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# Safety and utility of image-guided research biopsies in relapsed high-grade serous ovarian carcinoma—experience of the BriTROC consortium

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**Background:** Investigating tumour evolution and acquired chemotherapy resistance requires analysis of sequential tumour material. We describe the feasibility of obtaining research biopsies in women with relapsed ovarian high-grade serous carcinoma (HGSC).

**Methods:** Women with relapsed ovarian HGSC underwent either image-guided biopsy or intra-operative biopsy during secondary debulking, and samples were fixed in methanol-based fixative. Tagged-amplicon sequencing was performed on biopsy DNA.

**Results:** We screened 519 patients in order to enrol 220. Two hundred and two patients underwent successful biopsy, 118 of which were image-guided. There were 22 study-related adverse events (AE) in the image-guided biopsies, all grades 1 and 2; pain was the commonest AE. There were pre-specified significant AE in 3/118 biopsies (2.5%). 87% biopsies were fit-for-purpose for genomic analyses. Median DNA yield was 2.87  $\mu$ g, and was higher in biopsies utilising 14 G or 16 G needles compared to 18 G. TP53 mutations were identified in 94.4% patients.

**Conclusions:** Obtaining tumour biopsies for research in relapsed HGSC is safe and feasible. Adverse events are rare. The large majority of biopsies yield sufficient DNA for genomic analyses—we recommend use of larger gauge needles and methanol fixation for such biopsies, as DNA yields are higher but with no increase in AEs.

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The commonest subtype of ovarian cancer, high-grade serous carcinoma (HGSC), accounts for ~70% all cases, and 80% of deaths. Genomically, it is defined by near-universal *TP53* mutation (Ahmed *et al*, 2010; Köbel *et al*, 2016), widespread copy number alterations (Gorringe *et al*, 2010; TCGA, 2011) and loss of gene function through complex structural rearrangements (Patch *et al*, 2015).

HGSC is initially sensitive to platinum-based chemotherapy but relapse occurs very frequently, with progressive development of platinum resistance, resulting in poor long-term survival (Mackay *et al*, 2010; Oza *et al*, 2015). Spatial and temporal heterogeneity have been frequently observed in HGSC, and low-frequency subclonal populations, which are present at the time of diagnosis, can undergo expansion during chemotherapy to become the predominant resistant population (Forsheiw *et al*, 2012; Schwarz *et al*, 2015). In addition, secondary or revertant mutations in *BRCA1* and *BRCA2* may provide alternative mechanisms of abrogating platinum sensitivity (Edwards *et al*, 2008; Sakai *et al*, 2008; Patch *et al*, 2015).

Establishing accurate estimates of the prevalence of temporal heterogeneity and divergent evolution in HGSC will require sequential collection of tumour material at multiple time points during throughout course of the patient's disease, especially at relapse and progression. We and others have already demonstrated that image-guided biopsies can be obtained in patients with ovarian cancer (Spencer *et al*, 2006; Griffin *et al*, 2009; Swisher *et al*, 2017), whilst many early phase clinical trials now include translational research biopsies to investigate pharmacodynamic biomarkers. However, there is widespread variation in the reporting of outcomes of biopsy-driven studies (Freeman *et al*, 2013) and few data on the utility of these biopsies for complex genomic studies.

We have established BriTROC, a UK-based ovarian cancer consortium, which is investigating the acquisition of resistance in women with HGSC by focussing on the collection of sequential tumour biopsies, ctDNA and ascites from women with relapsed HGSC. We present here the interim data from the BriTROC-1 study in order to demonstrate the safety and feasibility of acquiring tumour biopsies in relapsed ovarian cancer across multiple academic centres. The secondary aim is to report the utility of methanol-fixed biopsies for next-generation sequencing analyses (Piskorz *et al*, 2016).

## PATIENTS AND METHODS

**Study conduct.** The BriTROC-1 study was funded by Ovarian Cancer Action and sponsored by NHS Greater Glasgow and Clyde. A Trial Management Group (TMG, see Supplementary Material 1) designed and ran the study. Ethics/IRB approval was given by Cambridge Central Research Ethics Committee (Reference 12/EE/0349). The primary objective was to demonstrate the safety and feasibility of acquiring tumour biopsies from women with relapsed ovarian cancer in multiple centres. The secondary endpoints were to examine genomic alterations in relapsed ovarian HGSC. All patients provided written informed consent—this consent included specific consent to biopsy, use of biopsy material (and ascites if present) for genomic studies and testing of germline DNA for *BRCA1/2* mutations. In addition, patients could optionally consent to a second biopsy upon disease progression and to be informed of germline *BRCA1/2* analysis results. A copy of the patient consent form is included in Supplementary Material 7.

Significant complications were continuously monitored during the study. These were defined as grade  $\geq 2$  events related to image-guided biopsy, specifically pain, haemorrhage, wound infection and

peritoneal infection. The monitoring process used a one-sided (upward) CUSUM chart (Lerch *et al*, 2007) both study-wide and for individual sites. CUSUM control limits were set using an acceptable significant complication rate of  $< 5\%$ , an unacceptable rate of  $> 10\%$ , a power of 88% and a significance level of 10%. If an individual site breached CUSUM control limits, that site was suspended pending detailed TMG review. If the whole study breached CUSUM control limits, recruitment was suspended to allow thorough review.

**Patients.** The study enrolled patients with recurrent ovarian high-grade serous or grade 3 endometrioid carcinoma who had relapsed following at least one line of platinum-based chemotherapy. Other histological subtypes were only allowed in patients with known deleterious germline *BRCA1* or *BRCA2* mutations. All patients had to have disease amenable either to image-guided or other interventional (e.g., endoscopy, bronchoscopy) biopsy, or secondary debulking surgery. Samples obtained at secondary debulking or other interventions were jointly classified as 'surgical' biopsies. Access to archival diagnostic formalin-fixed was also required, as well as snap frozen tumour material if available. Overall survival was calculated from the date of enrolment to the date of death or the last clinical assessment, with data cutoff at 1 December 2016. Full inclusion and exclusion criteria are listed in Supplementary Material 2.

Patients underwent biopsy (at least two cores, 14–16 G biopsy needle) or secondary debulking surgery, with tumour samples fixed in methanol (TissueTek Xpress, Sakura Finetek, Torrance, CA, USA) (Piskorz *et al*, 2016). If 14–16 G cores were felt not to be appropriate (e.g., due to site of disease), three 18 G cores were taken instead. For patients undergoing secondary debulking or other interventional biopsies, 14–16 G cores or a 1 cm<sup>3</sup> piece of macroscopically identified tumour tissue were taken. Retrospective registration was allowed only following consultation with the Chief Investigator. All samples were shipped within 24 h at ambient temperature to the University of Glasgow for processing.

All treatment following study entry was at the discretion of the treating oncologist. Patients optionally also consented to a further biopsy at subsequent progression.

### Sample processing, DNA extraction and quantification.

A detailed laboratory manual was provided to all study sites. After fixation, tumour samples were processed in a ThermoExcelsior Tissue Processor using optimised protocols (Piskorz *et al*, 2016). Tumour cellularity was determined on H&E staining by a pathologist with expertise in gynaecological pathology as the percentage of tumour cells in areas selected for dissection. Up to forty 10  $\mu$ m tissue sections per block were macro- or micro-dissected. DNA was extracted using QIAmp DNA Micro Kit (Qiagen, Manchester, UK). Quantification was performed by Qubit 2.0 fluorimeter (Life Technologies, Paisley, UK) using dsDNA BR Assay.

**Tagged-amplicon sequencing.** The coding regions of *TP53*, *PTEN* and hotspot regions in *EGFR*, *PIK3CA*, *KRAS* and *BRAF* were sequenced by tagged-amplicon sequencing (Forsheiw *et al*, 2012) on the MiSeq Sequencing System (Illumina, Cambridge, UK) using paired-end 125 bp protocols. Sequencing data analysis was performed as previously (Piskorz *et al*, 2016).

**Statistical analyses.** All statistical analyses were performed in Prism for Mac v.6 (GraphPad, La Jolla, CA, USA) and R (v.3.3.2). Adverse event (AE) rates were compared using Pearson's  $\chi^2$ -test, while DNA yields were compared using Wilcoxon rank sum test. All tests were two-sided and  $P < 0.05$  was considered significant. A complete R markdown document detailing all analyses is provided as Supplementary Material 8.

**RESULTS**

**Patients and recruitment.** Recruitment started on 4 January 2013 and involved 14 UK centres. Figure 1 summarises the recruitment and sample numbers. A total of 519 patients were screened (Figure 1A). One hundred and eighty-two patients were ineligible and 117 declined to participate. The most frequent cause of ineligibility was disease deemed not suitable for image-guided

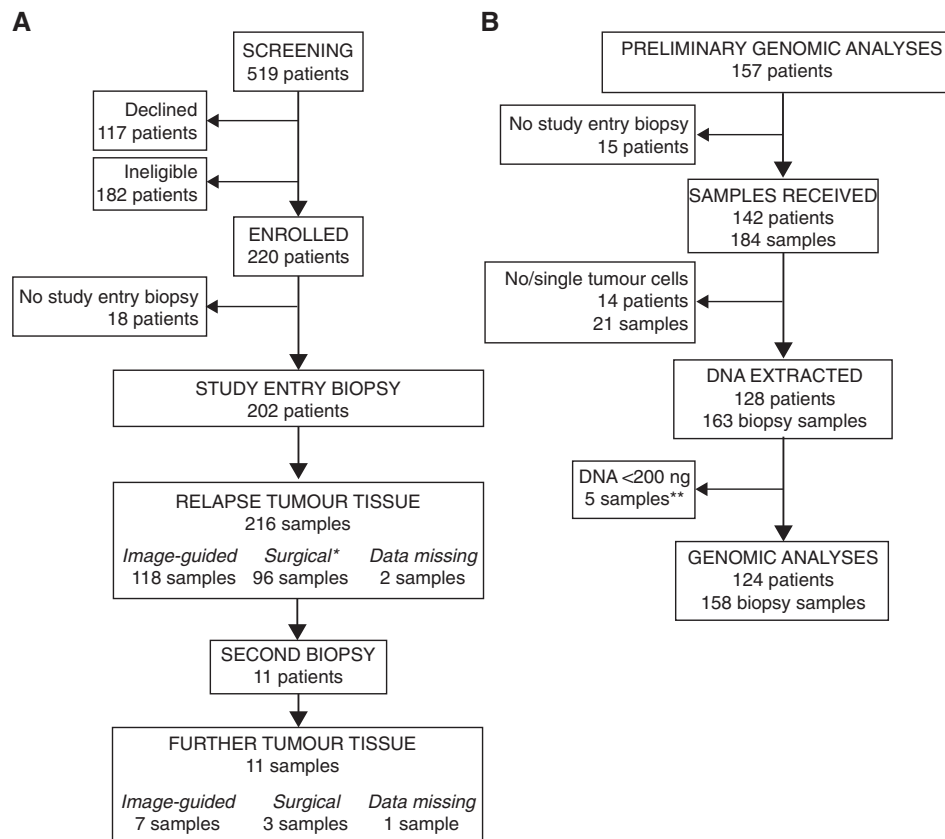
biopsy by expert radiologists. Supplementary Material 3 lists all reasons for ineligibility and for declining participation.

Table 1 summarises the clinical characteristics of the 220 enrolled patients, of whom 198 (90%) were registered prospectively and 22 (10%) retrospectively. The median number of prior chemotherapy lines was 1 (range 1–5) for platinum-sensitive patients and 2 (range 1–12) for platinum-resistant (Supplementary Material 4), and 17 patients (7.7%) had received prior bevacizumab. Median overall survival from study entry was 13.4 months for

**Table 1. Demographic and disease characteristics**

Characteristic	Platinum-sensitive (N=171)	Platinum-resistant (N=49)	Total (N=220)
Median age (range), years	69 (37–93)	66 (25–83)	68 (25–93)
Median time since diagnosis (range), months	32.2 (10.2–285.2)	24.4 (7.6–184.2)	30.6 (7.6–285.2)
Germline BRCA1/2 mutation, N (%) <sup>a</sup>			
BRCA1	11 (6.4)	3 (6.1)	14 (6.4)
BRCA2	7 (4.1)	5 (10.2)	12 (5.5)
Histology, N (%)			
High-grade serous	160 (93.6)	49 (100)	209 (95.0)
G3 Endometrioid	5 (2.9)	0	5 (2.3)
Carcinosarcoma	1 (0.6)	0	1 (0.4)
Missing	5 (2.9)	0	5 (2.3)
Number of prior treatment regimens			
Median number of regimens (range)	1 (1–5)	2 (1–12)	1 (1–12)
1, N (%)	120 (70.2)	17 (34.7)	137 (62.3)
2, N (%)	39 (22.8)	23 (46.9)	62 (28.2)
3, N (%)	6 (3.5)	1 (2.0)	7 (3.2)
4, N (%)	1 (0.6)	2 (4.1)	3 (1.4)
> 4, N (%)	3 (1.7)	6 (12.3)	9 (4.0)
Data missing	2 (1.2)	0	2 (0.9)

<sup>a</sup>Mutation status as recorded at time of study entry.



**Figure 1. Trial profile. (A)** Flow of patients and samples in the BriTROc-1 study. \*Patients undergoing other interventional biopsies are classified as ‘surgical’. **(B)** Sample flow from the first 157 patients. \*\*Two samples with <200 ng DNA were still sufficient for tagged amplicon sequencing analysis.

**Table 2. Biopsy locations by sample**

Anatomical location	N=	%
Lymph node <sup>a</sup>	64	28.2
Peritoneum	53	23.3
Omentum	26	11.5
Liver	17	7.5
Gynaecological organ <sup>b</sup>	14	6.2
Soft tissue (subcutaneous, chest or abdominal wall)	14	6.2
Bowel, serosa or mesentery	12	5.3
Diaphragm	4	1.8
Peri-splenic	4	1.8
Other <sup>c</sup>	19	8.4
Total	227	100.0

<sup>a</sup>Pelvic (14), para-aortic/retroperitoneal (13), other (37).  
<sup>b</sup>Vaginal vault (8), uterus, ovary/fallopian tube (6).  
<sup>c</sup>Bladder wall (3), brain (2), iliac fossa (2), pelvic/pelvic sidewall (2), breast (1), paracolic gutter (1), para-sternal (1), perinephric (1), pleural (1), retro-caecal (1), small bowel (1), trachea (1), lung (1), obturator fossa (1).

the platinum-resistant patients and 34.9 months for platinum-sensitive patients (Glasgow and Edinburgh patients,  $n = 68$ ; Supplementary Material 5).

**Biopsies.** Eighteen of the 220 (8.2%) consented patients did not complete study-entry biopsy, most commonly because disease was found to be unsuitable for biopsy during the procedure. Therefore, 202 patients completed study-entry biopsy procedure, including 118 image-guided biopsies, yielding a total of 216 relapse tumour samples. Eleven patients also underwent a second biopsy procedure (seven image-guided) upon subsequent progression, including one patient whose study entry biopsy had been unsuccessful. Thus, a total of 227 tumour samples were collected. Reflecting the pattern of spread of HGSC, these samples were obtained from multiple different anatomical locations (Table 2). The commonest sites were lymph node (64 samples), peritoneum (53 samples), omentum (26 samples) and liver (17 samples). Out of the 125 image-guided biopsy procedures (118 at study entry, 7 at second biopsy), needle size information was available for 120: 17/120 (14.2%) used 14 G needles, 49 (40.8%) 16 G and 54 (45.0%) 18 G.

Study-related AEs were recorded for image-guided biopsy procedures (A full listing is given in Supplementary Material 6). Twenty-two AEs of any grade were reported (20 baseline biopsy, 2 second biopsy) in 18/125 (14.4%) procedures (Table 3). Pain was the commonest AE (16/22, 72.7%; 13 grade 1, 3 grade 2). The frequency of AEs was not significantly higher for 14 G (five events in 17 biopsies) or 16 G (6/49) needles compared to 18 G (11/54;  $P = 0.228$ ). Pre-specified significant complications were reported in 3/125 (2.4%) biopsies; two for grade 2 pain and one for combined grade 2 pain and grade 2 haemorrhage (liver haematoma). The grade 2 haemorrhage was managed conservatively and did not require transfusion or any active intervention. There was only one other grade 2 AE, of vaginal discharge in one patient, which was also managed conservatively. CUSUM control boundaries were not crossed and all complications resolved without long-term sequelae.

**Quality of biopsies.** We analysed samples from the first 157 patients, of whom 15 did not successfully complete study-entry biopsy. The remaining 142 patients yielded 184 samples (Figure 1B). At pathology review, 21/184 (11.4%) samples from 14 patients were found to contain no or few tumour cells, leaving 163 tumour samples (88 from image-guided biopsies, 75 from surgical). Following dissection (41 macrodissection, 122 microdissection), median tumour cellularity was 65% (IQR 45%–75%). The median DNA yield was 2.87  $\mu\text{g}$  (IQR 0.78–8.19). Five samples

**Table 3. Adverse events in 125 image-guided biopsy procedures**

Event type <sup>a</sup>	Grade 1	Grade 2
Pain	13	3
Haemorrhage	3	1
Vaginal discharge	0	1
Other <sup>b</sup>	1	0

<sup>a</sup>One biopsy could have > 1 adverse events. These events were reported from 19 biopsy procedures in 18 patients.  
<sup>b</sup>Haematoma.

(3.1%) yielded <200 ng DNA, the minimum pre-specified for genomic analyses, although 2 of these 5 were still sufficient for tagged amplicon sequencing. For image-guided biopsies (Figure 2A), median DNA yield was 1.96  $\mu\text{g}$  (IQR 0.62–3.39), whilst surgical sample median was significantly higher (4.64  $\mu\text{g}$  (IQR 1.96–10.14);  $P < < 0.001$ ). In image-guided biopsies (Figure 2B), median yield was significantly higher for 14 G/16 G needles (2.86  $\mu\text{g}$  [IQR 0.85–5.54]) than for 18 G (0.89  $\mu\text{g}$  (IQR 0.47–2.92),  $P = 0.011$ ).

By anatomical location in image-guided biopsies specifically (Figure 2C), the highest median DNA yields were obtained from gynaecological organs (8.82  $\mu\text{g}$  (IQR 3.16–13.36  $\mu\text{g}$ )), peritoneum (2.86  $\mu\text{g}$  (IQR 0.62–4.56)) and lymph nodes (2.32  $\mu\text{g}$  (IQR 0.63–3.18)), although these differences did not reach statistical significance. There was a significant correlation (Spearman  $\rho = 0.304$ ,  $P < < 0.001$ ) between tumour cellularity and overall DNA yield (Figure 2D).

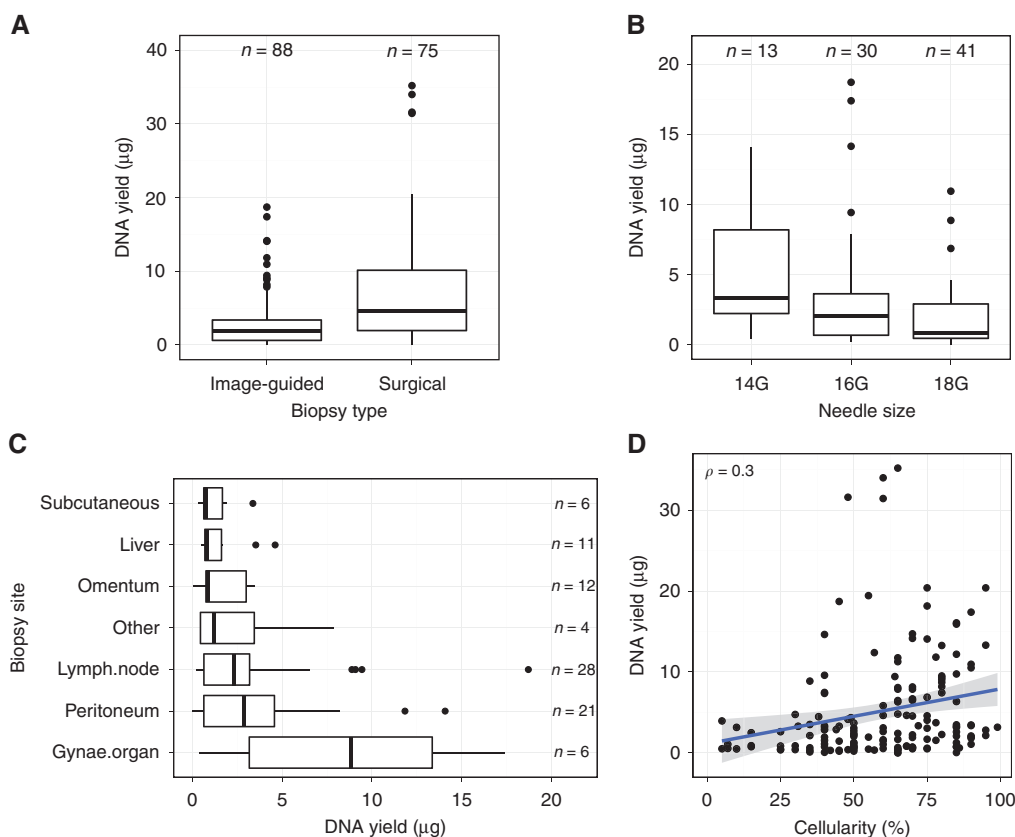
Using tagged amplicon next-generation sequencing (median read depth 1086  $\times$ ), *TP53* mutations were identified in 94.4% (118/125) patients (Figure 3A) with mean mutant allele frequency (MAF) 0.55. These included 71 (60.2%) nonsynonymous (missense), 41 (34.7%) loss of function mutations (nonsense, splicing and frameshift) and 6 (5.1%) inframe indels. Mutations in *KRAS*, *PIK3CA* and *PTEN* were identified in 4.8% (6/125), 2.4% (3/125) and 1.6% (2/125) patients respectively. No mutations were detected in *EGFR* or *BRAF*. Overall, we found a strong correlation between cellularity and *TP53* MAF (Figure 3B, Spearman  $\rho = 0.314$ ,  $P < < 0.001$ ) and a weaker, but still significant, correlation between DNA yield and *TP53* MAF ( $\rho = 0.220$ ,  $P = 0.0034$ ).

## DISCUSSION

In this study, we demonstrate that obtaining tumour biopsies in women with relapsed ovarian cancer is feasible and safe, and can be co-ordinated across multiple academic centres. Moreover, we demonstrate that patients with recurrent ovarian cancer are willing to undergo a research biopsy for purely altruistic purposes; treatment following the biopsy was at the local investigator's discretion and there was no investigational agent on offer.

In order to recruit 220 patients, we had to screen 519—the commonest reason for non-participation was disease thought not to be amenable to image-guided biopsy on review of imaging. Thus, our image-guided biopsies were obtained from women with relatively bulky disease. However, median OS (13.4 months and 34.9 months, respectively, for platinum-resistant and platinum-sensitive patients) was comparable to that in the control arms of recent large randomised studies (Aghajanian *et al*, 2012; Pujade-Lauraine *et al*, 2014), and tagged amplicon sequencing demonstrated near-universal *TP53* mutation and low-frequency mutations in *KRAS*, *PIK3CA* and *PTEN*, in keeping with previous data in HGSC (Ahmed *et al*, 2010; TCGA, 2011; Köbel *et al*, 2016). Thus, we believe that our patients are similar to other trial





**Figure 2. DNA yields.** (A) DNA yield in image-guided and surgical biopsies. \*\*\*\* $P < 0.0001$ . (B) DNA yield was significantly greater in image-guided biopsies using 14 G or 16 G needles compared to 18 G. \*\* $P = 0.0022$ . (C) DNA yields according to anatomical site of biopsy for image-guided procedures. (D) Correlation between DNA yield and tumour cellularity. Number represents Spearman rank correlation coefficient  $\rho$ .  $P < 0.001$ . Line represents linear regression  $\pm$  95% CI.

populations in relapsed ovarian cancer. Seven patients were found to have *TP53* wild-type tumours, three of which harboured *KRAS* mutations. Two of these cases were diagnosed before the binary grading of serous carcinomas was introduced and had been originally classified as 'grade 2 serous' tumours. In other cases, the reporting pathologists stated that grading was difficult or that there were areas of borderline change. Thus, it is likely that these cases represent low-grade serous carcinomas that were misclassified at the time of original diagnosis.

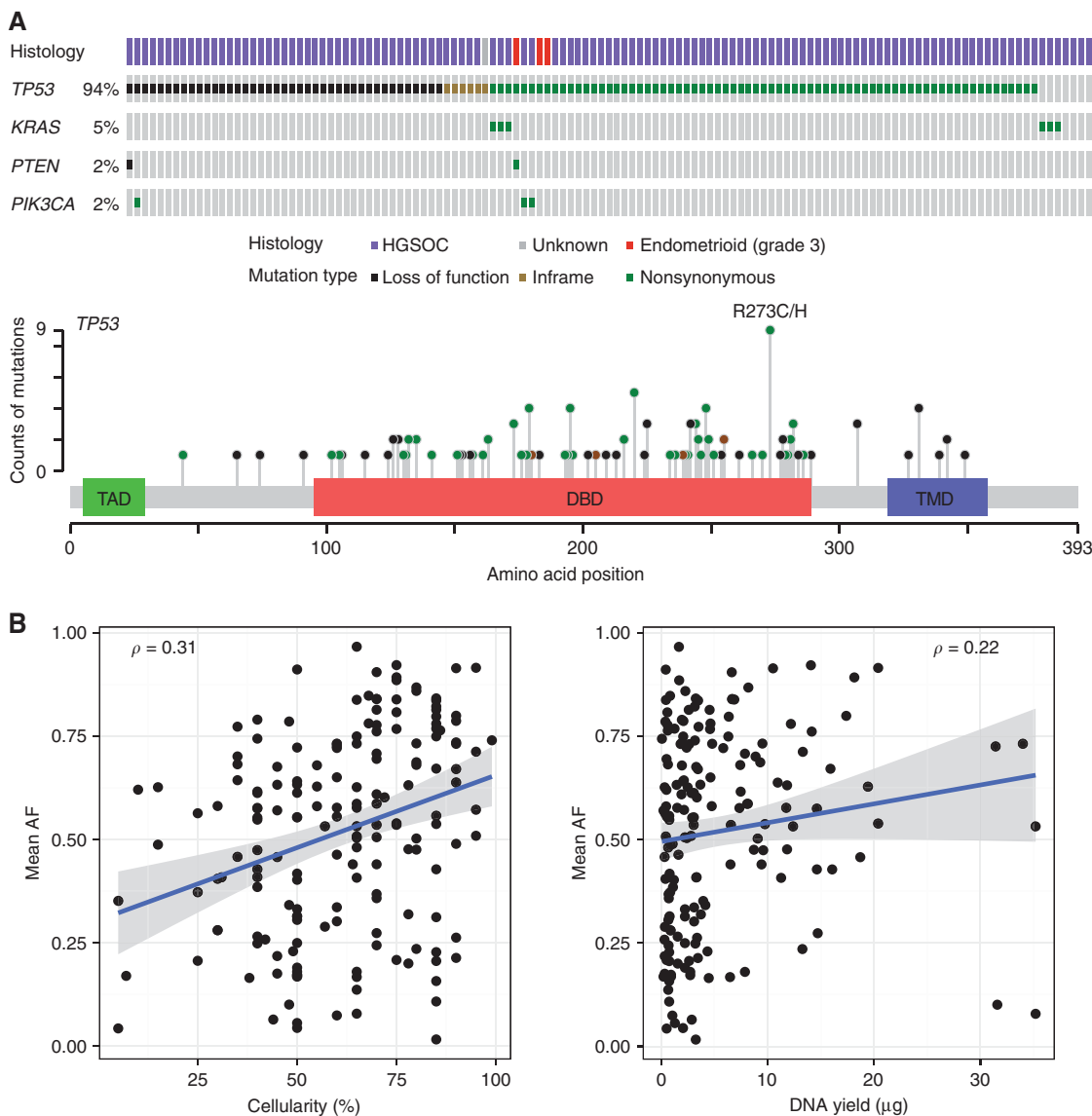
Adverse events were generally rare, with grade 1 pain as the commonest event following image-guided biopsy, recorded in 13 cases. There were only three expedited AEs, a figure comparable to results reported from large single centre series (El-Osta *et al*, 2011; Gomez-Roca *et al*, 2012), and one other grade 2 AE (vaginal discharge), in one patient. However, it is clearly important that patients are properly consented before research biopsy procedures, and these data provide robust safety information to inform the consent process in patients with relapsed ovarian cancer in future.

We fixed samples in a methanol-based fixative that we have previously shown to be superior to buffered formalin for genomic studies (Piskorz *et al*, 2016). In addition, methanol fixation allows samples to be transported and stored at ambient temperature and analysed by IHC, both distinct advantages over snap-frozen samples for a multi-centre study. We highlight that, although 11.4% (21/184) samples contained  $< 20\%$  tumour cells, only three of the remaining 164 samples yielded no usable DNA. Thus, overall 87% of the biopsies were fit-for-purpose. These figures are comparable to the ARIEL2 study, which investigated genomic LOH as a potential predictive biomarker of response to the PARP inhibitor rucaparib (Swisher *et al*, 2017). In ARIEL2, 136/152

(89.5%) relapse biopsies yielded sufficient DNA for LOH analyses. Three of the samples in our study with  $< 200$  ng yields utilised an 18 G needle—our results show that 14 G/16 G needles generate higher DNA yields but do not cause more frequent AEs. We thus recommend the use of larger gauge needles for biopsies where genomic analyses are planned.

In terms of anatomical biopsy location, experience from the MOSCATO-01 study indicated that bone biopsies were associated with the lowest rates of tumour cellularity (Tacher *et al*, 2016). In our series, there were no bone biopsies, given the pattern of dissemination of HGSC. The commonest site of biopsy was lymph node—the choice of biopsy site was at the discretion of the radiologist undertaking the procedure for image-guided biopsies. Thus, these were the most accessible sites in the participating patients rather than the only sites of relapse. We found that biopsies from gynaecological organs yielded the highest quantities of DNA in image-guided procedures, although the differences between biopsy sites were not significant. We found strong correlation between tumour cellularity and *TP53* mutant allele fraction, as well as correlation between DNA yield and cellularity. The correlation between total DNA yield and mutant allele fraction was still significant, although more weakly than the other two analyses. This suggests that, although DNA yield is important, dissection of samples to ensure high tumour cellularity is critical when processing biopsies for genomic studies.

The scientific purpose of the BriTROC programme will be to investigate changes in cancer biology as tumours recur and develop chemotherapy resistance. Intra-tumoural spatial heterogeneity is a potential concern when examining single core biopsies in patients with multi-site relapse, and the recent International Cancer Genome Consortium analysis identified multiple separate



**Figure 3. Mutation analysis.** (A) Mutations in *TP53*, *KRAS*, *PIK3CA* and *PTEN* identified in 125 patients by tagged amplicon sequencing with histological subtype (upper). No mutations in *EGFR* or *BRAF* were identified in any sample (Cerami *et al*, 2012; Gao *et al*, 2013). Schematic representation of p53 (lower) showing protein domains (Green, transactivation domain; red, DNA binding domain; blue, tetramerisation domain) with lollipops showing positions and counts of identified mutations. Mutation type is indicated by circle fill: black, loss of function (including nonsense, splicing and frameshift); brown, inframe indels; green, nonsynonymous. (B) Correlation between cellularity in dissected areas and *TP53* mutant allele fraction (MAF)—left, ( $P < <0.001$ ), and between DNA yield and *TP53* mutant allele fraction—right, ( $P = 0.0034$ ). Numbers represent Spearman rank correlation coefficient  $\rho$ . Lines represent linear regression  $\pm$  95% CI.

reversion events in post-mortem analysis of a germline *BRCA2* mutation carrier with HGSC (Patch *et al*, 2015). We have allowed patients undergoing secondary debulking surgery to enter BriTROc-1, and five of these patients have donated multiple samples.

In early phase trials, there is evidence that patients are broadly willing to undergo translational biopsies (Seah *et al*, 2013), although rates of take-up are highest for mandatory biopsies (El-Osta *et al*, 2011). Scheduling research biopsies in busy interventional radiology departments can be challenging—we demonstrate here that it is logistically possible to do so, and centres received a standard per-biopsy fee of £800 (approximately US\$1000, €950) to cover the cost of the scan (CT or ultrasound), the radiologist’s time, the biopsy needle and fixative. There is variation in the reporting of research biopsies (Freeman *et al*, 2013), and we believe that it is essential that biomarker biopsy studies are reported consistently to improve future trials. Such reports should include

complete details for biopsy sites, the number of biopsies, the protocol for tissue analysis and overall outcome. BriTROc-1 shows that research biopsies that are fit-for-purpose for genomic analyses can be readily and safely obtained from women with recurrent HGSC across multiple centres. Although the yields from surgical biopsies were higher, image-guided biopsies are satisfactory, less invasive and obviate the need for a general anaesthetic in patients not scheduled for secondary debulking surgery. From these results, we recommend the use of methanol fixation and of 14 G or 16 G needles for biopsies as they are associated with optimum DNA yield but no increase in AEs.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### AUTHOR CONTRIBUTIONS

Study design: IMcN, JDB, JP, TG, AP, DE and GM. Patient recruitment: IMcN, JDB, RMG, DK, ML, EB, AM, AW, SS, RE, GH, AC, CG, MH, SF, CF and HG. Pathological assessment: LM and MJL. Data acquisition: LAL, JS, CW, TG, AP, DE, GM and IMcN. Data analysis: JS, TG, DE, AP, GM, JP, IMcN and JDB. Manuscript preparation: IMcN, JDB, TG, DE and AP. All authors reviewed the manuscript before submission.

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