Current and Future Clinical Applications of ctDNA in Immuno-Oncology

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ABSTRACT

Testing peripheral blood for circulating tumor DNA (ctDNA) offers a minimally invasive opportunity to diagnose, characterize, and monitor the disease in individual cancer patients. ctDNA can reflect the actual tumor burden and specific genomic state of disease and thus might serve as a prognostic and predictive biomarker for immune checkpoint inhibitor (ICI) therapy. Recent studies in various cancer entities (e.g., melanoma, non–small cell lung cancer, colon cancer, and urothelial cancer) have shown that sequential ctDNA analyses allow for the identification of responders to ICI therapy, with a significant lead time to imaging. ctDNA assessment may also help distinguish pseudoprogression under ICI therapy from real progression. Developing dynamic changes in ctDNA concentrations as a potential surrogate endpoint of clinical efficacy in patients undergoing adjuvant immunotherapy is ongoing. Besides overall ctDNA burden, further ctDNA characterization can help uncover tumor-specific determinants (e.g., tumor mutational burden and microsatellite instability) of responses or resistance to immunotherapy. In future studies, standardized ctDNA assessments need to be included in interventional clinical trials across cancer entities to demonstrate the clinical utility of ctDNA as a biomarker for personalized cancer immunotherapy.

Introduction

Over the past decade, the identification of the molecular mechanisms by which tumor cells hamper immunity marked the coming of a new era in the management of cancer patients. Since first immune checkpoint inhibitor (ICI) approval in unresectable malignant melanoma (1), up to 15 different clinical entities, comprising both solid and hematologic malignancies, currently benefit from an FDA-approved indication for ICI-based treatment (2) and the field of applications is rapidly evolving. Notably, the repertoire of immune-oncology (IO) therapeutic options is constantly expanding by targeting additional immune checkpoints or costimulatory molecules, combining ICI with other therapeutic strategies (3, 4) and introducing innovative approaches based on T-cell bioengineering (5).

Early identification of relapse and early therapeutic intervention are essential determinants for improved overall survival. However, an objective biomarker associated with the efficacy of IO drugs is an urgent but still unmet clinical need.

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The past decade has also seen the advent of liquid biopsy (6, 7). Contrary to tumor tissue biopsy, liquid biopsy gives access to tumor material in a minimally invasive way, therefore offering the patient a more acceptable, safer, and easily repeatable option to monitor tumor response. Liquid biopsy applies to detecting tumor cells or tumor-derived products like tumor DNA (referred to as circulating tumor DNA, ctDNA) mainly shed in peripheral blood and other body fluids. The field of ctDNA clinical applications is mainly based on mutation detection and has greatly benefited from significant improvements of detection methods in terms of sensitivity and multiplexing. The utility of monitoring tumor genomics through plasma ctDNA analysis has been widely investigated over the past years in diverse clinical settings (8, 9).

This review will present the different clinical applications of ctDNA analysis in the specific context of IO. We will discuss the capability of ctDNA, quantified either before or during therapy, to identify patients who will benefit from the treatment. We will finally describe ctDNA as a privileged substrate to study and monitor the genetic determinants of immunotherapy response, such as tumor mutation burden or microsatellite instability and underline the value of ctDNA-based decision-making in cancer treatments.

Pretreatment Levels of ctDNA as a Prognostic Biomarker in IO

Clinical value of pretreatment ctDNA levels in metastatic patients

Supplementary Table S1 recapitulates the studies investigating the correlation of ctDNA measured before the treatment with the primary clinical endpoints. Most of the studies were conducted on melanoma and NSCLC populations who received ICI either as a first or later-line therapy, according to the timing of drug approval. Recent pan-cancer studies and hematologic malignancies, implementing new IO strategies, highlight the advantage of ctDNA to be implemented agnostic to cancer types and independent from a specific ICI treatment, as long as one mutation can be detected. There is a high level of heterogeneity between the studies about the number of included subjects, the types of clinical cohorts, and the methodology adopted to measure ctDNA,

including the detection of a single mutation—usually the driver—versus multiple with gene panels, different sensitivity thresholds, and quantification strategies. Notably, most of the studies so far have only demonstrated the clinical validity of ctDNA as a biomarker (10). The use of pretreatment ctDNA value as a biomarker in the clinic will therefore necessitate establishing precise pretreatment ctDNA cutoff points for each particular assay and for each particular tumor type. Moreover, interventional studies are needed to demonstrate the clinical utility of ctDNA measurements. Nevertheless, several investigations have identified a congruent association between undetectable ctDNA or low ctDNA levels [inferior to the cohort's median variant allele frequency (VAF)] and a longer progression-free survival (PFS) and overall survival (OS) in univariate analyses (11–18). Owing to the close relationship between ctDNA and tumor burden, well established in NSCLC (19–21) and melanoma (11, 12, 15, 22–26), the underlying influence of anatomic tumor disease burden in the duration of response to ICI therapy might partially explain the pretreatment ctDNA association to PFS or OS (27, 28). However, in the up-to-now most extensive study encompassing 16 different tumor types in 790 patients, ctDNA association with OS after adjustment for Eastern Cooperative Oncology Group (ECOG) performance status, baseline liver metastases, baseline lymph node metastases, smoking status, tumor burden, and tumor PD-L1 score suggests that ctDNA is not simply a surrogate marker for baseline tumor burden (29). In this line, the mutation selected to quantify ctDNA could also play a role in associating ctDNA levels to the clinical outcome since specific mutations identified in tumor tissues could have different prognostic values (refs. 30, 31; cf. chapter 4.1). As an additional confounding factor, the capability to equally detect all different mutations in ctDNA remains unclear, as reported in melanoma (17).

Contrary to PFS and OS, the association to objective response rate (ORR) is poorly reported. When mentioned, ORR and pretreatment ctDNA levels association was not significant (Supplementary Table S1). This observation rather confers to ctDNA pretreatment levels a prognostic value than a direct link to clinical efficacy. Of note, the pretreatment ctDNA levels discrimination of patients with durable or nondurable clinical benefit reported by Nabet and colleagues can be explained by a different evaluation model of clinical response from immune Response Evaluation Criteria In Solid Tumors (RECIST) criteria (32).

Pretreatment ctDNA levels associated with PFS and OS should also be differently examined between the first- or the second-line treatment setting. For instance in melanoma, pretreatment ctDNA levels are only associated with clinical outcome in patients receiving ICI therapy as a first line (13, 17). Brain metastasis development in patients who relapsed after first-line therapy might be one potential explanation for the limited discriminative capacity in the second-line setting due to an insufficient ctDNA detection. This observation merits further clinical investigations, notably in other tumor types where ICI therapy can be proposed in the second line.

Clinical value of pretreatment ctDNA levels in adjuvant immunotherapy

Adjuvant immunotherapy, by definition, is being applied to tumor-resected patients. Several reports in melanoma (33–36) or lung (20) and colorectal cancer (37) have shown that the prevalence of ctDNA-positive patients after resection is low despite the use of highly sensitive digital PCR techniques. To increase the sensitivity of ctDNA testing, one could particularly recommend the interrogation of multiple mutations with personalized gene panels based on the primary tumor sequencing, the analysis of higher volumes of plasma, and repeated sampling to increase the sensitivity of mutation detection (38). However, in the adjuvant setting, the low quantities of ctDNA and sequencing artifacts currently limit the usage of large sequencing panel assays. Error suppression strategies to reduce background error rate will be necessary to improve the analytical specificity of ctDNA assays (39). In this line, ctDNA detection via personalized profiling by cancer personalized profiling by deep sequencing (CAPP-seq) was associated with a better outcome in a cohort of 28 locally advanced NSCLC patients receiving ICI as consolidation therapy after adjuvant chemoradiotherapy (40). In a clinical trial comparing adjuvant administration of the anti-PDL1 antibody atezolizumab versus observation in operable urothelial cancer patients, ctDNA positivity (detected by patient-specific mutation) at the beginning of the treatment identified a high-risk population who will benefit from adjuvant ICI therapy (41). This study design paves the way for additional high level of evidence studies in other clinical entities aimed to achieve clinical utility of ctDNA testing in the adjuvant setting.

On-treatment ctDNA Measurement to Predict Clinical Outcome

ctDNA measurements can easily be repeated throughout therapy. On-treatment levels of ctDNA either were used to calculate ctDNA changes by comparison with ctDNA levels at baseline or were directly associated with clinical outcome. Supplementary Table S1 also details the corresponding studies.

Early ctDNA dynamics after the onset of systemic therapy

The terms ctDNA "dynamics," "kinetics," and "variations" denote changes in VAF or concentration measured between before the first and before subsequent treatment infusions. It is worth mentioning here that, besides tumor driver mutations, those encoding for neoantigens (42) or even chromosomal number aberrations (CNA; refs. 43, 44) were used to quantify ctDNA changes.

ctDNA decrease is associated with a higher ORR, PFS, and OS. However, studies significantly differ by the ctDNA change threshold (20%, 50%, nonspecified increase or decrease, complete clearance) and time point (after one infusion or more, between 4 and 8 weeks) to assess molecular response. In the future, it will be critical to harmonize the strategy to adopt by a precise definition of a cutoff and of the time point to compare with baseline. In addition, a better knowledge of ctDNA intraday variation (45, 46) and the reproducibility of the methods is necessary to identify actual biological ctDNA variations correctly. Again, the reported studies have provided evidence for the clinical validity of ctDNA monitoring while demonstration of its clinical utility is still pending (10).

In the metastatic setting, the superior association of early on-treatment ctDNA changes to clinical efficacy over baseline ctDNA values is noteworthy (32, 47). Indeed, as a direct reflection of tumor burden (11, 12, 22–26, 48–54), ctDNA changes would encompass all variables that contribute to overall tumor response.

CtDNA variations evaluated in the early course of therapy correlated to radiographic best response evaluated 5 to 12 weeks later, suggesting an exciting capacity to anticipate tumor response in NSCLC or metastatic melanoma (55–58). However, this conclusion can be inherently biased by the study's design (i.e., most studies report on radiologic evaluations performed in daily clinical routine later than ctDNA sampling). Anticipating tumor response presents several advantages for the clinician, notably in case of treatment interruption due to severe side effects or in patients presenting with stable disease at their first assessment, to identify those who will finally go in clinical

response (29, 32, 55). Nevertheless, the agreement between the first radiologic evaluation of tumor response and ctDNA evolution profile is not total (59, 60). For instance, 23% of the patients present discordant ctDNA kinetics from the first RECIST evaluation (59). Pseudoprogression, defined as a radiologic finding of disease progression before response caused by various immune cells infiltrating the tumor mass, thus contributing to increased tumor volume, can be one source of discordance (61). Although not frequent (incidence range, 0% to 9.7%), pseudoprogression is a specific challenge associated with ICI treatment. One study in 29 metastatic melanoma patients treated with PD-1 antibodies has demonstrated that decreasing ctDNA profiles can accurately differentiate pseudoprogression from a proper disease progression (62). First RECIST evaluation cannot be considered as an accurate predictor of clinical outcome for ICI (32, 55), mandating additional response assessment at later time points during tumor evolution. Therefore, it will be necessary to explore early ctDNA variations with clinical benefit determined several months later to better understand its potential to guide clinician decisions. In this line, Nabet and colleagues acknowledge that ctDNA early kinetics misclassified 25% of NSCLC patients for durable clinical benefit (32), highlighting the need for continuous monitoring of ctDNA throughout the therapy. The limited value of ctDNA as a biomarker of intracranial response suggests that ctDNA measurements and clinical imaging are not redundant but rather complementary. In metastatic melanoma, intracranial disease control did not associate with on-treatment ctDNA favorable profiles or undetectability (11, 12). Properly designed studies with simultaneous assessment of tumor response by both methods would provide interesting hints to understand this complementarity better and build more accurate models to predict clinical outcome (32, 55, 59). Exploring the cerebrospinal fluid as another compartment for liquid biopsy would also be a good alternative for clinicians (63).

Measuring ctDNA variations could also be applied to predict other immunotherapy regimes' efficacy. CtDNA clearance after the first cycles of treatment identified responders to adjuvant therapies in urothelial carcinoma or NSCLC (40, 41).

Recent advancements in immunotherapy have allowed treatment of relapsed or refractory diffuse large B-cell lymphoma thanks to CD19-targeted chimeric antigen receptor T cells (CAR T cells; ref. 64). In a pilot study of six patients, investigating specific clonotypic V(D)J rearrangements in ctDNA through the treatment could predict patient response to CAR-T cell therapy (65). In a subcohort of patients with metastatic cervical cancer treated with tumor-infiltrating lymphocyte therapy (TIL), a solid but transient HPV peak detected in cfDNA, immediately after the TIL therapy start was preferentially observed in patients with a complete and long-term response to TIL therapy (66). A similar post-TIL ctDNA "flair" was also observed in melanoma patients (67).

Association between on-treatment ctDNA concentrations and clinical outcome

Among the aforementioned studies, some have also directly correlated the ctDNA levels after the first cycles of ICI therapy to a clinical endpoint, with notable superiority to predict clinical efficacy over pretreatment levels of ctDNA (cf. Supplementary Table S1; refs. 11, 29, 47, 59, 68). Whether on-treatment levels or ctDNA variations is the most accurate way to predict the clinical outcome is still an open question. The disadvantage of ctDNA dynamics could be that, depending on its calculation mode, it can equalize patients presenting low pretreatment ctDNA and significant decrease with patients presenting high pretreatment ctDNA and smaller decrease. As such, an integrated metric defined as the ratio of on-treatment VAF to pretreatment VAF had a superior association with immunotherapy outcomes than on-treatment levels (29). On the other hand, ctDNA clearance was associated with the most favorable outcome profile (14, 29, 55, 59, 69), and conversely, detection of high levels of ctDNA is associated with future progression (14). Therefore, stratifying patients by both pretreatment and on-treatment levels and distinguishing ctDNA clearance should result in the most accurate evaluation of patient outcome, as initially suggested by Lee and colleagues in melanoma patients (12) or more recently by Zhang and colleagues (29). More studies comparing on-treatment ctDNA with on-treatment RECIST tumor evaluation would also be necessary to understand the complementarity between the two approaches better (70).

Even with favorable ctDNA kinetics, some of these patients will ultimately progress, and early ctDNA variations might not be able to discriminate long-term responders. Some studies have then evaluated ctDNA level at later time points of therapy. In NSCLC, 31 blood samples from patients achieving long-term benefit were collected at a median of 26.7 months after initiation of therapy (71). At this surveillance timepoint, 25/27 patients with undetectable ctDNA remained progression-free while all four patients with detectable ctDNA eventually progressed. A similar observation was reported in 38 melanoma patients evaluated after cessation of ICI therapy (72). Of the 28 patients with no progression, ctDNA was undetectable in 27 patients, and among the ten patients who progressed, four had detectable ctDNA at the time of treatment cessation. These independent observations corroborate the hypothesis raised by Bratman and colleagues in which ctDNA clearance at any time point during the therapy is associated with long-term survival (59). Concerning the lack of knowledge in the optimal treatment duration (and its consequences in terms of potentially severe side-effects exposure and financial costs), both studies pave the way for additional ctDNA evaluation later in therapy to better discriminate patient personal benefit. In this setting also, the usage of highly sensitive methods to detect ctDNA will be necessary to reduce the probability of false-negative results.

Genetic Determinants of Response to IO Therapies Assessed on ctDNA

In addition to a quantitative assessment, other genetic determinants of ICI therapy response can also be measured on ctDNA, such as the association of specific mutations to ICI therapy outcome, the assessment of tumor mutational burden and microinstability phenotype.

Status of specific cancer mutations relevant to therapy

As a surrogate of tumor tissue, plasma genotyping could also be used to directly evaluate the association of tumor-specific molecular alterations with response to ICI therapy or with the onset of immune-related adverse events. After excluding patients with no detectable ctDNA, Guibert and colleagues confirmed a better prognosis in patients harboring *TP53* or *KRAS* mutations and the detrimental effect of STK11 mutations and loss of *PTEN* compared with wild-type patients (30). Similarly, in 38 metastatic gastric cancer patients, the mutation status of *TGFBR2*, *RHOA*, and *PREX2* in ctDNA at baseline negatively influenced the PFS (31). In the same metastatic gastric cohort, patients with alterations in *CEBPA*, *FGFR4*, MET, or *KMT2B* detected in plasma at baseline had a greater likelihood of experiencing irAEs (31). In classic Hodgkin lymphoma (cHL), CHD8 mutation in ctDNA was only detected in patients with the longest PFS (73).

Repetitive sampling throughout therapy is the main advantage offered by ctDNA analysis. Large gene panels or whole-exome sequencing (WES) analysis on ctDNA depicting tumor clonal evolution can lead to identifying specific mutations implicated in resistance to immunotherapy. Mutation in *FOXL2* and *RHOA* genes and copy-number variation of *FGFR2* gene were identified as candidate resistant mechanisms after plasma analysis of 13 metastatic gastric cancer patients who had initially benefited from the treatment (31). Serial sequencing of ctDNA with a 329 pan-cancer--related gene panel and WES identified mutations in *PTCH1* and *B2M* genes in two out of four NSCLC patients with progressive disease (74). WES on ctDNA performed on eight different NSCLC patients reported alterations of Wnt-signaling pathway-related genes, an increase of copy-number aberrations in cancer-related genes, and loss of PTEN or B2M as molecular mechanisms associated with late progression (i.e., progression observed after six months of treatment) to ICI therapy (75). Considering the broader usage of comprehensive genome sequencing in the near future, one could strongly emphasize the need for additional studies across different clinical entities with regular plasma sampling to decipher the tumor molecular landscape at the onset of resistance to immunotherapy.

Tumor mutational burden

Following the hypothesis that the more nonsynonymous mutations are present in the tumor DNA, the more neoantigens will be presented at the surface of the tumor, tumor mutational burden (TMB; i.e., the number of somatic mutations per megabase of interrogated genomic sequence) has been extensively explored as an additional predictor of clinical benefit in ICI therapies. However, the correlation between a high TMB and better response to ICI therapy is still not completely established, varying between cancer entities (76–78). If WES would be the most accurate way to assess TMB in tumor tissue (named tTMB hereafter), panel sequencing-based estimates of TMB were mainly used in the clinic so far. Nevertheless, a lack of standardization in TMB score determination due to technical features (i.e., location and size of the sequenced regions, types of mutation detected, differences in the germline mutations filtering methods, and mode of calculation of TMB score) prevents TMB score comparison across platforms and tumor types (76, 77, 79–81) and has led to the recent initiative of establishing harmonization guidelines (82). Moreover, tTMB determination on a single biopsy can also be affected by intratumor heterogeneity and might evolve with treatment.

As an alternative to tissue determination, blood-based determination of TMB (bTMB) could overcome the double problem associated with repeated access to tumor material and tumor heterogeneity (83–86). However, bTMB assays face specific challenges, such as tumor-derived molecules' input varying upon cancer type and clonal hematopoiesis (87, 88). Importantly, standardization in bTMB assays is also currently lacking but should rapidly benefit from the harmonization efforts currently ongoing for tTMB determination.

Nevertheless, bTMB via ctDNA analysis with multiple gene panels was first evaluated as a surrogate for tTMB. Despite the use of different gene panels and independent cohorts of patients, a similar level of correlation (R around 0.6) between tissue and plasma was reported (89–91) The absence of a higher correlation between tTMB and bTMB could originate from the intratumor heterogeneity. However, a low VAF and an extended time interval between blood and tissue collection in some cases could also explain the reported level of correlation (90).

bTMB was then evaluated as a predictor of ICI therapy outcome. Like for tTMB, there is an association between a high bTMB score and a better ORR and improved PFS and OS in NSCLC patients (90, 92, 93). However, no association with OS was reported by several studies (94, 95), leading Wang and colleagues to question ctDNA-based TMB determination rationale. Patients with the highest amount of ctDNA have the highest number of mutations and the highest tumor burden, and both situations result in a contradictory effect on OS. Upon adjustment by VAF, bTMB-high eventually associated with improved ORR, PFS, and OS in uni- but also multivariate analysis (96). Still, prospective studies are needed to validate the predictive efficacy of low allele frequency bTMB. Interestingly, Nabet and colleagues recently addressed this issue by defining normalized bTMB as the ratio of bTMB and ctDNA level. Normalized bTMB was superior to both individual metrics (bTMB and ctDNA levels) for predicting durable clinical benefit (32).

Microsatellite instability

In colorectal cancer, microsatellite instability (MSI) was associated with a high Th1/CTL infiltration and upregulation of immune-checkpoint proteins, suggesting a link between MSI and response to ICI (97). Like for TMB, minimally invasive determination of MSI is highly desirable in a context of a constantly expanding usage of ICI therapy.

Next-generation sequencing (NGS)-based approaches can nowadays determine MSI by measuring the length of altered microsatellites sequences (98–101). Several NGS-based assays were recently developed on cfDNA to determine tumor MSI status by overcoming the technical challenges associated with detecting low-level allele length polymorphisms in coexisting excessive amounts of wild-type DNA and PCR originating errors on long mononucleotides repeats (98, 102–104). Despite a lack of consensus on the selected loci number and nature, the different NGS assays had a sensitivity around 0.1%–1% tumor fraction and presented a high concordance with tissue MSI status (102–104). Landscape studies performed in large plasma samples sets from cancer patients reported an MSI-high prevalence among tumor types similar to the one observed with tissue-based analyses (102, 104). This approach paves the way for a pragmatic strategy to identify better the subset of patients who might benefit from ICI therapies, especially in tumor types where the benefit of the IO treatment is not yet fully established. In small cohorts of gastrointestinal cancers treated by ICI therapy, patients detected with an MSI phenotype had significantly prolonged PFS (98, 102, 104), demonstrating clinical validity of the developed assays.

NGS-based methodologies present the advantage to enable simultaneous determination of the MSI status of the tumor together with detection of other genomic determinants of response to ICI therapy like TMB. The European Society for Medical Oncology (ESMO) recommendations on MSI tissue testing for immunotherapy in cancer stated that the relationships between MSI and TMB are complex and differ according to tumor types (105). Studies exploring the complementarity between these two biomarkers are needed to predict the outcome of ICI more finely. In this line, Willis and colleagues observed a significantly superior number of SNV in MSI-high than in microsatellite stable (MSS) patients (102). Wang and colleagues, in a pan-cancer plasma analysis, questioned this putative complementarity by dichotomizing the bMSS patient's cohort into bTMB-high and bTMB-low subsets. bMSS-TMB-high and the bMSI-high groups collectively predicted significantly improved outcome, indicating that bMSI combined with bTMB may maximize the scope of ICB therapy (104).

General Conclusions

The last years witnessed a growing body of evidence supporting the use of ctDNA's multiple features (e.g., ctDNA levels, mutations,

Table 1. Clinical applications of ctDNA in IO.

Biomarker	Clinical entities	Biomarker study type	Clinical trial number and ICI therapy evaluated	Refs.
Quantitative analysis of ctDNA[a]	Melanoma—metastatic	Evaluated in IO clinical trial[b]	NCT02374242 + NCT02089685 (nivolumab, pembrolizumab)	(12)
		Standard-of-care cohorts	Pembrolizumab/ipilimumab/nivolumab	(11, 13–18, 22, 58, 121, 122)
	NSCLC—metastatic	Evaluated in IO clinical trial	NCT01693562 + NCT02087423 (durvalumab)	(123)
			NCT01903993 + NCT02008227 (atezolizumab)	(32)
			NCT02475382 (nivolumab)	(124)
		Standard-of-care cohorts	Nivolumab, pembrolizumab	(30, 42, 47, 55, 57, 125, 126)
	NSCLC—localized	Evaluated in IO clinical trial	Durvalumab + NCT02525757 (atezolizumab)	(40)
	Gastric cancer—metastatic	Standard-of-care cohorts	Nivolumab, pembrolizumab, toripalimab, sintilimab	(31)
		Evaluated in IO clinical trial	NCT02589496 (pembrolizumab)	(89)
	Biliary tract—metastatic	Evaluated in IO clinical trial	SHR1210-GEMOX-BTC-IIT03	(95)
	Classic Hodgkin lymphoma	Evaluated in IO clinical trial	NCT03114683 (sintilimumab)	(73)
	Urothelial cancer—metastatic	Evaluated in IO clinical trial	NCT01693562 + NCT02087423 (durvalumab)	(123)
	Urothelial cancer – localized	PRCT designed to address clinical utility	NCT02450331 (atezolizumab)	(41)
	Multiple clinical entities—metastatic	Evaluated in IO clinical trial	NCT02644369 (pembrolizumab)	(59)
			NCT01693562 (durvalumab), NCT02087423 (durvalumab), NCT02261220 (durvalumab + tremelimumab)	(29)
Tumor mutational burden estimation	NSCLC—metastatic	Evaluated in IO clinical trials	NCT01903993 + NCT02008227 (atezolizumab)	(90, 91, 96)
			NCT02453282	(93)
			NCT02478931	(92)
			NCT02848651	(89)
	Biliary tract—metastatic	Evaluated in IO clinical trial	SHR1210-GEMOX-BTC-IIT03	(95)
Microsatellite instability estimation	Gastric cancer—metastatic	Evaluated in IO clinical trials	NCT02589496 (pembrolizumab)	(102)
	Gastrointestinal cancer—metastatic	Standard-of-care cohorts		(104)
	Prostate cancer—metastatic	Standard-of-care cohorts		(103)
	Multiple clinical entities—metastatic	Standard-of-care cohorts	NCT01876511 (pembrolizumab)	(98)

Note: This table summarizes the main clinical applications of ctDNA sorted by disease severity (localized or metastatic) and tumor type. For further details on the cited studies, please refer to Supplementary Table S1.
Abbreviation: PRCT, prospective randomized controlled trial.
[a]Please note that ctDNA quantification refers to both quantification before the treatment and early on treatment.
[b]The clinical trial was initially designed to measure drug efficiency/safety and ctDNA was measured as an observational parameter.

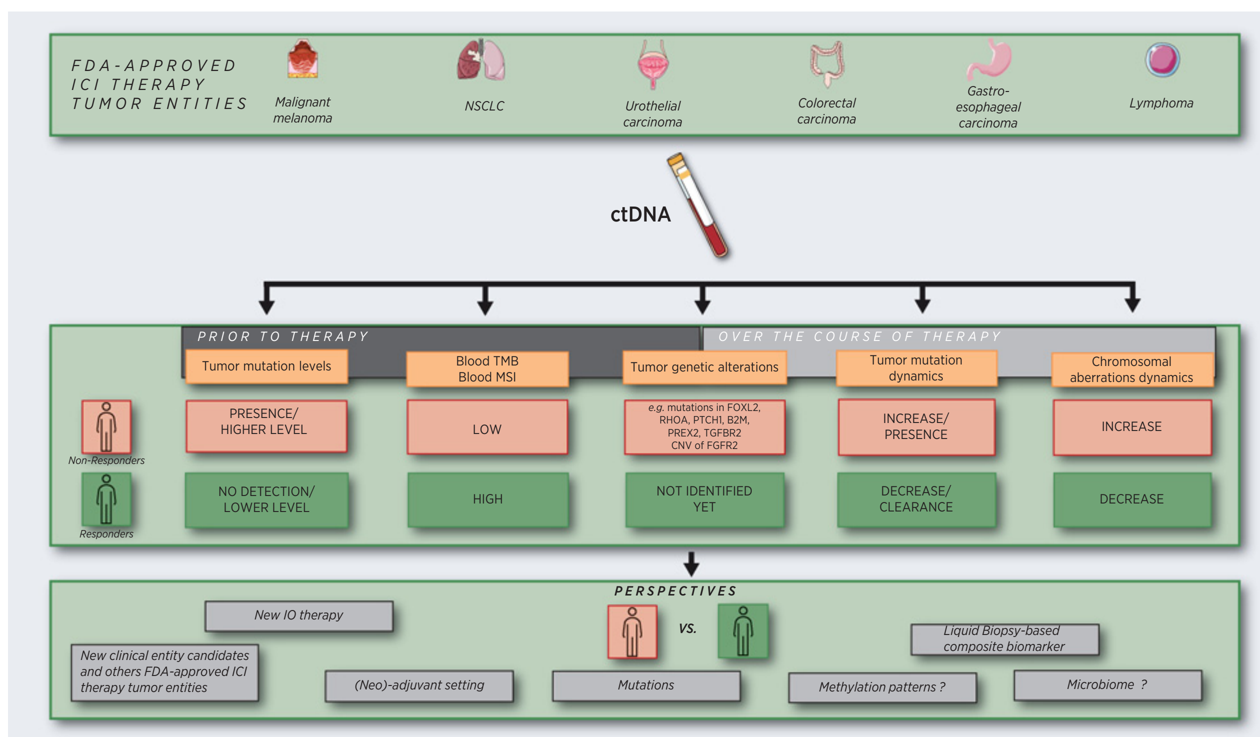

Figure 1.

Current strategies and perspectives of clinical applications of ctDNA analysis in IO. Besides the detection and quantification of tumor mutations, other features like chromosomal aberrations can be quantified as a score, with the advantage of not requiring any prior knowledge of tumor mutation in an individual patient. Performing low-coverage genome-wide sequencing of cfDNA may also present interests in comparison with high-depth sequencing of targeted panels, in terms of cost and time. As a future perspective, the same kind of ctDNA analysis could be applied to new clinical entities where ICI therapy will be approved, or in new clinical therapeutic settings (neo/adjuvant). Other fascinating perspectives offered by studying ctDNA will be to identify other determinants of response to ICI therapy such as tumor-derived methylation patterns and the signatures of the host microbiome.

bTMB, bMSI) for discrimination of patient response to ICI therapy (**Table 1** and **Fig. 1**).

Most data have been obtained in metastatic patients with different types of solid tumors in the context of IO clinical trials, establishing the clinical validity of ctDNA quantification (before and early on-treatment) as prognosticator for response to therapy. The few reports on patients with localized disease in NSCLC and urothelial cancers suggest the capacity of ctDNA measurement to discriminate response from failure to therapy also in the adjuvant setting. The standardization of tests including the harmonization of cutoff points to discriminate ctDNA responders from nonresponders is now the priority task of international consortia like the European Liquid Biopsy Consortium (www.elbs.eu) or the International Alliance of Liquid Biopsy Standardization (ILSA; ref. 106). Indeed, most of the work so far reported was performed on patients included in standard-of-care cohorts or in the frame of a clinical trial initially designed to measure drug efficiency/safety. To introduce ctDNA measurements into clinical practice, interventional ctDNA-based clinical decision trials need to be designed to demonstrate the clinical utility of this biomarker. In this context, it is worth to highlight the pioneering clinical trial in localized urothelial cancers where ctDNA detection was used to personalize treatment selection for patients. In the same line, several clinical trials in early-stage NSCLC or triple-negative breast cancer are currently ongoing, in which adjuvant or neoadjuvant treatment choice is based on ctDNA positivity status after surgery (NCT04966663, NCT04849364, and NCT04585490). To better predict clinical benefit, ctDNA monitoring of tumor response could also open new avenues in the management of side effects and treatment costs. In the metastatic setting, such monitoring could also help to determine the best time point for switching from first- to second-line treatment. The CAcTUS trial in metastatic melanoma (NCT03808441) is a good example of this strategy; based on the determination of BRAF-mutant ctDNA levels patients receiving targeted therapy as first-line therapy are switched to immunotherapy as second-line therapy. In future studies, one could also imagine trials where increasing ctDNA kinetics will guide a switch from PD-1 monotherapy to a more aggressive PD-1 and CTLA-4 combination therapy while decreasing ctDNA will guide a deescalation from combination to the less aggressive monotherapy. Finally, medico-economic comparison with conventional radioimaging technologies is now also needed.

Despite the current technical challenges discussed above, ctDNA can also be used to estimate bTMB and bMSI, two genetic determinants of ICI therapy response. However, the overall response to immunotherapy is not solely dependent on tumor genomics. Tumor escape mechanisms driven at the transcriptional level and host immune system features have been highlighted as additional parameters involved in treatment efficacy (107–109). Therefore, it is very likely that multicomposite biomarkers capable of integrating several metrics will present the highest accuracy to predict tumor response to ICI. Thus, peripheral blood, including circulating tumor cells, circulating cytokines, peripheral T cells population profiles, and extracellular vesicles could be an ideal source to encompass simultaneously all

parameters involved in tumor immune response, and that have already been separately demonstrated as a candidate biomarker of clinical efficacy (110–114). Likewise, Nabet and colleagues have recently developed the DIREct-On score (Durable Immunotherapy Response Estimation by immune profiling and ctDNA) to predict the response of NSCLC patients receiving ICI-based therapies that incorporates three pretreatment biomarkers (ctDNA-normalized TMB, PDL1 tissue expression, circulating CD8 T-cell fraction) but also ctDNA levels after a single cycle of ICI therapy. This score outperformed each metric on the clinical classification accuracy and prognostic value and was the only feature independently associated with PFS in the multivariate Cox proportional model comprising age, ECOG, and line of therapy (32).

Besides mutations, other valuable information like methylation of specific loci or methylation patterns could be extracted from ctDNA analysis (115). Recently, the EPIMUNNE signature based on methylome analysis of the tumor tissue was successfully correlated to the clinical outcome of NSCLC patients treated by immunotherapy (116). Moreover, with thousand copies per cell, mitochondrial DNA in plasma represents an abundant source to exploit, potentially providing valuable information on both tumor and microenvironment (117, 118). Other exciting perspectives of exploiting plasma information could come from the emergent possibility to dissect the microbiome in peripheral blood that would make sense in this context owing to the putative role of intratumor bacteria in response to ICI therapy (119, 120). Thus, liquid biopsy analysis expands the offer to interrogate several features originating from both the host and tumor in a minimally invasive way, leading to the development of a personalized biomarker of response to ICI therapy.

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Authors' Disclosures

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