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In-house homologous recombination deficiency testing in ovarian cancer: a multi-institutional Italian pilot study

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ABSTRACT

Aims Poly (ADP-ribose) polymerase (PARP) inhibitors (PARPIs) represent a standard of care for the clinical management of high-grade serous ovarian cancer (HGSOC). The recognition of homologous recombination deficiency (HRD) has emerged as a predictive biomarker of response for first-line PARPIs treatment in patients with HGOSC. On the other hand, this test is extremely complex and therefore it is often externalised. Regrettably, the reliability of outsourced HRD testing can be troubled by inconclusive results and high rejection rates. In this methodological study, we assessed the technical feasibility, interassay and interlaboratory reproducibility of in-house HRD testing using three different commercially available next-generation sequencing assays.

Methods A total of n=20 epithelial ovarian cancer samples previously analysed with MyChoice CDx were subjected to HRD retesting using three different platforms in three different major pathology laboratories, that is, SOPHiA DDM HRD Solution, HRD focus and Oncomine homologous recombination repair pathway predesigned panel. Concordance was calculated by Cohen's (dual) and Fleiss (triple) κ coefficients.

Results In-house *BRCA1/2* molecular testing yielded a concordance rate >90.0% among all participating centres. HRD scores were successfully calculated by each institution with a concordance rate of 76.5%. Concerning the external gold standard test, the overall percentage of agreement ranged from 80.0% to 90.0% with a positive percentage agreement ranging from 75.0% to 80.0% and a negative percentage agreement ranging from 80.0% to 100%.

Conclusions In-house testing for HRD can be reliably performed with commercially available next-generation sequencing assays.

WHAT IS ALREADY KNOWN ON THIS TOPIC
⇒ Homologous recombination deficiency (HRD) status has revolutionised the clinical management of patients with high-grade serous ovarian cancer.

WHAT THIS STUDY ADDS
⇒ In-house harmonised next-generation sequencing (NGS) procedures may represent a valid testing strategy for HRD evaluation.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ In-house harmonised NGS procedures for HRD testing lead an improvement of successful testing rate.

INTRODUCTION

Epithelial ovarian cancer (EOC) represents the leading cause of death among gynecological malignancies.[1] Particularly, high-grade serous ovarian cancer (HGSOC) is characterised by a high mortality rate linked to advanced stage at diagnosis.[2] The standard therapeutic schemes commonly rely on surgery combined with platinum-based chemotherapies.[3] Lately, the overall survival of these patients has gradually improved thanks to novel and emerging therapeutic strategies.[4] One of the most promising therapeutic approaches against HGSOC is the use of poly (ADP-ribose) polymerase (PARP) inhibitors (PARPIs). The mechanism of action of PARPIs is rather complex, involving the inhibition of PARP-mediated homologous recombination repair (HRR), upregulation of non-homologous end joining and PARP 'trapping'.[5–8]

Accounting for 20%–25% of HGSOC cases, *BRCA1/2* mutations hold a predictive value for the selection of platinum-sensitive patients with HGSOC eligible for PARPi treatment.[9–11] However, other key actors are involved in the HRR system.[12–14] In detail, functional defects in HRR, known as homologous recombination deficiency (HRD), promote the activation of error-prone DNA repair mechanisms that induce genomic instability and malignant cell transformation.[15 16] The role of HRD status in the clinical selection of patients with HGSOC for PARPIs treatment has been proved by several clinical trials showing the remarkable clinical benefits of PARPi treatment against HRR-deficient compared with HRR-proficient tumours.[16–19]

Conventionally, HRD status is determined by analysing the signature approached with the

evaluation of genomic instability (ie, genomic scars), including loss of heterozygosity (LOH), telomeric allelic imbalance (TAI) and large-scale state transitions (LST).[20 21] In clinical trials, HRD-induced genomic scars are generally assessed by centralised next-generation sequencing (NGS)-based assays. This implies that HRD testing requires outsourcing analysis—a circumstance that may drastically impact on the success rate of molecular tests in clinical practice. Moreover, adding to the complexity of achieving accurate and reproducible results is the increasing number of different NGS-based assays commercially available for HRD status evaluation and the ensuing lack of standardised protocols and data interpretation.[19] A possible solution to these issues could be the application of in-house testing for HRD. However, although in-house HRD testing should bridge the gap between molecular tests and diagnostic routine samples,[22] standardising analytical pipelines and harmonising results remain challenging. Indeed, even in this case, simplification of complex technical procedures and harmonisation of diverse data analysis workflows are essential steps to implement in-house HRD testing in routine clinical practice. In this retrospective study, we tested the feasibility of in-house HRD testing to evaluate HRD status in patients with HGSOC. Here, we evaluated the inter-assay and interlaboratory reproducibility of HRD scores of three commercially available HRD assays used by three Italian referral institutions to determine HRD status in EOC tissue samples. All patients had been previously diagnosed with the MyChoice CDx Myriad HRD test (Myriad Genetics, Salt Lake City, USA).

METHODS

Study design and samples

We retrospectively analysed a series of n=20 formalin-fixed paraffin-embedded (FFPE) tissue samples from patients with EOC. All samples, previously externalised for HRD testing with the Food and Drug Administration (FDA)-approved MyChoice CDx were retrieved from the institutional archives of the European Institute of Oncology, IRCCS, Milan, Italy. Each case was defined as HRD positive or negative according to the clinically validated cut-off Genomic Instability Score of 42 (table 1).

The study was conducted to evaluate in-house analysis of HRD status in three reference centres (Division of Pathology of the European Institute of Oncology, Milan; Department of Pathology of Federico II University, Naples; Department of Pathology, University of Padua, Padua, Italy) using three commercially available HRD panels (ie, SOPHiA DDM HRD Solution, SOPHiA Genetics, Saint-Sulpice, Switzerland; HRD focus panel, Amoy Diagnostics, Xiamen, Fujian, China; Oncomine HRR pathway predesigned panel, Thermo Fisher Scientific). In addition, a reference DNA sample (OncoSpan gDNA reference standard, Horizon Discovery, Cambridge, UK) containing 386 different variants across 152 cancer-related genes was also distributed to each participating centre to evaluate the technical reproducibility and concordance rate between each internal workflow (figure 1).

The centres were anonymised as centre #1, centre #2 and centre #3 (online supplemental table 1).

Nucleic acids purification

Seven unstained slides from representative FFPE tissue blocks were cut into 4 μm thick sections. In brief, DNA was extracted with the Maxwell RSC DNA FFPE Kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions and then quantified with the QuantiFluor ONE dsDNA System (Promega) on the Quantus Fluorometer (Promega).

In-house HRD testing

DNA samples were handled according to the internal standardised diagnostic procedures of each laboratory; in-house HRD testing was evaluated according to the manufacturer's instructions for each HRD panel. In detail, 1.25 ng/μL (corresponding

Table 1 List of 20 patients with ovarian cancer selected from internal archive of Pathology Division of European Institute of Oncology, Milan

Sample ID	Histotype	Specimen type	Site	Cellularity (%)	Neoadjuvant therapy	*BRCA1* status	*BRCA2* status	HRD
ID_1	HGSOC	Surgical resection	Adnexa uteri	75	0	WT	WT	NEG
ID_2	HGSOC	Surgical resection	Fallopian tube	40	1	p.Ser288Argfs*10	WT	POS
ID_3	HGSOC	Biopsy	Omentum	80	0	WT	WT	POS
ID_4	HGSOC	Surgical resection	Adnexa uteri	60	0	WT	WT	POS
ID_5	HGSOC	Surgical resection	Ovary	70	0	WT	WT	NEG
ID_6	EnOC	Surgical resection	Ovary	60	0	WT	WT	NEG
ID_7	HGSOC	Surgical resection	Ovary	70	0	WT	WT	NEG
ID_8	HGSOC	Surgical resection	Ovary	60	0	WT	WT	NEG
ID_9	HGSOC	Surgical resection	Ovary	60	0	WT	WT	NEG
ID_10	HGSOC	Surgical resection	Adnexa uteri	60	1	WT	WT	NEG
ID_11	HGSOC	Biopsy	Peritoneum	80	0	WT	WT	POS
ID_12	HGSOC	Surgical resection	Ovary	80	0	WT	WT	NEG
ID_13	HGSOC	Surgical resection	Ovary	60	0	WT	WT	POS
ID_14	Mixed OC	Surgical resection	Ovary	60	0	WT	WT	NEG
ID_15	EnOC	Surgical resection	Ovary	40	0	WT	WT	NEG
ID_16	HGSOC	Surgical resection	Ovary	60	0	WT	WT	POS
ID_17	HGSOC	Surgical resection	Omentum	60	0	WT	WT	NEG
ID_18	HGSOC	Surgical resection	Peritoneum	50	1	WT	WT	NEG
ID_19	HGSOC	Surgical resection	Ovary	60	0	WT	WT	POS
ID_20	HGSOC	Surgical resection	Fallopian tube	70	0	WT	WT	NEG

BRCA, BReast CAncer associated gene; EnOC, endometrioid ovarian cancer; GSS, Genomic Scar Score; HGSOC, high-grade serous ovarian cancer; HRD, homologous recombination deficiency; NEG, negative; POS, positive; VUS, variant of uncertain significance; WT, wild-type.
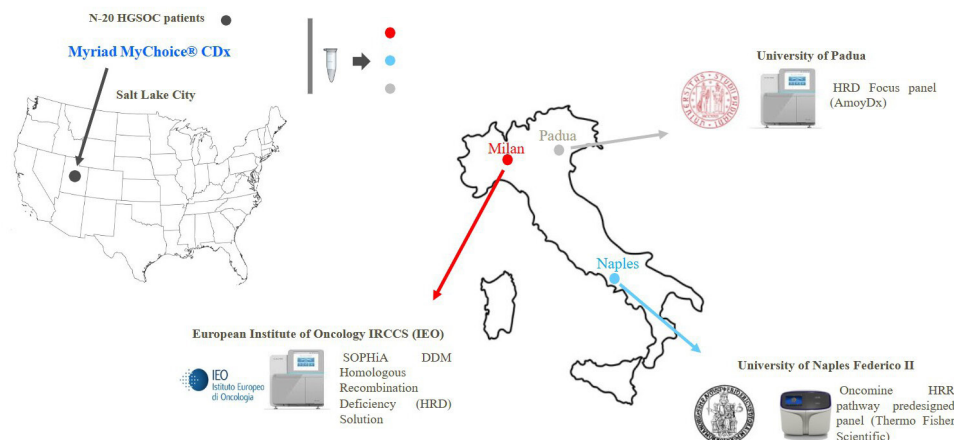
Figure 1 Study design. HGSOC, high-grade serous ovarian cancer; HRR, homologous recombination repair.

to 50 ng in 40 µL) of DNA was used for manual library preparation with the SOPHiA DDM HRD solution panel. This assay is a hybridisation-based approach able to combine the Genomic Integrity Index (GII) with deleterious mutations in n=28 hours (including BRCA1/2) complex-related genes. In addition, HRD genomic scar was calculated by an optimised analytic pipeline based on deep learning algorithms for the analysis of low-pass whole genome sequencing data. Sequencing was performed on the Illumina NextSeq 550Dx platform (Illumina, San Diego, California, USA). HRD score was calculated by proprietary software. In particular, a negative or positive GII score was indicative of HRD negative or positive status, respectively.

Generally, the Oncomine HRR pathway predesigned panel allows laboratory clinicians to analyse the high functional impact of point mutations, indels and CNV (Copy Number Variation) found in n=27 hours related genes.[23] In detail, an optimum of n=20 ng DNA input was achieved. Library preparation was manually performed as follows: 10 samples were amplified with the Ion AmpliSeq Library Kit Plus (Thermo Fisher Scientific, Waltham, Massachusetts, USA) under thermal conditions (25 cycles), as specified by the manufacturer's instructions. After two purification steps, libraries were diluted at 50 pM and pooled for template generation on an automatic platform (Ion Chef system, Thermo Fisher Scientific). Overall, two sets of n=10 pooled libraries were loaded on the Ion Chef platform by adopting Ion 540 Kit-Chef (Thermo Fisher Scientific), according to the manufacturer's instructions. Sequencing was performed on Ion 540 chip. Data were processed by using signal processing and base calling default parameters on Torrent Suite (V.5.0.2). Coverage analysis and variant calling were carried out with a customised workflow based on optimised bed files on Torrent Suite. In detail, a median depth coverage of 2000X and a quality score ≥20 were used to filter variants at 5% of allele frequency, as indicated by manufacturer's guidelines. Moreover, raw data were manually inspected on Golden Helix Genome Browser V.2.0.7 (Bozeman, Montana, USA) to confirm the molecular alterations automatically detected by a dedicated informatics pipeline. HRD score was calculated by a customised bioinformatics pipeline able to integrate LOH from referenced genes and CNV in BRCA1/2 genes. Particularly, HRD status was identified in accordance with ≥10 hotspot mutations in referenced genes and/or CNV-positive status. CNV was identified when

≥3 statistically significant unbalanced BRCA1/2 regions were detected in relation to normalised read counts.

Amoy HRD focus panel enables to detect SNVs (Single Nucleotide Variations) and indels in coding/non-coding regions from n=27 HRR-related genes and hotspot mutations in n=5 driver genes in solid tumours starting from tissue and liquid biopsy specimens.[24] Moreover, this assay covers BRCA1/2 large rearrangements from peripheral blood samples. In brief, target selection is based on the Halo-shape Annealing and Defer-Ligation Enrichment system, which is an improved version of the Molecular Inversion Probe technology. A set of n=10 samples (n=9 samples plus internal control) were simultaneously processed in each run. Library construction was performed with high-sensitive probes that hybridised the target region. After DNA extension from the corresponding probe arm, circular DNA was obtained and repaired by DNA ligation. Moreover, circular DNA was cleaned by exonucleases, which cleave single and double DNA strands, and finally amplified with a universal primer to create the template for the sequencing process on a NextSeq 550 system. Data analysis was automatically carried out with proprietary software able to identify BRCA1/2 molecular alterations and calculate the Genomic Scar Score (GSS). Samples with a GSS ≥50 were considered HRD positive.

Statistical analysis

Interassay and interlaboratory reproducibility was measured as the percentage of agreement between two in-house HRD testing methods. The overall percentage of agreement (OPA) was calculated to assess the concordance between the three in-house HRD testing panels and the outsourced HRD assay (reference standard). HRD status was considered as a categorical variable (positive vs negative) according to the HRD score cut-off of each testing method (online supplemental table 1).

RESULTS

Reference DNA samples were successfully analysed by each participating centre (online supplemental table 2). In detail, the total number of reads, percentage of aligned reads and uniformity of coverage were successfully achieved by each participating institution (online supplemental table 1). Overall, a concordance rate of 80.0% was observed for BRCA1/2 status. In particular, centres #1 and #3 detected four out of five (80.0%) clinically

Table 2 Schematic representation of *BRCA1/2* clinically relevant molecular alterations and HRD score (pathogenetic and VUS alterations according to ClinVar database) detected by each participating institution on reference DNA sample (OncoSpan gDNA reference standard, Horizon Discovery, Cambridge, UK)

Centre #1			Centre #2		Centre #3	
Gene	Results	GIS SOPHiA	Results	HRD score	Results	GSS Amoy
BRCA1	c.4327C>T: p.(Arg1443*) (pathogenetic)	−11.2 (NEG)	c.4327C>T: p.(Arg1443*) (pathogenetic)	NEG	c.4327C>T: p.(Arg1443*) (pathogenetic)	NEG
BRCA2	c.8021dup: p.(Ile2675Aspfs*6) (pathogenetic)		c.5073del: p.(Lys1691Asnfs*15) (VUS)		c.8021dup: p.(Ile2675Aspfs*6) (pathogenetic)	
	c.5073del: p.(Lys1691Asnfs*15) (VUS)		c.5351del: p.(Asn1784Thrfs*7) (pathogenetic)		c.5073del: p.(Lys1691Asnfs*15) (VUS)	
	c.5351del: p.(Asn1784Thrfs*7) (pathogenetic)				c.5351del: p.(Asn1784Thrfs*7) (pathogenetic)	

BRCA, BReast CAncer associated gene; GIS, Genomic Instability Score; GSS, Genomic Scar Score; HRD, homologous recombination deficiency; NEG, negative; VUS, variant of uncertain significance.

relevant *BRCA1/2* alterations harboured by the OncoSpan gDNA. Instead, centre #2 identified three out of five (60.0%) *BRCA1/2* referral alterations (table 2). Remarkably, all participating centres successfully calculated a negative HRD status for the reference DNA sample (table 2).

In particular, centres #1 and #2 successfully analysed *BRCA1/2* status and HRD scores in all 20 samples (success rate 100%); centre #3 analysed 17 out of 20 cases (success rate: 85%). Remarkably, only centre #2 detected a pathogenic *BRCA1* alteration in PD_6 (table 3).

All participating centres successfully calculated HRD scores in all the analysed ovarian cancer samples (table 4). Overall, a concordance rate in HRD status of 76.5% (13 out of 17) was detected by the three assays used in the three participating centres. Centres #1, #2 and #3 detected a positive HRD score in 7 out of 20 cases (35.0%), in 5 out of 20 cases (25.0%) and in 8 out of 17 cases (47.0%), respectively (tables 3 and 4). The Amoy HRD focus panel and SOPHiA DDM HRD Solution assay showed a concordance rate of 88.2% (15 out of 17 cases) for the evaluation of the HRD score. Similarly, the Amoy HRD focus

Table 3 List of *BRCA1/2* pathogenetic variations and HRD score on a retrospective series of n=20 patients with HGSOC inspected with commercially available NGS assays

Sample ID	Gene	Centre #1			Centre #2		Centre #3	
		Results	HRD SOPHiA	GSS Myriad	Results	HRD score	Results	GSS
ID_1	*BRCA1/2*	WT	3.4	40	WT	LOH*	WT	77.7
ID_2	*BRCA1/2*	WT	16.8	60	WT	NEG	WT	98.7
ID_3	*BRCA1/2*	WT	5.3	53	WT	LOH	WT	92.7
ID_4	*BRCA1/2*	WT	−1.6	47	WT	LOH	WT	88.3
ID_5	*BRCA1/2*	WT	−3.3	28	WT	NEG	WT	17.1
ID_6	*BRCA1/2*	WT	−11.4	3	p.Glu577AsnfsTer11	NEG	WT	4.1
ID_7	*BRCA1/2*	WT	−18.8	18	WT	NEG	WT	11.8
ID_8	*BRCA1/2*	WT	−10.7	16	WT	NEG	WT	7.2
ID_9	*BRCA1/2*	WT	−11	15	WT	NEG	WT	19
ID_10	*BRCA1/2*	WT	−1.4	23	WT	NEG	NA	NA
ID_11	*BRCA1/2*	WT	11.9	75	WT	NEG	NA	NA
ID_12	*BRCA1/2*	WT	−5.4	15	WT	NEG	WT	6.5
ID_13	*BRCA1/2*	WT	12.6	56	WT	NEG	WT	99.3
ID_14	*BRCA1/2*	WT	−15.5	9	WT	NEG	WT	7.9
ID_15	*BRCA1/2*	WT	−21.3	2	WT	NEG	WT	0.7
ID_16	*BRCA1/2*	WT	14.9	55	WT	LOH	WT	89.7
ID_17	*BRCA1/2*	WT	−5.7	34	WT	NEG	WT	50.1*
ID_18	*BRCA1/2*	WT	−12.4	19	WT	NEG	NA	NA
ID_19	*BRCA1/2*	WT	8.1	56	WT	LOH	WT	97.2
ID_20	*BRCA1/2*	WT	−9.6	25	WT	NEG	WT	19.3

HRD positive (green) and negative (red) status is shown.
*Borderline results.
BRCA, BReast CAncer associated gene; GSS, Genomic Scar Score; HRD, homologous recombination deficiency; ID, identify number; LOH, loss of heterozygosis; NA, not assessed; NEG, negative; NGS, next-generation sequencing; WT, wild-type.

Table 4 Schematic representation of HRD score evaluated by each participating institution by using commercially available NGS assays on a retrospective series of diagnostic HGSOC specimens

Sample ID	Centre #1		Centre #2	Centre #3
	Illumina NextSeq 550Dx	Illumina HiSeq 2500	Ion Gene Studio S5 Plus System	Illumina NextSeq 550 platform
	SOPHiA DDM HRD solution panel	MyChoice CDx Myriad HRD	Oncomine HRR pathway panel	Amoy HRD focus panel
ID_1	POS	NEG	POS*	POS
ID_2	POS	POS	NEG	POS
ID_3	POS	POS	POS	POS
ID_4	NEG	POS	POS	POS
ID_5	NEG	NEG	NEG	NEG
ID_6	NEG	NEG	NEG	NEG
ID_7	NEG	NEG	NEG	NEG
ID_8	NEG	NEG	NEG	NEG
ID_9	NEG	NEG	NEG	NEG
ID_10	NEG	NEG	NEG	NA
ID_11	POS	POS	NEG	NA
ID_12	NEG	NEG	NEG	NEG
ID_13	POS	POS	NEG	POS
ID_14	NEG	NEG	NEG	NEG
ID_15	NEG	NEG	NEG	NEG
ID_16	POS	POS	POS	POS
ID_17	NEG	NEG	NEG	POS*
ID_18	NEG	NEG	NEG	NA
ID_19	POS	POS	POS	POS
ID_20	NEG	NEG	NEG	NEG

*Borderline results.
HRD, homologous recombination deficiency; HRR, homologous recombination repair; ID, identify number; NA, not assessed; NEG, negative; NGS, next-generation sequencing; POS, positive.

Figure 2 Concordance rate between SOPHiA DDM HRD Solution assay and Amoy HRD focus between center #1 and center #3. HRD, homologous recombination deficiency.

HRD panel and the other NGS assays used to calculate HRD scores. Interestingly, a PPA and an NPA of 85.7% and 92.3%, 80.0% and 80.0%, 75.0% and 100.0% were observed between the MyChoice CDx Myriad HRD panel and the SOPHiA DDM HRD Solution assay, the Oncomine HRR pathway predesigned panel and Amoy HRD focus panel, respectively (figure 4).

DISCUSSION

HRD testing has become an essential predictive biomarker for all patients with HGSOC who may benefit from PARPi therapies.[24] However, the clinical need for widespread routine HRD testing remains challenging in Italian molecular diagnostic laboratories.[23 24] Although external clinically validated assays are available for routine HRD status analysis, not all patients can benefit from this approach mainly because it is costly and not reimbursable by the Italian Healthcare System. Moreover, centralised approaches show a significant rejection rate (30%–40%) due to the strict quality requirements of externalised procedures.[23] In addition, external analyses require increased turnaround time, which sometimes does not reflect the actual clinical need.[25] For these reasons, the identification of an alternative low-cost, highly reproducible, in-house assay for the evaluation of the HRD status in clinical samples would be an ideal alternative to guarantee this service to the highest possible number of patients. Several in-house HRD-testing strategies have been implemented,

panel and Oncomine HRR pathway predesigned panel showed a concordance rate of 82.4% (14 out of 17). Finally, the Oncomine HRR pathway predesigned panel and the SOPHiA DDM HRD Solution assay showed a concordance rate of 80.0% (16 out of 20 cases).

To investigate interlaboratory and interassay variability separately, we also evaluated the HRD status of the 20 EOC samples by using the same assay in two participating centres and two in-house HRD panels performed in a single institution. In detail, the use of the Amoy HRD focus panel yielded a concordance rate of 94.1% (16 out of 17 cases) between centre #1 and centre #3 (figure 2 and online supplemental table 2). Moreover, the use of both the Amoy HRD focus panel and the SOPHiA DDM HRD Solution assay by centre #1 yielded concordant HRD status results in 18 out of 20 cases, thereby showing a concordance rate of 90.0% (figure 3 and online supplemental table 3).

High concordance rates were also seen in the classification of HRD status between the reference MyChoice CDx Myriad HRD test and the other in-house panels. In particular, we observed an OPA of 88.2% (15 out of 17) between the reference standard and the Amoy HRD focus panel; an OPA of 80.0% (16 out of 20) between the reference standard and the Oncomine HRR pathway predesigned panel and an OPA of 90% (18 out of 20 cases) between the reference standard test and the SOPHiA DDM HRD Solution assay (table 3). In addition, positive percentage agreement (PPA) and negative percentage agreement (NPA) were also evaluated between the MyChoice CDx Myriad
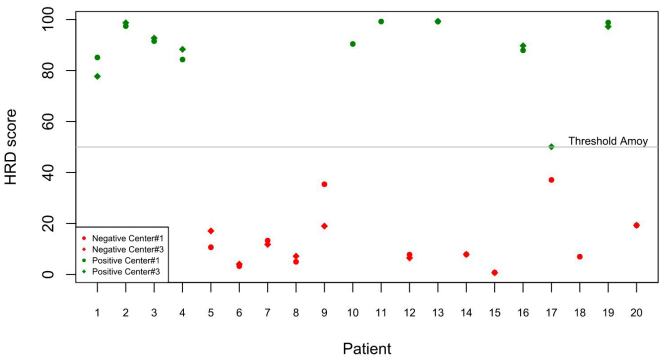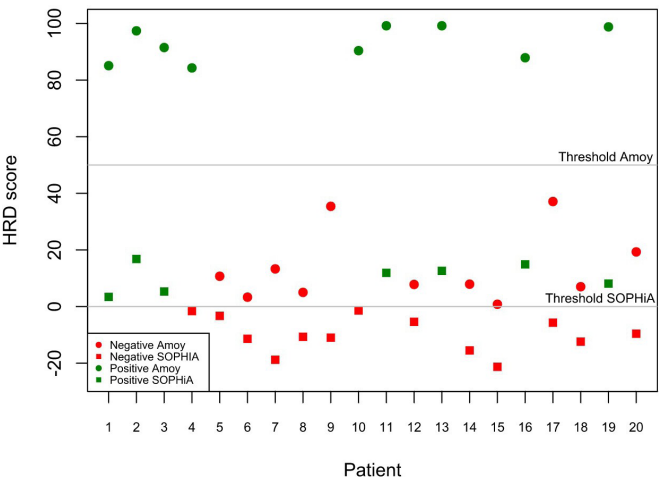

Figure 3 Concordance rate of center #1 by using SOPHiA DDM HRD Solution assay and Amoy HRD focus assay. HRD, homologous recombination deficiency.

		MyChoice® CDx Myriad HRD			
HRD focus panel		HRD positive	HRD negative	Total	– PPA:75.0%
	HRD positive	6	2	8	– NPA: 100.0%
	HRD negative	0	9	9	
	Total	6	11	17	

		MyChoice® CDx Myriad HRD			
Oncomine HRR pathway predesigned panel		HRD positive	HRD negative	Total	– PPA: 80.0%
	HRD positive	4	1	5	– NPA: 80.0%
	HRD negative	3	12	15	
	Total	7	13	20	

		MyChoice® CDx Myriad HRD			
SOPHiA DDM HRD Solution		HRD positive	HRD negative	Total	– PPA: 85.7%
	HRD positive	6	1	7	– NPA: 92.3%
	HRD negative	1	12	13	
	Total	7	13	20	

Figure 4 Negative percentage agreement (NPA) and positive percentage agreement (PPA) evaluated for each technical assay in comparison with gold standard externalised next-generation sequencing assay. HRD, homologous recombination deficiency; HRR, homologous recombination repair.

mainly involving different gene panels, bioinformatic pipelines, HRD score cut-off and sequencing platforms.[26]

In this retrospective study, we evaluated the HRD status in FFPE ovarian cancer samples using three commercially available in-house NGS-based assays performed in three laboratories. We observed concordance rates from 80% to 90% between in-house testing and the outsourced FDA-approved MyChoice CDx Myriad HRD test. The interassay and laboratory agreement rates were also encouraging, ranging from 71% to 94%. In addition, a reference standard DNA was also analysed by each participating centre to assess the technical validity of each in-house assay. Five out of six *BRCA1/2* pathogenetic alterations covered by the DNA reference standard were detected by the SOPHiA DDM HRD Solution assay and Amoy HRD focus panel. Only the Oncomine HRR pathway predesigned panel failed to detect one of these alterations. We speculate that this discrepancy was probably due to the fact that the low quality of the homopolymeric region was not adequately covered by the bioinformatics mutation calling pipeline of the amplicon-based and semiconductor NGS platforms.[26–28]

Remarkably, HRD status was successfully evaluated in 100% of cases analysed with SOPHiA DDM HRD Solution assay and the Oncomine HRR pathway predesigned panel, and in 85% of cases analysed with the Amoy HRD focus panel. Three cases were excluded from the molecular analysis performed by centre #1 because of the technical capability of multiplexing of sequencing cartridges (n=9 specimens plus internal run control). The overall agreement between the three in-house HRD testing assays was of 76.5% (13 out of 17 tested samples). It was observed that all technical approaches were concordant to successfully detect 4 out of 13 (30.8%) and 9 out of 13 (69.2%) HRD-positive and HRD-negative cases, respectively (table 4). Overall agreement is strongly dependent from the difference in terms of reference range between hybridisation-based assays and Oncomine HRR pathway predesigned panel.[29 30] Indeed, the technical performance of the Oncomine HRR pathway predesigned panel was slightly lower than that of the other two assays (concordance rate of 80.0%/82.4% vs 88.2%), especially for HRD-positive cases (only 20%). This difference was expected since the Oncomine HRR pathway assay is designed to detect only one (LOH) of the three genomic scars (LOH, LST, TAI),

which instead are covered by the other HRD panels.[29] At the sight of this critical issue, a non-negligible percentage of patients with HGSOC should be clinically administrated on the basis of false negative molecular results decreasing the population of patients with tumour that could benefit from PARPi administration. Moreover, whereas the HRD score was automatically calculated by proprietary software with the SOPHiA DDM HRD Solution and Amoy HRD focus assays, a customised analytical pipeline was implemented to define the HRD scores with the Oncomine HRR pathway predesigned panel. As of today, an automatised bioinformatics pipeline focused on the molecular fingerprint covered by NGS panels still remains an open challenge. However, some efforts are being made in this regard. For instance, Thermo Fisher recently announced that a genomic instability metric, able to calculate an HRD score in patients with HGSOC, is currently being investigated to optimise HRD calling in diagnostic specimens. Moreover, a comparative study will be performed to identify the most appropriate cut-off values for the clinical application of this approach.[30]

Overall, the Amoy HRD focus panel and SOPHiA DDM HRD Solution panel detected a positive HRD score in a substantial percentage of patients (from 30.0% to 45.0%). These data were consistent with the percentage of HRD-positive HGSOC evaluated in clinical trials.[7 17] A high concordance rate was also observed between HRD testing performed in a single laboratory using both the Amoy HRD focus and SOPHiA DDM HRD Solution panels (90%), and in two laboratories using the same panel (94.1%). These results highlight that in-house methods display a high interassay and interlaboratory reproducibility. Moreover, the comparison with a clinically validated HRD testing assay (MyChoice Cdx Myriad HRD test) revealed a high percentage of agreement for both the Amoy HRD focus panel (88.2%) and SOPHiA DDM HRD Solution panel (90%). The majority of the interassay and interlaboratory discordances were observed in cases with an HRD score close to the predefined cut-off value of the in-house assay. This aspect may be related to the technical variability observed in real-world practice.[31] Interestingly, the number of HRD-positive cases and overall agreement scores observed between in-house HRD testing and MyChoice Cdx Myriad HRD test were consistent with the percentages reported in previous studies.[32 33]

Although this study provides encouraging preliminary evidence for the possibility of implementing of in-house NGS-based HRD testing in routine clinical practice, it does have some main limitations. The first is that being a retrospective study based on a small number of selected cases, it did not allow us to compare the failure rate between in-house and outsourced HRD testing. The second limitation is that the small number of samples analysed yielded only preliminary results that will necessarily require further validation. Finally, we were unable to assess the clinical validity and utility of in-house HRD evaluation that was beyond the scope of this work aiming to verify the analytical reproducibility of in-house HRD testing. Therefore, building on these preliminary insights, we hope that future research will validate the clinical benefits that routine in-house HRD testing could have on patients with HGSOC.

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Patient consent for publication Not applicable.

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	Center#1			Center#2			Center#3		
	Reads	**Aligned Reads**	**Uniformity of coverage**	**Reads**	**Aligned Reads**	**Uniformity of coverage**	**Reads**	**Aligned Reads**	**Uniformity of coverage**
ID_1	30342484	95.00%	100.00%	3151680	95.78%	96.43%	43382964	96.98%	99.91%
ID_2	32051208	95.00%	100.00%	3009193	96.48%	95.60%	42443652	96.34%	99.91%
ID_3	32257448	96.00%	100.00%	2280014	96.24%	96.56%	40340238	95.99%	99.88%
ID_4	27997808	96.00%	100.00%	3083424	95.80%	94.46%	43434872	96.41%	99.92%
ID_5	27929198	96.00%	100.00%	3346566	95.56%	94.80%	38681522	96.65%	99.91%
ID_6	28519654	95.00%	100.00%	3025094	95.31%	96.08%	39567014	96.85%	99.89%
ID_7	26566174	95.00%	100.00%	2614340	95.41%	95.88%	36812004	96.85%	99.92%
ID_8	29026612	95.00%	100.00%	2160659	94.73%	96.15%	38378622	97.30%	99.90%
ID_9	28976660	97.00%	100.00%	3240796	95.98%	97.30%	41666208	96.80%	99.89%
ID_10	29974484	96.00%	100.00%	3226484	96.54%	88.66%	NA	NA	NA
ID_11	29498640	94.00%	100.00%	2987740	95.88%	96.13%	NA	NA	NA
ID_12	26728516	95.00%	100.00%	3077696	96.44%	92.03%	38811978	98.39%	99.90%
ID_13	25679184	96.00%	100.00%	2944272	95.95%	93.21%	37560378	98.15%	99.92%
ID_14	28749864	95.00%	99.94%	2980758	96.11%	97.57%	29447798	96.22%	99.94%
ID_15	25582666	95.00%	100.00%	2523669	94.45%	94.94%	30959056	96.44%	99.92%
ID_16	23514336	95.00%	100.00%	2175409	96.02%	91.01%	31234964	96.20%	99.93%
ID_17	24284426	95.00%	100.00%	2620747	95.85%	94.46%	35129310	96.13%	99.94%
ID_18	47376516	92.00%	98.59%	2482504	96.20%	91.90%	NA	NA	NA
ID_19	52442568	95.00%	100.00%	2657231	95.79%	97.43%	34807102	98.33%	99.93%
ID_20	47540742	93.00%	99.03%	2584802	95.85%	94.09%	36855122	98.05%	99.91%
C	25770988	95.00%	99.87%	6985476	95.15%	98.31%	34170392	98.10%	99.91%

Table x: Technical parameters from NGS analysis of each tested sample by participating institutions.

Abbreviations: *C* (Control); *ID* (Identify Number); *NA* (Not Assessed).

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AMOY GS		
	Center#1	**Center#3**
ID_1	85.10	77.70
ID_2	97.40	98.70
ID_3	91.50	92.70
ID_4	84.30	88.30
ID_5	10.70	17.10
ID_6	3.30	4.10
ID_7	13.30	11.80
ID_8	5.00	7.20
ID_9	35.40	19.00
ID_10	90.40	NA
ID_11	99.20	NA
ID_12	7.80	6.50
ID_13	99.20	99.30
ID_14	7.90	7.90
ID_15	0.80	0.70
ID_16	87.90	89.70
ID_17	37.10	50.10
ID_18	7.00	NA
ID_19	98.80	97.20
ID_20	19.30	19.30

Supplementary Table 3: Center#1 and Center#3 interlaboratory concordance on 20 HGSOC patients tested by adopting HRD focus panel assay (Amoy Diagnostics Co Ltd) for HRD evaluation. Concordant results (green) were also observed in 16 out of 17 tested samples. In a single instance (yellow) HRD positive score was detected.

Abbreviations: *GS* (Genomic Scar); *ID* (Identify Number); *NA* (Not Assessed).

Center#1				
Sample ID	BRCA	HRD Sophia	GSS Myriad	GS AMOY
ID_1	WT	3.4 POS	40 NEG	POS 85.1
ID_2	BRCA1 p.S288Rfs*10	16.8 POS	60 POS	POS 97.4
ID_3	WT	5.3 POS	53 POS	POS91.5
ID_4	WT	(-1.6) NEG	47 POS	POS 84.3
ID_5	WT	(-3.3) NEG	28 NEG	NEG 10.7
ID_6	WT	(-11.4) NEG	3 NEG	NEG 3.3
ID_7	WT	(-18.8) NEG	18 NEG	NEG 13.3
ID_8	WT	(-10.7) NEG	16 NEG	NEG 5
ID_9	WT	(-11) NEG	15 NEG	NEG 35.4
ID_10	WT	(-1.4) NEG	23 NEG	POS 90.4
ID_11	WT	11.9 POS	75 POS	POS 99.2
ID_12	WT	(-5.4) NEG	15 NEG	NEG 7.8
ID_13	WT	12.6 POS	56 POS	POS 99.2
ID_14	WT	(-15.5) NEG	9 NEG	NEG 7.9
ID_15	WT	(-21.3) NEG	2 NEG	NEG 0.8
ID_16	WT	14.9 POS	55 POS	POS 87.9
ID_17	WT	(-5.7) NEG	34 NEG	NEG 37.1
ID_18	WT	(-12.4) NEG	19 NEG	NEG 7
ID_19	WT	8.1 POS	56 POS	POS 98.8
ID_20	WT	(-9.6) NEG	25 NEG	NEG 19.3

Supplementary table 4: Interlaboratory concordance on 20 HGSOC patients tested by adopting two commercially available NGS assays for HRD evaluation. HRD score was also compared with externalized approach (MyChoice® CDxMyriad HRD).

Abbreviations: *BRCA* (Breast Cancer Associated gene); *GSS* (Genomic Scar Score); *HRD* (Homologous Recombination Deficiency); *ID* (Identify Number); *NEG* (Negative); *POS* (Positive); *WT* (Wild-Type).

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ID_7	13.30	11.80
ID_8	5.00	7.20
ID_9	35.40	19.00
ID_10	90.40	NA
ID_11	99.20	NA
ID_12	7.80	6.50
ID_13	99.20	99.30
ID_14	7.90	7.90
ID_15	0.80	0.70
ID_16	87.90	89.70
ID_17	37.10	50.10
ID_18	7.00	NA
ID_19	98.80	97.20
ID_20	19.30	19.30

Supplementary Table 3: Center#1 and Center#3 interlaboratory concordance on 20 HGSOC patients tested by adopting HRD focus panel assay (Amoy Diagnostics Co Ltd) for HRD evaluation. Concordant results (green) were also observed in 16 out of 17 tested samples. In a single instance (yellow) HRD positive score was detected.

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