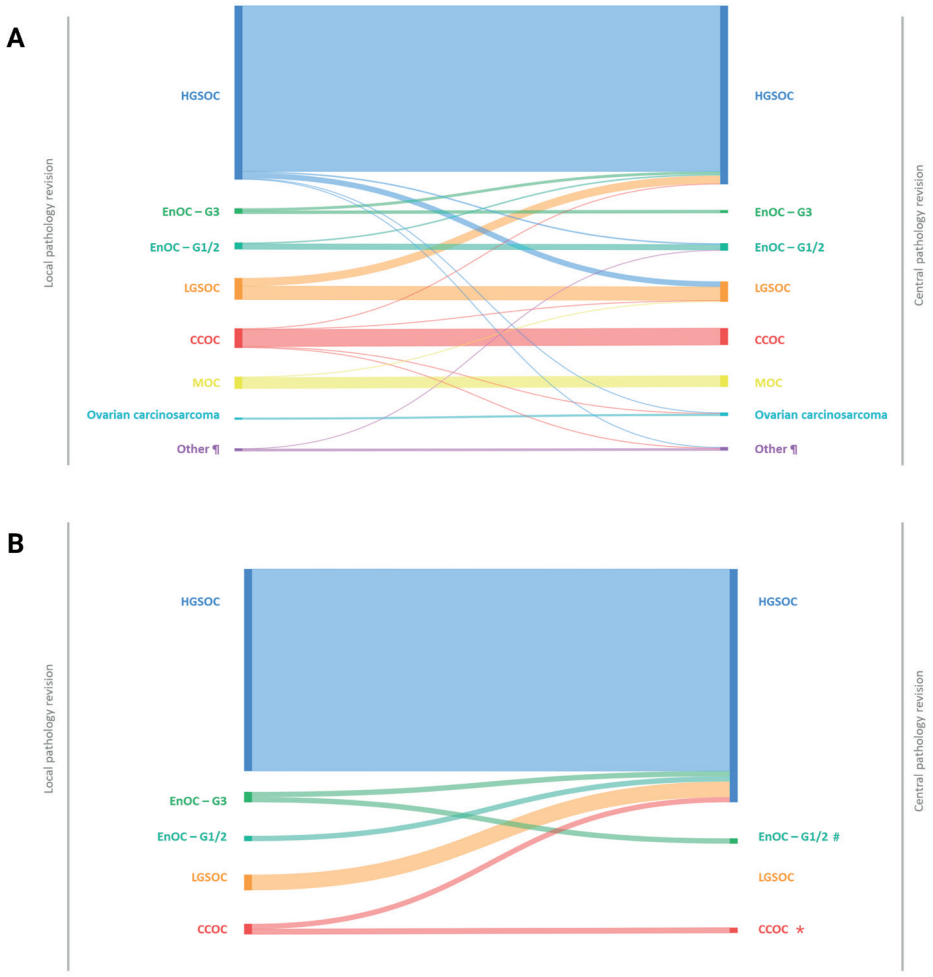

Figure 1. *Overview of the shifts in epithelial ovarian carcinoma histotyping between local and central pathology revision (n=362). Figure A shows the shifts in all epithelial ovarian carcinomas, whereas figure B highlights all epithelial ovarian carcinomas with a BRCA1/2 pathogenic variant. The agreement in locally- and centrally-assessed histotypes in all epithelial ovarian carcinomas was 90% (95% CI, 87% – 93%). Cohen's kappa value was 0.79, indicating moderate agreement among local and central pathologists.*[31] *¶ Including malignant Brenner tumour, mixed-type histology, mesonephric-like adenocarcinoma, undifferentiated carcinoma and small cell carcinoma. # The low-grade EnOC had a somatic BRCA2 pathogenic variant that was non-causal in carcinogenesis. The BRCA2 pathogenic variant was a passenger variant in an EnOC with a POLE pathogenic variant. * The CCOC had a somatic BRCA1 pathogenic variant that was non-causal in carcinogenesis. The BRCA1 pathogenic variant was a passenger variant in a microsatellite instable CCOC. Abbreviations: HGSOC, high-grade serous ovarian carcinoma; EnOC, endometrioid ovarian carcinoma; G1/2, grade 1 or 2; G3, grade 3; LGSOC, low-grade serous ovarian carcinoma; MOC, mucinous ovarian carcinoma; CCOC, clear cell ovarian carcinoma.*

Table 1. *Relationship between BRCA1-BRCA2 pathogenic variants and epithelial ovarian carcinoma histological subtypes after central revision.*

		***BRCA1/2* PV**	
	n	**Prevalence**	**95% CI**
All EOC	**125/946**	**13%**	**11–15%**
High-grade serous OC	**117/690***	**17%**	**14–20%**
Non-high-grade serous OC	**8/256***	**3.1%**	**1.0–5.3%**
Endometrioid OC – G3	3/20	15%	
Endometrioid OC – G1/2	1/40	2.5%	
Low-grade serous OC	3/67	4.5%	
Clear cell OC	1/64	1.6%	
Mucinous OC	0/39	0%	
Ovarian carcinosarcoma	0/13	0%	
Very rare EOC histotypes ¶	0/13	0%	

** Logistic regression analysis: OR: 0.16; 95% CI, 0.08 – 0.33; P<0.001; 94% (117/125) of all BRCA1/2 PVs were detected in HGSOC. ¶ Including malignant Brenner tumour, mixed-type histology, mesonephric-like adenocarcinoma, undifferentiated carcinoma and small cell carcinoma. Abbreviations: PV, pathogenic variant; n, total; EOC, epithelial ovarian carcinoma; OC, ovarian carcinoma; G1/2, grade 1 or 2; G3, grade 3; OR, odds ratio; 95% CI, 95% confidence interval.*

BRCA1/2 pathogenic variants in EOCs

Next, all 946 EOCs were prospectively screened for the presence of *BRCA1/2* PVs in tumour tissue. Assessment of *BRCA1/2* PVs in EOCs revealed an overall frequency of 13% (*n*=125/946; 95% CI: 11-15%, Table 1), predominantly consisting of *BRCA1* PVs (supplementary material, Table S2A,B). The two academic centres involved showed a comparable prevalence (supplementary material, Table S3A,B). Importantly, the wide distribution of identified variants across the *BRCA1/2* genes (supplementary material, Figure S2) reiterates the necessity of sequencing the genes in their entirety.

BRCA1/2 pathogenic variants across all EOC histotypes

When assessing the frequency of *BRCA1/2* PVs across histotypes, significantly more PVs were present in HGSOC (*n*=117/690; 17%, 95% CI 14 – 20%) compared to non-HGSOC (8/256; 3.1%, 95%CI 1.0–5.3%) (OR: 0.16; 95% CI, 0.08 – 0.33; *P*<0.001 Table 1). In line with previous studies,[32] for the HGSOC with a *BRCA1/2* PV, the majority showed loss of the wildtype allele (89% and 74% for *BRCA1* and *BRCA2*, respectively) (supplementary material, Table S4). Intriguingly, *BRCA1/2* PVs were found in other histotypes including high-grade EnOC, low-grade EnOC, LGSOC and CCOC (see Table 1 for details), whereas they were entirely absent from MOCs (*n*=0/36; 0%), ovarian carcinosarcomas (0/13; 0%)

and very rare EOC histotypes (0/13; 0%). Finally, PV in other EOC-susceptibility genes (i.e., *BRIP1, RAD51C, RAD51D*) were confined to HGSOC histologies (supplementary material, Table S5).

Table 2. *Re-evaluation of non-high-grade serous ovarian carcinomas carrying a BRCA1/2 pathogenic variant using H&E and ancillary immunohistochemistry to exclude misclassification.*

	Histotype		Immunohistochemical staining				
Case	**Initial**	**Re-evaluated**	**Nuclear PAX8**	**Nuclear WT-1+**	**Nuclear PR**	**Intracytoplasmic NapsinA**	**p53 ∞**
1	LGSOC *	HGSOC	+	+	+	-	Abnormal
2	EnOC – G3	EnOC – G3	+	-	-	-	Abnormal
3	EnOC – G3	EnOC – G3	+	+	+/-	-	Abnormal
4	LGSOC	LGSOC	+	+	+/-	-	Wildtype
5	LGSOC	LGSOC	+	+	+/-	-	Wildtype
6	EnOC – 1/2	EnOC – G1/2	+	-	Weakly +	-	Wildtype
7	EnOC – G3	EnOC – G3	+	Patchy	+/-	-	Abnormal
8	CCOC	CCOC	+	-	-	+	Wildtype

** Initially classified as 'serous carcinoma Silverberg grade 2'. Within the current 2020 WHO classification, this case was best classified as LGSOC. After re-evaluation, including representative H&E staining and full IHC panel, case 1 was classified as HGSOC. ∞ The complete absence, overexpression and cytoplasmic p53 staining pattern are considered 'p53 abnormal'. Abbreviations: -, negative; +, positive; +/-, partly positive; HGSOC, high-grade serous ovarian carcinoma; EnOC, endometrioid ovarian carcinoma; G1/2, grade 1 or 2; G3, grade 3; LGSOC, low-grade serous ovarian carcinoma; CCOC, clear cell ovarian carcinoma.*

Histological re-evaluation of non-HGSOCs with a BRCA1/2 PV

H&E slides from non-HGSOC histotypes were first re-evaluated to exclude histological misclassification. Histological re-evaluation by two expert gynaecopathologists confirmed that seven out of eight apparent non-HGSOC cases carrying a *BRCA1/2* PV (cases 2 to 8) were correctly classified (Table 2; Figure 2). Case 1 proved to be a misdiagnosed HGSOC (initial diagnosis: LGSOC; Table 2) showing loss of the *BRCA2* wildtype allele and an HRD phenotype (GIM: 16; CN-sig. 3: 32%; RAD51-FFPE score 6%; Table 3). Accordingly, the seven remaining cases (7/946; 0.8% overall) were considered anomalous, i.e., non-high-grade serous histology with a *BRCA1/2* PV.

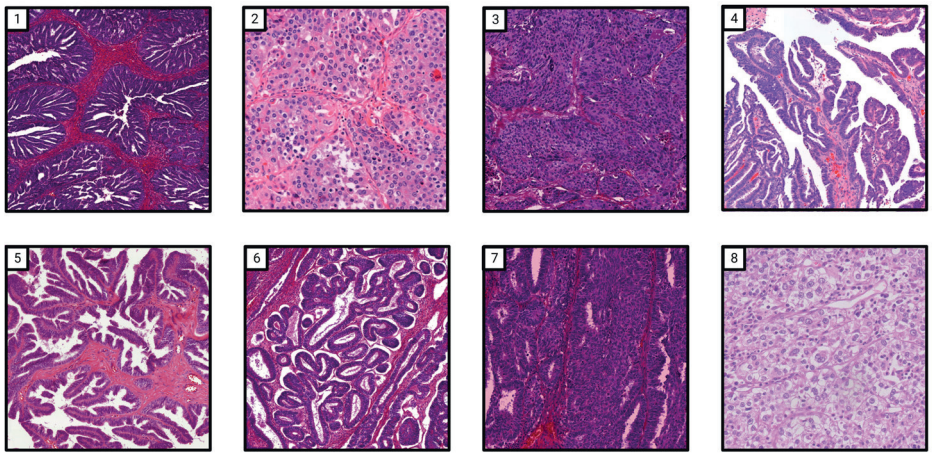

Figure 2. *Representative H&E staining of eight (case 1 – 8) non-high-grade serous ovarian carcinomas with a BRCA1 or BRCA2 pathogenic variant. After re-evaluation in the context of this study, case 1 was reclassified as a high-grade serous ovarian carcinoma.*

In-depth analyses of anomalous samples

LOH analysis of wildtype *BRCA1/2* alleles confirmed LOH in three samples (cases 2 – 4) (Table 3), two of which were p53-abnormal high-grade EnOCs (case 2, 3). The identified *BRCA2* PVs were of germline origin and the tumours showed clear evidence of HRD, consisting of a high genomic instability metric (case 2 – 28; case 3 – 33), high levels of gwLOH (case 2 – 52%; case 3 – 32%) and low RAD51 scores (case 2 – 5%; case 3 – 0%; Table 3). Only one of the 67 tested LGSOC (1.5%) harboured a *BRCA2* PV of somatic origin accompanied by LOH, with HRD characteristics (GIM: 18; CN-signature 3: 31%; Table 3). The functional relevance of the *BRCA2* PV in the p53-wildtype LGSOC could, however, not be assessed (Table 3). However, the yield of these potentially clinically relevant *BRCA2* PVs remains relatively low (*n*=1/67; 1.5%), which is comparable to the population frequency of *BRCA1/2* PVs.[33]

Table 3. *Overview of non-high-grade serous ovarian carcinomas harbouring a BRCA1/2 pathogenic variant.*

		PV					Presence of mutator	HRD assessment				
Case	**Re-evaluated histotype**	**Variant**	**VAF**	**LOH**	**Origin**	**TMB** *	***POLE* PV**	**MSI score**	**gwLOH**	**GIM ≈**	**CN-sig.3 ∞**	**RAD51-FFPE test Δ**
1	HGSOC £	*BRCA2* NM_000059.3*:* c.7419_7420del, p.(Cys2473*)	91%	Yes	Unknown$	1.0	Wildtype	1.3	N.E.	16	32%	6%
2	EnOC – G3	*BRCA2* NM_000059.3*:* c.635_636delGA, p.(Arg212Lysfs*2)	93%	Yes	Germline	15	VUS	0.9	51%	28	N.E.	5%
3	EnOC – G3	*BRCA2* NM_000059.3*:* c.3487delG, p.(Asp1163fs)	88%	Yes	Germline	5.7	Wildtype	0.6	32%	33	34%	0%
4	LGSOC	*BRCA2* NM_000059.3*:* c.7757G>A, p.(Trp2586*)	70%	Yes	Somatic ¶	7.6	Wildtype	7.1	N.E.	18	32%	N.E.
5	LGSOC	*BRCA1* NM_007294.4: c.1292dupT, p.(Leu431Phefs*5)	49%	No	Germline	1.9	Wildtype	0	0%	0	0%	28%
6	EnOC – G1/2	*BRCA2* NM_000059.3*:* c.4936G>T, p.(Glu1646*)	24%	No	Somatic	72	*POLE* NM_006231.4: c.1366G>C, p.(Ala456Pro)	2.3	0%	1	0%	26%
7	EnOC – G3	*BRCA2* NM_000059.3*:* c.6082G>T, p.(Glu2028*)	34%	No	Somatic	216	*POLE* NM_006231.4: c.857C>G, p.(Pro286Arg)	3.5	0%	0	0%	4%
8	CCOC	*BRCA1* NM_007294.4: c.5165C>T, p.(Ser1722Phe)	22%	No	Somatic	190	Wildtype	26 ±	0%	N.E.	0%	N.E.

** Number of mutations per 1 Mb. ≈ Genomic Instability Metric is based on the Oncomine Comprehensive Assay Plus.[28] A GIM score of ≥ 16 is associated with HRD. ∞ Shallow-whole genome sequencing-based CN signatures have been described by Macintyre et al.[29] High CN-signature 3 exposure has been associated with BRCA1/2 PV. Δ RAD51-FFPE test parameters were calibrated in Van Wijk et al.[30] An EOC with a RAD51-FFPE score ≤ 15% is considered HRD. £ After re-evaluation, including representative H&E staining and a full immunohistochemistry panel, case 1 was re-classified as HGSOC. $ The patient has been referred to the Department of Clinical Genetics in accordance with national guidelines, although the germline testing was not completed due to patient-related reasons. ¶ The patient has been referred to the Department of Clinical Genetics in accordance with national guidelines, although the germline testing results were not completed and therefore not available. However, the patient had both a low-grade serous ovarian carcinoma (LGSOC) and a uterine clear cell carcinoma. While both tumours were sequenced for BRCA1 and BRCA2, the specific BRCA2 PV was only identified in the LGSOC, supporting the somatic origin of the variant.' ± Tumour is MSI-high and carries an MSH6 PV (NM_000179.2: c.3964G>T, p.(Glu1322*)). Abbreviations: PV, pathogenic variant; VAF, variant allele frequency; LOH, loss of heterozygosity; TMB, tumour mutational burden; MSI, microsatellite instability; CN-sig. 3, copy-number signature 3; gwLOH, genome-wide LOH percentage; GIM, genomic instability metric; N.E., non-evaluable; HGSOC, high-grade serous ovarian carcinoma; EnOC, endometrioid ovarian carcinoma; G1/2, grade 1 or 2; G3, grade 3; LGSOC, low-grade serous ovarian carcinoma; CCOC, clear cell ovarian carcinoma; HRD, homologous recombination deficient; HRP, homologous recombination proficient; VUS, variant of uncertain significance.*

For the remaining four anomalous samples that showed retention of the *BRCA1/2* wildtype allele (case 5 – 8; Table 3), we carried out additional analyses to determine the impact of monoallelic *BRCA1/2* variants in all cases. In case 5, a patient with a *BRCA1* germline PV, an LGSOC in the right ovary was observed together with a clonally-unrelated HGSOC in the left vaginal wall (Figure 3). p53 IHC showed a mutant staining pattern in the HGSOC, while the LGSOC showed a wildtype staining pattern. In line with this observation, high levels of chromosomal instability (Figure 3) were present in the HGSOC but absent from the LGSOC. The *BRCA1* PV variant allele frequency (VAF) was considerably higher in the HGSOC (94%) compared to the LGSOC (49%), indicating loss of the *BRCA1* wildtype allele confined to the HGSOC. Similarly, the HRD-associated CN signature 3 (HGSOC 38%; LGSOC 0%) and a low RAD51-FFPE score (HGSOC 2%; LGSOC 28%) were restricted to the HGSOC (Figure 3), indicating that the *BRCA1* PV was not causal in the LGSOC.

The remaining three anomalous samples included a low-grade EnOC (case 6), a high-grade EnOC (case 7) and a CCOC (case 8). All three harboured somatic monoallelic *BRCA1/2* PVs with low VAFs (24%, 34%, 22%, respectively; Table 3). Subsequent analysis identified a high tumour mutational burden in all three tumours (Table 3), indicative of an alternative carcinogenic mechanism, i.e., a mutator-driven tumour. The low- and high-grade EnOC (cases 6 & 7) harboured *POLE* PVs, explaining the mutational burden, whereas the CCOC exhibited a high MSI score (Table 3). The observations above are strengthened by the presence in these cases of *POLE* PV- and MSI-associated mutational signatures, respectively (SBS signature 10a in cases 6 and 7; SBS signature 44 in case 8; Table 3 and supplementary material, Figure S3). The tumours did not show genomic-based HRD-associated characteristics (Table 3). In conclusion, two of the original 946 EOC samples (0.2%), or alternatively, 2 of 256 non-HGSOC histotypes (0.8%), harboured a likely causative germline *BRCA1/2* PV.

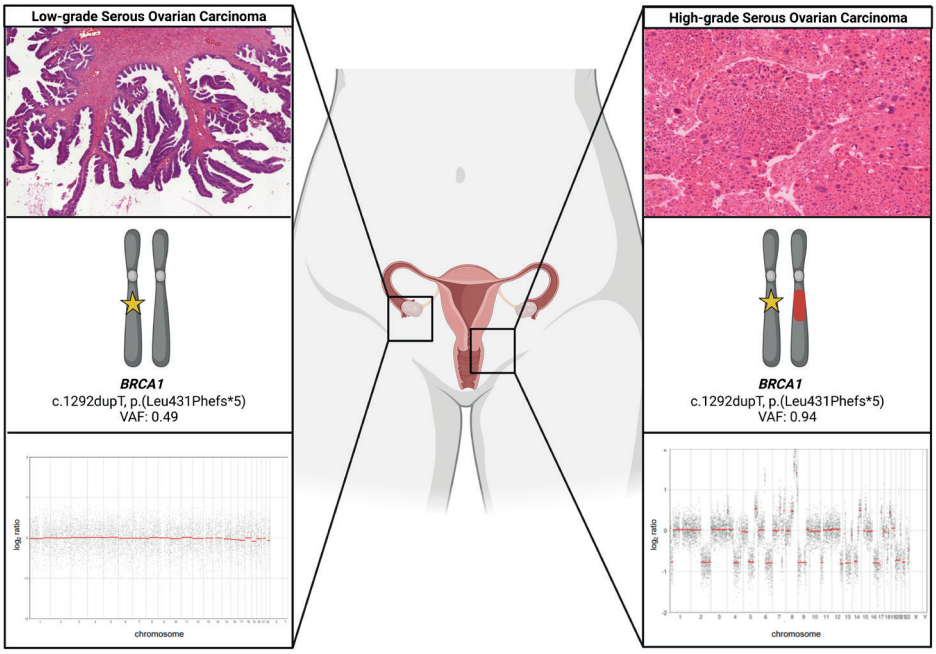

Figure 3. *Illustration of a germline BRCA1 PV without loss of the wildtype BRCA1 allele in a low-grade serous ovarian carcinoma. The patient has two tumours, 1) a high-grade serous ovarian carcinoma in the left vaginal wall, and 2) a low-grade serous ovarian carcinoma in the right ovary. Haematoxylin and eosin (H&E) staining reveals two distinct, unrelated tumour morphologies. The BRCA1 PV was identified in both epithelial ovarian carcinomas (visualized by yellow stars), while loss of heterozygosity (LOH) of the wildtype allele was only observed in the HGSOC (visualized by red area). The difference in variant allele frequencies of the BRCA1 PV was not explained by differences in tumour cell percentages. The relative copy-number profile of the p53-abnormal HGSOC showed chromosomal instability, which was absent in the p53-wildtype LGSOC. The figure was created with BioRender.com. Abbreviations: LGSOC, low-grade serous ovarian carcinoma; HGSOC, high-grade serous ovarian carcinoma; H&E, haematoxylin and eosin; VAF, variant-allele frequency.*

DISCUSSION

Although *BRCA1/2* PVs have been reported in all major EOC histotypes,[21, 22] PVs are nevertheless generally considered exclusive to HGSOC. In a large real-world series of prospectively sequenced EOCs, centrally revised by expert gynaecopathologists, we showed that this view needs to be qualified. We unequivocally demonstrated that causal and functionally relevant *BRCA1/2* PVs are also found in high-grade EnOCs but are absent from other histotypes. We showed that the *BRCA1/2* PVs identified in other non-HGSOC histotypes were unlikely to be drivers of EOC carcinogenesis and therefore can be considered incidental passenger variants.

To our knowledge, this is the first large, in-depth examination of *BRCA1/2* PVs across EOC histotypes. Even after taking into consideration the categorization of observed inter-pathologist agreement as 'substantial', our data show the relevance of an accurate diagnosis prior to tumour genetic screening, which may be achieved by central pathology review. This would become even more relevant, when introducing a histotype-directed approach towards tumour screening of *BRCA1/2.*

Furthermore, our findings have implications for future tumour sequencing approaches and therapeutic strategies in EOC. As anticipated, the majority of *BRCA1/2* PVs were identified in HGSOC, with a high fraction of specimens with a PV showing loss of the wildtype allele. Still, the fact that a subset retained the *BRCA1/2* wildtype allele emphasizes that, even within the context of HGSOC, the presence of a PV does not necessarily indicate causality. Intriguingly, *BRCA1/2* PVs were also identified in other EOC histotypes, but likely causative germline *BRCA1/2* PVs were only found in two cases of (p53-abnormal) high-grade EnOC.

In this context, it is important to emphasize that the differentiation of HGSOC and high-grade EnOC – based on H&E staining – is particularly challenging. The complexity of histotype differentiation is further aggravated by the fact that HGSOC with *BRCA1/2* PVs frequently display pseudo-endometrioid morphology, thereby resembling high-grade EnOC.[34] These factors, together with the data above, lead us to recommend the extension of genetic screening beyond HGSOC to include high-grade EnOC. Notably, this recommendation accords with inclusion criteria for practice-changing PARPi therapy trials which included both HGSOC and high-grade EnOC.[3, 4, 6, 8, 10] Our results therefore have important implications for the design of future clinical trials, confirming that there is no biological rationale for testing PARPi therapy in other EOC histotypes.

Another notable aspect of this study was our observation of likely passenger PVs in mutable EOC histotypes.[35-38] The somatic monoallelic *BRCA1/2* PVs with low VAFs observed in mutable EOC were probably secondary to a *POLE* PV or MSI. Additional evidence that such variants are a consequence rather than a cause of carcinogenesis could be provided by analysis of (functional) HRD status. This result emphasizes the importance of adopting a holistic view in biomarker assessment.

As sequencing costs continue to decline, *BRCA1/2* sequencing becomes more accessible, with the important caveat that the probability of identifying non-causal monoallelic PVs in *BRCA1/2* consequently increases. This underlines the necessity of accurate pre-selection of

cases in which *BRCA1/2* sequencing is relevant. If *BRCA1/2* PVs are nevertheless identified in non-*BRCA1/2*-associated histologies, as above, analysis of locus-specific LOH, as well as HRD, can help contextualize an identified PV.

The functional analyses described here are applicable well beyond EOC. Firstly, determining the causality of *BRCA1/2* PVs is crucial in the context of other cancers, including breast cancer,[32] endometrial cancer,[39] prostate cancer[40] and pancreatic cancer,[41] as retention of the wildtype *BRCA1/2* allele is a common occurrence in these cancers. In addition to assessment of LOH, the concept of passenger PVs in a mutator-driven tumour is relevant in other cancers, especially in *POLE*-mutated and/or MSI-high endometrial carcinoma.[42] Our results further underline the fact that the interpretation and clinical relevance of *BRCA1/2* PVs is highly dependent on the biological and clinical context of a particular cancer.

Despite the size of the cohort described here, to our knowledge the largest prospective series of EOCs yet sequenced for *BRCA1/2*, our study is not without limitations. First of all, while the series includes a large number of non-HGSOC histotypes, the overall number of individual histotypes remains limited, especially for the very rare EOC histotypes. Particularly, germline *BRCA1/2* PVs with LOH have been identified in a subset of ovarian carcinosarcomas.[43] The next two limitations are inherent to the design of analysing tumor-based sequencing results. Firstly, even though tumour sequencing is an established approach in routine pathology and demonstrates near-perfect sensitivity for identifying (challenging) *BRCA1/2* PVs,[17, 18, 44] PVs may have been missed.[45, 46] This limitation, however, applies to all EOC histotypes, and we do not anticipate bias specifically towards non-HGSOC histotypes. Second, we cannot completely rule out the possibility of selection bias, even in this consecutive series. Current guidelines recommend universal screening of EOCs and thus the series should include practically all newly-diagnosed EOCs. Nevertheless, pathologists may have prioritized sequencing of HGSOC at the expense of non-HGSOC histotypes. Finally, conducting a comprehensive cost-benefit analysis of the histotype-directed screening approach would be necessary before any potential changes are made to the screening guidelines.

In conclusion, we demonstrated that *BRCA1/2* PVs as putative drivers of EOC carcinogenesis are limited to HGSOC and high-grade EnOC. Even though some *BRCA1/2* PVs were identified in other major EOC histotypes, we confirmed a non-causal relationship. As non-HGSOC histotypes collectively represent a substantial proportion of all EOCs, our results seem to justify a transition from 'universal' towards a 'histotype-directed' approach when screening EOC for *BRCA1/2* PVs.

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SUPPLEMENTARY DATA

Table S1. *Specifications of sequencing pipelines in two large academic centres.*

Centre-1	DNA isolation is described in detail by De Jonge *et al.* [17]. In brief, DNA was isolated from formalin-fixed paraffin-embedded (FFPE) blocks in a routine diagnostic setting. In centre 1, either three 0.6 mm tissue cores or microdissected areas/whole slides (five 10 µm sections) were used as input material. During the study period (September 2017 – November 2022), multiple custom Ampliseq next-generation sequencing (NGS) panels were used: – BRCAv4 *n*=90/563 (16%) – BRCAv5 *n*=249/563 (44%) – HRDv2 *n*=224/563 (40%) BRCAv4 comprised full horizontal coverage of the coding exons of *BRCA1* and *BRCA2.* Large deletions and genomic rearrangements were not routinely tested. BRCAv5 further included the coding exons of *ATM, BARD1, BRIP1, CDK12, CHEK1, CHEK2, FANCL, PALB2, PPP2R2A, RAD51B, RAD51C, RAD51D, RAD54L*. In HRDv2, *TP53, PIK3CA,* and *ERBB2* were added. NGS was performed on the Ion Torrent S5 Genestudio platform (Thermo Fisher Scientific, Gilford, New Hampshire, United States of America). Variants in tumour tissue were called with a minimum variant allele frequency (VAF) of ≥ 3.5% and a read depth of ≥100. The pathogenicity of each *BRCA1/2* variant is evaluated in our routine molecular diagnostics by clinical molecular biologists at the department of Pathology and discussed with the clinical genetics department. The pathogenicity is registered in a local Geneticist Assistant (GA) database (SoftGenetics, State College, Pennsylviania, United States of America, Geneticist Assistant version 1.8.1) as has been described elsewhere [17]. The sequencing results from our study were imported in GA and the pathogenicity score is then automatically assigned to the variants.
Centre-2	The details of DNA isolation have been described by Wei *et al.* [47]. In centre 2, tumour DNA was isolated from areas with a high tumour cell percentage. Four to eight 10 µm FFPE tissue sections were used as input material. Within the scope of this study, multiple NGS panels were applied (July 2018 – June 2022): – NimaGen *n*=310/383 (80%) – Illumina *n*=73/383 (20%) NimaGen (EasySeq™ NGS smMIP Targeted Capture Kit) (NimaGen, Nijmegen, Gelderland, The Netherlands) comprised full coverage of the coding regions of *BRCA1* and *BRCA2.* NGS was performed on an Illumina NextSeq 500 (Illumina, San Diego, California, United States of America). Use of the TruSight Oncology 500 (Illumina) subsequently added the coding regions of *RAD51C, RAD51D* and *BRIP1*. Sequencing was performed on the Illumina NovaSeq 6000 (Illumina). Moreover, an additional MLPA test was performed (SALSA MLPA probe mix P002 BRCA1) (MRC Holland, Amsterdam, Noord-Holland, The Netherlands) according to the standard manufacturer's protocol, including the Coffalyser.Net™ MLPA analysis software (MRC Holland). Variants in tumour tissue were called with a minimum variant allele frequency (VAF) of ≥ 3.5% and a read depth of ≥100. The pathogenicity of each *BRCA1/2* variant is evaluated in our routine molecular diagnostics by clinical molecular biologists at the department of Pathology according to the ACMG standards and guidelines using Franklin (www.Franklin.genoox.com) and LOVD (www.LOVD.nl) and discussed with the Clinical Genetics department. The pathogenicity is registered in JSI seqnext (JSI medical systems GmbH, Ettenheim, Germany, version 5.4.0). Class 4 and class 5 PV's are reported.

***Table S2**. Frequencies of BRCA1 and BRCA2 pathogenic variants in epithelial ovarian carcinomas (A. BRCA1 and B. BRCA2). Δ Two EOC harboured a PV in BRCA1 and BRCA2. ¶ Including a malignant Brenner tumour, mixed-type histology, mesonephric-like adenocarcinoma, undifferentiated carcinoma and small cell carcinoma.*

A.	***BRCA1* Pathogenic Variant**		
	n	**Prevalence**	**95% CI**
All EOC	**78[Δ]/946**	**8.2%**	**6-10%**
High-grade serous OC	**76/690**	**11%**	**9-13%**
Other EOC histotypes	**2/256**	**0.8%**	**0-2%**
Endometrioid OC – G3	0/20	0%	
Endometrioid OC – G1/2	0/40	0%	
Low-grade serous OC	1/67	1.5%	
Clear cell OC	1/64	1.6%	
Mucinous OC	0/39	0%	
Ovarian carcinosarcoma	0/13	0%	
Very rare EOC histotypes ¶	0/13	0%	

B.	***BRCA2* Pathogenic Variant**		
	n	**Prevalence**	**95% CI**
All EOC	**49[Δ]/946**	**5.2%**	**4-7%**
High-grade serous OC	**43/690**	**6.2%**	**4-8%**
Non-high-grade serous OC	**6/256**	**2.3%**	**0-4%**
Endometrioid OC – G3	3/20	15%	
Endometrioid OC – G1/2	1/40	2.5%	
Low-grade serous OC	2/67	3.0%	
Clear cell OC	0/64	0%	
Mucinous OC	0/39	0%	
Ovarian carcinosarcoma	0/13	0%	
Very rare EOC histotypes ¶	0/13	0%	

Table S3. *Comparison of BRCA1/2 PV frequencies across epithelial ovarian carcinoma histotypes in two independent academic medical centres (A. centre 1 and B. centre 2). ¶ Including malignant Brenner tumour, mixed-type histology, mesonephric-like adenocarcinoma, undifferentiated carcinoma, and small cell carcinoma.*

A.	**Centre-1**		
	***BRCA1/2* Pathogenic Variant**		
	n	**Prevalence**	**95% CI**
All EOC	**75/563**	**13%**	**11-16%**
High-grade serous OC	**71/410**	**17%**	**14-21%**
Non-high-grade serous OC	**4/153**	**2.6%**	**0-5%**
Endometrioid OC – G3	1/11	9%	
Endometrioid OC – G1/2	0/19	0%	
Low-grade serous OC	2/42	5.1%	
Clear cell OC	1/34	2.9%	
Mucinous OC	0/32	0%	
Ovarian carcinosarcoma	0/7	0%	
Very rare EOC histotypes ¶	0/8	0%	

B.	**Centre-2**		
	***BRCA1/2* Pathogenic Variant**		
	n	**Prevalence**	**95% CI**
All EOC	**50/383**	**13%**	**10-16%**
High-grade serous OC	**46/280**	**16%**	**12-21%**
Non-high-grade serous OC	**4/103**	**3.9%**	**0-8%**
Endometrioid OC – G3	2/9	22%	
Endometrioid OC – G1/2	1/21	4.8%	
Low-grade serous OC	1/25	4.0%	
Clear cell OC	0/30	0%	
Mucinous OC	0/7	0%	
Ovarian carcinosarcoma	0/6	0%	
Very rare EOC histotypes ¶	0/5	0%	

Table S4. *Fraction of high-grade serous ovarian carcinomas with a BRCA1/2 pathogenic variant showing presence or absence of loss-of-heterozygosity.*

	BRCA1	***BRCA2***
LOH present	33/37 (89%)	17/23 (74%)
LOH absent	4/37 (11%)	6/23 (26%)

Table S5. *Relationship between BRIP1, RAD51C and RAD51D pathogenic variants and epithelial ovarian carcinoma histological subtypes after central revision. * BRIP1 (2x), RAD51C (2x), RAD51D (3x).*

	***BRIP1, RAD51C, RAD51D* Pathogenic Variant**		
	n	**Prevalence**	**95% CI**
All EOC	**7/546**	**1.3%**	**0.3 – 2.2%**
High-grade serous OC	**7/396***	**1.8%**	**0.5 – 3.1%**
Non-high-grade serous OC	**0/150**	**0%**	**0 – 0%**
Endometrioid OC – G3	0/11	0%	
Endometrioid OC – G1/2	0/18	0%	
Low-grade serous OC	0/42	0%	
Clear cell OC	0/34	0%	
Mucinous OC	0/27	0%	
Ovarian carcinosarcoma	0/8	0%	
Very rare EOC histotypes ¶	0/10	0%	

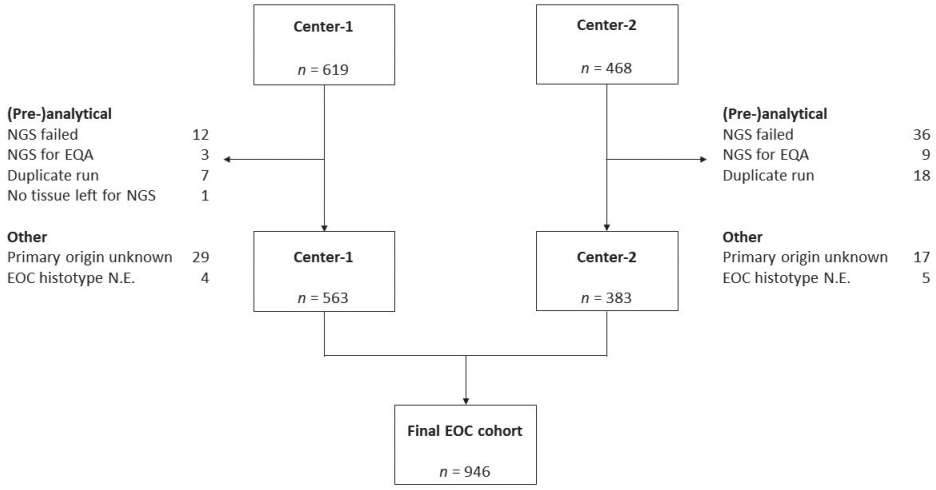

Figure S1. *CONSORT diagram of sample analysis.*

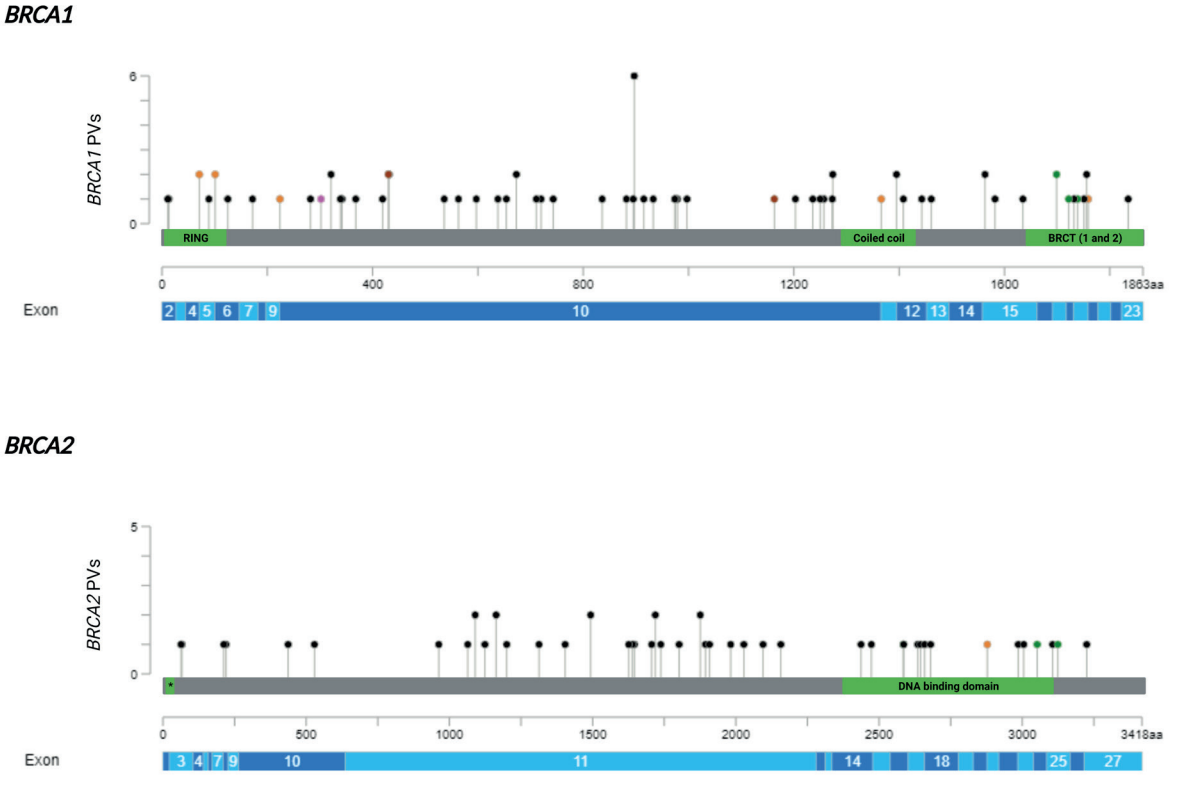

Figure S2. *Spectrum of BRCA1 and BRCA2 pathogenic variants detected in epithelial ovarian carcinomas. Each lollypop represents a specific variant and the height of the plot indicates the number of variants identified in the cohort. Lollypop colour indicates the mutation type; black = truncating, green = missense, brown = in-frame, orange = splice site. The reference sequences were NM_007294 and NM_000059 for BRCA1 and BRCA2, respectively. The cBioPortal MutationMapper application was used to create the figure (https://www.cbioportal.org/mutation_mapper) (accessed on 22 November 2022).*

Figure S3. *(A) Profiles of single-base substitution (SBS) mutational signatures in two endometrioid ovarian carcinomas (EnOC; case 6 & 7) with a POLE PV, and a clear cell ovarian carcinoma (CCOC; case 8) with a high microsatellite instability (MSI) score. (B) Exposure to SBS signatures. Both EnOCs have a considerable contribution of POLE-associated SBS signature 10a. The CCOC showed 100% exposure to MSI-associated SBS44.*