



Droplet digital PCR for large genomic rearrangements detection: A promising strategy in tissue *BRCA1* testing

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ABSTRACT

Background and aims: With the introduction of Olaparib as target therapy for High Grade Serous Ovarian Cancer (HGSOC) patients with germline and somatic *BRCA1/2* mutations, the genetic test performed on tumor tissue has become important like the germline test. In somatic testing the evaluation of Large Genomic Rearrangements (LGRs) represents the main challenge. We describe a droplet digital PCR (ddPCR) assay for the evaluation of target *BRCA1* LGRs on blood and formalin-fixed paraffin-embedded (FFPE)/Fresh Frozen Tissue (FFT) samples. **Materials and methods:** We analyzed blood, FFPE and FFT samples in a validation setting of $n = 78$ HGSOC patients. We applied the ddPCR to *BRCA1* exons 2, 20 and 21 as some of the most common *BRCA1* exons involved in LGRs in our cohort of patients.

Results: The ddPCR custom assays allowed the identification of LGRs in all sample types, including FFPE specimens. Moreover, we were able to clearly detect LGRs accounted as somatic event.

Conclusion: The introduction of ddPCR in a comprehensive workflow, encompassing both germline and somatic tests, represents an improvement in *BRCA1/2* testing. ddPCR can overcome challenges related to *BRCA* testing, especially on FFPE analysis. Finally, ddPCR represents a promising alternative strategy to the established standard methods currently used in clinical setting.

1. Introduction

BRCA1 (Breast Cancer 1, early onset) and *BRCA2* (Breast Cancer 2, early onset) are the two major susceptibility genes in Breast (BC) and Ovarian Cancer (OC) [1]. Even if most of *BRCA1/2* pathogenic variants (PVs) are Single Nucleotide Variants (SNVs) and insertions/deletions of few bases (Indels), it is known that Large Genomic Rearrangements (LGRs) represent a significant proportion of *BRCA1/2* alterations [2].

LGRs are deletions or duplications involving one or more exons resulting in a Copy Number Variation (CNV) of the gene. These are usually considered pathogenic due to the significant impact on protein structure and function [3]. The identification of constitutional *BRCA1/2* PVs is a major concern for genetic counselling in high-risk families, because it requires a dedicated path related to the management of family members of the mutated proband. However, the analysis of somatic PVs on tumor tissue DNA is steadily increasing, even if it does not exclude the

Abbreviations: *BRCA1*, Breast Cancer 1, early onset; *BRCA2*, Breast Cancer 2, early onset; **BC**, Breast Cancer; **OC**, Ovarian Cancer; **PVs**, Pathogenic Variants; **SNVs**, Single Nucleotide Variants; **Indels**, Insertions/deletions; **LGRs**, Large Genomic Rearrangements; **CNV**, Copy Number Variation; **PARPi**, Poly ADP Ribose Polymerase inhibitor; **HGSOC**, High-Grade Serous Ovarian Cancer; **tBRCA**, BRCA tissue test; **FFPE**, Formalin-Fixed Paraffin-Embedded; **FFT**, Fresh-Frozen Tissue; **CN**, Copy Number; **NGS**, Next Generation Sequencing; **MLPA**, Multiplex Ligation Probe Amplification; **MAQ**, Multiplex Amplification Quantification; **ddPCR**, droplet digital PCR; **gBRCA**, BRCA germline test; **RPL0**, Large Ribosomal Protein P0; **RFU**, Relative Fluorescence Unit; **SD**, Standard Deviation.

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confirmation at germline level [4]. In 2015, the Poly ADP Ribose Polymerase inhibitor (PARPi) Olaparib was licensed for the treatment of germline *BRCA*-mutated OC [5]. Olaparib is subsequently administered as maintenance therapy for patients with platinum-sensitive relapsed High Grade Serous Ovarian Cancer (HGSOC) carriers of germline or somatic *BRCA* PVs [6]. This new therapeutic indication has changed medical attitudes towards *BRCA1/2* testing and has led to new challenges for molecular evaluation. One of the major critical issues of molecular *BRCA1/2* tissue test (*tBRCA*) is the quality of DNA. Formalin-Fixed Paraffin-Embedded (FFPE) is the most widely available specimen type and the amount of DNA extracted from FFPE samples is often limited and characterized by poor quality [7]. Fresh-Frozen Tissue (FFT) samples generally provide better quality of DNA, even if it is not always available in a routine *BRCA* diagnostic workflow [8]. Moreover, tumor testing provides information on the average situation of the cells from which the DNA was extracted: therefore, a high sensitive Copy Number (CN) assay is needed to accurately discriminate differences in a so heterogeneous context. In our laboratory, *BRCA* genetic testing is routinely performed on blood, FFT and FFPE samples [9,10]. In many cases, we routinely analyse matched blood and tissue samples belonging from the same patient, in order to perform an efficient *BRCA1/2* test comprehensive of both germline and somatic evaluation. Given the diversity of sample types (blood, FFPE, FFT) and types of *BRCA* mutations (SNPs, indels, LGRs), we implemented our diagnostic pipelines in order to quickly respond to patient's clinical needs to integrate both germline and somatic *BRCA* assays and, moreover, to the present indications of PARPi. In the context of *tBRCA*, classical diagnostic tools may be affected from pitfalls that make the determination of LGRs a great challenge [11]. Next Generation Sequencing (NGS) is the recommended sequencing method for *tBRCA* [12–14] and bioinformatics predictive algorithms represent efficient tools for the identification of LGRs [15–17]. Multiplex Ligation Probe Amplification (MLPA) and Multiplex Amplification Quantification (MAQ) are, to date, the recommended methods for LGRs evaluation [18,19]. Few examples of integrative *BRCA1/2* diagnostic workflow applied to somatic LGRs are described in literature. Successful applications of NGS bioinformatics pipeline, MLPA and MAQ are reported [8,20], though no methodological guidelines are still available. Recently, Preobrazhenskaya *et al.* developed an efficient droplet digital PCR (ddPCR) assay to identify CNV in *BRCA1* gene on blood-derived DNA samples [21]. The ddPCR is a new technique used for absolute quantification of nucleic acids. This methodology can potentially improve *BRCA* CNV analysis due to its ability to quantify target DNA with high precision and accuracy [22]. In ddPCR, the target DNA molecules are separated into 20,000 individual nano-scale water-in-oil droplets that represent multiple PCR replicate reactions. Each droplet provides an independent digital measurement characterized by a fluorescent signal, indicating the presence of target DNA. The absolute quantification of gene CN is performed determining the number of droplets with an amplified signal for the target gene. The present preliminary study shows as ddPCR can be safely applied to the evaluation of target *BRCA1* LGRs on blood, FFT- and FFPE-derived DNA. Particularly, we applied the ddPCR to some of the most common *BRCA1* LGRs accounted in our cohort of patients, proving robust data about its usefulness in critical methodological conditions such as FFPE genetic testing. Our findings clearly show as an improvement of *tBRCA* can be easily achievable.

2. Materials

2.1. Sample selection

In this study we selected a group of HGSOC patients ($n = 78$) admitted at the Gynecologic Oncology Unit of the “Catholic University of the Sacred Heart” and routinely tested for *BRCA* germline tests (*gBRCA*) and *tBRCA*. All patients received oncologist counseling and a signed written informed consent before *BRCA* tests. The *BRCA* CNV

status of all patients was previously assessed by our pipeline [9,17,22]. In particular, we selected $n = 70$ patients that resulted wild type for *BRCA1* LGRs (CNV-), including the following sample types: $n = 30$ blood samples; $n = 20$ FFT samples; $n = 20$ FFPE samples. Moreover, we selected a set of $n = 8$ patients genotyped as positive for known *BRCA1* LGRs (CNV +) at germline and/or somatic level. Matched blood/tissue samples deriving from the CNV + patients were analysed in order to test the capability of the ddPCR method to discriminate LGRs of germline and somatic origin (Table 1). The study has been complied with all the relevant national regulations, institutional policies and in accordance to the Helsinki Declaration, and has been approved by the authors' institutional review board.

2.2. DNA extraction

Genomic DNA was extracted from peripheral blood samples using an automated method based on a commercial kit (QIAamp® mini DNA extraction kit, QIAGEN), according to manufacturer's procedures. Eosin-stained histology tissue slides were examined by pathologists to identify areas of at least 70% of HGSOC for tissue samples. DNA was extracted from 10 μ m thick unstained FFPE slides using MagCore® Genomic DNA FFPE One-Step kit (RBC Bioscience, New Taipei City, Taiwan) on the automated platform MagCore® HF16Plus (Diatech Lab Line, Jesi, Italy) following the manufacturer's instructions. For the isolation of DNA from FFT samples, the MagCore® Genomic DNA Tissue kit (RBC Bioscience, New Taipei City, Taiwan) was used according to manufacturer's instructions on the same automated instrument MagCore® HF16Plus (Diatech Lab Line, Jesi, Italy). The quantitation of the extracted DNA was performed using the Qubit dsDNA BR and HS fluorimetric assays (Life Technologies, Gaithersburg, MD, USA) for germline/FFT and FFPE DNA, respectively. Finally, the qualitative evaluation of the DNA integrity was performed using the DNA Fragmentation Quantification Assay (EntroGen Inc., CA, USA) on LightCycler® 480 Real-time system (Roche Diagnostics, Basel, Switzerland). Only tissue samples with at least 50% of percentage of amplifiable DNA ≥ 150 bp were analysed.

2.3. ddPCR workflow

All ddPCR experiments were carried out using the QX100 Droplet Digital PCR System according to the manufacturer's protocols (Bio-Rad Inc., Hercules, CA). Custom fluorescently labelled ddPCR primers and

Table 1

Patients carrier of *BRCA1* LGRs and corresponding analysed sample types. For each selected subject (ID), the table shows the samples types analysed by ddPCR, together with the characteristics of the *BRCA1* LGR.

Patient ID	Sample type	<i>BRCA1</i> LGR	LGR origin
ID1	Blood FFT	Exon 2–12 deletion	Germline
ID2	Blood FFT	Exon 20–21 deletion	Germline
ID3	Blood FFT	Exon 2 deletion	Germline
ID4	Blood FFT	Exon 2 deletion	Germline
ID5	Blood FFT ¹ FFT ²	WT WT Exon 2–12 deletion	Somatic
ID6	Blood FFPE	Exon 20–21 deletion	Germline
ID7	Blood FFPE	Exon 2 deletion	Germline
ID8	Blood FFPE	Exon 2 deletion	Germline

Footnotes: ¹primary tumor sample, ²secondary tumor sample.

FFT: Fresh-Frozen Tissue; FFPE: Formalin-Fixed Paraffin-Embedded; LGR: Large Genomic Rearrangement; WT: Wild Type. For patients ID1 and ID5, only *BRCA1* exon 2 deletion was confirmed by ddPCR assay.

TaqMan probes were designed for *BRCA1* target sequences and *RPLP0* reference gene (BioRad Inc., Hercules, USA). The *RPLP0* (Large Ribosomal Protein P0) gene was chosen from literature data among recommended genes for the normalization of real-time PCR data in OC tissue [23]. According to results coming from our patient cohort, we selected *BRCA1* exons 2, 20 and 21 as some of the most commonly *BRCA1* exons involved in CNV events. Primer pairs and probes sequences are shown in [Supplementary Material](#), Table 1. Optimized thermocycling conditions were determined by testing a temperature range. Particularly, the evaluation of the optimal annealing temperature was based on: 1) efficient separation of positive/negative events; 2) separation of clusters of at least 2000 Relative Fluorescence Unit (RFU); 3) no or minimal “rain” (e.g. droplets with unclassified fluorescence). The ddPCR reactions were performed in a final volume of 20 μ L, consisting of 10 μ L of 2x ddPCR Supermix for Probes (no dUTP) (BioRad Inc., USA), 1 μ L of target primers (900 nM) and target FAM-labeled probes (250 nM) mix, 1 μ L of reference primers (900 nM) and reference HEX-labeled probes (250 nM) mix, 1 μ L of DNA (previously diluted to 15 ng/ μ L) and ultrapure H₂O until final volume. Using the DG8 cartridge (Bio-Rad Inc., USA), each assembled ddPCR reaction mixture was loaded into the sample wells, while droplet generation oil for probes (Bio-Rad Inc., USA) was loaded to generate droplets using a QX200 droplet generator (Bio-Rad Inc., USA). Successively, the emulsions were transferred to a 96-well PCR plate and it was heat-sealed with pierceable foil seal using a PX1 PCR plate sealer (Bio-Rad Inc., USA) prior to loading in a T100 thermal cycler (Bio-Rad Inc., USA). The thermocycling consisted in an initial step of 10 min at 95 °C followed by 40 cycles of 94 °C step for 30 s and a 60 °C step for 1 min. The final extension step was performed at 98 °C for 10 min. The ramp rate was set to 2 °C/sec. For each ddPCR experiment, appropriate controls were used.

2.4. Data analysis

Fluorescence data analysis was performed on the QX200 droplet reader using QuantaSoft analysis software (Bio-Rad Inc., USA) using the CNV experiment type. Samples with <10,000 droplets were excluded. In the output graphs, each droplet was represented by a dot plotted according to its fluorescence amplitude into different populations. In the 1D-Amplitude dot-plot, data collected from channel 1 (FAM) for target *BRCA1* gene and channel 2 (HEX) for the reference gene are reported. The manufacturer’s software defines an automatic fluorescence threshold, allowing each droplet to be classified as positive or negative. The automatic classification of the fluorescence intensities was used for germline and FFT samples analysis. Moreover, the software also allows the setting of a user-defined threshold that can facilitate the binary read-out of the droplets. We decided to rely on the user-defined settings in the FFPE analyses. In particular, the automatic fluorescence thresholds obtained from the evaluation of n = 20 CNV- FFPE samples were used to calculate the mean and standard deviation (SD) of the corresponding manual fluorescence thresholds: 3000 \pm 100 RFU for *BRCA1* exon 2; 2000 \pm 50 RFU for *BRCA1* exon 20; 3000 \pm 60 RFU for *BRCA1* exon 21; 2000 \pm 50 RFU for the *RPLP0* sequence. In the clustering 2D-Amplitude scatter plot, the fluorescence signal of channel 1 (target *BRCA* gene) was plotted against channel 2 (reference gene). The absolute amounts (copies/ μ L) of each *BRCA1* target exon and reference gene were obtained applying the Poisson statistics, followed by the calculation of the gene CN for each *BRCA1* exon. To support the CN analysis obtained by fluorescence evaluation, the CN results belonging to the n = 30 blood-derived wild type DNA samples were used to calculate the mean, standard deviation (SD) and the corresponding range of ratios for CN wild-type status (mean \pm 2SD; 2n copies; CN = 2.0 \pm 0.2). Ratio values outside this reference range were considered as CN positive as follows: exon deletion (\leq mean - 2SD; CN \leq 1.8); exon amplification (\geq x + 2SD; CN \geq 2.2).

2.5. NGS and MLPA testing

All samples were previously analysed as described below. NGS analysis was carried out in order to detect SNVs and indels, and to perform the bioinformatics CNV prediction. Briefly, Devyser BRCA NGS kit (Devyser, Hågersten, Sweden) was used for library preparation, according to the manufacturer’s instructions. Sequencing were carried out on the Illumina MiSeq System (Illumina, San Diego, CA, USA) and CE-IVD Amplicon Suite Software (SmartSeq, Novara, Italy) was used for NGS data interpretation and CNV analysis. All positive CNV prediction were re-evaluated using MAQ assay, as previously reported. When necessary, MLPA assay was used as an alternative method [24].

3. Results

Custom ddPCR assays were performed on n = 78 samples (blood, FFT and FFPE) previously genotyped for *BRCA1* LGRs. The ddPCR assays correctly classified the positive samples, detecting the *BRCA1* rearrangements in all three different specimen types. Likewise, samples previously reported as CNV- were also confirmed as wild type by ddPCR, obtaining a specificity of 100%. For each analysed sample, the presence of the *BRCA1* CN alteration was primarily detected by the interpretation of fluorescence graphs. As an example, in [Figs. 1 and 2](#) we show the fluorescence plots associated to *BRCA1* exon 21 ddPCR assays performed for the wild type and the deleted germline samples, respectively. Instead, [Figs. 3 and 4](#) show the results of *BRCA1* exon 21 evaluations in wild type and deleted FFT samples, respectively. The ddPCR assays resulted to be useful for *BRCA* CNV evaluation: for each *BRCA1* ddPCR assay evaluated on DNA deriving from blood and frozen samples, we obtained a high-resolution droplets readout, resulting in a strong separation of clusters, with no adjustment of the manufacturer’s automated threshold ([Figs. 1 to 4](#), panels A). By viewing the results regarding the absolute concentration of both target and reference sequences ([Figs. 1 to 4](#), panels C), we obtained the corresponding CN ratio for each tested sample. Assuming that the reference gene sequence has an allele CN of 2 (2n), we can estimate the corresponding CN of *BRCA1* target sequence and consequently detect the deletion events (n) accounted in the sample. Moreover, we compared the CN ratio obtained from each sample with the calculated reference range of ratio: we were able to identify exon deletions with a full concordance with our previous genotyping. In [Fig. 5](#), we reported the absolute concentration and CN plots resulting from the evaluation of the CNV + patient ID5, characterized by a *BRCA1* macro-deletion involving exon 2 and accounted as a somatic event in the FFT secondary tumor sample. The ddPCR analysis allowed the identification of the wild type CN status for both germline (CN = 2.01; [Fig. 5](#), panel A) and FFT primary tumor sample (CN = 1.90; [Fig. 5](#), panel B), while a deletion was correctly detected from the FFT secondary tumor sample (CN = 0.99; [Fig. 5](#), panel C). When applied to FFPE samples, ddPCR analysis resulted in the typical occurrence of droplets graphically positioned in the intermediate region between the two clear positive/negative clusters. This phenomenon commonly occurs in FFPE evaluation and it is colloquially known as “rain”. In [Fig. 6](#) we reported the 1D-Amplitude plots obtained for *BRCA1* exon 21 analyses on blood, FFT and FFPE samples in order to show the fluorescence variability found while evaluating the FFPE wild type samples. The occurrence of this common artifact does not affect the automatic classification of CN status in our ddPCR assays. However, in order to limit the variability and the subjectivity of FFPE evaluation, we decided to set optimized fluorescence thresholds to filter out droplets not falling in the established cut-offs. The interpretation of fluorescence plots and the comparison with CN reference range allowed an efficient classification of the CNV status of FFPE samples. As an example, the ddPCR analysis obtained for the CNV + patient ID6 has been reported in [Fig. 7](#).

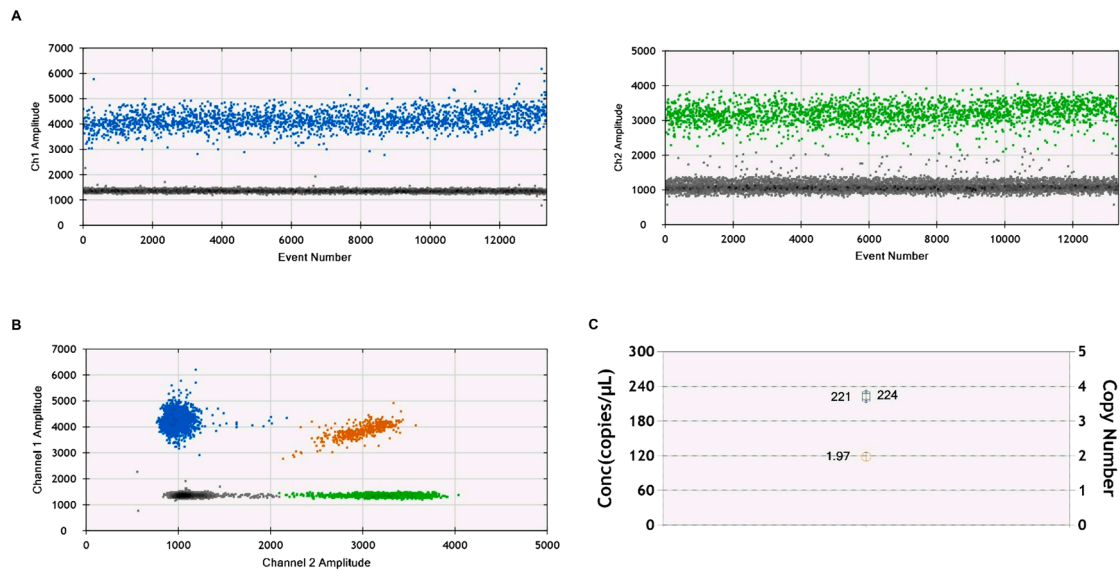


Fig. 1. ddPCR analysis of *BRCA1* exon 21 in wild-type germline sample. (A): 1D-Amplitude plots for *BRCA1*+ (blue, left panel) and for *RPLP0*+ (green, right panel). (B): 2D-Amplitude plot for *BRCA1*+ (blue), *RPLP0*+ (green), *BRCA1* + and *RPLP0*+ (orange). (C): Absolute concentrations (copies/μL) for *BRCA1* (blue) and *RPLP0* (green), with the corresponding CN results (orange). In black: below detection threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

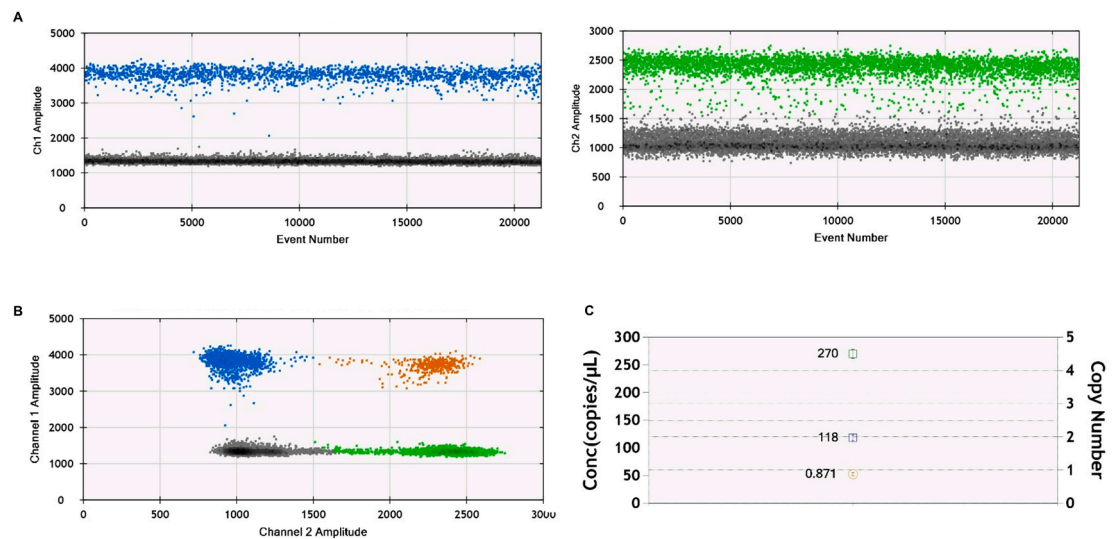


Fig. 2. ddPCR analysis of *BRCA1* exon 21 in CNV-positive germline sample. The figure shows the ddPCR germline results of patient ID2. (A): 1D-Amplitude plots for *BRCA1*+ (blue, left panel) and for *RPLP0*+ (green, right panel). (B): 2D-Amplitude plot for *BRCA1*+ (blue), *RPLP0*+ (green), *BRCA1* + and *RPLP0*+ (orange). (C): Absolute concentrations (copies/μL) for *BRCA1* (blue) and *RPLP0* (green). The CN result (orange) clearly shows the deletion of *BRCA1* exon 21 (CN = 0.871). In black: below detection threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion and conclusions

LGRs account for a significant proportion of all *BRCA* PVs and their evaluation is mandatory in a complete diagnostic molecular pipeline [25]. In our BC/OC cohort of selected patients, *BRCA1* LGRs account for 14.5% of all *BRCA1* PVs [24]. We extensively evaluated different molecular approaches for LGRs detection [2,24–26]. From our point of view, in *BRCA1/2* LGRs analysis, the continuous implementation of an integrated workflow with multiple approaches, characterized by different technical resolution, is preferable [15]. Tumor tissue is a heterogeneous sample type that leads to experimental biases for somatic LGRs detection [27]. Methodological considerations and technical adjustments are needed when deciding to apply a method commonly used for germline LGRs detection to tumor tissue testing [6]. Bioinformatics

prediction requires computational algorithms developed *ad hoc* and specific characteristics of sequencing raw data (e.g. maximum amount, coverage uniformity and sufficient reads depth) [16]. Furthermore, the sensitivity of NGS-based methods mostly depends on DNA quality, library preparation, type of algorithms and size of rearrangement. Due to these considerations, CNV prediction in tissue sample may be carefully applied and an alternative confirmatory test is required [28]. On the other hand, MLPA represents the gold standard method for CNV analysis at germline level and it is commonly adapted to somatic LGR analysis. In this context, DNA quality represents the most important variable and the tissue source, as well as DNA extraction method, could significantly influence the performance of the test [29,30]. In this regard, recommendations for *tBRCA* concerning methodological procedures needed to obtain DNA of suitable quality, are available [6]. Secondly, in order to

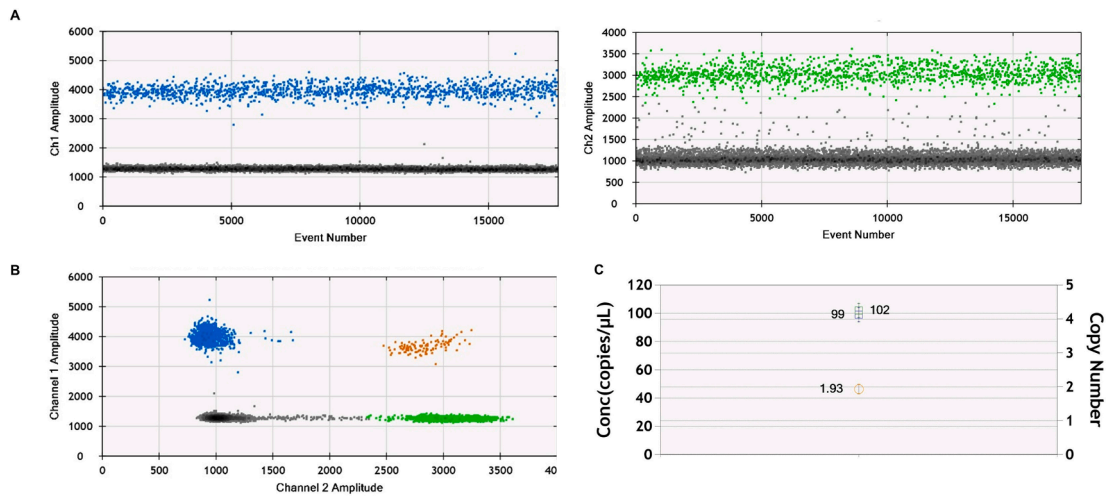


Fig. 3. ddPCR analysis of *BRCA1* exon 21 in wild type FFT sample. (A): 1D-Amplitude plots for *BRCA1*+ (blue, left panel) and for *RPLP0*+ (green, right panel). (B): 2D-Amplitude plot for *BRCA1*+ (blue), *RPLP0*+ (green), *BRCA1* + and *RPLP0*+ (orange). (C): Absolute concentrations (copies/μL) for *BRCA1* (blue) and *RPLP0* (green), with the corresponding CN result (orange). Black colour: below detection threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

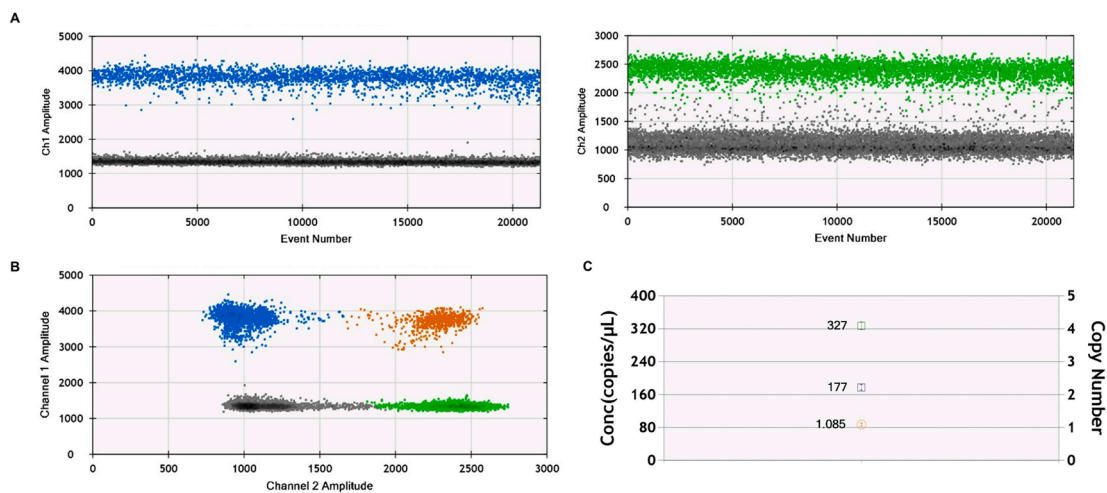


Fig. 4. ddPCR analysis of *BRCA1* exon 21 in CNV-positive FFT sample. The figure shows the ddPCR FFT results of patient ID2. (A): 1D-Amplitude plots for *BRCA1*+ (blue, left panel) and for *RPLP0*+ (green, right panel). (B): 2D-Amplitude plot for *BRCA1*+ (blue), *RPLP0*+ (green), *BRCA1* + and *RPLP0*+ (orange). (C): Absolute concentrations (copies/μL) for *BRCA1* (blue) and *RPLP0* (green). The CN result (orange) clearly shows the deletion of *BRCA1* exon 21 (CN = 1.085). Black colour: below detection threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

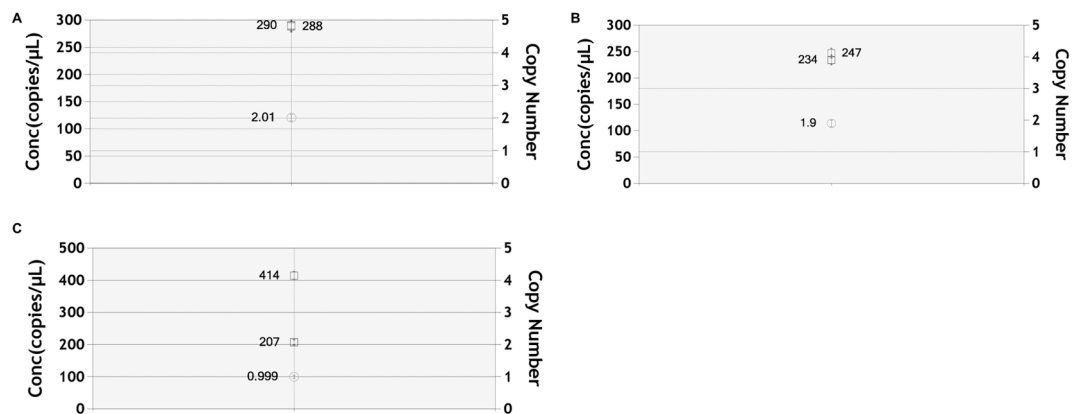


Fig. 5. ddPCR analysis of *BRCA1* exon 2 in patient ID5. The figure shows the absolute concentration (copies/μL) for *BRCA1* (blue) and *RPLP0* (green) with CN results (orange) resulting from the analysis of *BRCA1* exon 2 in germline sample (A), FFT primary tumor sample (B) and FFT secondary tumor sample (C) of patient ID5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

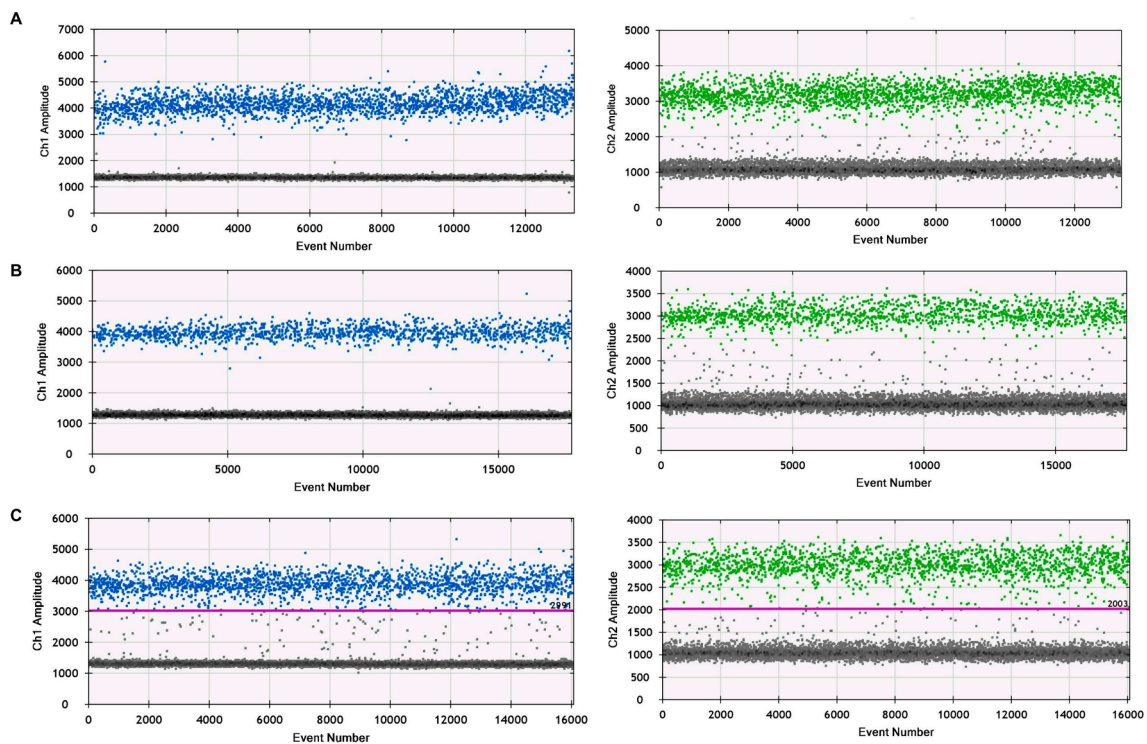


Fig. 6. ddPCR fluorescence variability in FFPE samples. The figure shows the 1D-Amplitude plots obtained for *BRCA1* exon 21 analysis performed on blood (A), FFT (B) and FFPE (C) wild type samples. In each plot, the fluorescence signal associated to *BRCA1* is indicated in blue (left panels) while *RPLPO* is indicated in green (right panels). The threshold fluorescence intensity is indicated by the pink line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

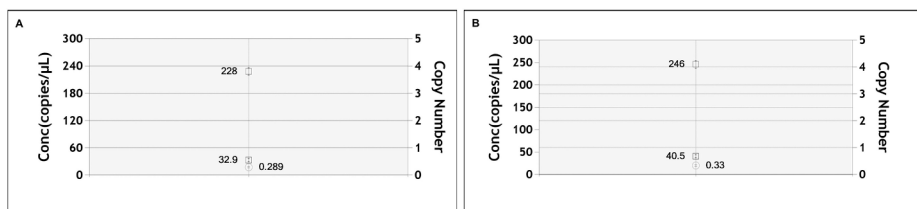


Fig. 7. ddPCR analysis of *BRCA1* exon 21 in CNV-positive FFPE sample. The figure shows the absolute concentration and CN plots resulting from the analysis of *BRCA1* exon 20 (A) and exon 21 (B) in FFPE sample of patient ID6. Absolute concentrations (copies/ μL) for *BRCA1* (blue) and *RPLPO* (green) with the corresponding CN results (orange) clearly show the deletion of both *BRCA1* exon 20 (CN = 0.289) and 21 (CN = 0.33). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

minimise the effects of contaminants, MLPA should be performed on sample and reference DNA derived from the same source (e.g., OC tissues and healthy ovarian tissue). Noteworthy, the LGRs analysis of *BRCA* genes needs a methodological resolution of single exon. In the case of single exon alteration, the accuracy of MLPA decreases and confirmatory methods, not often applicable to tumor derived-DNA, are needed [26]. Finally, both MLPA and MAQ are time-consuming methods that may give false-positive results for the deletion due to the occurrence of SNVs in the annealing region of MLPA probes or MAQ primers. To circumvent some of these issues and to describe an integrative workflow for *BRCA* testing, we applied ddPCR to the study of target *BRCA1* LGRs on DNA derived from blood/FFPE/FFT samples. The ddPCR is well suited for the evaluation of LRGs on DNA derived from tissue and shows different advantages. In ddPCR, the number of replicates (i.e., droplets) defines the dynamic range of target DNA quantitation. For 20,000 droplets, the ddPCR dynamic range spans from one to around 100,000 copies [31] with an accurate identification of fold differences in gene CN of 1.2X, demonstrating a high sensitivity for the evaluation of somatic molecular events [32,33]. Moreover, it represents an efficient technology which requires a low amount of starting DNA and works on short fragment size (<100 bp), that correspond to the most common critical issues when using FFPE in tumor testing [34]. Finally, the massive sub-partitioning

of DNA and PCR reactions into thousands of droplets ideally represents an enrichment method optimal for the detection of rare target LGRs events in a high background of non-target DNA. Unlike MLPA, ddPCR allows the absolute qualification without a reference calibration curve. As a consequence, any differences between reference and test source are minimized. In the present paper, we demonstrate the suitability of ddPCR in both germline and somatic evaluation of *BRCA1* LGRs. This technical option was investigated in order to respond to the increasing demands of sensitive and efficient tumor tissue assays in routine testing. Blood and FFT samples are easily analysed for LGRs using our ddPCR assays. The FFT from ovarian biopsies is the most common tissue sample type analysed in our laboratory. As with blood samples, also for tissue specimens an efficient analysis of *BRCA* genes is performed in order to address each patient to the proper target therapy [9]. Here we underlined that the ddPCR represents an attractive method for CNV evaluation in FFPE sample but optimization procedures may be necessary. The high degree of fragmentation of FFPE-derived DNA may result in the appearance of a “rain” fluorescence that affects the interpretation of ddPCR results. The droplets clustered in the “rain” area are associated to an inefficient fluorescent signal, with damaged positive droplets emitting a reduced signal [35]. In this work, we designed custom assays associated to short amplicon size (<80 bp) and we

introduced a quality check of the FFPE DNA integrity. By adopting these adjustments, we were able to obtain conclusive ddPCR analyses. In other instances, as commonly reported in literature, the user can manually set an appropriate threshold that allows an efficient classification [34,35]. The deep evaluation of fluorescence variability observed in multiple FFPE controls resulted as useful to standardize the analysis and reduce the occurrence of inconclusive results. Moreover, taking into account that a limited amount of FFPE tissues is often available for genetic testing, the detection of multiple target exons in a single ddPCR test could represent a significant improvement for the molecular workflow [36,37].

Additionally, the normalization of quantitative PCR data using stable reference genes is crucial to gain accurate results. This is particularly relevant in the evaluation of cancer tissue, wherein common reference genes can significantly vary in CN. In this context, new reference genes and also the combination of multiple reference genes, should be considered in the validation path of the assays. In the present preliminary study, we selected the *RPLP0* as reference gene taking into account the literature data available for HGSOc sample type. This gene, alone or in combination with other candidate genes, was proven as suitable for the normalization of quantitative PCR data in ovarian tissue-related studies [23]. Furthermore, as previously reported, *BRCA1/2* genes are relevant in the genetic predisposition to other cancers beyond OC, like breast, prostate and pancreatic ones. The re-evaluation, verification and validation of the *BRCA1* ddPCR assays also in these types of tissue will be necessary. In our *BRCA* genetic test workflow, each patient was analysed for using NGS. In case of positive or inconclusive LGRs results, MLPA/MAQ test were performed. Taking into account its advantages, ddPCR could be used as: 1) confirmatory method for MAQ, MLPA and bioinformatics prediction algorithms, especially in case of unclear results specifically dependent on DNA characteristics or single exon alterations; 2) target screening test in families with known hereditary LGRs; 3) target test for the sensitive monitoring of tumor-specific acquired LGRs, for example in relapsed cancer events. To note, both MLPA and ddPCR methods can be affected by the same artefacts, like SNVs that prevent probes hybridization. In the context of target use of ddPCR after gene sequencing, as in the case of our *BRCA* workflow, we underline as this issue should be kept under review due to the custom design of both probes and primer pairs and the acquired information concerning gene sequencing. The ddPCR offers significant advantages including simple instrumentation and protocols that are easily adoptable by any laboratory. However, a full automation is needed as a future prospective for a viable implementation of ddPCR into clinical routine. Moreover, the cost of ddPCR is higher than MLPA even if ddPCR can be used for several applications, such as the validation of somatic mutations identified by NGS that are under the limit of detection of Sanger sequencing. To our knowledge, this is the first report showing the utility of ddPCR for assessing somatic *BRCA1* LGRs on FFT/FFPE samples in a clinical molecular biology laboratory. The study provides evidence for the potential utility of the assays in a selected set of target exons. We underlined that this is the main limit of the study. However, taking into account the presented preliminary findings, we consider to apply ddPCR also to other common *BRCA* LGRs accounted in our patients, like the deletion of *BRCA1* exons 18–19. The combination of ddPCR with other high-throughput methods like NGS and MLPA could help solve the challenges related to somatic *BRCA1/2* diagnostic tests.

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CRediT authorship contribution statement

Elisa De Paolis: Conceptualization, Writing - original draft, Investigation, Data curation. **Maria De Bonis:** Investigation, Data curation. **Paola Concolino:** Supervision. **Alessia Piermattei:** Investigation. **Anna Fagotti:** Supervision. **Andrea Urbani:** Supervision. **Giovanni**

Scambia: Formal analysis. **Angelo Minucci:** Supervision. **Ettore Capoluongo:** Conceptualization, Writing - original draft, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cca.2020.12.001>.

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