Background: The incidence of oesophageal adenocarcinoma (OAC) is rapidly increasing and despite improvements in treatment, the 5-year survival rate remains poor. Prognostic biomarkers that address the genomic heterogeneity in this highly complex disease will aid the development of precision therapeutics and improve patient survival. The aim of this study was to determine whether circulating tumour DNA (ctDNA) has prognostic significance as a biomarker in OAC patients.

Patients and methods: We profiled 209 blood and tumour samples from 57 OAC patients. Using a panel of 77 cancer genes, we sequenced ctDNA in plasma samples \((n = 127)\) which were taken at multiple time points before and after therapy. In parallel, we sequenced matched tumour samples from 39 patients using the same gene panel. To assess whether the ctDNA profile reflected the tumour heterogeneity, we sequenced additional multi-region primary tumour samples in 17 patients. In addition, we analysed whole-genome and whole-exome sequencing data from primary tumours for a subset of 18 patients.

Results: Using a tumour-agnostic approach, we found that detectable ctDNA variants in post-treatment plasma samples were associated with worse disease-specific survival. To evaluate whether the ctDNA originated from the primary tumour, we carried out a tumour-informed analysis which confirmed post-treatment ctDNA variants were associated with worse survival. To determine whether ctDNA could be used as a clinical follow-up test, we assessed blood samples from multiple time points before and after treatment, in a subset of patients. Results showed that the variant allele frequency of ctDNA variants increased with disease recurrence.

Conclusion: This study demonstrates that ctDNA variants can be detected in patients with OAC and this has potential clinical utility as a prognostic biomarker for survival.

Key words: ctDNA, sequencing, biomarker, oesophageal adenocarcinoma, survival, prognostic
limitation is that variants are not all confirmed as tumour derived. In OAC, four small studies assessed the role of ctDNA as a biomarker of survival.\textsuperscript{14,15,32,33} These studies vary in their methodology limiting their clinical utility.

The primary aim of this study was to determine whether ctDNA detected perioperatively has prognostic or predictive significance in patients with locally advanced OAC (n = 57). The second aim was to determine whether the ctDNA profile is reflective of ITH in these patients. The third aim was to examine the ability of ctDNA to detect disease recurrence during follow-up. Using a CAPP-seq pan-cancer gene panel, we sequenced 183 tumour and plasma samples, which included multiple tumour biopsies for a subset of patients. Whole-genome sequencing (WGS) or whole-exome sequencing (WES) data were available for an additional 24 tumour biopsies. Using this data, we describe the impact of ITH on the development of ctDNA as a prognostic biomarker in OAC.

MATERIALS AND METHODS

Patient cohort

We retrospectively analysed plasma samples from 57 OAC patients treated at the Upper Gastrointestinal tract Clinic at the Princess Alexandra Hospital, Queensland, Australia, between September 2010 and October 2019. All participants provided written informed consent through the Cancer Evolution Biobank (HREC/10/PAH/152, UQ/2011001287). Ethics approval for this project has been granted by the Metro South Human Research Ethics Committee (HREC/2019/QMS/55554), the University of Queensland Ethics Committee (2019002466) and QIMR Berghofer Human Research Ethics Committee (P3559). Clinical data including stage, tumour size and therapy were collected for all participants (HREC/15/QPAH/614, Table 1, Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2022.100452).

Patients received standard of care including endoscopy for diagnosis, perioperative chemotherapy or neoadjuvant chemoradiotherapy and surgery. The median age of disease onset was 68 years (range 42-84 years), with clinical stage ranging from I to III. Progression-free survival (PFS) was the time between surgery and first recurrence of metastatic disease. Disease-specific survival (DSS) was the time from surgery until death from disease. The median PFS was 17.42 months (range 1.9-92.5 months). The median DSS was 27.5 months (range 5.1-92.5 months). The median follow-up for survivors was 37 months (range 7.4-92.5 months, Figure 1).

A baseline blood sample, described as B1, was taken at diagnosis and before treatment (Figure 1). An additional perioperative blood (B2) was collected (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2022.100452). Forty-five patients received neoadjuvant chemoradiotherapy. Twelve patients did not receive pre-operative therapy and went direct to surgery. For 10 patients, blood samples were taken at multiple time points during clinical follow-up (Figure 2A). In addition, up to two post-therapy blood samples (B3 and B4) were taken, up to 60 months after diagnosis.

For a subset of patients (n = 39), tumour tissue was collected at endoscopy, before surgery. Biopsies were taken at several sites within the primary tumour and are described as T1, T2 and T3. Tissue was stored in RNAlater. DNA and RNA were extracted using the Qiagen AllPrep DNA/RNA mini kit (Qiagen, Germany).

| Variables | Number of patients (n = 57) |
|-----------|-----------------------------|
| Age at diagnosis (years) | |
| < 60 | 16 (range 42.59) | 28.1 |
| < 90 | 41 (range 60.85) | 71.9 |
| Sex | |
| Male | 53 | 93.0 |
| Female | 4 | 7.0 |
| Clinical stage (AJCC 8th edition) | |
| Stage I | 7 | 12.3 |
| Stage II | 12 | 21.1 |
| Stage III | 38 | 66.7 |
| Median pathological tumour size (mm) | |
| < 10 | 20 (range 0-4) | 35.1 |
| 10-40 | 25 (range 12-35) | 43.9 |
| > 40 | 12 (range 45-80) | 21.1 |
| PFS, months | |
| < 12 | 15 (range 1.9-11.9) | 26.3 |
| > 12 | 42 (range 12-92.5) | 73.7 |
| DSS, months | |
| < 24 | 25 (range 5.1-21.7) | 43.9 |
| > 24 | 32 (range 25-92.5) | 56.1 |
| Neoadjuvant treatment | |
| Direct to surgery | 12 | 21.1 |
| Pre-operative CXRT | 26 | 45.6 |
| Pre-operative chemotherapy | 19 | 33.3 |

Table 1. Patient characteristics

Clinically relevant characteristics include age, gender, stage, tumour size and type of treatment. Patient survival is indicated in months.

AJCC, American Joint Committee on Cancer; CXRT, chemoradiotherapy; DSS, disease-specific survival; PFS, progression-free survival.

Figure 1. Study design. (A) Tumour-agnostic analysis on ctDNA variants from baseline (B1, orange squares) and post-treatment samples (B2, green squares). (B) Tumour-informed approach aligning the ctDNA variants to matched primary tumour samples (T1). For a subset of patients, an additional biopsy (T2) was taken from a different site within the one primary tumour. In the tumour-informed approach, a ctDNA variant was only considered positive if it was present in a matching primary tumour sample. (C) Analysis of ctDNA at multiple time points post treatment (B3 and B4). Blue squares represent pre-treatment tumour biopsies. Number of patients included in each analysis is indicated.

ctDNA, circulating tumour DNA.
**ctDNA extraction, amplification and analysis**

Plasma was isolated from either an Ariosa tube or EDTA tube. Ariosa tubes contain a preservation buffer and were processed within 3 days. EDTA tubes were processed within 6 h of collection. To ensure cellular debris did not contaminate downstream applications, an additional high g-force centrifugation step was carried out (16 000 × g, 10 minutes at 4°C).

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**Figure 2. Patient overview.**

(A) Swimmer plot. The clinical timeline is shown for each patient. Triangles indicate the blood collection time points. Where possible, tumours were sampled at time of diagnosis. The colour of each bar represents the DSS. (B) Variants detected in ctDNA and tumour DNA using a pan-cancer gene panel. The top panel shows DSS in months. Stage and treatment type are indicated. The number of variants per patient is shown, range 0 to 9 variants. Mutated genes are plotted for each patient. Overall, 45% of patients harboured a TP53 variant. The analysis approach is represented using a colour code (tumour-informed or tumour-agnostic approach).

CXRT, chemoradiotherapy; DSS, disease-specific survival; NSR, no sign of recurrence.
ctDNA was extracted from 1.5 ml to 5 ml of plasma using the AVENIO ctDNA Isolation kit (Roche Diagnostics, Switzerland) and underwent sequence library prep with the AVENIO Expanded panel (Roche Diagnostics, Switzerland), a 77-gene panel associated with cancer diagnostics. Based on published single-nucleotide variants (SNVs) associated with OAC, we estimated that the gene panel would identify ≥1 mutation in at least 95% of patients.

Tumour library prep was carried out using the AVENIO Tumor Expanded kit. We carried out a buffer exchange which decreased the EDTA content in the input DNA. To do this, two ethanol washes were carried out using 3 x volume of clean-up beads. DNA was then eluted in water for use with the kit.

Sequence libraries were quantified by Qubit fluorometer (Thermo Fisher Scientific, USA) and profiled using a Bioanalyzer (Agilent, USA).

The ctDNA and tumour libraries were sequenced using the Illumina HiSeq4000 (Illumina, USA) to a minimum read depth of 500X. The average read depth was 5444X for the plasma samples (range 763-9265) and 1957X for the tumour samples (range 523-3836) (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2022.100452). Variant analysis was carried out using the AVENIO ctDNA Analysis software version 2.

### Whole-genome and exome sequencing and analysis

WGS was carried out on matched tumour and buffy coat DNA from 11 patients on the HiSeqX-ten (Illumina, USA). For five patients, WGS data were available for multiple biopsies, with a total of 19 biopsies included in this study. The percent tumour content was assessed using Illumina SNP arrays and the qpure tool. All samples had tumour cellularity >40%. WGS was carried out to a targeted read depth of 60X in tumour and 30X in buffy coat samples. The average read depth was 50X for the tumour samples (range 40-66) and 25X for matched normal samples (range 22-30) (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2022.100452, Supplementary Table S1 available at https://doi.org/10.1016/j.esmoop.2022.100452).

WES was carried out for seven patients on the HiSeqX-ten. The average read depth was 23X for the tumour samples (range 22-24) and 21X for the matched normal samples (range 13-26) (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2022.100452, Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2022.100452).

WGS and WES data underwent adaptor trimming with Cutadapt48 (version 1.9) and sequence reads were mapped to GRCh37 using BWA-MEM49 (version 0.7.15) and SAMtools (version 1.9). Duplicated reads were marked with Picard MarkDuplicates (version 2.8.15). Using matched tumour and buffy coat pairs, SNV and indel variants were called using qSNP (v2.1.4) and GATK HaplotypeCaller (v3.8) and annotated with SnpEff.

### Pileup methods

For each patient, various types of sequence files (BAM files) were available. These included AVENIO Expanded panel data from plasma and/or tumour tissue as well as WES/WGS data derived from tumour tissue. A pileup approach was used to assess whether variants detected in ctDNA were present in other sequencing output from the same patient. Pileups were generated using the Adamalava tool qbasepileup v70 using pileup profile ‘DNA’ and filtered with the following parameters: CIGAR_M > 34, MD_mismatch ≤ 3, Flag_DuplicateRead = false and MAPQ > 10. Pileups determined the number of sequence reads at each variant genomic position across the patient samples.

### Criteria for tumour-agnostic and tumour-informed variant calling

Assessment of sequencing data was carried out using two different approaches. In the tumour-agnostic approach, only the ctDNA sequence data were analysed for each patient. This analysis is blinded to the primary tumour genomic profile which is acquired from the biopsy. For each patient, sequence files from the B1 and B2 blood samples were aligned using the pileup method (Figure 1). Using the pileup output, ctDNA sequencing data were filtered to remove germline variants, CHIP variants and false-positive calls. Common SNVs present in gnomAD (allele frequency > 0.1%) were excluded. ctDNA variants with variant allele frequency (VAF) > 20% were removed as they were considered germline. To remove potential false-positive calls, variants in <0.001% of reads or variants with >20 sequence reads were removed. Additionally, somatic variants with <60% variant reads were removed. Variants that were present in three or more patients were also excluded.

A second analysis, the tumour-informed approach, confirmed whether ctDNA variants detected in plasma were present in the primary tumour. For the tumour-informed approach, variants were first identified in tumour DNA using the AVENO platform or WGS/WEs. BAM files from the AVENIO/WGS/WES were included in the pileup analysis. For a ctDNA variant to be considered tumour derived, it needed to match the tumour variant with minimum 10 reads in the ctDNA.

### Statistical analysis

A log-rank (Mantel–Cox) test was used to assess ctDNA as a biomarker of survival in OAC (GraphPad Prism 8.3.1). The correlation between tumour regression, tumour variants and post-treatment blood variants was assessed using a chi-square test. For DSS data, hazard ratio was determined using a Cox proportional hazards regression model (R Foundation for Statistical Computing).

### RESULTS

**ctDNA detection using a tumour-agnostic approach**

We analysed plasma samples from a cohort of patients with OAC (n = 57) who were treated with a curative intent (Figure 2A). The clinicopathological characteristics are shown in Table 1 and Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2022.100452.
To determine whether ctDNA isolated from both baseline (B1) and after treatment (B2) plasma samples had prognostic significance, we used a tumour-agnostic approach (Figure 1). This method gives insight into whether ctDNA can be a useful clinical tool when the patient tumour sample is not available for genomic reference. The median time between the B1 and B2 blood samples was 3 months (range 1-7 months).

Using a CAPP-seq 77-gene pan-cancer panel, we analysed 114 plasma samples to detect tumour-derived ctDNA variants. We applied stringent filtering criteria to minimise the false-positive rate. To eliminate CHIP and germline variants, we excluded calls with VAF > 0.4. This is essential as CHIP variants are present in blood but are not tumour derived.

The median ctDNA concentration was 7.88 ng/ml (range 1.67-126.51 ng/ml, Table 1) at baseline and 8.02 ng/ml (range 1.71-115 ng/ml) after treatment. When we assessed the clinical features, we found no statistical association between ctDNA concentration and either clinical stage or pathological tumour size (data not shown). A pileup approach identified ctDNA variants in 22/57 patients (38%). We detected a total of 90 ctDNA variants (range 0-7 per sample) occurring in pre- and post-therapy plasma samples (Figure 2B). Recurrent variants (n = 14) between the baseline and post-treatment blood samples were observed in 12/57 (21%) patients.

cDNA detection using a tumour-informed approach

Previous studies have shown that CHIP variants may be detected in up to 23% of OAC patients. The remaining ctDNA variants are presumed to be tumour derived, although these were not verified as genuinely tumour derived. We have used an alternate tumour-informed approach, profiling 185 matched plasma and tumour samples to determine whether ctDNA variants originated from the tumour sample.

Pre-treatment ctDNA and primary tumour samples from 39 patients were sequenced using the AVENIO pan-cancer 77-gene panel. We detected 56 variants (median 2 per patient, range 1-9) in the primary tumour samples of 26/39 (67%) patients (Figure 2B). In a subset of patients (33%, n = 13), no variants were detected in the tumour samples. For 17 patients, an additional biopsy from the same tumour was sequenced to assess the macroscopic tumour heterogeneity. The spatially distinct tumour biopsies (1-2 cm apart) are referred to as T1, T2 and T3 (Figure 1). In 94% (16/17) of patients, where two tumour biopsies were sequenced with the AVENIO platform, we identified a variant in at least one of the tumour biopsies. In contrast, when only one tumour biopsy was sequenced, variants were observed in only 45% (10/22) of patients. Sequencing two tumour biopsies per patient increased the likelihood of identifying variants that might otherwise be missed due to ITH.

WGS/WES data (n = 26) from the tumour tissue were available for 18 patients. WGS data were available for multiple tumour sites in 6/18 patients (Figure 3A, Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2022.100452). We focused on the 77 genes that were represented on the AVENIO panel. For each patient, we carried out a sequence pileup to compare the sequence data from the primary tumour with all available ctDNA samples. In total, we detected 68 tumour variants in WGS/WES, 22% (15 variants) that aligned with ctDNA calls from the same patient (Figure 3A and B). We defined patients with these variants (n = 10) as ‘shedders’. For these patients, there is evidence of the tumour shedding DNA into blood.

We assessed the concordance between tumour calls on the AVENIO platform and the WGS/WES data. We found that 74% of the variants were in alignment (Figure 3).

Patient SOG062 had two variants—in genes TP53 and KRAS—that were concordant between tumour regions and across WGS and AVENIO platforms (Figure 3). Of note, only the KRAS variant was detected in the baseline blood. Similarly, SOG104, SOG179, SOG203 and SOG501 had concordant variants across platforms and multiple tumour regions. For SOG069, the AVENIO platform detected two distinct KDR variants which were not seen in the WGS data (Figure 3) or in the plasma. Similarly, in SOG143, the AVENIO platform detected three variants, occurring in the tumour and the plasma, which were not observed in the WGS data.

Patients SOG066 and SOG083 had no tumour variant detected in the 77 genes despite sequencing two tumour biopsies on each platform. The biopsies sequenced using WGS had high tumour content, with tumour cellularity ranging from 47% to 60%. The biopsies sequenced using the AVENIO platform had cellularity ranging from 22% to 60%. Similarly, SOG506 had no variants detected using WES or the AVENIO platforms.

OAC tumour heterogeneity

To determine whether ITH limits the application of ctDNA in the clinic, we aligned ctDNA data from patients with multiple tumour biopsies sequenced (Figure 3). Fifty patients were sequenced using the AVENIO capture alone. In SOG427, an RB1 variant was only detected in one of the biopsies and the baseline blood. In SOG415, T2 had variants in TP53, PIK3CA and CDKN2A. Only two of these were seen in T1. However, all three were detected in the pre- and post-therapy blood samples. SOG443 (Figure 3) contained the highest number of variants and showed heterogeneity across both tumour and blood samples. Three variants detected in T2 were shared with the pre-therapy blood (genes BRCA1, PIK3CA and NTRK2). Only two of these were shared with T1. The post-therapy blood only contained the NTRK1 variant. For 20 patients, we sequenced multiple tumour biopsies using a combination of the AVENIO and WGS/WES platforms (Figure 3). Fifty percent of patients (10/20) had concordant variants between the two biopsies. Forty percent of patients (8/20) had individual variant specific to one biopsy. In two patients, no variant was observed.

SOG143 tumour presented with ITH as T1 and T2 had a common PTCH1 variant but individual variants in BRCA2 and TP53 (Figure 3). In the corresponding blood samples,
Figure 3. Overview of variants detected using a pan-cancer gene panel.

VAF in tumour and blood samples analysed by WGS, WES and the AVENIO tumour and ctDNA platform. Samples run using WGS or WES are indicated. SOG066, SOG083 and SOG506 are not represented as they had no tumour variants detected using any platform. Biopsies (T1, T2 and T3) were taken from multiple sites within the primary sample. Blood samples were taken at baseline, B1 and after treatment—B2 and B3. Patient disease-specific survival is indicated in brackets. The asterisk indicates patients that are shedders.

cDNA, circulating tumour DNA; mo, months; NSR, no sign of recurrence; VAF, variant allele frequency; WES, whole-exome sequencing; WGS, whole-genome sequencing.

*Significance of the P values.

only the PTCH1 variant was detected. The tumour from SOG221 also displayed heterogeneity. While the TP53 variant was seen in all biopsies analysed (across both platforms), the RNF43 variant was only detected in two of three biopsies. None of these variants were observed in the blood samples.

**ctDNA positivity as a prognostic biomarker**

Previous studies have demonstrated that ctDNA from post-treatment bloods has prognostic value in detecting MRD in oesophageal cancer.32,33 First, we sought to determine whether ctDNA positivity has prognostic value when applying the tumour-agnostic approach. We analysed blood samples taken after treatment, either after neoadjuvant therapy or surgery. We detected 35 variants (range 0-7 variants per patient, Figure 2B). Univariate survival analyses demonstrated ctDNA-positive patients had significantly worse DSS (median 58.8 months, range 7.4-92.5 months, *P* = 0.0130, log-rank, Figure 4A) and a trend towards worse PFS (median 23 months, range 1.9-92.5 months, *P* = 0.1017, log-rank, Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2022.100452) when compared with ctDNA-negative patients (median DSS not reached, range 5.1-80.8 months and median PFS not reached, range 2.1-80.8 months). There was no association between baseline blood samples and PFS or DSS (data not shown).

Various ctDNA studies have used a cut-off of two variants to be classified ctDNA positive.32,40 We repeated our survival analysis splitting the cohort into two groups: patients with 0 or 1 variant versus patients with >1 variant, and the outcome remained significant (median 13.5, range 7.8-90.5 months, *P* = 0.0205, log-rank, Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2022.100452). Considering three categories—0 variant, 1 variant and >1 variant—a clear separation was observed between the groups (*P* = 0.0175). In our data, patients with at least one ctDNA variant had worse DSS than the zero variants group; therefore, patients with a minimum of one variant were considered ctDNA positive.

We assessed whether the number of ctDNA variants was associated with the pathological tumour size. We found that patients with small tumours, <10 mm, were less likely to have detectable variants in their post-treatment plasma (not significant (NS), *P* = 0.1211, Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2022.100452). We next examined whether tumour regression was associated with the number of ctDNA variants. We compared
patients that were ctDNA positive to those that were negative. Patients with major histological response (90%-100% tumour regression) were more likely to be ctDNA negative \( (P = 0.0556) \), chi-square test, Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2022.100452).

To determine whether the tumour-informed approach had prognostic significance, we analysed blood samples taken during the perioperative period, either after neo-adjuvant therapy or surgery. Patients with detectable variants in their tumour \( (n = 30) \) were split into two groups, shedders and non-shedders, where shedders had ctDNA variants verified by the primary tumour genomic profile. A third group was defined as patients with no variant detected in their tumour, \( n = 16 \) (Supplementary Figure S3, available at https://doi.org/10.1016/j.esmoop.2022.100452). The non-shedders and the patients with no variant detected were pooled, as univariable survival analysis indicated they had the same DSS (median survival not reached, range 5.1-92.6 months, \( P = NS \), log-rank). We examined the post-treatment bloods and found that shedders had worse DSS (median survival 10.6 months, range 8.0-37.6 months, ***\( p = 0.0007 \), log-rank, Figure 4B) and worse PFS (median survival 9.3 months, range 2.1-37.6 months, \*\( p = 0.0311 \), log-rank, Supplementary Figure S3, available at https://doi.org/10.1016/j.esmoop.2022.100452).
Additionally, we sequenced tumour samples from 46 cases in a cohort of 57 patients treated with curative intent. ctDNA isolated from pre- and post-treatment plasma samples was used to detect a panel of known biomarkers in patients with resectable OAC. We assessed the utility of ctDNA as a clinical biomarker.

**DISCUSSION**

High levels of several ctDNA variants were detected at baseline. However, after treatment, the levels of ctDNA variants increased after treatment; however, the longest time point available was only 1 month after resection. Similarly, SOG460 had short follow-up; however, a variant was detectable in all samples at low levels. In the post-surgery blood sample, 21 months after diagnosis, a variant was present, shedders had worse DSS (*P = 0.0473, log-rank, Figure 4C).

**ctDNA detection during following treatment and disease recurrence**

For 10 patients, we analysed multiple blood samples, one baseline and several post-treatment samples (Figure 2A). Five patients were alive with no sign of recurrence. Of this group, three patients had no variant detected in any of their blood samples using the AVENIO platform (Figure 4D). SOG062, the longest survivor, had no variant above the threshold for calling in the post-treatment blood samples; however, the KRAS variant identified by WGS/AVENIO tumour sequencing was detected with a low number of reads in the plasma. The KRAS variant was highest at diagnosis. After chemotherapy, the VAF reduced but was still detectable. After surgery, 60 months from diagnosis, the KRAS variant was detectable below the threshold for calling. SOG315 had an APC variant at baseline. In all post-treatment samples, this variant was detectable at low levels, below the threshold for calling. In the post-surgery blood sample, 21 months after diagnosis, a TP53 variant was identified at low levels. Neither variant was confirmed in the primary tumour; however, only one biopsy was available for sequencing.

Three patients are alive with disease. SOG425 had six variants detected in the baseline blood. None of these variants increased after treatment; however, the longest time point available was only 1 month after resection. Similarly, SOG460 had short follow-up; however, a MET variant was detectable in all samples at low levels. In SOG317, we observed a PIK3CA variant at baseline and after treatment. The VAF increased 21 months after surgery, reflecting disease progression which was confirmed by a computed tomography scan. Two patients died of their disease (SOG490 and SOG529). Both patients had low levels of ctDNA variants at baseline. However, after treatment, high levels of several ctDNA variants were detected (Figure 4D).

**DISCUSSION**

We present a study examining ctDNA as a prognostic biomarker in patients with resectable OAC. We assessed ctDNA isolated from pre- and post-treatment plasma samples in a cohort of 57 patients treated with curative intent. Additionally, we sequenced tumour samples from 46 patients to determine whether ctDNA captured ITH. While cohort size is a limitation of this study, we have demonstrated the utility of ctDNA as a clinical biomarker.

We carried out ctDNA sequencing using a commercially available kit. The panel provided very high-depth sequence reads, but was limited to 77 genes. In OAC, while there are frequently mutated genes, somatic mutations occur across the genome with no specific hotspot,

No standardised method exists to remove false-positive variants identified in ctDNA. To filter the variants, other studies have used patient PBMCs to eliminate CHIP variants. The lack of primary tumour sequence data is a limitation of these studies as acknowledged by the authors themselves. In our study, we hypothesised that focusing on a tumour-informed approach would give more accurate results when assessing ctDNA variants.

ITH is a well-described characteristic of OAC where the tumour harbours several clones with different mutation profiles,

We are the first to compare ctDNA variants detected in blood with multiple tumour biopsies from the same primary tumour. We showed that when only one tumour biopsy was sequenced, only few variants were identified in both ctDNA and the tumour, most likely due to ITH. When we sequenced multiple biopsies from each patient, there was greater concordance between the tumour and ctDNA genomic profiles. This finding is in line with Maron et al. who showed only 26% concordance between the genomic profile of primary tumours, metastatic tumours and ctDNA in gastrointestinal adenocarcinoma. This suggests that tumours may not shed ctDNA homogenously across the tumour mass, and raises the question of whether only certain sub-clones shed ctDNA.

TP53 is the most frequently mutated gene in OAC with mutations found across the length of the gene. Mutations occur in ~80% of primary tumours. Variants in TP53 are problematic for ctDNA studies as CHIP variants frequently occur in that locus. In our study, TP53 was the most frequently mutated gene in patients with detectable variants (43%). This aligns with a previous ctDNA study detecting TP53 in 50% of patients. This frequency is lower than expected and may be caused by factors such as ITH and non-uniform DNA shedding. Furthermore, it has been shown that in other hypermutated cancers, as few as 10% of ctDNA variants matched the tumour of origin.

In the literature, there are inconsistencies in the definition of ctDNA positivity with a cut-off of either one or two variants. In our study, the detection of one or two ctDNA variants was significantly associated with DSS. Our tumour-agnostic approach showed that the presence of any ctDNA variant after therapy was associated with worse DSS. One of the challenges of this approach is that ctDNA variants were not verified as specifically originating from the primary tumour. To address this, we carried out a tumour-informed analysis which confirmed ctDNA has prognostic significance. Furthermore, assessing the baseline blood samples using the tumour-informed approach, we observed...
that ctDNA could not discriminate patient prognosis. This suggests that even if ctDNA is detected at baseline, it does not indicate a palliative approach is appropriate, as the patient may still respond to treatment.

To determine whether serial time point analysis had prognostic significance, we assessed blood from multiple time points. Of the five patients with no recurrence, three had no ctDNA variants identified, and the other two patients had very low levels of ctDNA below the threshold for calling in their most recent blood sample. Using our pileup approach, we were able to detect these low-level variants. We hypothesise that the patient immune system has eliminated most of the tumour cells and these ctDNA variants may be good individual prognostic markers of disease. In the long term, these patients may benefit from close monitoring of these variants to anticipate disease recurrence.

In two out of three patients with progressive disease, ctDNA variants were detected in the most recent blood sample. For the third patient, SOG425, the baseline sample revealed very high ctDNA variant levels before chemoradiotherapy. After treatment, the ctDNA levels dropped. However, the final sample was collected <1 month after surgery, which limited the analysis. Tracking these variants at a later time point may have more clinical relevance.

The two patients who died of their disease had ctDNA variants with increasing VAF in their post-treatment blood samples. We hypothesise these patients had more aggressive disease reflected in their ctDNA profile.

**Conclusion**

This study provides a strong argument for the use of ctDNA as a personalised clinical tool. It has the potential to serve as a prognostic test after neoadjuvant chemo/radiotherapy and may inform treatment choices. ctDNA may also represent a viable liquid biopsy, for shedders specifically, as it captures the ITH associated with OAC. Indeed, these patients have the worst outcomes and it is of particular importance to improve prognostication and treatment options for this group. There is a need for standardisation of ctDNA quantification methods and time points to apply these findings to the clinic. In clinical trials, ctDNA-positive patients may be the ideal candidates for post-operative immunotherapy. In CheckMate 577,32 patients with MRD benefited from immunotherapy; perhaps patients that are ctDNA shedders should also be considered for therapy.

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**DISCLOSURE**

AB received a Roche Investigator initiated clinical research grant. NW is a co-founder and board member of genomIQa. All other authors have declared no conflicts of interest.

**DATA SHARING**

The data that support the findings of this study are available in the European Genome-phenome Archive, study ID EGAS00001002864, dataset ID EGAD00001008554. EGA identifiers can be found in Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2022.100452.

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