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Genetics and Genomics

Capturing breast cancers' copy-number landscape in routine pathology: Exploiting low-resolution, genome-wide sequencing to identify HRD and beyond

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BACKGROUND: Because breast cancer (BC) is molecularly heterogeneous, diagnosis and treatment will likely benefit from comprehensive genetic profiling. However, routine, high-resolution sequencing is not feasible yet, due to implementation challenges associated with whole-genome sequencing of formalin-fixed paraffin embedded (FFPE) BC samples. Therefore, we explored the potential of an alternative low-resolution, genome-wide testing approach that is able to capture the copy number (CN) landscape, including actionable alterations, in FFPE derived DNA.

METHODS: The performance of the genome-wide CN testing approach, including CN signatures/focal CN alterations, was evaluated in two phases: (i) exploration and (ii) feasibility phase. First, high-resolution sequencing data of a previously published triple-negative BC cohort ($n = 237$) was leveraged to benchmark the homologous recombination deficiency (HRD)-related CN signature using a comprehensive, multimodal approach incorporating both genetic and functional HRD tests. Secondly, the low-resolution testing strategy's feasibility was prospectively evaluated in a BC cohort of patients referred to clinical genetic services ($n = 147$).

RESULTS: Applying the HRD threshold that was established using both genomic and functional HRD data, we identified a 100% sensitivity for BC with *BRCA1/BRCA2/PALB2* pathogenic variants in the prospective cohort. Moreover, the success rate of the low-resolution testing approach proved high, regardless of input material. Finally, additional CN alterations were enriched in the HR-proficient BC population, indicating potential actionable CN-alterations beyond HRD.

CONCLUSIONS: In conclusion, low-resolution, genome-wide sequencing has shown high potential in capturing the CN landscape, including features associated with HRD, in BC patients. This preselection testing approach is likely to maximize potential for personalized medicine and genetic counseling.

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BACKGROUND

Breast cancer (BC) is a heterogeneous disease, exhibiting heterogeneity at the clinical [1, 2], molecular [3, 4], and genetic level [5–7]. Given the significant implications of this heterogeneity for both diagnosis and clinical decision making, this warrants capturing the genetic tumor profile in a comprehensive manner. Unfortunately, the high global incidence (2,000,000 new diagnoses of BC annually [8]) renders this financially and logistically unfeasible, even with significantly reduced sequencing costs taken into account.

While the role of various focal copy number (CN) alterations, particularly *ERBB2* [9], has been established in BC, recently, attention has been focused on defective homologous recombination (HR) DNA repair pathway (i.e., homologous recombination deficiency (HRD) [10–13]), which can be targeted through platinum-based chemotherapy or PARP inhibition (PARPi) [14, 15]. Additionally, the HRD phenotype identifies a BC subgroup that is enriched for germline pathogenic variants (PV) in one of the HR-related cancer-susceptibility genes, i.e., *BRCA1, BRCA2, PALB2* (referred to as: high-risk genes).

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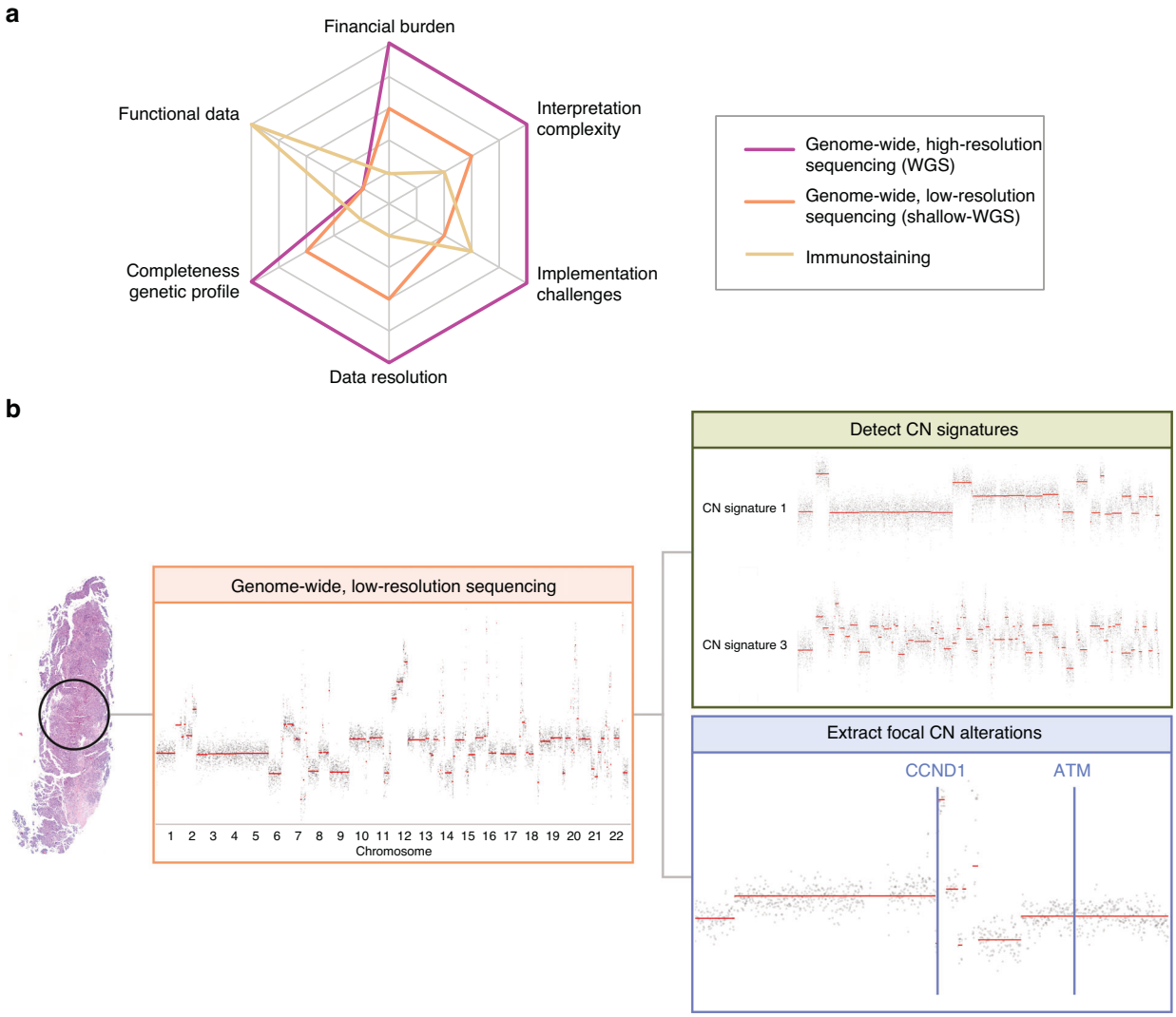


Fig. 1 Positioning and workflow of genome-wide low-resolution sequencing in breast cancer diagnostics. a Spider plot illustrating the positioning of various biomarker platforms (genome-wide, high-resolution sequencing, genome-wide, low-resolution sequencing, immunostaining) across six test specifications. **b** Workflow of genome-wide, low-resolution sequencing in routine breast cancer diagnostics. FFPE DNA of breast cancer specimens (resection, biopsy or ascites) was isolated and shallow-WGS was performed. Absolute CN profiles were used to (i) detect CN signatures, including HRD, and, (ii) extract focal CN alterations of interest. WGS whole genome sequencing, CN copy number, HRD homologous recombination deficiency.

Multiple studies aimed to capture an HRD phenotype through genomic-based [10, 11, 13] and/or functional [16–23] approaches. However, the lack of a gold standard to define HRD has hampered clinical implementation. Additionally, advanced and comprehensive models, including, but not restricted to, HRDetect [10, 12], predominantly require whole-genome sequencing (WGS). The inherent disadvantage of this sequencing platform lies in the current inability to implement WGS for all BC patients in routine diagnostic care due to the absence of fresh-frozen tissue, the commonly limited tissue quantity available for most patients, as well as high financial burden (Fig. 1a). Therefore, there is an urgent clinical need for an alternative testing approach in routine diagnostics that can be applied in a broad population of BC patients to select eligibility for targeted therapy e.g., potential targeting of focal CN alterations (as *ERBB2* [9], *CCNE1* [24], *MYC* gene amplification) and CN signatures [25], including those associated with HRD. Simultaneously, such an alternative test could aid in pre-selecting BC patients with HRD tumors for genetic counseling and germline testing at a clinical genetic service.

The solution to this unmet clinical need may lie in a testing approach that allows for sequencing the entire genome, albeit at a lower resolution (e.g., sequencing depth), and that is applicable to routinely available clinical tissue, typically FFPE tissue. A notorious example of such a platform is shallow-WGS. The additional benefit from a low- over high-resolution sequencing approach is twofold (Fig. 1a). First, genome-wide approaches allow for the identification of CN phenomena, including signatures and focal alterations, providing a more thorough genetic profile compared to smaller targeted sequencing panels (Fig. 1b). Furthermore, the reduced financial burden associated with low-resolution sequencing allows for a broader, more inclusive usability in routine BC diagnostics.

Here, we describe a genome-wide, CN-based tumor test, capable of detecting CN signatures, including an HRD-associated CN signature, and focal CN alterations which might identify additional therapeutic targets. The performance of this genome-wide CN testing approach in BC was first evaluated in a retrospective setting using a triple-negative BC cohort (TNBC; SCAN-B cohort ($n = 237$ [12, 26])) to establish the HRD read-out using multimodal benchmarking (i.e., exploration phase). Next, we

assessed the feasibility of implementing a low-resolution, genome-wide 'shallow-WGS' testing strategy in routine pathology using a prospective real-life scenario ($n = 147$; i.e., feasibility phase). Overall, we showed that the use of the proposed low-resolution testing approach is feasible for routine BC pathology and has the potential to capture both HRD as well as CN alterations, such as *ERBB2* gene amplification, with a high sensitivity.

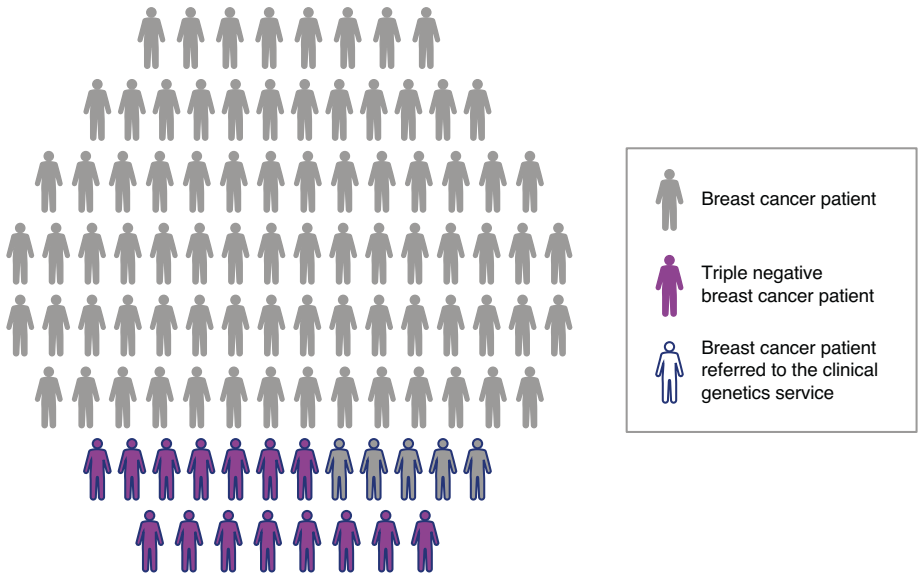
RESULTS

Exploration phase

The SCAN-B cohort [12, 26, 27], including $n = 237$ TNBC (Methods section; Fig. 2; Supplementary Fig. 1A; Supplementary Table 1), was positioned as an exploration cohort.

Benchmark homologous recombination deficiency. The exploration cohort was first used to benchmark HRD-associated CN signatures (quantified using WGS data; Methods section). First, CN signature contributions (CN signature 1–17) were extracted from whole genome sequencing data, as detailed by Drews and colleagues [25].

Since HRD extends beyond tumors with a PV in *BRCA1*, *BRCA2* or *PALB2*, we decided to benchmark beyond genetic HR defects. In light of the absence of a gold standard in HRD testing, we integrated three advanced HRD read-outs to establish an unbiased 'hypothetical ground truth', i.e., two DNA-based HRD tests (HRDetect [10] and HRD score [13]) and one functional HRD test (RAD51-FFPE [16]). Tumors were defined as either HRD or homologous recombination proficient (HRP) only if the three different read-outs agreed (hypothetical ground truth HRD; 93/237 (39%), hypothetical ground truth HRP; 34/237 (14%)), increasing the likelihood of an accurate classification



	Study design			Yield	
	Cohort (*n*)	Phase	Goal	HRD	Beyond
	237	Exploration	Multimodal benchmarking of HRD	67%	• Additional CN signatures
	147	Feasibility	Feasibility testing in real-life clinical scenario	56%	• Focal alterations (*CCND1*, *CCNE1*, *EGFR*, *ERBB2*, *MYC*, *PIK3CA*)

Fig. 2 Overview of study design. Circular plot represents the breast cancer population, with an annual global incidence of 2,000,000 patients [8]. The study, aimed to evaluate the performance of a low-resolution copy number testing approach comprises two phases, including selected BC populations: (i) an exploration phase, and, (ii) a feasibility phase. The first, exploration phase, includes triple-negative BC patients ($n = 237$), and is positioned to perform multimodal benchmarking of HRD. The second, feasibility phase, includes a BC population of patients that are referred to the clinical genetics service in The Netherlands [39]. In this second phase, the feasibility of the low-resolution testing approach will be tested in a real-life clinical scenario. The yield of potentially actionable CN alterations, including HRD and beyond, in the two selected BC populations are detailed in the table. Details on the yield of HRD and other potential actionable CN alterations in the unselected BC population are lacking, since studies exploring the prevalence of above-described CN events in unselected BC are sparce due to anticipated low yield and high costs. Overall, the low-resolution testing approach, as presented in this study, has a lower financial burden, and therefore aids in expanding accessibility to tumor testing in unselected BC. BC breast cancer, HRD homologous recombination deficiency, CN copy number.

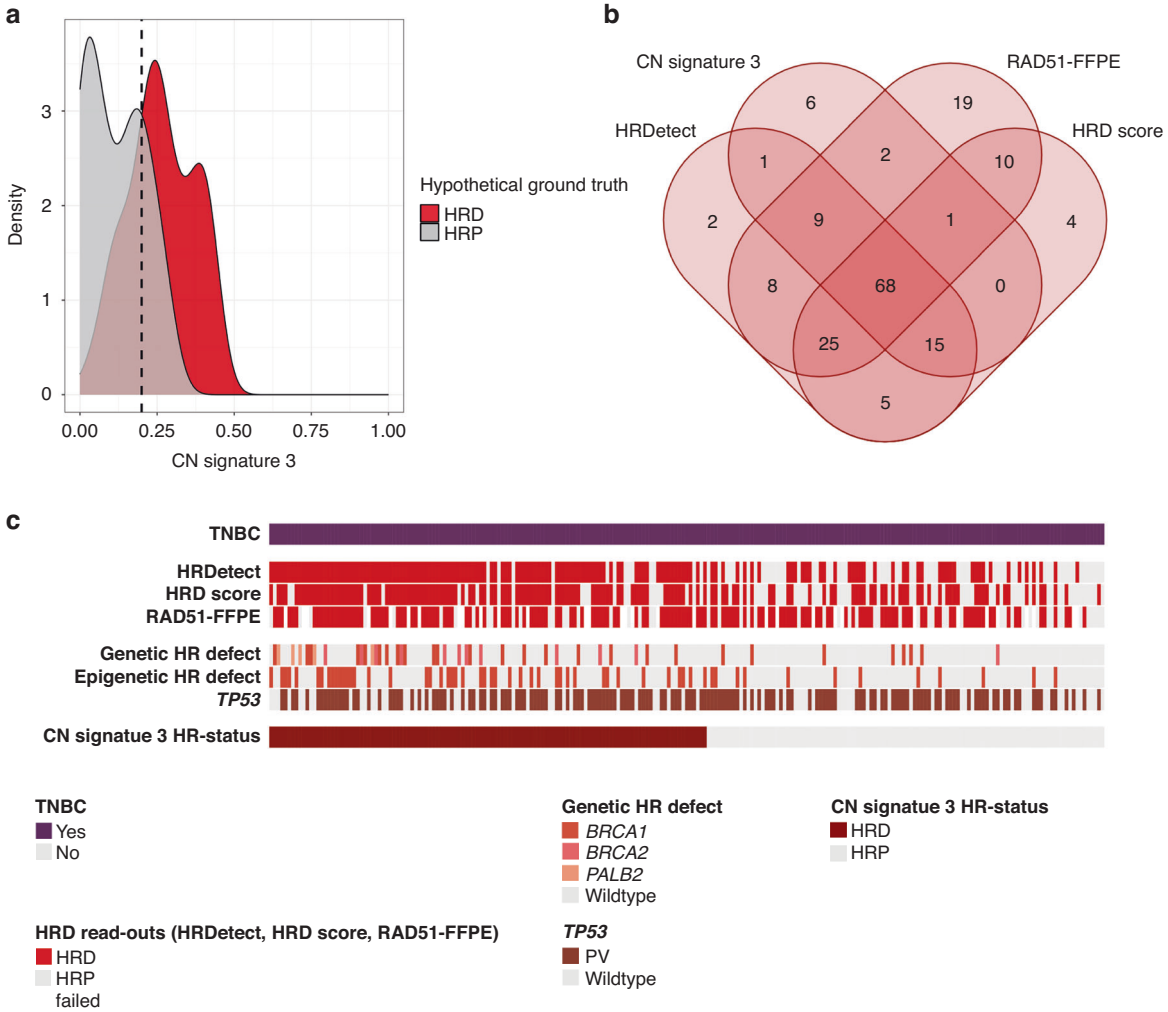


Fig. 3 Multimodal benchmarking of HRD (CN signature 3) in the exploration cohort, using both genomic and functional HRD read-outs. **a** In light of the absence of a gold standard in HRD testing, we integrated three advanced HRD read-outs to establish an unbiased 'hypothetical ground truth' (HRDetect [10]), HRD score [13], and RAD51-FFPE test [16]. Next, a density plot was visualized of CN signature 3 contributions in TNBC that were HRD (red) or HRP (grey), based on the hypothetical ground truth. The density plots are intersecting at a CN signature 3 contribution of 0.20 that was subsequently used as an HRD threshold. **b** Venn diagrams showing the identification of TNBC ($n = 175$) with HRD classification in at least one of four tests. In $n = 68/175$ (39%) of TNBC were classified as HRD by all four HRD tests. **c** Landscape of HRD read-outs in TNBC. The TNBC are ordered on CN signature 3 contribution. CN copy number, HR homologous recombination, HRD HR deficient, HRP HR proficient, PV pathogenic variant.

as either HRD or HRP. Notably, Drews et al. [25] have associated three CN signatures (CN signatures 2, 3, and 5) with HRD. To identify the CN signature with the highest feature importance in predicting the hypothetical ground truth, we employed a random forest model using these three CN signatures. We found that CN signature 3 had the highest feature importance, as indicated by high Gini score (Supplementary Fig. 2A). Consequently, we selected this CN signature (CN signature 3) as our HRD signature of interest.

The densities of CN signature 3 contributions in these 'ground truth' HRD and HRP tumors were clearly separated and intersected at 0.20, which was subsequently used as HRD threshold in our study (Fig. 3a; Supplementary Fig. 2B–D). Applying this threshold, the accuracy of CN signature 3 to reflect 'true' HRD and HRP TNBC, using the hypothetical ground truth was 75% (95% CI: 66–82%) in the exploration cohort (Supplementary Table 2C).

To evaluate the performance of the benchmarked CN signature 3 threshold, sensitivity and specificity were determined for three levels: (i) genetic HR defects, (ii) (epi)genetic HR defects and (iii) individual HRD read-outs (HRDetect, HRD Score, RAD51-FFPE test). These three levels provide decreasing certainty in explaining HRD. Importantly, the fact that our established threshold was determined using an 'hypothetical ground truth' allows for the tests' sensitivity to identify (epi)genetic HR defects to be tested in an unbiased manner, meaning not training on BC with a *BRCA1/2* PV only.

A sensitivity of 81% (95% CI: 67–92%) in detecting genetic HR defects was observed within the context of TNBC (Supplementary Fig. 3A–C; Supplementary Table 2). Additionally, when including epigenetic HR defects (*BRCA1* promotor hypermethylation) a comparable sensitivity (80%, 95% CI: 70–87%) was demonstrated for both (epi)genetic HR defects in high-risk genes (Supplementary Fig. 3; Supplementary Table 2). Finally, for the three HRD read-outs individually (HRDetect, HRD Score, RAD51-FFPE test), accuracies in HR-status of 58–75% were observed, with the highest accuracy noted when HRDetect was used as reference (75%, 95% CI: 69–81%; Supplementary Fig. 4; Supplementary Table 3). An overview of the aforementioned results is illustrated in Fig. 3b, c.

In an exploratory manner, the potential role of technical artifacts was examined to provide a possible explanation for discordant classifications between CN signature 3 and the consensus of the three HRD read-outs. Of note, low fractions of neoplastic cells in the

exploration cohort appeared to negatively affect HRD test concordance rates (visualized in Supplementary Fig. 5).

The correlations of the CN signature 3-based HRD test with individual components of HRDetect and HRD Score are detailed in Supplementary Fig. 6 and Supplementary Table 4. Moreover, the associations between HR-status and clinicopathological characteristics are specified in Supplementary Table 5.

CN landscape beyond HRD. With respect to potential therapeutic opportunities, we specifically explored the CN landscape beyond HRD in the TNBC population, including gene amplifications (*CCND1*, *CCNE1, EGFR, ERBB2, MYC, PIK3CA*) and other CN signatures. *EGFR* gene amplifications were significantly enriched in the HRP population (as defined by CN signature 3 contribution below the threshold of 0.20; HRP: 11% versus HRD: 2%; *P*-value < 0.01; Fisher's exact test; Supplementary Table 5A). Furthermore, contributions of CN signatures associated with chromosomal missegregation (CN signature 1; *P*-values < 0.001; Wilcoxon rank sum test) and replication stress (CN signatures 5, 9, 11 and 13; *P*-values < 0.001; Wilcoxon rank sum test) were significantly higher in HRP compared to HRD TNBC (Supplementary Fig. 7; Supplementary Table 6).

Feasibility phase

To test the feasibility of a genome-wide CN test in a real-life clinical scenario, we prospectively investigated the utility of this test in a BC patient population referred to the clinical genetic service for germline DNA testing ($n = 147$; Methods; Study design in Fig. 2; Supplementary Fig. 1B; Supplementary Table 7). Compared to the exploration cohort, the feasibility cohort was significantly younger at diagnosis (<50 versus ≥50 years; *P*-value < 0.001; Chi-Square test; Supplementary Tables 1 and 7), and included non-TNBC as well as males with BC.

In comparison to WGS, we used low-resolution WGS (i.e., shallow-WGS) which has the advantage of utilizing FFPE instead of fresh-frozen derived tumor DNA, thereby reducing the implementation challenges in routine Pathology as well as the financial burden (Fig. 1a). Using routine FFPE tumor DNA of BC specimens, a CN profile is obtained from which both CN signatures and focal CN alterations can be extracted (Fig. 1b). To ensure the applicability of our above-defined HRD threshold for CN signature 3 in both WGS and shallow-WGS data, we downsampled WGS data to simulate the shallow depth (30X to ~0.1X, detailed in Methods section) and observed a moderate correlation of CN signature 3 contributions in matched specimens (Spearman $R^2 = 0.52$; *P*-value < 0.001; Supplementary Fig. 8A). Applying the previously established HRD threshold of 0.20, the sensitivity of shallow-WGS in identifying WGS-classified HRD samples was 90% (95% CI: 82–95%).

Shallow-WGS-based absolute CN profiles were successfully fitted for $n = 123/147$ (84%) BC specimens (Supplementary Fig. 1B; Supplementary Fig. 8B; reasons for unsuccessful fitting detailed in Supplementary Table 8). Specimens with successful shallow-WGS exhibited heterogeneity in terms of tumor cell percentage, DNA concentration, and DNA quality (Supplementary Fig. 9). The observed success rate, despite heterogeneity in input quality and/or quantity, underscores the feasibility of the test for most of the FFPE BC specimens analysed routinely.

Identifying homologous recombination deficiency. In the feasibility cohort, the HRD threshold of 0.20 CN signature 3, established in the exploration cohort, was found to be highly sensitive in distinguishing between a BC population with and without a high-risk PV (Fig. 4; Supplementary Fig. 10). Critically, all BC with a genetic HR defect (i.e., germline or somatic PVs in *BRCA1* ($n = 8$), *BRCA2* ($n = 4$), *PALB2* ($n = 4$)) had a CN signature 3 score ≥ 0.20 (Supplementary Fig. 11), resulting in a 100% sensitivity (95% CI: 76% – 100%) to detect genetic HR defects. Notably, a total of 56% ($n = 69/123$) of the tumors were classified as HRD using this threshold, of which 53 out of 69 (77%) did not harbor *BRCA1/2* or *PALB2* PVs. Importantly, even in this highly selected population, HRD was not restricted to BC patients with a clear family history of hereditary breast and ovarian (HBOC)-related cancers (Fig. 4; Supplementary Table 10).

On a functional level, the sensitivity to detect CN signature 3-based HRD using the RAD51-FFPE test was 82% (95% CI: 63–93%; Supplementary Fig. 11; Supplementary Table 9). Notably, the sensitivity of the RAD51-FFPE test for detecting genetic HR defects was 70% (95% CI: 35–93%; Supplementary Table 9C). The 30% discordant cases are unlikely to be explained by restoration of HR status due to therapy-induced selection pressures, as the majority of BC biopsies in the study were treatment-naive.

CN landscape beyond HRD. In the HRP population ($n = 54/123$; 44%), potentially actionable focal CN alterations were observed in *ERBB2* (18/54; 33%), *CCND1* (9/54; 17%), *CCNE1* (1/54; 2%), *MYC* (10/54; 19%), and *PIK3CA* (3/54; 6%) (Fig. 4; Supplementary Table 10A). Notably, we observed an excellent concordance (97%) between IHC- and CN-based HER2/*ERBB2* assessment (detailed in Supplementary Table 11). CN signatures associated with chromosomal segregation (1 and 6) and replication stress [25] were significantly higher in HRP BC compared to HRD group (Supplementary Fig. 12; Supplementary Table 6). Taken together, these results demonstrate potential actionable CN alterations for selected BC lacking HRD characteristics.

DISCUSSION

Here, we aimed to exploit an accessible, low-resolution, genome-wide testing approach which captures actionable alterations on a CN level. Despite preliminary feasibility studies [28], widespread adaptation of WGS is currently limited in routine pathology due to (i) the technical complexities associated with sequencing FFPE tumor DNA and (ii) the high financial burden (Fig. 1a) [29–32]. These implementation challenges are particularly true in the context of BC, where the global incidence is extremely high [8].

To overcome this challenge, we explored an alternative approach using CN analyses on FFPE tumor DNA. We demonstrated that the low-resolution testing approach for BC has high potential in broadly capturing the genetic profile, including a high sensitivity for identifying HRD tumors. Beyond its performance, the success rate of the test was high in a real-life clinical cohort, regardless of quality and quantity of FFPE tumor DNA. A limiting factor for all CN-based analyses, however, is that a sufficient tumor cell percentage (>30%) is required to accurately characterize CNA. Notably, the vast majority of specimens were biopsies, highlighting the potential role of the CN-based testing approach in defining therapeutic opportunities and genetic cancer predisposition information in the diagnostic, preoperative, setting.

Recently, numerous attempts have been made to capture the HRD phenotype, employing different concepts (genomic-based and functional) and using diverse sequencing platforms (Fig. 1a). Crucially, apart from commercial tests, such as the Myriad MyChoice HRD test, there is currently no established standard to test HRD in the context of FFPE DNA. The strength of our approach lies in the comprehensive, multimodal benchmarking of a candidate HRD test for FFPE DNA, incorporating both genomic and functional HRD testing methodologies [10, 16, 23]. Importantly, even though, in contrast to aforementioned HRD read-outs [10, 16, 23], CN signature 3 has not been explicitly trained on *BRCA1/2*-driven tumors [25], a (near-)perfect sensitivity for identifying tumors with *BRCA1, BRCA2, PALB2* PVs was observed in our study.

The actionability of identifying HRD tumors is twofold, encompassing both therapeutic implications, such as platinum-based therapy and/or PARPi [14, 15] eligibility, and insights into

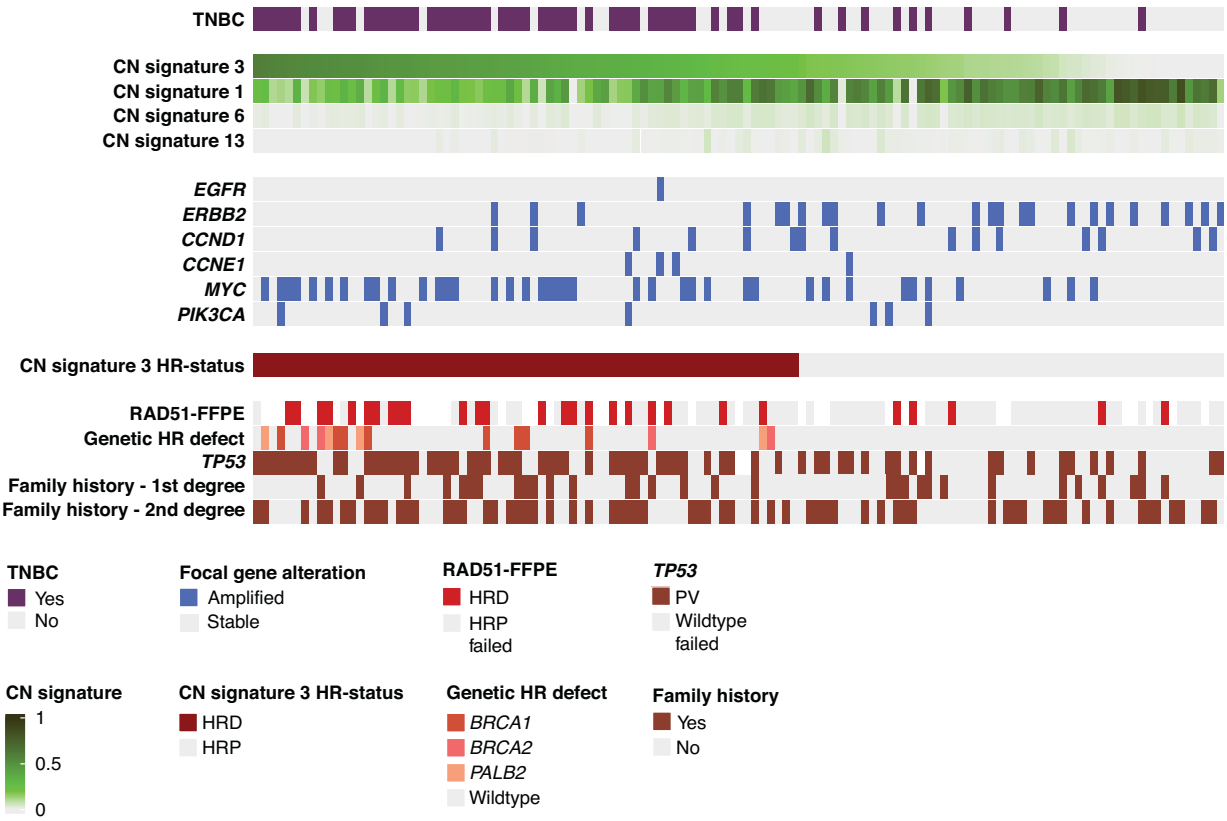


Fig. 4 Landscape of actionable CN alterations in the breast cancer specimens of the feasibility cohort. The actionable CN alterations include: CN signatures (i.e., CN signature 3-related HRD) and focal CN alterations that both are extracted from the genome-wide, low-resolution CN test. CN signatures that have significantly higher contributions in HR-proficient compared to HR-deficient breast cancers are visualized (Supplementary Fig. 12; Supplementary Table 6; CN signature 1, 6, 13). CN copy number, BC breast cancer, HRD homologous recombination deficiency.

cancer-susceptibility. The identification of germline PVs in *BRCA1*, *BRCA2* and *PALB2* is crucial for effective clinical management of both patients and their families. Historically, referral to germline testing has been based on clinicopathological criteria, including age of diagnosis, tumor type, and family history [33, 34]. Nevertheless, current referral guidelines have demonstrated suboptimal performance [35], as germline carriers who do not comply with the specified criteria may be missed, potentially having significant consequences. Despite the obvious relevance of HRD testing in BC patients, testing is not routinely performed, and studies exploring the prevalence of HRD in unselected BC are sparce due to anticipated low yield and high costs.

We acknowledge that the study design is not without limitations, the major one being the highly selected nature of the cohorts included in this study. Although this study provides a conceptual framework for the positioning of low-resolution, genome-wide sequencing as a preselection test, we strongly encourage validating the performance and feasibility in large, unselected BC populations. CN signature 3 captures only one genetic feature of HRD (i.e., the levels of loss-of-heterozygosity), in contrast to other genetic-based HRD tests, as HRDetect, Myriad MyChoice, HRD Score. Logically, CN signature 3 is less specific in defining HRD. However, for use in routine diagnostics as a 'preselection test', this lack of specificity is unlikely to be a significant issue, as our aim is to enrich for BC likely to harbor *BRCA1, BRCA2, PALB2* PVs. In this context, 'overcalling' HRD is not a major concern. Furthermore, defining the prevalence and patient and tumor characteristics associated with HRD tumors in a large, unselected BC population will be crucial for establishing accurate guidelines to optimize HRD testing in BC.

Importantly, a major advantage of a low-resolution, genome-wide testing approach described here, is the capability of capturing additional CN events beyond an HRD-related signature. Potential actionable alterations in the HRP population included focal CN alterations, as well as signatures associated with chromosomal missegregation and replication stress (potential targets described in Drews et al. [25]). Over time, *ERBB2* gene amplification has become a well-established predictive biomarker for response to anti-HER2 therapies (e.g., trastuzumab for HER2-positive early breast cancer). Importantly, a near-perfect concordance (97%) was observed between genetic (shallow-WGS) and IHC-based approaches, validating the feasibility of using low-resolution testing approaches to detect focal CN alterations for first-line therapy eligibility [36]. In a more exploratory context, the actionability of additional focal CN alterations is currently being investigated in various clinical trials focusing on advanced-stage disease (*MYC*: NCT05159518; *EGFR*: NCT01732276). Of note, a recent phase II study demonstrated promising results for Adavosertib in the context of solid tumors with *CCNE1* amplification, including BC [24].

In conclusion, the proposed low-resolution, genome-wide testing strategy to derive CN signatures and focal CN aberrations is likely to address current implementation and economic challenges associated with WGS in routine pathology. The CN-based test would offer inclusivity in capturing actionable alterations, including but not limited to HRD and the identification of potential *BRCA1*, *BRCA2* and *PALB2* germline carriers. The adaptation of this lower-resolution genetic testing approach for FFPE tumor DNA as a companion diagnostic test in pathology shows promise in expanding accessibility to tumor testing in BC,

thereby maximizing the potential for personalized medicine and genetic counseling.

METHODS

Study design – Exploration and feasibility phases

This study comprised of an exploration ($n = 237$) [12, 26, 27] and feasibility BC cohort ($n = 147$). The design of the study is visualized in Fig. 2.

Exploration phase

Patient and tissue collection. The SCAN-B TNBC cohort consists of a population-based consecutively enrolled series of patients diagnosed with initial primary resectable TNBC accrued in Sweden during 2010–2015 [12, 26]. In total, 340 patients were included in the SCAN-B study, and 254/340 (75%) patients were selected for whole genome sequencing (WGS), of which 237/254 (93%) passed the WGS-related QC as detailed in Staaf et al. [12]. Freshly collected specimens were treatment-naive at biopsy. Moreover, for 265/340 (78%) patients core biopsies from matched FFPE tumor material were taken for the preparation of TMAs as outlined in Aine et al. [37]. TMAs were kindly provided by J. Staaf, Lund University, Sweden. The SCAN-B study (ClinicalTrials.gov ID NCT02306096) was approved by the Regional Ethical Review Board in Lund, Sweden (applicable registration numbers 2009/658, 2015/277, 2016/742, 2018/267 and 2019/01252) [26, 27]. All patients provided written informed consent prior to enrollment.

Clinicopathological characteristics and whole genome sequencing data. Publicly available data (cohort summary, HRDetect scores, and CN profiles from ASCAT) were downloaded (https://data.mendeley.com/datasets/2mn4ctdpxp/1, version 3, accessed on December 2023) and used for further analyses. Genomic-related data was obtained using WGS (coverage tumor – Q1: 20.6x; median: 35.5x; Q3: 37.9x). With respect to HRDetect data, as assessed in a previous study [10], HRDetect 'high' and 'intermediate' scores were considered HRD. HRDetect has been successfully determined for all TNBC that passed the WGS quality control $n = 237/237$ (100%) (Supplementary Table 12) [12]. Additionally, an HRD score was obtained, with a threshold of ≥ 42 to define HRD [13].

RAD51-FFPE test. The RAD51-FFPE test (co-immunofluorescence staining of RAD51 and geminin) was performed within the scope of this study on Tissue Microarrays (TMAs) from the SCAN-B cohort, as described previously (Supplementary Methods) [16]. The TMAs contained 530 cores (core size 1 mm, tissue sections 4 µm), with two cores from each patient (265 patients) placed on a total of five microscope slides. RAD51/geminin IF-stained cores were manually scored by two experienced observers. Per core, geminin-positive cells were identified and ≥30 geminin-positive cells were selected at random for the calculation of a RAD51 score (percentage of geminin-positive cells with ≥2 RAD51 foci). A RAD51-FFPE score ≤ 15% was used to classify TNBC as HRD [16, 22]. Here, we focused on TNBC cases that passed the WGS QC ($n = 237$). Final RAD51-FFPE scores were obtained for $n = 204/237$ TNBC (84%) (Supplementary Table 12).

Copy number signatures. CN signatures were quantified, as described by Drews et al. [25], using absolute CN profiles. The absolute CN profiles for TNBC were previously determined by Staaf et al. [12] using ASCAT and were downloaded for CN signature quantification. CN signatures were successfully determined for $n = 231/237$ (97%) TNBC (Supplementary Table 12). For the remaining $n = 6$ (3%) TNBC fewer than 20 CN alterations were observed, for which signatures were not quantifiable.

Hypothetical ground truth in HRD testing. Considering that a gold standard in HRD testing is currently lacking, an 'hypothetical ground truth' was established by combining the HR-status of three HRD read-outs: HRDetect [10], HRD score [13], and RAD51-FFPE test [16]. In this context, TNBC were considered HRD or HRP only when they were consistently characterized as such across all three HRD read-outs. The densities of the HRD-TNBC and HRP-TNBC populations were visualized and the intersection was used as an HRD threshold (Fig. 3a).

Downsampling WGS data to shallow-WGS data. To investigate the impact of sequencing depth (WGS: 30X vs. shallow-WGS: ~0.1X) on CN signature 3 contributions, BAM files from the SCAN-B cohort were downsampled using SAMTOOLS [38], to simulate ~0.1X 'shallow' sequencing depth. For the downsampled BAM files, absolute CN fitting was performed using the same ploidy/purity solution previously determined with ASCAT (detailed above). Next, CN signatures were quantified [25], and the CN signature contributions derived from WGS and shallow-WGS were compared using Spearman R^2. Matched WGS and shallow-WGS data was available for $n = 191/237$ TNBC (81%).

Feasibility phase

Patient selection and tissue collection. BC patients were recruited during routine counseling at the clinical genetics service at the Leiden University Medical Center (Leiden, The Netherlands) [39]. Patients at age of ≥18 years old and diagnosed with BC were eligible for the study. For this clinical cohort, we included BC population of patients aged ≥18 years, likely enriched for PVs in *BRCA1, BRCA2, PALB2* by applying additional inclusion criteria: (1) diagnosis of BC ≤ 40 years (2) diagnosis of TNBC ≤ 50 years (3) men with diagnosis of BC. A representative FFPE block (ascites, biopsy or resection) was requested for central pathology review, in accordance with the most recent World Health Organization (WHO) guidelines, by an expert pathologist [40]. After central histopathological revision, including ancillary immunohistochemistry stainings if needed (e.g. ER/PR/HER2/Ki67), tumor DNA was isolated in routine diagnostic setting as described previously [10]. The inclusion period comprised June 2021 through November 2023. The study design was approved by the medical ethics committee of the Leiden University Medical Center (N20.183).

Clinicopathological characteristics. Clinicopathological characteristics, including cancer-specific (first and second degree) family history and germline testing results for high-risk (*BRCA1, BRCA2, PALB2*) and moderate-risk genes (*ATM, CHEK2*) were extracted from medical patient files.

Shallow-WGS: Copy number signatures and focal alterations. FFPE tumor DNA was isolated for all included BC patients (Fig. 1b; Supplementary Fig. 1B; Supplementary Table 2). Copy-number signatures were determined using shallow-WGS data of FFPE tumor DNA (Fig. 1b). For all included tumor specimens (CONSORT diagram in Supplementary Fig. 1B), shallow-WGS (Illumina NovaSeq6000 sequencing, single-end, 150 bp, sequencing reads tumor – Q1: 2.9 million; median: 3.3 million; Q3: 4.5 million; median coverage <0.20X) was analysed (Supplementary Fig. 9C). A bin size of 100 kilobase pairs was used, as it balances a high fraction of used sequencing reads with limited noise in sequencing. Quality-related read-outs for the sequenced cases are visualized in Supplementary Fig. 9.

Absolute CN profiles were fitted with the Rascal tool, using the variant allele frequency (VAF) of a driver gene (e.g. *TP53, PIK3CA, BRCA1/2*) as a surrogate of the tumor purity or selecting the only Rascal scenario (Supplementary Fig. 8; Supplementary Table 8C) [12]. Using objective parameters, such as the VAF, in selecting a purity/ploidy-scenario limits the chances of interobserver bias in the selection. When utilizing the VAF of a driver gene for fitting, Rascal predicts the VAF for the gene of interest to guide a decision on the optimal purity/ploidy-solution. Notably, a strong positive correlation was observed between the true *TP53* VAF (as assessed by NGS) and predicted *TP53* VAF (by Rascal) ($n = 71$; Pearson $R^2 = 0.96$, *P*-value < 0.001; Supplementary Fig. 13). In the absence of a bona fida driver PV, one of the following fitting strategies was applied: selecting the Rascal scenario with the smallest distance, using the estimated tumor cell percentage by an expert pathologist, or performing manual fitting (detailed in Supplementary Table 8B). Absolute CN fitting was successful for 127/147 (86%) (Supplementary Fig. 1B (CONSORT) + Supplementary Table 8A).

Absolute CN profiles were used to quantify CN signature contributions [13], following a similar approach to that used in the exploration cohort, which proved successful for $n = 123/127$ (97%) specimens. The remaining $n = 4$ cases had <20 CNA and were therefore classified as 'genetically silent' (representative examples in Supplementary Fig. 14).

Additionally, focal amplifications (absolute CN ≥ 6, corrected for DNA ploidy) of *EGFR, ERBB2* (relationship HER2 IHC and *ERBB2* CN assessment in Supplementary Table 11), *CCND1, CCNE1, MYC*, and *PIK3CA*, were accessed (representative examples in Supplementary Fig. 15).

RAD51-FFPE test. Unstained sections of the formalin-fixed paraffin embedded tissue were used for the RAD51-FFPE test (see details in Method section 'Exploration phase – RAD51-FFPE test') [1, 2]. Here, sections were scored by a single observer, aiming for at least 40 geminin-positive cells. In rare cases where fewer geminin-positive cells were present, a

minimum of 10 was scored to maximize yield. On average, 46 geminin-positive cells were scored in the feasibility cohort.

Next-generation sequencing of BC-susceptibility (high- and moderate-risk) genes. Additionally, targeted next-generation sequencing was performed using a custom Ampliseq panel (HRDv2, Ion Torrent S5 Genestudio platform), comprising full coverage of the coding exons of *BRCA1* and *BRCA2*, as well as *ATM, BARD1, BRIP1, CDK12, CHEK1, CHEK2, ERBB2, FANCL, PALB2, PIK3CA, PPP2R2A, RAD51B, RAD51C, RAD51D, RAD54L*, and *TP53* (detailed in Supplementary Methods; characteristics of sequencing panel in Supplementary Table 13).

Statistical analyses

Figures were created with Adobe Illustrator CC 2023 (Adobe Inc, San Jose, CA, USA) and R (version 4.2.3; packages: ggplot2, dplyr, ComplexHeatmap, Rascal, grid, ggsignif, circlize, ggridges, caret). Statistical analyses were performed using R (version 4.2.3). Test parameters, including accuracy, sensitivity, and specificity, were reported along with the 95% confidence interval (CI). Wilcoxon Rank Sum test was performed to test differences when numerical data was not normally distributed. Categorical data of groups were tested with Fisher's exact test or Chi-square test. Fisher's Exact test was selected when any expected value was less than one, or when more than 20% of the expected values were less than five. Correlations were performed using Spearman's rho (R^2) when the data were not normally distributed. Generally, a *P*-value of <0.05 was considered significant, however, for multiple tests, Bonferroni multiple testing correction was performed.

DATA AVAILABILITY

The WGS data within the exploration phase (SCAN-B cohort) were derived from the following public domain resource: https://data.mendeley.com/datasets/2mn4ctdpxp/1, version 3. The RAD51-FFPE data of the SCAN-B cohort were generated and are available upon reasonable request from the corresponding author (MV). For the exploration phase, the data generated are available upon reasonable request from the corresponding author (MV).

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AUTHOR CONTRIBUTIONS

Conceptualization: CK, KT, TB, CA, MV; Methodology: CK, TB, JS, HV, CA, MV; Formal analysis: CK, MV; Investigation: CK, LW, DR, SGV; Resources: KT, DC, TB, VS, NS, CA, JS, JVC; Data curation: CK, LW, DR, SV; Writing – original draft: CK, MV; Writing – review & editing: LW, DR, SGV, KT, NS, TW, JW, VS, DC, HV, JS, TB, CA, JVC; Visualization: CK, MV; Supervision: TB, CA, MV; Project administration: CK, CA; Funding acquisition: TB, CA, MV.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The SCAN-B study (exploration phase) (ClinicalTrials.gov ID NCT02306096) was approved by the Regional Ethical Review Board in Lund, Sweden (applicable registration numbers 2009/658, 2015/277, 2016/742, 2018/267 and 2019/01252) [26, 27]. All patients provided written informed consent prior to enrollment. For the feasibility cohort, the study design was approved by the medical ethics committee of the Leiden University Medical Center (N20.183). The study was performed in accordance with the Declaration of Helsinki.

ADDITIONAL INFORMATION

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