

**DIAGNOSTIC DETECTION OF DNA REPAIR PATHWAY  
ABERRATIONS IN CANCER**

**PhD thesis**

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**List of Abbreviations:**

AI - allelic imbalance

HR - homologous recombination

HRD - homologous recombination deficiency

NGS - next generation sequencing

NER- Nucleotide excision repair

WES - whole exome sequencing

WGS - whole genome sequencing

## 1. Introduction

DNA repair mechanisms are essential for the maintenance of the genomic integrity of normal cells. DNA repair deficiencies can lead to accumulation of cancer-initiating mutations and also contribute to the accumulation of mutations that enhance survival and fitness of cancer cells. However, the very same DNA repair pathway aberration that initiated and/or promoted cancer development may also constitute a significant therapeutic vulnerability. Since DNA repair pathway aberrations are common in tumor cells but are largely absent in normal cells, therapeutic agents that target DNA repair deficient cells may have a clinically exploitable therapeutic window. The most successful example stemming from this working hypothesis is the development of PARP inhibitors. Cells with homologous recombination deficiency due to alterations in genes such as BRCA1 or BRCA2 have ~1000-fold higher sensitivity to PARP inhibitor treatment compared to cells with intact homologous recombination [1]. This synthetic lethal relationship has been successfully exploited for the treatment of homologous recombination deficient ovarian, prostate, breast and pancreatic cancer.

In this work we investigated the potential therapeutic exploitation of the deficiency of two DNA repair pathways, homologous recombination and nucleotide excision repair.

Homologous recombination is one of the DNA repair mechanisms cells use to maintain genomic integrity when encountering double strand DNA breaks (DSBs). It is an error free process using the sister chromatid as a template and as such it is fundamentally different from other error prone mechanisms such as non-homologous end joining (NHEJ).

Nucleotide excision repair is a DNA repair mechanism that recognizes and corrects bulky DNA adducts. NER can be divided into two sub-pathways: global genome NER (GG-NER) and transcription coupled NER (TC-NER). GG-NER and TC-NER differ in how they recognize DNA damage, but they share the same process for DNA duplex incision, repair, and subsequent ligation, which we term here the “common NER pathway” [2]. GG-NER repairs damage in both transcribed and non-transcribed DNA strands across the entire genome and is activated by sensing proteins (such as DDB1/2) that recognize distortions in the DNA helix. Alternatively, TC-NER is activated when

RNA polymerase stalls at a lesion in transcribing DNA, with the blocked RNA polymerase serving as a damage recognition signal. GG-NER and TC-NER play a critical role in maintaining genomic integrity, and germline mutations in NER genes lead to increased mutation rate of normal cells and increased cancer incidence [2].

A major obstacle in the optimal exploitation of therapeutic agents that are synthetic lethal with above-described specific DNA repair pathway aberrations is the lack of reliable diagnostic methods to identify and quantify the various DNA repair pathway deficiencies in tumor biopsies. For homologous recombination deficiency lack of RAD51 foci formation as detected by immunohistochemistry was proposed to serve as diagnostic measure [3], but this method has not reached the level of general diagnostic introduction yet. For nucleotide excision repair deficiency there are no readily applicable histological diagnostic tests either.

Our work was aimed at providing solutions for this diagnostic problem. We developed methods for the accurate detection of homologous recombination deficiency in solid tumors, and thus improve the efficacy of PARP inhibitor-based therapy. We also developed methods to detect nucleotide excision repair deficiency in tumor biopsies. The core principle of our approach is based on the fact that in the absence of a given DNA repair mechanism specific genomic aberrations are accumulated in the genome and those can be identified and quantified by next generation (whole exome or whole genome) sequencing.

We started our work in the context of breast cancer. It was known that breast and ovarian cancers in patients carrying loss of function mutations in either the BRCA1 or BRCA2 genes are sensitive to platinum-based chemotherapy [4,5]. Pathogenic BRCA1 mutations together with loss of heterozygosity are present in about 1-10% (depending on ethnicity) of breast cancer cases. The majority of BRCA1 deficient breast cancer cases are triple negative (lack expression of estrogen and progesterone receptors and not amplified for the HER2 gene). Pathogenic BRCA2 mutations together with loss of heterozygosity are present in about 1-3% (depending on ethnicity) of breast cancer cases and they tend to be estrogen receptor positive.

We noticed that triple negative breast cancer cases share several characteristics independently from their BRCA1 mutational status [6,7]. They tend to share similar patterns of genomic aberrations, such as high levels of chromosomal aberrations,

including allelic imbalance (AI), the unequal contribution of maternal and paternal DNA sequences with or without changes in overall DNA copy number [8]. We hypothesized that some of the BRCA1 wild type triple negative breast cancer cases may also be homologous recombination deficient due to e.g. promoter methylation based suppression of BRCA1 expression. We also assumed that the homologous recombination deficient breast cancer cases will be similarly sensitive to platinum-based therapy and display similar genomic aberrations independently from their BRCA1/2 mutational status.

We had access to two relevant clinical trials, in which patients with operable triple negative breast cancer were treated with neoadjuvant cisplatin monotherapy. The neoadjuvant platinum treatment resulted in greater than 90% tumor reduction in 27 of 72 patients (37%) [9,10]. Of the 27 good responders only 10 patients carried BRCA1 or BRCA2 mutations. This has prompted us to investigate whether we can identify patients responding to platinum-based therapy in the absence of BRCA1/2 mutations.

We analyzed SNP array-based data from these two clinical trials and investigated which types of chromosomal abnormalities are most significantly associated with response to therapy.

We further investigated whether diagnostic methods to detect homologous recombination deficiency, which is associated with platinum or PARP inhibitor sensitivity, could be also applied in the metastatic setting of breast cancer, especially in the context of brain metastasis.

About 15%-20% of breast cancer patients develop brain metastases significantly mitigating improved survival that was achieved by therapeutic advances over the last several decades [11]. Due to its unique location, and perhaps its distinct biology, treatment options have been limited.

PARP inhibitors may offer therapeutic benefit in the case of homologous recombination deficient brain metastasis of breast cancer. Some of the clinically applicable PARP inhibitors such as niraparib and veliparib are known to cross the blood-brain barrier [12], making those potential candidates for the treatment of brain metastases. However, in order estimate the potential benefit of PARP inhibitors the frequency of homologous recombination deficiency needs to be estimated. For this we used HR deficiency induced mutational signatures, or DNA scarring signatures and compared the homologous

recombination deficiency status of paired primary breast cancer and their brain metastasis.

We played an active role in developing the first scoring system to quantify the degree of HR deficiency [10]. These first methods were based on data derived from hybridization microarrays such as SNP arrays and combined three different DNA aberration types. Our method, which is generally known as ntAI [10] is the number of AIs (unequal contribution of parental allele sequences) that extend to the telomeric end of a chromosome. Popova et al. derived the so-called the large-scale state transition or LST score [13], which is defined as the number of chromosomal breaks in the cancer genome between adjacent regions of at least 10 Mb, with a distance between them not larger than 3 Mb; Abkevich et al. developed the homologous recombination deficiency loss of heterozygosity (HRD-LOH) score [14], which is the number of loss of heterozygosity regions in the cancer genome that exceed the size of 15 Mb but less than the whole chromosome. It was shown later that these three measures carry complementary information about homologous recombination deficiency, therefore they were combined into a single measure, which is generally known as the HRD score and part of the myChoice HRD assay [15]. This method is approved by FDA for the prioritization of PARP inhibitor therapy in ovarian cancer. This measure was later converted into a next-generation sequencing (NGS) based diagnostic method [15].

Next generation sequencing revealed that loss of function of BRCA1 or BRCA2 is associated with a wide range of distinct mutational signatures that can be extracted from NGS data. Those include: (i) a single nucleotide variation-based mutational signature (COSMIC signature 3 or BRCA signature as labeled in the original publication [16]); (ii) a short insertions/deletions-based mutational profile, flanked by microhomology, a sign of alternative, POL $\theta$  mediated alternative end-joining [17]; and (iii) large scale rearrangements such as non-clustered tandem duplications of a given size range (mainly associated with BRCA1 loss of function) or deletions in the range of 1-10 kb (mainly associated with BRCA2 loss of function; [18]). All these can be also induced by the inactivation of BRCA1, BRCA2, or several other key downstream HR genes (XRCC2, XRCC3, RAD51, etc.; [17,19]).

It was shown that a composite mutational signature, HRDetect, that combines the various types of mutations listed above may represent a more accurate measure of HR deficiency than any of the mutational features alone [20,21].

We used the above-described mutational signatures to characterize the HR deficiency status of paired biopsies of primary breast cancer and brain metastasis.

Ovarian and breast cancer are the solid tumor types that are most often associated with mutations in BRCA1 and BRCA2, the key enzymes of a specific DNA repair pathway, homologous recombination (HR). Consequently, these were the first tumor types for which PARP inhibitors had been approved. However, other tumor types such as prostate cancer may also harbor BRCA1 or BRCA2 mutations. It was shown recently that PARP inhibitors, such as olaparib or talazoparib are also effective in prostate adenocarcinoma [22].

Homologous recombination deficiency can also be present in tumors with intact BRCA1/BRCA2 genes. The list of genes involved in homologous recombination includes several other genes beyond BRCA1/2 such as RAD51, PALB2, BARD1 etc. Recently other, so far unconsidered mechanisms, such as deletion of CHD1 was also implicated as a possible mechanism leading to homologous recombination deficiency in prostate adenocarcinoma [23]. SPOP mutations through the suppression of expression of BRCA2 genes by, may also contribute to homologous recombination deficiency in prostate cancer cells [24]. Therefore, simply sequencing a targeted panel of genes will not identify all homologous recombination deficient prostate cancer cases. This is especially important because tumors deficient in homologous recombination usually benefit from either PARP inhibitor or platinum-based therapy. The consequence of not identifying such tumors then is that treatment with these agents may be delayed or not given. In our work we investigated three related questions: (1) Do loss of function mutations of BRCA1 and BRCA2 coupled with loss of heterozygosity also lead to the homologous recombination deficiency associated mutational profiles usually seen in homologous recombination deficient ovarian and breast cancer? (2) Are there any prostate cancer cases with HRD-associated mutational signatures that do not harbor germline or somatic loss of function BRCA1 or BRCA2 mutations, and (3) whether whole genome sequencing and whole exome sequencing based mutational signatures are equally accurate to identify homologous recombination deficient prostate cancer cases.



Finally, we sought to extend the benefits of DNA repair pathway aberrations based synthetic lethality driven therapy to a solid tumor type with a particularly unfavorable outcome, bladder cancer.

Nucleotide excision repair (NER) is a highly conserved DNA repair pathway that recognizes and repairs bulky intrastrand DNA adducts formed by genotoxic agents, such as UV radiation and platinum chemotherapies [2]. NER is a complex mechanism that is initiated through two separate sensory mechanisms of lesion recognition: transcription-coupled repair (TC-NER) is activated by RNA polymerase stalling at lesions in transcribed regions, while global genome repair (GG-NER) recognizes distorted DNA structures throughout the entire genome. Following lesion recognition, TC-NER and GG-NER converge on a complex multi-step process that excises and then replaces the damaged DNA strand in an error-free manner.

Nucleotide excision repair deficiency is present in various solid tumor types as revealed by sequencing and functional studies. One salient example is the somatic missense mutations of ERCC2, encoding the DNA helicase, XPD, in a significant portion of (approximately 12-15%) of muscle-invasive bladder cancers [25]. These ERCC2 mutations are usually associated with increased sensitivity to platinum-based chemotherapy while they are not strongly prognostic in patients treated without chemotherapy, [26–28]. Consequently, mutations in ERCC2 and other DNA repair genes (such as ATM, FANCC, and BRCA1/2) are considered as predictors of therapy [29,30]. We also assumed that there are other mechanisms in bladder cancer that could induce nucleotide excision repair deficiency.

For muscle invasive and metastatic urothelial cancer cisplatin-based chemotherapy is often administered as first-line treatment. Despite the fact that 60%-70% of patients have an initial response, resistance develops in the majority of patients [31]. Another complicating factor is that due to medical comorbidities half of all patients with urothelial cancer are not eligible for cisplatin-based chemotherapy [32]. Immune checkpoint inhibitors are also approved for cisplatin-ineligible or cisplatin-resistant patients but less than 25% of patients show clinical response [33]. All these data suggest that novel agents with activity in post-cisplatin and cisplatin-ineligible patient populations are needed.

Irofulven is a semisynthetic DNA alkylating agent, which is derived from the natural product, illudin S, originally extracted from fungi [34]. Irofulven-mediated DNA damage is recognized by and activates TC-NER when the damage is encountered by RNA polymerase. Irofulven has an approximately 100-fold increased cytotoxic activity in nontumor cell lines with a TC-NER or common NER pathway gene defect compared with the isogenic NER-proficient line [35,36]. Irofulven is well-tolerated, but it showed modest clinical benefit when it was tested as a single agent in various phase I/II clinical trials [37,38]. This lack of efficacy was due to the fact that these trials were conducted in biomarker-unselected populations.

Despite the potential clinical actionability of tumor NER deficiency, currently there are no reliable, clinically applicable direct biochemical assays to quantify GG-NER/TC-NER deficiency in tumor biopsies [39]. A possible alternative is to use next generation sequencing based mutational signatures, since DNA repair deficiencies are frequently associated with increase in specific mutation types. We and others have successfully employed this strategy to identify homologous recombination deficient cancer cases for the prioritization of PARP inhibitor and platinum-based therapy.

For nucleotide excision repair, a specific single nucleotide variation (SNV)-based mutation signature (SBS5) has been associated with ERCC2 mutations in bladder tumors [40], but the signature was not associated with platinum response in tumors with intact ERCC2 status. One can improve the predictive utility of the various mutational signatures by combining them with other mutational features.

In this thesis, we identified a novel synthetic lethal relationship between NER deficiency and irofulven sensitivity. We show that inactivating mutations in genes of the TC-NER or common NER pathway are sufficient to drive irofulven sensitivity in vitro and in vivo, and we demonstrate that acquired cisplatin resistance does not induce cross-resistance to irofulven. We also defined a composite mutational signature of ERCC2 deficiency in bladder cancer that is strongly associated with cisplatin sensitivity, including in cases that lack an ERCC2 mutation, and may, therefore, be a useful tool to predict sensitivity to NER-targeting agents, such as cisplatin and irofulven.

## **2. Objectives**

- 1) Developing a clinically applicable diagnostic method to detect homologous recombination deficiency in tumor biopsies.
- 2) Diagnostic detection of homologous recombination deficiency in solid tumor types that are usually not associated with germline BRCA1 and BRCA2 mutations.
- 3) Diagnostic detection of homologous recombination deficiency in the metastatic setting.
- 4) Developing a clinically applicable diagnostic method to detect nucleotide excision repair deficiency in tumor biopsies.

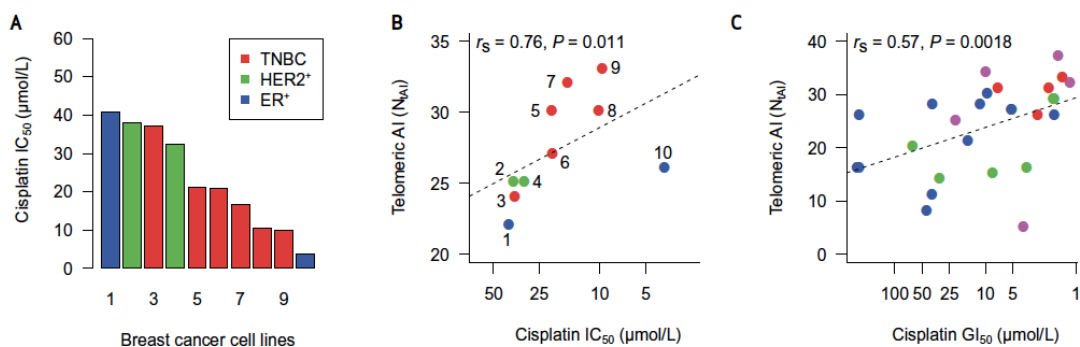
### 3. RESULTS

#### 1) Developing a clinically applicable diagnostic method to detect homologous recombination deficiency in tumor biopsies.

Homologous recombination deficiency was initially described and defined in the context of germline and somatic loss of function mutations of BRCA1 and BRCA2. However, it has become evident that tumors without mutations in these key homologous recombination enzymes may also be homologous recombination deficient. In the absence of this DNA repair mechanism (HR) specific mutational patterns accumulate in the tumor DNA and we identified the first such “DNA scarring signature”, telomeric allelic imbalance (see **publication 1**).

#### **Cisplatin Sensitivity Correlates with Burden of Telomeric AI in Breast Cancer Cell Lines**

We have downloaded raw single-nucleotide polymorphism (SNP) genotype array data from the Wellcome Trust Sanger Institute for a set of established BRCA1 wild-type breast cancer cell lines as listed in the figure. We determined the sensitivity of those cell lines to cisplatin treatment. Then we used the ASCAT algorithm to determine allele copy number changes and also to quantify allelic imbalance from the SNP array data.



**Figure 1.** Chromosomal aberrations and cisplatin sensitivity *in vitro*. The relationship between  $N_{AI}$  and cisplatin sensitivity was analyzed in breast cancer cell lines. **A** and **B**, 10 cell lines were included in this study, 1: CAMA-1, 2: HCC1954, 3: MDA-MB-231, 4: MDA-MB-361, 5: HCC1187, 6: BT-549, 7: HCC1143, 8: MDA-MB-468, 9: BT-20, and 10: T47D. **A**,  $IC_{50}$  values for each of the 10 cell lines. A proliferation assay was used to assess viability after 48 hours of cisplatin exposure, and  $IC_{50}$  was determined from the dose response curves. **B**, relationship between  $N_{AI}$  and cisplatin sensitivity. Breast cancer subtype is indicated as follows: ER<sup>-</sup>HER2<sup>-</sup>, red; HER2<sup>+</sup>, green; ER<sup>+</sup>HER2<sup>-</sup>, blue. **C**, relationship between  $N_{AI}$  and cisplatin sensitivity as determined by  $GI_{50}$  in breast cancer cell lines from Heiser and colleagues (18). Reported transcriptional subtype is indicated as follows: basal, red; claudin-low, pink; ERBB2Amp, green; luminal, blue. See Supplementary Methods for cell line identifiers.

(Source of the figure is own publication #1, Birkbak et al.)

Our focus was specifically directed at the end of the chromosomes since it is well-known that HR deficiency often leads to the formation of triradial and quadriradial

chromosome structures. These aberrant chromosomes need to be resolved at mitosis and this resolution often result in large regions of allelic imbalance and/or copy number changes extending from the cross-over to the telomere [42,43]. We hypothesized that the consequences of these of triradial and quadriradial chromosome structures can be detected by SNP array measurements in the form of either subtelomeric copy number variations or subtelomeric allelic imbalance. Furthermore, we also assumed that the presence of these subtelomeric aberrations reflect the inability of the cancer cells to resolve aberrant chromosomal structures by homologous recombination. Since lack of homologous recombination is usually associated with platinum sensitivity, we looked for an association between cisplatin sensitivity and the number of subtelomeric regions with allelic imbalance, copy gain, or copy loss.

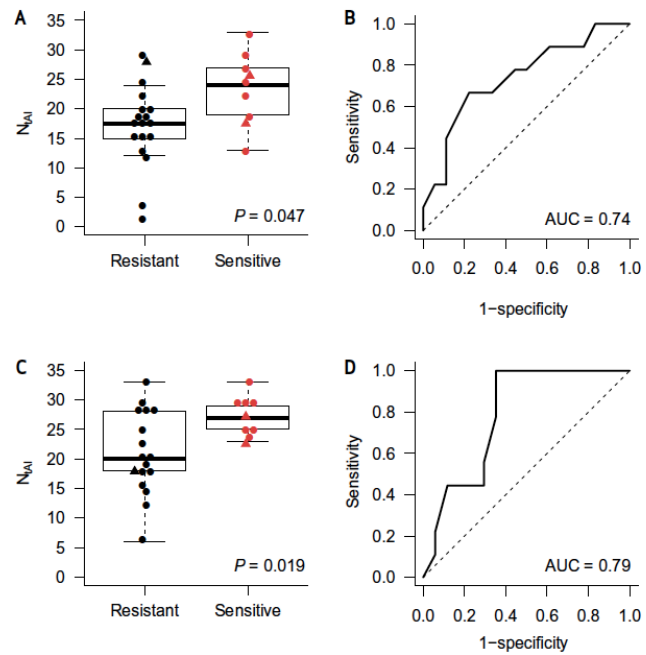
The number of regions of telomeric AI was the only summary genomic measure that was significantly associated with cisplatin sensitivity in the breast cancer cell lines ( $r_s = 0.76$ ,  $P = 0.011$ ; Fig. 1B); the correlation between NtAI and cisplatin sensitivity was stronger when the analysis was restricted to the TNBC lines (Fig. 1B, red circles;  $r_s = 0.82$ ,  $P = 0.0499$ ).

### **Tumors Sensitive to Cisplatin-Based Chemotherapy Have Greater Levels of Telomeric AI**

As the next logical step, we extended our analysis from cell lines to clinical cohorts. Remarkably, in the two clinical trials we analyzed there was also a significant correlation between NtAI in clinical tumor samples and cisplatin sensitivity (Figure 2). Sensitivity was measured by pathologic response determined after preoperative treatment.

We used ASCAT to calculate the NtAI values from SNP array data in the pretreatment tumor samples. Significant response was defined as a reduction of at least 90% in the content of malignant cells (cisplatin-sensitive) and such cases were compared to tumors with limited or no response to cisplatin (cisplatin-resistant, defined by tumor reduction of <90%).

**Figure 2.**  $N_{MI}$  and cisplatin response in breast cancer. In 2 clinical trials, patients with TNBC were given preoperative cisplatin (Cisplatin-1, **A-B**) or cisplatin and bevacizumab (Cisplatin-2, **C-D**). Cisplatin-resistant tumors are indicated in black; cisplatin-sensitive tumors are indicated in red. Tumors with germline mutations in BRCA1/2 are indicated with triangles. **A** and **C**, box plots showing  $N_{MI}$  distribution in cisplatin-resistant and -sensitive tumors. **B** and **D**, ROC curves showing the ability of  $N_{MI}$  to predict for sensitivity to cisplatin.



This method has become the first component of an FDA approved diagnostic mutational signature, the myChoice HRD assay. This is routinely used now for the prioritization of ovarian cancer patients for PARP inhibitor therapy. Our method was initially developed for SNP arrays. However, next generation sequencing has largely replaced this hybridization-based technology and we converted and validated our method for next generation sequencing based platforms (see **publication 2**).

## 2) Migrating the SNP array-based homologous recombination deficiency measures to next generation sequencing data

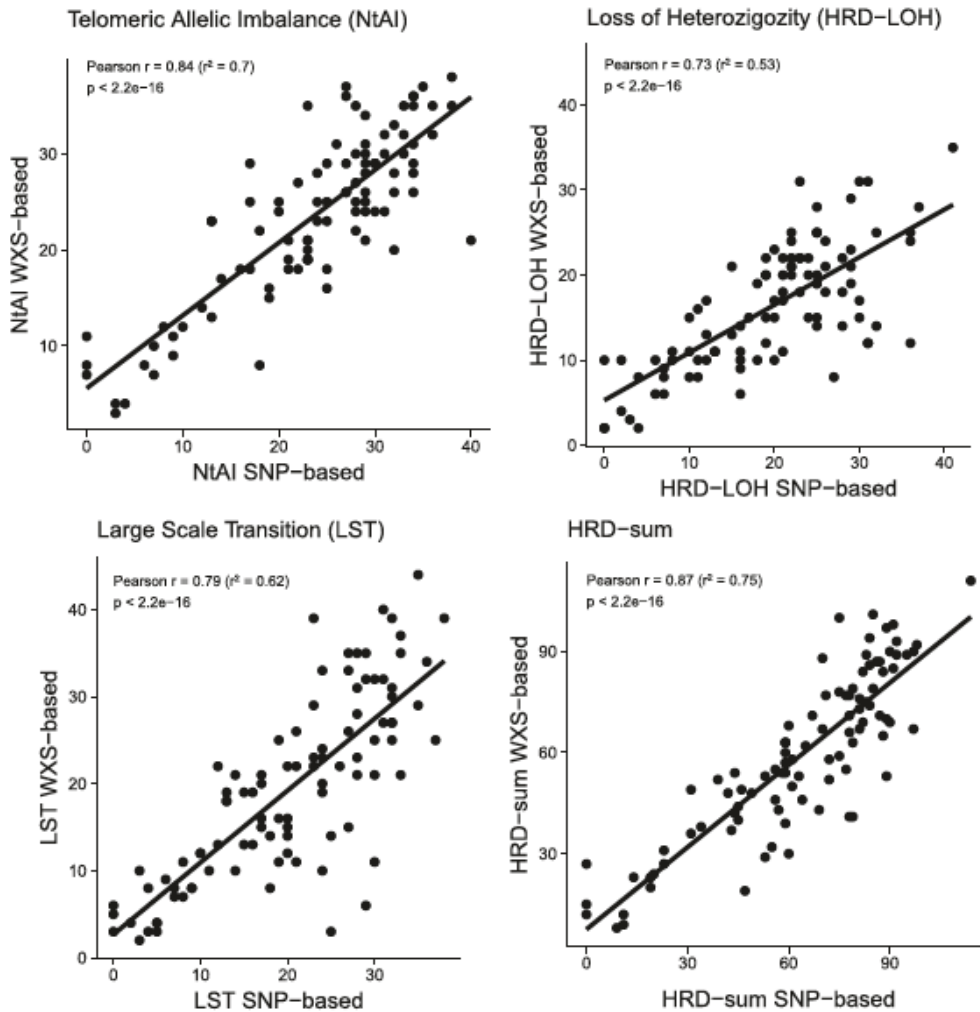


Figure 3: Correlation between Affymetrix SNP 6.0 array-based and whole exome sequencing-based measurements of homologous recombination deficiency (telomeric allelic imbalance, loss of heterozygosity, large-scale transitions, and the sum of these estimates)

(Source of figure is own publication #2, Sztupinski et al. )

As outlined above, the first genomic scar-based homologous recombination deficiency (HRD) measures were produced using SNP arrays. As array-based technology has been largely replaced by next generation sequencing approaches, it has become important to develop algorithms that derive the same type of genomic scar scores from next generation sequencing (whole exome ‘WXS’, whole genome ‘WGS’) data. In order to perform this analysis, we developed the scarHRD R package and show that using this method the SNP array-based and next generation sequencing-based derivation of HRD scores show good correlation (Pearson correlation between 0.73 and 0.87 depending

on the actual HRD measure) and that the NGS-based HRD scores distinguish similarly well between BRCA mutant and BRCA wild-type cases in a cohort of triple-negative breast cancer patients of the TCGA data set (Figure 3).

As the independent citations show, this method is gaining popularity and acceptance in the field as an increasing number of groups are using it to calculate HR deficiency scores from next generation sequencing data.



3) **Diagnostic detection of homologous recombination deficiency in solid tumor types that are usually not associated with germline BRCA1 and BRCA2 mutations.**

Homologous recombination deficiency was described in the context of the loss of function mutations of BRCA1 and BRCA2. Therefore, the efficacy of PARP inhibitors, the agents specifically targeting homologous recombination deficient tumors, was first explored in tumor types that are most frequently associated with such mutations, namely ovarian and breast cancer. Initially PARP inhibitors were evaluated for tumor cases in BRCA1 or BRCA2 mutation carriers and this strategy is still used, for example, in the case of prostate cancer. However, homologous recombination deficiency can be present even in the absence of BRCA1 or BRCA2 mutations in ovarian and breast cancer and those could be detected by the HR deficiency associated mutational signatures (see **publication 1**). We decided to determine the frequency of homologous recombination deficient cases, as detected by HR deficiency associated mutational signatures, in prostate cancer (**publication 4**).

It is already known that prostate cancers with mutations in key genes of the homologous recombination (HR) machinery, most commonly BRCA2, significantly benefit from PARP inhibitor and platinum-based chemotherapy [22,44]. We hypothesized that prostate tumors that do not harbor deleterious mutations in these particular genes can still be deficient in HR if the activity of HR genes are suppressed by other mechanisms such as promoter methylation. Such tumors are expected to show the specific HR deficiency associated mutational signatures and they may also be sensitive to HR-directed therapies such as PARP inhibitors.

As a positive control we identified all prostate cancer cases with inactivating mutations in BRCA1 or BRCA2 with a corresponding loss of heterozygosity. We found that these BRCA1/2-deficient samples showed all previously described HRD-associated mutational signatures in the WGS data. As we hypothesized, the HRD-associated mutational signatures were also detected in a subset of patients who did not harbor germline or somatic mutations in BRCA1/2 or other HR-related genes (Figure 4).

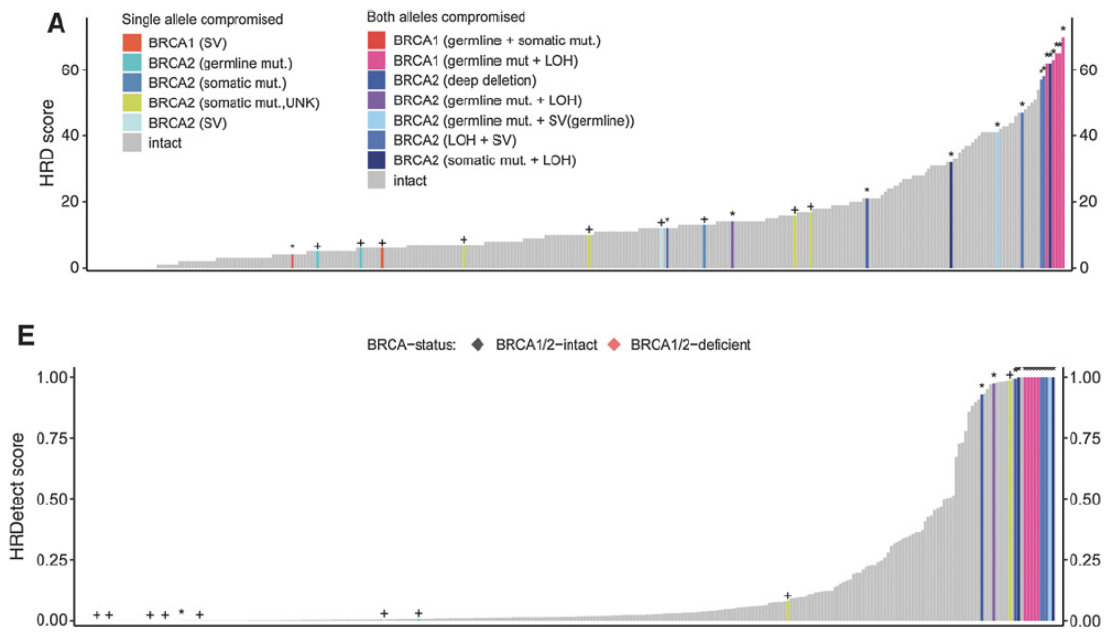


Figure 4: Summary of the HRD-related predictors in the whole-genome datasets. A: HRD score: the sum of the three allele-specific CNV-derived genomic scars; E: HRDetect.

(Source of figure own publication #3, Sztupinski et al. )

Similar results, albeit with lower sensitivity and accuracy, were also obtained from WES data.

These findings will probably expand the number of cases likely to respond to PARP inhibitor treatment. On the basis of the HR-associated mutational signatures, 5% to 8% of localized prostate cancer cases may be good candidates for PARP-inhibitor treatment (including those with BRCA1/2 mutations). Our findings are currently validated and evaluated in several PARP inhibitor based prostate cancer clinical trials.

#### 4) Diagnostic detection of homologous recombination deficiency in the metastatic setting.

We also investigated whether HR deficiency associated mutational signatures differ in primary tumors and their matched metastasis. In particular, we compared the various HR deficiency induced genomic aberrations in primary breast cancer and their patient matched brain metastasis (see **publication 3**).

All of the mutational signatures indicative of HR deficiency showed a significant increase in the brain metastases relative to their matched primary tumor in the previously published whole exome sequencing dataset. (Figure 5)

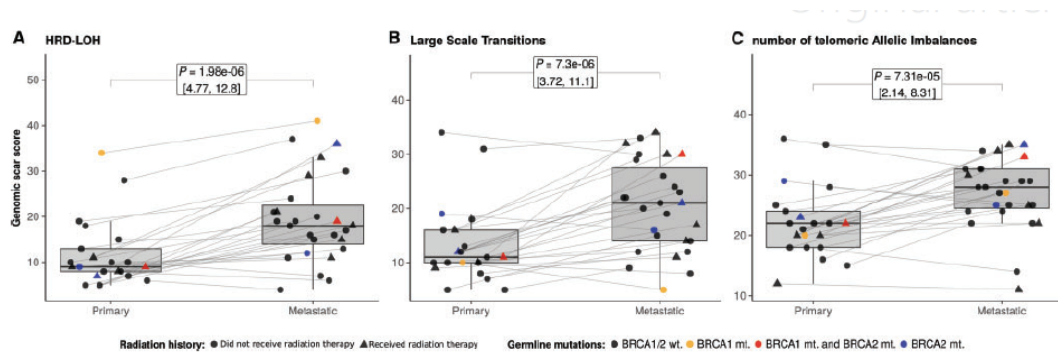


Figure 5: Genomic scar scores of the Brastianos et al. [41] breast cancer brain metastasis samples. Distributions of the homologous recombination deficiency–loss of heterozygosity (HRD-LOH) (A), large-scale state transitions (LST) (B) and number of telomeric allelic imbalances (ntAI) (C) scores. The corresponding primary-metastatic pairs are connected by thin lines. Scores for each of these measures were increased in metastases compared with primary tumors.

(Source of figure own publication #4, Diossy et al.)

In the independent validation cohort, the myChoice HRD assay showed an increased level in 87.5% of the brain metastases relative to the primary tumor, with 56% of brain metastases being HRD positive according to the myChoice criteria. (Figure 6)

The consistent observation that brain metastases of breast cancer tend to have higher HRD measures may raise the possibility that brain metastases may be more sensitive to PARP inhibitor treatment. This observation warrants further investigation to assess whether this increase is common to other metastatic sites as well, and whether clinical trials should

adjust their strategy in the application of HRD measures for the prioritization of patients for PARP inhibitor therapy.

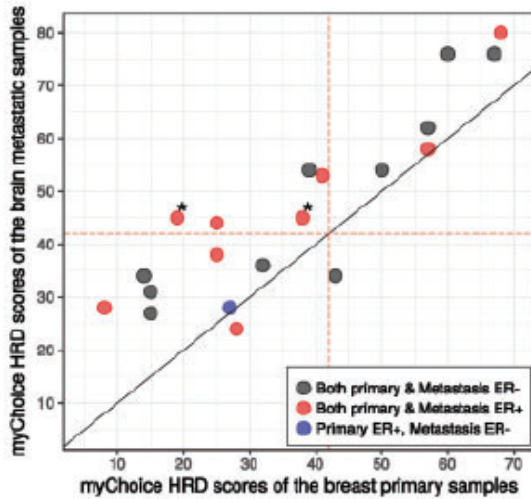


Figure 6: Summary of the analysis carried out on the validation cohort by Myriad Genetics. Genomic scar scores determined by Myriad Genetics summed together into a single HRD score. HRD scores were increased in metastases compared with primary tumors

(Source of figure own publication #4, Diossy et al.)

**5) Developing a clinically applicable diagnostic method to detect nucleotide excision repair deficiency in tumor biopsies (publication 5).**

We also explored whether specific diagnostic mutational signatures can be derived for other types of DNA repair pathway aberrations as well. We were particularly interested in nucleotide excision repair (NER) deficiency. Preclinical studies suggested that the experimental cancer therapy drug, iriffulven, may possess a synthetic lethal efficacy against NER deficient cancer similar to that seen for PPAR inhibitors in the context of HR deficient cancer. However, due to the lack of reliable identification of NER deficient cancer cases, this hypothesis could not be tested in advanced clinical settings. In fact, most phase 2 clinical trials of iriffulven showed only limited clinical efficacy, since patients were not prioritized based on their NER status.

Prior evidence suggested that NER deficiency is likely to be present in a subset of bladder cancer cases. Cisplatin-based chemotherapy is a first-line treatment for muscle-invasive and metastatic urothelial cancer. Approximately 10% of bladder urothelial tumors have a somatic missense mutation in the nucleotide excision repair (NER) gene, ERCC2, which confers increased sensitivity to cisplatin-based chemotherapy. However, a significant subset of patients is ineligible to receive cisplatin-based therapy due to medical contraindications, and no NER-targeted approaches are available for platinum-ineligible or platinum-refractory ERCC2-mutant cases.

We used a series of NER-proficient and NER-deficient preclinical tumor models to test sensitivity to iriffulven, an abandoned anticancer agent.

In addition, we used available clinical and sequencing data from multiple urothelial tumor cohorts to develop and validate a composite mutational signature of ERCC2 deficiency and cisplatin sensitivity. (Figure 7)

This composite mutational signature was derived as follows. We used the TCG bladder cancer WES data (396 samples) to determine which types of mutational signatures are associated with ERCC2 mutational status. As Figure 7/A shows we found that single nucleotide variation based mutational signatures 2, and 5, short indel signatures ID8, ID10, ID2, double nucleotide variation signature DSB4, and transcribed strand bias ratios were associated with ERCC2 mutational status. We combined these signatures into a logistic regression-based classifier (Figure 7/A).

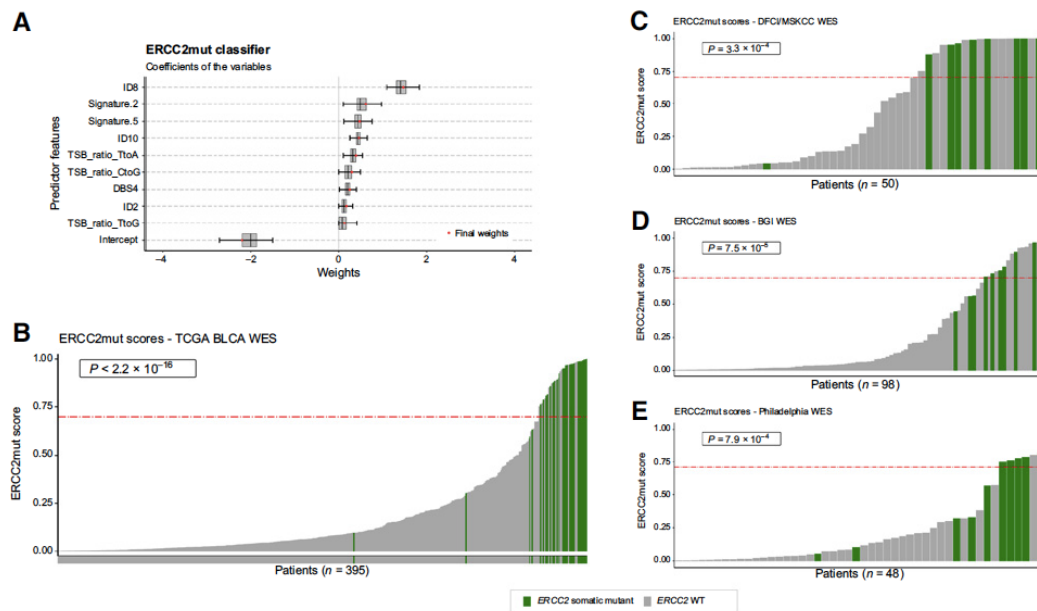


Figure 7: Development of the ERCC2mut logistic regression-based classifier and validation of the association between ERCC2mut scores and ERCC2 mutation status in three independent bladder cancer cohorts. Samples with an ERCC2 mutation are shown in green and WT ERCC2 samples are shown in gray. A, Nine mutational features were significantly associated with ERCC2 mutation status in TCGA bladder cancer WES cohort and were used to develop the composite ERCC2mut score. B, ERCC2mut signature scores are strongly associated with ERCC2 mutation status in TCGA bladder cancer cohort. A value of  $\geq 0.70$  maximally separates ERCC2-mutant from WT cases and is denoted by the red horizontal dash-dotted line. In each validation cohort, ERCC2 mutants were highly enriched among patients with a high ERCC2mut score ( $\geq 0.70$ ).

(Source of figure own publication #5, Borcsok et al.)

The ERCC2 mutational signature classifier was validated on three independent cohorts as shown on Figure 7 C, D and E.

It was notable that in addition to the 10-15% ERCC2 mutant cases an additional 10-15% of bladder cancer cases show robust signs of the same NER deficiency mutational signatures and those were also sensitive to platinum-based therapy, an indicator of NER deficiency. (Figure 8)

We identified a novel synthetic lethal relationship between tumor NER deficiency and sensitivity to irifolven. Irifolven specifically targets cells with inactivation of the transcription coupled NER (TC-NER) pathway and leads to robust responses in vitro and in vivo, including in models with acquired cisplatin resistance, while having minimal effect on cells with intact NER. We also found that a composite mutational signature of

ERCC2 deficiency was strongly associated with cisplatin response in patients and was also associated with cisplatin and irifolven sensitivity in preclinical models.

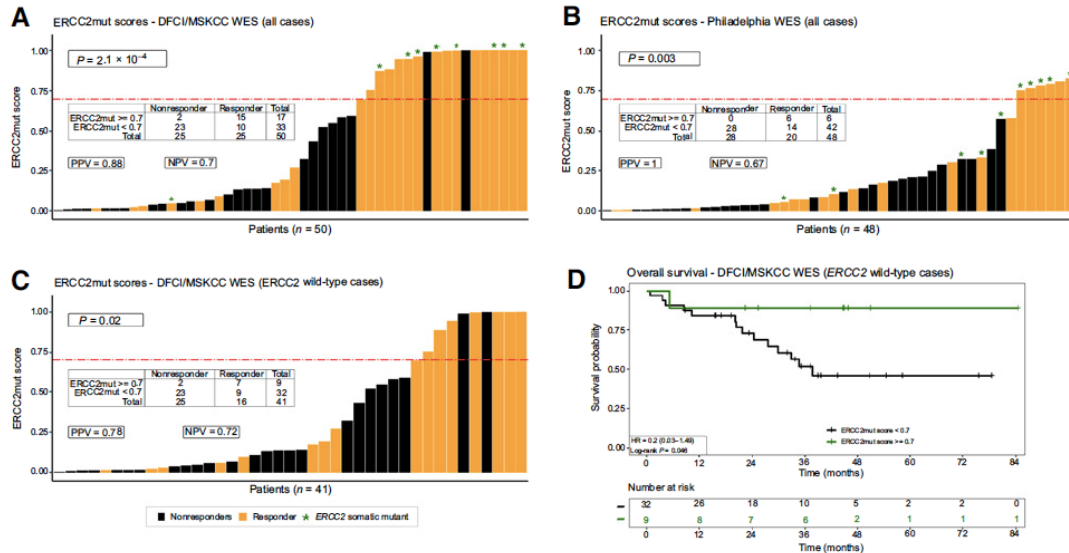


Figure 8: ERCC2mut signature scores are associated with cisplatin response, including among WT ERCC2 cases. Cisplatin responders are colored in orange and non-responders are in black. ERCC2-mutant cases are denoted by green asterisks. A, ERCC2mut signature scores for all cases in the DFCI/MSKCC cohort (n=50). B, ERCC2mut signature scores for all cases in the Philadelphia cohort (n=48). C, ERCC2mut signature scores for WT ERCC2 cases in the DFCI/MSKCC cohort (n=41); high ERCC2mut signature scores ( $\geq 0.70$ ) were significantly associated with cisplatin response. D, overall survival for patients with WT ERCC2 tumors in the DFCI/MSKCC cohort. Overall survival was significantly longer for WT ERCC2 patients with ERCC2mut signature scores  $\geq 0.70$ .

(Source of figure own publication #5, Borcsok et al.)

Tumor NER deficiency confers sensitivity to irifolven, a previously abandoned anticancer agent, with minimal activity in NER-proficient cells. A composite mutational signature of NER deficiency may be useful in identifying patients likely to respond to NER-targeting agents, including cisplatin and irifolven.

These observations form the basis of a soon to be started clinical trial of irifolven treatment in bladder cancer.

#### 4. DISCUSSION

We analyzed neoadjuvant platinum treated triple negative breast cancer (TNBC) clinical trials in which pathologic response at the time of surgery provided the clinical endpoint. Sporadic TNBCs are heterogeneous in their responses to cisplatin therapy, which are chemotherapeutic agents that depend on various DNA repair defects, such as homologous recombination deficiency or nucleotide excision repair deficiency, for their therapeutic activity [45]. In particular, loss of function aberrations of the BRCA1 or BRCA2 genes can also lead to platinum sensitivity; we hypothesized that the types of large scale chromosomal aberrations induced in the context of BRCA1/2 dysfunction might also be associated with platinum sensitivity in wtBRCA cancers.

Cell line experiments pointed in the direction of one particular chromosomal abnormality, telomeric AI. Such aberrations in the genomic data of pretreatment tumor biopsies were associated with pathologic response to cisplatin therapy. The summary measure of those aberrations, NtAI was significantly associated with response to platinum treatment in our TNBC cisplatin trials and in platinum-treated serous ovarian cancer. These findings established NtAI as a component of the clinically applicable measures of HR deficiency. Our findings also suggested that the burden of this genomic abnormality is induced by an underlying deficiency of DNA repair in the platinum-sensitive subset of these cancers. AI propagated from a given chromosomal location to the telomere suggests some form of error-prone processes, such as break induced repair, abnormal cross-overs or template switching events, rather than error-free DNA repair. In conclusion, a summary measure of telomeric chromosome aberrations in the tumor genome, NtAI, predicts sensitivity to platinum treatment and quantified HR deficiency.

Our work (publication 3) was the first demonstrating that the various DNA aberration- based HRD measures are significantly higher in brain metastases relative to their primary breast tumor counterparts. Our results were recently validated by independent groups and presented at the AACR conference: ([https://cancerres.aacrjournals.org/content/81/4\\_Supplement/PD13-01.short](https://cancerres.aacrjournals.org/content/81/4_Supplement/PD13-01.short))

This increase in homologous recombination deficiency has several clinical consequences. Ideally, only patients sensitive to a given therapy should receive that particular treatment. In the case of PARP inhibitors, it is likely that mainly patients with



HR deficiency benefit from this form of therapy. Therefore, correlating the clinical benefit of PARP inhibitor therapy with HR status of the tumors is an important field of investigation [47,48]. It is important to note that HR status is most often determined in biopsies derived from the primary tumors but clinical benefit is determined at a more advanced, often metastatic stage of the disease. Therefore, it is important to realize that if the HR deficiency status is significantly different in the brain metastases relative to the primary tumors then the observed correlations will be distorted leading to contradictory clinical results. For example, in the case of breast cancer repeated observations demonstrated a correlation between the nTAI score and response to platinum-based therapy [10]. The TNT trial [49], however, studied the efficacy of platinum-based therapy in locally advanced or metastatic triple-negative breast cancer and failed to find a correlation between the myChoice HRD score and response to carboplatin. It should be noted, however, that the myChoice HRD score was determined on the primary tumors and not on the metastases. The discrepancy between the myChoice HRD score of the primary and metastatic sites presented in our work may be partially responsible for this lack of correlation.

There are several clinical trials evaluating the potential clinical benefit of PARP inhibitor treatment in metastatic breast cancer, including NCT02723864 (<https://clinicaltrials.gov/ct2/show/NCT02723864>). This trial, however, excludes patients with active brain metastasis. Based on our results, it seems likely that breast cancer brain metastases might be a particularly sensitive population.

The SWOG trial S1416 is a Phase II Randomized Placebo-Controlled Trial of Cisplatin with or without ABT-888 (Veliparib) in Metastatic Triple-Negative Breast Cancer (TNBC) and/or BRCA Mutation-Associated Breast Cancer. This trial includes patients with brain metastasis but excludes patients with estrogen receptor positive breast cancer. As we showed in our validation cohort, the majority of estrogen receptor positive breast cancer brain metastasis cases showed high myChoice HRD scores. It might be worth establishing the proportion of HR deficient ER+ breast cancer cases on a larger cohort as those patients might be responding to PARP inhibitors as well.

The reassessment of frequency of HR deficient cases in prostate cancer may also lead to significant changes in clinical practice. Personalized therapy of prostate cancer has entered a new phase since the demonstration of the clinical efficacy of PARP

inhibitors and platinum in cases with mutations in the DNA repair pathway, especially homologous recombination [22,44]. However, some of the DNA damage checkpoint gene germline mutants do not benefit from PARP inhibitor therapy [50] and some cases without such mutations are sensitive to platinum-based therapy [44,51]. Therefore, the optimal use of these therapeutic approaches will require more accurate identification of HR deficient cases. We decided to investigate the utility of HRD-induced mutational signatures here. As a positive control we analyzed cases with inactivating mutations in key HR genes (such as BRCA1/2, RAD51, PALB2 etc.). Such cases showed all three types of HRD-induced mutational signatures. Surprisingly, we also found that there is a significant number of cases without inactivating mutations in HR genes that also demonstrate the same HRD-induced mutational signatures. This strongly suggests that there are additional prostate cancer cases that will likely benefit from PARP inhibitor-based therapy. A recent clinical report supports this assumption where they found at least one case without germline mutations of HR genes with exceptionally good response to platinum-based therapy showing HRD score based on WES analysis [51]. Clinical trials, prioritizing patients based on such molecular signatures, are currently underway at several clinical centers to further validate this hypothesis.

HR deficiency is not the only DNA repair that can potentially be targeted using a synthetic lethality-based strategy. We characterized a novel synthetic lethal relationship between tumor NER deficiency and the abandoned anticancer drug, irifolven, and we showed that NER deficient tumors have a unique mutational signature that may expand identification of patients likely to respond to NER targeted agents, such as cisplatin or irifolven.

Cisplatin-based chemotherapy is the most frequently applied treatment for patients with muscle-invasive or metastatic bladder cancer. Mutations in ERCC2 or other DNA repair genes are present in approximately 20% of bladder tumors and are associated with cisplatin response rates of 80%-100% [27,30]. However, despite the demonstrated activity of cisplatin in patients with tumor NER deficiency, 30%-50% of patients with bladder cancer are unable to receive cisplatin due to renal dysfunction or other medical comorbidities. For these patients, other agents that target tumor NER deficiency represent an attractive therapeutic strategy. On the basis of its mechanism of action and on its reported activity in NER-deficient nontumor models, we elected to investigate irifolven,

a semisynthetic alkylating agent previously tested as an anticancer agent in a number of phase I/II trials [37,38]. Irofulven response rates were very modest (10%-15%) in unselected patient populations. These clinical trials, however, did not prioritize patients for NER deficiency, due to a lack of appropriate diagnostic tools. Our functional analyses showed that NER-deficient tumor cells were profoundly sensitive to irofulven both in vitro and in vivo. Our diagnostic mutational signature will allow such prioritization and currently we are preparing a prospective clinical trial at the Rigshospitalet (Copenhagen, Denmark) of irofulven for patients with advanced urothelial tumors harboring predicted NER dysfunction as defined by NER gene defects (with or without LOH) or the presence of a high ERCC2mut signature score.

In summary, we identified a novel synthetic lethal relationship between tumor NER deficiency and sensitivity to the previously discarded anticancer agent, irofulven, and we show that NER deficiency is sufficient to drive sensitivity to irofulven in cisplatin-sensitive and cisplatin-resistant tumor models. Furthermore, we developed and validated a composite mutational signature of ERCC2 deficiency that is strongly associated with NER deficiency and irofulven sensitivity in preclinical models and also correlates with clinical cisplatin response, including in cases that lack an ERCC2 mutation.

## 5. CONCLUSIONS

We showed that specific DNA repair pathway aberrations (homologous recombination and nucleotide excision repair deficiencies) are associated with diagnostically applicable mutation signatures. We developed methods to detect such signatures from either fresh frozen or formalin fixed paraffin embedded (FFPE) material. Our methods are applicable both in whole exome and whole genome sequencing.

We showed that HR deficiency can be reliably detected in solid tumor biopsies and that the diagnostic detection of such signatures has clinical relevance. It can identify patients with PARP inhibitor sensitivity in ovarian, breast and prostate cancer.

We also showed that diagnostic signatures can be derived for another DNA repair pathway aberration, nucleotide excision repair deficiency. This signature will be essential to introduce irifolven therapy to bladder cancer patients, especially for those patients that are ineligible for platinum-based therapy.

## 6. SUMMARY

In summary, we developed diagnostic mutational signatures that can effectively direct the application of certain targeted therapies, which derive their efficacy from a synthetic lethal relationship with specific DNA repair pathway aberrations. Thus, we established clinically applicable combinations of companion diagnostics and DNA repair pathway aberration specific therapeutic agents.

We have significantly extended the use of diagnostic mutational signatures of homologous recombination deficiency for other solid tumor biopsies including brain metastases of breast cancer and prostate cancer.

We have also established a novel diagnostic mutational signature for nucleotide excision repair deficiency that could serve as a companion diagnostic for irifolven therapy.

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