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Original article

BRCA testing in metastatic castration-resistant prostate cancer: successes and troubles in a real world setting. An Italian Multicentric study

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Summary

Objective. Prostate cancer (PCa) is the most common cause of cancer-related deaths in men worldwide. *BRCA1/2* genes are reported altered in approximately 1% and 8% of PCa cases, respectively. To date, formalin-fixed paraffin-embedded (FFPE) tissues have a consolidate use in the clinical practice, but with a significant drawback related to DNA/RNA degradation during the pre-analytical process. The purpose of this study is to evaluate the feasibility of detecting *BRCA1/2* alterations in DNA extracted from FFPE tissues collected from PCa patients after various years of storage in seven Italian hospitals.

Methods. A total of 241 DNA samples were extracted from FFPE tissue with different storage times (1-12 y) and sequenced with NGS technology. *BRCA1/2* evaluability was assessed performing data analysis with a chi-square test to study the impact of the storage time on the DNA degradation.

Results. The data collected showed a strict relation not only between the storage time and the *BRCA1/2* evaluability, but even between the storage time and DNA degradation (DIN). Taken together, all the parameters considered decrease with an increase in the storage time.

Conclusions. Excessive FFPE tissues storage time (more than 3 years) can harshly affect DNA analysis and evaluability, hindering the achievement of a result useful in the clinical practice. Hence, it should be considered to perform the analysis as soon as possible to increase the evaluability of the test.

Key words: cancer, tissue, prostate, BRCA1, BRCA2

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Introduction

According to the GLOBOCAN database of the International Agency for Research on Cancer more than 1.4 million new cases of prostate cancer

(PCa) have been diagnosed worldwide in 2020 with 375,000 estimated deaths [1]. In Italy more than 41,000 new diagnoses were estimated in 2023 with a registered mortality for PCa of 8,400 deaths in 2022 [2].
Within three years from diagnosis about 15% of patients with metastatic PCa treated with androgen-deprivation therapy develop castration resistant prostate cancer (CRPC) [3].
It has been shown that cells deficient in homologous recombination repair (HRR) are susceptible to the synthetic lethality induced by poly (ADP-ribose) polymerase inhibitors (PARPi), specifically when they carry mutations in *BRCA1* and *BRCA2* genes [4,5]. *BRCA1* and *BRCA2* genes have been reported to be altered in 0.3% and 13% at somatic level, and in 0.9% and 5.3% at germinal level, respectively [6,7].
Apart from these genes, a series of alterations in gene involved in the HRR pathway, such as *ATM*, *RAD51*, *RAD52*, *RAD54*, *DSS1*, *RPA1*, *ATR*, *CHK1*, *CHK2*, *NBS1*, *FANCD2*, *FANCA* and *FANCC*, are identified in approximately 20% of metastatic castration resistant prostate cancer (mCRPC) patients [8]. Mutations in these genes are frequently associated with clinically aggressive PCa, and also revealed PARPi hypersensitivity to these cells [9]. The National Comprehensive Cancer Network (NCCN) [10] and the European Society for Medical Oncology (ESMO) guidelines [11] suggest that germline and somatic mutations in HRR genes may be predictive of the clinical benefit of PARPi in PCa patients. Olaparib, a progenitor of PARPi, has been approved as monotherapy in Italy in patients with mCRPC in progression, who carried germinal or somatic *BRCA2* and *BRCA1* mutations, after previous treatment with new hormonal agents (NHA) [https://www.aifa.gov.it/-/aggiornamento-registro-lynparza-mcrpc-].
The NCCN guidelines [12] recommend a germinal genetic screening using next generation sequencing (NGS) technologies, in order to detect alterations in *BRCA2*, *BRCA1*, *ATM*, *CHEK2*, *PALB2*, *MSH2*, *MSH6* and *PMS2*, in high risk patients or in PCa patients with family history.
On the other hand, the recent recommendations of the Italian Scientific Society of Oncology, AIOM [13] state that the somatic *BRCA* test should be performed on tissue samples from needle-biopsies or prostatectomies. Biopsies of metastatic sites may be challenging or not feasible and do not take into account the metastasis heterogeneity. Data obtained from the PROfound trial [14] showed that more than 30% of the formalin-fixed paraffin-embedded (FFPE) samples were not evaluable in NGS, because of DNA fragmentation due to prolonged storage. Indeed, FFPE samples are collected from patients affected by mCRPC at diagnosis and the time to progression to mCRPC can span many years. To overcome this problem the evaluation of circulating tumor DNA (ctDNA) may represent an alternative to tissue testing when it is not available or to monitor the disease during therapy, although this procedure underlies several concerns.
The main aim of this multicentric study is the evaluation of pathogenic alterations in *BRCA1* and *BRCA2* using DNA extracted from FFPE tissue samples obtained from mCRPC patients and sequenced with NGS platforms. Specifically, we focused on the FFPE sample feasibility related to the storage time.
To date, it is the only multicentric study performed in Italy and in a real-world setting on sequencing of samples of mCRPC.

Materials and methods

Seven diagnostic departments (Pathology, Molecular Biology and Genetics) representative of the entire national territory participated in the study (Tab. I).

Table I. Centers involved in the multicenter study.

Istituto Tumori "Giovanni Paolo II"IRCCS, Bari
IFO, Istituto Nazionale Tumori, Rome
Università degli Studi Federico II, Naples
Istituto Europeo di Oncologia IRCCS, Milan
Università di Pisa
Azienda Ospedaliera Santa Maria, Terni
Ospedale Policlinico San Martino, Genoa

Samples and clinical data of patients with mCRPC were re-evaluated according to regulations and guidelines and with Ethics Committee approval (protocol 1001/CE doc. 642/2022).
Briefly, 241 FFPE samples (217 prostatectomies and 24 diagnostic core-biopsies) collected from January 2007 to January 2022 were obtained from the seven centers. Patient data are summarized in Table II.
In all centers FFPE tissues were fixed in 10% neutral buffered formalin at room temperature overnight or up to 96 hours after surgery, depending on the size of the sample. Formalin penetrates at 1 mm/h, a rate which varies according to several factors (type of tissue, temperature, pressure). The process described implies that small specimens are rapidly and uniformly fixed, while in large tissue blocks the central areas could be rapidly reached by the fixative, which causes progressive autolysis. To avoid the degradative process, the radical prostatectomy samples were step sectioned at

Table II. Patient features by participating center. NA: Not Available data.

	Center 1	Center 2	Center 3	Center 4	Center 5	Center 6	Center 7
Total cases	32	12	32	31	81	32	21
Median Age	67.5	72	68	64	68	69	62
Histology							
Acinar	32	12	31	14	81	32	20
Ductal	0	0	0	1	0	0	0
Acinar and Ductal	0	0	1	1	0	0	0
NA	0	0	0	15	0	0	1
Dimension							
Needle biopsy	0	0	0	14	0	0	10
Average Volume (cm3)	75.9	94.8	109.4	37.4	64.7	64	78.5
NA	0	9	6	4	43	0	0
Gleason Score							
Low Grade	14	1	21	1	28	16	4
High Grade	18	10	10	30	52	16	14
NA	0	1	1	0	1	0	3
Stage							
T2	13	2	22	4	21	14	2
T3	19	2	10	19	18	17	9
T4	0	0	0	3	0	1	0
NA	0	8	0	5	42	0	10
Extracapsular Invasion							
Yes	19	2	8	11	20	18	11
No	13	3	24	0	47	13	2
NA	0	7	0	20	14	1	8
Vascular Invasion							
Yes	5	0	0	13	1	4	7
No	27	6	32	5	4	28	4
NA	0	6	0	13	76	0	10
Perineural Infiltration							
Yes	32	5	20	14	61	30	10
No	0	0	0	5	10	2	1
NA	0	7	12	12	10	0	10
Average storage time (yr)	2.7	2.9	3.6	4.3	4.8	3.3	6.1

5 mm intervals along the coronal plane and the resulting sections were immersed in fixative. Tissue sections were embedded in paraffin blocks, from which 4-5 µm-thick sections were prepared and stained with hematoxylin and eosin for routine histological analysis. The dominant tumor nodule was identified by an experienced pathologist and at least 6 consecutive unstained sections were macrodissected. The median tumour cell content was 50% (range 20-80%).

In order to understand if the number of unsuitable samples for NGS testing was correlated to the sample storage time, the whole cohort was divided in three groups: samples with less than 3 years of archive storage (group A), from 3 to 5 years (group B), and more than 5 years (group C).

Each participating center used its own extraction method (spin column-based or binding bead-based) to obtain the DNA from FFPE samples. DNA was quantified using a Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Inc.,

Waltham; MA, USA) and the quality was evaluated using the Agilent 2200 Tape Station System and the Agilent Genomic DNA Screen Tape Assay (Agilent Technologies, Santa Clara, CA, USA).
The library preparation was performed with 10 ng of genomic DNA, and the chip was automatically loaded on the Ion Chef System (ThermoFisher). The sequencing run was carried out with Ion S5 System (ThermoFisher). Data were analyzed using Ion Reporter Analysis Software. Only mutations with an allele frequency ≥ 5% and with adequate quality metrics were considered. Each relevant alteration was visually inspected using the Integrative Genomics Viewer (IGV) software (Broad Institute and the Regents of the University of California, Cambridge, MA, USA). The human reference genome GRCh37 (also called HG19) was used to obtain an accurate reads alignment. The molecularCoverageAnalysis (v5 12.0.0) was run in Torrent Suite™ Software with Variant Caller (v5 12.0.4#3) Plugins.
BRCA1 and *BRCA2* pathogenic mutations and variant of uncertain significance (VUS) were classified according to ENIGMA Consortium rules (https://enigmaconsortium.org/) and IARC classification (IARC genetic variant classification).

Statistical analysis

The impact of the storage time on the *BRCA1/2* evaluability, on the *BRCA1/2* status, and on the DNA quality and yield was evaluated with a chi-square test. The effect of storage time for the three different storage groups was analyzed performing a one-way ANOVA test.
Statistical analysis was handled using Excel (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism version 8.0.2 for Windows (GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com).

Results

A total of 241 tumor samples were screened to evaluate if the extracted DNA was still evaluable for *BRCA1/2* gene mutations. 170 samples (70.3%) were suitable for molecular analysis.
The number of not evaluable cases increased with increasing storage time: 13 samples (10.8%) were in group A, 26 (40%) in group B, 31 (55.3%) in group C (t-test p < 0.0001) (Fig. 1).
Pathogenic mutations in *BRCA1* were found in 3 (1.2%) patients whereas *BRCA2* mutations were detected in 19 (7.9%) (Fig. 2a). If we consider the *BRCA1* and *BRCA2* alterations together, 9 pathogenic mutations and 3 VUS were detected in group A, 9 mutations in group B and 4 mutations and 3 VUS in group C (Fig. 2b).

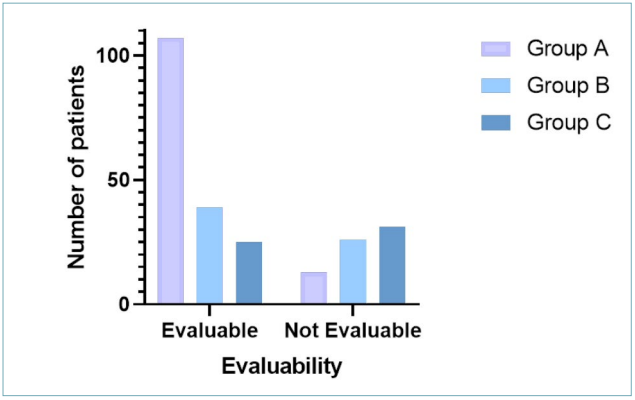

Figure 1. Age-related BRCA1/2 evaluability between samples with a storage time less than 3 years (Group A), with a storage time between 3 and 5 (Group B), and sample with a storage time more than 5 years (Group C). Chi-Square test p-value < 0.0001.

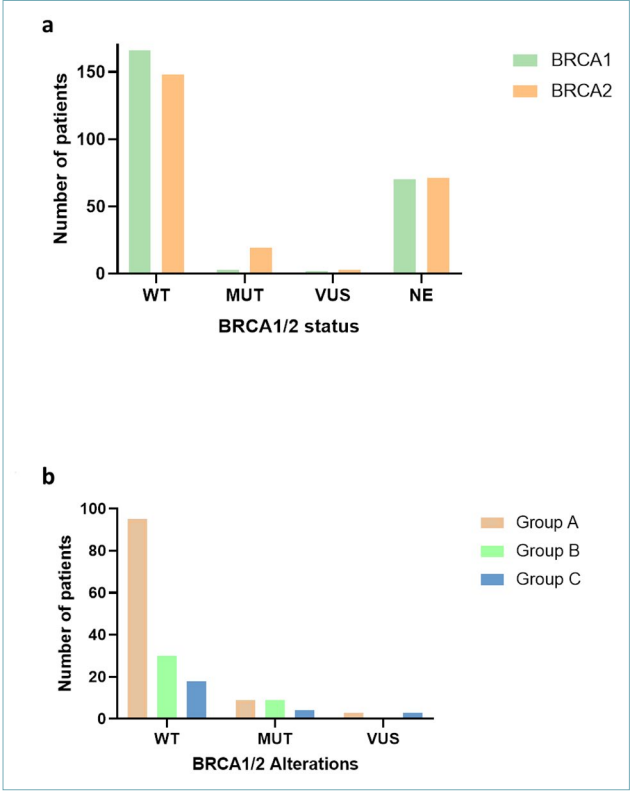

Figure 2. Gene alteration distribution: A. BRCA 1 and BRCA2 status in the analyzed series. B. BRCA1/2 status in patients with a storage time < 3 years (Group A), with a storage time between 3 and 5 (Group B), and sample with a storage time >5 years (Group C). WT: wild type samples; MUT: samples with pathogenic mutations; VUS: samples with variant of uncertain significance; NE: not evaluable samples. Anova test p-value = 0.0133.

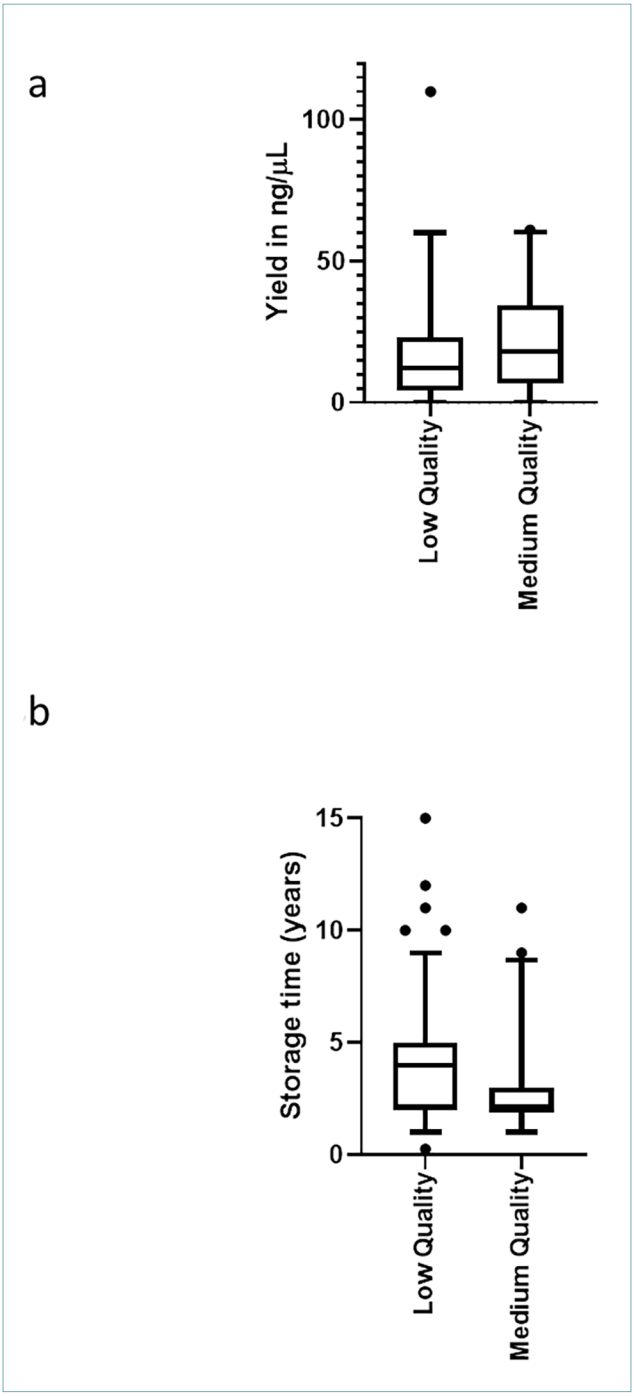

Figure 3. Quality of extracted DNA with respect to the extracted DNA (A) and storage time (B).

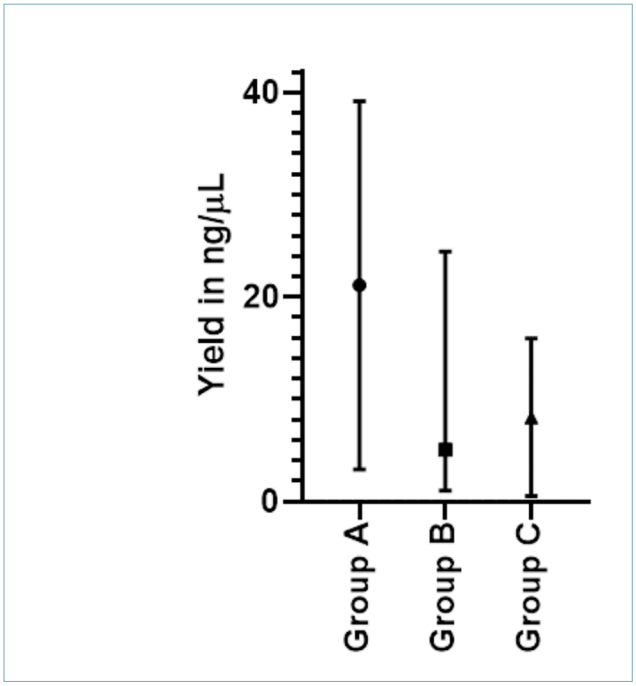

Figure 4. Correlation between the sample storage time and the DNA extraction yield in group A (•), group B (■) and group C (▲) as defined in M&M.

The median yield of extracted DNA for all the samples was 11.45 ng/µL (range 0.017-174). Thirty two samples (16 needle-biopsies) had a yield lower than 2 ng/µL (range 0.017-1.95).

The DNA integrity number (DIN) is a metric for assessing DNA degradation. It was calculated in 187 samples. The median value of DIN was 3.8. 132 had a DIN < 2 (low quality value) and 45 have a DIN between 2 and 5 (medium quality value). No high-quality samples were extracted. The medium quality samples had a higher mean extraction yield (mean = 22 ng/µL) compared to low quality samples (mean = 17 ng/µL), although this difference was not statistically significant (Fig. 3a). The mean storage time of the low-quality samples was higher than the medium quality samples (4 vs 2.9 years; p = 0.006 by chi-square test) (Fig. 3b). Finally, in order to estimate the age-related degradation of DNA we evaluated if the DNA quantity of the samples was linked to the storage time. The information on time storage and Qubit quantitation were available in 204 cases. The mean amount of DNA obtained from tissues of Group A was 21.14 ng/µL, 13.12 ng/µL for group B and 8.24 ng/µL for group C (p = 0.0006 by Anova test) (Fig. 4).

Discussion

In an effort to address the high lethality of mCRPC [15], new therapies are emerging. Tumors with pathogenic gene alterations, mainly in *BRCA1 and BRCA2 genes*,

contribute to inhibition of PARP through the mechanism of synthetic lethality [16,17]. Findings from a phase 2 trial of the PARPi olaparib in patients with mCRPC and homologous recombination deficiency were later confirmed in PROfound, a phase 3 randomized trial [18]. *BRCA1/2* gene mutations were investigated by NGS, but unfortunately a number of FFPE samples from prostatectomies or core biopsies were unsuitable for NGS. According to the study of Hussain et al. the reasons for the high percentage of sequencing failure are: insufficient/inadequate tumor tissue, tumor content or tumor nucleated cells, low DNA quality/quantity at extraction, failure at DNA library construction, hybridization capture and sequencing [18].

The molecular testing of FFPE tissue can be challenging due to the fragmentation of DNA enhanced by oxidation, by cross-linking between nucleic acid strands and proteins, by random breakings in sequence and excessive air exposure during the storage [19].

In 2018, Ellison et al. discussed the importance of an intact DNA for somatic *BRCA1/2* analysis for single nucleotide variants and copy number variation detection [20].

Watanabe et al. estimated the age-related DNA degradation extracted from FFPE tissue, and found a correlation between the storage time and the quantity of DNA available [21]. This observation was confirmed by Carlsson et al., who reported an association between DNA quality and quantity, and the storage time [22].

Groelz et al. evaluated the effect on DNA and RNA on mouse FFPE tissue stored at different temperatures and demonstrated that cross-linking reagents and room temperature can facilitate nucleic acid degradation [23].

Our real world multicentric study showed that the storage time (and probably the temperature and humidity conditions of the Pathology archives) was the main reason for the failure in molecular testing.

We showed a significant correlation between the storage time and DNA quality evaluated as DIN. Indeed, our data suggest that nucleic acid extraction should be performed as soon as possible in order to preserve its integrity for further analyses.

Extraction at the time of the diagnosis on a enriched sample provides an adequate quantity of nucleic acids with high quality. Extracted samples can be stored at -80°C for years without losing their quality and taking minimal space in freezer [24].

But what to propose to the patients when their samples are inadequate for NGS evaluation?

In a clinical setting a re-biopsy of a metastatic site could be challenging since mCRPC has a specific tropism for bones [25] and DNA extracted from bone biopsies had the lowest rates of NGS read-out (42.6%). Fresh-frozen samples could be a suitable sample type for genetic analysis, although in clinical settings this opportunity is limited to a few Academic centers with a clinically oriented biobank.

Recently Matsubara et al. [26] demonstrated that when tissue testing is not feasible or has failed, ctDNA testing may be a suitable alternative to identify patients with mCRPC carrying *BRCA* alterations who may benefit from olaparib [27].

The success of liquid biopsy may be limited by low ctDNA percentage in some patients, but in the near future its use will be more widespread and likely indicated for molecular characterization not only after failure of tissue analysis.

In addition, ctDNA percentage has recently been shown to be a strong predictor of overall survival, progression-free survival and treatment response in mCRPC patients, independent of therapeutic context [28].

Conclusions

In conclusion, prolonged storage of FFPE tissues (beyond 3 years) can significantly compromise DNA analysis and evaluability, impeding the generation of clinically useful results. Therefore, it is advisable to conduct the analysis promptly to enhance the evaluability of the test.

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Conflict of interest statement

The authors declare no conflict of interest.

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Author's contribution

Conception/design: ST, SB, MB.
Provision of study material or patients: AC, FAZ, AR, MP, UM, GT, GF, MB, DV, MC, SB, MC, VG.
Methodology: CAC, BP, EM, BC, GC, FP, RB, PF, SVT, SF, CC, MC.
Collection and/or assembly of data: CAC, ST, BP, AC.
Data analysis and interpretation: CAC, ST, MB.
Manuscript writing: CAC, ST, MB.
Final approval of manuscript: All authors.

Ethical consideration

This study was approved by the Institutional Ethics Committee of Istituto Tumori Giovanni Paolo II Bari (approval number/protocol number 1001/CE doc.642/2022). The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki. Written informed consent was obtained from each participant/patient for study participation and data publication.

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