Detecting liquid remnants of solid tumors treated with curative intent: Circulating tumor DNA as a biomarker of minimal residual disease (Review)

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Abstract. Circulating tumor DNA (ctDNA) has emerged as a promising biomarker of minimal residual disease (MRD) in solid tumors. There is increasing evidence to suggest that the detection of ctDNA following curative-intent treatments has high potential in anticipating future relapse in various solid tumors. Multiple liquid biopsy technical approaches and commercial platforms, including tumor-informed and tumor-agnostic ctDNA assays, have been developed for ctDNA-based MRD detection in solid tumors. Accurate ctDNA-based MRD analysis remains a critical technical challenge due to the very low concentration of ctDNA in peripheral blood samples, particularly in cancer patients following a curative-intent surgery or treatment. The present review summarizes the current key technical approaches that can be used to analyze ctDNA in the surveillance of MRD in solid tumors and provides a brief update on current commercial assays or platforms available for ctDNA-based MRD detection. The available evidence to date supporting ctDNA as a biomarker for detection of MRD in various types of solid tumors is also reviewed. In addition, technical and biological variables and considerations in pre-analytical and analytical steps associated with ctDNA-based MRD detection are discussed.

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1. Introduction

With rapidly growing morbidity and mortality, cancer remains the leading cause of mortality globally. Worldwide, there are ~19.3 million new cancer cases and 10 million cancer-related deaths annually (1). In recent decades, continuous efforts have been made to improve the outcomes and prognosis of cancer patients. However, following curative treatments, patients still face a high risk of disease recurrence, progression and therapeutic resistance, owing to relapse and the development of metastases. The ability to monitor disease recurrence or progression following primary curative treatment is key to providing immediate effective interventions to eliminate or control residual tumor cells in patients with a high risk of relapse. The current standard-of-care mainly relies on traditional diagnostic methods, such as regular clinical examination, and radiological imaging to detect disease recurrence or metastasis. However, a large proportion of patients with successful curative treatment may harbor clinical micrometastases or minimal residual disease (MRD) (2,3), a potential source of subsequent early relapse locally and/or metastatic relapse at distant sites, which cannot be reliably assessed and monitored by standard radiological imaging or regular clinical examination due to the lack of sensitivity. In various types of tumors, there is ample evidence to support the clinical validity of MRD detection in anticipating future recurrence in patients treated for early-stage cancer (4-8). MRD detection is well-established and widely employed in hematological cancers; however, it remains challenging in solid tumors, although MRD detection has been mentioned in the National Comprehensive Cancer Network (NCCN) guidelines for solid tumors. Performing serial biopsies of solid tumors for MRD detection is not practical, as it is invasive, and there is intratumor heterogeneity and difficultly in sampling once the tumor has metastasized to a distant site.

Liquid biopsy, which detects blood-based tumor-specific biomarkers, has exhibited good potential in identifying MRD in cancer patients. Analytes of liquid biopsies include circulating tumor cells, proteins, extracellular vesicles, cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), exosomes, circulating tumor RNA, metabolites, or other biological components that are shed into the bloodstream by tumor cells (9,10). Among these, ctDNA is a widely used biomarker as it carries the comprehensive genetic and epigenetic aberrations of cancers, including point mutation, deletion, amplification, gene fusion, hypermethylation and hypomethylation (10), which are representative of the entire tumor profile, including the intratumor heterogeneity of a patient. Therefore, it can be utilized as a substitution of tumor DNA to detect and characterize MRD in a number of cancer types. There are several potential benefits of detecting MRD using ctDNA as a biomarker compared with traditional diagnostic methods. First, ctDNA-based MRD detection can predict disease relapse several months or even years before current radiological imaging procedures (11,12). Second, ctDNA may depict a more comprehensive genetic and epigenetic landscape of tumor heterogeneity, possibly allowing for the quantitative and qualitative real-time evaluation of the characteristics of multiple cancer types in a particular patient. Third, ctDNA-based MRD detection is safer, easier to obtain, less invasive, and less dangerous and painful than a tumor biopsy. Finally, ctDNA-based MRD detection may monitor the molecular evolution of residual tumor cells in real time during tumor progression, thereby aiding in precision treatment to delay or eliminate metastatic recurrence.

Herein, the current approaches and commercial platforms of plasma-based ctDNA MRD assays are discussed, and the evidence of using ctDNA as a promising biomarker for the detection and characterization of MRD in solid tumors is summarized. The present review also discusses the experimental workflow considerations and the technical and biological challenges of ctDNA-based MRD analysis using plasma and its future horizon.

2. Biology of cfDNA and ctDNA

cfDNA was first discovered by Mandel and Métais (13) in human blood plasma in 1948. cfDNA are fragments of extracellular nucleic acids, which are released into biological fluids through multiple mechanisms, such as cellular apoptosis, necrosis, phagocytosis and possibly active secretion (14,15) (Fig. 1). cfDNA exists in various bodily fluids, including blood plasma (16), saliva (17), urine (18), cerebrospinal fluid (19) and pleural fluid (20), but mostly in blood plasma. cfDNA is typically double-stranded, highly fragmented and ~150-200 base pairs (bp) in length. The prominent length of cfDNA fragments is 166 bp, corresponding to the length of DNA wrapped in a chromatosome (21). The half-life of cfDNA is relatively short, from 16 min to 2.5 h (22). This may be prolonged up to 10-fold by binding to protein complexes, cell membranes, or extracellular vesicles (22,23). In healthy individuals, cfDNA mainly originates from cells of hematopoietic lineage, such as erythrocytes, leukocytes and endothelial cells (24-26). The concentration of cfDNA is usually low, ranging from a negligible amount to 10 ng/ml of plasma, and is rarely >30 ng/ml (27). Under certain physiological and pathological conditions, including intense exercise, trauma, pregnancy, inflammation, infection and cancer, the concentration of cfDNA significantly increases (28,29). In a malignant state, the levels of cfDNA may increase 50-fold over the normal levels to 50-1,000 ng/ml plasma (14), presumably due to a higher cellular turnover rate.

ctDNA, released by tumor cells, is a fraction of cfDNA. A higher concentration cfDNA was found in cancer patients by Leon *et al* (30) in 1977, and Stroun *et al* (31) first identified tumor-derived cfDNA, referring to it as ctDNA in 1989. The fraction of ctDNA is highly variable, ranging from <0.05% (32) to 90% (14). It is influenced by a number of factors, including the type of tumor, tumor size and staging, localization and vascularization, tumor microenvironment, antitumor treatments, and hepatic, splenic and renal clearance (33). ctDNA is more fragmented than healthy cfDNA, with a shorter fragment length distribution of 132-145 bp, and the mean length is ~143 bp (34). ctDNA analysis is considered as a 'real-time' reflection of tumor burden for the short half-life. ctDNA can be enriched through some DNA extraction and fragment enrichment procedures, considering the size difference between ctDNA and healthy cfDNA. To prevent the degradation, purified ctDNA should be stored at -20˚C, with no more than one freeze-thaw cycle.

3. Current technologies/approaches and commercial platforms for ctDNA-based MRD analysis in solid tumors

Strategies for ctDNA-based MRD assays are based on the detection and/or tracking of tumor-specific genomic alterations in the ctDNA of patients with definitive therapy. The detection of ctDNA of MRD requires a significantly higher sensitivity to increase the likelihood of detecting low-variant allele frequency (VAF). The relatively lower concentration of ctDNA in plasma following curative-intent treatments, even <0.01% of total cfDNA (9), poses a key challenge to MRD detection. With immense efforts being made over the past decades, multiple approaches based on different technologies have been developed and utilized for ctDNA-based MRD detection (Fig. 1, bottom panel). These include PCR-based methods, such as allele-specific PCR, droplet digital PCR (ddPCR), and beads, emulsions, amplification and magnetics (BEAM)ing-PCR (35); PCR amplicon-based next-generation sequencing (NGS) approaches, such as Safe-Sequencing (Safe-SeqS) (36), Signatera (Natera Inc), ArcherDX's personalized cancer monitoring assay and RaDaR assay (Inivata); hybridization capture-based NGS approaches, such as Cancer Personalized Profiling by Deep Sequencing (CAPP-Seq), AVENIO assay (Roche Diagnostics), and Phased variant Enrichment and detection Sequencing (PhasED-Seq) (37); and whole-genome sequencing (WGS), whole-exome sequencing (WES), and other approaches based on epigenetic features, such as the Guardant Reveal assay. PCR-based methods, particularly ddPCR, have a high possibility to reliably detect known genomic alterations with high sensitivity, down to a VAF of 0.01% (38). PCR-based methods to assess MRD have been broadly used in hematological malignancies with a or several specific mutations, such as BRAF V600E in hairy cell leukemia (39). In addition, promising results were observed in one study on solid tumors with well-established hotspot

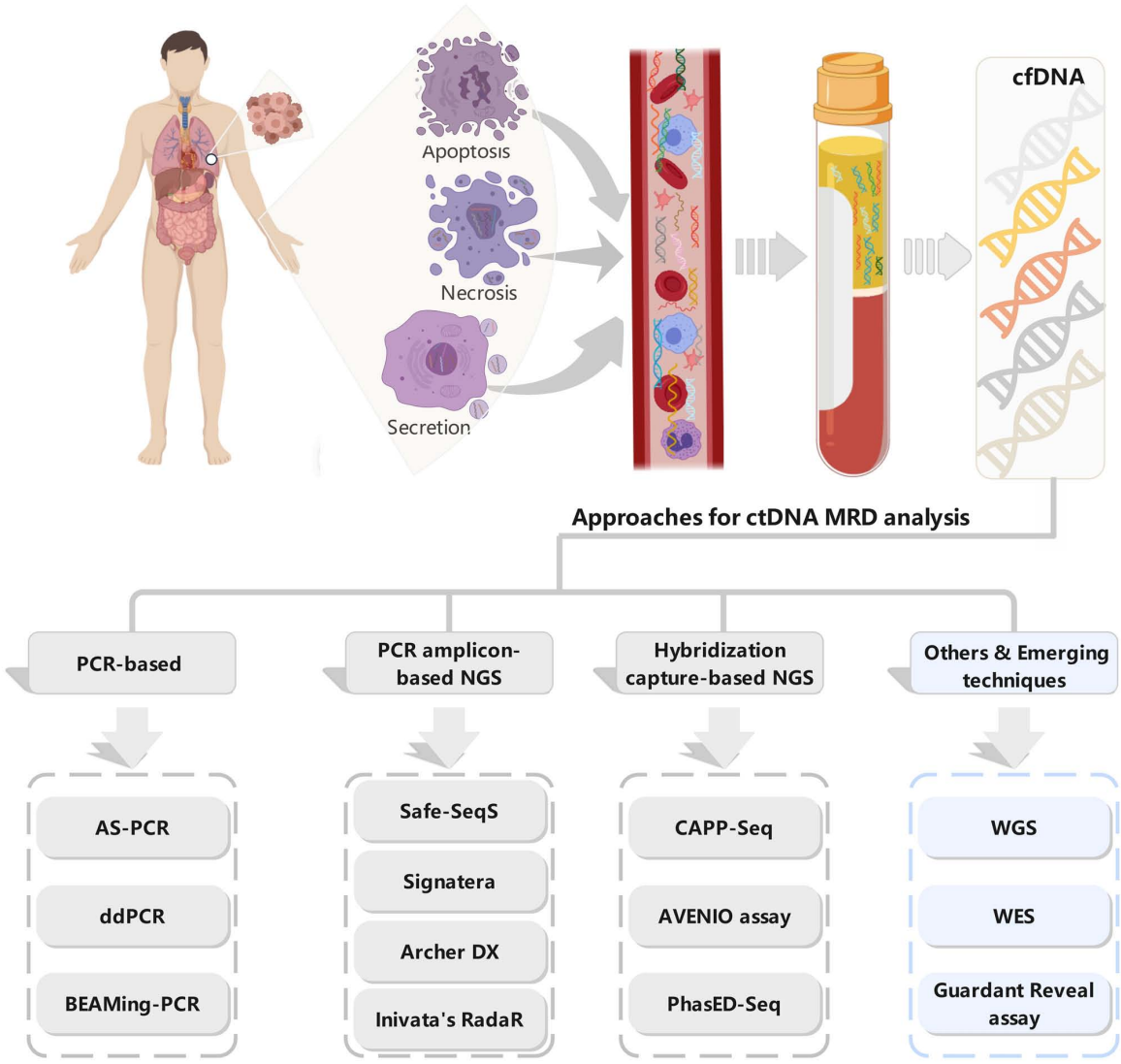

Figure 1. Origins of ctDNA and technologies for ctDNA MRD detection. Top panel: ctDNA, released from tumor cells via apoptosis, necrosis, and active secretion, can be extracted from the plasma of patients with cancer. Tumor-associated genetic aberrations can be analyzed in the isolated ctDNA. Bottom panel: Several different technologies for ctDNA MRD analysis in solid tumor patients with definitive therapy.cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; MRD, minimal residual disease; NGS, next-generation sequencing; AS-PCR, allele-specific PCR; ddPCR, droplet digital PCR; BEAMing-PCR, Beads, Emulsions, Amplification, and Magnetics-ing PCR; Safe-SeqS, Safe-Sequencing; CAPP-Seq, Cancer Personalized Profiling by Deep Sequencing; PhasED-Seq, Phased variant Enrichment and detection Sequencing; WGS, whole-genome sequencing; WES, whole-exome sequencing.

driver mutations (40). The NGS-based approaches with high-throughput ability and hypersensitivity are currently the most broadly used ctDNA detection techniques (16,41). All those approaches can be classified into two broad categories: tumor-informed and tumor-agnostic approaches.

Tumor-informed ctDNA-based MRD approaches. Tumor-informed approaches are based on prior knowledge of the tumor-specific mutational profile. Thus, the sensitivity and specificity can be enhanced by tracking patient-specific genomic alterations expected to be present in plasma vs. *de novo* potential false-positive alterations originated from non-tumor, such as clonal hematopoiesis of indeterminate potential (CHIP) (42,43). Typically, tumor biopsy or surgical tissue is sequenced by WGS, WES, or a large NGS panel to identify the genomic alterations that were not found in the corresponding germline sample, and the identified alterations are monitored by tumor-informed fixed or bespoke panels, or custom-based PCR assays in post-treatment plasma. The use of WGS, WES, or a large NGS panel to identify somatic mutations detected as ctDNA in plasma by custom-based panel broad WGS, WES, or large NGS panel and/or custom-based panel integrated as a combinatorial approach has demonstrated the feasibility to detect MRD in previous clinical studies (44-46). The outstanding advantage of this design is the comprehensive exploration of genomic alterations of the corresponding tumor to provide multiple follow-up targets in the subsequent plasma, thereby improving the sensitivity and reliability of detection. Moreover, using a small target-sized patient-specific panel allows for a lower limit of detection (LOD) by deep sequencing at a lower cost. Some approaches, such as Safe-SeqS (36) and targeted digital sequencing (TARDIS) (46), which combine ultra-deep sequencing with unique molecular identifier (UMI) barcoding, or MRDetect (47) and the Integration of Variant Reads (INVAR) pipeline (48), which increase the number of informative targets in an assay, can detect tumor VAF as low

as 0.002%. However, the selection of follow-up tumor variants (tumor variants that should be included in the patient-specific panel) and the threshold of ctDNA positivity (the limit number of variants per sample to follow) are subjective or arbitrary, mostly based on investigator's personal criterion and/or disease-pertinence bioinformatic algorithms. The number of tracking variants ranges from 1 to 115 in the currently used tumor-informed approaches (36,49,50). A previous study found that tracking at least two variants in plasma increased the ability to identify MRD by 87.5% (6). The sensitivity of MRD detection by tracking multiple somatic variants (both driver and passenger) has been found to be significantly higher than that of MRD detection by tracking a single mutation (94 vs. 58%, respectively) (51).

The tumor-informed approach offers a cost-effective solution to examine only tumor-specific genomic alterations present in the primary tumor in ctDNA, which lowers the risk of false-positive findings, decreases demands for blood volume, and increases sensitivity compared with the tumor-agnostic approach. However, the tumor-informed approach requires tumor biopsy or surgical tissue for genotyping and may pose several limitations. First, the turnaround time (TAT) is longer, and more resources are required for custom personalized assay development compared with the tumor-agnostic approach, where generally only plasma is required. Second, in numerous cases, biopsy or surgical tumor specimens with limited tumor cellularity, particularly in tumors with neoadjuvant therapy, are insufficient for molecular analysis, which hinders the application of the tumor-informed approach. In a previous study, up to 9% of the specimens were reported as inadequate for tissue sequencing, given their insufficient tumor content, DNA yield, or DNA quality (52). Third, NGS panels based on a particular part of the tested tissue cannot capture the whole genomic alteration view due to tumor heterogeneity (subclones in different parts of the same tumor or distant metastases). A patient-specific panel developed according to the genomic profile of primary tumor may miss new potentially informative targets in the plasma that occur during the evolution of tumor metastases or the natural selection of tumor clones during treatment. Despite these drawbacks, the tumor-informed approach still has immense potential to become the gold standard of ctDNA MRD detection for its ability to reliably detect very low quantities of tumor variants (53).

Tumor-agnostic ctDNA-based MRD approaches. Tumor-agnostic or tumor-uninformed (tumor-naïve) approaches detect plasma only and are conducted without a prior tumor genomic profiling in the tumor of a particular patient. Currently available liquid biopsy assays designed for identifying actionable tumor mutations in advanced tumors are not suitable for ctDNA-based MRD detection due to a low reliability (<0.5% VAF) (54). Tumor-agnostic approaches need to be developed for the primary purpose of ctDNA-based MRD detection, with high specificity and low LOD. NGS combined with specific enrichment strategies and/or other supplementary methods, e.g., using UMI allows tumor-agnostic assays to maintain high specificity and accuracy, while detecting the lower ctDNA allele fraction. Targeted sequencing approaches examining a larger number of loci of interest with deep sequencing are optimal for tumor-agnostic ctDNA MRD detection. Typically, multiple PCR amplicons [e.g., simple, multiplexed, PCR-based barcoding of DNA for sensitive mutation detection using sequencing or SiM Sen-seq (55)] and hybrid capture with molecular barcoding [e.g., CAPP-Seq (56)] are the two main enrichment strategies.

Multiplexed PCR-based assays generally demand multiple separate reaction pools running simultaneously to cover the large genomic space of designed gene panels, considering the limited multiplex-ability of PCR in a single reaction pool up to 2,000 bp of genomic breadth (57,58), which may be challenging when the total cfDNA quantity is limited. In comparison, hybrid capture-based assays have the ability to enrich and sequence tens of thousands to millions of bp in a single reaction. However, these assays may have a higher false-positive rate (due to the increased opportunity for artifactual mutations to arise across a large genomic space), and a longer TAT for target enrichment compared with PCR-based assays (58). Incorporation with single-strand and/or double-strand UMI or other molecular barcodes and bioinformatics pipelines, can reduce the artifactual mutations and technical noise derived from enrichment and sequencing errors, allowing for the detection of molecular alterations present at very low allele frequency in high sequencing depth. The CAPP-seq approach enables the detection of a ctDNA mutant allele fraction from 0.1% to as low as ~0.0001% and yields very promising results in specific cancer-type MRD detection (56). Tumor-agnostic assays integrating both standard genomic alterations and DNA methylation status, such as the Guardant Health Reveal test (59), exhibit an increased sensitivity compared with those addressing genomic alterations alone.

Commercial platforms of ctDNA-based MRD detection. With the advancement and distinct advantages of each strategy, multiple companies have adopted these technologies to develop their own ctDNA-based MRD assays. To date, several commercial platforms are available, including tumor-informed assays Signatera™ (Natera), PCM™ (ArcherDX), RaDaR™ (Inivata), MRDetect™ (C2i Genomics) and PhasED-Seq™ (Foresight Diagnostic), and tumor-agnostic assays predicineALERT™ (predicine), AVENIO™ (Roche), Guardant reveal™ (Guardant Health) and NavDX™ (Naveris) (Table SI).

Tumor-informed commercial platforms. Signatera™, which has received three US Food and Drug Administration (FDA) breakthrough device designations for MRD testing (one in May, 2019 and two in March, 2021), utilizes a patient's top 16 somatic variants from the primary tumor detected by WES to personalize a 16-plex multiplex PCR-based NGS approach for the detection of MRD in plasma. Each target can achieve an average depth of 100,000x with ultra-deep sequencing, and this approach has exhibited a sensitivity >98% at 0.01-0.02% ctDNA concentrations (60). The total TAT of the Signatera™ assay is between 4-5 weeks, including ~2 weeks for tumor sequencing results, 1 week for designing the patient-specific PCR primers and assay, and 1-2 weeks for post-treatment blood sample testing. Promising results and evidence of clinical validity were observed using the Signatera™ assay in several studies for multiple solid tumor types, including colorectal cancer (CRC), non-small cell lung cancer (NSCLC), breast cancer and bladder cancer. Moreover, recurrence was detected by the Signatera™ assay prior to clinical evidence

by a median lead time of 2.3-10.1 months, depending on the tumor type (11,61-63).

ArcherDX received a breakthrough device designation by the FDA in January, 2019 for its Personalized Cancer Monitoring (PCM™) assay that uses the company's proprietary anchored-multiplex PCR technology for ctDNA MRD detection. PCM™ uses patient-specific anchored-multiplex PCR enrichment panels developed based on somatic variants via WES of the resected tumor to identify MRD in plasma, which has served as companion diagnostics in the MERMAID-1 study (NCT04385368). Inivata's RaDaR™ assay, a multiplex PCR-based assay based on InVision® platform, was granted breakthrough device designation by the FDA in March, 2021 as an assay for ctDNA MRD detection. RaDaR™ tracks a set of up to 48 patient-specific variants for assessing ctDNA MRD in various types of solid tumor, with a LOD of 0.0011% VAF reported by Inivata. Similar to Signatera™, the total TAT of RaDaR™ is 5 weeks, including 4 weeks for initial bespoke personalized assay design and development and 7 calendar days for plasma cfDNA analysis (64).

MRDetectTM is a WGS-based cfDNA assay for MRD detection, and all somatic single-nucleotide alterations and copy number alterations (CNAs) of the tumor tissue via WGS are used to inform each personalized ctDNA assay. For integrating genome-wide genomic signature with machine-learning artificial intelligence-based error suppression models, this assay requires only 2-3 ml peripheral blood and exhibits a great LOD of 0.001% tumor fraction at a genome-wide sequencing depth of 35x (47). PhasED-Seq, a hybrid capture-based sequencing assay, detects the phased variants [two or more SNVs that occur with 150 bp regions on the same individual DNA molecule (in cis)] to improve the sensitivity, allowing a 100-fold improvement over traditional SNV-based ctDNA MRD assays, with a LOD of below 1 part-per-million (<0.0001%) (ppm) tumor fraction (37). With high sensitivity, PhasED-Seq offers the potential for detecting ctDNA during and immediately after curative intent treatment.

Tumor-agnostic commercial platforms. Predicine ALERT™ is an integrated MRD assay, including a targeted panel covering hotspot mutations and important genes, PredicineCNB™ (a companion LP-WGS assay for copy number burden), and PredicineEPIC™ (a whole-genome methylation assay). It detects the genomic variants, aberrant methylation and chromosomal abnormalities simultaneously for ctDNA MRD detection. With a LOD of 0.005% VAF, Predicine ALERT™ can be a personalized assay based on the molecular profile of the patient's plasma, urine, or tissue or a baseline-agnostic assay with a simple blood draw to predict residual disease (65). The AVENIO ctDNA Surveillance kit, one of three assays in the AVENIO ctDNA assay portfolio, is a hybrid capture-based NGS assay designed and optimized for the longitudinal monitoring of tumor burden in lung cancer and CRC. The surveillance kit, containing 197 genes with a panel size of 198 kb, exhibits >99% specificity and >99% positive predictive value (PPV) for all four classes of alterations (SNVs, indels, fusions, and CNAs) with a LOD of 0.1% VAF. The clinical utility and validity of the platform are currently being investigated. In a previous study on 24 patients with oligometastatic CRC, the use the tumor-agnostic approach based on the AVENIO assay to detect plasma and urine samples yielded a sensitivity and specificity of 95 and 100% for plasma-based ctDNA analysis, respectively, and a sensitivity and specificity of 64 and 100% for urine-based ctDNA analysis, respectively, due to the lower ctDNA levels in urine (~11-fold lower than in plasma) (66). Guardant Reveal™, a 500 kb hybrid capture-based gene panel, is a blood-only MRD assay for MRD assessment in early-stage CRC, breast cancer and lung cancer. Utilizing Guardant Health proprietary bioinformatics software, Guardant Reveal™ can query the genomic and epigenomic signatures simultaneously and filter out biological noise derived from CHIP without the paired blood mononuclear cell sequencing data. In early-stage CRC, Guardant Reveal™ exhibits 91% sensitivity at a LOD of 0.01%. In addition, favorable sensitivity and specificity for recurrence were observed in a prospective observational study that enrolled 103 patients with stage I-IV CRC with curative-intent therapies (59).

4. Evidence supporting ctDNA MRD in solid tumors

The significant development of cfDNA-sequencing methods expands the potential clinical use of ctDNA profiling for the detection of MRD and molecular relapse. Multiple studies using a variety of ctDNA-based MRD approaches in various types of solid tumors have indicated that ctDNA has the potential as a predictor of cancer recurrence with high specificity and sensitivity, and the detailed performances are shown in Table SII and Fig. 2.

Lung cancer. The TRACERx study (NCT01888601), a multicenter prospective trial that aimed to enroll >800 patients with primary stage I-III NSCLC treated with surgery for tracking tumor evolutionary dynamics in ctDNA through cancer relapse and metastases, was one of the first studies demonstrating the clinical utility of ctDNA MRD in patients with early-stage NSCLC following curative-intent treatment. In 2017, based on the analysis of a subgroup of 24 patients using a tumor-informed multiplex-PCR NGS approach (Signatera™ assay), Abbosh *et al* (50) reported that 13/14 (93% sensitivity) patients with confirmed NSCLC relapse had positive ctDNA detection prior to or at clinical relapse. The median lead time of ctDNA detection prior to NSCLC relapse confirmed by radiographic evidence was 70 days (range, 10-346 days); one patient was ctDNA-positive among 10 patients without clinical evidence of relapse (90% specificity) (50). The updated data of that study using Archer DX technology revealed that 37/45 (82.2%) patients who suffered relapse of their primary NSCLC were ctDNA-positive before or at the clinical relapse, with a median ctDNA lead time of 151 days (range, 0-984 days). For 23 relapse-free patients at a median of 1,184 days of study follow-up, ctDNA was detected in 1 of 199 time points analyzed, reflecting the specificity of the ctDNA MRD assay (67).

In a surveillance study on 40 patients treated with curative intent for stage I-III localized lung cancer, ctDNA MRD detection using CAPP-seq ctDNA analysis with a LOD of ~0.002% VAF revealed that 94% sensitivity and 100% specificity were achieved by tracking multiple somatic variants each patient at the MRD landmark (defined as first ctDNA detected within 4 months of treatment completion). Compared

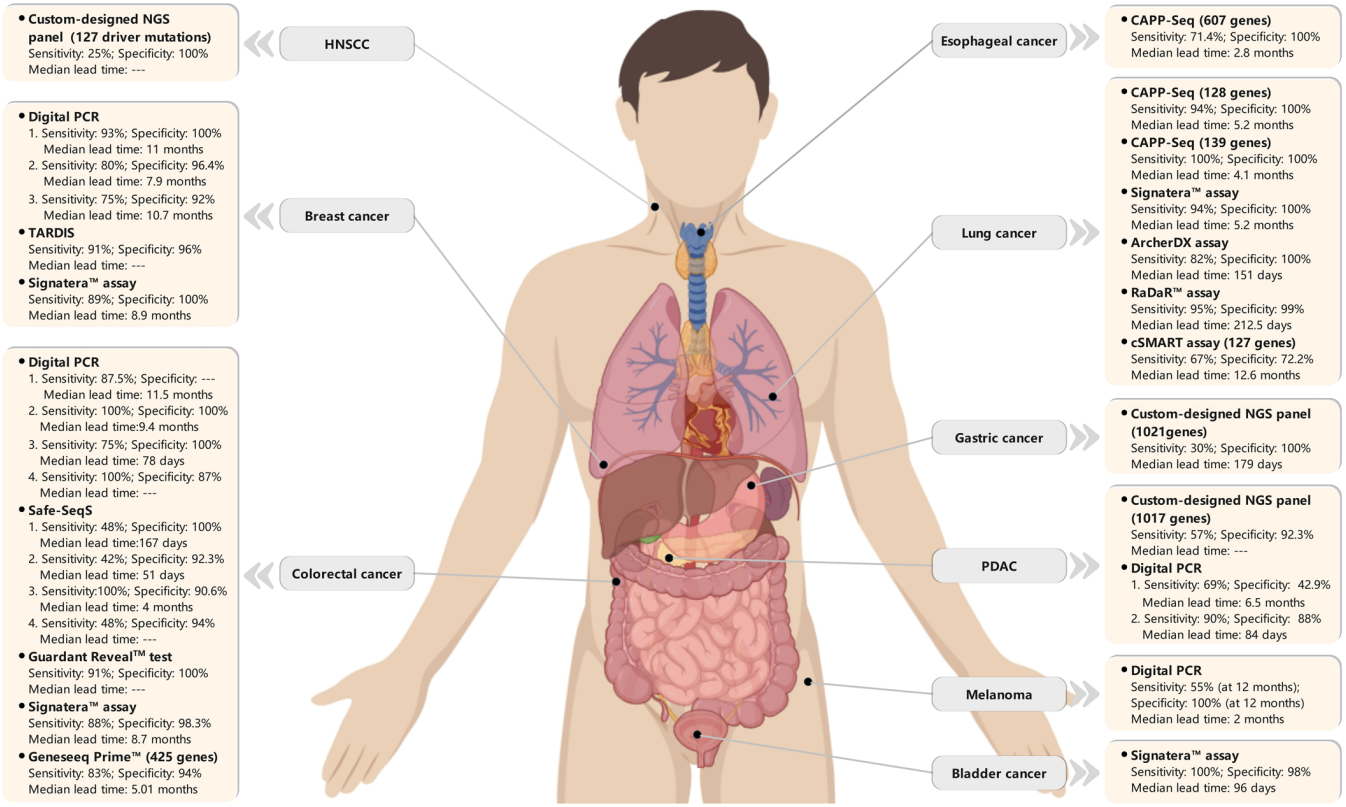

Figure 2. Performances of ctDNA-based MRD approaches in various types of solid tumors. ctDNA, circulating tumor DNA; MRD, minimal residual disease; HNSCC, head and neck squamous cell carcinoma; PDAC, pancreatic ductal adenocarcinoma; CAPP-Seq, Cancer Personalized Profiling by deep Sequencing; TARDIS, targeted digital sequencing; Safe-SeqS, Safe-Sequencing System; cSMART, circulating single-molecule amplification and resequencing technology.

with patients with detectable ctDNA at the MRD landmark, ctDNA-negative patients had an improved disease-specific survival and overall survival (OS). The serial ctDNA analysis of 37 patients with detectable pretreatment ctDNA revealed that 20 patients (54%) were ctDNA-positive at least at one post-treatment time point during follow-up, and all of these 20 patients ultimately recurred. Recurrence detected by ctDNA was earlier than that detected by radiographic imaging in 72% of the recurred patients by a median of 5.2 months (4). Peng *et al* (68) found that among 19 patients with recurrent NSCLC, 17 were ctDNA-positive within 2 weeks after surgery, and post-operative ctDNA detection preceded the radiographic findings by a median of 12.6 months. A significantly improved progression-free survival (PFS) and OS were observed in patients with undetectable postoperative ctDNA (68). In a recent surveillance study on 88 patients with localized early-stage (IA-IIIB) NSCLC, serial ctDNA in post-operative plasma samples was analyzed using personalized RaDaR™ assay to detect residual disease and recurrence. ctDNA MRD detection yielded 95% PPV and 98.7% specificity for relapse prediction of primary tumor, with a median lead time of 212.5 days relative to clinical recurrence (69).

CRC. Although the majority of CRC cases are diagnosed at early stages (I-III), a higher recurrence rate (30-40%) remains for patients with stage IIB and higher CRC following surgical resection plus adjuvant chemotherapy, and in the majority of cases (80%), recurrence occurs within the first 2 years of surgery (70). Several studies have demonstrated that ctDNA MRD detection can predict the risk of relapse in patients with resected, localized CRC with 82-100% sensitivity and 89-100% specificity (Table SII). In a cohort of 230 patients with stage II CRC treated with surgical resection, ctDNA in plasma was detected by designed personalized Safe-SeqS assay. ctDNA was detected in 14/178 (7.9%) patients without chemotherapy treatment, and 11/14 (78.6%) had clinical recurrence. In 164 of 178 (92.1%) post-operative ctDNA-negative patients, disease recurrence occurred in 16 (9.8%) patients. The post-operative ctDNA status was an independent predictor of inferior recurrence-free survival (RFS). ctDNA-based MRD analysis yielded a 48% sensitivity and 100% specificity in predicting recurrence at 36 months after surgery. The median lead time of ctDNA over radiological recurrence was 167 days (81-279 days), longer than that of carcinoembryonic antigen elevation (61 days; 0-207 days) (71). In another prospective study on 130 patients with stage I-III CRC following definitive therapy, longitudinal ctDNA analysis using Signatera™ technology identified 14 of 16 (87.5%) patients with clinical recurrence, and ctDNA-positive patients exhibited 40-fold increased risk of recurrence. Serial ctDNA analyses with 88% sensitivity and 98% specificity for relapse provided a median lead time of 8.7 months (0.8-16.5 months) relative to the radiographic recurrence (11).

Recently, Parikh *et al* (59) provided a plasma-only MRD assay (Guardant Reveal™) in patients with stage I-IV CRC post-definitive therapy. At the landmark time point (blood drawn 1 month after definitive therapy; median, 31.5 days), 15 patients were ctDNA-positive, and all of them recurred at

a median of 162 days following definitive therapy. The assay demonstrated 55.6% sensitivity and 100% specificity for landmark recurrence. Integrating serial longitudinal (blood drawn subsequently after the 'landmark' time point) and surveillance (blood drawn within 4 months of clinical recurrence) analyses improved the sensitivity from 55.6 to 69.0 and 91%, respectively, with the specificity remaining at 100%. Incorporating epigenomic signatures improved MRD detection sensitivity by 25-36% compared with the assessment of genomic variants alone. The results revealed that the sensitivity of ctDNA-based MRD for recurrence prediction could be improved by combining the genomic and epigenomic signatures and by integrating serial longitudinal and surveillance samples. This assay with acceptable assay performance characteristics is currently commercially available for clinical use to identify CRC patients at high risk of recurrence after curative-intent resection and is used in multiple ongoing prospective studies to collect additional datasets for further validation of the assay performances and clinical utility. ctDNA-MRD analysis is also a powerful prognostic factor in patients with resectable colorectal liver metastasis (CRLM), as well as in patients with non-metastatic CRC. The serial analysis of ctDNA using Safe-Sequencing (Safe-SeqS) assay in 54 patients with resectable CRLM revealed that post-operative ctDNA clearance was significantly associated with an improved PFS. All 8 patients with persistently detectable ctDNA of serial analysis during adjuvant chemotherapy recurred (72).

Breast cancer. An initial study on ctDNA MRD detection in breast cancer was performed in 2015 by Garcia-Murillas *et al* (73). In this prospective cohort of 55 patients with high-risk early-stage breast cancer with neoadjuvant chemotherapy (NAC), personalized tumor-specific dPCR assays were designed for each somatic mutation identified in the corresponding primary tumor. Tracking these mutations in the single or serial blood samples obtained at different post-curative therapy time points yielded a high early relapse prediction accuracy (hazard ratio, 25.1). Mutation tracking in serial blood samples improved the sensitivity of relapse prediction compared with a single postoperative sample (from 50 to 80%), with a median lead time of 7.9 months (range, 0.03-13.6 months) relative to clinical relapse (73). In the same year, a retrospective study on 20 patients with primary breast cancer revealed that serial ctDNA analysis using a quantitative ddPCR-based personalized rearrangement analysis had a 93% sensitivity and 100% specificity for recurrence prediction, with a median lead time of 11 months (range, 0-37 months) relative to the clinical recurrence in 86% (12/14) of patients (51). In another pilot study on 38 patients with early-stage triple-negative breast cancer who received multiple-agent NAC, the Oncomine Research Panel consisting of 134 cancer genes was used to detect the mutations in the primary tumor and track the mutations in the following plasma. A total of 4 of the 33 patients with mutations identified in their primary tumors were ctDNA-positive in their plasma, and all four patients had disease recurrence (100% specificity) within 9 months. However, sensitivity was limited to detect only 4 of 13 patients who clinically relapsed (31% sensitivity) (74). Another study revealed that the detection of ctDNA during follow-up was associated with the risk of relapse in all early-stage breast cancer subtypes, with a median lead time of 10.7 months (8.1-19.1 months) prior to clinical relapse. A total of 22 of 23 patients (96%) with distant extracranial metastatic relapse were ctDNA-positive compared with 1 of 6 (17%) patients with brain-only metastasis, suggesting that relapse sites may affect the sensitivity of ctDNA MRD detection (75).

In a study on 49 patients with high-risk with stage I-III breast cancer, serial plasma ctDNA analysis by Signatera™ yielded 88.9% sensitivity and 100% specificity for predicting relapse, with a lead time of up to 2 years (median, 8.9 months; range, 0.5-24.0 months) ahead of clinical or radiologic relapse (63). A personalized ctDNA analysis using TARDIS, developed by McDonald *et al* (45), demonstrated excellent accuracy for identifying molecular response and residual disease in patients with stage I-III breast cancer treated with curative intent. In addition, a novel, ultrasensitive assay for tracking hundreds of patient-specific mutations to detect MRD in patients with early-stage breast cancer revealed that tracking a larger number of individualized tumor mutations in cfDNA could increase the reliability and improve the sensitivity of ctDNA MRD detection (76). This approach demonstrated a 100-fold higher sensitivity than ddPCR when tracking 488 mutations. The presence of ctDNA MRD soon after curative surgery and at a post-operative landmark (1 year after surgery) was highly predictive of distant relapse. The median lead time of ctDNA detection over clinical relapse was 18.9 months (3.4-39.2 months) (76). In the multicenter I-SPY 2 trial (NCT01042379), serial ctDNA testing was able to predict pathological complete response (pCR) and metastatic recurrence risk in high-risk early breast cancer patients treated with NAC. Patients who were ctDNA-positive at 3 weeks following the initiation of paclitaxel treatment were significantly more likely to have residual disease after NAC compared with patients with cleared ctDNA (83% non-pCR vs. 52% non-pCR). Among the 43 patients who failed to achieve pCR, 14% patients who were ctDNA MRD-positive experienced a significantly higher risk of metastatic recurrence. Notably, the remaining 86% patients with cleared ctDNA had a favorable prognosis, similar to those who achieved pCR. The lack of ctDNA clearance was a strong predictor of poor treatment response and higher metastatic recurrence (46).

Other solid tumors. ctDNA-based MRD detection has also shown the ability of reliably predicting recurrence in a number of other solid tumors, such as pancreatic, bladder, head and neck, and esophageal cancer. In a study on 68 patients with localized advanced bladder cancer treated with NAC and surgery, serial ctDNA analysis by Signatera™ during surveillance following cystectomy demonstrated 100% sensitivity (13/13 patients) and 98% specificity (48/49 patients) in identifying metastatic relapse, with a median lead time of 96 days relative to radiographic imaging (61). Positive ctDNA at diagnosis before chemotherapy, after chemotherapy and before cystectomy, and during disease surveillance after cystectomy was significantly associated with a poor DFS and inferior OS, and in a multivariate analysis, positive ctDNA was the strongest predictor of RFS after cystectomy [hazard ratio (HR), 129.6; $P<0.001$] (61). In a prospective study that enrolled 45 patients with localized esophageal cancer (ESCA)

treated with esophagectomy or chemo-radio therapy (CRT), ctDNA analysis used a designed CAPP-Seq ESCA panel targeted 802 regions of 607 genes (77). The detected ctDNA post-CRT was significantly associated with an increased risk of disease progression (HR, 18.7), distant metastasis (HR, 32.1) and disease-specific mortality (HR, 23.1). The detection of ctDNA post-CRT was able to detect relapse on an average of 2.8 months before radiographic evidence, with 71.4% sensitivity and 100% specificity for recurrence prediction (77).

The value of ctDNA in predicting relapse was investigated using panel-captured sequencing in patients with pancreatic ductal adenocarcinoma following surgical treatment (78). Specifically, 8/9 (88%) patients with detected post-operative ctDNA ultimately recurred, and post-operative positive ctDNA was an independent prognostic factor for DFS (HR, 3.60) and was significantly associated with disease relapse in univariate analysis and multivariate analyses (78). For gastric cancer, ctDNA MRD detection could identify early-stage patients with a high risk of recurrence and facilitate new adjuvant therapy studies to improve survival in the adjuvant treatment setting (12). In a prospective cohort study on 46 patients with stage I-III, resectable gastric cancer, positive ctDNA after surgery was significantly associated with a higher risk of relapse. All patients with ctDNA positivity in the immediate postoperative period eventually recurred, with a median time of 179 days prior to radiographic recurrence. Positive ctDNA at any post-operative subsequent longitudinal time point was associated with a poor DFS and OS. Post-operative ctDNA positivity yielded time-dependent sensitivity and specificity in predicting recurrence, with 30% sensitivity and 100% specificity at 30 months after surgery (12). In the chemotherapy vs. chemoradiotherapy after surgery and preoperative chemotherapy for resectable gastric cancer (CRITICS) study (NCT00407186), all 11 patients with operable gastric cancer with ctDNA clearance at the median post-operative 42-month follow-up were alive and free of recurrence, and 6/9 patients with detected ctDNA at the post-operative time point experienced disease recurrence and succumbed due to metastatic disease. Moreover, patients with detectable tumor-specific mutations after surgery exhibited a significantly shorter median OS and event-free survival (28.7 months and 18.7 months vs. median not reached, respectively), as well as a 21.8-fold higher risk of relapse. ctDNA analysis determined disease recurrence at 1.3 months, with a median lead time of 8.9 months relative to clinical recurrence (79).

In another study, serial ctDNA analyses were conducted in 20 patients with locally advanced head and neck squamous cell carcinoma (HNSCC) treated with definitive radio-chemotherapy (80). Furthermore, 2/8 (25%) patients suffering from a relapse were ctDNA-positive, and both had a significant number of tumor fragments with ctDNA MRD scores of 12.16 (recurrence within 101 days) and 2.44 (distant relapse after 833 days). All 8 patients (100%) who were relapse-free were ctDNA-negative. The same dynamic properties were observed in circulating HPV DNA (cvDNA) and ctDNA levels during treatment in that study (80). In another single-center prospective cohort study of 17 patients with stage III-IVb HNSCC, plasma ctDNA was detected in all 5 patients with clinical recurrence prior to disease progression, with the lead time ranging from 108 to 253 days (44). In a study on 133 patients with resected melanoma, positive ctDNA at the post-operative time point was a stronger predictor of recurrence than that at the baseline time point and was significantly associated with distant metastasis-free survival. All patients with post-operatively detected ctDNA eventually experienced recurrence. The time-dependent accuracy of post-operative ctDNA in predicting clinical relapse was observed during 6-30 months of follow-up after resection, and, 55% sensitivity and 94% specificity at 12 months after resection were observed (81).

5. Technical considerations and challenges of ctDNA analysis

Extensive research has demonstrated the high potential of ctDNA in determining MRD in solid tumors; however, ctDNA-based MRD detection is still complex as there are a number of technical and biological challenges to its widespread clinical application. An accurate ctDNA analysis remains a critical technical challenge, particularly in patients with curative treatments, due to the ultra-low ctDNA level in body fluids. Pre-analytical workflows are crucial for reliable ctDNA MRD analysis and analytical approaches. All parameters of the entire experimental workflow that may impact the accuracy and reproducibility of the final result, including pre-analytical factors, should be considered (Fig. 3).

Pre-analytical variables and considerations. The timing of sample collection, sample collection tubes, storage and transportation conditions, centrifuge processing and extraction protocols are the main pre-analytical variables of plasma ctDNA MRD detection (Fig. 3). The ctDNA level and fraction may be affected by various factors, such as the physiological condition of a patient and concurrent inflammatory processes. The timing of post-treatment sampling is significantly associated with the clinical sensitivity and specificity of ctDNA MRD assay, particularly in studies employing an MRD landmark analysis (82). The blood draw timing after the completion of curative therapy was heterogenous across the reported ctDNA MRD studies, and there is still no standard for the first and serial longitudinal blood draw timing. The European Society for Medical Oncology (EMSO) recommends that for ctDNA MRD detection after surgery, the ideal timing of blood sampling is at least 1 week after surgery and at least 2 weeks for major surgeries for longer healing time (83). Plasma is the preferred source for cfDNA analysis in blood, given that cfDNA in serum is usually contaminated with larger DNA fragments originating from the *ex vivo* rupture of leukocytes and other cells during coagulation. Storage conditions, extraction options, sample integrity, and the quality and quantity of cfDNA are highly dependent on the blood collection tube type. There are various blood plasma collection tubes available, and the choice of collection tubes should be compatible with the ctDNA assays to be deployed. Ethylene diaminetetraacetic acid (EDTA, K_2EDTA and K_3EDTA) tubes are widely used non-preservative collection tubes for blood, which require plasma isolation within 6 h, as recommended in the majority of studies if stored at room temperature following venipuncture (84). If stored at 4˚C, the time can be extended to 24 or 48 h, as previously reported (85). Cell-preservative blood collection tubes, such as Cell-Free DNA BCT (Streck) and PAXgene Blood ccfDNA tubes (PreAnalytix), allow the

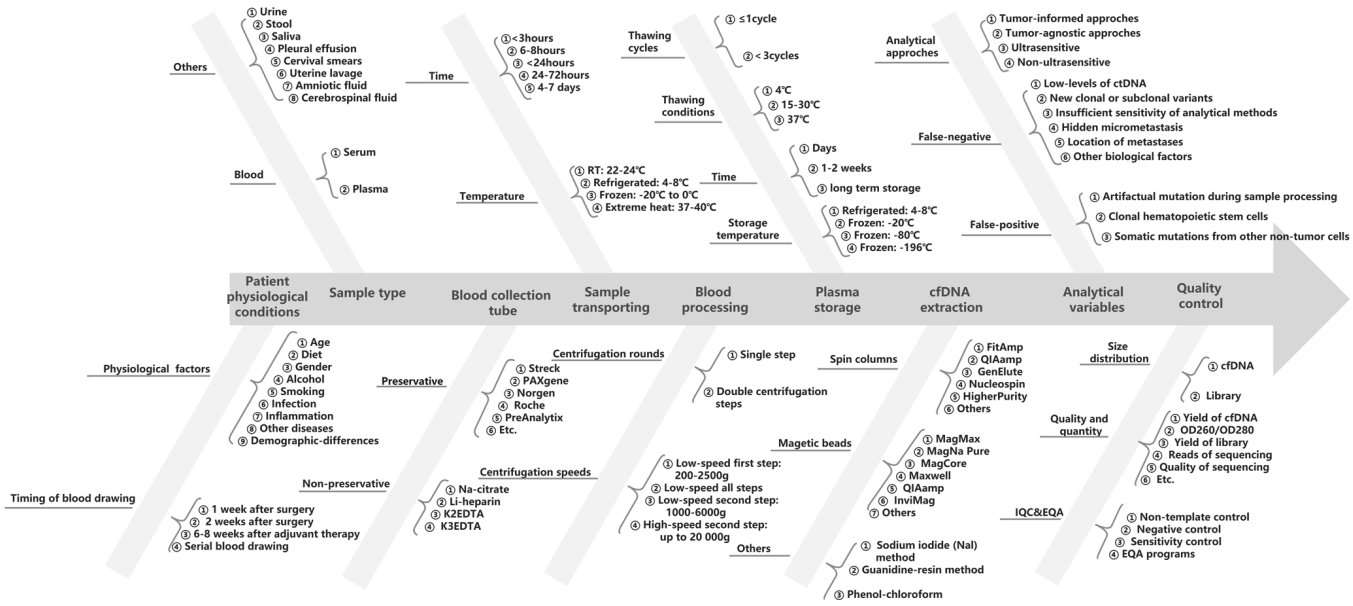

Figure 3. Overview of variables and considerations of preanalytical and analytical steps in ctDNA MRD measurements. RT, room temperature; IQC, internal quality control; EQA, external quality assessment.

extension of the time required for plasma isolation to several days or weeks (7-30 days) (86).

An initial slow centrifuge speed (3,000 x g), followed by a high-speed centrifuge (>10,000 x g), is a recommended and broadly used protocol to isolate plasma from blood. There is also a report demonstrating that a second low-speed centrifuge at 3,000 x g provides similar cfDNA yields as second high-speed centrifuge (87). Temperature variations or exposure to high temperatures may lead to damage and the degradation of cfDNA; thus, plasma should be stored at -80˚C and freeze-thawing should be avoided. The DNA integrity index is significantly decreased after three freeze–thaw cycles compared with only one cycle (88). Numerous commercial cfDNA extraction methods are available, including the manual and automated methods. The majority of these use silicon membrane-based spin columns or magnetic beads. Size discrimination, reproducibility and recovery efficiency are highly variable among these methods. Generally, magnetic bead-based methods appear to have a higher recovery efficiency for small cfDNA fragments (50-250 bp) than silicon membrane-based spin columns (86). Selecting the cfDNA extraction methods or kits needs to be based on the consideration of their compatibility with the blood collection tubes used and the analytical approach to be used. The optimization and standardization of pre-analytical procedures are as critical as the analytical approach. More importantly, the whole pre-analytical sample handling and processing should always follow validated standard operation procedures and be performed in dedicated areas of laboratories to reduce the risk of contamination.

Analytical variables and considerations. There are multiple technical and biological factors that may generate false-negative or false-positive results in ctDNA MRD analysis. A low level of ctDNA in the limited volume of plasma is one of the most common causes of false-negative results and a great technical challenge of ctDNA MRD analysis. NGS panels with high-depth sequencing, monitoring numerous patient-specific mutations, and serial testing may improve the assay sensitivity and enhance the reliability of plasma ctDNA MRD detection. Several ctDNA analysis methods with ultra-deep sequencing are being developed, which can detect ctDNA as low as ~0.0001% (37,56). The emergence of new clonal or subclonal variants is another cause of false-negative results, particularly in tumor-informed ctDNA-based MRD approaches. In addition, some biological factors, such as variable ctDNA levels between tumors or even between patients with the same tumor, hidden micrometastasis and the location of metastasis itself, can cause unavoidable false-negative results. Higher ctDNA levels have been found in patients with CRC with liver metastases than in those with nodal or lung metastases (89). Moreover, ctDNA MRD analysis has revealed a higher sensitivity for distant metastasis than for local recurrence in some studies (77,90). The biological mechanism remains unclear, although it is hypothesized that a higher tumor burden may exist in metastases than in residual local disease.

The introduction of artifactual mutations during sample processing, such as unrepaired DNA polymerase errors arising during PCR amplification and/or oxidative DNA damage in library preparation and sequencing, is still the main cause of false-positive results. Several error-suppression strategies, such as using unique molecular identifier, duplex sequencing, or the *in silico* elimination of stereotypical background artifacts (91,92), have been developed and utilized to decrease the artifactual alterations arising *ex vivo* during the various cfDNA profiling steps. Somatic alterations derived from CHIP are one of the most common biological sources of false-positive mutations. The number of variants, allele fractions and genes involved in CHIP is prone to change over time, and is associated with smoking, previous cancer therapy and an increasing age (93,94). These false-positive mutations can be reduced by the paired

sequencing of white blood cell (WBC) DNA or matched tumor tissues or by utilizing advanced bioinformatics analysis. The synchronous profiling of plasma ctDNA and paired WBC DNA to exclude CHIP-associated variants is highly recommended in ctDNA MRD analysis, particularly in plasma-only approaches. Nevertheless, accurately separating all non-tumor variants from tumor variants remains difficult. Somatic mutations from diverse non-malignant cell types, such as epithelial, endothelial and stromal cells, can be detected in cfDNA. A previous study found that ~10% of variants detected in cfDNA were not present in matched WBCs (95). Tumor-informed approaches that track specific mutations identified in tumor tissues can effectively guard against these sources of biological background.

6. Conclusions and future perspectives

There is rapidly increasing evidence demonstrating the capability of ctDNA-based MRD to predict future relapse several months or even years prior to clinical or radiologic recurrence in various types of solid tumors, with high sensitivity and specificity. However, there are still several hurdles to be overcome before this approach can be integrated into the current clinical practice workflow. First, although the clinical validity of ctDNA-based MRD testing has clearly been demonstrated through a number of studies (11,51,63,68,69,71,72,74,78,81), the clinical utility remains to be fully established. Although preliminary data on the clinical benefit of ctDNA-based MRD testing for personalizing consolidation systemic therapies or adjuvant in ctDNA-positive patients are promising, the available studies involved mostly a small proportion of participants, restricted to limited applications and lacked validation cohorts. In addition, there is less evidence of the use of ctDNA to guide the de-escalation or discontinuation of adjuvant, consolidation systemic therapies in ctDNA-negative patients. Hence, multiple carefully designed large-scale prospective randomized clinical trials are critical to firmly establish and validate the clinical utility of ctDNA-based MRD analysis for treatment personalization in various types of solid tumors. Second, the lack of standardization is another major issue for ctDNA-based MRD detection in clinical practice. As aforementioned, multiple pre-analytical and analytical factors have an effect on the sensitivity and specificity of ctDNA MRD. Optimizations of the standardization of pre-analytical variables, NGS standards and post-analytical ctDNA interpretation are the key improvement areas to use ctDNA MRD as robust standalone blood-based biopsy in solid tumor management. The basic standardization of pre-analytical conditions has been established in ctDNA MRD commercial assays (e.g., plasma is the required source and Streck tubes are the required blood collection tubes) (96). However, multiple aspects, such as assay timing, number of collection time points, variable ctDNA shedding between cancer types and patients, and LODs of the detection approaches, still have to be optimized and considered by commercial and academic partners before ctDNA MRD is incorporated into clinical practice workflow.

Multiple studies (12,46,50,59,61,67,73,77,80) suggest that ctDNA is a stronger predictor of relapse, and ctDNA MRD following curative-intent treatments has high PPV for the risk of recurrence in multiple types of solid tumors. With immense efforts being made over the past decade, several academic and commercial ctDNA MRD assays/platforms have been developed and implemented in clinical trials/practice. Although ctDNA MRD assays are still facing multiple technical difficulties due to the very low ctDNA concentration at post-treatment time points, no standardized methods, the lack of validated large-scale clinical trials and other obstacles, there is no doubt that ctDNA MRD assays will play an increasing role in the personalized management of consolidation systemic therapies and/or adjuvant for various types of solid tumors in the coming years.

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Authors' contributions

Both authors (HC and QZ) were involved in the collection and interpretation of data to be included in the review, in reviewing the literature, as well as in drafting and revising the manuscript. Both authors agree to be accountable for all aspects of the work. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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