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REVIEW

Genomic instability in breast and ovarian cancers: translation into clinical predictive biomarkers

Marieke A. Vollebergh · Jos Jonkers · Sabine C. Linn

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Abstract Breast and ovarian cancer are among the most common malignancies diagnosed in women worldwide. Together, they account for the majority of cancer-related deaths in women. These cancer types share a number of features, including their association with hereditary cancer syndromes caused by heterozygous germline mutations in *BRCA1* or *BRCA2*. BRCA-associated breast and ovarian cancers are hallmarked by genomic instability and high sensitivity to DNA double-strand break (DSB) inducing agents due to loss of error-free DSB repair via homologous recombination (HR). Recently, poly(ADP-ribose) polymerase inhibitors, a new class of drugs that selectively target HR-deficient tumor cells, have been shown to be highly active in BRCA-associated breast and ovarian cancers. This finding has renewed interest in hallmarks of HR deficiency and the use of other DSB-inducing agents, such as platinum salts or bifunctional alkylators, in breast and ovarian cancer patients. In this review we discuss the similarities between breast and ovarian cancer, the hallmarks of genomic instability in *BRCA*-mutated and BRCA-like breast and ovarian cancers, and the efforts to search for predictive markers of HR deficiency in order to individualize therapy in breast and ovarian cancer.

Keywords Breast cancer · Ovarian cancer · *BRCA1* · *BRCA2* · Genomic instability · Predictive markers · Double-strand break-inducing agents

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M. A. Vollebergh · J. Jonkers (✉) · S. C. Linn (✉)
Division of Molecular Biology,
Netherlands Cancer Institute,
Antoni van Leeuwenhoek Hospital,
Plesmanlaan 121, 1066 CX Amsterdam,
The Netherlands
e-mail: j.jonkers@nki.nl

S. C. Linn
e-mail: s.linn@nki.nl

M. A. Vollebergh
e-mail: m.vollebergh@nki.nl

M. A. Vollebergh · S. C. Linn
Division of Medical Oncology,
Netherlands Cancer Institute,
Antoni van Leeuwenhoek Hospital,
Amsterdam, The Netherlands

Abbreviations

8-OHdG	8-Hydroxy-2′-deoxyguanosine
aCGH	Array comparative genomic hybridization
AC	Adriamycin–cyclophosphamide
AR	Androgen receptor
AT	Doxorubicin–docetaxel
BCSS	Breast cancer-specific survival
BER	Base excision repair
BLBC	Basal-like breast cancer
CIN	Chromosomal instability
CK	Cytokeratin
CNA	Copy number aberration
CS	Cockayne syndrome
CMF	Cyclophosphamide–methotrexate–5-fluorouracil
DFS	Disease-free survival
DSB	Double-strand break
E2	Estrogen
ERE	Estrogen responsive element
ER	Estrogen receptor

FA	Fanconi anemia
FAC	5-Fluorouracil–adriamycin–cyclophosphamide
FEC	5-Fluorouracil–epirubicin–cyclophosphamide
FFPE	Formalin-fixed paraffin-embedded
HER2	Human epidermal growth factor receptor-2
HR	Homologous recombination
HRT	Hormone replacement therapy
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
LBD	Ligand binding domain
MMR	Mismatch repair
MRN-complex	MRE11–RAD50–NBS1 complex
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
PARP	Poly(ADP-ribose) polymerase
PARPi	PARP inhibitors
pCR	Pathological complete remission
PFS	Progression-free survival
PgR	Progesterone receptor
PRE	Progesterone binding element
OS	Overall survival
RCT	Randomized controlled trial
RFS	Recurrence-free survival
SSB	Single-strand break
TTD	Trichothiodystrophy
TN	Triple-negative
XP	Xeroderma pigmentosum

Introduction

Breast and ovarian cancer comprise approximately 10 and 3% of all cancers among women worldwide, respectively. Together, they account for the majority of cancer-related deaths in women [1, 2]. Every year, more than 1.6 million women are diagnosed with breast or ovarian cancer and over 598,000 women die of these malignancies [1, 2]. For both cancer types, treatment generally consists of surgery followed by systemic therapy. Most guidelines for current systemic therapies rely on results of large randomized controlled trials (RCTs) in the general breast or ovarian cancer population. However, these trials do not take into account the molecular heterogeneity of these diseases and consequently many patients might not benefit from these general treatment guidelines. Insights into the molecular biology of these cancers may not only yield novel biomarkers to guide treatment choices, but also novel molecular drug targets that permit development of new targeted therapies. Well-known targets in the treatment of breast cancer patients are the hormone receptors and the epidermal growth factor receptor-2 (ERBB2, i.e., HER2) [3]. Expression of the estrogen receptor (ER) predicts improved outcome after endocrine therapy [4], while expression of HER2 indicates the presence of HER2 amplification and predicts improved survival after HER2 targeting drugs, such as trastuzumab [5, 6]. Recently, a new targeted agent has been introduced in the form of poly(ADP-ribose)-polymerase inhibitors (PARPi) [7, 8], an agent which selectively targets homologous recombination (HR)-deficient cells, such as cells with mutations in breast cancer susceptibility genes 1 or 2 (*BRCA1* or *BRCA2*) [9]. Patients carrying germline mutations in *BRCA1* or *BRCA2* have long been recognized for their predisposition to familial breast and ovarian cancer [10–12]. Recent trials have shown that *BRCA1/2*-mutated breast and ovarian cancer patients are indeed sensitive to PARPi [13, 14]. However, this sensitivity is not restricted to *BRCA*-mutated tumors but likely applies to all cells with any molecular defect resulting in HR deficiency. These recent findings have led us to evaluate genomic instability as one of the hallmarks of HR deficiency in breast and ovarian cancers.

In this review, we will discuss shared features of breast and ovarian cancers, including the DNA repair deficiencies that give rise to genomic instability and chemotherapy sensitivity in specific breast and ovarian cancer subtypes. We also discuss the underlying mechanisms and opportunities to exploit features of HR deficiency as predictive markers to select patients for systemic therapies.

Similarities between breast and ovarian cancer

Besides the fact that breast and ovarian cancers share epidemiologic risk factors and both originate from hormone-responsive tissues, they share many additional features such as tumor heterogeneity, spectrum of mutations and degree of genomic instability (Table 1).

Tumor subtypes in breast and ovarian cancer

Both breast and ovarian are further characterized by their heterogeneity of disease (Table 1). Firstly, this is illustrated by the histological variety present in both diseases [15, 16]. The prognostic relevance of these histological subtypes is indicative of different molecular biological backgrounds within the same disease [16–18]. Secondly, gene expression microarray studies revealed even further heterogeneity by identifying additional subtypes within both cancers. Further insights into the molecular biology of breast cancer were offered by the hallmark paper of Perou and colleagues on the molecular portraits of breast cancer, and follow-up studies in which Sorlie et al. reported the

Table 1 Similarities and differences between breast and ovarian cancer

Features specific for either breast or ovarian cancer		
Features	**Breast cancer**	**Ovarian cancer**
Epidemiologic risk factors	- Period immediately after pregnancy (duration dependent on age of mother) - Recent oral contraceptives use	
Tumor heterogeneity	- Molecular subtype distribution roughly according to hormone receptor and HER2 status	- Molecular subtype distribution roughly according to histology and grade
Familial Cancer	- *BRCA1* mutation risk: 60 – 80% - *BRCA2* mutation risk: 40 – 80%	- *BRCA1* mutation risk: 40 – 50% - *BRCA2* mutation risk: 10 – 20%
Features shared between breast and ovarian cancer		
Epidemiologic risk factors	- Young age at menarche - Older age at menopause - Nulliparity - Inverse correlation with breastfeeding - Hormonal Replacement therapy	
Tumor heterogeneity	- Great histological variation - Different types of histology have different prognosis	
Mutations	- *PI3KCA* mutations (somatic) - *TP53* mutations (somatic) - *BRCA1* or *BRCA2* mutations (germline)	

HER2 human epidermal growth factor receptor-2

influence of these molecular subtypes on prognosis [19–21]. These papers used gene-expression microarray data to classify breast cancer into five subtypes, which roughly followed the distribution of hormone receptor and HER2 status. The luminal A and B subtypes expressed ER and genes associated with luminal epithelial cells, which was confirmed by positive cytokeratin (CK) 8/18 staining using immunohistochemistry (IHC); the HER2-positive subtype expressed genes associated with the HER2 gene; the normal-like subtype showed many similarities with normal breast tissue on gene expression and the basal-like breast cancer (BLBC) subtype was characterized by high expression of basal CK5/6 and CK17, which was verified by positive IHC staining for CK5/6 [19–21]. Furthermore, BLBCs showed large overlap (70–80%) with tumors lacking expression of ER, progesterone receptor (PgR) and HER2, also known as triple-negative (TN) breast cancers [22–24]. The BLBC subtype was further characterized by IHC and was found to have the highest concordance (81%) with ER- and HER2-negative and either CK5/6-positive or EGFR-positive staining breast tumors (BLBC–IHC) [25].

Whereas molecular subtypes in breast cancers clustered on hormone receptor and HER2 status, gene-expression profiling of ovarian cancers yielded subtypes that generally followed histology. Unsupervised clustering of epithelial ovarian cancers clearly distinguished clear cell carcinoma and mucinous carcinoma from serous carcinomas; the endometrioid subtype showed overlap with all other histological types [26]. Additionally, Tothill et al. [27] performed gene expression profiling of endometrioid and serous ovarian carcinomas and found classification by grade. Low-grade tumors showed activated signaling of the TP53 pathway that was not observed in high-grade tumors [28].

Mutations reveal similarities between breast and ovarian cancer subtypes

Some mutations are restricted to ovarian cancer only. Examples of these are *BRAF* or *KRAS* mutations, which were found with a high frequency (~60%) in low-grade serous and borderline ovarian carcinomas suggesting a similar etiology. However, these mutations are uncommon in breast cancers and other ovarian subtypes (high-grade serous or clear-cell) [29, 30], except for endometrioid ovarian carcinomas, which show a modest frequency of *BRAF* mutations of 24% [30]. More recently, inactivating mutations in *ARID1A* have been reported in 46% of all clear-cell ovarian carcinomas and 30% of all endometrioid ovarian cancers [31]. However, no studies have thus far reported on *ARID1A* mutation frequency in breast cancer and it therefore remains to be determined whether *ARID1A* mutations are specific for these ovarian cancer subtypes.

Interesting parallels can be drawn between breast and ovarian cancer subtypes regarding shared mutations (Tables 1, 2). In both cancer types, *PI3KCA* mutations appear common but seem to predispose to specific subtypes (Table 2). In ovarian cancer, these mutations were mainly

present in clear-cell ovarian carcinomas [32, 33]. In breast cancer, *PI3KCA* mutations were significantly enriched in invasive lobular carcinomas (ILC), a histological subtype of breast cancer that intriguingly metastasizes to the ovaries and gastro-intestinal tract [17, 34, 35]. Besides mutations, these subtypes seem to share other features, such as chemoresistance [36–38] and poor prognosis [16, 18]. Interestingly, clear-cell ovarian cancers show relatively few DNA copy number aberrations (CNAs) when visualized by array comparative genomic hybridization (aCGH), especially when compared to high-grade serous carcinomas [39]. Similarly, ILC breast cancers nearly always cluster among the luminal A subtypes [40] in which a low degree of genomic instability is also observed [41, 42].

Breast and ovarian cancers also display frequent mutations in *TP53*, predominantly in specific subtypes, which again share certain characteristics (Table 2). The frequency of *TP53* mutations is approximately 25% in breast cancer [43, 44] and ~50% in ovarian cancer [45–48]. However, this frequency is significantly higher in high-grade serous ovarian carcinomas [46, 48] and in BLBCs [20, 49]. BLBCs are known to be poorly differentiated, high-grade tumors with a poor prognosis and a high degree of chemosensitivity [21, 23], as are serous ovarian carcinomas [36, 50]. Furthermore, both tumor subtypes display a high level of genomic instability shown by the high number of CNAs present in these tumors [41, 42, 51]. The chemosensitivity and genomic instability phenotypes suggest that a substantial fraction of serous ovarian carcinomas and BLBCs are defective in error-free DNA repair. Fitting with this notion is that *BRCA1* germline mutations predispose to both cancer subtypes. Furthermore, it has been shown that most *BRCA1*-mutated breast and ovarian cancers harbor *TP53* mutations [49, 52, 53].

Table 2 Similarities between *subtypes* of breast and ovarian cancer

Similarities between basal-like breast cancer and serous ovarian carcinoma
High frequency of *TP53* mutations (82–92% in BLBC and ~50% in high-grade serous carcinoma)
Poorly differentiated (high grade)
Chemotherapy sensitivity
Poor prognosis
Genomic instability
Tumors of *BRCA1* mutation carriers are often found within these subtypes
Similarities between invasive lobular breast carcinoma and clear-cell ovarian carcinoma
High frequency of *PI3KCA* mutations (46–52% in ILC and 33–46% in CCC)
Well or moderately differentiated (low or intermediate grade)
Chemotherapy resistance
Elevated risk of recurrences or metastases
Absence of genomic instability

BLBCs basal-like breast cancers, *ILC* invasive lobular carcinoma, *CCC* clear cell carcinoma

Germ-line mutations in BRCA1 and BRCA2 predispose to breast and ovarian cancer

It has been long recognized that within familial breast cancer families the incidence of ovarian cancers was very high [54]. The discovery that this familial predisposition is caused by germline mutations in *BRCA1* or *BRCA2* is probably the foremost reason why breast and ovarian cancers are mentioned together (Table 1) [11, 12]. Germline mutations in *BRCA1* or *BRCA2* confer a life-time risk of 40–80% for breast cancer and respectively 25–65 and 15–20% for ovarian cancer [55, 56]. *BRCA* germline mutations also increase the risk for other cancer types such as prostate and pancreatic cancer [57, 58]. Although *BRCA1* has been implicated in multiple cellular processes, both *BRCA1* and *BRCA2* are mostly known for their role in the HR-pathway, which is responsible for error-free repair of double-strand breaks (DSBs) in the DNA; in case of defects in this repair pathway, i.e., due to *BRCA1/2* mutations, cells call upon alternative error-prone pathways, such as non-homologous end joining (NHEJ), resulting in genomic instability and predisposition to cancer [59]. However, the increased risks associated with *BRCA1/2* mutations are relatively specific for ovarian and breast epithelium in women. This gender- and tissue-specificity cannot be explained by the housekeeping function of *BRCA1* and *BRCA2* in DNA repair. In the next sections, we will give a short overview of the DNA repair pathways and their relation to genomic instability and therapy sensitivity. We will also review potential causes for the tissue- and gender-specificity of *BRCA*-associated cancers.

Role of DNA repair pathways in therapy response and genomic instability

Already more than a century ago, Boveri [60] suggested that cancer might arise as a consequence of abnormal segregation of chromosomes to daughter cells. The link between genomic instability and cancer was further elucidated when it became clear that many inherited defects in DNA repair genes lead to genomic instability and predispose to malignancies, illustrating the importance of DNA repair pathways for maintaining genomic integrity and preventing cancer [61]. In general DNA repair can be divided into pathways that repair damage of one of the DNA strands (mismatches, subtle base modifications, bulky adducts, single-stranded breaks or gaps) or damage

that affects both DNA strands (crosslinks, double-stranded breaks).

Repair of DNA double-stranded breaks

In the presence of a DNA double-strand break (DSB), repair systems no longer can depend on the complementary strand for correct repair. DSBs are mostly induced by free radicals, ionizing radiation, chemotherapeutics forming DNA interstrand crosslinks (ICLs) and the conversion of SSBs into DSBs by replication fork collapse during DNA replication [61]. The presence of a DSB is sensed by the MRN complex of MRE11/RAD50/Nijmegen Breakage Syndrome 1 (NBS1) (MRN-complex), which localizes to both DNA ends and subsequently recruits ataxia telangiectasia mutated (ATM), which is responsible for checkpoint activation and cell cycle arrest through TP53. ATM also phosphorylates histone H2AX (γH2AX) resulting in chromatin remodeling around the break and recruitment of DNA damage response (DDR) factors such as BRCA1. Depending on the phase of the cell cycle, DSBs are repaired either by NHEJ, which takes place in G0–G1 phase, or by HR, which takes place in the S or G2 phase. For an extensive review on both NHEJ and HR, see [62, 63].

Non-homologous end joining

Non-homologous end joining (NHEJ) is an error-prone mechanism for ligation of DNA DSBs. In brief, after phosphorylation of γH2AX, a heterodimer of KU70/KU80 binds to both DNA ends and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The DNA-PKcs proteins on either end of the DSB interact, forming a bridge between both DNA ends [62, 63]. The MRN complex has been suggested to play an additional role in NHEJ, probably in stabilizing the two DNA ends [64]. Lastly, the break needs to be sealed by ligating the DNA ends back together; the complex of XRCC4/Ligase 4 is responsible for this step [62, 63]. Since NHEJ fuses DNA ends without taking into account the missing DNA or a template, this pathway is error-prone.

Repair by homologous recombination

In contrast to NHEJ, DNA DSB repair by HR is error-free, since the homology of the sister chromatid is used for repair. To search for this homology, a long 3′end DNA overhang needs to be created. For this, the MRN complex is again needed, which interacts with CtBP-interacting protein (CtIP), EXO1 and the helicase Bloom syndrome protein (BLM) helicase [62, 63]. *BRCA1* seems to play a role in the interaction between CtIP and MRN [65]. The created single-stranded DNA ends are subsequently coated with RPA; however, to start the search for sequence homology, RPA needs to be replaced by RAD51. This process is directly mediated by BRCA2 (also called FANCD1) [62, 63]. To facilitate this replacement, a complex of BRCA1/BARD1, together having an E3 ubiquitin ligase function, needs to be present. The exact interaction remains unknown, but it is thought that PALB2 (partner and localizer of BRCA2, also known as FANCN) may connect BRCA2 and BRCA1/BARD1. RAD51 subsequently invades the sister chromatid, resulting in partial displacement of the non-complementary strand (D-loop). If the second end of the DSB is also captured in the D-loop, a structure called a Holliday junction is formed, enabling DNA synthesis using the sister chromatid as a template. Lastly, the DNA structures formed by the D-loop or Holliday junction are resolved by proteins such as BLM, topoisomerase IIIa, GEN1 and probably also the Werner syndrome protein (WRN) [62, 63].

In the presence of DNA ICLs, an additional pathway comes into play, consisting of Fanconi anemia (FA) proteins. Upon DNA damage, ATM and ATR activate a complex of eight FA proteins, which function as an E3 ubiquitin ligase that monoubiquitinates FANCD2 and FANCI. These seem to be involved in the recruitment of BRCA2 and RAD51 at the site of the break, although the exact mechanism remains to be resolved [66].

Repair of single-strand lesions

Several DNA repair pathways exist for repair of different types of single-strand lesions such as DNA adducts and mismatched bases. These pathways use the intact complementary DNA strand for error-free repair.

Nucleotide excision repair

The nucleotide excision repair (NER) pathway is responsible for clearing helix-distorting lesions from the DNA, such as those induced by ultraviolet radiation or chemotherapeutics causing bulky intrastrand DNA adducts [61]. Using a broad range of proteins, the NER pathway (1) unwinds $\sim$30 base pairs of DNA around the damage site through helicases, (2) cleaves the DNA using endonucleases, excising the nucleotides of the unwound stretch of DNA including the damaged site, and (3) fills the resulting gap using the complementary DNA strand as a template (see review by Cleaver et al. [67]). Inherited defects in the NER pathway are associated with three autosomal-recessive diseases: xeroderma pigmentosum (XP; mutations in *XPA–XPG*), Cockayne syndrome (CS; mutations in *CSA* and *CSB*), and trichothiodystrophy (TTD; mutations in *XPD* (*ERCC2*)). In general, patients with XP have a strongly increased risk of developing skin cancers with a

small increased risk of other cancers, while persons heterozygous for the mutation do not show this phenotype [68, 69]. Patients with CS and TTD do not show cancer predisposition but are characterized by neurodegeneration resulting in mental and physical retardation, as well as brittle hair, nails, and scaly skin in case of TTD. In contrast, mouse models with engineered mutations specific for CS and TTD do display an increased risk of skin cancer after ultraviolet exposure [70, 71]. It has been proposed that this difference is caused by the fact that patients with CS or TTD rarely live long enough to develop cancer, whereas mice display a milder, non-lethal neurodegenerative phenotype that allows enough time for cancer to occur [67]. The genomic instability seen with NER loss has been classified by Lengauer et al. [72] as "subtle sequence instability".

Base excision repair

In contrast to NER, base excision repair (BER) takes care of non-bulky base modifications in the DNA; most frequently this consists of oxidative modifications, methylation, or alkylation [73]. Furthermore, BER repairs single-strand breaks (SSB) in the DNA, which can be caused by, for example, ionizing radiation or a result from intrastrand crosslinks formed by platinum agents [74–76]. In brief, DNA glycosylases specific for different types of DNA damage cleave the DNA around the damaged base and remove the damaged base from the helix but not from the sugar phosphate backbone. Subsequently, apurinic-apyrimidinic endonuclease-1 (APE1) incises the backbone after which polymerase β fills the single nucleotide gap using the complementary DNA strand as a template (short patch BER) and the nick is sealed by the XRCC1–ligase3 complex. In case of a 2–12 nucleotide gap (long-patch BER) additionally polymerase δ/ε, proliferating cell nuclear antigen (PCNA), flap endonuclease 1, replication factor C, and DNA Ligase1 (LIGI) are being used [61, 77, 78]. PARP1 is thought to function as a SSB damage sensor binding to the SSB after which repair via mostly long-patch BER can take place [79]. Although homozygous deletion of some of the BER genes, such as *Xrcc1*, leads to embryonic lethality in mice [80], only one cancer syndrome has been linked to a defect in the BER pathway. Germline mutations in *MUTYH*, one of the DNA glycosylases, have been shown to increase the risk of colorectal tumors in an autosomal-recessive manner [81, 82].

Mismatch repair

The DNA mismatch repair (MMR) pathway specifically recognizes and repairs erroneous mis-incorporated bases and insertion/deletion loops that can occur during DNA replication; these loops originate from incorrect replication of repetitive sequences, also called microsatellites. In short, four steps have been recognized in MMR (1) hMSH proteins form heterodimers (hMSH2/hMSH6 and hMSH2/hMSH3), which recognize the mismatched bases or loops; (2) these heterodimers recruit a protein complex consisting of MLH1/PMS2, MLH1/PMS1, or MLH1/MLH3 heterodimers, which are thought to facilitate excision of the mismatched DNA by recruitment of an exonuclease; (3) using the complementary strand, which is stabilized by replication protein A (RPA), polymerase δ and PCNA resynthesize the DNA; (4) the remaining nick in the DNA is sealed by LIGI (see [83, 84] for extensive review).

The MMR pathway is most commonly associated with familial colorectal cancers. Inherited defects in the MMR pathway (specifically mutations in *MSH2*, *MSH6*, *MLH1*, or *PMS2*) result in Lynch syndrome, formerly called hereditary non-polyposis colorectal cancer (HNPCC). Even though mouse models defective for different MMR genes are all viable (for example [85, 86]), HNPCC is an autosomal-dominant syndrome. Heterozygous germline mutations in MMR genes (generally resulting in a truncated protein) predispose to mainly colorectal cancer, but also endometrial, gastric, and bladder cancer [84]. Inactivation of the remaining wild-type allele by mutation, loss of heterozygosity, or promoter methylation results in loss of MMR [84], leading to the accumulation of point mutations (mismatches) and insertions and deletions in repetitive sequences (microsatellite instability), finally resulting in cancers displaying the "subtle sequence instability" phenotype [72, 87].

DNA repair defects and therapy sensitivity

Several error-free DNA repair pathways are involved in the repair of DNA lesions induced by anticancer drugs (Table 3). Defects in DNA repair pathways may therefore offer potential new therapeutic targets, since failure to repair DNA lesions should lead to accumulation of damage and eventually cell death due to apoptosis or mitotic catastrophe, Two studies have used this concept to identify DNA-damaging drugs that selectively kill cells with specific DNA repair deficiencies. Martin et al. [88] screened a library of clinically approved drugs for compounds with selective toxicity in MSH2-deficient cells. They found that agents that cause oxidative DNA damage, such as methotrexate, were able to specifically kill MSH2-deficient cells because of their inability to remove the oxidized base 8-hydroxy-2′-deoxyguanosine (8-OHdG) [88]. These oxidative lesions are known to increase G–T transversions, which are thought to persist in the absence of an effective MMR, leading to cell death [88]. Secondly, Evers et al. [89] screened for compounds with selective toxicity in

Table 3 Involvement of major error-free DNA repair pathways in repair of DNA lesions induced by anticancer drugs

Drug class	Examples	DNA lesions	Error-free repair pathways
Monofunctional alkylators	Monofunctional nitrogen mustards Temozolomide	Base damage, adducts	HR, BER
Bifunctional alkylators	Bifunctional nitrogen mustards Platinum drugs	DSBs, adducts, crosslinks	HR, NER, FA
Topoisomerase I inhibitors	Camptothecins	SSBs, DSBs	HR
Topoisomerase II inhibitors	Anthracyclines, etoposide	SSBs, DSBs	HR
PARP inhibitors	Olaparib	SSBs, DSBs	HR
Replication inhibitors	Aphidicolin, hydroxyurea	DSBs	HR
Antimetabolites	Base analogs, antifolates	Base damage	BER, MMR

SSBs single-strand breaks, *DSBs* double-strand breaks, *HR* homologous recombination repair, *BER* base excision repair, *NER* nucleotide excision repair, *FA* Fanconi anemia, *MMR* mismatch repair

BRCA2-deficient cells. They found that bifunctional alkylators, which form DNA ICLs and subsequently DSBs, were specifically lethal to BRCA2-deficient cells because of their defect in HR-mediated DSB repair [89].

Paradoxically, many proteins involved in DNA repair are tumor suppressors and their loss of function has been proven difficult to target. To solve this, the concept of synthetic lethality has been introduced, which is based on the fact that two events may not affect cell viability when they occur separately from each other but induce lethality when they occur simultaneously. Thus, inhibition of a specific DNA repair pathway may be relatively harmless for normal cells but induce specific killing of tumor cells with defects in another DNA repair pathway. This concept of synthetic lethality potentially provides a large therapeutic window for the treatment of DNA repair-defective cancers with small molecule inhibitors of DNA repair pathways. For example, the G–T transversions that accumulate in MMR-deficient cells upon oxidative damage can be repaired by BER during replication. Consequently, inhibition of the BER polymerases β and γ induced selective toxicity in MSH2- and MLH1-deficient cells, respectively [90]. Similarly, targeting BER and SSB repair through PARP1 inhibition induces selective toxicity in HR-deficient cells due to the accumulation of SSBs that may be converted into DSBs following replication fork stalling during the S-phase [91]. Whereas wild-type cells can repair these DSBs via homology-directed repair, *BRCA1*- and *BRCA2*-mutated cells will accumulate unrepaired DSBs or DNA rearrangements generated by error-prone repair DSB repair, ultimately leading to mitotic catastrophe [92].

DNA lesions induced by cytotoxic chemotherapy drugs and PARP inhibitors are resolved by several major error-free DNA repair pathways, including HR, BER, NER, MMR, and FA (Table 3). However, since proliferating cells will convert persisting SSBs into DSBs due to replication fork stalling during the S-phase, one could argue that targeting the HR pathway would be most effective for selective killing of rapidly dividing tumor cells. This might also explain why *BRCA1*-mutated and TN breast cancers are generally more sensitive to chemotherapy agents, although the level of sensitivity differs for the different classes of compounds, as has for example been shown by the synthetic lethality screen in *BRCA2*-deficient cells [89]. Agents that directly or indirectly induce DSBs might not only be useful for the treatment of BRCA-mutated breast and ovarian cancers, but also for other cancers with defects in HR [93]. For example, genomic analysis of high-grade serous ovarian carcinomas showed that 51% of all cases contained (epi)genetic alterations in one or more HR genes [94]. Similarly, it has been reported that pancreatic cancer patients with a BRCA2 or PALB2 mutation showed a good response to mitomycin C, a DSB-inducing bifunctional alkylator [95, 96]. Lastly, also non-BRCA related defects in HR, such as ATM deficiency in leukemia and lymphoma cells, have been shown to cause sensitivity to PARPi [97], and overexpression of FANCF has been shown to lead to resistance to melphalan, a bifunctional alkylator, in multiple myeloma cells [98].

In addition to hypersensitivity to DSB-inducing agents, HR-defective tumors have other features that might facilitate their identification. One of these is their genomic instability phenotype.

HR deficiency and genomic instability

Failure of HR will result in error-prone repair of DSBs, resulting in gross chromosomal rearrangements such as deletions and translocations, resulting in genomic instability. The severity of this defect is illustrated by the cancer predisposition seen in syndromes such as ataxia telangiectasia (AT; caused by mutations in *ATM*), Nijmegen breakage syndrome (*NBS1*), Bloom syndrome (*BLM*),

Werner syndrome (*WRN*), Fanconi anemia (*FA* genes), and familial breast and ovarian cancers (*BRCA1* or *BRCA2*). These syndromes are caused by mutations in genes mainly involved in HR. All syndromes are autosomal-recessive, except for familial breast and ovarian cancer, which is caused by a heterozygous germline mutation in *BRCA1* or *BRCA2*. However, a homozygous mutation in *BRCA2* has been described, giving rise to a Fanconi anemia phenotype rather than breast or ovarian cancer [99]. The clinical presentation of these autosomal-recessive syndromes is diverse with congenital abnormalities, immunodeficiency, neurodegeneration as some of the features. However, all syndromes are characterized by a strongly increased risk for cancer, with a preference for hematological malignancies, at a young age and hypersensitivity to ionizing radiation (syndromes are reviewed in [100, 101]).

It remains puzzling why little elevated cancer risk is observed in heterozygous relatives of patients with these autosomal-recessive syndromes [102, 103], especially since heterozygosity for *BRCA1* or *BRCA2* germline mutations results in a strongly increased breast and ovarian cancer risk. This could suggest that there is to some extent redundancy for genes of the above-mentioned syndromes but not for *BRCA1* and only to a very limited extent for *BRCA2*. In this case, tumorigenesis in *BRCA*-mutation carriers would require cell-intrinsic or -extrinsic mechanisms to promote the survival of cells with a second-hit in *BRCA1* or *BRCA2* [104]. It is tempting to speculate that cell-extrinsic survival mechanisms are also responsible for the gender- and tissue-specificity of *BRCA1*- and *BRCA2*-associated cancers, as hormonal signaling might foster proliferation and/or survival of BRCA-deficient breast and ovarian epithelial cells [104].

Gender- and tissue-specificity of BRCA-associated cancers

Mechanisms that have been implicated in gender- and tissue-specificity of *BRCA*-associated cancers are the ER, PgR, androgen receptor (AR), and the X-chromosome.

Tissue specificity and the estrogen receptor

In mice, puberty and pregnancy but also supplementation with estrogen (E2) and progesterone after ovariectomy induced *Brca1* expression in the mammary gland, linking BRCA1 with hormonal signaling [105, 106]. In addition, *BRCA1* expression was shown to cause reduced expression of estrogen-responsive element (ERE)-containing luciferase reporter genes and endogenous E2 responsive genes after E2 stimulation. This might be mediated by direct interaction of ERα with the amino-terminal region of BRCA1 [107, 108], which contains the RING domain required for BRCA1–BARD1 interaction and E3 ubiquitin ligase activity. Interestingly, ERα is an in vivo substrate of the BRCA1–BARD1 ubiquitin ligase [109] and mono-ubiquitinated ERα is targeted for degradation [110]. Together, these findings suggest a model in which ERα activation by E2 stimulates proliferation through transcriptional activation of target genes including *BRCA1*, which subsequently counteracts this signal by monoubiquitinating ERα and targeting it for degradation. Hence, functional loss of BRCA1 would result in both defective HR and sustained ERα signaling due to loss of a negative feedback loop. However, this model does not explain the fact that most *BRCA1*-mutated breast cancers are ERα-negative [111, 112] and the recent finding that *BRCA1*-associated breast cancer originates from luminal ERα-negative progenitor cells [113, 114].

Functional links between BRCA2 and ER have been less well studied. It has been reported that ERα may activate transcription of *BRCA2* through histone deacetylation [115], but the significance of this finding for the tissue specificity of *BRCA2*-associated tumorigenesis remains unclear.

Tissue specificity and the progesterone receptor

BRCA1 has also been linked to PgR signaling. Exogenous *BRCA1* expression was found to reduce transcriptional activity of PgR-responsive genes, possibly through direct interaction between BRCA1 and both isoforms of PgR [116]. In conditional *Brca1*-deficient mice, BRCA1 was found to regulate PgR stability through polyubiquitination [117]. Moreover, treatment of these mice with PgR antagonist RU486 abrogated mammary tumorigenesis, suggesting a causal relation between the PgR signaling and *BRCA1*-associated tumorigenesis [117]. In patients, contradicting evidence has been found. Although one study reported that PgR expression in adjacent tissue was significantly higher in *BRCA1*-mutated breast tumors compared to sporadic tumors [118], another study showed a reduced expression of PgR in normal breast tissue of *BRCA1* or *BRCA2* mutation carriers [119]. Moreover, since PgR is an ERE [120], it remains difficult to determine the individual effects of ERα and PgR on *BRCA*-associated breast cancer risk.

Although the precise interactions remain to be elucidated, a picture emerges in which extensive crosstalk exists between *BRCA1/2* and ER or PgR. Some of these interactions may explain the specific susceptibility of E2/progesterone-responsive proliferating tissues such as breast and ovaries to *BRCA*-associated tumorigenesis. The importance of this hormonal stimulation is further illustrated by the finding that BRCA1-deficient mice showed

substantially decreased tumorigenesis after ovariectomy [121]. In patients, meta-analysis showed that bilateral salpingo-oophorectomy in *BRCA1/2*-mutation carriers reduced the risk of ovarian/fallopian tube cancer with 80% and breast cancer with 50% [122].

Tissue specificity and the androgen receptor

Besides the female sex hormones, androgens have also been suggested to play a role in the tissue specificity of *BRCA*-associated breast and ovarian cancers. Both *BRCA1* and *BRCA2* can act as co-activators of androgen receptor (AR) mediated transcription [123–125]. Furthermore, *BRCA1*-mutated breast cancers were shown to have reduced AR expression compared to sporadic or *BRCA2*-mutated breast cancers [126].

Tissue specificity and X chromosome dosage

Evidence that the X-chromosome dosage is linked to breast cancer was provided by studies on cancer incidence in patients with numerical sex chromosome abnormalities. Men with Klinefelter syndrome, caused by an extra X-chromosome (XXY), were strongly predisposed to developing male breast cancer [127, 128]. In contrast, women with Turner syndrome, caused by lack of one X-chromosome (X0), were found to be at a decreased risk for breast cancer [129]. Interestingly, in a small study it was noted that none of the 62 Turner women who received 20–40 years of continuous HRT developed breast cancer, even though this would have been expected based on breast cancer incidence and increased risk after HRT [130].

Another link between X chromosome dosage and tissue specificity was forged by the observed loss of the Barr body in breast and ovarian cancers (reviewed by Pageau et al. [131]). The Barr body is the heterochromatic inactive state of the X-chromosome (Xi), which is triggered by *X-inactivation specific transcript* (*XIST*), a non-coding RNA, in order to control gene dosage of X-linked genes. Loss of Xi and reduced *XIST* levels have both been linked to *BRCA1*-mutated hereditary breast cancer and sporadic BLBC, the molecular subtype resembling *BRCA1*-mutated breast cancer [132, 133]. Although, BRCA1 seemed to co-localize with XIST [132], 3D analysis revealed that BRCA1 was located adjacent to XIST [134, 135]. Nevertheless, it remains possible that BRCA1 has an indirect effect on the localization of *XIST* to the Xi [131].

Effects of BRCA1/2 founder mutations on tissue specificity

Given the large number of reported associations between *BRCA1/2* and ER, PgR, AR, or the X-chromosome, it is possible that multiple factors underlie the tissue specificity of *BRCA*-associated tumorigenesis. Moreover, the tissue specificity might vary with specific sites of a *BRCA1* or *BRCA2* mutation. For example, it was found that different *BRCA1* founder mutation sites (N-terminal, central, or C-terminal) confer different risks to breast and ovarian cancers [136]. Furthermore, there might well be an interplay between general breast cancer risk factors (present for example on the X-chromosome as suggested by the Klinefelter and Turner syndrome studies), which may be enhanced in the presence of *BRCA1/2* mutations, and more direct mechanisms involving ER, PgR, and/or AR signaling. All these issues complicate the elucidation of the exact mechanisms underlying gender- and tissue-specificity of *BRCA*-associated cancer.

Identification of predictive markers for HR deficiency and their clinical utility in breast and ovarian cancer

The development of PARP inhibitors (PARPi) offers new opportunities for treatment of *BRCA1*- and *BRCA2*-related breast and ovarian cancers. Recent clinical trials have indeed shown sensitivity to PARPi for this specific subgroup [13, 14, 137]. However, sensitivity is likely not restricted to familial *BRCA*-mutated cancers. All tumors incapable of error-free DSB repair, i.e., tumors with a defect in the HR repair pathway, should be sensitive to PARPi. A recent placebo-controlled study with the PARP inhibitor olaparib (AZD2281) in ovarian cancer patients with relapsed platinum-sensitive, high-grade serous ovarian cancer showed a significant improvement in progression-free survival after olaparib treatment compared to the placebo-arm [138]. This is indicative that there is a subgroup of sporadic ovarian cancers in which sensitivity to PARPi might be caused by other defects in the HR pathway. Indeed, integrated genomic analyses of 489 high-grade serous ovarian carcinomas showed that 51% of all cases contained defects in HR genes [94]. In breast cancer, the HR-deficient phenotype (BRCAness) is thought to be present in $\sim$30% of all cases [93]. Conversely, HR-deficient cancer cells are likely not only sensitive to PARPi but to all agents that directly or indirectly cause DNA DSBs. Examples of these DSB-inducing agents are bifunctional alkylators or platinum agents, which are known to form DNA crosslinks that cause DSBs. Adequate selection of patients with HR-deficient cancers might place these "old" classes of chemotherapeutics in a new perspective.

An important question is how to adequately select HR-deficient breast or ovarian cancer patients who may benefit from targeted therapy involving PARPi or other DSB-inducing agents. For this review, we evaluated three strategies for selecting predictive markers for DSB-inducing

agents in breast or ovarian cancer patients: (1) use of knowledge-driven, mostly gene-based markers; (2) use of genomic instability as a general hallmark of HR deficiency; and (3) use of functional readouts of HR activity (Fig. 1). Below we will describe these different approaches to identify predictive markers for DSB-inducing agents described in the literature. Although some agents (e.g., bifunctional alkylating agents and platinum agents) are considered to be stronger DSB-inducing agents than others, we will consider a wide range of chemotherapeutics in our overview.

Predictive versus prognostic markers

For the description of these approaches, it is important to distinguish between predictive markers and prognostic markers. Prognostic markers are informative of the natural outcome of disease irrespective of treatment. In other words, prognostic markers will tell us who will have a recurrence, metastasis, or die of disease, and they will therefore tell us *who should be treated* (Fig. 2a). In contrast, predictive markers are treatment-specific: these markers will tell us which tumors will respond to a specific therapy and which will not, and therefore tell us *how to treat* (Fig. 2b) [139]. Ideally, predictive markers do not show any differential effect on outcome in the absence of treatment but only in the presence of the specific treatment.

In general, there are two settings to study predictive markers: the neoadjuvant setting and the adjuvant setting. In neoadjuvant studies, systemic treatment is given *before* surgery. Response to neoadjuvant therapy can directly be measured by comparing tumor size on radiologic imaging before therapy to the pathologic tumor size after therapy. Response in the neoadjuvant setting is a good measure of the sensitivity or resistance of the bulk of the tumor, and complete disappearance of disease (pathologic complete remission, pCR) is predictive of good outcome of disease [140]. However, response to neoadjuvant therapy does not inform us of total disease eradication and therefore does not eliminate poor outcome. In the adjuvant setting, chemotherapy is given after surgery and the endpoint of study is usually disease-free, progression-free, recurrence-free or overall survival (DFS, PFS, RFS, or OS, respectively), the ultimate proof of disease eradication. To study predictive markers in both settings, patients should be randomized between two treatments (RCTs). For markers associated with improved outcome (adjuvant) or pCR (neoadjuvant), the RCT design permits discrimination between markers that are prognostic or predicting general chemosensitivity and markers that are predicting response to specific treatments. Prognostic markers are related to natural outcome of disease and will therefore also correlate with improved outcome regardless of treatment. Markers for general chemosensitivity will show similar correlation with increased survival or pCR in both treatment arms. On the other hand, predictive markers that are related to a specific treatment will correlate with increased survival or pCR for one specific treatment arm. Sargent et al. [141] has described four clinical adjuvant RCT designs for predictive marker studies. Up till now, most predictive marker studies were not part of the adjuvant RCTs design, and therefore not prospectively performed. However, predictive markers can be assessed retrospectively in adjuvant RCTs by testing

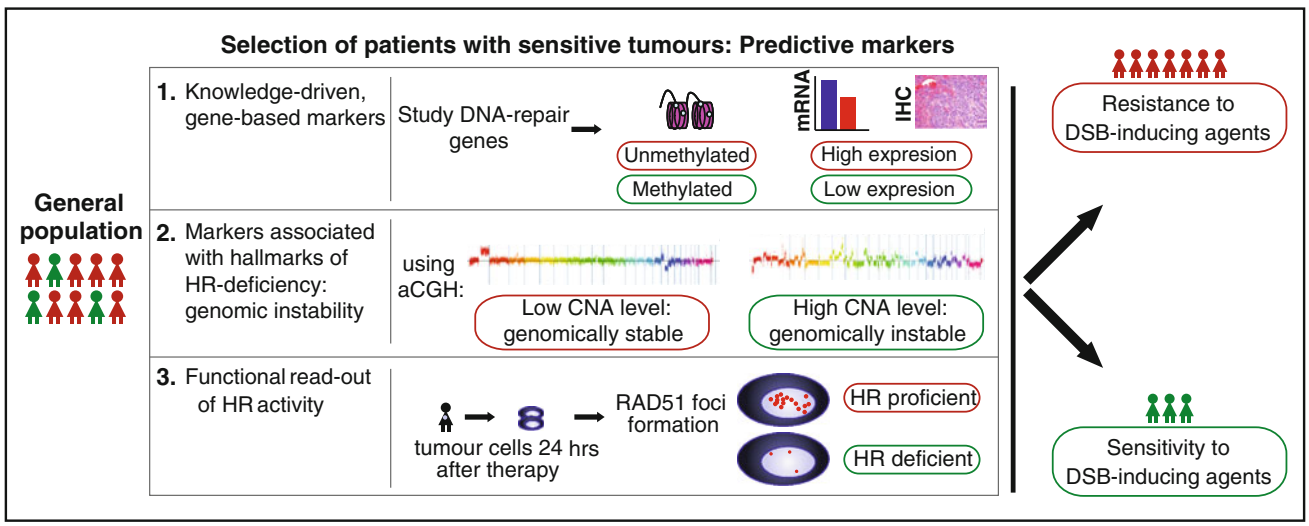

Fig. 1 Predictive markers are needed to adequately select those patients benefiting from DSB-inducing agents (such as bifunctional alkylators, platinum salts, or PARPi) out of the general breast or ovarian cancer populations. In general, three ways of predictive marker studies can be distinguished. Examples are given for all three options (in example 2, aCGH plots are depicted, with on the *x*-axis the chromosomes, and on the *y*-axis the log2-ratio of tumor DNA over normal DNA). *IHC* immunohistochemistry, *CNA* copy number aberration, *HR* homologous recombination, *hrs* hours, *DSB* double-strand break

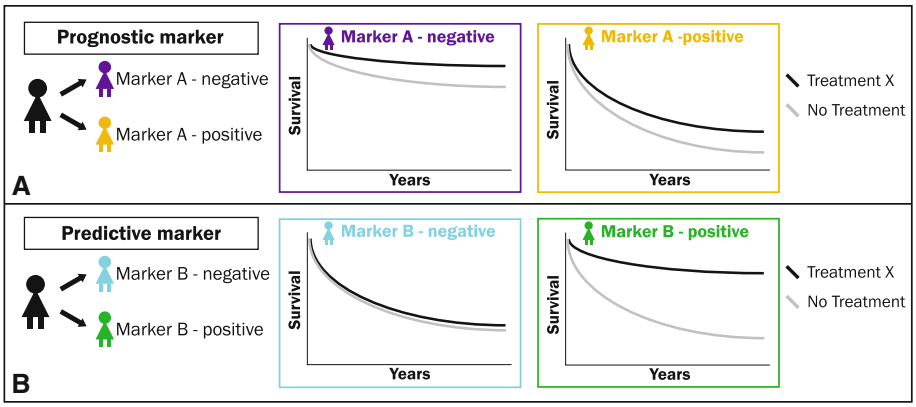

Fig. 2 **a** A prognostic marker predicts outcome of the natural history of a disease, regardless of treatment and therefore tells you *whom* to treat. (In this example: Marker A is a prognostic marker, as it predicts for a worse survival for Marker A-positive cases compared to Marker A-negative cases, irrespective of treatment. Consequently, Marker A-positive cases should be treated as these are more likely to die of disease. However, the marker does not tell how to treat). **b** A predictive marker predicts outcome in the presence of a specific therapy only but not in the absence of that specific treatment. It therefore tells you *how* (with what specific therapy) the patient should be treated (in this example: Marker B is a predictive marker, as it predicts an improved outcome to treatment X over no treatment in Marker B-positive cases, while no such benefit is seen in Marker B-negative cases; This difference in treatment effect between Marker B-positive and -negative cases should be significant on a test of interaction)

whether the treatment effect on survival observed in the presence of the marker is significantly different from the treatment effect observed in the absence of the marker, using a statistical test for interaction [141]. We used four criteria to evaluate predictive marker studies: (1) Preclinical/clinical evidence existed that the marker of interest plays a role in the pathway targeted by the treatment of interest; (2) The marker was studied in at least two different treatment populations; (3) The marker studied was reproducible; (4) The marker–treatment relation was studied with regard to survival (see Table 4). Although predictive markers can only truly be evaluated in an RCT, we will also consider single-arm treatment studies in this overview, since they can be considered as hypothesis-generating. A comprehensive overview of all predictive marker studies cited is shown in Supplemental Table 1.

Knowledge-driven studies to identify predictive markers: BRCA1 and BRCA2

BRCA mutation status

Early on it was observed in ovarian cancer patients that BRCA-mutation carriers had a better survival rate compared to non-carriers after cisplatin treatment [142, 143]. Interestingly, survival rates also seemed to differ by mutation site although the numbers were small [142]. The initial good response of ovarian cancers to platinum-based chemotherapy might be explained by the relative high frequency of BRCA germline mutations of ~13% [144]. Moreover, the presence of somatic BRCA mutations was recently found to raise the frequency of *BRCA* mutations to 23% in high-grade serous ovarian cancers [53]. However, since the standard of care for ovarian cancer consists of treatment with a platinum agent, it was difficult to distinguish whether the observed association of *BRCA* mutation status with increased survival was due to sensitivity to the specific treatment (predictive) or favorable tumor features (prognostic) [143]. Since regimens without a platinum agent or a bifunctional alkylator are not often used, it is difficult to compare regimens with and without DSB-inducing agents. Further evidence that BRCA-mutated ovarian cancers are sensitive to DSB-inducing agents is provided by the recent PARPi studies. In the phase I study of the PARPi olaparib, nine out of 15 *BRCA*-mutated ovarian cancer patients showed an objective response [137], an astonishing result for a phase I study, which was confirmed in a phase II study [13].

Evidence that *BRCA*-mutation status forms a predictive marker for DSB-inducing agents for breast cancer is complicated by the fact that bifunctional alkylators or platinum agents are not commonly used as systemic treatment, except for cyclophosphamide, which is mostly given in a relatively low dose. Until now, only two neoadjuvant studies have reported on the use of platinum in *BRCA*-mutated breast cancer. Byrski et al. showed in a cohort study that ten out of 12 *BRCA1*-mutated breast cancer patients achieved a pCR after neoadjuvant cisplatin, while this was much lower (7–22%) after other treatments [cyclophosphamide–methotrexate–5-fluorouracil (CMF), doxorubicin–docetaxel (AT), doxorubicin–cyclophosphamide (AC) and 5-fluorouracil–AC (FAC); 145]. However, direct comparison could not be made since this study was not randomized and cisplatin-treated patients had more favorable patient characteristics compared to patients with other treatments (for example previous chemotherapy was given in ~18% of other treatments compared to 0% in the cisplatin arm) [145].

Table 4 Criteria that can be used to judge or set up predictive marker studies

Criteria essential for predictive marker studies
1. *Preclinical and/or clinical data show involvement of the marker in the pathway targeted by the treatment of interest*
Has a relevant treatment regimen been studied with regard to the marker of interest?
Is the dosing of the drug of interest relevant with regard to the marker of interest?
2. *The marker was studied in at least two different treatment populations, preferentially in an RCT*
Was the marker tested in patients treated *with* the therapy of interest and in a similar patient population *without* the therapy of interest (or preferably but uncommon: without any treatment)?
Was the treatment-effect tested in a marker-negative population?
In case the study design did not consist of a RCT: was the marker tested in a well-matched case–control study (i.e., matching of patient characteristics treated with the therapy of interest to those without the therapy of interest)?
3. *The marker studied was reproducible*
Were the results concordant when tested twice on the same patient population with respect to the marker?
Were the results from independent studies concordant with respect to the marker?
4. *The marker–treatment relation was studied with regard to either pCR rate but preferably survival*
Was the outcome (RFS, DFS, PFS, OS) or pCR rate improved in marker-positive patients treated with the therapy of interest, compared to those without the therapy of interest? And was this treatment-related survival benefit or increased pCR rate absent in the marker-negative population (i.e., was the statistical test for interaction significant)?
The ultimate goal of treatment remains long-term survival as this is the most objective read-out
Although pCR after neoadjuvant therapy has been related to long-term survival, the survival of partial responders cannot be predicted

RCT randomized controlled trial, *pCR* pathological complete remission, *RFS* recurrence-free survival, *DFS* disease-free survival, *PFS* progression-free survival, *OS* overall-survival

Furthermore, the fact that no comparison was made to non-mutated cases precluded evaluation of *BRCA1* mutation status as a predictive marker. Silver et al. [146] showed that 2/2 BRCA1-mutated breast cancer patients achieved a pCR in a neoadjuvant study in which TN breast cancer patients received cisplatin and markers for response were investigated. However, numbers remain small and perhaps the most convincing hint that BRCA-mutated breast cancers are sensitive to DSB-inducing agents is the phase II PARPi study. In this study of metastatic breast cancer patients, an objective response rate of 41% was seen in the olaparib 400 mg arm [14].

Mutations are not the only cause of functional loss of BRCA1 or BRCA2 and many studies have investigated different read-outs of BRCA1 and BRCA2 and their influence on chemotherapy response/outcome. Since immunohistochemical analyses of BRCA1 and BRCA2 proteins have given conflicting results due to lack of specificity of antibodies [147], we did not include them in this overview.

BRCA1 promoter methylation

Studies investigating ovarian cancers for *BRCA1* promoter hypermethylation have given contradictory results; patients with BRCA1-methylated ovarian cancers were shown to have a worse survival [148] but also a better clinical response [149, 150] after platinum-based chemotherapy. However, these studies were all hampered by small numbers of tumors (max methylated tumors $n = 15$), a different mix of control patients compared to methylated cases, and different outcome measurements (clinical response versus adjusted survival analyses). In one study, in which control patients were matched to patients with *BRCA1*-mutated ($n = 40$) and -methylated ($n = 19$) tumors, little difference in survival after platinum agents was found between subgroups [151]. However, control patients were also matched for residual disease, which might have biased this study [151], since residual disease itself relates to survival. In breast cancer, one study investigated methylation of *BRCA1* and 13 other genes in patients treated with neoadjuvant doxorubicin and found no association with breast cancer specific survival (BCSS) [152]; this might be due to the type of treatment (doxorubicin) used. Silver et al. tested multiple markers for response in TN breast cancer patients who received neoadjuvant cisplatin [146]. *BRCA1*-promoter methylation, but also low *BRCA1* mRNA expression, was significantly associated with better tumor response in the neoadjuvant setting. However, since this was a small study ($n = 28$) investigating multiple markers without any multivariate adjustments, it should be considered as hypothesis-generating [146].

Next to *BRCA1*-related markers specific types (nonsense or frameshift) of *TP53* mutations were shown to be predictive of a good response [146]. Interestingly, specifically these types of *TP53* mutations have been found in high frequencies in *BRCA1*-mutation carriers [52] and might function as an alternative marker for *BRCA1* deficiency. In line with this reasoning, it was found that a yeast-based screen for functional *TP53* mutations could predict for pCR in a cohort of three different series of breast cancer patients treated with neoadjuvant intensified cyclophosphamide (a bifunctional alkylator), while it predicted resistance to anthracyclines alone [153].

BRCA1 mRNA expression

In breast cancer, two studies examined *BRCA1* mRNA expression as a predictive marker with mixed outcomes

(i.e., high levels better clinical response [154] and low levels prolonged survival [155]). However, both studies used different read-outs (clinical response vs. survival), used an arbitrary cut-off for low versus high expression [154], and consisted of small subgroups (highest $n = 17$). In ovarian cancer, Quinn et al. observed first in cell lines that loss of *BRCA1* mRNA expression increased cisplatin sensitivity and taxane resistance [156]; subsequently, they found in ovarian cancer patients ($n = 70$) that low *BRCA1* mRNA expression gave a survival benefit after platinum-based chemotherapy (significant after adjustment for potential confounders) [156]. A similar association of low *BRCA1* expression and improved survival was seen in ovarian cancer patients with little residual disease after surgery [157]; however, this study was small ($n = 51$) with the number per subgroup unknown and a heterogeneous population regarding treatment and stage of disease [157].

The above-mentioned studies regarding *BRCA1* promoter methylation or gene expression as markers of response to DSB-inducing agents were all limited by small numbers of patients, and in some cases also by the treatment schedules and statistical shortcomings (for details see Supplementary Table 1), making firm conclusions impossible. Larger studies, preferably within RCTs, with a statistical sound set-up should be performed to investigate the performance of these markers.

Knowledge-driven studies: genome-wide effects of BRCA1 or BRCA2 deficiency

Gene expression classifiers

Instead of studying *BRCA1* mRNA expression levels, several studies used genome-wide gene expression profiling as starting point to identify predictive markers in sporadic tumors. In breast cancer patients, a previously published gene expression signature of BRCA1-mutated breast cancer [158] was used to develop a DNA repair gene expression profile [159]. This profile was associated with pCR after anthracyclines in TN breast cancer patients from two neoadjuvant studies and with resistance after a neoadjuvant taxane-based regimen [159]. However, the experimental design of this study was flawed for various reasons, for example resistance was not defined and patient characteristics (and subsequently adjustment for potential confounders) were not shown but, more importantly, ability of the signature to identify *BRCA1*-mutated cases was not investigated [159]. A somewhat similar strategy in ovarian cancer was employed, in which differences in gene expression between *BRCA1/2*-mutated and sporadic ovarian cancers were used to develop a BRCAness profile [160]. The presence of this BRCAness profile in sporadic ovarian cancers ($n = 70$) was associated with a significant longer DFS and OS after platinum-based chemotherapy [160].

aCGH classifiers

We employed a similar genome-wide strategy by using DNA copy number aberrations (CNAs) as predictors for BRCAness [161, 162]. In a retrospective analysis of an RCT in which breast cancer patients were randomized between intensified carboplatin–thiotepa–cyclophosphamide (CTC) and conventional FEC chemotherapy, we tested the performance of an aCGH classifier characteristic for *BRCA1*-mutated breast cancer (BRCA1-like^{CGH}) as a predictive marker for recurrence-free survival after CTC [162]. We found that HER2-negative breast cancer patients with a BRCA1-like^{CGH} tumor were eight times less likely to have a recurrence after CTC compared to FEC, while no difference in treatment was observed in non-BRCA1-like^{CGH} breast cancer patients (test for interaction $p < 0.01$) [162]. While this BRCA1-like^{CGH} profile was highly associated with TN status, it was still able to predict outcome *within* TN breast cancer patients only, making this marker more than just a readout for TN breast cancer. Although the use of intensified chemotherapy in breast cancer is controversial [163], our data strongly suggest that certain subgroups may specifically benefit from this treatment regimen [153, 164]. However, whether this survival benefit is due to the type of DSB-inducing agents used in these intensified regimens or the dosing itself, should be further investigated. In accordance with the FEC results of the above-mentioned RCT, this BRCA1-like^{CGH} profile was not correlated with pCR in TN patients after anthracycline-based chemotherapy as part of a neoadjuvant trial [161]. Interestingly, in this study, an aCGH profile for *BRCA2*-mutated breast cancer [165] identified both ER-positive and TN breast cancer patients. Furthermore, this BRCA2-like profile showed a trend for prediction of pCR after anthracycline-based chemotherapy in ER-positive breast cancer patients [161]. In line with the fact that *BRCA1*-methylated breast cancers were shown to display similar aCGH patterns as *BRCA1*-mutated breast cancers [166], the BRCA1-like^{CGH} profile identified several sporadic breast cancer patients with a *BRCA1*-methylated tumor [161, 162].

DNA and histone methylation

Since *BRCA1* has additional functions besides DNA repair, these features could also be explored in the search for predictive markers. As such, *BRCA1* has been shown to play a role in chromatin remodeling through interaction

with HDAC1 and HDAC2 [167] and the SWI/SNF-complex [168]. Moreover, multiple studies have shown that *BRCA1*-mutated breast cancers show less DNA methylation when compared to sporadic breast cancers [169] or other familial breast cancers [170]. In a study including a larger number of sporadic breast cancers it was observed that this lower methylation state was not restricted to *BRCA1*-mutated cancers but also present in sporadic BLBCs, suggesting that a BRCA1-like defect might also be present in these tumors [171]. In this study, no difference between methylation frequency in sporadic and *BRCA1*-mutated breast cancer could be found. Mixed results have been reported for patterns of gene promoter methylation in ovarian cancers and their association with response or survival after platinum-based chemotherapy [149, 150, 172]. Increased numbers of methylated genes were associated with a shorter PFS [172], platinum sensitivity (PFS interval of >12 months) [150] but not with clinical response [149]. However, these studies did not measure genome-wide methylation or investigate the same set of genes. It therefore remains to be determined whether general methylation status can be used as a predictive marker.

Identification of chromatin remodeling factors as predictive markers might become even more interesting since it was recently shown that cells could become reversibly drug-tolerant, i.e., survive treatment, through demethylation of H3K4 [173]. Moreover, histone deacetylase inhibitors could eradicate these drug-tolerable populations by inducing γH2AX [173]. These findings offer a new perspective and candidate therapeutic targets for disease eradication.

Knowledge-driven markers: other DNA repair genes

Besides the *BRCA* genes, other genes involved in DNA repair have been studied for their predictive potential for sensitivity to DSB-inducing agents. These include genes involved in MMR, NER, BER, and HR.

HR genes

Although most predictive marker studies have focused on the *BRCA* genes, a few studies have investigated other HR genes in relation to DSB-inducing agents. Two separate studies on the same dataset, consisting of an RCT in which breast cancer patients were randomized between CMF or local radiotherapy used IHC to measure expression of firstly, BRCA1, BRCA2, and RAD51, and secondly, MRE11, NBS1, RAD50, and ATM [174, 175]. It was found that patients with tumors with low RAD51 expression or with high expression of nuclear ATM or RAD50 benefited significantly from radiotherapy compared to CMF, while no differential benefit was seen in patients with tumors expressing high RAD51 or low ATM/RAD50 (no significant test for interaction) [174, 175]. While the RAD51 data was consistent with the hypothesis that lack of RAD51 corresponds to impaired DNA repair and sensitivity to radiotherapy [174], the ATM/RAD50 data are counterintuitive [175]. As stated by the authors, the ATM/RAD50 data could be explained by the fact that the MRN/ATM complex is responsible for cell cycle arrest and subsequently apoptosis. Lack of this complex could therefore result in radiotherapy resistance due to failure to induce apoptosis. In ovarian cancer, sensitivity to platinum agents was further linked to *FANCF* methylation [176]. Although *FANCF* methylation was shown to be variable and thought to disappear on progression of disease [176], it was found in ~20% of ovarian cancers [176, 177]. Unfortunately, only one study investigated association of *FANCF* methylation with survival, and in this study only seven methylated cases were found [178], resulting in too small numbers to draw any conclusion.

A recent study in high-grade serous ovarian carcinomas has illustrated that many defects in HR-related genes have thus far been undiscovered. In this study, using integrated genomic analyses of 489 high-grade serous ovarian carcinomas, it was found that (epi)genetic somatic alterations/mutations in BRCA1, BRCA2, EMSY, RAD51C, ATR, ATM, PALB2, and several FA genes were present in 51% of all the cases [94]. Identifying these patients will be essential, since they will have selective benefit of DSB-inducing agents.

NER genes

Since NER is responsible for the repair of bulky DNA adducts or intrastrand crosslinks, many studies investigated whether loss of NER through reduced ERCC1 activity in ovarian cancers would result in sensitivity to crosslinking agents such as platinum drugs. Most studies indicate that good platinum response is associated with low levels of ERCC1 [measured by mRNA expression, IHC or analysis of single nucleotide polymorphisms (SNPs) associated with low mRNA expression; 179–183]. However, these studies again have some limitations, making it impossible to draw firm conclusions from the reported data. While some studies investigated relatively small subgroups [179, 180, 183], others showed association of ERCC1 with CA-125 levels response but showed no difference in survival [182].

Besides ERCC1, ERCC5 has also been studied as a predictive marker in ovarian cancer patients [184]. Using genome-wide LOH analysis, LOH at a region on chromosome 13q was found to correlate with a prolonged PFS after platinum-based chemotherapy. Based on the biological functions of genes present in this 13q region, *ERCC5*

was selected for further investigation. Reduced ERCC5 expression was associated with a prolonged PFS after platinum-based chemotherapy [184].

In conclusion, although published studies suggest involvement of *ERCC* genes in platinum response in ovarian cancer patients, it remains difficult to draw firm conclusions on the predictive value of these genes. However, the potential involvement of NER in platinum response is supported by studies in other cancer types such as lung cancer [185]. Additional studies, preferably RCTs, in larger cohorts of ovarian cancer patients would therefore remain interesting.

BER genes

In the hope of identifying new potential drug targets next to PARP1, other BER genes have been tested for their predictive capacity of chemotherapy response. A good example is an exploratory study that investigated APE1 in ovarian cancer patients treated with platinum compounds [186]. Although nuclear expression of APE1 was found to be associated with worse OS and platinum resistance (i.e., progression on therapy or relapse within 6 months after the start of therapy), this study should be considered as hypothesis-generating, as it consisted of a heterogeneous patient population and no adjustment for potential confounding factors was performed [186]. Two breast cancer studies investigating SNPs in BER genes yielded opposing associations of XRCC1 variants with survival: the _AA variant of XRCC1_1196G>A was associated with favorable survival [187] but also with a worse survival [188]. However, both studies suffered from relatively heterogeneous treatment regimens and small subgroups (for details see Supplementary Table 1), leaving the true association between these XRCC1 SNPs and survival unsolved.

MMR genes

Loss of MMR has been associated with resistance to platinum agents by loss of MMR-dependent apoptosis in vitro [189]. Consequently, multiple studies investigated MMR deficiency in breast and ovarian cancer (via IHC, methylation, or microsatellite instability detection) in relation to response or outcome after chemotherapy [172, 190–197]. These studies reached opposing conclusions based on associations of low MMR gene expression with both clinical progressive disease (e.g., [191]) and longer OS (e.g., [197]). The reason for these contradictory results is that the quality of all studies was compromised by analysis of small subgroups within heterogeneous patient populations (for example varying chemotherapy regimens within studies [190, 195]), risk of unnoticed associations with other potential prognostic patient characteristics, or studying only clinical responses (e.g., [196]; for details regarding the above-mentioned studies, see Supplementary Table 1). Interestingly, prospective analysis of *hMLH1* methylation in ovarian cancer patients as part of a RCT showed an increased methylation frequency in DNA isolated from blood plasma recovered at time of progression compared to plasma DNA recovered prior to carboplatin treatment [193]. This increase was associated with an increase of microsatellite instability, a more direct read-out of MMR activity [193]. Moreover, the acquisition of *hMLH1* methylation was associated with a shorter progression-free interval (PFI) and a worse OS [193]. Unfortunately, it could not be studied whether *hMLH1* methylation status in blood plasma reflected methylation status in the actual tumor. Nevertheless, these results suggest an acquired resistance mechanism via loss of MMR, which is supported by the in vitro data and warrants further investigation.

Markers based on general hallmarks of HR deficiency

Alternative strategies for developing predictive markers of DSB-inducing chemotherapy sensitivity in breast and ovarian cancer focus on general aspects of homology-directed DSB repair deficiency rather than on activity of single genes. Several studies have investigated genomic instability as a central hallmark of HR deficiency.

DNA copy number aberrations

One way to assess genomic instability is by measuring DNA copy number aberrations (CNAs) in tumors. It has been shown in multiple studies that *BRCA1*- and *BRCA2*-mutated breast cancers display characteristic DNA copy number gains and losses [165, 198–200]. Subsequently, it has been found that the total number of CNAs, also referred to as the chromosomal instability (CIN) score or the genomic instability index, differs between *BRCA*-associated familial cancers and sporadic tumors. Mainly *BRCA1*-mutated breast tumors were observed to have the highest number of CNAs compared to other tumors [198–200]. Furthermore, the type of CNAs differed between familial and sporadic tumors, with a higher frequency of large deletions being present in the *BRCA*-mutated breast cancers [199]. Interestingly, high numbers of CNAs were also found in sporadic ER-negative/TN/basal-like breast cancers [42, 199–201] and these tumors clustered with the *BRCA1*-mutated group [42, 199]. Similarly, the number of CNAs in ER-positive breast cancers was found to be highest in luminal B tumors, which co-clustered with *BRCA2*-mutated breast cancers [42, 201]. These findings suggest that a subset of sporadic breast cancers share

features of *BRCA*-mutated cancers, which could include HR deficiency. Evidence of this is provided by the aCGH patterns of sporadic *BRCA1*-methylated breast cancers, which were shown to resemble *BRCA1*-mutated breast cancers [162, 166, 199] and to display a high genomic instability index [199]. Whether genomic instability measured by number of CNAs can be employed for response prediction has thus far only been investigated in one small study, in which the total number of chromosomal breakpoints was associated with response to neoadjuvant cisplatin in breast cancer patients [202]. Also in ovarian cancer, specific CNAs as well as type of CNA (large deletions) are associated with *BRCA1*-mutation status [203]; however, these features have never been related to therapy response.

DNA rearrangements

With the advent of next-generation massively parallel sequencing (MPS) techniques, new ways of investigating genomic instability can be explored, especially since this technology also permits the evaluation of copy number-neutral alterations such as point mutations, balanced translocations, and inversions [204]. In-depth analysis of cancer genomes using MPS might reveal association of specific types of DNA rearrangements or mutations associated with HR deficiency, which would enable development of sequencing-based tests for identifying genomic instability and predicting response to DSB-inducing agents. Two studies have used MPS to catalogue genomic rearrangements in breast cancer. Using paired-end MPS of nine breast cancer cell lines and 15 human breast cancer samples, Stephens et al. [205] showed that intrachromosomal rearrangements and tandem duplications were most frequent in TN breast tumors ($n = 4$) compared to ER- or HER2-positive breast tumors but not in *BRCA*-mutated cancers ($n = 4$). This high frequency of tandem duplications was not observed in a subsequent paired-end MPS analysis of genetically engineered mouse mammary tumors recapitulating *BRCA*-mutated hereditary breast cancer and *BRCA*-proficient sporadic breast cancer [206]. Furthermore, neither the type nor the frequency of genomic rearrangements was different between *BRCA*-deficient and -proficient tumors, suggesting that HR deficiency can actually not be identified using this method [206]. However, in the mouse study, it seemed that microhomology in non-amplicon related rearrangements was higher in BRCA1-deficient tumors compared to BRCA1-proficient tumors [206]. It is difficult to draw firm conclusions from these two studies, because the numbers of tumors analyzed were very low. Furthermore, the heterogeneity was very high, even between tumors from the same breast cancer subtype or genetically engineered mouse model. More extensive studies involving larger collections of tumors will be required to evaluate the potential of MPS for the identification of HR deficiency and development of predictive markers.

Functional assays for homologous recombination status

Until now, we have described markers that are indirectly related to HR deficiency or DSB-inducing agent sensitivity. Identification of these markers might be hampered by the fact that control groups are likely to be contaminated with sporadic HR-deficient tumors, thereby diminishing the chances of finding predictive markers. This could be the case in for example sporadic TN breast cancers, of which $\sim$60% showed a BRCA1-like aCGH profile [162].

Thus far, only a few studies have pursued the identification of markers that directly test the functionality of HR. One study used seven pre-treatment breast cancer biopsies for ex vivo analysis of radiation-induced RAD51, BRCA1, and FANCD2 nuclear foci [207]. Although defective foci formation was found in four cases, of which three were TN tumors, the quantification of foci formation was shown to be heterogeneous [207]. Similarly, Asakawa et al. [208] analyzed γH2AX, BRCA1, and RAD51 nuclear foci in breast cancer biopsies obtained prior to treatment and 18–24 h after the first cycle of epirubicin–cyclophosphamide. They found that clinical response was negatively correlated with the presence of RAD51 foci post chemotherapy or the presence of BRCA1, γH2AX, or RAD51 foci prior to chemotherapy [208]. Unfortunately, correlations between more reliable endpoints, such as pathological response or survival, were not studied [208]. In ovarian cancer, PARPi-induced formation of γH2AX and RAD51 foci was studied on in vitro cultured cells from ascitic fluid from patients [209]. An increase in RAD51 foci formation after PARPi treatment was found to be associated with increased cell survival and reduced growth inhibition; however, it is unclear how this in vitro response to PARPi translates to PARPi responses in the actual patients [209]. Recently, a neoadjuvant study investigating breast cancer biopsies 24 h after anthracycline-based chemotherapy found that low RAD51 foci formation in proliferative cells correlated with TN status and a higher pCR rate [210]. However, numbers were again small, and therefore this marker could not be tested next to markers known to be associated with pCR (high grade and TN status).

Although these first results indicate that functional assays hold promise as potential markers to identify all sensitive patients based on HR deficiency, implementation in the clinic might be difficult because most of these assays require extra biopsies. Furthermore, inter- and intra-tumor

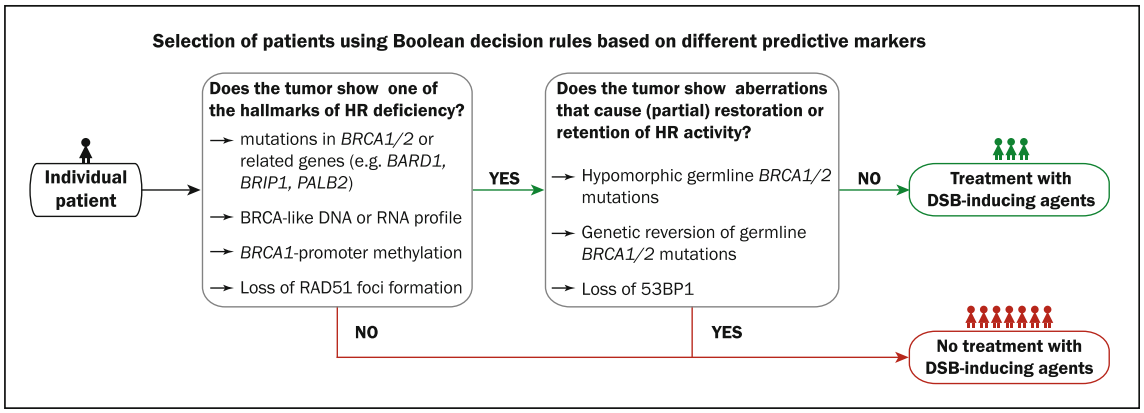

Fig. 3 In the future, therapy choice might be guided by combinations of predictive biomarkers. In the presented example, the presence of one feature and the absence of a second feature might guide treatment with DSB-inducing agents. *HR* homologous recombination, *DSB* double-strand break

heterogeneity might complicate unambiguous tumor classification based on numbers of nuclear foci.

Challenges for the future

In the material above, we have tried to summarize most of the efforts that have been undertaken so far to identify predictive markers for HR deficiency and sensitivity to DSB-inducing agents. For this overview, we have restricted ourselves to studies performed in patient settings. Although the level of evidence differs substantially per marker, it is clear that some candidate markers are very promising and should be studied with high priority in translational prospective studies. Based on the evidence obtained in breast and ovarian cancer, interesting candidate markers are: expression of BRCA1/2 and other HR genes, *BRCA1* promoter hypermethylation, aCGH classifiers and functional approaches using RAD51 foci induction.

However, even with highly specific and sensitive predictive markers for sensitivity to DSB-inducing agents, many challenges lie ahead (Fig. 3). Firstly, previous studies have shown that even the presence of *BRCA* mutations (supposedly the gold standard for HR deficiency) does not guarantee lasting therapy sensitivity, since secondary mutations in *BRCA1/2* can restore expression of functional protein and thereby confer resistance to DSB-inducing agents [211, 212]. Secondly, it could well be that even within *BRCA*-mutated cancers, the level of HR deficiency might affect therapy sensitivity. The fact that risk of breast and ovarian cancer differ by *BRCA* mutation position [136] suggests that different *BRCA* founder mutations might also be associated with differences in the level of HR deficiency. In support of this, studies in ovarian cancer patients showed that different *BRCA* founder mutations correlated with different survival rates after platinum-based chemotherapy [142]. This phenomenon could also contribute to the variation in response to PARPi observed in the phase II trials [13, 14]. Thirdly, a recent study showed that restoration of HR and concomitant therapy resistance in *BRCA*-mutated tumors might also be caused by other DDR pathway aberrations, such as loss of 53BP1 [213]. Bouwman et al. [213] showed that loss of 53BP1 in *BRCA1*-deficient cells restored partial functionality of HR. Furthermore, it was shown in breast cancer patients that 15 and 11% of *BRCA1*- and *BRCA2*-mutated tumors, respectively, had reduced 53BP1 expression as measured by IHC; and this frequency was even higher in TN breast cancer [213]. Fourthly, the current focus has been on genes and pathways directly related to DNA repair; however, it has been proposed that loss of PTEN might also lead to HR deficiency and sensitivity to DSB-inducing agents [214]. Lastly, acquired resistance mechanisms might greatly influence the response and outcome of metastatic disease in patients, even when predictive markers are capable of adequate selection of (neo)adjuvant therapy. It has for example been shown that upregulation of P-glycoprotein confers acquired resistance to the clinical PARPi olaparib in a mouse model of *BRCA1*-associated breast cancer [215].

Concluding remarks

In conclusion, specific subgroups of breast and ovarian cancer show remarkable resemblance in terms of hormonal regulation, driver mutations, genomic instability, and chemotherapy sensitivity. Hence, knowledge obtained in one cancer type might very well be applicable to the other cancer type, thereby accelerating the process of developing tailored therapies and companion diagnostic biomarkers for prediction of therapy response. Many studies have investigated predictive markers in breast and ovarian cancer for sensitivity to DSB-inducing agents, such as platinum salts, bifunctional alkylators and recently PARPi. However, the identification of truly

predictive markers has proven difficult since most studies were not based on RCTs or lacked a control group. With the growing interest in personalized cancer therapy, correct trial designs will become increasingly important. However, given all of the challenges presented above, one could envision that one biomarker will not suffice. Instead, decision rules based on a combination of multiple markers using Boolean operators might be applied to treatment decisions in clinical practice in the future (Fig. 3). While these biomarker combinations are expected to improve the outcome of breast and ovarian cancer patients, they will also present a challenge with regard to cost-effectiveness and implementation in clinical practice.

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