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Review

Perioperative Use of ctDNA to Guide Treatment for Urothelial Carcinoma: The Future is Now

Tyler F. Stewart[a,*], Healther Chalfin[b], Nicholas Simon[b], Alan Tan[c], Andrea Apolo[b] and Rana R. McKay[a]
[a]*Department of Medicine, University of California San Diego, La Jolla, CA, USA*
[b]*National Cancer Institute, NIH, Bethesda, MD, USA*
[c]*Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA*

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Abstract. Muscle-invasive bladder cancer represents a potentially curable disease, yet often disease recurs and is ultimately fatal. Outcomes for patients with localized urothelial carcinoma are heterogeneous with some patients cured with surgery alone, deriving no benefit from perioperative systemic therapy, while others are left with residual disease and may benefit from additional therapy. Neoadjuvant chemotherapy increases cure rates but comes with significant toxicity. Recently, adjuvant nivolumab has demonstrated significant improvement in disease free survival (DFS), and overall survival analysis is pending. With more therapies approved for urothelial cancer within the last 5 years than ever before, there is incredible potential to improve clinical outcomes and potentially cure more patients with integrated multimodal therapy. Biomarkers are needed to dichotomize those most likely to benefit from perioperative systemic therapy for residual disease, and de-escalate therapy for those likely to be cured with surgery alone. Ultrasensitive assays for circulating tumor DNA (ctDNA) have emerged as a method to identify patients at high risk of recurrence after definitive therapy and may benefit from escalated therapy, while also identifying those least likely to benefit from systemic therapy. Studies have demonstrated that the presence of ctDNA after surgery is prognostic of disease recurrence across multiple cancer types, including bladder cancer, but questions remain as to the utility of these tests, and whether they can be predictive of benefit of adjuvant therapy. Although these liquid biopsies hold significant promise to transform perioperative treatment, prospective studies are needed to validate their utility as prognostic and predictive biomarkers. To bridge this knowledge gap, contemporary clinical trials are incorporating ctDNA as an integral biomarker to guide therapy for MIBC.

Keywords: Bladder cancer, ctDNA, circulating tumor DNA, muscle-invasive bladder cancer, adjuvant therapy, perioperative therapy

BODY: CURRENT

The treatment landscape for locally advanced urothelial cancer (LAUC, including muscle-invasive bladder cancer (MIBC) and upper tract urothelial carcinoma (UTUC)) is rapidly changing. Platinum-based chemotherapy has been a mainstay of perioperative systemic therapy for years, typically given in the neoadjuvant setting if cisplatin eligible, improving 5-year overall survival by 5–10%, while 40% of patients are likely to recur [1–3]. Multiple trials have or are currently investigating the addition of perioperative novel agents for LAUC (e.g. BLASST-1, AURA, NIAGARA, KEYNOTE-866, ImVigor010, CheckMate-274, Ambassador, EV-303, EV-304) [4–11]. To date, data for adjuvant

*Correspondence to: Tyler F. Stewart, MD, Department of Medicine, University of California San Diego, La Jolla, CA, USA. E-mail: tstewart@health.ucsd.edu.

immunotherapy have been mixed. Adjuvant atezolizumab demonstrated no benefit for the intention to treat population, while adjuvant nivolumab demonstrated improvement in disease free survival (HR DFS 0.70 98.22% CI, 0.55–0.90) [8, 12]. While studies are largely focused on adding agents and intensifying therapy to improve outcomes, many patients with localized disease may be cured with local therapy alone, and the addition of systemic therapy may lead to medical, financial and psychologic toxicities without benefit.

The cancer community has long been seeking biomarkers in the perioperative setting that identify patients most likely to benefit from escalation of therapy to increase cure rates and improve long-term outcomes, and to identify those least likely to benefit from systemic therapy to prevent overtreatment and unnecessary toxicities. Circulating tumor DNA (ctDNA) is poised to be that marker. Recent studies have highlighted the potential of ctDNA as a prognostic biomarker, able to identify patients with minimal residual disease (MRD) after definitive therapy. As the field of perioperative systemic therapy for bladder cancer changes, the use of these biomarkers may play a crucial role in stratifying risk of recurrence and future therapy. Indeed, multiple studies are ongoing or on the horizon with ctDNA as *integral* biomarkers, around which treatment decisions are made. As these biomarkers are incorporated into studies, it is imperative to better understand these tools.

The purpose of this article is to 1) review the current literature regarding the use of ctDNA as a prognostic and predictive marker for risk of recurrent disease with LAUC, 2) highlight current clinical trials incorporating ctDNA as an integral biomarker, and 3) outline future directions for the use of ctDNA to guide adjuvant and early systemic therapy.

Although this review will focus on plasma based ctDNA assays, data to date and future applications, urine-based biomarkers including ctDNA assays are of increasing importance for bladder cancer. Such assays are already being used to identify cancer, monitor for recurrence and could potentially be used in bladder preservation protocols [13, 14].

BIOLOGY AND APPLICATIONS OF CELL FREE DNA AND CIRCULATING TUMOR DNA

Cell free DNA (cfDNA) is DNA released into the circulation upon cell death. The presence of this non-cellular component was originally described by Mandel and Metais in 1948 [15]. In the late 1980s, Stroun et al demonstrated that a subset of cfDNA were derived from cancer cells, so-called "plasma DNA of the neoplastic type" [16]. In the 1990s, investigators began to detect specific DNA mutations in the plasma that corresponded to the exact DNA mutations in patients' tumors [17, 18]. Subsequently over the last 25 years, with the development of novel techniques to analyze cfDNA, there has been an explosion of studies evaluating the clinical utility of these assays.

CtDNA refers to the fraction of cfDNA that is derived from tumor tissue. These tumor derived ctDNA fragments are not only found in blood, but may be detected in saliva, urine, stool and cerebrospinal fluid [19]. The amount of ctDNA in blood at any given time in a patient with cancer is dependent on tumor type, stage and total disease burden, and can range from <0.01% to >90% of cfDNA [20, 21]. Current research suggests that cfDNA is released from cells through apoptosis and necrosis, and possibly secretion, thus cells with rapid turnover and higher levels of necrosis may be linked to higher levels of ctDNA (Fig. 1) [22, 23]. The half-life of cfDNA is generally less than 2 hours making ctDNA, which is more stable in circulation given association with circulating proteins, an ideal candidate to monitor dynamic changes in tumor burden [21, 24].

The breadth of the uses of ctDNA have been thoroughly discussed in multiple reviews and is beyond the scope of this article [25–28]. Briefly, ctDNA use falls into two broad categories: qualitative and quantitative. Qualitative uses include identification of predictive/actionable mutations and detection of mechanisms of resistance. One such example is the detection of targetable mutations such as mutations in fibroblast growth factor receptors (*FGFR*) in advanced urothelial carcinoma. More recently, quantitative uses, quantifying presence, and degree of ctDNA, have been increasingly investigated throughout the disease spectrum including diagnosis, monitoring for disease recurrence after definitive therapy, and monitoring for response to treatment. Early studies have demonstrated that quantitative evaluation of ctDNA may differentiate between progression and so-called "pseudoprogression" with immune checkpoint therapy [29–31]. Such a differentiation may improve patient care, informing clinicians to continue patients on checkpoint inhibitors who are truly benefiting, and changing to alternative therapies earlier than typical for those who are not responding. Studies have also suggested a role for ctDNA

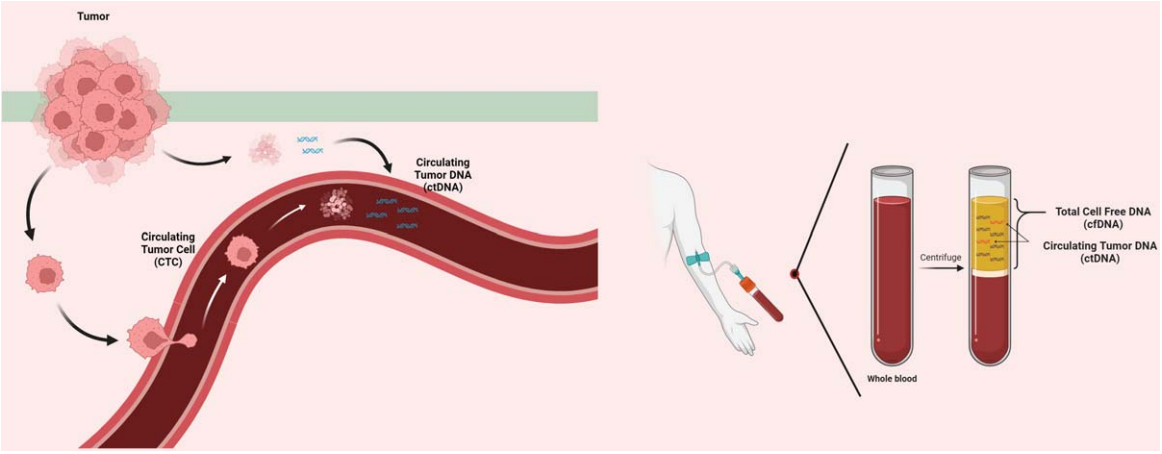

Fig. 1. Circulating Tumor DNA.

Table 1
Examples of ultrasensitive ctDNA assays

Methodology		Limit of Detection	Examples
Genomic Variants	Digital PCR	0.1–0.01%	Droplet Digital PCR BEAMing
	Modified NGS – Hybrid Capture – Amplicon	0.01–0.001%0.1%	CAPP-Seq, PhasED-Seq, Signatera, ArcherDx, Avenio, RaDaR, Tam-Seq
Epigenetic	Methylation	0.1%	GRAIL Galleri
Combination	Genomic and Epigenetic	0.1%	Guardant Infinity

as a cancer screening tool, and a tool to monitor for progression from early to locally advanced bladder cancer [32–34]. As technology has become more sensitive, significant efforts are underway to study ctDNA as a marker of minimal residual disease (MRD) after definitive therapy, and as a surveillance tool to identify early disease recurrence, before radiographic recurrence.

CTDNA ASSAYS TO DETECT MINIMAL RESIDUAL DISEASE

The efforts to use ctDNA to identify MRD and monitor for recurrence hinge on the ability to detect ctDNA at very low levels. Although ctDNA may represent a substantial portion of cfDNA in patients with overt metastatic disease, when assessing for MRD, the fraction of ctDNA among total cfDNA is significant lower. Ultrasensitive assays aim for sensitivity magnitudes higher than classic assays. Classic assays to assess ctDNA such as sanger sequencing, quantitative polymerase chain reaction (PCR), and next-generation sequencing (NGS) are generally not sensitive enough to identify ctDNA at concentrations less than 1% [27]. In addition to the need for enhanced sensitivity, variations in DNA such as somatic mosaicism and clonal hematopoiesis of indeterminate potential (CHIP) may lead to particular challenges with tumor specificity.

Over the last 10 years new technologies have been developed that significantly increase the sensitivity to detect ctDNA, so called ultrasensitive assays (Table 1). Further modifications such as assessing for multiple abnormalities within the same tumor (e.g. multiple tumor-specific somatic mutations), adding unique tags to DNA prior to DNA amplification to reduce technical artifacts (background noise), and combining multiple modalities have led to increased sensitivity and specificity. Other techniques to increase sensitivity involve the use of genome wide sequencing of cfDNA (MRDetect) and detection of phased variants [35, 36]. These advances decrease rates of false positives from errors in PCR, or interference of CHIP with tumor specificity.

Two of the major considerations that go into the design of an ultrasensitive assay for MRD are (1) personalization and (2) methodology. A personalized approach integrates *a priori* knowledge of the

genetic abnormalities in the specific patient's tumor, and targets these abnormalities for ctDNA detection. Customized approaches require sequencing of tumor tissue; however, NGS from plasma prior to definitive therapy may be sufficient if ctDNA is identified. The Signatera assay, for instance, requires whole exome sequencing (WES) of a patient's tumor tissue to identify 16 patient-specific mutations, and subsequent ctDNA analysis is performed by a personalized assay that probes for these 16 mutations [37]. A patient-specific, personalized approach may increase sensitivity and specificity but comes with the need for primary tissue/tumor sample, additional time to create the specific assay, and increased cost. Alternatively, non-personalized approaches rely on measurement of genomic or epigenomic aberrations that are commonly found in specific cancer types. Epigenomic features such as cancer-specific methylation patterns are being pursued as alternative signals for MRD assessment (e.g., Guardant Infinity, GRAIL Galleri).

In addition to the approach, the methodology of the assay plays a critical role in sensitivity and specificity.

- *Digital PCR:* Real-time quantitative PCR (qPCR) is common in medical laboratories, however the sensitivity for very small quantities of ctDNA is limited. Digital PCR partitions the PCR reaction into thousands of individual reaction vessels prior to amplification allowing for direct quantification which significantly improves sensitivity [38]. Further developments on this technique including droplet digital PCR (ddPCR) and BEAMing have increased sensitivity, with detection rates down to 0.01% [21, 39–41].
- *Next-Generation Sequencing:* Unlike PCR which is used for a limited number of genes, NGS testing allows for analysis of a much larger number of genes through massive parallel sequencing of short fragments of DNA. Standard NGS is generally able to detect DNA variants at concentrations down to 1%. Targeted capture NGS techniques employ a target panel of genes and additional factors to increase sensitivity and specificity. Available products include multiplex PCR-based NGS (e.g. Safe-SeqS and Signatera) and hybrid capture-based NGS (e.g. CAPP-Seq, TARDIS) assays [42–45].
- *Methylation Sequencing:* Due to the limited number of somatic nucleic acid alterations that distinguish cancer cells from their non-malignant counterparts, alternative changes in DNA that occur during carcinogenesis have been explored to identify ctDNA. Epigenetic methylation alterations have been well described in cancer, and because much of the genome is involved in abnormal methylation in tumor cells, the absolute quantity of tumor-derived DNA may be more robust compared to detection of a single or array of somatic mutations. This allows for detectability of ctDNA at very low levels [46]. Recent studies have suggested that ctDNA incorporating methylation signature may be more sensitive than PCR based assays [47]. The combination of ultrasensitivity and detection of tissue of origin has led to work using methylation-based ctDNA assays to screen for malignancy in a general population [34]. Many ultrasensitive ctDNA assays have moved to this methodology, either alone or in combination with NGS testing.

EFFICACY AND PITFALLS OF CTDNA AS A MARKER FOR MINIMAL RESIDUAL DISEASE

The specific, quantifiable, and dynamic properties of ctDNA have led to significant interest in its use as a marker for MRD across multiple malignancies. MRD refers to the persistence of tumor despite curative intent procedure. Typically, MRD refers to residual microscopic or molecular disease in the absence of overt radiographic or visible tumor detection. The precise definition of MRD and systematic criteria to define MRD including ctDNA thresholds are still being developed for solid organ malignancies. Early studies performed to assess the dynamic qualities of ctDNA demonstrated that the presence of ctDNA after definitive therapy corresponds to high risk of recurrence. In Diehl's early landmark study, the presence of ctDNA on days 13–56 after definitive therapy for colon cancer (including post metastatectomy for oligometastatic disease) was associated with a 94% recurrence rate (15 of 16 patients). Conversely, of those who were negative for ctDNA after surgery, none of the four patients developed recurrence, suggesting this assay may provide sensitive and specific testing for MRD.

Over the next 15 years, a series of studies tested the prognostic significance of ctDNA and demonstrated that ctDNA positivity is associated with increased risk of recurrence (Table 2). These data thus far sug-

Table 2
Selected studies assessing prognostic role of ctDNA

Study	Disease	N	Assay	Setting	Major Findings (ctDNA+ vs ctDNA–)
Patel (2017) [63]	Bladder	12	Tam-Seq	After 1 cycle neoadjuvant chemotherapy	RR: 83% vs 0%
Birkenkamp-Demtroder (2017) [48]	Bladder	24	ddPCR	Post-cystectomy	RR: 67% vs 0%
Christensen (2019) [37]	Bladder	68	Signatera	Diagnosis	RR: 46% vs 3%
				After Neoadjuvant Chemotherapy	RR: 75 % vs 11%
				Post-cystectomy	RR: 76% vs 0%
Lindskrog (2023) [50]	Bladder	102	Signatera	Diagnosis	RFS HR 3.4 (1.7–6.8)
				Post-cystectomy	RFS HR 17.8 (3.9–81.2)
Powles (2021, ImVigor010) [53]	Bladder	581	Signatera	Post-cystectomy, observation	DFS HR = 6.3 (CI 4.45–8.92) for ctDNA+
				Post-cystectomy, adjuvant atezo	DFS HR = 3.36 (CI 2.44–4.62) for ctDNA+
				Post-cystectomy, CtDNA-	DFS HR for ctDNA- treated with atezo vs obs: 1.14 (CI 0.81–1.62)
				Post-cystectomy, CtDNA+	DFS HR for ctDNA+ treated with atezo vs obs: 0.58 (CI 0.43–0.79)
Powles (2023, ImVigor010) [54]	Bladder	396	Foundation Medicine, Signatera	Post-cystectomy, CtDNA-	DFS HR for ctDNA- treated with atezo vs obs: 1.28 (CI 0.88–1.88)
				Post-cystectomy, CtDNA+	DFS HR for ctDNA+ treated with atezo vs obs: 0.56 (CI 0.38–0.83)
Powles (2022, ABACUS) [53]	Bladder	40	Signatera	After Cystectomy	RFS HR = 78; no relapses in ctDNA- patients
Sfakianos (2024) [64]	Bladder	109	Signatera	Post-cystectomy	RR: 44% vs 8%; HR 6.93 (CI 2.4–20.05)
Nakano (2022) [65]	UTUC	50	Oncomine TagSeq	Pre-surgical	RFS HR = 4.6 (CI 1.4–14.5)
				Post-surgical, of pre-surgical CtDNA+	RFS HR = 4.6 (CI 1.4–14.5)
Tie (2016) [66]	Colon	178	Safe-SeqS	After Surgery Alone	RR: 79% vs 10%
Tie (2019) [67]	Colon	88	Safe-SeqS	After Adjuvant Chemotherapy	3-Year DFS: 30% vs 77%
Reinert (2019) [68]	Colon	125	Signatera	Post op, day 30	RR: 70% vs 12%
				Post Adjuvant chemotherapy	RR: 100% vs 13.7%
				serial follow up	RR: 93% vs 3%

(*Continued*)

Table 2
(*Continued*)

Study	Disease	N	Assay	Setting	Major Findings (ctDNA+ vs ctDNA–)
Tie (2019) [69]	Rectal	159	Safe-SeqS	Prechemotherapy After chemoradiation, 4–6 weeks Post operative, 4–10 weeks	RR: No difference, HR 1.1, 95% CI 0.42–3.0 RR: 50% vs 11% RR: 58% vs 8.6%
Pietrasz (2017) [70]	Pancreatic	31	ddPCR: KRAS	After Surgery,	ctDNA+ DFS 4.6 months; OS 19.3 months ctDNA– DFS 17.6 months; OS 32.2 months
Cabel (2019) [71]	Gastric	32	ddPCR	Baseline Post chemotherapy Post Surgery	RR: 25% vs 40% All patients found to be CtDNA– ($n = 18$); 39% recurred RR: 100% vs 33%
Lee (2018) [72]	Melanoma	161	ddPCR: BRAF/NRAS	After surgery, within 12 weeks	RFS HR = 3.12 (1.79–5.47)
Eroglu (2023) [73]	Melanoma	69	Signatera	After surgery ($n = 30$)	RR: 60% vs 4%
Gale (2022) [74]	Lung	88	RaDaR	Landmark post-surgical	RFS HR = 14.8 (CI 5.82–37.48)
Jung (2023) [75]	Lung	278	ddPCR	Multiple time points Group A: ctDNA– at baseline and post surgery; Group B: ctDNA+ at baseline and ctDNA– post surgery; Group C: ctDNA+ at baseline and post surgery	3 year DFS: Group A: 84% Group B: 78% Group C: 50%
Garcia-Murillas (2019) [57]	Breast	101	digital PCR	At Baseline At serial follow up	RFS HR 5.8 (95% CI, 1.2–27.1) RFS HR 16.7 (95% CI, 3.5–80.5)
Lipsyc-Sharf (2022) [76]	Breast	103	RaDaR	At serial follow up	Distant Recurrence: 75% vs 0%
Chera (2020) [77]	HPV+ Oropharynx	45	digital PCR	serial follow up 2 ctDNA testing required for "positive"	RR: 50% vs 0% RR: 100% vs 0%

Key: RR = risk of recurrence; ddPCR = droplet digital PCR; DFS = disease free survival; RFS = relapse free survival; HR = hazard ratio; UTUC = upper tract urothelial carcinoma; OS = overall survival; FFP = freedom from progression. *CtDNA negative here was CtDNA fraction <2%.

gest that ctDNA presence after definitive therapy identifies patients at high risk of recurrence, and this biochemical persistence/recurrence often precedes radiographic progression by months to years.

Specifically for bladder cancer, the utility of ctDNA to predict disease recurrence has been investigated in prospective studies and retrospective analysis of large randomized clinical trials [37, 48, 49]. In one of the largest prospective study to date ($n = 68$), Christensen et al studied serial ctDNA in patients with locally advanced bladder cancer undergoing neoadjuvant chemotherapy using the Signatera assay. At a median follow up of 21 months, the presence of plasma ctDNA at time of diagnosis, prior to chemotherapy, was associated with a 46% chance of recurrence, whereas the ctDNA-negative population had only a 3% rate of recurrence. Impressively, 10 of 12 (83%) of those found to be ctDNA-positive before day 160 after cystectomy demonstrated radiographic recurrence by 1 year [37]. Additionally, serial testing detected ctDNA in all patients with recurrent disease, with an average lead-time of 96 days [37].

In a follow up of this study, Lindskrog et al reported clinical outcomes based on ctDNA status for 102 patients with MIBC that were not treated with neoadjuvant chemotherapy [50]. CtDNA was associated with worsened relapse free survival and OS before surgery (HR 3.4 (1.7–6.8); HR 1.7 (1–3)) and after surgery (HR 17.8 (3.9–81.2); HR 3.5 (1.4–9.1)) [50]. In an analysis of patients who were ctDNA negative at baseline, in the NAC cohort, none of these patients experienced recurrences. For those were ctDNA negative at baseline and did not receive NAC, 5 of 50 patients developed disease recurrence within 1 year [50]. This suggests that although ctDNA at baseline is prognostic, even patients who are ctDNA negative at baseline may be at risk for disease recurrence after surgery and should be considered for NAC. Additionally, in the cohort that did not receive NAC, 4 patients were ctDNA positive prior to surgery and were ctDNA negative after surgery, suggesting that ctDNA may come local disease alone [50].

In the phase 2 ABACUS study of neoadjuvant atezolizumab for 2 cycles, 27 of 88 (31%) patients with MIBC (pT2-4aN0M0) had a pathologic complete response [51]. CtDNA was assesses in 40 patients at baseline, after neoadjuvant atezolizumab, and after cystectomy [52]. CtDNA negative patients had significantly improved recurrence-free survival and all timepoints and ctDNA conversion from positive to negative was associated with response or absence of relapse [52].

Powles et al. demonstrated the prognostic and predictive potential of ctDNA in urothelial carcinoma in a pre-specified ctDNA analysis of the ImVigor010 study (locally advanced urothelial carcinoma followed by resection +/– adjuvant atezolizumab; total population $n = 809$, ctnda subset $n = 581$) [53]. While the study demonstrated no benefit of adjuvant atezolizumab in the intention to treat population (HR DFS 0.89, 95% CI: 0.74–1.08), subset data based on outcomes by ctDNA status demonstrated remarkable differences. Using the Signatera® platform, in the observation arm, patients who were ctDNA-positive after cystectomy exhibited high rates of disease recurrence (12-month DFS only 20%), while ctDNA-negative patients had a 12-month DFS of 75%. Most strikingly, when dichotomized by ctDNA status after resection, there was a benefit for adjuvant atezolizumab in the ctDNA-positive patients (HR OS 0.59, 95% CI 0.41–0.86), while no benefit was observed for the ctDNA-negative patients (HR OS 1.31, 95% CI: 0.77–2.23). These data highlight the prognostic ability of ctDNA in urothelial cancer and ability of ctDNA positivity to predict response to IO therapy.

More recently, a similar analysis on the ImVigor010 study was performed using the Signatera® platform; however, instead of performing WES, the Foundation Medicine comprehensive genomic profiling platform was used to identify up to 16 abnormal variants [54]. This analysis used a computational algorithem to filter out non-tumor derived variants which allowed it to avoid the germline analysis used in WES methods. Of the 809 total ITT population, 396 were included in the biomarker-evaluable population (BEP). Similar to the original analysis, there was an improvement in DFS with adjuvant atezolizumab compared to observation alone in the ctDNA positive cohort (HR DFS 0.56, 95% CI 0.38–0.83). There was no observed DFS benefit in the ctDNA negative cohort (HR 1.28, 95% CI 0.88–1.88) [54].

With these recent data, the FDA has granted breakthrough device designation and as of July 2022, the Centers for Medicare & Medicaid Services (CMS) has agreed to cover the Signatera assay for patients with MIBC. This assay, and similar assays, are now being incorporated into the clinic while many questions remain about the prognostic and predictive value and the clinical management in response to a positive or increasing ctDNA report. Despite the significant findings from the subset analysis from ImVigor010 analysis, this approach remains investigational, as

many limitations remain for these assays that need to be carefully considered. In addition to the assay itself, many clinical characteristics are important to consider for the practical application of this test. These factors include:

- *Tumor Type:* CtDNA relies on tumors shedding DNA into the blood stream, and this varies by volume of disease and disease type [55, 56]. An assay therefore may be very sensitive for one tumor type, but not another. For instance – if ctDNA by a specific assay is found to be a marker for MRD for colon cancer, it does not necessarily translate as a successful marker for kidney cancer. Additionally, and particularly for non-targeted ctDNA assays, these require use of a classifier, a bioinformatics platform trained to identify specific tumors, and call cancer from non-cancer. Therefore, utility of an assay must be based on tumor type, bioinformatics, and clinical setting.
- *Location of Disease:* Location and volume of disease are important determinants for sensitivity. CtDNA may not be sensitive to detect recurrences in certain sanctuary sites, such as the central nervous system. In an analysis of ctDNA to detect recurrence of breast cancer after definitive therapy, 23 of 29 patients who relapsed were positive for ctDNA before radiographic/clinical progression [57]. Of the 6 patients who were not detected, all 6 had a single site of disease, 3 of whom had a single brain met, 2 with solitary local recurrence and 1 with an ovarian metastasis. Therefore, local recurrence and recurrence in some sanctuary sites may not be as readily picked up by ctDNA. Recently, ctDNA from plasma by methylation signature was able to identify intracranial tumors and discriminate between specific pathologies, however this data is early, and whether one assay may be better to identify intracranial lesions, or whether these tumor types were characteristically different needs further study [58].
- *Timing:* Timing of assessment of MRD after definitive surgery is critical to the interpretation of the assay. The detection of ctDNA depends not only on the absolute amount of ctDNA, but on the ratio of ctDNA to cfDNA. Henriksen et al demonstrated that cfDNA levels increased 3-fold and 8-fold post-operatively for patients with colon cancer and bladder cancer, respectively. The cfDNA appeared to normalize around weeks 4–6 [59]. Therefore, in studies with ctDNA as an integrated or integral biomarker, appropriate timing must be considered as to optimize the sensitivity, but remain within an appropriate therapeutic window. Moreover, there is no significant data on the use or kinetics of ctDNA in patients after definitive radiation for MIBC. For radiation, there may be residual tumor death over weeks to months and the optimal timing to assess for MRD is unknown.
- *Perioperative Systemic Therapy:* Even less is known about the predictive ability of ctDNA for MRD in patients who receive perioperative systemic therapy and those that do not. Although pretreatment ctDNA status does correspond to prognosis, as does ctDNA status after definitive surgery, it is unclear if a ctDNA-negative status in patients that receive perioperative systemic therapy has the same value as ctDNA-negativity without perioperative therapy. Perioperative therapy will likely minimize ctDNA and therefore the utility in the context of systemic therapy is unknown.
- *Sample Collection:* Sample collection and processing is critical for ctDNA analysis. Plasma samples are superior to serum as cfDNA is 2–24-fold higher in serum, likely from release of DNA from leukocytes during the clotting process [28]. Given detection of ctDNA is dependent on the ratio of ctDNA to cfDNA, the lower background amount of cfDNA makes plasma the preferred source.
- *Sensitivity:* Across the board, the positive predictive value of ctDNA to predict recurrence has been demonstrated. However, ctDNA-negative status does not rule out risk of recurrence. In the ctDNA analysis of ImVigor010 referenced above, nearly one-third of patients who were ctDNA negative developed disease recurrence, suggesting while the PPV is excellent, the NPV of this assay in this specific clinical setting leaves room for improvement [49]. For future clinical trials which aim at de-escalating therapy for patients who are ctDNA-negative, the most sensitive assay (in serial assessments), will be needed to avoid withholding therapies to patients likely to benefit.
- *Tissue Acquisition for a Targeted Approach:* Once again, in the ImVigor010 dataset, 28% of patients were not evaluable for the biomarker, 83% of which was due to insufficient tumor or plasma sample [49]. Although studies and clin-

ical practice moving forward will likely adapt to this requirement, the practical aspect is that tumor assessment may not be possible for some patients, and untargeted assays may be more practical for some.

INCORPORATING CTDNA AND OPTIMIZING CLINICAL TRIALS, THE FUTURE IS NOW

Adjuvant trials have historically been necessarily large, often requiring several hundreds or thousands of patients to test benefit. Additionally, adjuvant trials are large and take years to accrue and to demonstrate clinically meaningful benefit. Out of a numerous of adjuvant trials for locally advanced UC, few trials have demonstrated significant DFS benefit (EORTC 30994, POUT and Checkmate 274) [8, 60, 61]. Both EORTC 30994 and POUT did not fully accrue to assess overall survival, and we still await overall survival data for Checkmate 30944.

These large, adjuvant trials are often negative despite reasonable design in part due to the inclusion of the patients who are cured with local therapy alone, poor accrual, and the use of therapies that are only effective in a small subpopulation. Efforts to optimize patient selection have largely relied on clinical prognostic markers such as tumor size, lymph node involvement, pathologic response after resection or genetic risk assessment (e.g. OncotypeDx score for breast cancer). These surrogate markers are of varying accuracy. Direct markers of residual disease such as ctDNA may refine this selection conundrum. Further, ctDNA could be used as a surrogate endpoint itself. In the ctDNA analysis from ImVigor, ctDNA clearance from cycle 1 to cycle 3 was strongly associated with improved outcomes [53].

In order to move the field forward, two types of studies are required to test and validate the use of CtDNA: prospective collection studies (including ongoing clinical trials with ctDNA as an integrated biomarker) and perioperative trials with ctDNA as an integral biomarker.

Prospective collection studies

Large, prospective collection studies are essential to the study of biomarkers. With the exception of the ImVigor010 study, that performed the ctDNA analysis post-hoc, these studies have been small. Larger prospective studies are necessary to demonstrate generalizability and validate findings in MIBC. Additionally, prospective studies generally evaluate only one ctDNA assay, rather than comparing multiple assays. Studies combining and comparing assays are critical to demonstrate the optimal assays for a specific tumor type and condition. It is certainly possible that a methylation-signature assay may be a more sensitive and specific assay for some tumors, where a targeted NGS or combination assay may be more appropriate for a different tumor type. Cross trial comparisons will not be able to compare sensitivity or lead-time analysis as patient populations are likely to differ. Rather, simultaneous blood collections for multiple ctDNA platforms are necessary for comparison.

Such collection protocols may be performed independent of any therapeutic trial (such as the ORACLE trial, investigating Guardant's MRD assay); however, collection protocols with ctDNA as an integrated biomarker embedded within neoadjuvant/adjuvant trials can accelerate the field significantly. As exemplified by the ImVigor010 data, trials which incorporate pre-specified subgroup analyses at optimal timepoints for patients who are ctDNA-positive (and therefore likely to recur) are likely to demonstrate a larger absolute benefit by not incorporating those likely to be cured. Moreover, these prespecificed subgroup analyses may identify a patient population likely to benefit from therapy, despite an overall trial being negative. We await the results of subgroup analysis of the Checkmate-274 trial as well as the AMBASSADOR trial (with a planned ctDNA-based subgroup analysis).

Prospective collection studies are also critical to consider de-escalating therapy. Indeed, in the analysis by Christensen et al which evaluated ctDNA for patients with MIBC, only 3% of patients who were ctDNA negative at the time of diagnosis developed recurrent disease over the course of the study. However, all patients received neoadjuvant therapy, and therefore it's not clear if those patients benefitted from neoadjuvant therapy or not. A potential clinical trial design would be to evaluate ctDNA at diagnosis, and restrict neoadjuvant therapy to individuals with the presence of ctDNA. Yet a trial de-escalating therapy may be unethical without prospective data to demonstrate that this biomarker may define a patient population unlikely to benefit from neoadjvuant chemotherapy. Therefore, a prospective collection study for patients that are deemed ineligible to receive neoadjuvant or adjuvant chemotherapy may provide adequate data to assess prognosis and design de-escalation or intensification trial. Given a substantial

Table 3
Selected ongoing/recent trials using ctDNA as integral biomarker for adjuvant treatment decisions

Disease	Trial	Disease State	Assay	N	Type	Schema	Key Endpoint(s)/Outcome
Bladder	TOMBOLA	MIBC	Signatera	282	Single Arm	All patients are monitored after cystectomy for ctDNA. If ctDNA+ at any point, treated with atezolizumab	CR after treatment with atezolizumab *CR defined as ctDNA negative status combined with regular imaging (CT) after treatment.
	IMvigor011	MIBC	Signatera	495	Randomized	All patients are monitored after cystectomy for ctDNA. If ctDNA–, observation. If ctDNA+, randomized to 1 year atezolizumab or placebo	DFS
	MODERN	MIBC	Signatera		Randomized	All patients receive ctDNA assessement after cystectomy. If ctDNA–, patients are randomized to immediate nivolumab or surveillance with ctDNA and nivolumab upon ctDNA conversion. If ctDNA+ after cystectomy, randomized to either nivolumab or nivolumab plus relatlimab.	DFS, OS
Breast	C-TRAK TN	TNBC	ddPCR	208	Randomized	All patients receive ctDNA every 3 months, randomized if ctDNA+ at any time point before 12 months: Intervention arm: pembrolizumab Control: continued surveillance	Positive ctDNA detection by 12 months Positive ctDNA detection by 24 months Absence of detectable ctDNA or disease recurrence 6 months (24 weeks) after commencing pembrolizumab Results: CtDNA+ by 12 months: 27.3%
	PERSEVERE	TNBC		197	Multi-Arm	All patients receive neoadjuvant chemotherapy followed by resection. If ctDNA+, adjuvant therapy is guided by genomics (combination of talazoparib, capecitabine, atezolizumab, inavolisib)	2-year DFS
	APOLLO	TNBC		460	Randomized	Patients are assessed for pCR and ctDNA after neoadj chemotherapy. Adjuvant therapy based on pCR and ctDNA status	5-year DFS
	ZEST	TNBC or BRCA+ BC	Signatera	800	Randomized	If ctDNA+, randomized to niraparib vs placebo	DFS
Colorectal	BESPOKE	Stage II/III colon cancer	Signatera	2000	Prospective	Prospective Arm: CtDNA-informed decision on adjuvant therapy	Percent of patients who have their adjuvant treatment regimen increased or decreased after the treating physician evaluates the results from the post-surgical ctDNA test Rate of recurrence of patients diagnosed with CRC while asymptomatic using ctDNA
	DYNAMIC	Stage II colon cancer		450	Randomized	Intervention Arm: adjuvant therapy if ctDNA+; no adjuvant therapy if ctDNA– Control: Adjuvant chemotherapy by physician discretion	To evaluate whether an adjuvant therapy strategy based on ctDNA results may affect the number of patients treated with chemotherapy and recurrence-free survival. Results: 2-year DFS: Intervention 93.5%; Control 92.4%; Received chemotherapy: Intervention 15%; control 28%

DYNAMIC III	Stage III colon cancer		1000	Randomized	Intervention Arm: Escalation/De-escalation of adjuvant chemotherapy based on ctDNA status Control Arm: Standard of care chemotherapy by physician discretion	To evaluate the impact of de-escalation/escalation treatment strategies as informed by post-op ctDNA analysis. a. For ctDNA– cohort: if a de-escalation treatment strategy is non-inferior to standard of care treatment as measured by the rate of 3-year recurrence-free survival. b. For the ctDNA+ cohort: if an escalation treatment strategy is superior to standard of care treatment as measured by the 24 month recurrence-free survival
DYNAMIC-RECTAL	Rectal cancer		408	Randomized	Intervention Arm: Adjuvant therapy based on pathologic risk assessment or if ctDNA+ Control Arm: Adjuvant therapy based on pathologic risk assessment alone	To evaluate whether an adjuvant therapy strategy based on ctDNA results in addition to standard pathologic risk assessment may affect the number of patients treated with chemotherapy.
ACT-3	Stage III colon cancer	Guardant Lunar	500	Randomized	All patients receive adjuvant FOLFOX. At completion, ctDNA is assessed: Intervention Arm: CtDNA+ receives additional adjuvant therapy based on genomics (FOLFIRI, encorafenib/binimetinib/cetuximab, nivolumab) Control Arm (CtDNA+): surveillance Control Arm (CtDNA–): surveillance	DFS, clearance rate of ctDNA
COBRA	Stage IIA colon cancer	Guardant Lunar	1408	Randomized	Intervention: if ctDNA+, then adjuvant chemo; if ctDNA–, then surveillance Control: surveillance	CtDNA clearance in ctDNA+ patients treated with or without adjuvant chemotherapy RFS in “ctDNA detected” patients treated with or without adjuvant chemotherapy
ALTAIR	Stage III colon cancer		240	Randomized	CtDNA+ patients are randomized: Intervention: Adjuvant TAS-102 Control Arm: Placebo	DFS
CIRCULATE	Stage II colon cancer	Signatera	4812	Randomized	ctDNA+: Randomized 2 : 1 to chemotherapy	DFS of ctDNA+ patients
CIRCULATE	Stage II colon cancer		1980	Single arm	Patients enroll in the study and are checked for ctDNA. If ctDNA+, randomized:	3-year DFS in ctDNA positive patients

(*Continued*)

Table 3
(*Continued*)

Disease	Trial	Disease State	Assay	N	Type	Schema	Primary Endpoint/Outcome
	CIRCULATE-IDEA	Stage II/III colon cancer	Signatera	1240	Randomized	Patients with high risk stage II and stage III colon cancer, after resection and ctDNA– are randomized to adjuvant chemotherapy vs observation	DFS
	COLUMBIA-2	Stage II/III colon cancer	Signatera	160	Randomized	CtDNA+ patients are randomized to FOLFOX +/– novel agents	ctDNA clearance is defined as the change from ctDNA positive status at baseline to ctDNA negative post baseline
	MEDOCC-CrEATE	Stage II colon cancer	PGDx elio	1320	Single arm	All patients randomized to control arm vs intervention: Intervention: ctDNA+ offered adjuvant CAPEOX; ctDNA– standard follow up Control arm: no adjuvant chemotherapy	Percent of patients who are ctDNA that choose to receive adjuvant chemotherapy
NSCLC	NCT04367311	NSCLC		100	Single Arm	CtDNA+ patients after defintive therapy undergo adjuvant therapy with cisplatin-based chemotherapy plus atezolizumab	CtDNA clearance after 4 cycles of adjuvant therapy
	NCT04585477	NSCLC	Avenio	80	Single Arm	ctDNA+ patients after definitive therapy undergo treatment with adjuvant durvalumab	ctDNA reduction after 2 cycles of adjuvant thearpy
	PACE-LUNG	EGFR+ NSCLC		50	Single arm	Patiets with stage IIIB/IV EGFRm lung cancer, initiated on osimertinib without ctDNA clearance will be given chemotherapy in addition to osimertinib	PFS
	MERMAID	NSCLC		332	Randomized	ctDNA+ after localized therapy randomized to chemotherapy vs chemohterapy plus durvalumab	ctDNA+ after localized therapy randoimzed to chemotherapy vs chemohterapy plus durvalumab
Pancreas	DYNAMIC-Pancreas	Resectable Pancreatic Cancer		438	Randomized	Adjuvant chemotherapy based on ctDNA status. Those who received neoadjuvant chemotherapy are randomized to either surveillance or ctDNA-based adjuvant therapy.	2-year RFS: a comparison of ctDNA-driven treatment outcomes (Cohorts B and C) against standard of care management treatment outcomes in the control arm (Cohort A).

Key: MIBC = muscle-invasive bladder cancer; CR = complete response; ctDNA = circulating tumor DNA; DFS = disease free survival; OS = overall survival; RFS = recurrence free survival; PFS = progression free survival.

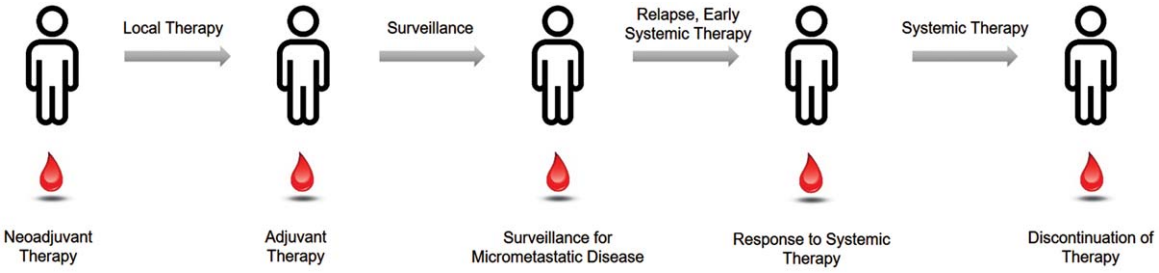

Fig. 2. Potential uses of CtDNA as a marker of minimal residual disease. Ultrasensitive and specific ctDNA assays may be used for treatment decisions throughout the treatment course for patients with localized disease. These include the decision to pursue neoadjuvant or adjuvant chemotherapy, escalation of adjuvant therapy, monitoring for early disease relapse and early initiation of systemic therapy, monitoring treatment response, and consideration to discontinue therapy.

amount of CR rates after neoadjuvant therapy, ctDNA may also be incorporated with direct visualization and advanced imaging to define a population that could be spared from cystectomy.

CtDNA also has potential as an early study endpoint. Christensen et al reported ctDNA being positive with a median lead time of 3 months compared to radiographic progression, and importantly, none of the patients who were ctDNA negative post cystectomy had disease recurrence (0 of 47 patients). Early surrogate markers for clinically meaningful endpoints allow for earlier trial readout, however these are difficult to demonstrate. Metastases free survival is surrogate for overall survival for advanced prostate cancer, and is now used as a study endpoint for many landmark trials [62]. For localized bladder cancer, DFS has been used as the primary endpoint in major adjuvant trials [8, 12]. If ctDNA is to be a marker of recurrence, response, or progression, robust prospective studies are needed to correlative clinically meaningful endpoints.

Studies with CtDNA as an integral biomarker

To validate the use of ctDNA to guide therapy, studies with ctDNA as an integral biomarker are necessary. As an integral biomarker may be used in two specific ways: to modify treatment decisions, and as stated above, a study endpoint. Table 3 lists selected ongoing studies incorporating ctDNA as an integral biomarker for treatment decision, with more currently under development. These trials aim at escalating and de-escalating therapy based on ctDNA using a variety of assays to improve outcomes. These studies are critical to demonstrate the utility of ctDNA, not only as a prognostic biomarker, but as a predictive biomarker.

There are many trial designs that incorporate ctDNA as an integral marker. Figure 2 demonstrates a schematic of major timepoints where presence or absence of ctDNA may be used to guide therapy.

CONCLUSION

As the field moves forward with new therapeutic advances, refining patient selection and personalizing therapy based on validated biomarkers to guide therapy selection will be key. CtDNA has significant potential to identify patients at high risk of recurrence most likely to benefit from additional therapy. Techniques for ultrasensitive ctDNA are evolving and likely today's assays will be outperformed over the next 1–2 years, and therefore while investigating these assays today, significant effort should be placed in banking samples for the newer assays of tomorrow. Although ctDNA has significant potential to guide therapy, limitations include sensitivity at early post-surgical time points, limited disease particularly in sanctuary sites, and lack of large scale, reproducible validation. Despite these limitations, significant potential remains and ongoing clinical trials are already incorporating ctDNA as integral biomarkers to guide therapy.

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