SIMSEN DIAGNOSTICS

Introduction

The presence of cell-free DNA in blood (cfDNA) was first reported in 1948. The first reports of tumor-specific mutations in cfDNA were published in 1994 when *KRAS* and *NRAS* mutations were observed in cancer patients. Recent work indicates that DNA fragments containing tumor-specific mutations are shed into the bloodstream and other body fluids by most cancer types. This tumor-derived fraction of cfDNA is called circulating tumor DNA (ctDNA). The association of ctDNA with clinical variables has now been investigated in many cancers, and it is well established that ctDNA levels are associated with stage, response to therapy, prognosis, and tumor burden[1–3].

Drug development for solid tumors in the early stage, non-metastatic setting typically involves large trials and multiple years of conduct and follow-up with time-to-event endpoints. Certain patients with early-stage solid tumors can be cured by surgery or another localized therapy. Yet, many others require (neo)adjuvant systemic therapy to be cured or will progress to fatal metastatic disease despite surgery and systemic treatment.

The amount of detectable ctDNA varies among individuals and depends on the type of tumor, location, stage, tumor burden, and response to therapy. ctDNA as a biomarker has several potential regulatory and clinical uses that may assist and expedite drug development. In the early-stage cancer setting, ctDNA may be used to detect a specific targetable alteration, to enrich a high- or low-risk population for study in a trial, to reflect a patient's response to treatment, or potentially as an early marker of efficacy.

The evidence supporting the clinical validity or clinical utility of ctDNA varies across solid tumor malignancies, patient populations, and treatment modalities. However, multiple studies suggest that residual ctDNA after surgery or completion of standard systemic therapy confers a poor prognosis and selects a population at high risk of relapse, *i.e.*, those with molecular residual disease (MRD).

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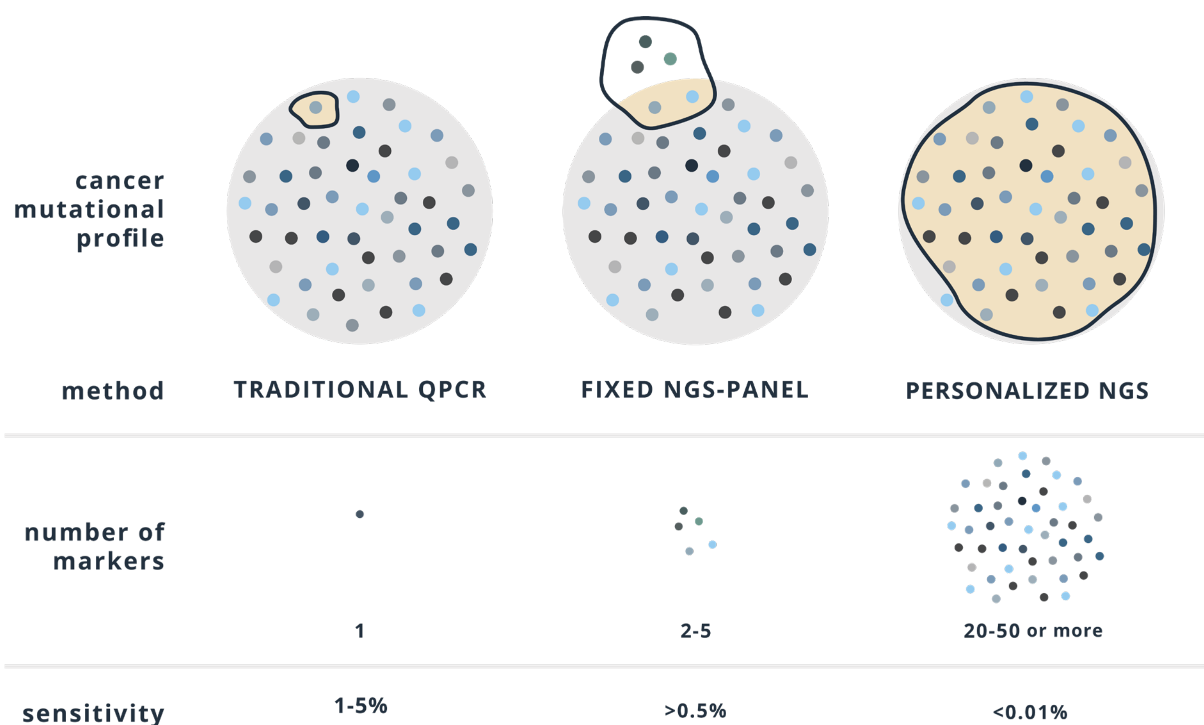

Figure 1. *Sensitivity of ctDNA detection methods.*

Tumor-informed vs. tumor-naïve ctDNA detection

An important axis for classifying ctDNA assays is whether they are tumor-informed or tumor-naïve. Tumor-informed assays are tailor-made designs based on the genetic information provided by a tumor biopsy. Tumor-naïve assays are typically fixed panels containing primers or probes targeting highly recurrent mutations present in specific tumor types, for example, breast or colon cancer (Figure 1). Thus, tumor-informed assays can only be used post-diagnosis, and often post-surgery. Additionally, they normally require at least targeted sequencing, and ideally, exome sequencing, of the primary tumor to identify a sufficiently large number of high-quality, somatic, tumor-specific mutations. In return, tumor-informed assays have orders-of-magnitude greater sensitivity than tumor-naive multigene panels, as the latter typically detect only an average of 2 - 5 variants per patient despite using large panels (e.g., >100 genes). Several properties of both types of tests are summarized in Table 1.

Although multigene panels provide improved sensitivity relative to a single marker (e.g., a qPCR assay), a tumor-naïve panel may not cover variants found in some patients, especially for cancer types with a highly heterogeneous tumor mutational landscape and few recurrent mutations between tumors. By contrast, prior knowledge from assessment of variants in tumor tissue allows tracking of a greater number of high-quality variants, enhancing sensitivity, whereas tumor-naïve approaches assay many regions unlikely to contain a relevant variant, increasing the chance of false-positive results. False positives are a major clinical concern and a barrier to adoption if a therapeutic intervention is to follow a positive test.

A significant challenge to maintaining the specificity of ctDNA testing is confounding by clonal hematopoiesis of indeterminate potential (CHIP)[4]. CHIP mutations originate from clonally expanded hematopoietic methodology used to detect and define CHIP progenitor cells carrying cancer-associated genetic variants, such as those found in the tumor suppressor gene *TP53*. Recent studies have reported that 14% of patients with early-stage lung cancer and 25% of patients with late-stage solid tumors harbor CHIP mutations[5]. Because of the difference in the

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__Table 1.__ Comparison of tumor-informed and tumor-naïve ctDNA detection methods.

COMPARISON	TUMOR-INFORMED	TUMOR-NAÏVE
Sensitivty	<0.01% VAF	>0.5% VAF
Specificity	High	Medium
CHIP confounding	Highly unlikely	Requires filtering of CHIP variants
Tumor DNA limited, unavailable or low quality	Not suitable	Suitable
Acquired resistance mutations	If present in the tumor or if added to each panel	Can be added to the panel
Cost	Additional cost for genotyping and personalized design	Plasma cost and single design only
Turnaround time	Longer time for initial test due to design and genotyping	Depends on turnaround time for the plasma test only

variants between these studies, comparing results does not permit inferences about CHIP mutation frequency by cancer type and stage. However, the high frequency of CHIP variants observed in both studies underlines how misclassifying CHIP variants as ctDNA variants may reduce specificity for MRD detection. Approaches to address this misclassification include sequencing paired peripheral blood cells for in silico filtering of variants common to peripheral blood cells and ctDNA and using tumor-informed methods to identify clonal tumor variants.

ctDNA-detection in clinical trials

Tumor-informed liquid-biopsy-based detection of ctDNA can be used during all stages of a clinical trial (Figure 2) to determine if adjuvant therapy is required after surgery, identify cancer recurrence, months to years before clinical symptoms merge, evaluate the impact of 2nd or 3rd line treatments, and track the emergence of treatment resistance mutations after targeted therapy.

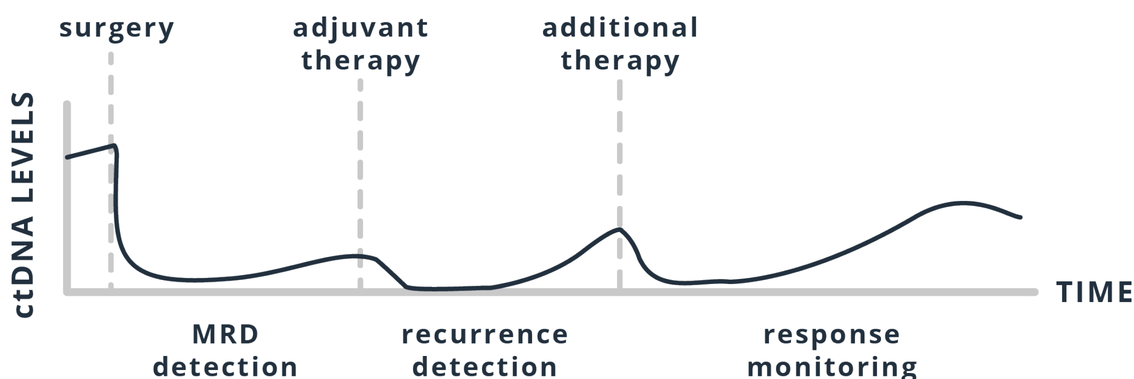


__Figure 2.__ Use cases for ctDNA in clinical trials.

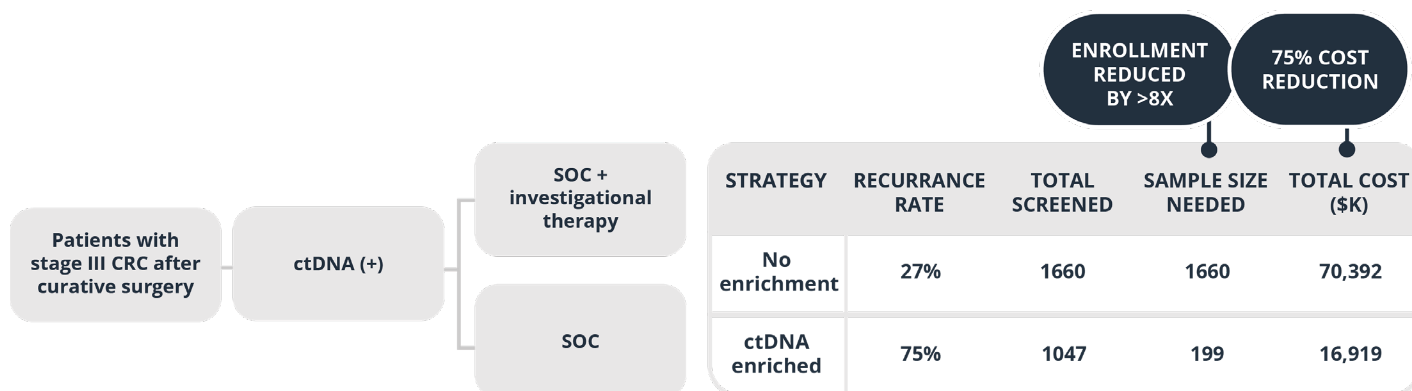
Reliable detection of MRD has substantial implications for clinical trial design. Identifying patients at high risk of recurrence through ctDNA testing can significantly reduce trial sample size, as enriching trials with patients likely to recur increases statistical power. Another potential role for ctDNA is as a surrogate endpoint for treatment response in settings where conventional response biomarkers are unavailable, for instance during adjuvant therapy. This could indicate treatment efficacy earlier than conventional measures such as progression-free (PFS) and overall survival (OS). These gains in trial efficiency can reduce study costs leading to expedited approval of new therapies. ctDNA-based testing also provides the opportunity to conduct trials where MRD status guides treatment. These trials could determine whether MRD-positive patients benefit from early therapeutic interventions.

ctDNA Molecular Residual Disease for Patient Enrichment

ctDNA can be used as a marker of MRD after definitive surgery and after (neo)adjuvant therapy to enrich a trial for patients with higher-risk disease and increased events of disease recurrence or death. Since the recurrence rate drives statistical power, much smaller sample sizes are possible for ctDNA-positive cohorts. There is substantial evidence that ctDNA-based MRD detection can stratify patients into high-risk and low-risk groups, which allows for more efficient trials by targeting high-risk patients for enrollment. Numerous retrospective studies across multiple cancer types have reported that ctDNA-based MRD detection is sensitive and specific for recurrence in both postoperative and serial testing scenarios. For some cancers, the latter can improve the sensitivity of ctDNA testing relative to the postoperative setting.

A hypothetical clinical trial enrolling of ctDNA-positive stage III patients with CRC in the adjuvant setting is shown in Figure 3. The example assumes 19% of patients are ctDNA-positive, of which 75% will experience recurrence, compared to patients enrolled irrespective of ctDNA status, with a recurrence rate of 27%. Since the recurrence rate drives statistical power, smaller sample sizes are possible for ctDNA-positive cohorts. This results in an 8-fold reduction in enrollment and a 75% reduction in per-patient costs after accounting for treatment and ctDNA screening [6].

Recent phase III adjuvant clinical trials that have enrolled thousands of patients further highlight the benefit of enriching trials with high-risk patients. For example, the PALLAS study enrolled 5,760 patients with early-stage breast cancer with a planned ten years of follow-up to determine whether a CDK4/6 inhibitor added to endocrine therapy improves disease-free survival (DFS). Similarly, the APHINITY study randomly assigned 4,805 patients with breast cancer to investigate the combination of pertuzumab with chemotherapy and trastuzumab. Both trials only showed modest results and could have substantially reduced sample size and costs if reliable biomarkers to identify patients at high risk for recurrence were available at enrollment[7] (Figure 4).



STRATEGY	RECURRANCE RATE	TOTAL SCREENED	SAMPLE SIZE NEEDED	TOTAL COST ($K)
No enrichment	27%	1660	1660	70,392
ctDNA enriched	75%	1047	199	16,919

Figure 3. *ctDNA enrichment reduces clinical trial cost using a hypothetical colorectal cancer trial adapted from Kasi et al.*[5]

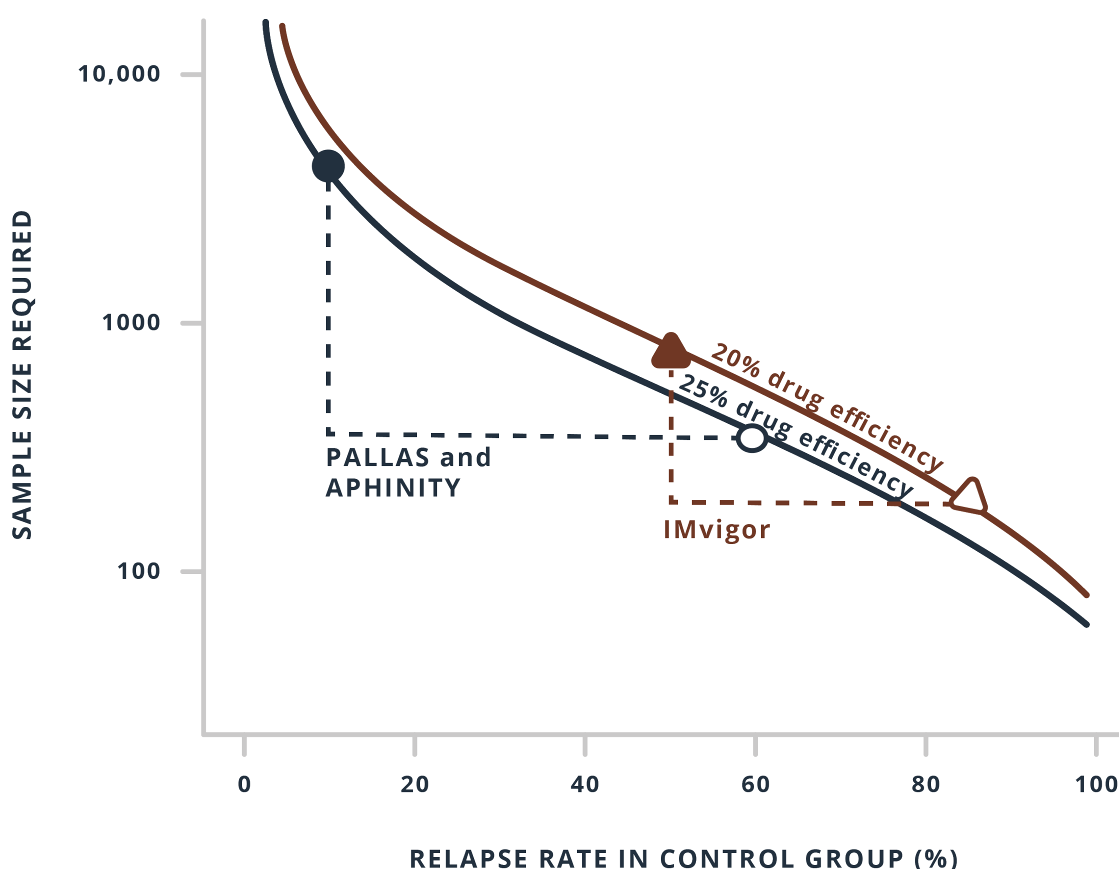


Figure 4. *Simulation of real-world clinical trial size reduction using ctDNA enrichment. Full shaded circle and triangle denote the actual sample sizes of the PALLAS/APHINITY and IMvigor trials, respectively. The hollow circle and triangle show the reduced clinical trial sizes if patients had been enriched using ctDNA using relapse rates and fraction of ctDNA positive patients based on the available scientific literature. Adapted from Kasi et al.*[6]

Several registered trials are underway where ctDNA-positivity informs enrollment. Power calculations for the MEDOCC-CrEATE trial indicated a sample size of 60 ctDNA-positive patients, which could be obtained from testing 1,320 patients, was sufficient to analyze recurrence rates[8]. The DARE trial (NCT04567420) estimated that 100 of 1,000 screened patients were needed to satisfy study power requirements for comparing recurrence across treatments. As screened populations are representative of sample size requirements for all-comers studies these trials point to a 10- to 20-fold sample size reduction for ctDNA-based enrichment trials, consistent with scenarios described in Figures 3 and 4, and further support the use of ctDNA-based enrichment studies to improve trial efficiency.

Therefore, the following represent potential avenues for using ctDNA in clinical trial designs based on currently available evidence[6,9]:

1. ctDNA testing after surgery or (neo) adjuvant therapy to select a biomarker-positive population.
2. ctDNA status at baseline as a stratification factor in a study enrolling both ctDNA negative and positive patients.
3. Escalation design of adding an experimental therapy to the standard of care compared to the standard of care alone for patients with ctDNA positive status (higher risk) or a de-escalation design based on ctDNA negative status (lower risk population).

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ctDNA as a Measure of Response

Studies that monitored ctDNA status during treatment or tested ctDNA postoperatively have shown that MRD detection in the adjuvant setting generally precedes recurrence detection by standard methods. Stage I-III CRC studies reported ctDNA-detected median lead times of 1.8-11.5 months[10,11]. For patients with breast cancer, lead times of 8.9-11.0 months were reported[1,12]. Median lead times of 2.3-8.9 months were observed for lung, esophageal, gastric, and bladder cancer[13,14]. As lead times are influenced by intervals between ctDNA testing and imaging, which vary across studies, these results must be interpreted with some caution. Older studies likely used less sensitive methods and, due to probabilistic sampling near the limit of detection, more frequent sampling will improve ctDNA detection sensitivity at low tumor burden[9] (Figure 5).

A significant new development in clinical cancer research is using ctDNA to detect molecular residual disease (MRD) and molecular relapse. MRD here means any molecular evidence of disease, typically when detected shortly after surgery or definitive treatment, or molecular evidence of disease found later during treatment or surveillance, also called molecular relapse.

Studies across numerous cancer types indicate that ctDNA-based MRD detection predicts recurrence with high sensitivity and specificity, preceding standard imaging by months. Thus, ctDNA could be used in early-phase clinical trials to aid in determining drug activity and aid pharmaceutical companies in their drug development plans.

ctDNA as a surrogate endpoint

Associations of ctDNA dynamics and clearance with response and survival outcomes are consistently reported across practically all cancer types in both neoadjuvant and adjuvant settings. These observations support using ctDNA status as a surrogate endpoint that could act as an early indicator of clinical benefit, reducing trial length and accelerating approval of new therapeutics. A surrogate endpoint validated against an established endpoint can provide insight into the benefit of new therapeutics, facilitating accelerated approval. A confirmatory trial with a potentially large sample size must be ongoing at the time of approval. Pathologic complete response (pCR) is a well-known surrogate endpoint, and the FDA (Food and Drug Administration) approved the use of pCR for accelerated approval in the neoadjuvant setting for high-risk, early breast cancer in 2013.

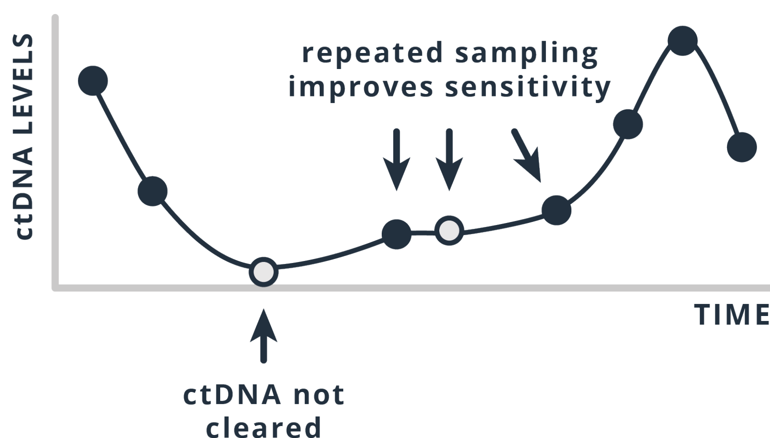

Figure 5. *Repeated ctDNA sampling improves sensitivity. Grey dots indicate a negative ctDNA test result, dark blue dots indicate a positive ctDNA result. Adapted from Cohen et al.[9]*

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A retrospective analysis of the I-SPY-2 trial found that ctDNA status was strongly associated with pCR, and lack of ctDNA clearance was a predictor of poor response and metastatic recurrence. Importantly, ctDNA clearance was associated with improved survival in patients who did not achieve pCR, indicating ctDNA testing might provide an added benefit over pCR alone[15]. However, further evidence is needed to support the use of ctDNA as a surrogate endpoint in clinical trials, including meta-analysis to aggregate the data from many currently ongoing smaller trials, such as ctMoniTR[16].

ctDNA and immunotherapy

Immune checkpoint blockade (ICB) therapy designed to target PD-1, PD-L1 and CTLA-4 have shown to improve survival in numerous cancer types[17]. Although only less than 20% respond to ICB, durable clinical benefit has been observed in patients who do respond[18]. Atypical responses such as pseudoprogression and hyperprogression can also occur, which can make it difficult to achieve or confirm therapeutic efficacy. There is a growing body of evidence indicating that ctDNA measurement may help in the interpretation of clinical response for patients receiving ICB therapy. ctDNA levels at baseline and shortly after commencement of treatment are predictive of response to ICB treatment in advanced-stage patients[18]. ctDNA might also serve as a biomarker to delineate the different types of responses to immunotherapy described below. Similarly, ctDNA could be used to enrich clinical trials in patients with a particular response type to investigate new therapies more efficiently to aid the majority of patients who currently do not receive a durable benefit from ICB.

Pseudoprogression

Pseudoprogression is an apparent increase in tumor size as a response to treatment, due immune cells infiltrating the tumor tissue. Pseudoprogression occurs in approximately 10% of solid tumours treated with immune checkpoint blockade[17]. Pseudoprogression makes it challenging for clinicians to determine whether the patient is responding, or an alternative treatment should be considered.

Currently, the distinction between pseudo- and true progression is defined by immunotherapy RECIST guidelines, where immune unconfirmed progressive disease of >20% in the sum of the diameter of the lesions is followed up at least 4 weeks later by imaging, to confirm progressive disease[19]. Importantly, ctDNA has been shown to identify pseudoprogression accurately and in real time at the molecular level, without the need for a 1 to 2 months follow-up period[18,20]. An unconfirmed radiological progression may be accompanied by a decrease in ctDNA level, resulting in eventual ctDNA clearance. However, current data on the clinical utility of ctDNA in this setting has been limited to a handful of smaller retrospective cohorts.

Timely distinction of pseudoprogression from true progression may help avoiding either premature discontinuation of an effective therapy or exposing patients to ineffective and costly treatments. Furthermore, in cases of true progression, ctDNA status can provide rationale for switching to an alternative therapy more quickly.

Hyperprogression

Recent studies have reported hyperprogressive disease in 4 - 29% of patients with solid tumours who receive immune checkpoint blockade therapy, which may lead to a shorter overall survival[21]. Key criteria for hyperprogression include time to treatment failure of less than two months, with a two-fold or greater increase in disease progression and at least a doubling of the patient's tumour burden compared with pre-baseline imaging. It is anticipated that large, rapid increases in ctDNA could potentially identify hyperprogression[22]. However, currently there is little data relating ctDNA dynamics to hyperprogression in the immunotherapy setting. Larger studies are needed to establish whether ctDNA can

effectively distinguish hyperprogression from other forms of progression and the potential utility for managing patients undergoing immunotherapy.

Exceptional responders

Patients with unusually favourable responses to a specific treatment protocol are defined as exceptional responders. Rapid clearance of ctDNA is known to be associated with exceptional treatment response[18]. Identification of exceptional responders may aid in determining treatment duration, allowing for earlier discontinuation, and sparing patients from treatment-associated toxicities and costs. Prospective studies and clinical trials evaluating the implications of longitudinal changes of ctDNA are needed to validate the benefits of discontinuing treatment in exceptional responders defined by ctDNA.

Immune-related adverse events

While checkpoint blockades are designed to activate immune responses against tumour cells, they can also induce immune responses against other tissues leading to sometimes severe side-effects in patients[23]. When these immune-related adverse events occur, ctDNA monitoring may assist in determining whether immunotherapy should continue. Cessation of immunotherapy, regardless of disease grade, could potentially spare patients from harmful side effects and reduce costs for patients and the healthcare system. A smaller study of 46 gastric cancer patients suggests that ctDNA could be used as a biomarker to detect immune-related adverse events[24]. However, the role of ctDNA in this space remains to be investigated further.

References

1. Coombes, R. C. *et al.* Personalized Detection of Circulating Tumor DNA Antedates Breast Cancer Metastatic Recurrence. *Clinical Cancer Research* **25**, 4255–4263 (2019).
2. Bettegowda, C. *et al.* Detection of Circulating Tumor DNA in Early- and Late-Stage Human Malignancies. *Sci Transl Med* **6**, (2014).
3. Stroun, M. *et al.* Neoplastic Characteristics of the DNA Found in the Plasma of Cancer Patients. *Oncology* **46**, 318–322 (2009).
4. Marnell, C. S., Bick, A. & Natarajan, P. Clonal hematopoiesis of indeterminate potential (CHIP): Linking somatic mutations, hematopoiesis, chronic inflammation and cardiovascular disease. *J Mol Cell Cardiol* **161**, 98–105 (2021).
5. Coombs, C. C. et al. Therapy-Related Clonal Hematopoiesis in Patients with Non-hematologic Cancers Is Common and Associated with Adverse Clinical Outcomes. *Cell Stem Cell* **21**, 374-382.e4 (2017).
6. Kasi, P. M. et al. Impact of Circulating Tumor DNA–Based Detection of Molecular Residual Disease on the Conduct and Design of Clinical Trials for Solid Tumors. *JCO Precis Oncol* e2100181 (2022) doi:10.1200/PO.21.00181.
7. Miller, K. D. Questioning Our APHINITY for More. *New England Journal of Medicine* **377**, 186–187 (2017).
8. Schraa, S. J. *et al.* Circulating tumor DNA guided adjuvant chemotherapy in stage II colon cancer (MEDOCC-CrEATE): Study protocol for a trial within a cohort study. *BMC Cancer* **20**, (2020).
9. Cohen, S. A., Liu, M. C. & Aleshin, A. Practical recommendations for using ctDNA in clinical decision making. *Nature* **619**, 259–268 (2023).
10. Reinert, T. *et al.* Analysis of Plasma Cell-Free DNA by Ultradeep Sequencing in Patients With Stages I to III Colorectal Cancer. *JAMA Oncol* **5**, 1124 (2019).
11. Tie, J. *et al.* Circulating Tumor DNA Analysis Guiding Adjuvant Therapy in Stage II Colon Cancer. *New England Journal of Medicine* **386**, 2261–2272 (2022).
12. Olsson, E. *et al.* Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease . *EMBO Mol Med* **7**, 1034–1047 (2015).
13. Chaudhuri, A. A. *et al.* Early Detection of Molecular Residual Disease in Localized Lung Cancer by Circulating Tumor DNA Profiling. *Cancer Discov* **7**, 1394–1403 (2017).
14. The TRACERx consortium *et al.* Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. *Nature* **545**, 446–451 (2017).
15. Magbanua, M. J. M. *et al.* Circulating tumor DNA in neoadjuvant-treated breast cancer reflects response and survival. *Annals of Oncology* **32**, 229–239 (2021).
16. Vega, D. M. *et al.* Changes in Circulating Tumor DNA Reflect Clinical Benefit Across Multiple Studies of Patients With Non–Small-Cell Lung Cancer Treated With Immune Checkpoint Inhibitors. *JCO Precis Oncol* e2100372 (2022) doi:10.1200/PO.21.00372.

17. Borcoman, E. *et al*. Novel patterns of response under immunotherapy. Annals of Oncology vol. 30 385–396 Preprint at https://doi.org/10.1093/annonc/mdz003 (2019).
18. Bratman, S. V et al. Personalized circulating tumor DNA analysis as a predictive biomarker in solid tumor patients treated with pembrolizumab. *Nat Cancer* **1**, 873–881 (2020).
19. Seymour, L. *et al*. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. *Lancet Oncol* **18**, e143–e152 (2017).
20. Lee, J. H. *et al*. Association between circulating tumor DNA and pseudoprogression in patients with metastatic melanoma treated with anti–programmed cell death 1 antibodies. in *JAMA Oncology* vol. 4 717–721 (American Medical Association, 2018).
21. Han, X., Alu, A., Xiao, Y., Wei, Y. & Wei, X. Hyperprogression: A novel response pattern under immunotherapy. *Clin Transl Med* **10**, (2020).
22. Sehgal, K. Hyperprogression in Patients with Cancer Receiving Immune Checkpoint Inhibitors. *JAMA Network Open* vol. 4 Preprint at https://doi.org/10.1001/jamanetworkopen.2021.1839 (2021).
23. Yin, Q. et al. Immune-related adverse events of immune checkpoint inhibitors: a review. *Frontiers in Immunology* vol. 14 Preprint at https://doi.org/10.3389/fimmu.2023.1167975 (2023).
24. Jin, Y. *et al*. The predicting role of circulating tumor DNA landscape in gastric cancer patients treated with immune checkpoint inhibitors. *Molecular Cance*r vol. 19 Preprint at https://doi.org/10.1186/s12943-020-01274-7 (2020).

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