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Original Article

Selective utilization of circulating tumor DNA testing enables disease monitoring in endometrial and ovarian carcinomas

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ABSTRACT

Objective: Biomarkers reflecting real-time response to therapy and recurrence are lacking. We assessed the clinical value of detecting cell-free circulating tumor DNA (ctDNA) mutations in endometrial cancer (EC) and ovarian cancer (OC) patients.
Methods: EC/OC patients undergoing primary surgery were consented for tissue banking and 2-year serial blood draws. Tumor tissue DNA and plasma ctDNA underwent next generation sequencing using a targeted gene panel for somatic mutations.
Results: Of 44 patients (24 EC, 17 OC, 2 synchronous endometrial and ovarian carcinomas [SEOC] and 1 endocervical adenocarcinoma [EA]) at least one somatic mutation was identified in tumor tissue in 40 (91%, 20/24 EC, all OC/SEOC/EA), and in preoperative plasma ctDNA in 12 (27%) patients (6/24 [25%] EC and 6/17 [35%] OC). Detection of preoperative ctDNA mutations was associated with advanced stage, higher preoperative CA125, and subsequent disease recurrence. In 5/12 (42%) patients with preoperative ctDNA mutations, examination/imaging suggested clinical stage I however final pathology revealed stage II/III. In 11 patients where serial timepoints were assessed during treatment for ctDNA and CA125, ctDNA clearance preceded normalization of CA125. Thirteen patients developed recurrent disease (4 EC, 8 OC, 1 EA); 8 in whom ctDNA mutations were detected postoperatively, and 4 followed through time of recurrence with ctDNA mutations identified 2–5 months prior to clinical/radiologic/biomarker progression in 3.
Conclusion: ctDNA can reflect larger tumor volume/metastases, treatment response and subsequent disease recurrence in EC and OC. Careful patient selection is critical to direct resources to patients most likely to benefit, considering disease burden and risk group.

Keywords: Endometrial Cancer; Ovarian Cancer; Mutations; Circulating Tumor DNA

Synopsis

Detection of preoperative circulating tumor DNA (ctDNA) mutations was associated with advanced stage, higher CA125, and subsequent disease recurrence. ctDNA clearance in treatment preceded CA125 normalization. In patients with recurrent disease who had ctDNA monitoring, ctDNA detection preceded clinical/radiologic/biomarker evidence of recurrence.

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Conflict of Interest

M McConechy and T Dowhy are previous employees of Imagia Canexia Health and D Huntsman is a previous founder and Chief Medical Officer of Imagia Canexia Health. The other authors have nothing to disclose.

Author Contributions

Conceptualization: M.J.N., H.D.G.; Data curation: J.A., M.M.K., L.A., S.J., D.T., H.D.G., M.J.N.; Formal analysis: J.A., M.M.K., D.T., M.J.N.; Funding acquisition: M.J.N.; Investigation: J.A.; Methodology: M.M.K., L.A., S.J., M.J.N.; Resources: L.A., S.J.; Supervision: M.J.N.; Visualization: D.T.; Writing - original draft: J.A., M.M.K., M.J.N.; Writing - review & editing: H.D.G.

INTRODUCTION

Ovarian cancer (OC) and endometrial cancer (EC) represent the most lethal and the most common gynecologic malignancies in North America, respectively [1,2]. Biomarkers associated with real-time response to therapy and earlier detection of disease recurrence are lacking in both diseases. Serum tumor marker CA125 has been used as a monitoring tool in OC treatment and surveillance for decades, however it lacks the sensitivity and specificity for monitoring treatment response and early detection of recurrence [2,3]. Despite the increasing worldwide incidence of EC and the poor overall survival of patients who recur, there is currently no blood-based biomarker for detecting and monitoring EC recurrence and progression during routine follow-up [1,4,5].

Technological advancements have expanded the opportunities for mutational profiling of formalin-fixed paraffin-embedded (FFPE) tumor tissues and liquid biopsy applications using plasma specimens to identify cell-free circulating tumor DNA (ctDNA) mutations. This technology provides an opportunity to assess tumor burden, identify potentially targetable mutations, monitor response to therapy and/or recurrence in real time [3,6-8]. Liquid biopsy technology is considered minimally invasive and potentially involves less risk for the patient. This is compared to obtaining multiple tissue biopsies, and frequent radiation exposure of clinical imaging required for disease monitoring. Several studies have shown the feasibility and utility of ctDNA monitoring in solid tumors [6,9-12], but the integration of the liquid biopsy into standard of care practice is variable for different tumor types and health care jurisdictions. In this study, we assessed the utility of using a liquid biopsy assay to detect ctDNA mutations in patients with newly diagnosed OC and EC who were then followed longitudinally through their disease course in order to determine if ctDNA mutations could be utilized as biomarkers for disease monitoring.

MATERIALS AND METHODS

1. Recruitment of cohort

With Institutional Review Board approval from the University of British Columbia, patients were approached who were undergoing primary surgical staging for suspected OC or had EC confirmed by biopsy at Vancouver General Hospital from June 2016 to June 2018. Recruitment was restricted to individuals that were presumed to have easy access to a specialized laboratory that handles ctDNA samples for serial post operative blood draws, both as an inpatient and outpatient. Blood draws for ctDNA monitoring were planned as follows: preoperatively, postoperative day 1, 2, 3, 4, 5 (if still hospitalized), day 14, at 6-week follow up, and every 3–4 months thereafter for 2 years. Blood was initially drawn into 2 Ariosa tubes (Roche, San Jose, CA, USA) with a transition to STRECK™ in February 2018. Tissue and blood sample analysis were undertaken after study completion, blinded to pathology and clinical outcomes. As final diagnosis was not known at time of patient consent, the initial recruitment captured many patients with masses of unknown etiology which were ultimately found to be benign or borderline ovarian tumors on final pathology. These patients were discontinued from the study once final the pathology was reported (**Fig. S1**).

2. Specimen processing and DNA extraction

At each time point, 2 blood tubes were drawn and processed for the isolation of plasma and buffy coat within 7 days. Normal buffy coat and frozen tumor tissue DNA was extracted

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using the Qiagen DNeasy blood and tissue extraction protocol. Frozen plasma samples were extracted for ctDNA using the Promega Maxwell® RSC Circulating DNA Purification Kit, using an optimized protocol. FFPE tumor tissue was extracted using the Qiagen GeneRead DNA FFPE kit with the UNG enzyme to aid in the removal of cytosine deamination artifacts.

3. Targeted gene analysis of tumor tissue and plasma ctDNA

Tumor tissue DNA, buffy coat DNA, and plasma ctDNA were sequenced using the Imagia Canexia Health liquid biopsy assay Follow It™ and solid tumor assay Find It™. Both the Find It and Follow It targeted gene assays contained the same content and PCR-based workflow. The panel included over 146 hotspots in 30 cancer associated genes (*AKT1*, *ALK*, *AR*, *BRAF*, *CTNNB1*, *DDR2*, *EGFR*, *ERBB2*, *ESR1*, *GNA11*, *GNAQ*, *GNAS*, *HRAS*, *IDH1*, *IDH2*, *KIT*, *KRAS*, *MAP2K1*, *MAP2K2*, *MET*, *NRAS*, *PDGFRA*, *PIK3CA*, *POLE*, *PTCH1*, *PTEN*, *ROS1*, *SMO*, *TP53*), encompassing single nucleotide variants (SNVs), deletions and insertions (up to 24 bp). Final amplified library pools were sequenced on an Illumina MiSeq™. The proprietary Imagia Canexia Health bioinformatics analysis pipeline used a machine learning random forest model to statistically identify low variant frequency SNVs. This model was trained to identify 0.5%–1% variant allele frequencies (VAFs). The Strelka algorithm [13] was used to identify indels 1%–5% VAF. In FFPE tumor tissue the threshold for SNVs and indels was 1% VAF. To identify plasma ctDNA mutations, the threshold for SNVs was 0.5% VAF and 1% VAF for indels. Driver mutations identified in the FFPE tumor tissue were used to observe ctDNA mutations in the serial plasma timepoints at VAFs below the validated thresholds. Normal buffy coat DNA was used to determine the somatic status of the tissue and plasma ctDNA mutations. All germline variants and SNPs were filtered out of the analysis. For further details on specimen processing, DNA extraction, targeted gene panel, bioinformatics analysis and statistical analysis see **Data S1**.

RESULTS

1. Study cohort

Fig. S1 summarizes how the final cohort (n=44) was obtained and followed (17 OCs, 24 ECs, 2 synchronous endometrial and ovarian cancer [SEOC], and 1 endocervical adenocarcinoma [EA] [preoperatively reported as an endometrial primary]). **Table 1** shows the tumor site, histotype and International Federation of Gynecology and Obstetrics (FIGO) stage of the total cohort.

2. Tumor and preoperative ctDNA detection

At least one somatic mutation in tumor tissue was identified in 40/44 (91%) of patients (20/24 ECs, 17/17 OC, 2/2 SEOC, 1 EA) (**Table 1**). The 4 cases where no tumor mutations were detected were small volume ECs. There were 12 patients with mutations detected in both tumor tissue and preoperative plasma ctDNA; 6/15 (40%) patients with high grade serous OC, 4/13 (31%) with endometrioid EC, and 2/10 (20%) with serous EC (**Table 1**). When comparing patients with preoperative ctDNA mutations to patients with no mutations, the mean largest tumor diameter was 6.9 cm compared to 5.2 cm (p=0.075), mean preoperative serum CA125 level was 1,220 vs. 165 U/mL (p=0.048), and advanced (FIGO stage III–IV) disease was 67% vs. 29% (p=0.038). **Table 2** demonstrates the clinicopathological characteristics of patients with the following scenarios for somatic mutation detection; 1) mutations in tumor tissue and preoperative plasma ctDNA, 2) tumor tissue and postoperative ctDNA, 3) mutations in ctDNA at any time point and not identified in the tumor tissue. There were 2 patients (1 with high

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Table 1. Tumor site, histotype and FIGO state of the total cohort is shown with the detection of somatic mutations in tumor tissue, preoperative and postoperative plasma ctDNA

Tumr site and histotype	Total cases	FIGO stage	Pre-op imaging showing distant disease	Tumor tissue mutations detected	Pre-op mutations in plasma ctDNA detected	Post-op mutations in plasma ctDNA detected
Endometrioid endometrial carcinoma	13	I–II: 10/13 (77%), III–IV: 3/13 (23%)	2/13 (15%)	11/13 (85%)	4/13 (31%)	1/13 (8%)
Serous endometrial carcinoma	10	I–II: 4/10 (40%), III–IV: 6/10 (60%)	0/10	9/10 (90%)	2/10 (20%)	2/10 (20%)
Clear cell endometrial carcinoma	1	I–II: *1/1, III–IV	0	0	0	0
High grade serous ovarian carcinoma	15	I–II: 4/15 (27%), III–IV: 11/15 (73%)	8/15 (53%)	15/15 (100%)	6/15 (40%)	8/15 (53%)
Ovarian endometrioid carcinoma	1	I–II: 1/1, III–IV	0	1/1	0/1	0/1
Mixed endometrioid and clear cell ovarian carcinoma	1	I–II: 1/1, III–IV	0	1/1	0/1	0/1
Synchronous ovarian and endometrial carcinoma	2	I–II: 2/2, III–IV	0	2/2	0	0
Endocervical adenocarcinoma	1	I–II, III–IV: 1/1	0	1/1	0	0
Total cases	44	I–II: 23/44 (53%), III–IV: 21/44 (47%)	10/44 (23%)	40/44 (91%)	12/44 (27%)	11/44 (25%)

ctDNA, cell-free circulating tumor DNA; FIGO, International Federation of Gynecology and Obstetrics.
*No residual tumor on hysterectomy specimen.

grade serous OC and 1 serous EC) with *TP53* mutations found in the preoperative ctDNA but not identified in the tumor.

In 5/12 (42%) of patients with preoperative ctDNA mutations detected, preoperative examination and imaging had suggested stage I disease but subsequent staging and final pathology revealed more advanced stage (2 stage II, 3 stage III) disease. Of the 12 patients with mutations detected both in tumor tissue and preoperative plasma ctDNA, 6 (50%) recurred from disease within the 2 years following surgery.

There were 30 (68%) patients who had no preoperative ctDNA mutations detected; 17 EC (13 stage I, 1 stage II), 10 OC (3 stage I), 2 SEOC, 1 EA. Eight of 30 (27%) patients with no preoperative ctDNA mutations detected experienced a disease recurrence within 2 years.

3. Tumor and postoperative ctDNA mutation detection

The majority of patients (82%) fulfilled early postoperative period blood draws but with only 18% documented to completion (**Fig. S1**). Reasons for discontinuation of serial blood draws were disease or treatment related symptoms, or geographic distance/transportation challenges.

We detected mutations in ctDNA post operatively in 11/44 (25%) patients (**Table 2**) e.g., in both tumor tissue and postoperative ctDNA. For 9/11 of these patients, ctDNA mutations were identified in the first 1–5 days postoperative (1 serous EC and 8 high grade serous OCs). Notably, all 8 patients with high grade serous OC had residual disease at primary surgery documented in their operative notes. The 2 other patients had postoperative ctDNA detected at later time points (day 55/ctDNA002 and 18 months/ctDNA013) and their clinical course is described in **Table 3.** Clearance of mutations in ctDNA was rapid for all 11 of these cases and preceded normalization of traditional tumor markers.

There were 13 patients who recurred in the total cohort (4 EC, 8 OC, and 1 EA) and 8 deaths from disease at 2 years (4 EC, 3 OC, and 1 EA). ctDNA mutations were detected postoperatively in 8/13 (62%) of cases who recurred. The other 5 patients had serial draws

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Table 2. Clinicopathological characteristics of patients in whom somatic mutations were detected in tumor tissue, preoperative plasma ctDNA and/or postoperative plasma ctDNA

ID#	Tumor type (molecular subtype)	Pre-op clinical stage	Pre-op imaging showing distant disease	Pre-op CA125	FIGO stage	Largest tumor size (cm)	Residual disease after surgery (Y/N, location)	Recurrence within 2 years after surgery (Y/N, location)	Mutation detected in both tumor tissue and pre-op ctDNA	Mutation detected in both tumor tissue and post-op ctDNA	Mutation detected in ctDNA at any time point and not found in tumor tissue
013	EEC (*NSMP*)	I	No	91	IIICI	3.7	No	Yes, vagina	PTEN p.R130G, BRAF p.K601E	PTEN p.R130G, BRAF p.K601E (mo 18, 24)	TP53 p.R249G (mo 24)*
021	EEC (*NSMP*)	I	No	107	IB	5.5	No	No	KRAS p.G12C	None detected	None detected
031	EEC (*NSMP*)	IIIA	Yes	147	IIIA	5.5	Yes, miliary	No	CTNNB1 p.D32H, AKT1 p.E17K	None detected	None detected
062	EEC (*MMRd*)	IIIA	Yes	590	IIIC2	4.5	Yes, miliary	No	PTEN p.G127E	None detected	None detected
002	SEC (*p53abn*)	IIIB	No	44	IIIB	4.5	No	Yes, pelvis	PIK3CA p.E545D, TP53 p.S241T	PIK3CA p.E545D, TP53 p.S241T (day 55)	None detected
017	SEC (*p53abn*)	I	No	11	IA	2.0	No	No	None detected	None detected	TP53 p.N131S (pre-op)†
046	SEC (*p53abn*)	I	No	135	IIICI	2.8	No	No	TP53 p.V272M	TP53 p.V272M (day 2)	None detected
014	HGSOC	IIIC	Yes	13767	IIIC	11	Yes, >2 cm peritoneal	No	None detected	TP53 p.R280G (day 5)	None detected
020	HGSOC	IIC	Yes	720	IIIC	12	Yes, >2 cm nodal	Yes, nodal	None detected	TP53 p.R273H (day 1, 2, 3, mo 6, 12)	None detected
024	HGSOC	IIIC	Yes	110	IIIC	5.0	Yes, miliary	Yes, abdominal/ pelvis	TP53 p.V274D	TP53 p.V274D (day 1, 15)	None detected
035	HGSOC	I	No	1687	IIB	2.0	Yes, <1 cm rectosigmoid	Yes, vagina	TP53 p.C176F	TP53 p.C176F (day 1)	TP53 p.T256I, ERBB2 p.W835 (wk 6)*
042	HGSOC	I	No	494	IC	8.5	No	No	TP53 p.Y220H	None detected	None detected
048	HGSOC	I	No	202	IIB	9.0	Yes, miliary	No	TP53 p.V157G	None detected	None detected
054	HGSOC	IIIC				8.5	Yes, <1 cm rectosigmoid	Yes, abdominal/ pelvis	None detected	TP53 p.A86fs (day 1)	None detected
055	HGSOC	IIIA	Yes	2000	IIIB	10.0	Yes, >1 cm nodal	Yes, nodal, abdominal	TP53 p.D281E	TP53 p.D281E (day 1, 2, 3, 15)	None detected
056	HGSOC	IIIC	Yes	20	IIIC	6.0	Yes, <1 cm peritoneal	No	None detected	TP53 p.S106fs (day 1)	TP53 p.C238Y (pre-op, day 3)†
060	HGSOC	I	No	1752	IIB	11.0	Yes, miliary	Yes, nodal	TP53 p.I195fs	None detected	None detected
061	HGSOC	IIIC				3	Yes, <1 cm peritoneal	Yes, abdominal	None detected	TP53 splice (day 1, 2)	None detected

Details on mutation loci and timing of postoperative ctDNA mutation detection is also shown.
ctDNA, cell-free circulating tumor DNA; EEC, endometrioid endometrial carcinoma; FIGO, International Federation of Gynecology and Obstetrics; HGSOC, high grade serous ovarian carcinoma; SEC, serous endometrial carcinoma.
*There were 2 cases with emergence of new mutations detected at later timepoints; †There were 2 cases that had *TP53* mutations found in the preoperative ctDNA but were not identified in the tumor tissue.

discontinued prematurely, therefore we were unable to evaluate the sensitivity of ctDNA mutations at recurrence. **Fig. 1** illustrates the 13 patients that recurred with the timeline of longitudinal serial blood draws, timepoints of detectable ctDNA mutations, and clinical, radiologic or tumor marker progression. **Fig. 2** demonstrates the relationship between pre and postoperative ctDNA mutations and serum CA125 tumor marker levels in the 8 patients who had a disease recurrence and who had ctDNA plasma drawn and mutations detected postoperatively. A detailed description of the clinical course and ctDNA detection and clearance in these 8 patients is given in **Table 3**.

4. New mutations identified through ctDNA testing

Two patients demonstrated the emergence of new mutations, detected at later timepoints (**Table 2**). This included 1 patient with high grade serous OC with *TP53* p.C176F mutation identified in tumor tissue, preoperative ctDNA and postoperative ctDNA on day one, then emergence of a new *TP53* p.T256I mutation and *ERBB2* mutation at postoperative week 6. There was also a patient with stage IIICI no specific molecular profile (NSMP) endometrioid EC

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Table 3. Detailed description of the clinical course and ctDNA detection and clearance in the 8 patients who had a disease recurrence and who had ctDNA plasma drawn and mutations detected postoperatively

Patient ID	Detailed description
ctDNA002	An 80-year-old with stage IIIA serous EC declined adjuvant therapy following surgery. Tumor testing and preoperative ctDNA identified *TP53* p.S241T and *PIK3CA* p.E545D somatic mutations. At 8 wk postoperative, ctDNA *TP53* and *PIK3CA* mutations were identified, but the patient was asymptomatic and clinically free of disease. There were no other blood timepoints for ctDNA analysis taken. CA125 was mildly elevated preoperatively (42 U/mL), but was normal when repeated at 10 wk postoperative. By 4 mo postoperative, the patient was symptomatic and PET confirmed disease recurrence. CA125 performed at this time was also elevated. In this case, ctDNA *TP53* and *PIK3CA* mutations preceded clinical, imaging, and tumor marker evidence of recurrence. On retrospective pathology review, the vaginal resection margin was positive for carcinoma on the hysterectomy specimen, suggesting microscopic residual disease may have been present. This possibly explains detection of ctDNA in the initial postoperative period and the early elevation in ctDNA mutations, preceding clinical recurrence.
ctDNA013	A 67-year-old with stage IIICI grade 3 NSMP endometrioid EC received adjuvant chemoradiation following staging surgery. Tumor testing and preoperative plasma ctDNA identified *BRAF* p.K601E and *PTEN* p.R130G mutations, which were not detected in the day one postoperative plasma sample. Disease recurrence was detected on clinical exam at 14 mo. At the 18 mo blood draw, the *PTEN* ctDNA mutation was identified, and at 24 mo the *PTEN* and *BRAF* mutations VAF increased. Interestingly, at 24 mo there was an emerging *TP53* p.R249G ctDNA mutation that was not previously identified in any other blood timepoints or in the baseline tissue sample. Unlike the earlier detection of ctDNA mutations, CA125 and did not become elevated until 22 mo postoperative (8 mo after disease recurrence was diagnosed).
ctDNA020	A 47-year-old with stage IIIC high grade serous OC with primary debulking surgery and >1 cm residual disease. *TP53* p.R273H mutation was identified in the tumor and plasma ctDNA on postoperative days 1, 2, and 3 consistent with her residual disease following surgery. Germline testing was negative for *BRCA1/2* mutations and she progressed on first line chemotherapy with platinum refractory disease. The *TP53* ctDNA mutations became undetectable on first and second line chemotherapy (first line chemotherapy given from 2 wks–5 mo postoperative, and second line from 6–12 mo). This clearance was short lived, followed by rapid increase in ctDNA *TP53* levels before chemotherapy was completed, consistent with platinum refractory disease. This rapid increase in ctDNA *TP53* levels occurred with both first and second line chemotherapy, and preceded CA125 relapse.
ctDNA024	An 81-year-old with stage IIIC high grade serous OC underwent primary debulking surgery with no residual disease. *TP53* p.V274D mutation was identified in the tumor and in preoperative ctDNA, then rapid clearance of ctDNA levels following primary debulking surgery compared to the slow decline in CA125. This patient had no further ctDNA detected beyond 2 weeks postoperative and had imaging detect recurrence at 23 mo postoperative.
ctDNA055	A 55-year-old with stage IIIB high grade serous OC underwent primary debulking surgery with no residual disease. *TP53* p.D281E mutation was identified on tumor testing and preoperative ctDNA. Preoperative CA125 was 2000 and took 8 weeks postoperative to normalize, compared to the rapid clearance of ctDNA following surgery. The ctDNA mutation VAF% increased at day 15 postoperative, where the patient had not yet started adjuvant carboplatin/paclitaxel chemotherapy. The ctDNA mutation was not detected at 3 mo likely because the patient was receiving adjuvant chemotherapy. No further ctDNA monitoring was performed beyond this point, and at 11 mo radiologic recurrence was detected.
ctDNA061	A 40-year-old with stage IIIC high grade serous OC underwent primary debulking surgery with <1 cm residual disease. *TP53* ctDNA splice site mutation was detected on day one and 2 postoperative blood samples, but not in the preoperative sample. There were no further plasma timepoints after 2 wks postoperative, and the patient showed radiologic recurrence at 7 mo.
ctDNA035, ctDNA054	ctDNA035 and ctDNA054 were patients with high grade serous OC; the former stage II disease with no residual disease after surgical debulking, and the latter with stage IIIC and residual estimated at <1 cm in multiple sites. Both patients were treated with platinum-based chemotherapy with clearance of ctDNA mutations within 2 days (ctDNA035) and within 3 mo (ctDNA054) of surgery, whereas normalization of CA125 took 2 weeks and 5 mo, respectively. Unfortunately, patient ctDNA035 missed multiple blood draw time points, and although blood was successfully collected at 6 mo the specimen was mishandled and could not be evaluated. We therefore missed the opportunity to assess these 2 individuals for ctDNA mutations prior to recurrence.

The relationship between pre and postoperative ctDNA mutations and serum CA125 tumor marker levels is shown for these 8 patients in **Fig. 2**.
ctDNA, cell-free circulating tumor DNA; EC, endometrial cancer; NSMP, no specific molecular profile; OC, ovarian cancer; VAF, variant allele frequency.

with mutations in *PTEN* p.R130G and *BRAF* p.K601E found in tumor tissue, preoperative and postoperative ctDNA at 18 and 24 months, with emergence of a new *TP53* mutation at 24 months.

5. Molecular subtypes of the endometrial carcinomas

Of the 24 ECs included in this study molecular classification identified 2 *POLE*mut, 5 MMRd, 11 p53abn and 6 NSMP. The detection of ctDNA mutations in this series was most commonly observed in NSMP and p53abn ECs (**Table 2**). Interestingly, one of the p53abn ECs was a patient with stage IA grade 2 endometrioid EC who at the time of treatment in 2017 was classified as low risk (molecular subtype unknown) and treated with surgery alone. This patient had a distant recurrence (peritoneal disease and lung metastases) and died from disease within 2 years. The 2 *POLE* mutated EC cases did not recur and no ctDNA mutations were identified in any timepoints.

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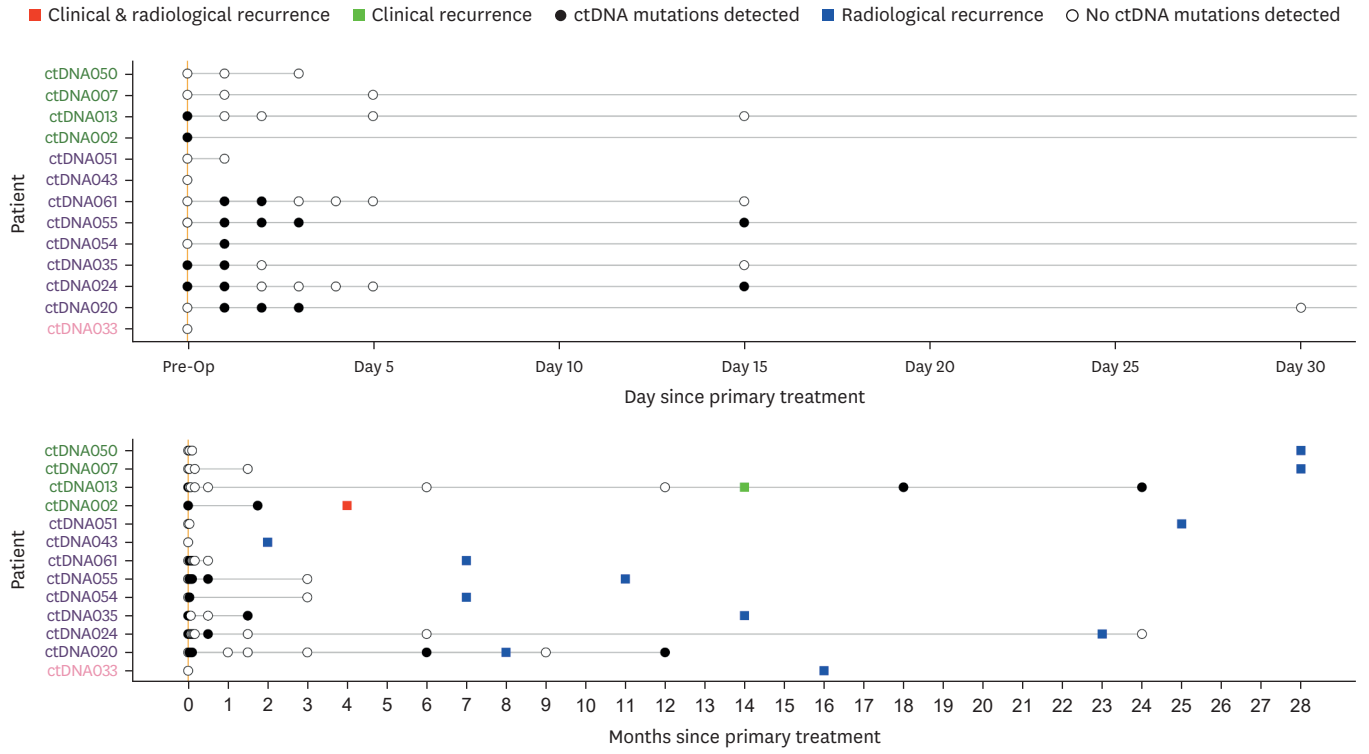

Fig. 1. Description of the 13 patients who recurred in the cohort. Green IDs represent endometrial cancer patients, purple represents ovarian cancer patients and the red ID (ctDNA033) is a patient with endocervical adenocarcinoma. The squares represent disease recurrence events (either identified through imaging, clinical examination, tumor markers, or combinations) and the circles represent blood draws with ctDNA mutations detected (solid) or not detected (empty). The horizontal lines for each case demonstrate the duration of serial blood draws since surgery (top graph x-axis measured in days since surgery and bottom graph x-axis represents months since surgery).
ctDNA, cell-free circulating tumor DNA.

DISCUSSION

Liquid biopsies are a precision oncology tool with the ability to assess tumor burden, identify driver and targetable mutations, monitor response to therapy and/or disease recurrence in real time [6,8-12,14]. We sought to determine the utility of detecting plasma somatic ctDNA mutations in patients with newly diagnosed OC and EC using a small targeted gene panel and next-generation sequencing (NGS). The detection of ctDNA mutations at any timepoint was most frequent in patients with high grade serous OC. Consistent with previous studies, the detection of ctDNA mutations was less common in EC [7,8,14-17], likely due to the sensitivity and limited gene panel used in this study, especially in patients with small and early-stage tumors. For both OC and EC, detection of preoperative ctDNA mutations was more likely with advanced stage disease, larger initial tumor burden, and higher preoperative CA125 levels. Postoperative ctDNA was associated with residual disease following primary surgery, where we observed 8 of 9 patients with residual disease and postoperative day 1–5 ctDNA mutations detected. These data suggest ctDNA mutation detection reflects the volume of tumor burden both pre and post surgery.

We identified multiple scenarios where ctDNA mutation detection would have useful clinical applications. Firstly, identification of patients with clinically occult advanced stage disease unrecognized at the time of diagnosis. In this series, we found 42% of patients who harbored detectable preoperative ctDNA mutations with clinically apparent stage I disease

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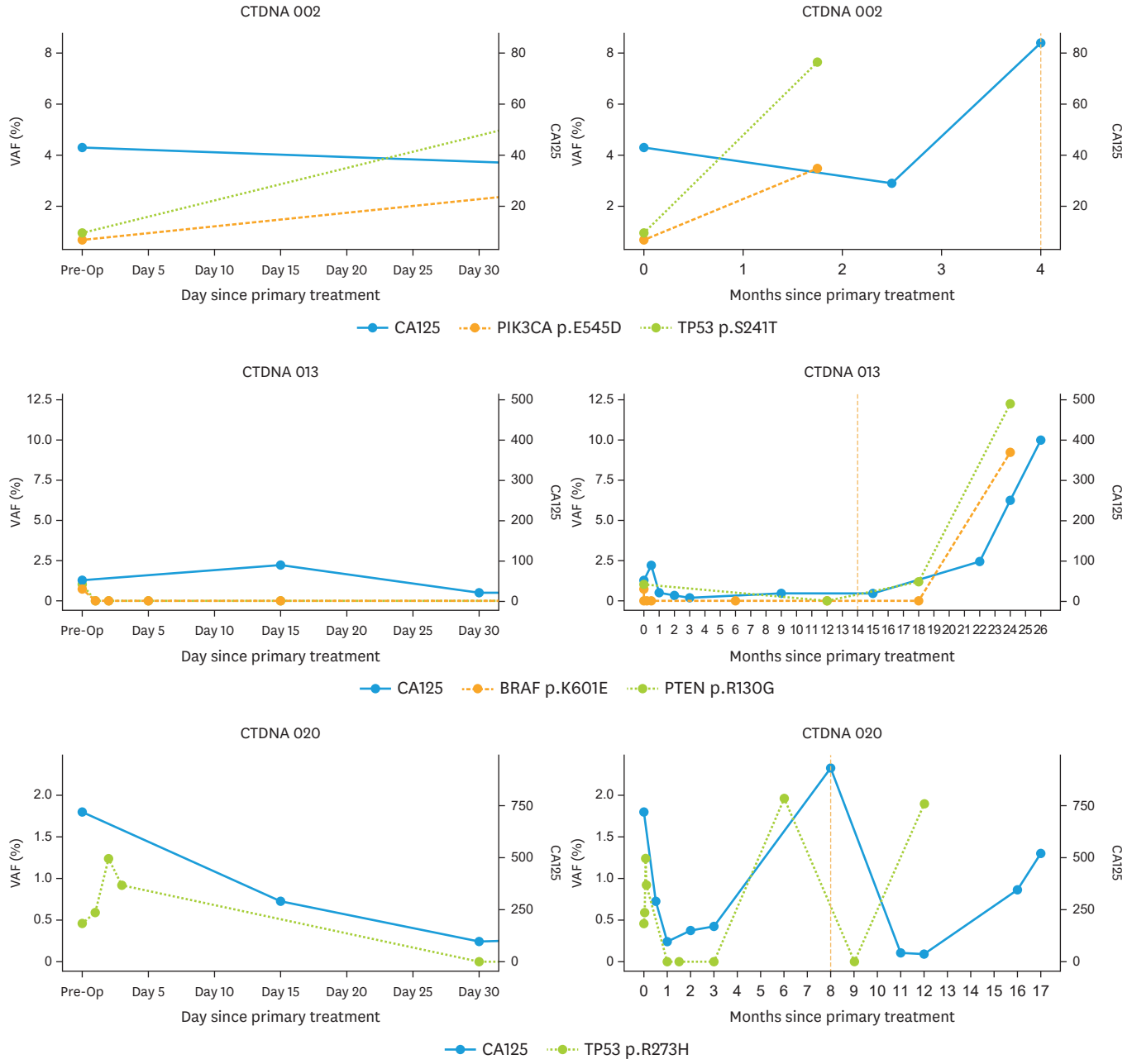

Fig. 2. The relationship between pre and postoperative ctDNA levels (VAF% shown on the left y-axis) and serum CA125 tumor marker levels (U/mL shown on the right y-axis) over time. Data is shown for the 8 patients who had a disease recurrence and who had ctDNA mutations detected postoperatively. Each row represents a patient, with measured values preoperatively and in days since surgery in the left graph, and months since surgery in right graph with changes reflecting response to treatment and/or disease status. The vertical yellow dashed line represents the time of documented disease recurrence.
ctDNA, cell-free circulating tumor DNA; VAF, variant allele frequency.
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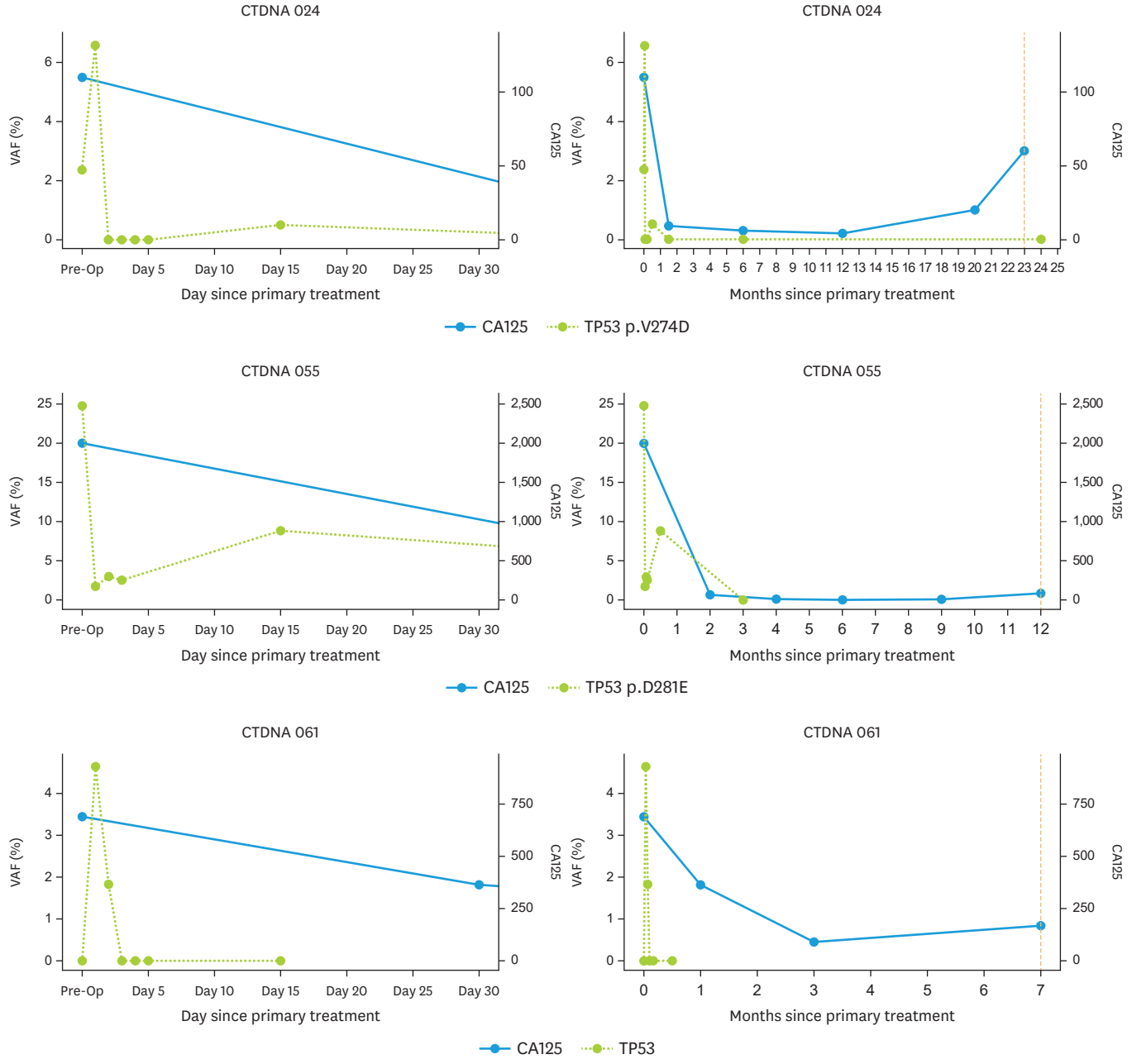

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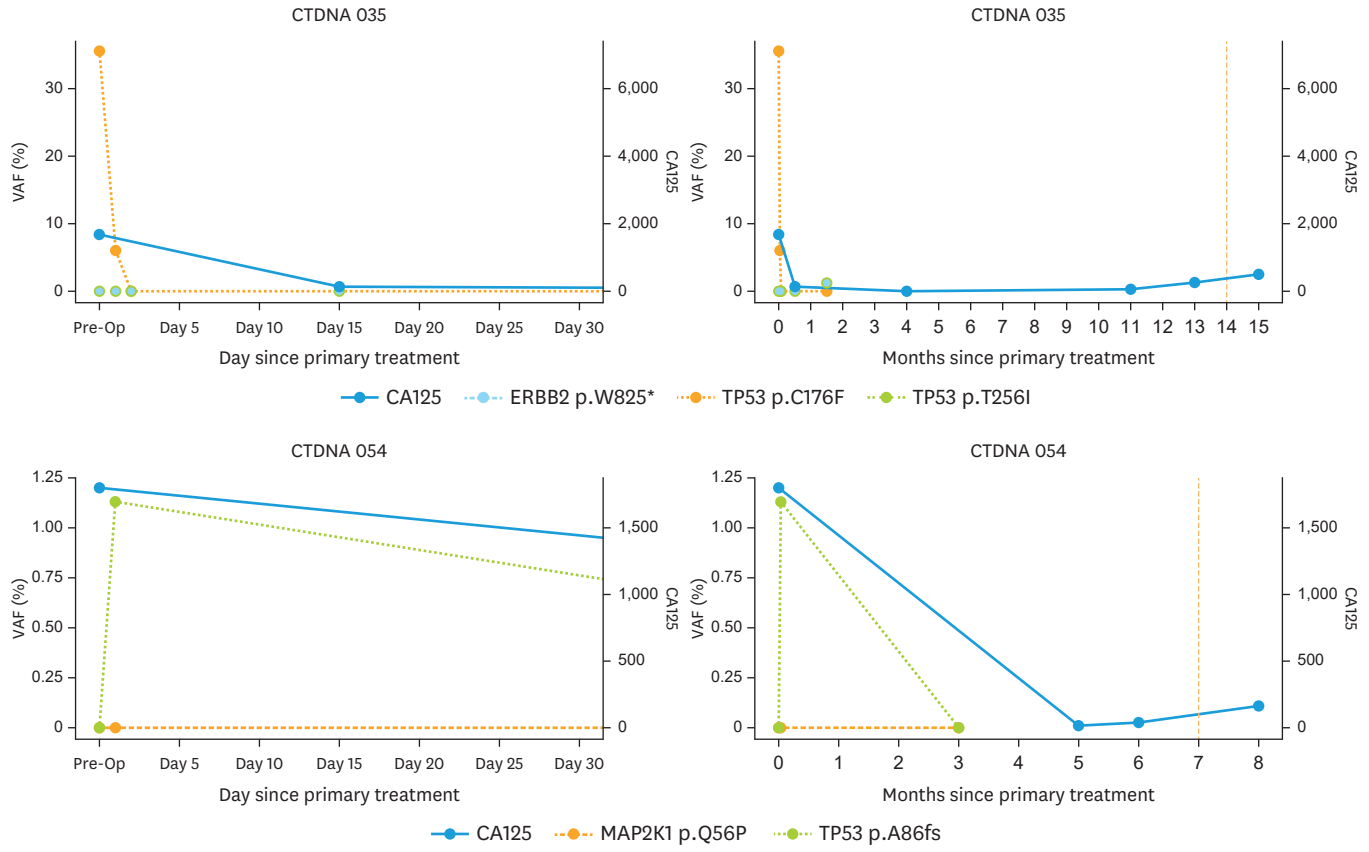

Fig. 2. (Continued) The relationship between pre and postoperative ctDNA levels (VAF% shown on the left y-axis) and serum CA125 tumor marker levels (U/mL shown on the right y-axis) over time. Data is shown for the 8 patients who had a disease recurrence and who had ctDNA mutations detected postoperatively. Each row represents a patient, with measured values preoperatively and in days since surgery in the left graph, and months since surgery in right graph with changes reflecting response to treatment and/or disease status. The vertical yellow dashed line represents the time of documented disease recurrence. ctDNA, cell-free circulating tumor DNA; VAF, variant allele frequency.

and preoperative imaging negative for distant disease, were subsequently found to have stage II/stage III disease at surgery and on final pathology review. One of these cases was a low grade NSMP EC (ctDNA013), a molecular subtype known to be associated with very favorable outcomes [4,18,19] and in some centers would be managed in the community with a hysterectomy and bilateral salpingo-oophorectomy without lymph node assessment. This patient had presumed stage I disease based on clinical exam and imaging with preoperative ctDNA detected, and was found to have metastases to pelvic lymph nodes (FIGO stage IIICI on final pathology). Hence the detection of ctDNA preoperatively could be used to flag a patient who potentially had larger volume disease and/or extra-uterine disease and trigger referral to manage in a tertiary cancer center. Identifying patients with advanced disease preoperatively can also impact management decisions when considering upfront surgery vs. neoadjuvant chemotherapy with delayed debulking surgery. Suboptimal tumor debulking at primary surgery is associated with worse survival outcomes for both EC and OC [2,20].

Secondly, a major clinical challenge in EC management is the lack of blood-based biomarkers to assess real time response to treatment [4,5]. Serum CA125 has been used as a monitoring tool in OC treatment for decades, but this lacks sensitivity and specificity [2,3], as changes in CA125 often lag behind tumor response. In this study, we demonstrated clearance of postoperative plasma ctDNA mutations in patients receiving adjuvant therapy that preceded

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the normalization of CA125 levels (**Fig. 2**; ctDNA013, 020, 024, 055, 061, 035, and 054). In one high grade serous OC patient, we observed *TP53* ctDNA mutations become undetectable on first and second line chemotherapy. This clearance was short lived, followed by a rapid increase in ctDNA *TP53* levels before chemotherapy was completed, consistent with the patient's platinum refractory disease (ctDNA020). Pereira et al. [3], assessed a similar sized cohort of EC and OC patients and found that ctDNA was a more accurate predictor of treatment response compared to imaging and CA125. Undetectable levels of ctDNA at 6 months following initial surgery and adjuvant therapy was associated with improved progression free and overall survival [3]. Similarly, Ashley et al. [15], found in 44 patients with EC, that changes in ctDNA VAFs closely mirrored therapy response in 6 patients with recurrent disease.

Thirdly, we currently lack tools in EC to identify earlier disease recurrence, at a time where recurrence is localized and potentially still salvageable/curable. In this study, there were 2 EC cases where postoperative ctDNA mutations were identified to increase in patients later diagnosed with recurrent disease. In ctDNA002, mutation detection preceded clinical, radiological and conventional tumor marker identification of disease recurrence. In ctDNA013, clinical recurrence was diagnosed prior to ctDNA mutation detection, however ctDNA mutations were identified 4 months prior to the elevation in CA125. Ashley et al. [15], also found 2/6 EC patients with recurrent disease show a rise in plasma ctDNA VAFs prior to the clinical detection of disease recurrence. Pereira et al. [3], showed that changes in ctDNA levels predicted disease recurrence with a 7-month lead time over CT imaging. We also demonstrate that preoperative ctDNA mutations is associated with higher risk of subsequent disease recurrence, with the recurrence rate almost double that of patients who did not have preoperative mutations, therefore the presence of preoperative ctDNA could flag patients that need closer monitoring for disease recurrence. In OC care, earlier detection of disease recurrence has become especially important following the publication of the DESKTOP III randomized trial [21]. This trial showed improved survival outcomes following secondary surgical debulking in select patients with platinum sensitive recurrent OC, if the recurrence is found early or at a time when compete resection is feasible [21].

There are also several scenarios for future applications of ctDNA. The use of larger and more comprehensive panels, not limited to targeted hotspots, that includes copy number assessments, genomic signature analysis and a broader range of targetable mutations that can be used to monitor disease progression and recurrence, will likely become an essential part of gynecological cancer care in the future. There is also the potential to track the emergence of new mutations in tumor evolution, disease recurrence and therapeutic resistance. As an example, *BRCA* reversion mutations detected in post-progression plasma ctDNA has been shown to predict acquired resistance to PARP-inhibitor therapy in patients with high grade serous OC [22].

Furthermore, with increasing targeted treatments options for patients, and our understanding that cancers evolve over time, the accurate assessment of relapsed disease, as opposed to the primary tumors they emerged from, will be essential for determining next lines of therapy. We have recently demonstrated that p53abn ECs, the most aggressive ECs, have targeted treatment options in 75% of tumors, such as HER2 overexpression, *CCNE1* amplification, homologous recombination deficiency, or *FBXW7* or *PPP2R1A* mutations [23]. The efficacy of these treatments in p53abn EC need to be assessed in clinical trials, and ctDNA monitoring could be used to help determine order of therapy.

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Further work is needed to assess the health economic comparisons of serial ctDNA sampling in selected patients, acknowledging the cost of blood draws, NGS panels and interpretation. These costs of ctDNA surveillance need to be countered with an appreciation that current surveillance methods (blood draws for conventional tumor markers, regular patient clinic visits, surveillance imaging) are also expensive and can be inconvenient, especially for patients that live large geographic distances from cancer centers. It will be critical to select the patients most likely to benefit from this technology.

The major limitations of this study include the small sample size, high variation in longitudinal postoperative blood draws for each patient, and limitation of the targeted gene panel used. Although we demonstrated that consent for multiple blood draws was acceptable, with an initial discontinuation rate of 8%, we did observe inconsistencies in obtaining serial blood draws. This was attributed to the health status of patients including symptoms associated with recurrent disease, and treatment side effects such as fatigue. Geographical location of patients also contributed to attrition. Compliance may increase if patients perceive a direct personal benefit from serial blood draws for disease monitoring in a real-life clinical situation rather than a research study. Samples were retrospectively sequenced and analyzed, therefore we were not able to ensure serial blood draws were undertaken in patients most likely to recur. Consequently, within the cohort that experienced disease recurrence, compliance with serial draws was low, with only 3 patients with blood draws beyond 6 months post-surgery (**Fig. 1**). This underestimated the ability of ctDNA mutations to detect earlier disease recurrence prior to clinical, radiological or tumor marker detection. Additionally, due to the small size of the targeted gene panel used in this study, it is also possible that ctDNA mutations were missed in specific timepoints, especially in patients with small and early-stage tumors.

In conclusion, we have demonstrated that analysis of plasma for ctDNA mutations can assist in identifying individuals with occult more advanced stage disease, monitoring real-time response to treatment, and earlier identification of disease recurrence in EC and OC. There are other opportunities not able to be tested in this series, such as identifying options for targeted therapies or indicators of drug resistance. Careful patient selection, identifying individuals with higher disease burden and/or higher likelihood of recurrence can direct resources towards diligent serial sampling for ctDNA analysis in some patients and spare cost in those unlikely to benefit.

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SUPPLEMENTARY MATERIALS

Data S1

Detailed supplemental methods

Fig. S1

Cohort description and proportion of patients who continued serial blood draws through study duration.

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