Homologous recombination DNA repair deficiency and PARP inhibition activity in primary triple negative breast cancer

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Triple negative breast cancer (TNBC) encompasses molecularly different subgroups, with a subgroup harboring evidence of defective homologous recombination (HR) DNA repair. Here, within a phase 2 window clinical trial, RIO trial (EudraCT 2014-003319-12), we investigate the activity of PARP inhibitors in 43 patients with untreated TNBC. The primary end point, decreased Ki67, occurred in 12% of TNBC. In secondary end point analyses, HR deficiency was identified in 69% of TNBC with the mutational-signature-based HRDetect assay. Cancers with HRDetect mutational signatures of HR deficiency had a functional defect in HR, assessed by impaired RAD51 foci formation on end of treatment biopsy. Following rucaparib treatment there was no association of Ki67 change with HR deficiency. In contrast, early circulating tumor DNA dynamics identified activity of rucaparib, with end of treatment ctDNA levels suppressed by rucaparib in mutation-signature HR-deficient cancers. In ad hoc analysis, rucaparib induced expression of interferon response genes in HR-deficient cancers. The majority of TNBCs have a defect in DNA repair, identifiable by mutational signature analysis, that may be targetable with PARP inhibitors.
Triple negative breast cancer (TNBC) may have diverse defects in HR DNA repair, through germline mutations in BRCA1, BRCA2 and PALB2, somatic mutations in BRCA1 and BRCA2, promoter methylation of BRCA1 and RAD51C, and other as yet to be identified mechanisms. Over the last decade, advances in whole-genome sequencing (WGS) have lead to the identification of mutational processes that leave a characteristic imprint, a mutational signature on the cancer genome. These have revolutionised our understanding of cancer and has the capability to improve diagnosis and treatment of cancer.

Cancers with defects in HR-based DNA repair have characteristic chromosomal changes reflecting the use of alternative error-prone repair pathways, including measures of genomic instability; loss of heterozygosity, telomeric allelic imbalance and large-scale state transitions to accurately identify BRCA1/2 tumours, and their combination to form the HRD Score has allowed identification of HR-deficient tumours (HRD Score >42), independent of BRCA1/2 deficiency within a sporadic TNBC population. Recent work has identified WGS signatures of HR deficiency with BRCA1/2 deficient tumours associated with distinct mutational signatures. The mutational signatures and chromosomal instability markers of HR deficiency have been aggregated into the HRDetect score, robustly identifying BRCA1/2 tumours with potential greater accuracy than indexes such as HRD-score.

Whether mutational signature-based scores such as HRDetect, can be used to direct therapy in the clinic is unknown, in part as there is limited direct evidence that cancers classified as HR deficient by these scores have a functional defect in HR. Breast cancers with BRCA1 and BRCA2 germline mutations are highly sensitive to PARP inhibitors, which target the underlying HR DNA repair defect in these cancers. However, no activity was observed with PARP inhibitors in the treatment of heavily pre-treated un-selected advanced TNBC. The extent to which this PARP inhibitor efficacy may translate to sporadic TNBC is unknown, as is the best way to identify HR-deficient TNBC. To address these questions, we designed a translational clinical trial, the RIO trial (EudraCT 2014-003319-12), with the objective of identifying biomarkers of PARP inhibitor activity in sporadic TNBC.

Results

Biomarkers of HR deficiency in primary TNBC. Patients with newly diagnosed, treatment naïve TNBC were treated with the PARP inhibitor rucaparib for 2 weeks prior to surgery or neoadjuvant chemotherapy. A total of 43 patients were entered into the trial between August 2015 and August 2017. Blood and tissue biopsies were taken prior to, and at the end of treatment, for molecular analysis (Fig. 1a). Within the trial, a subset of germline BRCA1/2 patients were recruited as a control population. The trial prospectively examined three potential biomarkers of PARP inhibitor activity, a molecular signature of HR deficiency.

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**Fig. 1 RIO study CONSORT diagram and HRDetect analysis.** a RIO study CONSORT diagram. b Effect of rucaparib on Ki67 expression assessed by immunohistochemistry (IHC). The change in proportion of tumor cells expressing Ki67 between baseline and EOT, in patients that had assessable pairs of baseline and EOT samples. BRCA mutation cancers had no evidence of decreased Ki67. e Effect of rucaparib on cleaved PARP expression assessed by immunohistochemistry, as a marker of apoptosis. The change in proportion of tumor cells expressing cleaved PARP between baseline and EOT, in patients that had assessable pairs of baseline and EOT samples. BRCA mutation cancers had no evidence of increased cleaved PARP expression. Grey bars, BRCA wild type patients; Blue bars, BRCA germline mutant patients. Orange line, >30% but <50% reduction; Red line, >50% reduction.
Table 1 RIO study patient demographics.

|                              | n  | %   |
|------------------------------|----|-----|
| **Age (mean (standard deviation))** | 54.6 (13.9) |
| Age group (years)            |    |     |
| <40                          | 7  | 16.3|
| 40-49                        | 13 | 30.2|
| 50-59                        | 9  | 20.9|
| 60-69                        | 5  | 11.6|
| 70+                          | 9  | 20.9|
| **BRCA status**              |    |     |
| Triple negative, known BRCA1/2 carrier at registration | 2 | 4.7 |
| Not TN, known BRCA1/2 mutation carrier at registration | 1 | 2.3 |
| Triple neg, no BRCA mutation | 35 | 81.4|
| Triple negative, BRCA1/2 mutation identified while on trial | 5 | 11.6|
| **Planned standard treatment after rucaparib** |    |     |
| Neoadjuvant chemotherapy     | 32 | 74.4|
| Surgical resection           | 11 | 25.6|
| ER & PR negative             | 42 | 97.7|
| **Hormone receptor status**  |    |     |
| ER positive & PR negative    | 1  | 2.3 |
| G1                           | 0  | 0   |
| G2                           | 12 | 27.9|
| G3                           | 24 | 55.8|
| Not known                    | 7  | 16.3|
| **Histological type**        |    |     |
| Infiltrating ductal          | 38 | 88.4|
| Infiltrating lobular         | 4  | 9.3 |
| Mixed ductal & lobular       | 1  | 2.3 |
| **DCIS present**             |    |     |
| Yes                          | 7  | 16.3|
| No                           | 35 | 81.4|
| Not known                    | 1  | 2.3 |
| **Tumour size by ultrasound**|    |     |
| <1.5                         | 2  | 4.7 |
| 1.5                          | 1  | 2.3 |
| >1.5 & ≤2                    | 10 | 23.3|
| >2 & ≤5                      | 26 | 60.5|
| >5                           | 4  | 9.3 |
| **Lymph node involvement**   |    |     |
| Yes                          | 16 | 37.2|
| No                           | 27 | 62.8|
| **Side of tumour**           |    |     |
| Left                         | 18 | 41.9|
| Right                        | 25 | 58.1|
| **Evidence of metastatic disease** | 0 | 0   |
| Yes                          | 43 | 100 |

*One patient was locally assessed as triple negative but central assessment noted weak PgR score of 3/8 by Alfred.

using HRDetect, RAD51 focus formation in a tumor biopsy at the end of treatment, and BRCA1 methylation. The primary activity end point was a fall in Ki67 on the end of treatment biopsy, with circulating tumor DNA dynamics as a prospectively planned exploratory end point of activity. Patient demographics were as expected for this population (Table 1). Rucaparib was well tolerated with adverse effect profile similar to previous clinical studies. We assessed rucaparib activity and the relationship with prospectively planned biomarkers. The primary end point was assessed in tissue samples, using Ki67 suppression after two weeks assessed by immunohistochemistry, as a potential biomarker (Fig. 1b). A drop in Ki67 by 50% in the triple negative patients without a known BRCA1/2 mutation at trial entry was seen in 12% tumors (95% CI: 2.5–31.2; n = 3/25). In secondary end point analysis, one additional patient with known BRCA mutation at trial entry was assessed and did not have a 50% drop in Ki67 (Fig. 1b). No association was observed between Ki67 change with BRCA1/2 mutated cancers (Fig. 1b). Similarly, no association was observed with cleaved PARP levels as a marker of apoptosis with BRCA1/2 mutated cancers (Fig. 1c).

In secondary end point analysis, baseline biopsies of patients entering the trial, we interrogated the prevalence of HR deficiency in primary TNBC (Fig. 2 and Supplementary Table 2). We performed whole-genome sequencing analysed with the HRDetect assay, identifying mutational processes characteristic of HR deficiency in 69% locally assessed TNBC (18/26 with score >0.70, herewith called HRDetect+ve; Fig. 2a,b and Supplementary Fig. 1a) as well as an additional control ER-positive cancer with a known germline BRCA2 mutation. In ad hoc analysis, we individually determined the mutational status of BRCA1, BRCA2 and PALB2, and promoter methylation of BRCA1 and RAD51C (Fig. 2a). Of the HRDetect+ve cancers, 74%(14/19) had a detectable underlying mutation of BRCA1/2 and PALB2 or gene promoter hypermethylation of BRCA1 or RAD51C. None of the eight HRDetect−ve cancers had an underlying genetic/epigenetic defect (p = 0.0005, Fisher’s exact test; Fig. 2a). A loss of heterozygosity (LOH)/copy-number-based HRD score was positive in more cancers than HRDetect, with the HRD score identifying cancers with genomic but without rearrangement signatures and indels at microhomology. None of the HRD score high but HRDetect low tumours had detectable pathway aberrations (Supplementary Fig. 1b), suggesting that HRDetect was more specific.

HRDetect identified all cancers with known HR pathway defects, as well as additional sporadic cancers with no single detectable defect (Fig. 2a). In secondary end point analysis, we next addressed whether HRDetect+ve cancers had an underlying functional defect in HR DNA repair, using RAD51 focus formation in the end of treatment (EOT) biopsy. When cells are exposed to genotoxic agents such as PARP inhibition, RAD51 is recruited to sites of DNA damage and stalled replication forks, mediating the search for a homologous sequence during HR. Using the HRDetect assay, identifying mutational processes characteristic of HR deficiency occurring in the majority of TNBC.

**Rucaparib activity assessed by ctDNA dynamics.** Circulating tumour DNA (ctDNA) is released from the tumor, allowing for serial sampling through the course of treatment. Early changes in ctDNA dynamics represent an early biomarker of drug
activity, as cancers that respond to treatment rapidly suppress the level of ctDNA in plasma\textsuperscript{23–25}. Analysis of ctDNA was prospectively planned as an exploratory end point of rucaparib activity, in part, as Ki67 change has only been validated as an activity end point in endocrine based therapies\textsuperscript{26–28}. To assess ctDNA in RIO, the primary tumor was sequenced in 35 patients, with somatic mutations identified in 31 patients, and personalised digital PCR used to track changes in ctDNA levels between baseline and end of treatment plasma (EOT). Change in ctDNA was assessable in the 19 patients with sufficiently high baseline
**Fig. 2 HRDetect analysis.** a HRDetect scores were established with whole-genome sequencing in baseline biopsies of 26 patients with untreated primary triple negative breast cancer (locally assessed) entering the RIO window clinical trial. HRDetect positive cancers (HRDetect score >0.7) were enriched for inactivating mutations and promoter methylation of HR genes compared to HRDetect negative cancers (p = 0.0005, Fisher’s exact test). Star, an additional patient with ER-positive breast cancer and BRCA2 germline mutation is shown. 2 stars, locally assessed as TNBC but centrally assessed as PR-positive breast cancer with BRCA1 methylation. Magenta bars, BRCA1/2 germline mutation; Blue bars, BRCA1 methylation; Yellow bars, PALB2 germline mutation; Turquoise bars, RAD51C methylation; Grey bar, None/unknown.

b Examples of genome plots for a sample with low (top) and high (bottom) HRDetect scores. The histograms associated with each circos plot show mutation counts for each mutation class: the topmost histogram shows the number of mutations contributing to each substitution signature; the middle histogram represents indel patterns; and the bottom histogram shows the number of rearrangements contributing to each rearrangement signature.

c Association between RAD51 foci proficiency and HRDetect scores in 18 patients, p = 0.0146 Mann–Whitney U Test. Line indicates median level.

**Fig. 3 Biomarkers of homologous recombination (HR) repair deficiency in TNBC.** a RAD51 focus assessment in paired baseline and on-treatment biopsies, p = 0.0016 Wilcoxon test. Inset right, example immunohistochemistry images of two cancers, one deficient and one proficient in RAD51 focus formation (brown), in nuclei stained for geminin (blue). Scale bars show 40 µm. b RAD51 immunohistochemistry score (>20% RAD51 foci proficient), as a functional assessment of HR proficiency, was assessed in end of treatment biopsies after 2 weeks of rucaparib in 25 patients. RAD51 foci deficient cancers are enriched for inactivating mutations and promoter methylation of HR genes compared to RAD51 proficient cancers (p = 0.037 Fisher’s exact test). *ER-positive breast cancer and BRCA2 germline mutation is shown. **Locally assessed TNBC but centrally assessed as PR-positive breast cancer with BRCA1 methylation. Magenta bars, BRCA1/2 germline mutation; Blue bars, BRCA1 methylation; Yellow bars, PALB2 germline mutation; Turquoise bars, RAD51C methylation; Grey bars, None/unknown. c Association between RAD51 foci proficiency and HRDetect scores in 18 patients, p = 0.0146 Mann–Whitney U Test. Line indicates median level.
ctDNA to assess change (Figs. 1a and 4a and Supplementary Tables 4 and 5). In contrast to the tumor biopsy-based data, a substantial proportion of patients suppressed ctDNA after rucaparib treatment (Fig. 4a).

Patients with germline BRCA1/2 mutations did not suppress Ki67, nor induce PARP cleavage, in the end of treatment biopsy (Fig. 1). In contrast, patients with germline BRCA1/2 mutations had a greater suppression of ctDNA than patients without germline mutations (n = 19, p = 0.021, Mann–Whitney; Fig. 4b), validating ctDNA dynamics as a marker of rucaparib activity.

Cancers with deficient RAD51 foci formation (n = 12, p = 0.033, Mann–Whitney) and HRDetect+ve cancers had greater ctDNA suppression (n = 15, p = 0.027, Mann–Whitney; Fig. 4b). In ad hoc analysis patients with suppressed ctDNA after two weeks rucaparib (ctDNA ratio (CDR) <0.25, methods) were enriched for germline mutations of BRCA1/2 and PALB2 and gene promoter methylation of BRCA1 and RAD51C (Fig. 4b). These data illustrate the potential of ctDNA analysis to transform window trials, presenting a simple and robust assay of drug activity, without the potential sampling challenges involved with repeat biopsies. However, analytical challenges associated with low plasma DNA levels and low purity tumor samples, will benefit from further technological development.

PARP inhibition induces an interferon response in HR-deficient cancers. Having demonstrated that HRDetect identifies sporadic TNBC in which PARP inhibitors have activity, in ad
We investigated the mechanisms of PARP inhibition in these cancers with RNA exome sequencing in 20 paired tumour samples, baseline and end of treatment. The majority of tumours were basal-like by PAM50 (75%, 15/20), with HRDetect scores >0.7 found in 80% (12/15) basal-like TNBC and none (0/4) of the non-basal TNBC, with one non-basal BRCA1 mutant ER+ve tumour having an HRDscore >0.7 ($p = 0.031$ Fisher’s exact test, Fig. 5a). Analysis of the immune micro-environment of baseline samples with CIBERSORT suggested higher levels of follicular helper T cells ($p = 0.0126$, $n = 20$ patients) in HRDetect+ve cancers along with higher levels of activated macrophages (Supplementary Fig. 3a and b).
Cancers with ctDNA suppression (CDR < 0.25) had more substantial changes in gene expression than cancers without ctDNA suppression (CDR > 0.25), with 415 compared to 37 significantly differentially expressed genes respectively (Fig. 5b), demonstrating stability of gene expression through rucaparib treatment in HR-proficient cancers. For individual genes, a significant decrease in gene expression was noted in PARP1 mRNA (q = 0.023, n = 20, DESeq2 with false discovery rate (FDR) correction), MKI67 mRNA (Ki67, q = 0.027, n = 20, DESeq2 FDR correction), as well as induction in gene expression of both CDKN1A mRNA (p21, q = 0.002, n = 20, DESeq2 FDR correction) and TMEM173 mRNA (STING, q = 0.05, n = 20, DESeq2 FDR correction; Fig. 5c). In HRDetect-ve cancers (Supplementary Fig. 4) and cancers with associated ctDNA suppression (Fig. 5d) pathway analysis demonstrated significant gene expression changes in pathways that regulate proliferation, apoptosis and immune function. There was decreased expression of G2M checkpoint genes, reflecting cell cycle arrest with rucaparib (Fig. 5d). There was substantial increase in interferon response genes, which along with TMEM173 mRNA expression, suggested PARP inhibition activated the cGAS–cGAMP–STING pathway in HR-deficient cancers (Fig. 5d and Supplementary Fig. 4). In contrast, HRDetect-ve cancers did not induce interferon response genes, nor had expression changes of cell cycle arrest, on rucaparib (Supplementary Fig. 4).

Discussion

Results from the RIO trial demonstrate that a majority of primary TNBC have defects in HR-based DNA repair, reinforcing the companion population-based TNBC study by Staaf et al.14. The primary end point of RIO, suppression of Ki67 in on-treatment biopsies, was infrequent. In pre-planned secondary analyses, we show that cancers with HR deficiency can be robustly identified with the mutational-signatures based classifier HRDetect (Fig. 2 and Supplementary Fig. 1), which identifies cancers with a functional deficiency in HR (Fig. 3b), and with evidence of activity of PARP inhibitors restricted to these cancers using ctDNA analysis (Fig. 3c). In ad hoc analysis, HRDetect was more specific to underlying HR deficiency than HRD scores (Supplementary Fig. 1b), suggesting that mutational signature assessment might be more accurate in identifying cancers that would benefit from platinum chemotherapy or PARP inhibition.30–32

Induction of RAD51 nuclear foci after neoadjuvant chemotherapy and PARP inhibition can measure the holomorphic recombination functionality in breast cancer biopsies,20,33–36 with an association to loss of heterozygosity measures of HR deficiency.34,37 Studies have shown that cells with deficient BRCA1/2 or other HR proteins, do not efficiently form RAD51 foci which could be used as a marker for PARP inhibitor sensitivity using FFPE tumour samples.35,38,39 The dynamics of DNA repair alter throughout tumour evolution and a functional RAD51 assay can be used as a dynamic readout of tumour HR status at the specific time for treatment decision-making.33,40,41 Current studies are using immunofluorescence (IF) on FFPE samples which can be labour intensive, and here we develop a RAD51 foci immunohistochemistry (IHC). Our cutoff <20% for HR deficiency is consistent with the RECAP test which has recently shown to be effective in ascertaining HR deficiency in metastatic breast tumours treated with ionising radiation.36 Further validation of this novel HR deficient biomarker is required and if clinically validated could be a useful tool in the clinic.

One of the main limitations of the study was lower recruitment into the trial than was anticipated. This was possibly due to patient preference to start treatment without the possibility of delaying for short-term trial therapy. Additionally, failure of tissue biopsies containing enough tumour content impacted end point analysis. We therefore demonstrate the advantage of non-invasive analysis, and the use of ctDNA as a potentially reliable and effective surrogate end point to assess response. This will require further validation.

We demonstrate that PARP inhibitors induce a pro-inflammatory/interferon response in HR-deficient TNBC, likely though the cGAS–cGAMP–STING pathway. Consistent with our findings, PARP inhibition has previously been shown to induce T-cell recruitment through activation of the cGAS–cGAMP–STING pathway in a BRCA1-null mouse model of TNBC.42 Similarly, PARP inhibitors have been shown to upregulate interferon response in TNBC cell lines with BRCA2 deletion,43 or mutant BRCA1.44 Furthermore, Sceney et al.45 recently demonstrated using mouse models of TNBC, that immune dysfunction characterised by decreased interferon signalling and decreased antigen presentation was abrogated by a STING agonists. Together these findings underline the potential of exploiting immune dysfunction in the context of HR deficiency, notably in BRCA1/2 mutant tumours, and TNBC more generally.

Our findings illustrate the potential of using whole-genome sequencing mutational signatures to guide cancer treatment, advocating for clinical trials of PARP inhibitors, potentially in combination with PD(L)1 targeting immune checkpoint antibodies, in sporadic TNBC with HR deficiency.

Methods

Study Design. Conducted in 10 hospitals throughout the United Kingdom, the window study of the PARP inhibitor rucaparib in patients with primary triple negative or BRCA1/2 related breast cancer (The RIO study; EudraCT 2014-003319-12; Cancer Research UK trial CRUK/12/034) was a single-group, open-label, phase II window of opportunity trial assessing rucaparib efficacy in patients with primary triple negative or BRCA1/2 mutant breast cancer prior to commencing primary treatment (neoadjuvant chemotherapy or surgery). The trial was co-sponsored by the Institute of Cancer Research and the Royal Marsden Hospital NHS Foundation Trust. Ethical approval for The RIO trial was given by the NRES Committee London - Fulham Research Ethics Committee (REC ID: 14/LO/2181) and informed consent was obtained from all patients enrolled in the study. Key eligibility criteria include breast tumour size ≥2 or <2 cm with cytologically/ histologically confirmed axillary lymph nodes, WHO performance status 0–2, no prior history of ipsilateral breast cancer within 5 years and no prior treatment with PARP inhibitors. Patients received rucaparib 600 mg twice daily for 12–14 days. Baseline bloods (EDTA and STRECK) and core biopsies (FFPE and RNAlater®) were collected at time of diagnostic biopsy or following trial entry. End of
treatment bloods (STRECK) and biopsies (FFPE and RNAlater) were taken at surgery or prior to neo-adjoint chemotherapy within 24–48 h of the last rucaparib dose. The primary end point was Ki67 response from baseline to end of treatment defined as a ≥25% decrease. Secondary endpoints (pre-specified endpoints in the trial protocol) were association between baseline biomarkers of BRCA1 methylation and a genomic predictor of HR deficiency (HRDetect) with Ki67 response to rucaparib, apoptosis induction following 12–14 days of rucaparib, the proportion of sporadic TNBC that fail to induce RAD51 foci on end of treatment biopsy (RAD51 score), safety and tolerability of rucaparib, association of biomarkers with RAD51 score, the proportion of patients with a change in circulating tumour DNA levels in response to rucaparib, association between change in circulating tumour DNA levels with biomarkers, and proportion of patients with BRCA1 and BRCA2 germline mutation related cancers with a Ki67 response to rucaparib and the proportion that have reduced RAD51 score and increased apoptosis induction. Pathological complete response was not assessed as an end point, as the two weeks window was considered to be too short for this end point. Where molecular analysis was not pre-specified in the trial protocol, this is highlighted as being ad hoc analysis.

Ki67 IHC and Scoring. Immunohistochemistry for Ki67 was performed and scored according to Leung et al.46. Tissue sections were deparaffinised and rehydrated prior to antigen retrieval using low pH (pH 6.0) Target Antigen Retrieval Solution (K8004, Dako UK Ltd). Tissue sections were stained for Ki67 using mouse monoclonal anti-Ki67 antibody (MIB-1 clone, Dako, M7240) diluted 1:50 in EnVision reagent buffer (K8006, Dako UK Ltd). Sections were washed using wash buffer (Dako, S3006) before colour development using REAL Kit (Dako, K5001). Tissues sections were counter stained with hematoxylin and coverslips mounted using DPX. The percentage of Ki67 positive cells was calculated from between 200–400 invasive tumour cells according to the method of Leung et al.46. A Ki67 response was determined as a ≥50% fall in Ki67 at EOT compared to baseline.

Cleaved PARP IHC and Scoring. IHC for cleaved PARP (cPARP) was performed using a rabbit polyclonal antibody specific for PARP cleaved at Asp214. Tissue sections were deparaffinised and rehydrated prior to antigen retrieval using High pH (pH9.0) Target Antigen Retrieval Solution (K8004, Dako UK Ltd), in PT-LINK (PT101, Dako UK Ltd). Tissue sections were stained for cPARP using rabbit monoclonal anti-cPARP antibody (Asp214, clone D64E10, Cell Signaling Technology, #s2565) diluted 1:100 in EnVision Antibody Diluent (K8006, Dako DK Ltd). Sections were washed using wash buffer (Dako,S3006) before colour development using anti-rabbit link reagent EnVision FLEX LINKER (K8019, Dako UK Ltd). Tissue sections were counter stained with hematoxylin and coverslips mounted using DPX. The percentage of cPARP positive cells was calculated from a minimum of 500 invasive tumour cells. If fewer than 500 invasive tumour cells were present the sample was recorded as insufficient invasive tumour (IFT). Sample processing. All samples were processed by the central laboratory as part of the RIO trial. Fresh tumour samples were collected in RNALater™ tubes, processed within 24 h and stored at -80°C until required for extraction. Baseline biopsies were sectioned using a cryostat. One section was cut for H&E and 16 sections were cut stained with Nuclear Red (NFR). A single H&E section was cut at the end of the series. H&E sections were reviewed and marked by a pathologist for macro-dissection. If the baseline biopsy did not have tumour the IHC for PARP was performed to confirm the presence of cancer. The targeted panel, “ABC-Bio” panel, has been validated in the ABC-Bio (molecular screening for patients with advanced breast cancer) trial and comprises of 41 genes commonly mutated in breast cancer34. Libraries were run on a MiSeq (Illumina) using MiSeq Reporter (MSR v2.5.1). All internally developed code is accessible on request. A 500-bp insert genomic libraries were constructed according to Illumina’s protocol (https://support.bioinformatics. babraham.ac.uk/projects/trim_galore/). Each read was aligned using pairwise alignment using Bowtie v2.2.6. Somatic mutations identified in paired-end sequencing using Picard (https://picard.sourceforge.net/). Primers for the BRCA1 and RAD51C methylation, bisulfite analysis.

Targeted tissue sequencing. Paired tumor and buffy coat DNA were sent to the Centre of Molecular Pathology at The Royal Marsden Hospital for sequencing using a targeted capture-based approach designed to detect mutations associated to breast cancer. The targeted panel, “ABC-Bio” panel, has been validated in the ABC-Bio (molecular screening for patients with advanced breast cancer) trial and comprises of 41 genes commonly mutated in breast cancer34. Libraries were run on a MiSeq (Illumina) using MiSeq Reporter (MSR v2.5.1). All internally developed code is accessible on request. The targeted panel, “ABC-Bio” panel, has been validated in the ABC-Bio (molecular screening for patients with advanced breast cancer) trial and comprises of 41 genes commonly mutated in breast cancer34. Libraries were run on a MiSeq (Illumina) using MiSeq Reporter (MSR v2.5.1). All internally developed code is accessible on request. A 500-bp insert genomic libraries were constructed according to Illumina’s protocol (https://support.bioinformatics. babraham.ac.uk/projects/trim_galore/). Each read was aligned using pairwise alignment using Bowtie v2.2.6. Somatic mutations identified in paired-end sequencing using Picard (https://picard.sourceforge.net/). Primers for the BRCA1 and RAD51C methylation, bisulfite analysis.

AVENIO sequencing. ctDNA samples from six patients from whom adequate tumour samples were not obtained were sent to Rochè for sequencing using the AVENIO ctDNA targeted tumour profiling kit (Rochè) and 2 samples were run on the AVENIO ctDNA expanded kit (77 genes), using HighOutput 300 cyc kit on a NextSeq 500 (Illumina) using NextSeq system suite (v2.2.0). Data was analysed using the AVENIO Oncology analysis software (v1.0.0 and v1.1) available from Rochè. BRCA1 and Rad51C methylation, bisulfite sequencing. The promoter region of BRCA1 and RAD51C was identified using the Eukaryotic Promoter Database (http://epd.vital-it.ch/index.php). BRCA1 promoter was amplified with forward-TATTTTGAGGATTCGTCATGATTAG and reverse-CTAAAAACCCCCACCAACCTCATCC primers. Analysis of BRCA1 methylation was pre-planned, and RAD51C methylation was added ad hoc to BRCA1 methylation prior to association with subsequent end points, as the potential importance of RAD51C methylation for TNBC was only recognised after the start of the trial25. The RAD51C promoter was amplified with forward-TGGTAATTTGGTATGCTGTGTTG and reverse-TCTCTA TAAATACAGCTTTACT primers. BRCA1 and RAD51C PCR conditions were optimised for multiplex assay using Thermofisher Scientific AccuPrime Hi-fidelity Taq. Human methylated and unmethylated DNA (Zymo Research, Human HCT116DKO non-methylated DNA and HCT116DKO methylated DNA) primers were used as a control. DNA from RNA LNA Later samples were subjected to bisulfite sequencing (Zymo Research Methylation Gold spin column kit D5005). Total DNA was eluted from 10 to 500 ng DNA was eluted from 10 to 500 ng and DNA was eluted from 10 to 500 ng and DNA was eluted from 10 to 500 ng using a QIAQuick PCR purification kit (ID:28104) and quantified using Qubit 3.0 fluorometer. Samples were subjected to Illumina Nextera Ultra II library preparation. Total library input ranged from 10–116 ng and PCR cycles were adjusted accordingly, and sequenced on the Illumina MiSeq platform with MiSeq kit (v2.1.1) available from Rochè. Mean number of reads for BRCA1 amplicon was 36999 (range 9654–60084) and RAD51C amplicon 48879 (range 28474–71129) with a mean 47% of reads on target for the methylation sequencing run. Bioinformatics analysis of methylation followed a similar workflow to previous studies35. Paired overlapping reads were merged into a single sequence using flash after adaptor trimming using trim-galore (https://www.bioinformatics. babraham.ac.uk/projects/trim_galore/). Each read was aligned using pairwise alignment using Bowtie v2.2.6. Somatic mutations identified in paired-end sequencing using Picard (https://picard.sourceforge.net/). Primers for the BRCA1 and RAD51C methylation, bisulfite analysis.

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calculating the unconverted cytosine count at non CG sites as well as reads where all CG sites in the read were not C or T. Reads were assessed as being methylated when >99% of the cytosine site were methylated. For RAD51C methylation, 2 sites in the RAD51C amplicon were removed as these were found to be consistently methylated in all samples.

Immunohistochemistry for RAD51/Gemin (GMNN) double staining. FFPE samples taken 24–48 h after the last dose of rucaparib were cut, deparaffinised, rehydrated and stained with hematoxylin and eosin (H&E) and double stained with GMNN and RAD51. Pre- and post-radiotherapy-induced squamous cell carcinoma were used as negative and positive controls.

Antigen retrieval was performed and RAD51 primary antibody (mouse monoclonal, Genetex, GTXT0230) was diluted 1/200 in Dako antibody diluent (K0068) and applied. Slides were incubated with Dako Envision Flex HRP (K0002). Geminin (GMNN) antibody (rabbit polyclonal, ProteinTech 10802-1-AP) diluted 1/1500 in Dako antibody diluent was applied and incubated with Dako Envision Flex HRP (K0002). Sections incubated with Vector TMB blue (SK4400) and counterstained with Gills 1 hematoxylin, air dried and dehydrated in xylene before mounted and cover slipped with Vectamount.

Five random fields at 40× magnification were identified and marked in PathXL. GMNN staining was identified with blue/green staining and RAD51 was identified by the presence of brown nuclear foci. Scoring was done by 2 scorers blinded to each other, time point and clinical details.

The number of tumour cells, GMNN-positive cells and RAD51-positive cells were counted. Cells with 5 or more RAD51 foci were classified as RAD51 positive. A minimum of 300 tumour cells and 30 GMNN-positive cells were a minimum requirement for inclusion. Raw data were collected and the proliferation fraction (no. of GMNN-positive cells / total number of tumour cells) × 100) and RAD51 score (no. of RAD51-positive cells / no. of GMNN-positive cells) × 100) was calculated.

Circulating tumor DNA analysis. For each trial subject, dPCR assays were designed for the mutations identified by tissue sequencing according to the method of Garcia-Murillas et al.22 Assays were optimised with temperature gradients and patients with more than one mutation had multiplex assays optimised. If optimisation was not achieved with multiplexes, a singleplex assay was used.

Mutation analysis was done using dPCR assays specific for each patient’s mutation(s) on a QX200 system (Bio-Rad) running Quantasoftware v1.7. 50 μL DNA (2 ml plasma equivalent) was used divided equally into 2 wells from each sample. DNA was dried at 60 degrees for 100 min before preparing the PCR reactions to a volume of 20 μL. Three NTCs and a negative control of the patient’s buffy coat DNA were included for each dPCR assay. Only paired samples with at least 4 positive droplets in baseline samples were analysed for change in ctDNA levels. The circulating DNA ratio at day 15 (CDR15) was assessed as a ratio of the ctDNA copies/ml at EOT copies/ml compared to ctDNA copies/ml at baseline. Where more than one mutation was tracked a weighted mean of ctDNA change was calculated.

The CDR15 cutoff <0.25 for ctDNA suppression, was pre-specified in a separate study in metastatic breast cancer, that validated this cutoff to predict response free survival on cytotoxic paclitaxel therapy25.

RNA sequencing. Paired RNAlater™ samples were identified and sectioned using a cryostat. One section was cut for H&E and 10 sections were cut and stained with nucleic acid staining for Nuclear Fast Red. A second H&E section was cut at the end of the series. H&E sections were reviewed and marked by a pathologist for tumour content. NFR stained sections were micro-dissected and RNA was extracted using the Qiagen RNeasy Mini kit according to the manufacturer’s instructions. RNA was eluted twice into separate 50 μL RNA free water and stored at −80°C before quantification. RNA was quantified using Qubit 3.0 fluorimeter using the Qubit™ RNA HS Assay Kit (Q28528, ThermoFisher Scientific).

Extracted RNA (~1 μg) was sent to Eurofins Genetic Services Limited for RNA Exome sequencing. Total RNA was subjected to RiboZero depletion and Illumina TruSeq RNA Exome library preparation. Libraries were pooled and sequenced on an Illumina HiSeq 2500 (+4 chemistry) running HiSeq Control Software (HCS) v2.26.8. Samples were aligned to the GCh37 genome using STAR aligner (https://github.com/alexdobin/STAR)51 with a mean of 44,117,169 reads per sample (range 140,107,52–91,470,225). Gene counts were established using htseq (https://htseq.readthedocs.io/en/master/index.html), Biostrings R package. RNA sequencing: STAR aligner (https://github.com/alexdobin/STAR), Htsq (https://github.com/simon-anders/htseq), DeSeq2 (https://doi.org/10.18129/B9.bioc.Deseq2), fgsca (https://doi.org/10.18129/B9.bioc.fgsca), AIMS (https://doi.org/10.18129/B9.bioc.AIMS), TNBCtype (http://cbbc.mc.vanderbilt.edu/tnbc/) and Cibersort (https://cibersort.stanford.edu/index.php).

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Code availability
The code used for the analysis of sequencing data is detailed here with links and references where appropriate.

Whole-genome sequencing: CaVEMan (Cancer Variants Through Expectation Maximization: http://cancerit.github.io/CAVEMan/), Pindel version 2.0 (http://cancerit.github.io/cgpPindel/), BRASS (Breakpoint AnalysisSS) (https://github.com/canceriter/BRASS), and ASCAT (v2.1.1). Annotation was to Ensembl build 75. Code for targeted tumour sequencing is available upon request. AVENIO ctDNA sequencing: AVENIO Oncology analysis software (v1.0.0 and v1.1) available from Roche. Bisulfite sequencing: Eukaryotic Promoter Database (http://epd-vital-it.ch/index.php), trim-galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), Biostrings R package. RNA sequencing: STAR aligner (https://github.com/alexdobin/STAR), Htsq (https://github.com/simon-anders/htseq), DeSeq2 (https://doi.org/10.18129/B9.bioc.Deseq2), fgsca (https://doi.org/10.18129/B9.bioc.fgsca), AIMS (https://doi.org/10.18129/B9.bioc.AIMS), TNBCtype (http://cbbc.mc.vanderbilt.edu/tnbc/) and Cibersort (https://cibersort.stanford.edu/index.php).

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analysed immunohistochemistry. H.R.D., A.D. and S.N.Z. performed whole-genome sequencing and HRDetect analysis. R.R., S.C., A.T., A.S., and A.E. were principal investigators at trial sites. N.T., N.C. and A.P. wrote the manuscript and all authors contributed comments and corrections.

Competing interests
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Additional information
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