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Posted Date: 24 July 2025

doi: 10.20944/preprints202507.2066.v1

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 doi:10.20944/preprints202507.2066.v1

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Detection of Clinically Significant BRCA Large Genomic Rearrangements in FFPE Ovarian Cancer Samples: A Comparative NGS Study

Alessia Perrucci [1,2,§], Maria De Bonis [1,2,§], Giulia Maneri [1,2], Claudio Ricciardi Tenore [1,2], Paola Concolino [1,2], Matteo Corsi [1,2], Alessandra Conca [1,2], Jessica Evangelista [1,2], Alessia Piermattei [3], Camilla Nero [4,5], Luciano Giacò [6], Anna Fagotti [4,5], Elisa De Paolis [1,2] and Angelo Minucci [1,2,*]

[1] Departmental Unit of Molecular and Genomic Diagnostics, Fondazione Policlinico Gemelli IRCCS, Rome, Italy
[2] Genomics Core Facility, G-STeP, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome, Italy
[3] Pathology Unit, Department of Woman and Child's Health and Public Health Sciences, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, 00168, Rome, Italy
[4] Unit of Oncological Gynecology, Department of Women, Children and Public Health Sciences, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome, Italy
[5] Università Cattolica del Sacro Cuore, Rome, Italy
[6] Bioinformatics Research Core Facility, Gemelli Science and Technology Park (G-STeP), Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome, Italy
* Correspondence: angelo.minucci@policlinicogemelli.it
§ **These authors contributed equally to this work.**

Abstract

Background: Copy number variations (CNVs), also referred to as large genomic rearrangements (LGRs), represent a crucial component of *BRCA1/2 (BRCA)* testing. Next-generation sequencing (NGS) has become an established approach for detecting LGRs by combining sequencing data with dedicated bioinformatics pipelines. However, CNV detection in formalin-fixed paraffin-embedded (FFPE) samples remains technically challenging, and it cannot always be guaranteed that such information will be reliably obtained. Therefore, optimization is needed, and implementing a robust analysis strategy for routine clinical practice could provide significant advantages. **Methods:** This study evaluated 40 FFPE ovarian cancer (OC) samples from patients undergoing *BRCA* testing. The performance of the amplicon-based NGS *Diatech Myriapod® NGS BRCA1/2* panel (Diatech Pharmacogenetics, Jesi, Italy) was assessed for its ability to detect *BRCA* CNVs, and results were compared to two hybrid capture-based reference assays. **Results:** Among the 40 analyzed samples (17 CNV-positive and 23 CNV-negative for *BRCA* genes), the Diatech pipeline showed high concordance with the reference methods. In a clinical diagnostic setting, the evaluated method achieved an overall accuracy of about 96%, with a sensitivity of 94% and specificity of 96%. Despite one inconclusive result due to low sequencing quality and one sample with a somatic CNV in *BRCA1* that was not detected, the *Diatech Myriapod® NGS BRCA1/2* panel kit demonstrated strong potential for routine clinical application in CNV detection from FFPE tissue. **Conclusions:** These findings support the clinical utility of NGS-based CNV analysis in FFPE samples when combined with appropriate bioinformatics tools. Integrating visual inspection of CNV plots with automated CNV calling improves the reliability of CNV detection and enhances the interpretation of results from tumor tissue. Accurate CNV detection directly from tumor tissue may reduce the need for reflex germline testing and improve turnaround times. Nevertheless, blood-based testing remains essential to determine whether detected *BRCA* CNVs are of germline or somatic origin, particularly in cases with a strong clinical suspicion of a germline CNV.

Keywords: *BRCA* genes; NGS; large genomic rearrangements; ovarian cancer; copy number variations; FPG500; *Diatech Myriapod® NGS BRCA1/2* panel kit; SOPHiA DDM™ homologous recombination solution

 doi:10.20944/preprints202507.2066.v1

1. Introduction

Hereditary breast and/or ovarian cancer syndrome has traditionally been the primary criterion for genetic counseling, followed by germline *BRCA1/2 (BRCA)* testing [1]. However, over the past decade, numerous clinical studies have demonstrated that ovarian cancer (OC) patients harboring germline or somatic pathogenic *BRCA* variants (PVs) show sensitivity to poly (ADP-ribose) polymerase inhibitors (PARPi) and platinum-based chemotherapy [2,3]. In addition, functional defects in homologous recombination repair genes, collectively referred to as homologous recombination deficiency (HRD), have been clinically validated as predictive biomarkers for PARPi treatment in OC [4]. As a result, *BRCA* and/or HRD testing on formalin-fixed paraffin-embedded (FFPE) tumor samples, which allow simultaneous detection of both somatic and germline PVs, has become increasingly important in the molecular management of OC patients [5–7].

Copy number variations (CNVs), also referred to as large genomic rearrangements (LGRs), such as deletions or duplications larger than 1,000 base pairs, have been identified in *BRCA* genes. Their prevalence varies widely among populations, ranging from less than 1% to more than 24% [8]. Consequently, LGRs account for a substantial proportion of *BRCA* PVs and are now an integral component of *BRCA* and HRD testing [6].

Next-generation sequencing (NGS) is a now well-established method for comprehensive *BRCA* screening from blood, enabling the simultaneous detection of single nucleotide variants (SNVs), insertions/deletions (*indels*), and CNVs [9–11]. However, CNV detection in tumor tissue presents specific challenges, including tumor heterogeneity, low tumor cellularity, the absence of a matched normal baseline, poor DNA quality, and the presence of PCR contaminants or artifacts. These factors can lead to uneven sequencing coverage across genomic regions, impairing the accurate identification of CNVs. As a result, NGS-based CNV detection may generate false positives or, more critically, false negatives, particularly when using workflows that lack validated and dedicated bioinformatics pipelines for CNV calling [10,11].

Among various NGS protocols, hybrid capture-based approaches have demonstrated greater reliability for CNV detection compared to amplicon-based PCR protocols. Nonetheless, several *BRCA* CNV assays are currently available, and not all are fully validated for clinical use or supported by robust bioinformatics pipelines [12].

The aim of this study was to evaluate the ability of different NGS bioinformatics pipelines to accurately identify and call *BRCA* CNVs from FFPE tumor samples. To this end, 40 OC samples were selected, including 17 samples harboring clinically significant LGRs. CNV calls from two hybrid capture-based NGS protocols were compared with the amplicon PCR-based *Diatech Myriapod® NGS BRCA1/2* panel kit.

Finally, an analysis strategy was proposed to improve the interpretation of NGS data for reliable identification of CNVs in FFPE samples, in a clinical setting (Figure 1).

 doi:10.20944/preprints202507.2066.v1

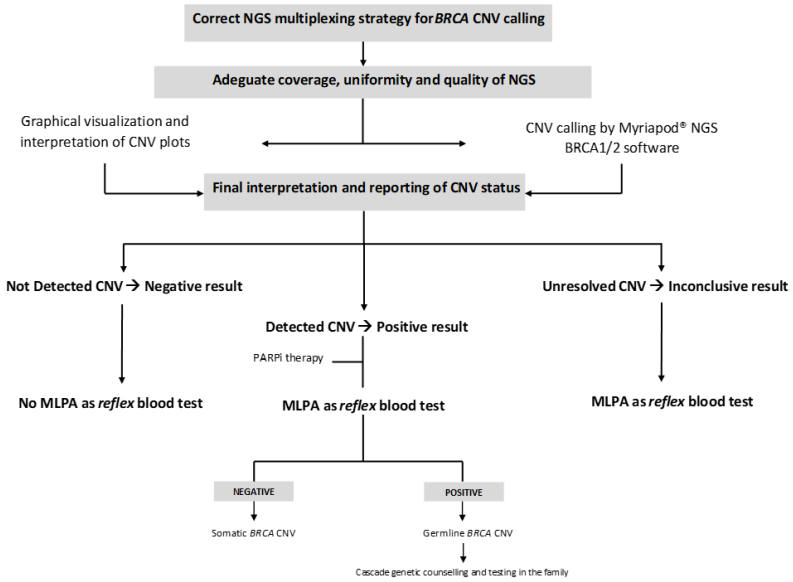

Figure 1. Strategy for interpreting CNV calls from NGS data in FFPE samples, optimized for real-world clinical implementation.

2. Materials and Methods

2.1. Patient Cohort and Study Design

This retrospective cohort study was conducted at the Genomics Core Facility, G-STeP, Fondazione Policlinico Universitario Agostino Gemelli IRCCS. Formalin-fixed paraffin-embedded (FFPE) tumor blocks from patients with advanced or relapsed platinum-sensitive OC were collected as part of routine clinical care for *BRCA* status assessment through the FPG500 program [13] and HRD testing performed in our institution. The same FFPE samples were evaluated for CNV calling using the *Diatech Myriapod® NGS BRCA1/2* panel kit (Diatech Pharmacogenetics, Jesi, Ancona, Italy).

A total of 40 patients were included in the study: 17 with *BRCA* CNV-positive results and 23 with *BRCA* CNV-negative results. All CNVs corresponded to clinically significant LRGs (Table 1).

All participants provided informed consent to participate in the study (Study ID: FPG500; Ethics Committee Approval No. 3837), which was conducted in accordance with the Declaration of Helsinki. Patient characteristics are summarized in Table 1.

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Table 1. Clinical and molecular characteristics of patients enrolled in the study.

ID	Disease	Timing	Age of the sample	Tumor content (%)	CNV Status	Reference assay	Gene	Exons	Type of CNV	Status of CNV*
1	HGSC	PRIMARY	2024	90	Positive	TSO500HT	BRCA2	2-3	Intragenic deletion	Somatic
2	HGSC	PRIMARY	2024	90	Positive	TSO500HT SOPHiA DDM HRD HRD	BRCA1	19	Intragenic deletion	Somatic
3	HGSC	PRIMARY	2024	60	Positive	TSO500HT SOPHiA DDM HRD	BRCA1	8-11	Intragenic deletion	Germline
4	HGSC	PRIMARY	2024	70	Positive	TSO500HT SOPHiA DDM HRD	BRCA1	20	Intragenic deletion	Germline
5	HGSC	RELAPSE	2023	90	Positive	TSO500HT SOPHiA DDM HRD	BRCA1	2	Intragenic deletion	Germline
6	HGSC	RELAPSE	2024	90	Positive	SOPHiA DDM HRD	BRCA1	16-17	Intragenic deletion	Germline
7	HGSC	RELAPSE	2024	70	Positive	TSO500HT SOPHiA DDM HRD	BRCA1	4-7	Intragenic deletion	Germline
8	HGSC	PRIMARY	2023	90	Positive	TSO500HT SOPHiA DDM HRD	BRCA2	19-21	Intragenic deletion	Somatic
9	ENOC	PRIMARY	2024	90	Positive	TSO500HT	BRCA1	11	Intragenic deletion	Somatic
10	HGSC	PRIMARY	2024	95	Positive	TSO500HT SOPHiA DDM HRD	BRCA1	2-3	Intragenic deletion	Somatic
11	HGSC	PRIMARY	2024	60	Positive	TSO500HT SOPHiA DDM HRD	BRCA1	19	Intragenic deletion	Germline
12	HGSC	RELAPSE	2024	80	Positive	SOPHiA DDM HRD	BRCA1	2	Intragenic deletion	Germline
13	HGSC	RELAPSE	2024	80	Positive	SOPHiA DDM HRD	BRCA1	2- 19	Intragenic deletion	Somatic
14	HGSC	PRIMARY	2024	80	Positive	TSO500HT	BRCA1	15	Intragenic deletion	Somatic
15	HGSC	PRIMARY	2024	55	Positive	TSO500HT SOPHiA DDM HRD	BRCA1	1-24	Whole gene deletion	Germline
16	HGSC	PRIMARY	2025	30	Positive	TSO500HT SOPHiA DDM HRD	BRCA2	11-27	Intragenic deletion	Somatic
17	HGSC	PRIMARY	2024	80	Positive	TSO500HT	BRCA1	3-23	Intragenic deletion	Somatic
18	OCS	RELAPSE	2024	60	Positive	TSO500HT SOPHiA DDM HRD	-	-	-	-
19	HGSC	PRIMARY	2024	80	Negative	TSO500HT SOPHiA DDM HRD	-	-	-	-

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20	HGSC	PRIMARY	2025	20	Negative	TSO500HT	-	-		
21	HGSC	PRIMARY	2024	80	Negative	TSO500HT SOPHiA DDM HRD	-	-	-	-
22	HGSC	PRIMARY	2024	80	Negative	TSO500HT	-	-	-	-
23	CCOC	PRIMARY	2024	80	Negative	TSO500HT	-	-	-	-
24	ENOC	PRIMARY	2024	90	Negative	TSO500HT	-	-	-	-
25	HGSC	PRIMARY	2024	25	Negative	TSO500HT	-	-	-	-
26	HGSC	PRIMARY	2024	80	Negative	TSO500HT SOPHiA DDM HRD	-	-	-	-
27	HSGC	RELAPSE	2024	95	Negative	SOPHiA DDM HRD	-	-	-	-
28	ENOC	RELAPSE	2024	30	Negative	SOPHiA DDM HRD	-	-	-	-
29	HSGC	RELAPSE	2024	30	Negative	SOPHiA DDM HRD	-	-	-	-
30	HSGC	RELAPSE	2024	35	Negative	SOPHiA DDM HRD	-	-	-	-
31	HGSC	PRIMARY	2023	70	Negative	TSO500HT SOPHiA DDM HRD	-	-	-	-
32	CCOC	PRIMARY	2023	80	Negative	TSO500HT	-	-	-	-
33	HGSC	PRIMARY	2024	40	Negative	TSO500HT SOPHiA DDM HRD	-	-	-	-
34	HGSC	PRIMARY	2024	36	Negative	SOPHiA DDM HRD	-	-	-	-
35	HGSC	PRIMARY	2023	80	Negative	SOPHiA DDM HRD	-	-	-	-
36	HGSC	PRIMARY	2025	70	Negative	TSO500HT SOPHiA DDM HRD	-	-	-	-
37	CCOC	PRIMARY	2025	20	Negative	TSO500HT	-	-	-	-
38	HGSC	PRIMARY	2025	30	Negative	TSO500HT SOPHiA DDM HRD	-	-	-	-
39	CCOC	PRIMARY	2025	70	Negative	TSO500HT	-	-	-	-
40	HGSC	PRIMARY	2025	25	Negative	TSO500HT	-	-	-	-

* CNV status was determined using MLPA as a reflex test on peripheral blood samples; Abbreviations: HGSC: High grade serous carcinoma; ENOC: endometrioid ovarian cancer, OCS: Ovarian carcinosarcoma; CCOC: Clear Cell Ovarian Carcinoma.

 doi:10.20944/preprints202507.2066.v1

2.2. Patient Selection

Patient selection for the study was performed using the *TruSight Oncology 500 High Throughput* (TSO500HT) (Illumina Inc., San Diego, CA) and the SOPHiA DDM™ Homologous Recombination Solution (SOPHiA DDM™ HRD) (SOPHIA Genetics). Both tests were performed on 19 samples, while 21 samples were analyzed with only one of the two assays (Table 1).

Multiplex Ligation-dependent Probe Amplification (MLPA) analysis on blood samples was carried out to confirm whether the CNVs were of germline origin, using the SALSA P002 BRCA1 and SALSA P045 BRCA2 MLPA kits (MRC Holland), as previously described [12].

The reference sequences used for CNV reporting were NG_005905.2/NM_007294.3 and NG_012772.3/NM_000059.3 for *BRCA1* and *BRCA2* respectively (Table 1).

2.3. DNA Extraction

For DNA extraction, FFPE tissue samples containing >20% tumor cells and <10% necrosis, as determined by the local pathologist, were selected. DNA was extracted using the Qiagen AllPrep DNA/RNA FFPE Kit on the EZ2 Connect workstation (Qiagen), following the manufacturer's protocol. DNA quantity and quality were assessed using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. BRCA Testing with Myriapod® NGS BRCA1/2 Panel Kit and Primary Sequencing Strategy

The *Myriapod® NGS BRCA1/2* panel kit is an in vitro diagnostic assay that enables the detection of SNVs, *indels* and splice variants in the *BRCA* genes. CNV analysis and calling were conducted using a proprietary algorithm part of toghe Myriapod® NGS data analysis software.

With the aim to evaluate the performance of CNV detection algorithm of *Myriapod® NGS BRCA1/2* panel kit considering "stressed" testing conditions, a "primary CNV calling strategy" was defined. In this strategy, four NGS runs, each including 10 FFPE samples, were performed on the MiSeq System, using MiSeq Micro Flow Cell (Illumina Inc., San Diego, CA). Specifically, two runs included 5 *BRCA* CNV-positive and 5 *BRCA* CNV-negative cases, while the other two runs consisted of 4 *BRCA* CNV-positive and 6 *BRCA* CNV-negative samples, respectively.

Data analysis was performed using Myriapod® NGS Data analysis software (Diatech Pharmacogenetics, Jesi, Ancona, Italy).

2.5. Re-Evaluation of CNV Calling Using Diatech Software by Simulating a Diagnostic Setting

To further evaluate the CNV calling performance of the *Myriapod® NGS BRCA 1-2* kit in association with the *Myriapod® NGS* data analysis software , all 40 samples were re-analyzed to simulate a routine clinical setting. Specifically, the analysis was designed to reflect a scenario in which CNVs occur with an estimated prevalence of less than 10% in the general population, corresponding to the likelihood of detecting at most one positive case per sequencing run of 10 samples.

2.6. Read Coverage and Comparative Analyses

Sequencing performance was evaluated across the 4 NGS runs, with the aim of optimizing CNV calling. Key quality metrics assessed included mean coverage, percentage of uniformity and on-target reads. Results were analyzed separately for each sequencing run and summarized as mean ± standard deviation (SD) across all samples within each run.

In parallel with the assessment of sequencing quality, statistical analyses were conducted to evaluate CNV calling performance using the *Myriapod® NGS BRCA1/2* panel kit in association with *Myriapod® NGS* data analysis software. Overall accuracy, sensitivity, and specificity of the *Myriapod® NGS BRCA 1-2* solution were calculated and compared to those of the *TSO500HT* and *SOPHiA DDM™ HRD Solution* kits.

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2.7. Data Analysis

Sequencing data were processed and interpreted using *Myriapod® NGS* Data Analysis Software, a CE-marked in vitro diagnostic application for targeted NGS assays within the Diatech NGS Applications portfolio. The software automatically generates an initial variant report, incorporating both SNVs, *indels* and CNVs analysis. For CNV detection, it plots each gene on an independent chart, assigning a copy-number score for each exon or amplicon.

3. Results

A total of 40 OC patients, selected from tests previously performed in-house as part of routine clinical care, were enrolled in the study. As shown in Table 1, 17 samples were *BRCA*-CNV positive and 23 were *BRCA*-CNV negative. The performance of CNV detection using the *Myriapod® NGS BRCA1/2* panel in combination with the *Myriapod® NGS data analysis software* was assessed at three distinct levels:

(a) Graphical visualization and interpretation of CNV plots;
(b) CNV calling by the Myriapod® NGS data analysis software;
(c) Final interpretation and reporting of CNV status, as a decision-making result integrating the two previous analysis levels.

3.1. Concordance Analysis Between TSO500HT/SOPHiA DDM HRD and Myriapod® NGS BRCA 1-2 Pipeline in Primary CNV Calling Strategy (5/4 BRCA CNV-Positive vs 5/6 BRCA CNV-Negative Samples in the Same NGS Run)

3.1.1. BRCA-CNV-Negative Samples

Based on the graphical visualization and interpretation of CNV status, out of the 23 CNV-negative samples, 16 could be considered negative for both genes. Four samples were CNV-negative for *BRCA2* but showed a potential CNV in *BRCA1*. One sample was CNV-negative for *BRCA1* with an inconclusive CNV result in *BRCA2*, while another was CNV-negative for *BRCA2* with an inconclusive CNV result in *BRCA1*. Finally, one sample was negative for *BRCA1* and showed an "other CNV" in *BRCA2*.

According to the CNV calling performed by the Diatech software, 7 samples were classified as *CNV Not Positive* for both genes. Nine samples were *CNV Not Positive* for *BRCA2* but showed a *Potential CNV* in *BRCA1*. Three samples were identified as *Potential CNV* for both genes, and 4 samples showed a *Potential CNV* in *BRCA2* and were *CNV Not Positive* for *BRCA1*.

The final interpretation and reporting of CNV status, based on both graphical visualization and software based CNV calling, led to the classification of 18 samples as *negative*, 3 samples as showing a *Potential CNV* in *BRCA1* and *Negative* in *BRCA2*, 1 sample as *Inconclusive* in *BRCA2* and *Negative* in *BRCA1*, and 1 sample with a potential CNV in *BRCA2* and CNV negative in *BRCA1* (Table 2)

Considering the final interpretation and reporting of CNV results, out of the 23 negative samples, 18 would be considered completely negative, and 5 would be referred for confirmatory testing.

Table 2. Concordance analysis between TSO500HT/SOPHiA DDM HRD and *Myriapod® NGS BRCA1/2* software results in *primary* CNV calling strategy (5/4 *BRCA*-CNV positive *vs* 5/6 *BRCA* CNV-negative samples).

ID samples	Graphical visualization and intepretation of CNV plots		CNV calling by Diatech software		Final interpretation and reporting CNV	
	BRCA1	***BRCA2***	***BRCA1***	***BRCA2***	***BRCA1***	***BRCA1***
***BRCA* CNV-negative samples**						
18	cCNV	oCNV	*Potential CNV*	*Potential CNV*	Negative	oCNV

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19	oCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Negative	Negative
20	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Negative	Negative
21	cCNV	cCNV	*Potential CNV*	*Potential CNV*	Negative	Negative
22	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Negative	Negative
23	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Negative	Negative
24	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Negative	Negative
25	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
26	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
27	oCNV	cCNV	*Potential CNV*	*CNV Not Positive*	oCNV	Negative
28	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
29	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
30	oCNV	cCNV	*Potential CNV*	*CNV Not Positive*	oCNV	Negative
31	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Negative	Negative
32	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Negative	Negative
33	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
34	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
35	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Negative	Negative
36	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Negative	Negative
37	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
38	iCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Negative	Negative
39	cCNV	iCNV	*CNV Not Positive*	*Potential CNV*	Negative	iCNV
40	oCNV	cCNV	*Potential CNV*	*Potential CNV*	oCNV	Negative
***BRCA* CNV-positive samples**						
1	oCNV	cCNV	*Potential CNV*	*Potential CNV*	Positive	Positive
2	cCNV	cCNV	*Potential CNV*	*Potential CNV*	Positive	Negative
3	cCNV	cCNV	*Potential CNV*	*Potential CNV*	Positive	Negative
4	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Positive	Negative

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5	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Positive	Negative
6	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Positive	Negative
7	cCNV	oCNV	*CNV Not Positive*	*Potential CNV*	Negative	iCNV
8	oCNV	cCNV	*Potential CNV*	*Potential CNV*	oCNV	Positive
9	fCNV	fCNV	*CNV Failed*	*CNV Failed*	fCNV	fCNV
10	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Positive	Negative
11	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Positive	Negative
12	cCNV	iCNV	*CNV Not Positive*	*Potential CNV*	Positive	iCNV
13	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Positive	Negative
14	cCNV	cCNV	*Potential CNV*	*Potential CNV*	Positive	Positive
15	ntCNV	iCNV	*CNV Not Positive*	*Potential CNV*	Negative	iCNV
16	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Negative	Positive
17	ntCNV	iCNV	*CNV Not Positive*	*Potential CNV*	iCNV	iCNV

Abbreviations: oCNV: other CNV; cCNV: confirmed CNV; fCNV: failed CNV; iCNV: inconclusive CNV; ntCNV: not detected CNV.

3.1.2. BRCA-CNV-Positive Samples

Based on graphical visualization and interpretation of CNV status, among the 17 CNV-positive samples, 10 were confirmed as *Positive* in both genes. In 2 samples, a CNV was confirmed in *BRCA2*, while a *Potential CNV* was suspected in *BRCA1*. In 1 sample, the CNV was confirmed in *BRCA1* and considered *Inconclusive* in *BRCA2*. In 2 samples, the CNV was not confirmed in *BRCA1* and was *Inconclusive* in *BRCA2*. In another sample, a CNV was confirmed in *BRCA1*, and an additional CNV was suspected in *BRCA2*. Finally, one sample was considered a complete CNV calling failure in both genes. According to CNV calling by the Diatech software, one sample was classified as *failed*.

In 9 samples, the expected CNV was correctly identified. Of these, 5 also showed a *Potential CNV* in the other gene, where a negative result was expected. In the remaining 4, only the expected CNV was detected. In 7 samples, the expected CNV was not detected, but a *Potential CNV* was identified in the other gene.

The final interpretation and reporting of CNV status, integrating graphical visualization with software-based calling, resulted in 13 samples being classified as definitively CNV-positive. Two samples were negative for the expected CNV but showed inconclusive findings in other genes. One sample was interpreted as *Inconclusive* for CNVs in both target genes, and one sample was classified as *failed* (Table 2).

Considering the final interpretation and reporting of CNV results, out of the 17 positive samples, 13 would be considered *Positive*, and 3 samples as *Negative* and 1 *Inconclusive*.

Overall, under these analytical conditions, the *Myriapod® NGS BRCA1/2* pipeline shows a sensitivity, specificity, and accuracy of about 80% compared to the reference assays (Table 4).

Table 4. Concordance analysis of CNV calling between the *Myriapod® NGS BRCA1/2 Panel* and TSO500HT and the SOPHiA DDM™ HRD assays.

 doi:10.20944/preprints202507.2066.v1

A) Primary CNV Calling Strategy and concordance analysis (5/4 *BRCA* CNV-positive *vs* 5/6 *BRCA* CNV-negative samples).

***Diatech Myriapod® NGS BRCA1/2* panel kit**	***TSO500HT/SOPHiA DDM™ HRD***	
	CNV Positive	**CNV Negative**
CNV Positive	13	5
CNV Negative	3	18
Inconclusive	**1**	
Analytical Performance	**Value (%)**	
Sensitivity	**81.25**	
Specificity	**78.26**	
Positive predictive value	**72.22**	
Negative predictive value	**85.71**	
Accuracy	**79.49**	

B) CNV Calling in a simulated BRCA diagnostic setting (1 *BRCA* CNV-positive *vs* 9 *BRCA* CNV-negative samples)

***Diatech Myriapod® NGS BRCA1/2* panel kit**	***TSO500HT/SOPHiA DDM™ HRD***	
	CNV Positive	**CNV Negative**
CNV Positive	15	1
CNV Negative	1	22
Inconclusive	1	
Analytical Performance	**Value (%)**	
Sensitivity	**93.75**	
Specificity	**95.65**	
Positive predictive value	**94.87**	
Negative predictive value	**93.75**	
Accuracy	**95.65**	

3.2. CNV Calling in a Simulated Diagnostic Scenario (1 BRCA CNV-Positive vs 9 BRCA CNV-Negative Samples in the Same NGS Run)

3.2.1. BRCA-CNV-Negative Samples

Based on the graphical visualization and interpretation of CNV status, out of the 23 CNV-negative samples, 22 could be considered negative for both genes. One sample was *Negative* for *BRCA2* but showed a *Potential CNV* in *BRCA1*.

According to the CNV calling performed by the Diatech software, 13 samples were classified as *CNV Not Positive* for both genes. Four samples were *CNV Not Positive* for *BRCA1* but showed a *Potential CNV* in *BRCA2*. Four samples showed a *Potential CNV* in *BRCA2* and were *CNV Not Positive* for *BRCA1*. Two samples were identified as *CNV-Positive* for both genes.

The final interpretation and reporting of CNV status, integrating both graphical visualization and software-based CNV calling, led to the classification of 22 samples as *Negative*, and 1 sample as showing a *Potential CNV* in *BRCA1* with CNV *Negative* in *BRCA2*. Based on the final interpretation and reporting, among the 23 samples initially classified as CNV-negative, 22 were considered definitively negative, while 1 sample was recommended for confirmatory testing.

3.2.2. BRCA-CNV Positive Samples

Based on the graphical visualization and interpretation of CNV status, among the 17 CNV-positive samples, 14 were confirmed as *Positive* for both genes. One sample showed a *confirmed CNV* in *BRCA1* and an *Inconclusive* result in *BRCA2*. One sample did not show a CNV in *BRCA1* as expected and was also *Inconclusive* in *BRCA2*.

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According to the CNV calling performed by the Diatech software, one sample was classified as *failed*. In 12 samples, the expected CNV was correctly identified. Of these, four also showed a *Potential CNV* in the other gene, where a negative result was expected. In the remaining eight samples, only the expected CNV was detected. Four samples did not show the expected CNV in *BRCA1* but instead showed a CNV in *BRCA2*.

The final interpretation and reporting of CNV status, integrating graphical visualization with software-based calling, resulted in 15 samples being classified as definitively CNV-positive. One sample was interpreted as *Negative* in *BRCA1* (compared to the expected result) and *Inconclusive* in *BRCA2*. One sample was classified as failed (Table 3).

Overall, under these analytical conditions, the Diatech pipeline showed a sensitivity, specificity, and accuracy of about 95% when compared to the reference assays (Table 4).

Table 3. Concordance analysis between TSO500HT/SOPHiA HRD DDM and Diatech software results in CNV calling in a simulated BRCA diagnostic setting (1 *BRCA* CNV-positive *vs* 9 *BRCA* CNV-negative samples.

ID samples	Graphical visualization and intepretation of CNV plots		CNV calling by Diatech software		Final interpretation and reporting CNV	
	BRCA1	*BRCA2*	*BRCA1*	*BRCA2*	*BRCA1*	*BRCA1*
***BRCA* CNV-negative samples**						
18	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Negative	Negative
19	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Negative	Negative
20	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Negative	Negative
21	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Negative	Negative
22	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
23	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
24	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
25	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
26	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
27	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Negative	Negative
28	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
29	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
30	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Negative	Negative
31	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
32	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
33	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative

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34	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
35	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
36	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Negative	Negative
37	cCNV	cCNV	*CNV Not Positive*	*CNV Not Positive*	Negative	Negative
38	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Negative	Negative
39	oCNV	cCNV	*Potential CNV*	*Potential CNV*	oCNV	Negative
40	cCNV	cCNV	*Potential CNV*	*Potential CNV*	Negative	Negative
***BRCA* CNV-positive samples**						
1	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Negative	Positive
2	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Positive	Negative
3	cCNV	cCNV	*Potential CNV*	*Potential CNV*	Positive	Negative
4	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Positive	Negative
5	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Positive	Negative
6	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Positive	Negative
7	cCNV	iCNV	*CNV Not Positive*	*Potential CNV*	Positive	iCNV
8	cCNV	cCNV	*Potential CNV*	*Potential CNV*	Negative	Positive
9	fCNV	fCNV	*CNV failed*	*CNV failed*	fCNV	fCNV
10	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Positive	Negative
11	cCNV	cCNV	*Potential CNV*	*CNV Not Positive*	Positive	Negative
12	cCNV	cCNV	*Potential CNV*	*Potential CNV*	Positive	Negative
13	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Positive	Negative
14	cCNV	cCNV	*Potential CNV*	*Potential CNV*	Positive	Negative
15	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Positive	Negative
16	cCNV	cCNV	*CNV Not Positive*	*Potential CNV*	Negative	Positive
17	ntCNV	iCNV	*CNV Not Positive*	*Potential CNV*	Negative	iCNV

Abbreviations: oCNV: other CNV; cCNV: confirmed CNV; fCNV: failed CNV; iCNV: inconclusive CNV; ntCNV: not detected CNV .

3.3. Sequencing Metrics and Performance

The distribution of sequencing quality metrics across individual samples in different runs is shown in Figure 2. Mean coverage was uniformly high, reflecting adequate sequencing depth

 doi:10.20944/preprints202507.2066.v1

consistent with expected performance in somatic sequencing. Coverage uniformity confirmed efficient and balanced target representation. The percentage of on-target reads showed minimal variation between samples reflecting the specificity and overall performance of the protocol.

Figure 2. Comparison of sequencing metrics between NGS runs: Mean Coverage (X); On target reads (%); Uniformity (%).

Table 5 compared the summary statistics (mean ± standard deviation) of mean coverage, and on-target read percentage across the different sequencing runs, as well as the overall averages. Mean coverage was maintained at elevated levels across all four runs, with a total average of 6680 ± 1346×. The mean uniformity across all samples was 94.9% ± 0.88, suggesting efficient and balanced coverage of the targeted regions. The percentage of on-target reads was remarkably high across all runs, with minimal variability with a total mean of 99.8% ± 0.05. Taken together, these results demonstrate a high level of technical reliability across runs, with all quality metrics falling within expected and acceptable thresholds.

Table 5. Summary statistics (mean ± standard deviation) across the different NGS runs: Mean coverage (X); On -target read (%).

	Mean ± Standard Deviation		
ID Run	**Mean Coverage**	**Uniformity (%)**	**On target reads (%)**
1	5231±876	96.1±1.9	99.9±0.03
2	6514±1083	94.6±2.3	99.9±0.03
3	8490±2167	94±6.12	99.8±0.2
4	6488±2732	94.9±2.6	99.8±0.07
Total	6680±1346	94.9±0.88	99.8±0.05

4. Discussion

This study evaluated the analytical performance of different NGS strategies for the detection of *BRCA* CNVs in FFPE samples from OC patients. Specifically, we focused on the concordance of CNV calls between two hybrid capture-based protocols and the amplicon-based *Diatech Myriapod® NGS BRCA1/2* panel. Particular attention was given to the clarity and reliability of result interpretation, as well as the practical feasibility of integrating these methods into routine clinical diagnostics. A key emphasis was placed on the crucial role of bioinformatics pipelines in enabling accurate and robust

 doi:10.20944/preprints202507.2066.v1

CNV detection, especially when dealing with the technical challenges posed by FFPE-derived DNA, which is often degraded and affected by tumor heterogeneity [15]. Amplicon-based sequencing protocols are widely used in clinical diagnostics due to their high efficiency in detecting SNVs and *indels*. However, several studies have highlighted the limitations of these methods in accurately detecting CNVs, particularly in FFPE samples [12].

An interesting aspect was the strategy used to evaluate the *Diatech Myriapod® NGS BRCA1/2* solution. Specifically, the bioinformatics pipeline was first intentionally stressed by assessing CNV calling under diagnostic conditions with up to 5 *BRCA* CNVs in a run of 10 samples. Subsequently, a more clinically realistic setting was simulated, in which only one CNV might be present in an NGS run of 10 OC samples. In both analytical conditions, the pipeline demonstrated high performance. Notably, in the second analysis mode, CNV calling using the *Diatech Myriapod® NGS BRCA1/2* panel achieved a maximum accuracy of approximately 96% compared to hybrid capture-based reference methods in successfully identifying CNVs. Excluding one sample that failed sequencing, all germline CNV-positive cases were correctly identified. Among the somatic CNVs, only one case was missed. We recognize that this limitation may arise particularly in the context of somatic CNVs, where different NGS strategies can exhibit variable detection performance. However, missing a somatic CNV is generally considered less critical than failing to identify a germline CNV, which can have significant implications for patient management and familial risk assessment [16].

From a diagnostic perspective, our findings suggest that graphical visualization and software-based interpretation should be considered complementary tools. Therefore, a multi-step approach combining algorithmic CNV calling, graphical visualization, and expert review is confirmed as the most reliable strategy, particularly for CNV detection in amplicon-based protocols using FFPE samples (Figure 1). It is, however, essential to note that the manufacturer's instructions require that samples automatically classified by the software as CNV-potential positive must always be confirmed by an orthogonal method.

The reliability of CNV detection is also intrinsically linked to the quality of sequencing data. Accurate variant calling requires that key quality metrics, such as mean coverage, coverage uniformity, and on-target read percentage, meet established thresholds. When these metrics fall below recommended levels, the risk of inconclusive or incorrect calls increases, primarily due to insufficient read depth or uneven read distribution. In our study, sequencing metrics remained consistently high across runs, with mean coverage exceeding 6,600× and on-target rates approaching 100%, supporting the technical robustness of the workflow and likely contributing to the successful CNV detection.

Among the strengths of this study are the use of real-world clinical FFPE samples, comparison across different NGS platforms, and simulation of practical diagnostic scenarios. However, certain limitations must be acknowledged. First, the sample size was relatively small and may not fully capture the heterogeneity of *BRCA* CNVs observed in routine clinical practice. Second, orthogonal validation methods (e.g., MLPA, qPCR) were not employed for all discordant or borderline cases, which may have introduced uncertainty in result interpretation.

Looking ahead, integrating automated quality control checkpoints and confidence scoring for CNV calls could reduce the burden of manual review while enhancing overall reliability. Furthermore, continued development and validation of dedicated CNV detection algorithms specifically optimized for amplicon-based sequencing will be essential for broader clinical implementation.

5. Conclusions

Our study highlights the importance of having validated NGS workflows and bioinformatics pipelines for the accurate detection of *BRCA* CNVs in FFPE tumor samples. The amplicon-based *Myriapod® NGS BRCA1/2* panel in combination with *Myriapod® NGS data analysis software* proved effective in identifying CNVs, demonstrating strong concordance with hybrid capture-based

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approaches when combined with optimized bioinformatics analysis and expert interpretation and reporting.

These elements are essential for the reliable molecular identification of *BRCA* CNVs, which carry critical implications for the management of OC patients, including therapeutic decisions involving PARP inhibitors and cascade testing in hereditary cancer syndromes. For these reasons, it is possible to hypothesize a decision-making workflow, as described in Figure 1, where suspected CNV detections can guide either a therapeutic approach or germline screening as for other PVs in *BRCA* genes. Therefore, the accurate and reliable detection of these alterations remains a fundamental requirement in the genomic evaluation of OC patients.

Acknowledgements: We gratefully acknowledge the Diatech team for their technical support, in contributing to the results presented. We also thank the Genomics Core Facility Group at the Gemelli Science and Technology Park (G-STeP) for their valuable contribution. This work is dedicated to the memory of Professor Giovanni Scambia, whose untimely passing represents a profound loss to the scientific community. His exceptional dedication, visionary leadership, and significant contributions to the field continue to inspire and guide both this project and future research efforts.

Competing interests: The authors declare that they have no competing interests.

Abbreviations: CNVs: Copy number variations; **LGRs**: large genomic rearrangements; **BRCA**: *BRCA1/2;* **NGS:** Next generation sequencing; **FFPE**: Formalin-fixed paraffin-embedded; **OC**: Ovarian cancer; **PVs**: Pathogenic variants; **PARP-1**: Poly-(ADP-ribose) polymerase; **HRD:** Homologous recombination repair**; SNVs:** single nucleotide variants (SNVs); **INDELS**: Insertions/deletions; **MLPA**: Multiplex ligation-dependent probe amplification; **SD**: Standard deviation

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