Clinical Epigenetics

REVIEW

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BRCA1 & *BRCA2* methylation as a prognostic and predictive biomarker in cancer: Implementation in liquid biopsy in the era of precision medicine

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Abstract

Background BReast CAncer gene 1 (*BRCA1*) and BReast CAncer gene 2 (*BRCA2*) encode for tumor suppressor proteins which are critical regulators of the Homologous Recombination (HR) pathway, the most precise and important DNA damage response mechanism. Dysfunctional HR proteins cannot repair double-stranded DNA breaks in mammalian cells, a situation called HR deficiency. Since their identification, pathogenic variants and other alterations of *BRCA1* and *BRCA2* genes have been associated with an increased risk of developing mainly breast and ovarian cancer. Interestingly, HR deficiency is also detected in tumors not carrying *BRCA1/2* mutations, a condition termed "BRCAness".

Main text One of the main mechanisms causing the BRCAness phenotype is the methylation of the *BRCA1/2* promoters, and this epigenetic modification is associated with carcinogenesis and poor prognosis mainly among patients with breast and ovarian cancer. *BRCA1* promoter methylation has been suggested as an emerging biomarker of great predictive significance, especially concerning Poly (ADP-ribose) Polymerase inhibitors (PARP inhibitor-PARPi) responsiveness, along with or beyond *BRCA1/2* mutations. However, as its clinical exploitation is still insufficient, the impact of *BRCA1/2* promoter methylation status needs to be further evaluated. The current review aims to gather the latest findings about the mechanisms that underline BRCA1/2 function as well as the molecular characteristics of tumors associated with *BRCA1/2* defects, by focusing on DNA methylation. Furthermore, we critically analyze their translational meaning and the validity of *BRCA* methylation biomarkers in predicting treatment response.

Conclusions We believe that *BRCA1/2* methylation alone or combined with other biomarkers in a clinical setting is expected to change the scenery in prognosis and predicting treatment response in multiple cancer types and is worthy of further attention. The quantitative *BRCA1* promoter methylation assessment might predict treatment response in PARPi and analysis of *BRCA1/2* methylation in liquid biopsy might define patient subgroups at different time points that may benefit from PARPi. Finally, we suggest a pipeline that could be implemented in liquid biopsy to aid precision pharmacotherapy in *BRCA*-associated tumors.

Keywords BRCA1, BRCA2, DNA promoter methylation, Breast cancer, Ovarian, Cancer, Liquid biopsy, PARP inhibitors

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Background

BReast CAncer gene 1 (*BRCA1*) and BReast CAncer gene 2 (*BRCA2*) encode for proteins that are well-known mediators of DNA damage response and particularly of double-strand breaks (DSBs) through Homologous Recombination (HR) [1, 2]. Since the discovery of *BRCA1* and *BRCA2* genes in the early 1990s, it has been demonstrated that individuals carrying germline *BRCA1/2* mutations had a much higher lifetime risk of developing a malignancy such as Breast Cancer (BrCa), Ovarian Cancer (OvCa), Prostate Cancer (PrCa), and Pancreatic Cancer (PaCa) compared to the general population [3–7]. Interestingly, the risk probability of carcinogenesis varies and depends on the type and position of the mutation within the *BRCA1/2* genes [8, 9]. While the correlation between *BRCA1/2* mutations and higher risk for tumorigenesis is well established, the conclusions regarding impact on the survival are still under debate and characterized by conflicting results [10–14]. Later, the major importance of *BRCA1* promoter methylation was also highlighted in different types of cancer as it represents an alternative silencing mechanism of the *BRCA1* gene [15–17]. In general, aberrant epigenetic regulation affecting gene expression independently of DNA sequence is very common in cancer [18]. Specifically, DNA hypermethylation of the 5′ promoter region of genes is a frequent and early epigenetic event in cancer cells, leading to gene silencing [19, 20]. Interestingly, *BRCA1* promoter methylation was identified only in tumor tissue, indicating its potential oncogenic role [15, 16]. Different clinical studies have demonstrated that patients with BrCa, OvCa, PrCa, and PaCa harboring *BRCA1/2* mutations or other aberrations leading to malfunction could receive clinical benefit with the use of PARPi, such as olaparib and rucaparib, thus succeeding a major advance of precision medicine for these tumor types [21–27]. Today, additional pieces of clinical research have shown the impact of *BRCA1/2* on a person's lifetime risk of developing specific types of cancer and highlight the potential of the aberrant methylation of these genes as prognostic and predictive biomarkers [28–31]. Therefore, on the eve of precision medicine, understanding the tumors with *BRCA1/2* aberrations and their distinct traits remains of utmost significance.

Main text

BRCA1/2 molecular mechanism of DNA damage response during Homologous Recombination

BRCA1 and BRCA2 are proteins with a critical role in maintaining genomic stability by responding to DSBs through the HR pathway [1, 32]. It is of note, that BRCA1 targets effectively every DSB through HR [1]. Repair through HR takes place in the late S phase and G2 of the cell cycle [33]. The ATR and ATM kinases recognize this DNA damage and initiate the repair process by phosphorylating downstream DNA repair-related targets such as BRCA1 [34]. BRCA1 is a multifunctional nuclear phosphoprotein composed of diverse domains such as BRCA C-terminal (BRCT) domain, which participates in many biological processes [1, 35]. HR is considered an error-free DNA damage response mechanism and is mediated by BRCA1/2 and other effectors, as follows: BRCA1 binds to the DSBs through a protein complex composed of Double-Strand Break Repair Nuclease Mre11, DNA repair protein Rad50, and Nibrin (NBS1), known as MRN complex, as well as CtIP [36]. Then, this BRCA1-containing multi-protein complex promotes DNA resection at the 5' end of DSBs, creating single-strand DNA (ssDNA) [37]. Then, BRCA1 employs Rad51, an important recombinase factor, through its interaction with BRCA2 and PALB2 and drives it to the ssDNA, where the HR process takes place [38]. A schematic representation of the main events of HR is illustrated in Fig. 1.

BRCA1/2-mutated cancers

BRCA1-mutated tumors include all tumors which exhibit a pathogenic mutation and not a Variant of Unknown Significance (VUS) in the *BRCA1* gene [39]. Mutations in *BRCA1* gene are detected in different cancers, such as in BrCa (about 5% to 10% of all cases), OvCa (about 20% of cases), PaCa (about 5% to 10% of all cases), and to a lesser extent in PrCa (about 1% to 5% of all cases) [40–45]. In clinicopathological settings, these tumors display some distinct features: *BRCA1*-mutated BrCa is more often associated with the basal-like triple-negative phenotype (ER-/PR-/HER2-), mutated *p53*, immune cell infiltration (mainly T-cell lymphocytes), whereas *BRCA2*-mutated BrCa presents the following features: luminal type, ER + / PR + /HER2- profile, intense immunogenicity and better survival rates [39, 46–48]. Apart from mutations, other genetic aberrations of *BRCA1/2* take an active role in carcinogenesis. For example, researchers analyzed 36 Formalin-Fixed Paraffin-Embedded (FFPE) OvCa samples by Next-Generation Sequencing (NGS) and found 15 *BRCA1* and 12 *BRCA2* variants as well as important loss of function due to copy number variations (CNV) of *BRCA1/2* genes [49].

Loss of Heterozygosity (LOH) is also a key concept in tumorigenesis. It refers to the loss of an allele, usually through a mutation, and then the loss of the second allele due to genetic imbalance/rearrangements, epigenetic regulation, or other mechanisms [50]. LOH is strongly correlated to *BRCA1/2* status as it was found to be relatively frequent in BrCa and OvCa bearing *BRCA1/2* mutations [51]. Moreover, *BRCA1* mutation carriers presented *BRCA1* promoter methylation and to a great extent exhibited LOH as well. In *BRCA1/2*

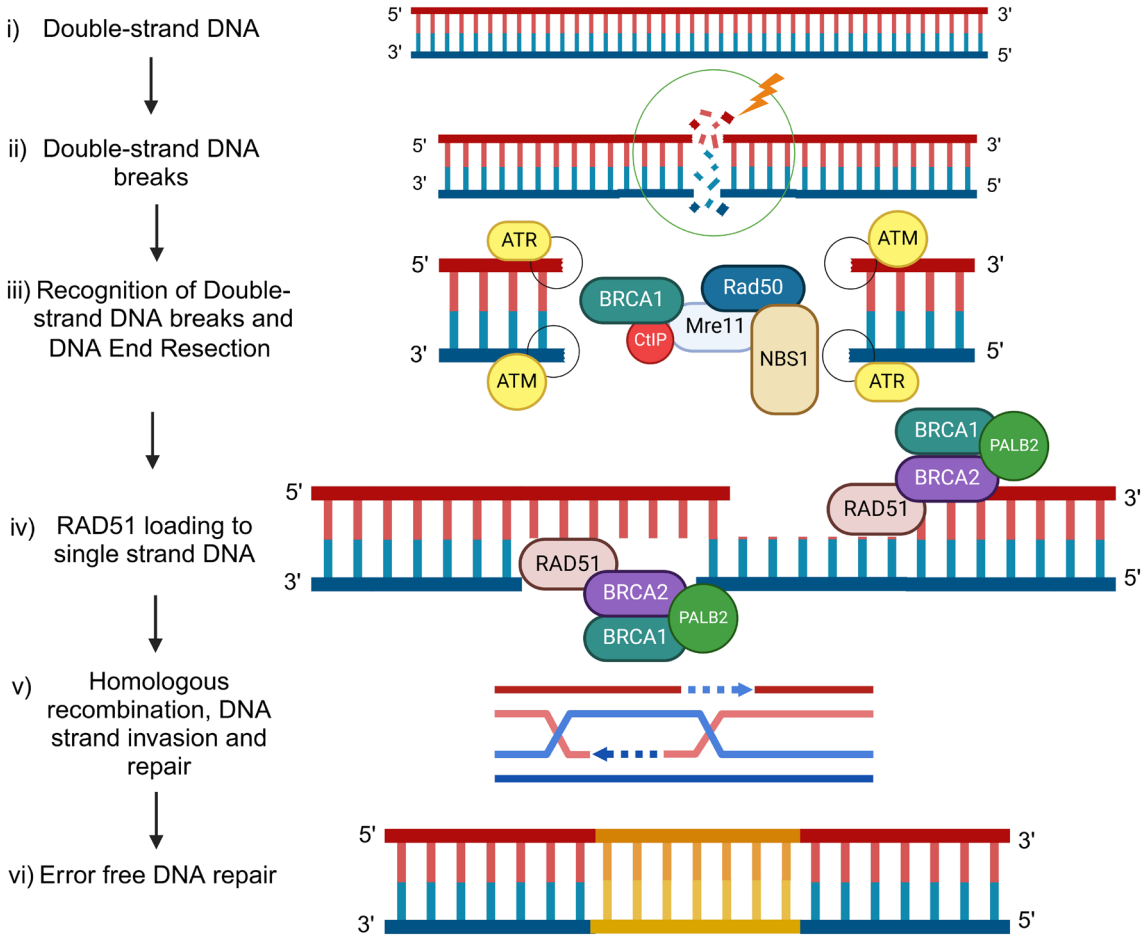

Fig. 1 BRCA1 and BRCA2 molecular mechanisms of DNA damage response during homologous recombination. (i) A double-strand DNA molecule without errors. (ii) The DSB of DNA is typically caused by external factors such as ionizing radiation or chemotherapeutic drugs but also naturally due to the accumulation of reactive oxygen species. Most of the time, this DNA damage leads to the uneven loss of numerous nucleotides resulting in two DNA strands that are incompatible near the breakage point. (iii) ATM and ATR kinases are activated in the presence of DSB initiating the repair process by phosphorylating a set of DNA repair targets. One of the main phosphorylation targets is the BRCA1, which in turn associates with the Mre11, Rad50, and NBS1 (MRN complex) as well as CtIP, eventually forming a multi-protein BRCA1 complex. This complex approaches the DSBs and initiates DNA end resection, creating single-strand DNA (ssDNA) overhangs. (iv) After DNA end resection is completed, BRCA1 recruits PALB2 and BRCA2 which in turn promotes the loading of the recombination enzyme RAD51 to the ssDNA. (v) The second BRCA1-based protein complex starts the DNA strand invasion and homologous repair mechanism, restoring the genetic information lost at the breakage. vi) After completing this multi-step but accurate process, two identical or almost identical DNA double strands are formed. Figure was designed in Biorender software (https://app.biorender.com/gallery). Abbreviation: DSB: Double-Strand Breaks (DSB)

mutation carriers, LOH is associated with better survival rates and therapeutic implications, as the absence of BRCA1/2 function due to LOH renders tumors sensitive to PARPi and mainly to platins [51], as LOH is necessary for tumor sensitivity to platins and LOH absence is connected with a worse prognosis under this type of treatment [51].

BRCA1-like cancers

Following the establishment of the pivotal role of *BRCA1/2* mutations in carcinogenesis, another emerging group of tumors was associated with BRCA1 dysfunction, the so-called *BRCA1*-like (or BRCAness) tumors, which do not exhibit a distinct mutation in the *BRCA1* gene but share common traits [52, 53]. *BRCA1*-like tumors are HR deficient and present chromosomal breaks, DNA methylation, CNV, and genomic instability [52, 54]. Recent studies focus on describing every aspect of the *BRCA1*-like tumors, to identify cancer subgroups with distinct characteristics rendering them candidates for efficient therapeutic strategies [48, 54].

A broad spectrum of DNA damage response genes directly or indirectly linked to HR status has been identified including *ATM*, *STK11*, *TP53*, *PTEN*, *CDH1*, *CHEK2*,

BARD1, *BRIP1*, *MRE11*, *RAD50*, *NBS1*, *RAD51C/D*, *ATR*, *BAP1*, *BLM*, *CDK12*, *FANCA*, *FANCC*, *FANCD2*, *KRAS*, and *PALB2* [3, 53, 55]. However, only a few of the aforementioned genes are often found mutated, such as *TP53*, which is mutated in 84% of all *BRCA1*-like tumors and could serve as a valuable biomarker for stratifying *BRCA1*-like tumors [48]. Takamatsu et al., showed that wild type (WT) *BRCA1/2* cancers which present alteration in other HR genes associated with elevated genomic scar scores (model predicting HR deficiency). This score differed significantly by sex and the presence of somatic *TP53* mutations and was associated with HR deficiency and treatment response to DNA-damaging agents [56]. Alternatively, the evaluation of foci formation (a biomarker of HR repair) of four key HR proteins (BRCA1, Rad51, γH2AX and 53BP1) on DNA is recommended to detect possible HR deficiency and *BRCA1*-like tumors [57]. As mentioned above, BRCA1 and Rad51 are key mediators of HR, and thus their foci formation is present in HR-proficient cells, whereas γH2AX and 53BP1 as conventional DNA damage markers build foci in HR-deficient cells [58]. Interestingly, researchers proved that the positive BRCA1 and RAD51 foci formation is associated with non-response to olaparib therapy in a study featuring patient-derived xenograft (PDX)-derived triple-negative breast cancer (TNBC) samples with BRCA1/2 defect and could be used as a predictive marker in the TNBC [59]. The above studies point out the importance of analyzing a panel of HR genes to identify HR deficiency. Also, identifying a *BRCA1*-like tumor and distinguishing it from a *BRCA1*-mutated tumor is not as simple as anticipated on the genetic level, making it clear that a multidimensional approach would be more suitable in studying *BRCA1*-like tumors.

BRCA1/2 promoter methylation

It is widely accepted that aberrant gene promoter methylation represents an epigenetic event exhibiting an oncogenic role by repressing gene expression in numerous cancers [60–63]. Specifically, locus-specific hypermethylation takes place on sites rich in CG dinucleotides of the promoter region (termed CpG islands, CGI) of tumor suppressor genes such as *BRCA1*, leading to *BRCA1* transcripts and BRCA1 protein levels downregulation [64]. On the other hand, *BRCA2* promoter hypermethylation is rarely encountered in BrCa, OvCa and PaCa, confirmed in only 4.6% of OvCa cases and from 0.0% to 7.6% in PaCa, and no statistically significant correlation has been observed in relation to clinical end-points [28, 31]. Therefore, the bulk of available relevant information comes from studies focusing on *BRCA1* methylation.

From a technical point of view, the overall approach for the quantification of *BRCA1* promoter methylation differs between studies likely due to the determination of different cut-offs, different handling and pre-analytical procedures, lack of a common validation assay and quality of the biomaterial, and differences in the promoter loci targeted, eventually leading to discrepancies in calling a sample hypermethylated or not [65]. For example, in one study, 5% of the TNBC tissue samples showed methylation levels over 80% and were classified as high-methylated, while 25% of them demonstrated methylation levels between 30 and 80%, respectively, classified as low-methylated [66]. In another study focusing on OvCa tissues, researchers considered as a cut-off value the 15% methylation for calling a sample methylated [31]. There are also different methodologies to determine methylation (pyrosequencing, Combined Bisulfite Restriction Analysis CoBRA, methylation-specific PCR, droplet digital PCR, Genome-Wide Methylation Assays) and therefore the results have to be interpreted according to the used assay to avoid discrepant results between studies [31, 64]. As per the gene promoter CG targeted in each case, many studies read the CGs close to the Transcription Start Site (TSS) (approximately between -600 to + 100), using basically sequencing and to a lesser extent methylation-specific PCR [67–70]. In another study, researchers used bisulfite sequencing to read the CpG islands of a larger region, between -2,000 and + 1,000 bp from the TSS of *BRCA1* gene. They found that 49% of breast cancer and 22% of paired non-cancerous tissue samples were methylated for the studied CpG islands [71]. Figure 2 represents a part of *BRCA1* promoter (-700 bp to + 100 bp), including also TSS and Translation Start Site (TLS) (+ 1 bp to + 1326 bp), based on U37574 (NIH, nucleotide) along with the CG enrichment, the CpG island near the TSS and CGs that have been mainly studied for their methylation.

It is clear that *BRCA1* promoter methylation should be examined quantitatively and in relation to methylation zygosity, as samples, are often misidentified as hypermethylated without adequate methylation levels [64]. Methylation zygosity describes the methylation status of all epialleles (alleles that are variably expressed due to epigenetic modifications). "Homozygous methylation" refers to the situation when all epialleles in a cell have highly methylated promoters resulting in gene silence. "Heterozygous methylation" describes a mix of highly methylated and unmethylated epialleles coexisting within each cell. In these cells, gene expression is active due to the presence of unmethylated epialleles, despite the presence of highly methylated epialleles [72]. An important factor that affects the methylation rate determination is neoplastic cellularity. Tumor cells exhibit drastically different methylation levels thus; sufficient tumor cellularity will lead to higher mean methylation in cancerous tissue

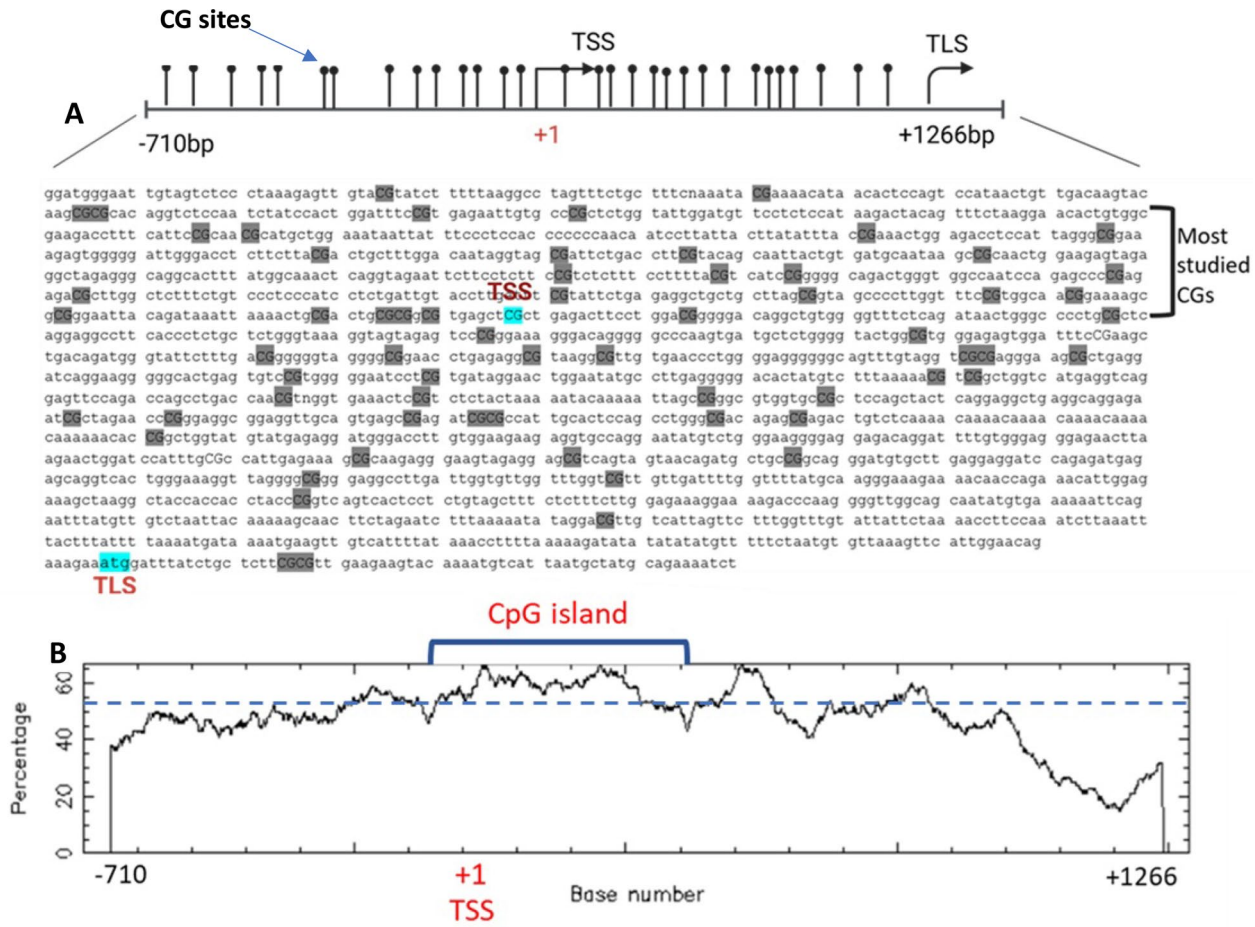

Fig. 2 *BRCA1* promoter and CGs targeted in methylation studies. Overview of a part of the promoter (-700 bp to + 100 bp), TSS, and TLS (+ 1 bp to + 1326 bp) of *BRCA1* showing A. the region upstream and downstream of TSS and TLS, with CGs highlighted as gray and indication of most studied CGs. B. A diagram with CG percentages (Y axis) between -700 bp to + 1326 bp (X axis), showing a CpG island with a GC content greater than 50%. CG percentages analysis was carried out by EMBL-EBI tool (https://www.ebi.ac.uk/about). Part of the figure was designed in Biorender software (https://app.biorender.com/gallery). Abbreviations: TSS: Transcription Start Site, TLS: Translation Start Site

compared to healthy tissue [29, 31, 64]. We suggest the analysis of all epialleles via sequencing of *BRCA1* promoter, which may have a clinical value. Collectively, for what is concerned with measuring *BRCA1* methylation, for valid conclusions to be drawn, the establishment of a widely accepted unified analytical procedure is of utmost importance.

BRCA1/2 promoter methylation in different *cancer* types

The following section will provide insight into current studies linking the *BRCA1/2* promoter methylation status and other *BRCA1/2*-related genetic modifications to certain cancer types. Data from major studies assessing *BRCA1* promoter methylation levels among patients with different types of cancer have been included, to frame the whole spectrum of *BRCA1* promoter methylation applications in clinical settings. Table 1 presents the percentages of *BRCA1* methylation reported in BrCa, OvCa, PrCa, and PaCa and correlations that have been made with the disease state.

Breast *cancer*

In BrCa tissues, *BRCA1* promoter hypermethylation has been identified in 9 to 24% of all cases [73, 76]. In particular, the prevalence of *BRCA1* promoter methylation is increased in TNBC [74, 77, 78]. In general, an individual with *BRCA1* promoter methylation, an event being particularly encountered in East Asia than Caucasians, has a 4.6 higher risk of developing BrCa than baseline, according to a meta-analysis [28] including 19,084 individuals, which associated *BRCA1* promoter methylation with BrCa occurrence, recurrence, prognosis, and therapy response [28, 86]. *BRCA1* promoter methylation was present in 44.4% of malignant and 9.7% of normal tissues [28]. Despite the strong evidence that hypermethylation of *BRCA1* promoter is detected mainly in cancer tissue,

Table 1 *BRCA1* methylation percentages in tissue and blood cells among cancer types and their correlations with the disease's clinically significant end-points

Cancer type	Biomaterial	*BRCA1* methylation (%)	Correlation	Methylation detection method	References
Breast Cancer, NOS	Tissue	9.1 (13/143)	Estrogen Receptor negativity, Diagnosis at a young age	Methylation-specific PCR	Birgisdottir et al. [73]
		3.0 (29/965)	Improved Survival after chemotherapy	Bisulfite Pyrosequencing	Stefansson et al. [74]
		26.0 (139/536)	Worse Survival	Methylation-specific PCR	Chen et al. [75]
TNBC	Tissue	24.1 (57/237)	Improved Survival after chemotherapy	Bisulfite Pyrosequencing	Glodzik et al. [76]
	WBC	12.4 (79/637)	Incidence of TNBC	NGS-based methylation sequencing	Lonning et al. [77]
	Tissue	20.6 (27/131)	Improved Survival after chemotherapy	NGS-based methylation sequencing	Brianese et al. [78]
Ovarian Cancer, NOS	Tissue	16.3 (430/2.636)*	Young age, Advanced stage, Improved Survival#	Methylation-specific PCR, NGS	Kalachand et al. [43]
		19 (15/79, high methylation) 14 (11/79, low methylation)	High methylation with high GIS and PARPi treatment	Droplet digital PCR	Blanc-Durand et al. [79]
		89.9 (62/69)	None	Methylation-specific PCR	Pradjatmo et al. [80]
		19.3 (17/88)	None	Bisulfite Pyrosequencing	Sahnane et al. [31]
HGSOC	Tissue	5.2 (2/38)	Partial BRCAness prediction	Epic BeadChip array	Aref-Eshgi et al. [49]
		14.8 (38/257)	Young age	Methylation-specific PCR	Ruscito et al. [81]
Prostate Cancer, NOS	Tissue	100.0 (22/22)	None	Methylation-specific PCR	Rabiau et al. [17]
		0.0 (0/31)	None	Methylation-specific PCR	Bednarz et al. [82]
Pancreatic Cancer, NOS	WBC	0.3 (-)	None	Methylation-specific PCR	Zhou et al. [83]
PDAC	Tissue	60.3 (35/58)	Poorer tumor differentiation	Methylation-specific PCR	Peng et al. [84]
PDAC	Tissue	0.0 (0/121)	None	Methylation-specific PCR	Abdalah et al. [85]

BrCa: Breast Cancer; TNBC: Triple-Negative Breast Cancer; OvCa: Ovarian Cancer; HGSOC: High-Grade Serous Ovarian Cancer; PaCa: Pancreatic Cancer; PDAC: Pancreatic ductal adenocarcinoma; GIS: Genomic Instability Score; PARPi: PARP inhibitor; NOS: Not otherwise specified

* The 16.3% (430/2636) is a pooled rate; # Improved Survival was noted only in the subgroup using methylation-specific PCR

several studies presented contradictory results, suggesting that *BRCA1* promoter methylation levels in normal tissue might be equal to or exceed the methylation levels of cancer tissues [87–89].

In addition to the study mentioned above [73], a meta-analysis by Wu et al. featuring data from 3,205 women suffering from BrCa, reported that *BRCA1* methylation in tumor tissues was statistically significantly correlated to poor prognosis in terms of overall survival [91]. Interestingly, the researchers also concluded that the handling and storage of cancerous tissue could affect the tissue quality, thereby influencing the methylation results [91]. In another study by Chen et al., 139/536 (26.0%) tumor samples deriving from patients with sporadic BrCa exhibited *BRCA1* promoter methylation. Moreover, the scientists observed a worse 5-year Disease Free Survival (DFS) for patients bearing tumors with *BRCA1* methylation in a statistically significant manner [75]. A meta-analysis in patients with BrCa showed that *BRCA1* promoter methylation status was similar between tumor tissue and peripheral blood cells, thus encouraging its potential use as a blood-based biomarker [28]. However, a study that analyzed *BRCA1* methylation in the blood of early BrCa in younger patients found that only 2 out of 154 blood cell samples presented hypermethylation of *BRCA1* promoter [92]. According to these findings, one could speculate that *BRCA1* promoter methylation is a rare event in the early onset of BrCa, but more studies are needed for definite conclusions to be drawn. TNBC is a subtype of BrCa lacking the ER, PR, and HER-2 receptors, and thus not responding to hormonal therapy (like tamoxifen or aromatase inhibitors) or therapies that target HER2 receptors (like Herceptin) [93]. TNBC accounts for about 10% to 20% of all BrCa cases and may be either hereditary or sporadic [94]. TNBC is stimulated by mechanisms, such as point mutations, large rearrangements, and gene promoter methylation,

and interestingly shares the same clinicopathological characteristics with the *BRCA1*-mutated tumors [28, 78]. Multiple studies confirmed that *BRCA1* promoter methylation and *BRCA1* mutation status are almost mutually exclusive, thus tumors featuring *BRCA1* promoter methylation are not linked to *BRCA1* gene mutations, although there are some rare exceptions observed [28, 29, 31, 43, 74, 77, 78, 95–97]. Interestingly, according to one study, 62% of *BRCA1*-mutated and 50% of *BRCA1* promoter-methylated cancers appear to be TNBC, whereas 40% to 70% of TNBC is estimated to be HR deficient [74]. Another study analyzed 237 TNBC tissues identifying hypermethylation of *BRCA1* promoter in the 57/237 (24.1%) of samples [76]. Interestingly, 89.5% of the hypermethylated cases harbored concurrent LOH of *BRCA1,* and patients with TNBC harboring *BRCA1* promoter methylation presented a significantly longer DFS than non-*BRCA1* altered patients [76]. An immense potential of *BRCA1* methylation as an early biomarker for TNBC (also HGSOC), was highlighted in a study showing that *BRCA1* promoter methylation aberrations can be detected in white blood cells almost 5 years earlier than usually expected, paving the way for timely interventions and a better therapeutic outcome [77].

It is clear that *BRCA1* promoter methylation is a strong candidate both as a prognostic and a predictive biomarker; nevertheless, intratumor heterogeneity and differences in epialleles render *BRCA1* promoter methylation as a marker only partially effective. It is well known that the dynamic evolution of a tumor leads to different tumor cell subpopulations with distinct genetic, epigenetic, and phenotypic traits. The different epialleles in these subpopulations could determine the response to treatment as in the case of *BRCA1* mutations. Scientists now focus their attempts on sequencing to catch all sample epialleles. In a relevant study, researchers using bisulfite sequencing found lower methylation in the epialleles of core breast tumors than in the tumor periphery [98]. These methylation differences were rendered to the hypoxic microenvironment of the tumor's, core leading to this heterogeneous phenotype; such tumor biology aspects need to be considered for developing effective treatment schemes [98]. On the other hand, the combination of the *BRCA1* promoter methylation status with other markers has been used to assess prognosis and therapy response with more accuracy. In TNBC, researchers revealed that the combination of low pRb expression levels, high p16 expression levels, PTEN absence, and *BRCA1* promoter methylation exhibited a similar phenotype to *BRCA1*-mutated tumors [86].

Collectively, *BRCA1* promoter methylation is detected frequently in BrCa, especially in TNBC, and has been associated with survival and other prognostic and therapy response end-points. Further studies analyzing all epialleles at a cellular level and/or combined with additional markers are awaited toward the establishment of *BRCA1* promoter methylation as a useful tool in the clinical management of BrCa.

Ovarian *cancer*

Although first identified in breast cancer, *BRCA1* mutations and other gene aberrations were soon shown to have a significant role also in OvCa [99]. The presence of germline *BRCA1* and *BRCA2* mutations in patients with OvCa ranges from 5 to 20%, also somatic mutations are rare (2% and 8%, respectively) [99]. OvCa is the second cancer type that has been extensively studied as regards *BRCA1* promoter methylation status. A recent meta-analysis of 15 studies concluded that *BRCA1* promoter methylation was present in 430/2.636 tumors (16.3%). However, methylation percentages were not consistent between studies, ranging from 6.2% to 73.7% [43], and this is probably attributed similarly to breast cancer to variations in analytical methods and different methylation cut-offs used in each study. In a relevant study, researchers analyzed ovarian tumors by methylation-specific sequencing and found that only 24 CGs out of 31, that were located at the promoter and near TSS (-600 to + 90) were consistently methylated, 4 of them presenting high methylation percentages (positions -37, -29, -567, -565) [67]. Nevertheless, *BRCA1* promoter-methylated tumors share similar clinicopathological characteristics with the *BRCA1*-mutated as they are associated with younger age and advanced disease but no correlation with survival or platinum sensitivity has been reported [43]. In general, studies are not in agreement regarding a possible correlation between *BRCA1* methylation and survival [79–81]. OvCa patients with homozygous *BRCA1* promoter methylation showed higher Progression-Free Survival (PFS) than patients bearing *BRCA1*-mutated tumors [43, 49]. Another study showing *BRCA1* promoter hypermethylation in 17/88 (19.3%) OvCa and *BRCA2* methylation in 4/86 (4.6%) reported no correlation with clinicopathological characteristics (age, stage, histology type) [31]. Interestingly, *BRCA1/2* promoter methylation is never observed in non-neoplastic ovarian tissue at any histological type, confirming its cancer-specific role [31].

High-Grade Serous Ovarian Cancer (HGSOC), a most lethal OvCa subtype accounting for 70% to 80% of OvCa cases is linked to rapid intraperitoneal spread [100]. Most *BRCA1* promoter methylation cases concern younger patients with advanced stage HGSOC [43]. A study including 172 HGSOC tissues concluded that the

combined examination of *BRCA1/2* sequencing, CNVs, and methylation could lead to a more accurate diagnosis of "BRCAness" phenotype, with an estimated Area Under the Curve (AUC) of 0.77 and accuracy of 0.75, thus worthy to be validated in bigger cohorts of patients [49]. Interestingly, another study using HGSOC-derived PDX models harboring *BRCA1* mutations showed a response to rucaparib and so did two chemo-naive HGSOC-PDX models with homozygous *BRCA1* methylation [101]. Moreover, the donor-patients responded to rucaparib as well [101]. On the other hand, two PDX models with heterozygous *BRCA1* methylation presented some *BRCA1* mRNA and protein expressions and failed to respond to the rucaparib, suggesting that it is homozygous *BRCA1* methylation that predicts PARPi sensitivity [101]. The above results again highlight the significance of assessing *BRCA1* methylation zygosity very carefully to predict clinical outcomes. The zygosity status is thus considered an emerging factor of clinical significance to support decisions for different therapeutic strategies [29, 49].

Collectively, these results point out a potential predictive and to a lesser extent prognostic role for *BRCA* gene methylation in OvCa. Survival rates in relation to *BRCA1* methylation should be further studied for conclusions to be drawn. For sure, a determining factor is the quantitative analysis in terms of methylation zygocity as it is of utmost importance for guiding treatment options.

Prostate *cancer*

PrCa is the most frequent cancer in men. Although the majority of PrCa cases present an indolent clinical course, PrCa remains a leading cause of cancer-related deaths [102]. Germline mutations in *BRCA1/2* genes increase significantly the risk of developing PrCa. Although *BRCA2* mutations have been found only in 1–3% of cases, *BRCA2* mutation carriers are two-fold to four-fold more likely to develop an aggressive tumor at a younger age compared to the general population [103]. Genetic alterations affecting *BRCA1* gene and representing part of BRCAness phenotype also seem to play a role in PrCa development and metastasis [104]. In PrCa, *BRCA1* promoter methylation status has not been considered of the same clinical importance as in BrCa and OvCa, as there are controversial results between studies. *BRCA1* promoter methylation was absent in all of the 31 prostate cancer samples examined, although other *BRCA1* aberrations, such as *BRCA1* imbalance, could bear some value in evaluating PrCa prognosis [82]. Another study examined *BRCA1* promoter methylation both in non-malignant and malignant tissues, reporting contrasting results. Specifically, *BRCA1* promoter methylation was observed in all malignant tissues (prostate intraepithelial neoplasia, peri-tumor tissue, and adenocarcinoma) but also in 15/17 normal samples [17]. Clearly, further studies are required to enlighten the topic and reveal any significance.

Pancreatic *cancer*

Generally, PaCa is characterized by a poor prognosis [105]. Pancreatic Ductal Adenocarcinoma (PDAC), the predominant form of PaCa is a highly aggressive tumor with rising incidence and the lowest survival rate among all the major cancers [106]. Germline *BRCA1/2* mutations are detected in approximately 5–10% of cases of hereditary PDAC and approximately 3% of cases of sporadic PDAC [45]. Similarly, with PrCa, *BRCA2* mutations seem to be associated with an increased risk of PDAC development [45]. Regarding *BRCA* mutations and survival, the few studies exploring possible associations have presented controversial findings [45, 107–109]. Moreover, the findings supporting the role of *BRCA1* promoter methylation in PaCa are not conclusive yet. Indeed, Peng et al. examined surgical samples of PDAC, reporting *BRCA1* promoter methylation in more than half of the cancerous samples (60.3%) [84]. However, Zhou et al. evaluated the promoter methylation status of *BRCA1* and *BRCA2* in the peripheral blood lymphocytes of 655 patients suffering from PaCa and reported *BRCA1* promoter methylation levels ranging from 0.0% to 3.3%. As the mean values were extremely low (0.3% and 0.1% respectively), the researchers considered the occurrence of *BRCA1* and *BRCA2* promoter methylation in PaCa as highly unlikely [83]. Abdallah et al. assessed the promoter methylation levels of *BRCA1* in 121 FFPE PDAC samples by using different analytical methods to exclude possible low sensitivity and observed no methylation in any of the PDAC samples [85]. In 2022, Zhen-Lin et al. examined tissue samples using sequencing from patients with PDAC and reached similar conclusions. The mean *BRCA1* promoter methylation at CGs near TSS of BRCA1 was found to be low (3.62%). To ensure the results, an additional detection method was used by which the unmethylated status of *BRCA1* promoter was confirmed. Thus, they concluded in concordance with previous studies that *BRCA1* promoter methylation was rather unusual [70].

BRCA1/2 methylation in liquid biopsy as a predictive biomarker

The emergence of liquid biopsy has revolutionized clinical oncology, introducing an alternative to traditional tissue sampling for exploring genetic aberrations and dynamic changes in the tumor [110–113]. Some of its most significant advantages are its non-invasive character and the powerful potential for effective disease monitoring by repeated sampling for controlling therapy efficacy and resistance onset [114, 115]. In cancer, circulating

tumor DNA (ctDNA) is an important blood component released in the bloodstream by dying tumor cells, reflecting molecular patterns of the cancer cells. It is mainly comprised of around 150 bp nucleic acid fragments and because of its relatively short length; an increased tumor volume is required for accurate assessment [92, 115]. The application of liquid biopsy in assessing the *BRCA1* promoter methylation status is on the rise, especially in OvCa. A study evaluating *BRCA1* promoter methylation status in plasma cell-free DNA (cfDNA) from patients with OvCa before and during treatment observed occurrence at 60% before treatment, and a 24% epigenetic shift to the unmethylated state during treatment, which was correlated to OvCa recurrence. Researchers concluded that *BRCA1* promoter methylation in cfDNA can be used as a marker for treatment monitoring [97]. In a relevant study, researchers found cfDNA hypermethylated *BRCA1* in about 57% of OvCa patients of all cancer stages, suggesting its use as a diagnostic and prognostic marker [116]. Similarly, the hypermethylation of *BRCA1* and *RASSF1A*[117], was detected in 68% of the tumor tissues but also in the corresponding cfDNA in all stages of OvCa, being present in the majority of early-stage OvCa cfDNAs, suggesting an early event in OvCa [118] and making *BRCA1* an ideal marker for OvCa monitoring in liquid biopsy. Melnikov et al., used the methylation of a five-gene panel (*BRCA1, HIC1, PAX5, PGR* & *THBS1*) for OvCa detection in cfDNA, reaching a sensitivity of 85% and a specificity of 61% [119]. These results indicate the importance of using multiple methylation biomarkers in cfDNA to achieve maximum effectiveness in cancer detection.

As far as BrCa is concerned, there are fewer studies of *BRCA1/2* methylation in liquid biopsy. Cristall et al., introduced the mDETECT method for detecting ctDNA to manage TNBC. This assay examined many common hypermethylated genome regions including *BRCA1* promoter, reaching an AUC of 0.97 for detecting a tumor with a sensitivity of 93% and a specificity of 100%. Interestingly, *BRCA1* promoter methylation was present in cfDNA of about 25% of TNBC cases and 5% of healthy samples [120]. Liu et al. found that cfDNA methylation frequency was higher (but still low) in patients with breast ductal cancers than in healthy individuals [121]. Low cfDNA *BRCA1* methylation frequency (below 5%) was also reported in BrCa by Sturgeon et al. However, *BRCA1* methylation was more often present in lymph-node-positive patients [122]. According to a meta-analysis, the hypermethylation of *BRCA1* in cfDNA, among other markers, was associated with poor prognosis in ER + /PR + BrCa [123]. In a recent work by Yen et al., researchers introduced the Guardant INFINITY, a cfDNA-based test that simultaneously examined *BRCA1* methylation and genomic alterations for the management of advanced BrCa. In specific, 3% of patients had germline mutations in *BRCA1, BRCA2,* or *ATM* and almost 9% of patients had methylated the *BRCA1* gene. Only one patient presented concomitant methylation and mutation at the *BRCA1* gene [124]. Interestingly, methylation of *BRCA1* was not detected in the 3210 cancer-free samples, implying the great specificity of *BRCA1* methylation as a biomarker for cancer detection and monitoring.

In PaCa, only one recent study in cfDNA is available. Unlike PaCa tissue where methylation is low, Koukaki et al. identified high methylation levels of *BRCA1* and *BRCA2* in plasma cfDNA, ranging between 46 and 63% in a group of 105 PaCa patients, associated further with poorer survival [125]. The evaluation of Circulating Tumor Cells (CTC), although challenging as CTCs are extremely few (1 cancer cell:10 billion healthy cells) [126] presents another source of liquid biopsy. *BRCA1* loss is linked to vimentin and cytokeratin-positive CTCs, showing an EMT stimulation through *BRCA1* loss [82]. Unfortunately, there is no available study examining the *BRCA1* methylation status in CTCs. This could be due to technical reasons as CTCs counts are low. Perhaps, analysis of methylation in CTCs could be applicable in metastatic cancer where CTCs are more abundant.

Based on these limited observations presented above, the highest *BRCA1/2* methylation percentages in liquid biopsy were reported in PaCa. Interestingly, BRCA1/2 methylation events were more often detectable in OvCa than BrCa, but more studies are needed to confirm the results. In OvCa, *BRCA1* methylation correlated with diagnosis and treatment monitoring but in BrCa the detectable methylation was correlated to specific cancer subtypes and poor prognosis. We believe that it is of utmost importance the design of new larger liquid biopsy-based studies in those and other cancer types, such as in PrCa, to explore *BRCA1/2* methylation as predictive liquid biopsy biomarkers to aid treatment decisions in a minimally invasive manner, which also allows dynamic monitoring.

BRCA1/2 methylation and treatment strategies

Through the evaluation of *BRCA1* promoter methylation in tumor tissue or liquid biopsy and as this assessment becomes more concrete in terms of methylation zygosity and methylation levels, specific groups of patients are identified, who are likely to benefit clinically from a specific treatment strategy [31]. PARPis (including olaparib, rucaparib, veliparib, talazoparib, niraparib) are considered a primary treatment option for patients with *BRCA1* mutations and especially for TNBC and HGSOC [29, 30, 48, 49, 59, 66, 77, 127–129]. However, not all tumors in these cancer subtypes are sensitive to

PARPi, due to tumor heterogeneity [130, 131]. Consequently, not all TNBC patients carrying WT *BRCA1* will benefit from PARPi, as much as non-TNBC patients, carrying a *BRCA1* mutation [46, 130]. Different findings including limited data from clinical studies have shown that PARPi might also be effective in patients presenting homozygous *BRCA1* methylated tumors [39, 49, 66, 129, 132–134]. Thus, all alleles of *BRCA1* must be evaluated. Homozygous *BRCA1* methylation carriers (and not heterozygous) show similar treatment outcomes as *BRCA1* mutation carriers [135]. In general, *BRCA1*-methylated tumors present similarities to *BRCA1*-mutated tumors as regards to the HR pathway activity but are substantially less differentiated according to their pathological traits [74, 77]. A study recommends that methylation levels for multiple genes engaged in the HR pathway need to be evaluated, to recognize eligible patients for PARPi treatment [49]. Interestingly, secondary *BRCA1* mutations occurring within the *BRCA1* ring domain can lead to platinum and PARPi resistance [136, 137]. Partially predictive for PARPi effectiveness are also the LOH status of *BRCA1/2* mutations implying a defective HR [39, 66]. To identify a possible correlation between *BRCA1* promoter methylation status and LOH, studies in PDX models were conducted using a suitable scoring system for measuring LOH. They confirmed that LOH is linked to homozygous *BRCA1* promoter methylation that could induce sufficient HR deficiency to permit PARPi activity [29]. The truth is that heterozygous *BRCA1* promoter methylation carriers cannot have a significantly improved clinical status under PARPi treatment due to remaining BRCA1 activity. It is of note, that low methylation levels may be attributed either to a monoclonal cancer with heterozygous *BRCA1* promoter methylation status or a heterogenous cancer with some cells exhibiting homozygous *BRCA1* promoter methylation status [51, 66]. The complete or almost complete loss of BRCA1/2 system activity is a requirement for HR deficiency and thus PARPi sensitivity [51]. According to a study, BRCA1/2 deficient status and consequently HR deficiency can be determined through the absence of BRCA1 and Rad51 [59, 92]. Other studies suggest the simultaneous evaluation of *BRCA1* methylation and BRCA1 protein expression or *PALB2* promoter methylation alone as predictive for therapy response [59, 130, 138–140].

In OvCa, patients having *BRCA1* hypermethylation are very likely to have high genomic instability, being good candidates for PARPi therapy. On the other hand, low levels of methylation were associated with poor outcomes post-platinum [79]. In a relevant study, TNB patients with *BRCA1*-methylated tumors were sensitive to adjuvant chemotherapy and had better survival as compared with TNB patients with *BRCA1*-unmethylated tumors [30]. A patient with TNBC presenting high *BRCA1* promoter methylation levels and a *BRCA2* VUS experienced a complete response after Olaparib/Eribulin combination treatment [66]. Rucaparib was evaluated in nine cell lines of BrCa, OvCa and PaCa of various *BRCA1/2* statuses such as methylation, LOH, and mutation [141]. Particularly, cytotoxic effect was caused in UACC3199, a BrCa cell line methylated at *BRCA1* promoter, being equal to or even exceeding carboplatin efficiency. The importance of *BRCA1* promoter methylation for PARPi efficiency is thereby confirmed. Furthermore, a study noted that *BRCA1* and *BRCA2* methylation frequencies varied between CG sites across their promoters. Some CG sites were methylated more frequently in *BRCA1/2* mutated cancers, while others were more often methylated in sporadic carcinomas, suggesting the use of *BRCA* methylation as a screening test to identify patients with *BRCA* germline mutation or BRCAness who may benefit from therapies such as PARPi [142].

In contrast to mutations, methylation status can change due to tumor microenvironment over the lifespan of a tumor or during treatment [143]. This might lead to the emergence of PARPi treatment resistance either during treatment or at recurrence [39, 43, 97, 144]. Retention of homozygous *BRCA1* methylation, a shift to heterozygous *BRCA1* methylation, or complete loss of *BRCA1* methylation may be observed following chemotherapy, e.g., under cisplatin/rucaparib treatment [29]. Loss of *BRCA1* promoter methylation restores BRCA1 function and thus HR activity [29, 97, 144], driving PARPi treatment resistance. Methylation reversion in recurrent tumors is associated with resistance and shorter PFS, as illustrated in studies of paired primary-recurrent ovarian tumors [144]. To the best of our knowledge, studies analyzing *BRCA1* methylation in cfDNA in relation to treatment response are missing. Only in one recent report, researchers used methylation and mutation analysis to assess how clinical resistance to PARPis developed in a cohort of 35 metastatic BrCa bearing *BRCA1/2* mutations. Guardant INFINITY (explained above) was employed to analyze tumors' DNA and corresponding cfDNA. Results showed that the most common resistance mechanism was *BRCA1/2* reversion mutation and less frequent alterations in the 53BP1-Shieldin pathway [145]. Liquid biopsy seems to be also promising in PrCa, but available data concern only gene sequencing results and no methylation. In a phase II study of abiraterone acetate in chemotherapy-naive metastatic castration-resistant prostate cancer patients, the targeted sequencing of *BRCA1, BRCA2*, and other 11 genes in cfDNA after one cycle of treatment could be indicative of cancer prognosis and treatment response [146].

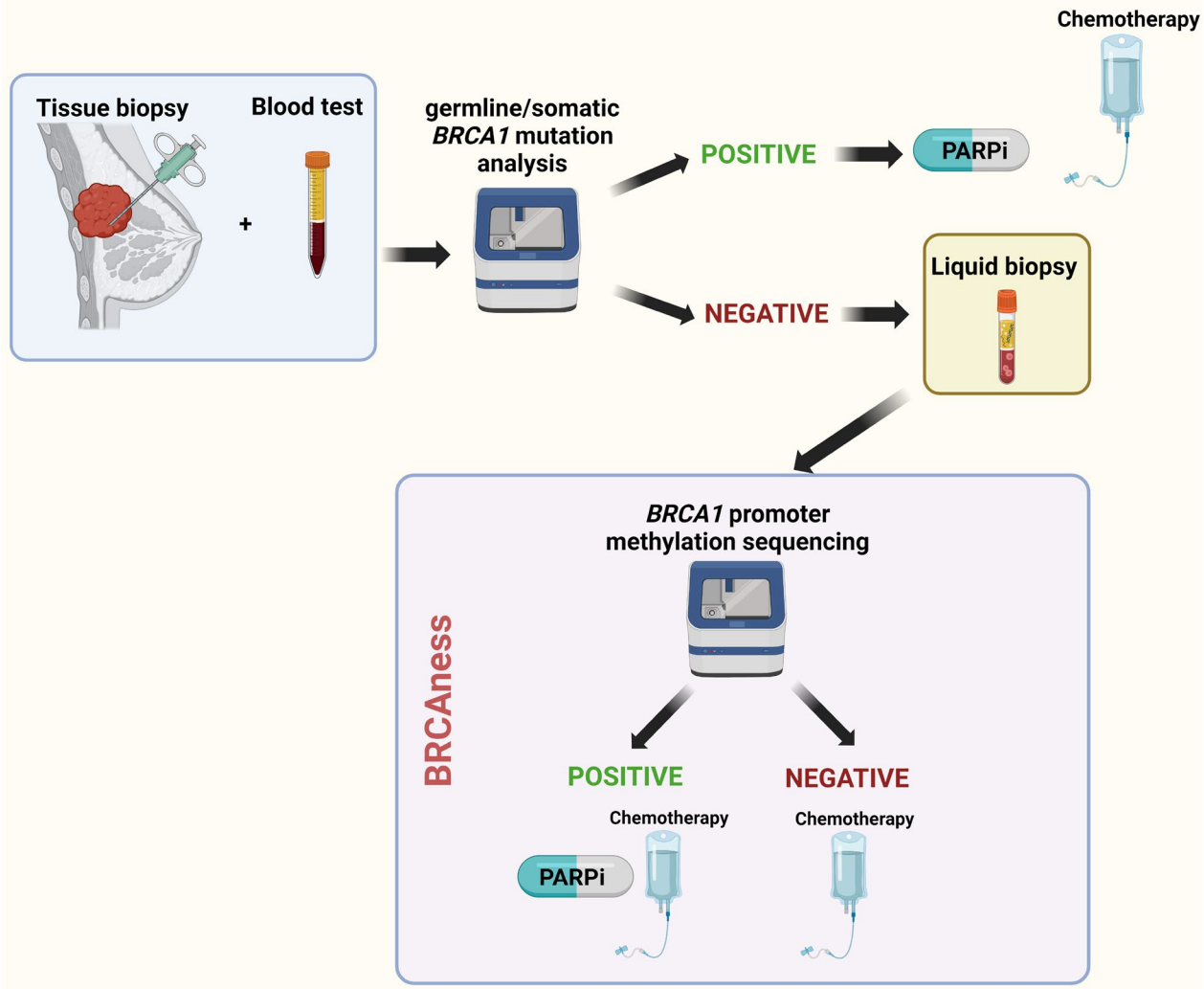

Fig. 3 A suggested diagnostic pipeline to identify BRCA1/2 related cancer patients who might benefit from PARPi treatment. Figure was designed in Biorender software (https://app.biorender.com/gallery). Abbreviation: PARPi: PARP inhibitors

Conclusions

BRCA1 promoter methylation status is a promising predictive and prognostic biomarker in breast cancer and ovarian cancer but is also worthy of further attention in prostate and pancreatic cancer. Apart from germline/somatic *BRCA1/2* mutations, other aberrations can lead to tumors bearing similar features, a phenotype called *BRCA1*-like or "BRCAness". Specifically, *BRCA1* promoter methylation, a cancer-specific mechanism, accounts for most cases of BRCA1-like tumors. It has become clear from several studies that to predict treatment response in PARPi, *BRCA1* promoter methylation needs to be assessed quantitatively, both concerning methylation levels and in terms of methylation zygosity. This is why some researchers point out the term hypermethylation, thus showing that methylation levels must exceed a certain cut-off, to be of clinical, prognostic, or therapeutic significance. A combination of a comprehensive evaluation of *BRCA1* methylation, Rad51 foci formation, and BRCA1 protein expression analysis in tumor samples is considered predictive for "BRCAness", although other genes may be of significance as well, e.g. *PTEN*. Currently, liquid biopsy as a cancer monitoring tool has attracted particular interest in clinical oncology. Evaluating BRCA1/2 in tumor-derived material in the blood can demonstrate an early diagnosis and predict therapy response thus, leading to personalized solutions for effective treatment. The analysis of *BRCA1/2* methylation in liquid biopsy could reveal how methylation patterns are influenced by cancer evolution and treatment and define patient subgroups at different time points that may benefit from PARPi. In Fig. 3, we suggest a diagnostic pipeline that could be implemented in liquid biopsy

to aid precision pharmacotherapy in BRCA-associated tumors. PARPi is a relatively new therapy with a particular effect in tumors with identified BRCA1/2 or HR deficiency. PARPi therapy is often combined with other chemotherapy agents and stands in the epicenter targeting the underlying molecular mechanisms. As genetic testing becomes less expensive and more comprehensive, validation, optimization, and unifying of assays analyzing *BRCA1/2* methylation alone or combined with other biomarkers in a clinical setting are expected to change the scenery in prognosis and predicting treatment response in multiple cancer types.

Abbreviations

BRCA1	BReast CAncer gene 1
BRCA2	BReast CAncer gene 2
HR	Homologous Recombination
PARP inhibitor-PARPi	Poly ADP-ribose Polymerase inhibitors
DSBs	Double-Strand Breaks
BrCa	Breast Cancer
OvCa	Ovarian Cancer
PrCa	Prostate Cancer
PaCa	Pancreatic Cancer
BRCT	BRCA C-terminal
ssDNA	Single-strand DNA
VUS	Variant of Unknown Significance
LOH	Loss of Heterozygosity
CNV	Copy Number Variations
PDX	Patient-Derived Xenograft
TNBC	Triple-Negative Breast Cancer
DFS	Disease Free Survival
AUC	Area Under the Curve
PDAC	Pancreatic Ductal Adenocarcinoma
ctDNA	Circulating tumor DNA
WT	Wild Type

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Availability of data and materials
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Declarations

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