DNA repair deficiency as circulating biomarker in prostate cancer

Martina Catalano[1], Daniele Generali[2]*, Marta Gatti[3],
Barbara Riboli[3], Leda Paganini[3], Gabriella Nesi[4]
and Giandomenico Roviello[4]

[1]School of Human Health Sciences, University of Florence, Florence, Italy, [2]Department of Medical,
Surgical and Health Sciences, University of Trieste, Cattinara Hospital Trieste, Trieste, Italy, [3]Servizio di
Citogenetica e Genetica - Azienda Socio-Sanitaria Territoriale (ASST) di Cremona, Cremona, Italy,
[4]Department of Health Sciences, University of Florence, Florence, Italy

Deleterious aberrations in DNA repair genes are actionable in approximately 25% of metastatic castration-resistant prostate cancers (mCRPC) patients. Homology recombination repair (HRR) is the DNA damage repair (DDR) mechanism most frequently altered in prostate cancer; of note *BRCA2* is the most frequently altered DDR gene in this tumor. Poly ADP-ribose polymerase inhibitors showed antitumor activity with a improvement in overall survival in mCRPC carrying somatic and/or germline alterations of HHR. Germline mutations are tested on peripheral blood samples using DNA extracted from peripheral blood leukocytes, while the somatic alterations are assessed by extracting DNA from a tumor tissue sample. However, each of these genetic tests have some limitations: the somatic tests are related to the sample availability and tumor heterogeneity, while the germline testing are mainly related to the inability to detect somatic HRR mutations. Therefore, the liquid biopsy, a non-invasive and easily repeatable test compared to tissue test, could identified somatic mutation detected on the circulating tumor DNA (ctDNA) extracted from a plasma. This approach should better represent the heterogeneity of the tumor compared to the primary biopsy and maybe helpful in monitoring the onset of potential mutations involved in treatment resistance. Furthermore, ctDNA may inform about timing and potential cooperation of multiple driver genes aberration guiding the treatment options in patients with mCRPC. However, the clinical use of ctDNA test in prostate cancer compared to blood and tissue testing are currently very limited. In this review, we summarize the current therapeutic indications in prostate cancer patients with DDR deficiency, the recommendation for germline and somatic-genomic testing in advanced PC and the advantages of the use liquid biopsy in clinical routine for mCRPC.

KEYWORDS

DNA damage repair, prostate cancer, liquid biopsy, circulating biomarker, circulating tumor DNA

1 Introduction

Deleterious aberrations in DNA repair genes are found in a considerable rate of patients with advanced prostate cancer (PC) (1–3). With the advent of target therapy such as ribose polymerase poly-ADP inhibitors (PARPis) and immune checkpoint inhibitors (ICIs), genomic testing has become part of the clinical practice in metastatic castration resistant prostate cancer (mCRPC) patients with DNA damage repair (DDR) (4). Homology recombination repair (HRR) is the DDR mechanism most frequently altered in PC and mutation of the *BRCA2* gene is the most frequently detected among the DDR genes (5). Oppositely to *BRCA1* involvement, the germline *BRCA2* mutations have been associated with a 2 to 6 fold increase in the risk for PC (6). *BRCA2* mutant patients seems to have a more aggressive phenotype, and a significant reduction in survival times compared to the non-mutated patients (7–9). Others germline mutations such as ataxia mutated telangiectasia (*ATM*), checkpoint kinase 2 (*CHEK2*), and the partner and locator of *BRCA2* (*PALB2*) seems to correlate, albeit to a lesser extent, with an increase of the risk of PC (3, 10, 11). Currently, the peripheral blood samples are preferentially used to detect germline mutations; while somatic alterations are assessed by extracting DNA from the tumor tissue sample, whose detection could be affected by the sample availability and by tumor heterogeneity.

Liquid biopsy is a new approach increasingly used in clinical setting allowing the rapidly and/or simultaneously detection/capture of cell-free DNA or circulating tumor cells (CTCs) or DNA (ctDNA), and extracellular vesicles. The liquid biopsy is a less invasive molecular profiling resource able to obtain intratumoral heterogeneity and to track dynamic changes and resistance mechanism occurring during therapies (12). The ctDNA has become a viable option to perform genomic testing in PC patients, receiving Food and Drug Administration (FDA) approval in the last years (13). However, although the advantages are now known, several limitations to the use of the ctDNA test are still present.

Here, we review the role that liquid biopsy currently plays in PC, the reliability of the ctDNA test in detecting DDR mutations and the evidence in favor of its routinely introduction in clinical practice.

2 DNA damage repair deficiency and mutations in prostate cancer

The DNA repair process is a fundamental mechanism for identifying and correcting the DNA damage induced by environmental factors or normal cellular metabolic processes. DNA damage induces a complex cascade of signals involving various checkpoints capable of interrupting the cell cycle to guarantee the repair of the lesion or, if not possible, to induce senescence and apoptosis (14).

This process is critical for cell survival, as it promotes genomic stability and reduces the risk of inheriting damage during cell division. DNA repair pathways include single-stranded break (SSB) defect repair mechanisms (base excision repair, nucleotide excision repair and mismatch repair (MMR) and repair mechanisms for damage to the double stranded (DSB) (homologous recombination (HR) and non-homologous end joining) as dispatched in Figure 1. Other mechanisms such as direct chemical inversion and crosslink repair between strands, although less common, may be coincided in the removal of damage (15). However, the presence of cells with alterations in these pathways are related to fallible repair mechanisms with consequent accumulation of cellular mutations and tumor transformation.

MMR is a proteins system including MLH1, MSH2, MSH6, and PMS2, recognizing and repairing erroneous insertion, deletion, and mis-incorporation of bases caused by DNA polymerase during the DNA replication (16). Its alteration, represented phenotypically as microsatellite instability (MSI), has been firstly identified in tumors from patients with Lynch Syndrome and subsequently in different types of cancers becoming an overall biomarker of response to treatments (17).

FIGURE 1
Main mechanisms of DNA repair.
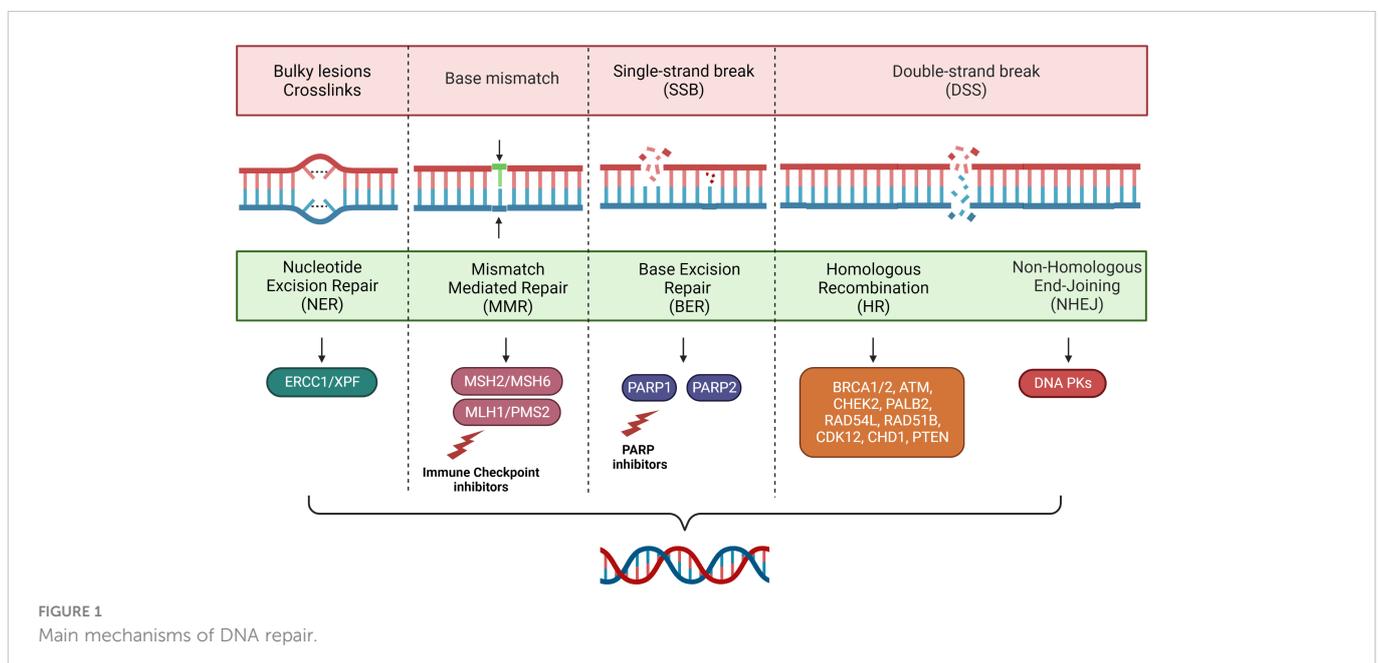
In the Memorial Sloan Kettering Cancer Center (MSKCC) cohort, the MSI and defective MMR (dMMR) have been identified in approximately 5% of PC patients (18). Another prospective case series reported MSI-H/dMMR in 3.1% of PC patients, while an unselected cohort study of 3,607 patients with personal history of PC reported the presence of mutations germline in MLH1, MSH2, MSH6 or PMS2 in 1.7% of cases (19, 20). On 60 rapid tumor autopsies from metastatic PC patients, 12% resulted dMMR/MSI-H (21), while in another study on 150 mCRPC patient's tumor biopsy detected an MSI-H frequency of 3% (22). This high variable frequency of dMMR/MSI-H ranged from 1 to 12% in patients with mCRPC may be in part explained by the diversity of assays used to detect tumors with dMMR (23, 24).

HR is a complex DNA double helix repair system, allowing one stretch of the DNA double helix to serve as a template to restore lost or damaged information in the other stretch. The germinal alterations in various genes belonging to HR, mainly BRCA1 and BRCA2, have been associated with the development of familial tumors, primarily involving breast and ovarian cancer, and subsequently prostate and pancreas cancer (25). Failure in the HR repair system can compromise the elimination of genome mutations, favoring the accumulation of DNA damage events and oncogenesis (26).

Actionable molecular alterations and aberrations in HR and MMR pathways occur in a considerable fraction of localized prostate cancers and, even more frequently in metastatic disease (22).

Regarding the inactivation of HR-associated genes (i.e., BRCA1/2, ATM, CHEK2, PALB2, RAD54L, RAD51B, CHD, CDK12 or PTEN), a number authors have reported the frequencies of somatic and germline mutations at several disease stages of PC (27). Somatic mutations were recorded in 19% of localized PCs and 23% of mCRPC cancers, with the highest incidence in the BRCA2 and ATM genes (22, 23). BRCA2 somatic mutations are associated with germline mutations in 42% of patients with mCRPC (22) and in 60% of localized PC (23). Recent data indicate that 11.8% of patients with metastatic prostate cancer (mPC) have germline mutations in 1 of 16 DNA repair genes: (BRCA1, BRCA2, ATM, CHEK2, PALB2, RAD51D, ATR, and NBN, PMS2, MSH2, MSH6, GEN1, RAD51C, MRE11A, BRIP1, or FAM175A) (28). In the Cancer Genome Atlas (TCGA) cohort, patients with high-risk localized PC had a rate of germline DNA repair mutations of 6% versus 2% in those at low/intermediate risk (23, 28). Another study reported a rate of pathogenic germline mutations in MUTYH, ATM, BRCA1, BRCA2 and BRIP1 of 7.2% in patients with high-risk, very high risk or metastatic PC (29). Other authors have reported varying incidence rates of DDR mutations ranging from 11 to 28%. Robinson et al. found a rate of 22.7% of germline DDR mutations or somatic mutations in BRCA1, BRCA2, ATM, FANCA, CDK12, RAD51B and RAD51C in patients with mCRPC (22), while a rate of less than 11.8% of germline mutations in at least one DDR gene has been reported by Pritchard et al. in the same context of patients (28). In all stages of PC, Abida et al. found germline or somatic alterations in BRCA1/2, TMJ and CHEK2 in 27% of patients (30). More recently in the PROfound study, 28% of mCRPC patients had alterations in 15 genes with direct or indirect roles in HR (31).

3 Recommendations for germline and somatic genomic testing in advanced prostate cancer

A family history of prostate cancer, as well as the hereditary breast and ovarian cancer syndrome (HBOC) due to germline mutations in HR genes and Lynch Syndrome increases the risk of PC (32–34). However, approximately 30% of patients with mPC carrying the germline DDR had no family history of cancer. An increased risk of PC has been found in Ashkenazi Jews in whom more than 2% carry germline mutations in BRCA1 or BRCA2 and in PC with intraductal histology that appear to have greater genomic instability compared to those with adenocarcinoma histology (35–38). Moreover, a correlation between clinical pathological features (Gleason score ≥8, lymph node and distant metastases to the diagnosis) and germline BRCA2 mutations has been observed, although the mutation cannot be excluded in the other patients (39). Below reported the main recommendations on carrying out the genetic and/or somatic test (40–42).

3.1 Germline testing

National Comprehensive Cancer Network (NCCN) panel recommends germline genetic testing, with or without pretest genetic counseling, for patients with PC and any of the following: a positive family history (multiple family members diagnosed with castration sensitive PC at the age <60 years, a family member died from PC, family history of high-risk germline mutations or of multiple cancers on the same side of the family); high-risk, very-high-risk, regional, or mPC regardless of family history; Ashkenazi Jewish ancestry and intraductal histology. Germline testing should include proteins of MMR and the HR genes (i.e., BRCA2, BRCA1, ATM, PALB2, and CHEK2). A cancer predisposition next-generation sequencing (NGS) panel testing, at a minimum including who consider other genes in addition (i.e., HOXB13) to the above, and guided by clinical context can be considered (40, 43, 44).

3.2 Somatic tumor testing

Alternatively somatic tumor test follows these recommendations: tumor testing for somatic HR gene mutations (i.e., BRCA1/2, ATM, PALB2, RAD51D, FANCA, and CHEK2) and MSI/dMMR can be considered in patients with regional or mPC; multigene molecular testing can be considered for patients with low- and favorable-intermediate risk PC and life expectancy ≥10 years; the Decipher molecular assay can be considered as part of counseling for risk stratification in patients with prostatic specific antigen (PSA) resistance/recurrence after radical prostatectomy. If HR mutations, especially BRCA1/2, ATM, CHEK2, or PALB2 are found, patient should be referred for genetic counseling to assess for the possibility of hereditary tumors such as HBOC. MSI testing should be performed using an NGS assay validated for prostate cancer and if positive, the patient should be referred for genetic counseling to assess for the possibility of Lynch Syndrome (40, 45–47).

Overall, the current recommendations are summarized below:

- Germline testing for DDR genes associated with cancer predisposition syndromes (especially *BRCA2*) is recommended for patients with a family history of cancer and should be considered in all patients withmPC;

- Somatic testing for HR and MSI genes should be considered in all patients with mCRPC;

- Patients with pathogenic mutations detected on somatic testing should be referred for germline testing and genetic counselling;

- In patients with localized prostate cancer, tissue molecular tests can be considered in the presence of suspected clinicopathological factors to aid decision making.

Notably European Association of Urology (EAU) reported genetic testing on circulating tumor DNA (ctDNA) as an alternative to tissue testing although still less common (42).

4 Damage of DNA damage repair as therapeutic target in prostate cancer

4.1 PARP inhibitors

PARP system is a nucleolar proteins complex involved in DNA repair, genomic stability and programmed cell death (48). Its main role consists in detecting and initiating an immediate cellular response to SSB damage (48). PARP inhibitors have developed as a possible therapeutic strategy in patients with DDR.

The anticancer effect of these drugs is attributed to the catalytic inhibition of PARP that interfere with efficient DNA damage repair inducing tumor cells death (49). While in normal cell PARP inhibition is tolerated, in tumors cells with concomitant HR alteration, the effect of PARPi are notable (50):the defective enzymatic function of PARP results in the accumulation of SSB that promote the accumulation of damage in the potentially lethal DSB, preferentially repaired by HR (51). The concomitant loss of PARP function in cancer cells with altered HR proteins involved in HR deficient repair with the accumulation of DSBs and subsequent cell death (51).

Based on this synergistic effect numerous PARP inhibitors (i.e., olaparib, rucaparib, niraparib, talazoparib and veliparib) have been tested firstly in patients with germline mutations in *BRCA1* or *BRCA2* (52, 53). Afterwards, sensitivity to PARPi has been proved in tumor with loss of other tumor suppressor DNA repair proteins (*e.g., ATR, ATM, RAD51, CHEK1/2, and PALB2*), suggesting the validity of this therapeutic strategy also in patients intrinsically deficient in HR without *BRCA1/2* mutations (54–57). The outcome benefits observed with PARPi in DDR mutated breast and ovarian cancers, led to the evaluation of PARPi efficacy in PC.

TOPARP-A was the first phase II study to test in 2014 olaparib in patients with mCRPC regardless of DDR mutations. Fourteen of the 16 patients with aberrations of the DNA repair genes (*BRCA2, ATM, BRCA1 or CHEK2 and HDAC2*) achieved a response to treatment measured by a composite methodology including the decline of CTCs (58). Based on these findings, olaparib received the FDA's breakthrough PC therapy designation (59).

A subsequent phase II study, TOPARP-B, further examined the anticancer effects of olaparib in mCRPC patients with DDR

mutations who had progressed to an earlier line of therapy (60). Patients positive for pathogen mutation or homozygous deletion in a DDR gene tested with NGS on primary tumor biopsies received olaparib 300 or 400 mg twice daily. Subgroup analysis showed that patients with the *BRCA1/2* mutation predicted greater responses and a longer median radiographic progression-free survival (PFS), with an overall response rate (ORR) of 83.3%. In patients with *ATM* and *PALB2* mutations, rate of radiographic objective responses was 8.3% and 33,3%, respectively; while PSA declines of at least 50% were detected in 5.2 and 66.6% of patients with alteration of *ATM* and *PALB2*, respectively, suggestinga susceptibility of *PALB2*to PARP inhibition. Limitations in obtaining accurate and timely somatic genetic testing in this trial allowed to enlist only 13.7% (98/711) of the screened patients. Data derived from these trials showed the antitumor effects of olaparib both when used to treat mCRPC patients with certain DDR genetic aberrations and in some patients with non-BRCA mutations (60).

The phase III study, PROfound, tested the efficacy of olaparib versus androgen receptor signaling inhibitors (ARSi) (abiraterone or enzalutamide) in patients with mCRPC and mutations in 15 HR-associated genes (*BRCA1, BRCA2, ATM, BRIP1, BARD1, CDK12, CHEK1, CHEK2, FANCL, PALB2, PPP2R2A, RAD51B, RAD51C, RAD51D* and *RAD54L*) (31). The primary endpoint examined was imaging-based PFS. The patients were divided into two cohorts: the cohort A included patients with alterations in *BRCA1/2* or *ATM*: the cohort B included patients with alterations in any of the other 12 genes. All patients received 300 mg olaparib twice daily versus second ARSi in a 2:1 ratio. In the overall population (cohorts A and B), significantly longer PFS was recorded in patients treated with olaparib compared to control arm (5.8 vs 3.5 months; hazard ratio [HR], 0.49; 95% confidence interval [CI], 0.38-0.63; p<0.001). An even longer PFS was recorded in cohort A in the olaparib group compared to control (7.4 vs 3.6 months; HR, 0.34; 95% CI, 0, 25-0.47; p <0.001) as well as a better OS (18.5 vs 15.1 months; HR, 0.64; 95% CI, 0.43 to 0.97; p=0.02). Notably, failure to sequence DNA occurred in approximately 31% of the tumor samples. Based on these results, FDA recently approved olaparib for patients with mCRPC progressed to enzalutamide and/or abiraterone who have deleterious germline alterations in *BRCA1/2* or somatic deleterious alteration in *BRCA1/2, ATM, BARD, BRIP, CDK12, CHEK1, CHEK2, FANCL, PALB2, RAD51B, RAD51C, RAD51D*, and *RAD54L* (61).

Another PARP inhibitor, rucaparib, has been evaluated for the treatment of patients with mCRPC who have germline or somatic mutations in the DDR genes (62). Phase II study, TRITON2 enlisted patients with any mutation in the HR genes, showing initial efficacy and safety results that allowed for the designation of rucaparib as a breakthrough therapy by the FDA. Preliminary data from this study showed promising results: 43.9% of patients with *BRCA* achieved a confirmed radiographic response, and lasting responses (62). Partial radiographic responses have been observed in 10.5% of patients with non-*BRCA* DDR genes and patients with *ATM* mutations. Two patients with *CHEK2* aberrations had a confirmed partial response and a confirmed PSA response. No objective response was observed in patients with *CDK12* mutations. In the 13 patients whit other mutations including *FANCA, PALB2, BRIP1*, or *RAD51B*, ORR was 38.5% with one complete response and four partial responses. The FDA recently approved rucaparib for the treatment of mCRPC

patients with germline or somatic *BRCA1/2* mutations (63). Is ongoing a phase III study, TRITON3, comparing rucaparib with standard of care treatments, enrolling only patients with mCRPC and mutations in *BRCA1/2* and *ATM* (NCT02975934).

The GALAHAD study evaluated the activity of niraparib in patients with mCRPC and DDR gene alterations received three or more systemic therapies for mCRPC (64). In this phase II trial niraparib was tolerable and showed anti-tumour activity in heavily pretreated patients, particularly in those with *BRCA* alterations with an ORR of 34.2%. Niraparib combined with abiraterone acetate/prednisone versus abiraterone acetate/prednisone for patients with mCSPC and deleterious germline or somatic HRR gene mutated is under evaluation in the phase III trial, AMPLITUDE (NCT04497844).

Finally, talazoparib has been tested in TALAPRO-1 phase II study enrolling patients with measurable soft tissue disease, progressive mCRPC, and DDR mutated (*ATM, ATR, BRCA1/2, CHEK2, FANCA, MLH1, MRE11A, NBN, PALB2, RAD51C*), treated with one or more taxane-based chemotherapy regimens and ARSi for mCRPC to receive oral talazoparib 1 mg/day until radiographic progression, unacceptable toxicity, consent withdrawal, or death (65). Talazoparib monotherapy has encouraging antitumor activity in docetaxel-pretreated mCRPC patients with *BRCA1/2* alterations and was generally well tolerated. The efficacy and safety of talazoparib and enzalutamide combination in mCRPC patients with or without DDR mutations is currently under evaluation in the phase III trial TALAPRO-2 (NCT03395197). The phase III study, TALAPRO-3, is comparing talazoparib plus enzalutamide versus placebo plus enzalutamide in patients with mCSPC and DDR alterations (NCT04821622).

As the use of PARPi is limited by primary resistance mechanisms and the onset of secondary resistance in sensitive patients'numerous efforts have been aimed at developing combined treatment approaches (66)

PROpel is a phase III trial randomizing patients with mCRPC regardless of HRR status to receive olaparib or placebo and abiraterone plus prednisone or prednisolone (67). The primary endpoint was investigator-assessed radiographical (rPFS), OS was one of the multiple secondary endpoints. Treatment with olaparib plus abiraterone significantly prolonged rPFS in patients with mCRPC regardless HRR status compared to control (24.8 vs 16.6 months; HR, 0.66, 95% CI, 0.54-0.81; p <0.0001). The safety and tolerability profile of combination was consistent with the known safety profiles of the individual drugs.

MAGNITUDE is a randomized, double-blind phase III study enrolling mCRPC patients (≤4 months of prior abiraterone acetate/prednisone for mCRPC was allowed) with or without HRR biomarker positive (*ATM, BRCA1/2, BRIP1, CDK12, CHEK2, FANCA, HDAC2, PALB2*) to receive niraparib 200 mg once daily plus abiraterone acetate/prednisone or placebo plus abiraterone acetate/prednisone (68). The primary endpoint was rPFS. assessed by blinded independent central review in the *BRCA1/2* group then in all patients with positive HRR biomarkers. The preplanned futility analysis in HRR mutations negative patients showed no benefit of adding niraparib to abiraterone acetate/prednisone in the prespecified composite endpoint (PSA progression or rPFS: HR 1.09, 95% CI 0.75-1.59). Niraparib plus abiraterone acetate/prednisone significantly improved the primary clinical outcome in HRR biomarker positive patients, with a manageable safety profile and health-related quality of life. Therefore, while PROpel trial showed a global benefit of PARPi and ARSi without the need for HRR stratification in untreated mCRPC patient, the MAGNITUDE study results support the combination strategy only for patients with alterations in HRR genes.

4.2 Immune checkpoint inhibitors

Prostate cancer belong to those tumors whose microenvironment is defined as immune-excluded, as it is characterized by a low mutational load, a reduced expression of neoantigens, hyperactivity of myeloid-derived suppressor cells and T-regulator cells, loss of major histocompatibility complex class I expression and abnormal IFN-1 signaling (69). However, like other solid tumors, it has been shown that dMMR or MSI-H prostate cancer may respond better to the immune checkpoint blockade (70).

Based on the results of a phase II trial, KEYNOTE 158, pembrolizumab received the first tissue agnostic approval for an antineoplastic therapy granted by the Food and Drug Administration (71). Patients enrolled in this study presented several types of cancer, including PC in two cases, with MMRd and had received at least one prior therapy. Objective radiographic responses have been reached in 46 (53%) of patients, with 18 (21%) achieving a complete response. Two subsequent placebo-controlled, phase III trials testing CTLA-4 inhibitor, ipilimumab, did not find improvement in OS in mCRPC patients, while pembrolizumab as single-agent showed a low response rate of 3%-5% post chemotherapy (72–74). A phase II trial combined ipilimumab with nivolumab showed an overall response of 26% in mCRPC chemotherapy naïve, although with an significant rate of grade ≥3 adverse events (75). Follow-up studies have largely confirmed pembrolizumab's efficacy in men with MMRd prostate cancer. In a study conducted at MSKCC, 3.1% of enrolled mCRPC patients were characterized by MSI-H PC and 11 of these received ICI-based therapy (19). About half of the patients achieved a PSA decline of at least 50% from baseline (PSA50 response), and four patients achieved a radiological response. A small sample size-based study by Schweizer et al. showed that 4 out of 10 ductal PC patients were dMMR, and 3 of them were also characterized by MSI-H. Notably, one of these dMMR/MSI-H patients with ductal PC achieved a significant reduction in PSA levels during treatment with pembrolizumab (76). Several other series of studies enrolling patients with PC with MMRd reported a PSA50 response, ranging from 50 to 65% when treated with ICI monotherapy, with long term responses observed (77).

5 DNA damage repair deficiency testing type

5.1 Tissue and whole blood testing

Tests for identifying mutations in DNA repair genes can be detected on two main tissues: blood or tumor tissue. The main difference between these two strategies is based on the type of mutation that can be detected. Through blood analysis we can detect genomic rearrangements significant for patients and their family, without indications on somatic mutations. Both alterations

can be identified through tissue testing (78). The blood test has numerous advantages including the easy availability of the sample, the minimum invasiveness of the procedure and the repeatability of the test. Tissue testing can be performed on both surgical or biopsy specimens of the prostate and on metastatic sites, although with some limitations. Firstly, the multifocal nature of PC, which may result in the core biopsy analyzed not representing the metastatic disease clone (79). The execution of the biopsy on the metastatic site is an invasive procedure and not free from complications for the patient, in addition the frequent bone involvement as a metastatic site in patients with PC considerably reduces the probability of success of the test (78). Secondly, is related to quantity and quality of sample. In fact, the small size of prostate primary tumor biopsies and progressive degradation of DNA in paraffin after years, are two conditions to be carefully considered for the choice of the test. Contrary to the high quality of blood sample, quality in the tissue is low and variable in relation to factors including carrying out different molecular tests, presence of necrosis, high infiltration of inflammatory cells, degradation related to aging, poor formalin fixation and cauterized tissue (80, 81). This making up to 20-30% of core biopsies unsuitable for NGS testing using commercial platforms (31).

Regarding MSI the gold standard for determining MSI/dMMR status is immunohistochemistry (IHC) and polymerase chain reaction (PCR) testing performed on tumor tissue samples. IHC is highly sensitive and specific in Lynch Sindrome-associated tumors by exploring the expression of the four major MMR proteins or just the MSH6/PMS2 doublet (82, 83). The MSI-PCR method based on PCR amplification of microsatellite regions followed by capillary electrophoresis is a reliable alternative to IHC. PCR helps also to recover cases that can escape IHC due to preanalytical problems, indeterminate results, as well as false negatives (non-truncating missense mutations in MMR genes associated with intact antigenicity) (84). Recently, they have emerged new molecular approaches (histopathology-based approach, PCR-based test, NGS-based approaches computational tools for MSI diagnosis) on tumor tissue samples that could improve sensitivity and specificity compared to conventional tests, representing a valid future option.

To date, circulating free DNA (cfDNA) can also be used to accurately determine MMRd and MSI status with the advantage of being easily obtainable compared to a metastatic biopsy; however, there are technical limitations of ctDNA-based sequencing approaches such as the low tumor burden which can results in indeterminate results.

5.2 Liquid biopsy and ctDNA

In the last year liquid biopsy has emerged as a promising surrogate for tumor biopsy, capable of overcoming spatial and temporal heterogeneity by allowing longitudinal monitoring of the disease through iterative sampling (85–87). It is an emerging field in the management of patients with cancer and its relevance as a potential diagnostic, prognostic, monitoring, and therapeutic tool makes it an attractive strategy in the management of these patients (87–89). However, liquid biopsy still has some limitations, although seem to be within the reach of technological development soon. This strategy has shown to be able to reliably represent the tumor

microenvironment and its modification. Different biomarkers such as CTCs, extracellular vesicles, ctDNA, circulating tumor RNA can be analyzed through liquid biopsy on the blood or on the other human fluids (i.e., urine, sperm, etc.) for diagnostic, prognostic, and predictive purposes (88).

The detection of cell-free DNA (cfDNA) as a part of liquid biopsy in PC has been widely explored, despite its diagnostic value for PC remains controversial. cfDNA is the total amount of circulating DNA found in blood plasma representing the total DNA released by normal and cancer cells. Its concentration may be increased in stroke, trauma, myocardial infarction, and autoimmune diseases (90–92), and even more in patients with advanced cancer (93, 94). Circulating tumor DNA is plasmatic DNA derived specifically from the primary or secondary site of the tumor, or from circulating tumor cells. It can represent 0.01% up to 90% of total free DNA, with an inherent patient variability due to various factors such as location, size, vascularity, tumor stage and response to therapy. The ctDNA level is higher in metastatic cancer than in localized disease and appears to correlate with disease progression (95–98). The release of free DNA from circulating cells can occur passively, during apoptosis or necrosis, or through active secretion. The DNA fragments released during apoptosis differ from those poured into the circulation in case of necrosis in the shorter length (99). A smaller amount of ctDNA is released through active secretion from extracellular vesicles, such as exosomes and prostasomes (100).

Liquid biopsy and specifically ctDNA testing can ensure monitoring of tumor evolution during therapy bypassing the intratumoral heterogeneity that limits tissue testing, especially if performed on the primary site. This allows to ctDNA to also detect resistance mutations. Both somatic and germline mutations can be detected through the ctDNA test, considering the pros and cons of the test (Table 1). Among the advantages of this method, there are: readiness in obtaining samples, repeatability during therapy or disease progression and rapidity, 1-2 weeks for the ctDNA test compared to 2-4 weeks for the examinations of the blood (101–103). The main disadvantages are related to amount of DNA and ctDNA levels (104). The amount of DNA in ctDNA is usually a very small fraction of cell-free DNA, especially in the early stages of the disease (105). The level of ctDNA is critical for performing the test, indeed it may vary during treatment and appears to closely correlate with tumor response (106). Some authors have shown how ctDNA determination changes at various stages of treatment. CtDNA was detected in 74% of patients before anti androgen therapy (ADT) initiation versus 59% of patients who received ADT prior to collection, with significantly higher ctDNA fraction in treatment-naïve patients (1.0% vs 11%; p = 0.02). The reduction in the ctDNA fraction was more pronounced after one week of ADT (107, 108).

Another critical point lies in the interpretation of the test result. Indeed, a negative result does not exclude the presence of a mutation in the patient's tumor, while a positive result for gene alterations does not distinguish between germinal and somatic origin. In the former case the patient should receive a confirmatory tissue test, in the latter case they should be referred to an appropriate confirmatory test if a germline mutation is suspected.

Some authors have evaluated the false positive rate linked to specific ctDNA tests in healthy controls, recording a rate of 0.82% in unique short variants. These false positive results may derive from

TABLE 1 Pros and cons of tissue, blood, and ctDNA-based HHR gene tests.

	Tissue	Blood	ctDNA
Mutation detected	Somatic and germline	Germline	Somatic and germline
Sample quantity	Medium	High	Low
Sample quality	Low	High	Variable
Time to response	2-8 weeks	2-4 weeks	1-2 weeks
Advantages	Archivial tissue for tumor histology	Easy to obtain samples Feasiblility in all cases Minimally invasive Easly repeatable	Easly to onbtain samples Better representative o ftumor heterogeneity and metastatic sites Minimally invasive Easly repeatable
Limitations	Tumor heterogeneity Invasive procedure to obtain samples High percentage of tests failed	Does not detect HRRm of somatic origin Does not capture the potential changing genetic profile of disease progression	Low concentration of ctDNA Type of sentitive tests False positive Adeguate amount of ctDNA particularly in early stages.
Genetic counselling	After germline test confirmation	Required	After germline test confirmation

ctDNA, circulating tumor DNA; HRR, homologous recombination repair.

somatic non-tumor changes in genes derived from clonal hematopoiesis indeterminate potential (CHIP), including *ASXL1, ATM, CBL, CHEK2, DNMT3A, JAK2, KMT2D, MLL2, MPL, MYD88, SF3B1, TET2, TP53 and U2AF1* (109–113). Despite tumor biopsy represents the reference tissue for the determination of MSI, clinically it has several limitations mainly related to the complexity of the procedure and to the spatial heterogeneity of the disease (114). Furthermore, in some rare cases, sporadic tumors may have a late onset of MMR defects that tissue biopsy cannot detect, leading to a misclassification of the MSI. It is now known the clinical potentiality of liquid biopsy in establishing tumour molecular diagnostics albeits data regarding its utility in determining MSI status which are still unclear, especially in the prostate cancer.

6 Circulating tumor DNA in prostate cancer

In PC, tissue testing is currently the test of choice for the analysis of tumor genomic profiles, although several critical issues have emerged in the main studies. In fact, in 30% of PC cases in which the tissue test was performed before enrollment, it failed due to problems in the pathological review, and during and after DNA extraction (31, 115–117). Therefore, the possibility of evaluating molecular alterations using ctDNA has made its way among pathologists and clinicians (118). NGS of ctDNA from plasma provides a minimally invasive method to identify genomic profile and resistance mechanisms in patients with mCRPC (119). However, the fraction of ctDNA in mCRPC patients and the clinical validity of the genomic alterations detected in plasma remain still unclear.

Several authors have studied the level of agreement between the plasma and tissue testing (Table 2). Firstly, Wyatt et al. in their study reported a high concordance rate between the ctDNA test and the metastatic tissue test. A ctDNA rate greater than 2% of the cfDNA was present in 75.6% of the samples (123). In these patients, all somatic mutations identified in metastatic tissue biopsies were simultaneously present in the ctDNA. The concordance results stratified by variant, showed a high positive agreement for substitutions (92%) and indels (95%) and a much lower agreement for rearrangements and copy number loss. Negative concordance was of 100%. In several patients, ctDNA sequencing revealed robust changes do not present in solid biopsy including clinically relevant alterations in the AR, WNT and PI3K pathways (123). Similarly, Vandekerkhove et al., reported a rate of 80% of concordance for mutation detection in diagnostic prostate tissue and ctDNA (107).

De Bono et al. reported a very high agreement between tissue and ctDNA testing for the detection of deleterious alterations in *BRCA1* or *BRCA2* with a positive percentage agreement of 88% and a negative percentage agreement of 95%. Some degree of discrepancy has been attributed to biological differences and sampling times between tumor tissues and plasma samples (31). Likely, Mateo et al. reported a similar prevalence between NGS over 470 primary tumors and metastatic site biopsy findings in patients who later developed mCRPC (60).

In their large study of ctDNA in 3334 mCRPC patients Tukakinsky et al. showed a high agreement between the alterations identified by liquid biopsy and those detected by tissue biopsy (119). The 94% of patient plasma samples had detectable ctDNA. In 79.5% of all patients, liquid biopsy identified at least one genomic alteration (*Tp53, RA, BRCA2/1, PI3K/AKT/mTOR, WNT/β-catenin pathway genes, RAS/RAF/MEK, MSI-H*). Regarding BRCA mutations, 67 (8%) *BRCA1/2* alterations were detected in both tissue and liquid biopsy, 5 (0.6%) exclusively in tissue biopsy (in 4 samples the ctDNA fraction was less than 1%) and 20 (2.4%) exclusively in liquid biopsy. The 20 cases detected only with liquid biopsy, may represent secondary alterations to the collection of the tissue sample. The concordance between BRCA mutation identified by blood test and ctDNA analysis was 100%.

Warner et al. demonstrated that the frequency of harmful *BRCA2, ATM* and *CDK12* changes detectable in plasma ctDNA was like those observed in the population with metastatic tissue biopsy in a large cohort of mCRPC, supporting minimally invasive liquid biopsy as approaches to identify responders to PARP inhibitors (120).

TABLE 2 Summary of the studies evaluating concordance between tissue and cDNA testing.

Study (ref)	Patients	Number of samples	Types of tests	Method	Results
Wyatt et al., 2017 (118)	mCRPC	45	Metastatic tissue and ctDNA	Targeted sequencing across 72 clinically relevant genes	All the somatic mutation identified in matched metastatic tissue biopsies were concurrently present in ctDNA; concordance of 88.9% for individual gene CAN.
Vandekerkhove et al., 2019 (119)	mCSPC	53	Diagnostic prostate tissue and ctDNA	Targeted sequencing strategy capturing the exon of 73 driver genes	80% of concordance for mutation detection in the matched samples. Combined ctDNA and tissue analysis identified potential driver alterations in 94% of patients; ctDNA or prostate biopsy alone failed in the 36% of cases.
Tukakinski et al., 2021 (120)	mCRPC	3334 (1674 screening samples from TRITON2/3 trial)	Tissue biopsy and ctDNA	Plasma assay with 62 (FoundationACT) or 70 genes (FoundationOne Liquid)	93% of concordance between BRCA 1/2 mutations detected in tissue biopsy and those identified by ctDNA 100% of concordance for germline variants. In 20 patients, BRCA 1/2 gene alterations were identified using ctDNA but not tissue testing.
Schweizer et al., 2021 (121)	mCRPC	51	Primary prostate tissue, metastatic tissue and ctDNA	Plasma assay with 324 (FoundationOne CDx) or 70 genes (FoundationOne Liquid)	Of the 53 paired samples, at least partial concordance in DDR genes was identified in 43 cases (84%) Concordance was numerically higher between ctDNA primary pairs compared with metastatic primary pairs; however, this difference was not statistic ally significant (92% vs 79%. 2 monoallelic DDR gene alterations only found in primary tissue.
Warner et al., 2021 (122)	mCRPC	1615	Archival primary tissue and ctDNA	Plasma assay with 22 genes (Illumina MiSeq or HiSeq 1500/2500 machine)	DDR gene status was concordant (94%) between archival primary tissue taken at cancer diagnosis and serial ctDNA-positive samples collected in the mCRPC setting.

mCSPC, metastatic castration sensitive PC; mCRPC, metastatic castration resistant prostate cancer; ctDNA, circulating tumor DNA; DDR, damage DNA repair.

Finally, Schweizer at al. in their study, showed as primary prostate tissue accurately reflected the mutational status of activatable DDR genes in metastatic tissue. After excluding probable CHIP events, the ctDNA profile accurately detected DDR mutations including alterations suggesting potential related mechanisms of resistance (122). Only one patient developed a *BRCA2* alteration later, while two cases *BRCA1/2* mutation positive in the primary sample, got lost in downstream samples. This may be reconducted to the intraprostatic genomic heterogeneity. However, it is also plausible a selective therapeutic pressure in the first case and an eradication of clones sensitive to DNA-damaging therapies in the second.

The confirmation of the high prevalence of HRR-associated gene mutations in advanced PC has led to some controversy regarding the use of archival primary prostate tumor biopsies for genomic profiling once patients have developed mCRPC (30, 121, 124).

Recently, Hussain et al. tried to outline the correct use of tissue for mutational analyzes by formulating the following recommendations (125): -in presence of more samples with similar tumor content, the choice must fall on the younger sample; -if the samples available exceed 5 years from collection, it is necessary to use those with the highest tumor content and high yield of DNA such as lymph nodes; -for the samples just collected, the recommendation is to optimize the fixation and storage of formalin and avoid descaling.

Regarding MSI, a good overall agreement was observed between conventional tissue-based tests and newly developed ctDNA-based approaches (126–128). This suggested that ctDNA-based MSI diagnosis could be performed as part of clinical practice to identify patients who might benefit from immune checkpoint inhibitors when tissue samples are unavailable or scarce (126, 127, 129). In Nakamura et al., changes in basal MSI levels during ICI treatment has been correlated well with those of other ctDNA markers and reliably reflected tumor response to treatment (128). Longitudinal analysis

of ctDNA also allowed to detect the acquisition of somatic MSI that can appear during cancer evolution in patients initially diagnosed with MSS tumors (130). At the present state of knowledge, there are few cases in which the MSI phenotype is acquired during the disease (19, 130). This phenomenon could be partly explained by the fact that most cancers are screened for MSI only at the time of diagnosis, underestimating cases. Further studies are needed to evaluate the true impact of such an acquired MSI phenotype in clinical practice.

In addition to a predictive value, a prognostic value may be recognized to ctDNA. Already in localized disease, *BRCA1/2* germline changes have been associated with poor outcome, including disease progression among patients under active surveillance (131) or a higher risk of recurrence and death among patients undergoing salvage therapy (132). In a retrospective study of mCRPC profiled patients with a 70-gene NGS cfDNA panel an alteration was recorded in over 94% of cases, and a greater number of ctDNA alterations were associated with a shorter time to treatment failure with chemotherapy (HR, 1.05, p=0.026) or androgen inhibitors. In the study conducted by the detection of a ctDNA fraction greater than 30% was strongly associated with a poor response to enzalutamide or abiraterone therapy even after adjustment for other clinical prognostic factors (133). In this study, a ≥50% reduction in cfDNA concentration after eight weeks of therapy was independently associated with longer OS suggesting free DNA concentration as predictive factor to PARPi response.

7 Conclusion and future perspectives

Given the significant percentage of mCRPC patients with DNA repair genes mutations and the therapeutic possibilities currently available, the most important guidelines recommend the performance of genomic testing in all these patients. The test can be performed on

various samples mainly whole blood and tissue, with relative advantages and limitations. In recent years, ctDNA has become a viable option for performing genomic testing receiving FDA approval in 2020 (13). The ctDNA can overcome the limitations of tissue testing, which can fail in up to a third of cases. Additionally, ctDNA testing can be performed longitudinally by detecting new alterations and resistance mutations that may emerge during disease progression.

Several authors demonstrated high agreement between tissue testing and ctDNA testing suggesting that the analysis on ctDNA is sufficient to identify all DNA alterations and be used as a guide for patient management with mCRPC. Ideally, the combined use of the two techniques could ensure the study of the molecular subtype, paving the way for the implementation of precision therapy, but still far from possible clinical practice.

To consider the ctDNA test results as reliable as possible, it should be performed in certified institutions using the standard NGS procedure. New sequencing technologies such as PacBioScience and Oxford Nanopore allow for the acquisition of additional information, such as large intermediate chromosomal aberrations that appear to correlate with a worse prognosis of PC (134, 135). These new technologies, still burdened by a high sequencing error rate and high costs, will enable the generation of more complete and easy-to-read data.

The large proportion of patients with a rich genomic signal from ctDNA and the sensitive and specific detection of *BRCA1/2* alterations position liquid biopsy as a compelling clinical complement for comprehensive tissue genomic profiling for mCRPC patients. However, despite the findings, there are still several barriers limiting the clinical implementation of genomic sequencing, including cost, access, and feasibility based on often limited tissue availability or quality. Furthermore, only a fraction of patients with PC and genomic aberrations respond durably to targeted therapy.

The integration of analyzes that combine genomics with transcriptome, epigenome and tumor microenvironment study could help identify patients who have more likely to benefit from targeted therapies. In the future, these integrated systems, combined with clinical information, will ensure a further push towards precision oncology.

Author contributions

GR had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: GR, DG. Acquisition of data: GR, MC, MG, BR, LP. Analysis and interpretation of data: GR, Drafting of the manuscript: GR, MC Critical revision of the manuscript for important intellectual content: DG Statistical analysis: None Obtaining funding: None. Administrative, technical, or material support: None. Supervision: DG, GN. Financial disclosures: None. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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