

Review

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Circulating tumor DNA in patients with cancer: insights from clinical laboratory

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Abstract: Blood-based circulating tumor DNA (ctDNA) analysis has emerged as a highly relevant non-invasive method for molecular profiling of solid tumors, offering valuable information about the genetic landscape of cancer. Somatic mutation analysis of ctDNA is now used clinically to guide targeted therapies for advanced cancers. Recent advancements have also revealed its potential in early detection, prognosis, minimal residual disease assessment, and prediction/monitoring of therapeutic response. In recent years, significant progress has been made with the development of various PCR and NGS-based methods designed for assessing gene variants in ctDNA of patients with cancer. However, despite the transformative

possibilities that ctDNA analysis presents, challenges persist. Standardization of preanalytical and analytical protocols, assay sensitivity, and the interpretation of results remain critical hurdles that need to be addressed for the widespread clinical implementation of ctDNA testing. In addition to somatic mutations, emerging studies on DNA methylation (epigenomics) and fragment size patterns (fragmentomics) in several types of biological fluids are yielding promising results as non-invasive biomarkers for effective cancer management. This review addresses the clinical applications of somatic gene variants in ctDNA, emphasizes their potential as cancer biomarkers, and highlights essential factors for successful implementation in clinical laboratories and cancer management.

Keywords: biomarkers; cancer; ctDNA; liquid biopsy; somatic gene variants

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Introduction

In recent years liquid biopsy has emerged as a non-invasive method for molecular tumor profiling through the analysis of circulating tumor components in several biological fluids, primarily in plasma (Figure 1) [1, 2]. This approach offers significant potential for cancer management, including early detection and screening, prognosis, minimal residual disease (MRD) detection, and monitoring of therapy response [1, 3]. Among the components analyzed, circulating tumor DNA (ctDNA) stands out for its ability to detect somatic mutations, providing valuable clinical insights [4]. Although somatic mutations are the most advanced molecular biomarkers for clinical implementation, other ctDNA features, such as DNA methylation [5] and fragment size patterns [6], are providing promising results for cancer management. Recent advancements in ctDNA assays have demonstrated their potential to guide targeted therapies for advanced cancers, with growing evidence supporting their

integration into routine clinical practice. However, there are still relevant challenges in the field, such as standardization, preanalytical and analytical procedures, and result interpretation [4]. This review highlights the clinical applications of ctDNA, emphasizing its potential as a cancer biomarker. It also addresses preanalytical and analytical factors, detection assays, result interpretation and reporting aspects, as well as alternative fluids for ctDNA analysis. Altogether, we provide an overview of the clinical utility of ctDNA, and discuss challenges and future opportunities for its implementation in clinical practice.

Circulating DNA

Characteristics of blood-based ctDNA

Cell-free DNA (cfDNA) consists of nuclear and mitochondrial small fragments of double-strand DNA [7], which is mainly

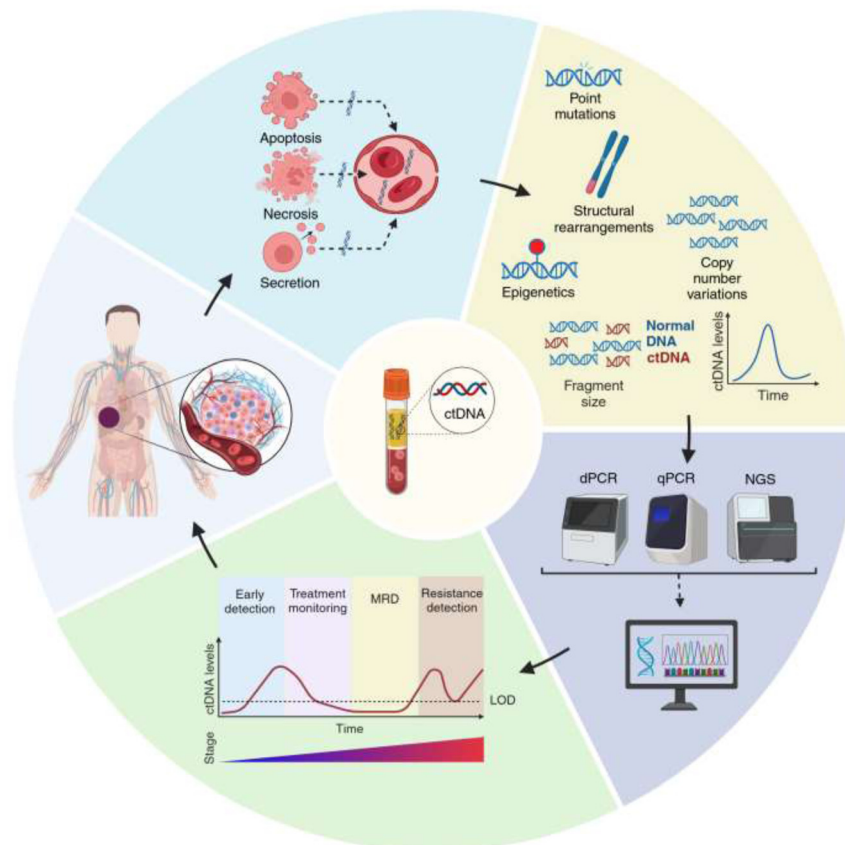


Figure 1: Schematic representation of ctDNA analysis in cancer patients. Solid tumors release ctDNA into circulation (apoptosis, necrosis, secretion), which carries specific genetic and epigenetic alterations representative of tumor molecular landscape. DNA fragments in circulation allow for potential evaluation of ctDNA features, including point mutations, structural rearrangements, copy number variations, epigenetics (DNA methylation), fragment size, and ctDNA levels. Current molecular assays for the study of ctDNA offers the possibility of analysing specific somatic genetic alterations using dPCR or qPCR, or molecular genotyping with NGS-based technologies. Potential clinical applications in oncology encompass screening/early cancer detection, treatment monitoring, detection of MDR, and resistance detection. CtDNA, circulating tumor DNA; dPCR, digital PCR; qPCR, quantitative PCR; NGS, next-generation sequencing; LOD limit of detection, MRD, minimal residual disease. Figure created with biorender (<https://BioRender.com/x96k221>).

released into bloodstream by the haematological system in healthy individuals [8]. These DNA fragments range from approximately 40 to 200 base pairs (bp) in length, with a peak around 166 bp, corresponding with nucleosome-associated DNA fragments [7, 9]. In patients with cancer, cfDNA also contains a small subset of shorter fragments (~145 bp) released into bloodstream by tumor cells known as ctDNA [10], which contributes to the elevated cfDNA levels observed in patients with cancer [11]. ctDNA is usually more fragmented than cfDNA [12] and it is characterised by presenting specific genetic and epigenetic features that provide information about the tumor of origin [10]. The release of ctDNA into circulation can be produced through various mechanisms (Figure 1), including passive and active processes [12, 13]. Passive release of ctDNA primarily occurs through apoptosis and necrosis [13]. Apoptosis is thought to be the major mechanism of shedding of ctDNA in most cancers, leading to a ladder-like pattern of DNA fragments [14] with a periodicity of 10 bp [11]. Although necrosis has a variable contribution to ctDNA shedding, this mechanism usually releases larger fragments of ctDNA, mainly >200 bp and even longer than 10,000 bp, along with an oligonucleosomal ladder-like pattern [13, 15, 16]. In addition, ctDNA can also be actively released via extracellular vesicles (e.g. exosomes, microvesicles or apoptotic bodies) [17], representing a promising area of active research [18]. However, its exact contribution to ctDNA is still under debate [19, 20]. On the other hand, it is important to note that ctDNA has a short half-life in circulation (less than 2 h) [21], which is influenced by enzymatic cleavage in the bloodstream, its clearance by the liver, and to a lesser extent, by the kidney [22]. Classically, the gold standard for molecular profiling of solid tumors has been the analysis of direct tissue biopsy. Nevertheless, in the last decade, blood-based analysis of ctDNA has emerged as a useful alternative [12], with increasing clinical evidence supporting its utility for the non-invasive molecular profiling of solid tumors [23–27]. ctDNA offers an opportunity for non-invasive analysis of tumors, providing the possibility of serial sampling to follow tumor evolution, with faster turnaround times, lower costs, and a more simplified process compared to tissue biopsy. Moreover, ctDNA allows the analysis of clonal evolution or mutational load of the tumor [28], and can capture heterogeneity better than tissue biopsy, which is inherently localized, and in some cancers difficult to obtain [12, 29]. In fact, ctDNA is able to capture tumor heterogeneity with up to 80–90 % sensitivity [30], depending on anatomical localization, and ctDNA abundance [31].

The analysis of ctDNA features can be useful for detecting a wide range of tumor characteristics (Figure 1) [4, 12]. Most publications have focused on the analysis of

gene variants, such as single nucleotide variants (SNVs), insertions and deletions (indels), copy number variations (CNVs), and fusions. However, additional features of ctDNA have recently gained significant attention [12]. For example, ctDNA levels correlate with tumor size and can be used to assess disease progression [7, 28] with lower levels associated with better outcomes [21]. In addition, the fragment size pattern analysis of cfDNA and ctDNA in patients with cancer can potentially provide valuable information about the tissue of origin [32], and it can be applied to improve ctDNA detection [33]. Importantly, the methylation profile of ctDNA can also offer valuable information about the tumor [5, 34], such as tissue of origin [12, 35].

ctDNA in non-blood body fluids

In addition to blood, ctDNA can be analyzed in alternative biological fluids, which may offer increased sensitivity in certain situations [36, 37], with distinct benefits and limitations [7, 37]. Nonetheless, its applicability in routine clinical analysis is hampered by its lack of standardization.

Urine is a promising non-invasive source of ctDNA for the detection of genitourinary tumors [38–41], since it can improve the detection and management of these tumor types [42–44]. Another non-invasive source of ctDNA is saliva, allowing serial sampling for the analysis of head and neck tumors [45–48]. Some studies suggest salivary ctDNA as the preferred sample for oral tumors, which may also be used in combination with blood-based ctDNA for other head and neck tumors [37, 49]. On the other hand, cerebrospinal fluid (CSF), is especially valuable for diagnosis and tracking of central nervous system primary cancers and intracranial metastasis [50–54]. Since the blood-brain barrier severely limits the passage of ctDNA between the bloodstream and the central nervous system [55], cerebrospinal fluid (CSF) represents a specific potential source of intracranial ctDNA [37]. This approach eliminates the need for brain tumor biopsy and allows serial sampling [56]. In addition to these fluids, the analysis of ctDNA in effusions can also provide some advantages, since these fluids present a better ratio of ctDNA-cfDNA than blood for proximal tumors [57]. For example, pleural ctDNA can offer fast detection of actionable mutations in lung cancer with more sensitivity than blood ctDNA [37]. Whereas peritoneal ctDNA has proven to be useful in the detection of peritoneal metastatic disease [37, 58]. Other types of fluid sources, such as bronchial lavages, offer the possibility of ctDNA analysis, providing valuable opportunities for lung cancer diagnostics [59]. This type of fluid contains higher ctDNA levels than blood, making it valuable for molecular profiling of lung tumors [59–61].

Furthermore, other potential sources of ctDNA with future clinical applicability are under investigation, including bile for pancreatic cancer [62], and breast milk from pregnant and postpartum women for breast cancer [63], among others.

Preanalytical considerations of ctDNA analysis

Preanalytical considerations (Table 1) are critical for the reliable analysis of ctDNA, as improper handling or processing can lead to its contamination, degradation, or low yield [4, 64].

Specimen types

For ctDNA analysis, plasma is preferred over serum. The coagulation process in serum can release genomic DNA (gDNA) from leukocytes, increasing contamination, and complicating the detection of low-frequency mutations.

Table 1: Preanalytical considerations for ctDNA analysis.

Step	Considerations
Sample type	Plasma rather than serum is recommended.
Blood collection	EDTA tubes require processing within 2–4 h. Cell preservation tubes maintain sample integrity for several days at room temperature.
Blood transport	Agitation and temperature fluctuation should be avoided. EDTA tubes should arrive the laboratory before 2–4 h. Cell preservation tubes can be transported at room temperature for up to several days without significant degradation. For longer transport times, plasma should be separated and frozen.
Plasma separation and QC	Typically involves two centrifugation steps. Plasma should be obtained without disturbing the buffy coat or red blood cells. Hemolysis, lipemia, and icterus should be avoided.
Plasma storage conditions	For long-term storage (months): –80 °C. For short-term storage (up to 30 days): –20 °C. Avoid repeated freeze-thaw cycles that can lead to ctDNA fragmentation and a loss of analytical sensitivity.
Extraction methods	Manual or automated extraction methods can be used. Yield and purity is relevant for choosing the methodology.
QC and storage of ctDNA	Fluorometric or quantitative PCR are usually used to determine ctDNA concentration. CtDNA is usually store at –80 °C if not used immediately, and repeated freeze-thaw cycles should be avoided.

QC, quality control.

Plasma minimizes gDNA contamination and provides more reliable results for detecting low-abundance alterations [65].

Blood collection and transport

It is essential to consider the timing of blood collection depending on the specific application of ctDNA analysis. Collecting the sample before treatment helps establish a baseline for ctDNA and initial tumor burden, during treatment contributes to monitor therapeutic efficacy and detect potential resistance, and after treatment enables the early identification of recurrences and progression [5, 21, 66]. For blood collection, tubes with or without preservatives can be used [67]. Among the tubes without preservatives, those containing potassium ethylenediaminetetraacetate (K2EDTA) as an anticoagulant are the preferred choice for the analysis of ctDNA. Of note, blood with K2EDTA tubes require to be processed within 2–4 h after extraction to prevent cell lysis and gDNA contamination. On the other hand, tubes with preservatives (e.g. Streck) are specifically designed to stabilize nucleated blood cells, allowing for the preservation of ctDNA in collected blood samples for up to several days at room temperature [1, 68, 69]. Blood tubes must be transported to the laboratory without agitation and protected from temperature fluctuations to prevent hemolysis and cellular damage [70]. When working with external laboratories, it's important to consider the use of cell preservation tubes and adhere to proper storage times and temperatures [64].

Plasma separation and quality control

Plasma separation typically involves two centrifugation steps: $\sim 1,600 \times g$ at 4 °C for 10 min, and a second centrifugation at $\sim 16,000 \times g$ at 4 °C for 10 min to obtain cell-free plasma [5]. This process helps eliminate cellular debris and improves ctDNA purity [65]. Factors like hemolysis, lipemia, and icterus can affect ctDNA analysis. Therefore, visual inspection of plasma after separation is recommended. To minimize hemolysis, gentle venipuncture and immediate inversion of collection tubes are advised [4, 64]. It is recommended to reject samples with hemolysis [67, 70], since it can promote the release of gDNA, interfering with the extraction process and reducing the proportion of ctDNA [67, 71]. Regarding lipemia and icterus, additional studies are required to determine the effect of elevated bilirubin levels, or hyperlipidemia impact on ctDNA levels.

Plasma storage conditions

Plasma can be stored at -20°C for up to 30 days if analysis is to be performed soon [1, 65], but it should be kept at -80°C for long-term storage. Proper aliquoting is essential to avoid repeated freeze-thaw cycles, which can fragment DNA and reduce ctDNA yield, impacting assay accuracy [72].

Extraction methods of ctDNA

Extraction methods should be tailored to the characteristics of ctDNA, which is typically found in low concentrations and as small fragments. Several commercial kits are available for ctDNA extraction, ensuring good recovery and reproducibility [65]. Laboratories should choose the most appropriate method, considering both yield and purity for low-molecular-weight DNA. Manual or automated procedures may be used, depending on the platform’s performance and the specific needs of the laboratory [64].

Quality assessment and storage of ctDNA

Assessing ctDNA quality is critical for downstream analyses. Fluorometric quantification is usually used to measure ctDNA concentration, while electrophoresis-based methods are useful to verify cfDNA fragment size and confirm the absence of gDNA contamination. When not used immediately, ctDNA should be stored at -80°C in multiple aliquots to prevent degradation from repeated freeze-thaw cycles [72, 73].

Analytical considerations of ctDNA analysis

When analyzing ctDNA, we must keep in mind several analytical factors (Table 2) that often interfere with the

results of somatic variants, such as clonal hematopoiesis, and the presence of germline variants. Additionally, it is important to consider the use of both internal and external quality controls, and to conduct thorough analytical validation to optimize ctDNA detection in routine practice, ensuring that assays are reliable and clinically applicable.

Clonal hematopoiesis

Clonal hematopoiesis of indeterminate potential (CHIP) is an age-related process in which somatic mutations in hematopoietic stem cells cause clonal expansion [4, 74]. As cfDNA largely originates from hematopoietic cells, CHIP complicates ctDNA interpretation by potentially generating false positives, particularly in genes typically associated with solid tumors, including *KRAS*, *GNAS*, *NRAS*, and *PIK3CA* [75]. For accurate variant interpretation, it is important to properly differentiate between CHIP-related and tumor-derived mutations, which usually implies sequencing of both cfDNA and peripheral blood mononuclear cells (PBMCs) [76]. Approaches based on analyzing cfDNA fragment size also may improve the accuracy of variant interpretation [33, 77].

Germline variants

Incidental detection of pathogenic germline variants (PGVs) should be considered when evaluating ctDNA results, particularly in NGS-based tests that include cancer predisposition genes (such as *BRCA1*, *BRCA2*, *PALB2*) [4, 78, 79]. In this regard, the presence of a variant allelic frequency (VAF) in circulation between 40 % and 60 % suggests germline origin, while somatic variants typically have lower VAFs [80]. However, we also have to take in mind that ctDNA may show increased VAFs due to the presence of high tumor burden or loss-of-heterozygosity [78, 81]. Only variants classified as Pathogenic and Likely Pathogenic according to American College of Medical Genetics and Genomics

Table 2: Analytical considerations for ctDNA assays.

Feature	Considerations
Clonal hematopoiesis (CHIP)	CHIP complicates ctDNA interpretation by potentially generating false positives. Sequencing of PBMCs avoids confounding results from CHIP.
Internal and external QC	Internal and external QC help assess the quality of ctDNA analysis.
Germline variants	Pathogenic germline variants in cancer predisposition genes can be detected through ctDNA testing.
Analytical validation	Analytical sensitivity (limit of detection) and analytical accuracy are key analytical performance parameters to evaluate for validation of ctDNA assays.
Analytical limitations	Limited sensitivity respect to tissue genotyping due to: i) low VAF and high fragmentation of ctDNA, ii) clonal heterogeneity, characterized by multiple tumor clones with different mutations at low VAF, iii) elevated levels of cfDNA that dilute ctDNA, and iii) limited shedding in early-stage cancers and low tumor burden.

QC, quality control; PBMCs, peripheral blood mononuclear cells.

guidelines [82], ClinVar, and other sources, should be considered as potential PGV [79]. According to European Society for Medical Oncology (ESMO) recommendations, when a potential germline variant is suspected, reflex germline testing with a validated assay should be carried out to confirm their germline or somatic origin [4]. Consequently, it is essential to alert clinicians to the potential detection of germline mutations or those related to CHIP, particularly in ctDNA assays that target frequently mutated genes.

External and internal QC

The implementation of internal and external QCs is crucial to ensure accuracy and reproducibility in detecting gene variants in ctDNA from patients with cancer. Internal controls, such as the Structural Multiplex cfDNA Reference Standard (HD786) from Horizon Discovery, are commercially available and help assess the quality of ctDNA analysis [83]. Additionally, external quality assessments (EQA), like those offered for example by the European Molecular Genetics Quality Network (EMQN), provide quality evaluation programs for ctDNA variant detection (e.g., “LUNG CANCER (NSCLC) [Plasma],” “cfDNA Multiple Biomarkers”). The use of these controls can help identify technical errors and ensure reliable clinical results, essential for biomarker-driven therapeutic decisions. Continuous validation through internal standards and comparisons with external proficiency schemes ensures the robustness of ctDNA analysis [83–85].

Analytical validation

Analytical validation must be established to optimize ctDNA detection in routine clinical practice, and it should be tailored to the specific patient population and the medical indication for the test. Recommendations and protocols for ctDNA assay validation include evaluating analytical performance parameters, such as analytical sensitivity, accuracy, repeatability, precision, and reproducibility [86, 87]. Laboratories should define and evaluate the limit of detection for at least each variant class to ensure reliable results at low frequencies [86]. Analytical accuracy can be assessed by method comparison (comparing results to an orthogonal method) or with known reference standards [87]. Orthogonal assay confirmations for analytical validation may include quantitative PCR, digital PCR (dPCR), droplet digital PCR (ddPCR), NGS, or any method with sensitivity equal to or greater than that of the assay being validated [86].

Analytical limitations

The primary limitation of ctDNA analysis is its lower sensitivity compared to tissue genotyping, leading to a higher rate of false negatives. This reduced sensitivity stems from several factors, including the extremely low concentration and high fragmentation of cfDNA in plasma, as well as the low proportion of ctDNA within the total cfDNA pool, typically ranging from 0.01 to 0.1%. Additionally, clonal heterogeneity and elevated concentration of normal cfDNA, often arising from non-malignant conditions or post-operative inflammation, further dilute ctDNA, making the detection of low-frequency variants more challenging [88, 89]. Detection reliability is especially compromised for mutations with low VAFs. Other contributing factors include early tumor stage, low tumor burden, and non-shedding tumors, all of which can reduce detection rates. The quantity of cfDNA input is also a critical variable, since higher input is associated with enhanced fragment depth, sensitivity, and reproducibility [90]. On the other hand, the detection of false positives in ctDNA analysis is relatively rare. Although false positives tend to occur in low-frequency variants, they can be minimized by employing unique molecular identifiers (UMIs) and setting a minimum VAF threshold, typically above 0.05%, that reduces sequencing error impact [90].

Methods for the analysis of gene variants in ctDNA

In recent years, both the scientific community and diagnostic companies have developed multiple methodologies to study ctDNA in solid tumors (Figure 1). However, the complexity and limitations of these molecular analyses have confined their use primarily to clinical research, with only a few approved for *in vitro* diagnostic (IVD). Although several regulatory-approved tests are available for outsourcing to private foreign laboratories (e.g., Guardant360 CDx, FoundationOne Liquid CDx) for various clinical applications [91], we will focus on tests and technologies that can be integrated into clinical laboratories for routine use. Table 3 summarizes the most frequent commercial methods, detailing their underlying technology/equipment, regulatory status, molecular markers, assay specifications, turnaround times, and applications.

Current molecular technologies for ctDNA analysis include PCR-based methods and NGS technologies. PCR-based techniques are designed to identify specific genetic alterations and encompass real-time quantitative PCR (qPCR) and dPCR. The main advantage of these techniques

Table 3: Common commercially available ctDNA tests for clinical laboratories.

Test – company	Technology	Equipment	Regulatory status	Molecular markers	Key test specifications ^a	Clinical and research application ^a
Cobas® EGFR Mutation Test v2 – Roche	RT-PCR	Cobas z 480 analyzer	CE-IVD/US-IVD	Mutations/indels <i>EGFR</i>	LOD: less than 100 copies of mutant DNA per mL of plasma Turnaround time: 4 h from blood extraction to reporting	Lung cancer
Idylla Mutation Test assays – Biocartis	RT-PCR	Biocartis Idylla™ system	RUO	Mutations <i>KRAS</i> <i>NRAS/BRAF</i> <i>EGFR</i>	LOD < 5 % for all <i>KRAS</i> mutations and for most prevalent <i>EGFR</i> mutations Turnaround time: 3 h from cell-free DNA to results	Lung and colorectal cancer
Therascreen PCR kits – Qiagen	RT-PCR	Rotor-Gene® Q MDx 5plex HRM	CE-IVD/US-IVD/ RUO ^b	Mutations <i>PIK3CA</i> <i>EGFR</i>	Overall percent agreement plasma-tissue 72 % Turnaround time: 1–2 days from blood extraction to reporting	Breast and lung cancer
Plasma-SeqSense™ Kits – Sysmex	Multiplex PCR – NGS	Illumina NextSeq 500/550 and MiSeq sequencing platforms	CE-IVD/RUO ^b	Mutations/indels Solid cancer kit (<i>BRAF</i> , <i>EGFR</i> , <i>KRAS</i> , <i>NRAS</i> and <i>PIK3CA</i>) Breast cancer kit (<i>AKT1</i> , <i>ERBB2</i> , <i>ESR1</i> , <i>KRAS</i> , <i>PIK3CA</i> and <i>TP53</i>) NSCLC kit (<i>EGFR</i> , <i>KRAS</i> , <i>BRAF</i> and <i>PIK3CA</i>) Colorectal cancer kit (<i>KRAS</i> , <i>NRAS</i> , <i>BRAF</i> and <i>PIK3CA</i>)	LOD: 0.06 % MAF Turnaround time: 2 days from cell-free DNA to results, including sequencing time	Solid cancer, lung, colorectal and breast cancer
Oncomine NGS panels – ThermoFisher	NGS – Amplicon-based libraries	Ion GeneStudio S5 system and the ion Torrent Genexus System	RUO	Mutations/fusions/indels/CNVs Pan-cancer cell-free assay (52 genes) Precision assay (50 genes) Lung cftNA assay (12 genes) Breast cftDNA assay (14 genes) Colon cftDNA assay (14 genes)	LOD: down to 0.1 % VAF for SNV hot-spots and indels Turnaround time: 1–3 days from blood extraction to reporting	Pan-cancer, lung, breast, and colorectal cancer
Avenio NGS panels – Roche	NGS – Hybrid-capture based libraries	Illumina NextSeq 500/550 sequencing platform	RUO	Mutations/fusions/indels/CNVs Targeted kit (17 genes) Expanded kit (77 genes) Surveillance kit (197 genes)	LOD: down to 0.5 % VAF Turnaround time: 5 days from cftDNA extraction to reporting	Pan-cancer
TruSight Oncology 500 ctDNA – Illumina	NGS – Hybrid-capture based libraries	Illumina NovaSeq X/NovaSeq 6,000 sequencing platform	RUO	Mutations/fusions/indels/CNVs/ MSI/TMB TruSight oncology 500 ctDNA (523 genes)	LOD for small variants: 0.5 % VAF Turnaround time: 3–4 days from purified nucleic acid to variant report	Pan-cancer
Guardant360 CDx – Guardant Health	NGS – Hybridation based capture libraries	Commercial outsourced application	FDA approved	Mutations/indels (74 genes)/fusions (6 genes)/amplifications (18 genes)/ MSI	LOD for SNVs varies with cftDNA input: 0.2 % VAF at 30 ng, and 1.8 % VAF at 5 ng Turnaround time: 7 days from sample receipt to results	Solid cancer. Test used to identify patients eligible for targeted therapies.

Table 3: (continued)

Test – company	Technology	Equipment	Regulatory status	Molecular markers	Key test specifications ^a	Clinical and research application ^a
FoundationONE Liquid CDx – Foundation Medicine	NGS – Hybridation based capture libraries	Commercial outsourced application	FDA approved	Mutations/indels (311 genes)/rearrangements (4 genes)/amplifications (3 genes)/MSI/TMB	Median LOD for short variants ranges from 0.4 to 0.8 % VAF, depending on the genomic region. Turnaround time: 8 days from sample receipt to results LOD: 0.01 % VAF	Solid cancer. Test used to identify patients eligible for targeted therapies.
Signatera – Natera	Whole exome sequencing + multiplex PCR-based NGS	Commercial outsourced application	Breakthrough Device Designation	Custom-built assay – based on the unique mutation signature of each patient's tumor	Turnaround time: 3 weeks for initial tumor sequencing and personalized assay design; 1–2 weeks for MRD results from sample receipt	Multi-cancer. Test used for treatment monitoring and MRD assessment

^aAccording to manufacturer specifications. ^bRegulatory status depends on the specific test. IVD, *in vitro* diagnostic; RUO, research use only; LOD, limit of detection; RT-PCR, real-time PCR; NGS, next-generation sequencing. Gene symbols are shown in italics for emphasis, following common editorial conventions.

over NGS-based sequencing panels lies in their high sensitivity and specificity for detecting variants, with the ability to identify VAFs at 0.1 % or below [92]. However, PCR-based methods can screen only a limited number of known variants, whereas NGS facilitates simultaneous screening of multiple markers and samples in the same run.

Currently, several commercial qPCR-based products are available. Real-time qPCR tests offer greater ease of use and are designed for specific clinical applications, some of which have been approved for routine clinical use. DPCR-based assays include numerous assays developed for the BioRad QX200/QX600 Droplet Digital PCR System and Thermo Fisher Scientific Absolute Q dPCR System, both of which offer similar sensitivity [93]. However, both reagents and equipment are currently only available for research use only (RUO) and these assays are currently limited to clinical research. Rigorous analytical and clinical validation is indispensable for their use in clinical settings.

NGS-based ctDNA methods allow for the detection of alterations across a broad spectrum of genes. Commercially available NGS panels for ctDNA analysis include the OncoPrint NGS assays (Thermo Fisher), Avenio ctDNA kits (Roche), TruSight Oncology 500 ctDNA (Illumina), and QIAseq Targeted cfDNA Ultra Panels (Qiagen), among others. These panels differ in the genes or regions covered, the types of alterations they can detect, and their sensitivity for detecting variants. However, ctDNA mutations above 0.5 % are generally detected by these assays with high sensitivity, precision, and reproducibility [90].

In clinical practice, given the variety of assays currently available for ctDNA analysis, selecting the most appropriate test should be based on availability, reimbursement status, and the number of actionable genetic aberrations within a tumor-specific context [4].

Comprehensive interpretation of gene variant results in ctDNA assays

Recommendations for identifying, interpreting, and reporting variants in cfDNA analysis should align with established criteria for somatic variant interpretation and oncogenicity classification [94]. However, it is essential to account for the unique characteristics of ctDNA and adhere to the specific guidelines tailored to ctDNA analysis across various tumor types [4, 95].

Variant identification in ctDNA analysis involves detecting SNVs, indels, fusions and CNVs. Although many

software tools automate this process, clinical laboratories must be aware of their limitations, as ctDNA analysis presents challenges in accurately for the identification of certain genetic aberrations, such as CNVs or fusion variants [4, 96]. Key metrics, including sequencing depth (coverage) and VAF, are essential for accurate interpretation and should be carefully evaluated [97]. When interpreting ctDNA findings, it is crucial to take into account that these assays have lower sensitivity compared to tissue profiling, which may increase the likelihood of false negative results. It is also important to consider the possibility of false positive results in ctDNA analysis due to the identification of CHIP variants, which can be detected at low VAF (0.1–5%), leading to their misinterpretation as tumor-derived variants [98]. As sporadic benign conditions can contain somatic alterations in cancer driver genes, interpretation of ctDNA assays should be done in the context of clinical information. For example, V600E variant has been found in plasma DNA not only in patients with cancer but also in individuals with benign nevi [99].

It is recommended to classify gene variants by their actionability, using current evidence to guide diagnostics, prognostics, and eligibility for FDA/EMA-approved therapies or clinical trials. In line with this, the Association for Molecular Pathology (AMP) Tier system and the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT) both evaluate genetic alterations based on clinical relevance. AMP tier-based classification categorizes somatic variants into four tiers based on their level of clinical significance: Tier I includes variants with strong clinical relevance, Tier II encompasses variants with potential clinical significance, Tier III includes variants of unknown clinical significance, and Tier IV consists of variants considered benign or likely benign. Levels of evidence A or B (Tier I) and C or D (Tier II) are weighted based on their significance in guiding clinical decision-making [97]. On the other hand, ESCAT categorizes molecular aberrations into Tiers I to V and X, based on the available evidence supporting their value as clinical targets. Tier I include molecular alterations with a recommended specific drug suitable for routine use, while other levels of clinical evidence (ESCAT Tier II to V) require additional data, restricting the clinical application to clinical trials. No clinical or preclinical evidence supports ESCAT Tier X alterations and should not be considered for clinical decisions [100].

Databases like COSMIC, ClinVar, and OncoKB are essential tools for interpreting ctDNA analysis results of patients with cancer (Table 4). These platforms provide context for detected genetic variants by compiling data on somatic variants, pathogenicity, and clinical relevance

across different cancer types, contributing also to identify variants of uncertain significance (VUS).

To accurately interpret gene variants detected in ctDNA assays, it is essential to establish tumor molecular boards composed of a diverse team of healthcare professionals. Laboratory professionals within these multidisciplinary teams are crucial for evaluating the clinical relevance of molecular findings. They ensure that genetic alterations are interpreted accurately and in the context of the patient's overall clinical scenario. Molecular tumor boards offer critical insights, particularly in complex cases with uncertain or conflicting data. These collaborative efforts enhance the quality of patient care by integrating various perspectives and expertise, ultimately leading to better treatment outcomes [101].

Clinical applications of ctDNA

Currently recommended applications: advanced disease

In clinical practice, ctDNA assays are considered reliable for genotyping advanced cancers and directing molecularly targeted therapies, especially in situations where tissue biopsies are suboptimal, or time is crucial [4]. The clinical utility of these assays in guiding therapy for Tier I actionable variants is supported by recent large prospective ctDNA-based studies, which have demonstrated high accuracy for SNVs (referring to tissue-plasma comparisons) across various types of cancer [27, 102–104].

In ctDNA assays, high sensitivity is achieved for SNVs and small indels. However, other aberrations such as fusions, CNVs, or microsatellite instability (MSI) may exhibit reduced sensitivity and should only replace tissue assessment when tissue testing is not feasible [4, 104]. In this context, a negative result for an actionable genetic alteration should be considered non-informative if there is no additional evidence of sufficient ctDNA levels in the assay. In such cases, confirmation with tissue testing is recommended [4, 105]. While tumor mutation burden (TMB) has shown potential as a predictive biomarker for immunotherapy, it remains an area of ongoing research [4, 106].

Nowadays, general recommendations for the use of ctDNA across various tumor types primarily target patients who lack tissue-based genomic test results when genomic testing is indicated, archival tissue is unavailable, or new tumor biopsies are not feasible [4]. Table 5 presents specific ESMO recommendations for the use of ctDNA assays in

Table 4: Common databases used for interpretation of cancer-related gene variants.

Database	Description	URL
Cancer Genome Interpreter (CGI)	Includes tumor alterations that drive the disease and may be therapeutically actionable, relying on computational methods such as in silico saturation mutagenesis of cancer genes (BoostDM and OncodriveMu) [109].	https://www.cancergenomeinterpreter.org
Cancer Hotspots	Provides significant recurrent mutations identified in large-scale cancer genomics data, detected in tumor samples using the described algorithm [110].	https://www.cancerhotspots.org
cBioPortal	Interactive, open-source platform designed for the visualization, exploration, and analysis of genomic cancer data and somatic variants across various tumor types [111].	https://www.cbioportal.org
CiVIC (clinical interpretation of Variants in Cancer)	Provides clinically relevant interpretations of cancer genetic variants to aid therapeutic decision-making, facilitating collaboration among researchers, clinicians, and patients advocates [112].	https://civicdb.org
CKB Core (Jackson Laboratory Clinical Knowledgebase)	Dynamic digital resource for interpreting complex cancer genomic profiles in the context of protein impact, therapies, and clinical trials [113].	https://ckb.jax.org
ClinVar	Public archive cataloging human genetic variations associated with diseases, drug responses, and malignancies; enhancing communication and supporting reevaluation of variant classifications [114].	https://www.ncbi.nlm.nih.gov/clinvar
COSMIC (Catalogue of Somatic Mutations in Cancer)	Source of expert-curated somatic mutation information related to human cancers, offering a comprehensive catalog of somatic variants and associated genes in oncology [115].	https://cancer.sanger.ac.uk/cosmic
DoCM (Database of Curated Mutations)	Curated repository that aggregates gene/variant information for variants with prognostic, diagnostic, predictive, or functional roles from various resources and individual publications [116].	https://docm.info
Franklin	AI-powered platform that automates the workflow from raw sequencing data (FASTQ/VCF) to clinical variant reporting; providing comprehensive variant analysis, literature evidence, automated ACMG-based classification, along with annotations and assessment tools [117].	https://franklin.genoox.com
My Cancer Genome	Provides insights into the clinical impact of molecular biomarkers on drug use in oncology, based on FDA labels, NCCN guidelines, clinical trials, and peer-reviewed publications, using data from tumor samples in the AACR project GENIE database [118].	https://www.mycancergenome.org
PMKB (Precision Medicine Knowledgebase)	An interface for collaborative editing and sharing of clinical-grade cancer mutation interpretations, designed to support the collection, maintenance, and reporting of interpretations for clinical cancer genomic testing [119].	https://pmkb.weill.cornell.edu
OncoKB	Focuses on precision oncology, providing biological and clinical data on genomic alterations in cancer. Alterations and tumor type-specific therapeutic implications are classified using the OncoKB™ levels of evidence system [120].	https://www.oncokb.org
VarSome Clinical	A platform for variant discovery, annotation, and interpretation of NGS data, integrating public databases and algorithms to provide detailed information on variant pathogenicity, population frequency, and clinical significance [121].	https://clinical.versome.com/

AACR, American Association for Cancer Research; ACMG, American College of Medical Genetics and Genomics; FDA, Food and Drug Administration; NCCN, National Comprehensive Cancer Network; NGS, Next-Generation Sequencing.

routine clinical practice, including Tier I actionable molecular markers (ESCAT scale) and associated FDA-approved drugs.

Beyond the currently recommended use of ctDNA in routine clinical practice, recent studies further support other clinical utilities in the context of advanced disease. In this regard, ctDNA tumor fraction has been established as an independent prognostic biomarker across multiple cancers [107], and ctDNA molecular profiling has shown utility in selecting patients for early-phase targeted therapies [108].

Potential applications

Despite recent advances, there are clinical applications of liquid biopsy that are not yet routinely implemented in the clinic. This is the case for example of early diagnosis/screening, detection of minimal residual disease (MRD), and monitoring of disease during treatment. Although ctDNA assays can improve diagnostic processes and help identify early-stage cancers, several challenges need to be resolved for their implementation in the clinic [4]. Achieving high

Table 5: ctDNA applications of Tier I variants (ESCAT) in clinical setting for advanced cancer disease.

Tumor type	Gene	Aberrations	Drugs/therapy ^a	ESMO recommendation for ctDNA analysis [4]
Non-small cell lung cancer	EGFR	T790M mutation	Osimertinib	Genotyping recommended in treatment-naïve cancer patients and resistance upon prior TKIs. Fusion detection is suboptimal and should be repeated in tissue where possible
	EGFR	Exon 19 in-frame deletions, L858R	Erlotinib, Erlotinib + Ramucirumab, Afatinib, Dacomitinib, Gefitinib, Osimertinib, Amivantamab + Lazertinib	
Colorectal cancer	EGFR	Exon 20 in-frame insertions (762_823ins)	Amivantamab	KRAS/NRAS/BRAF ^{V600E} /MSI for chemotherapy-naïve metastatic colorectal cancer when tissue not available or urgent therapeutic decision making. KRAS/NRAS/BRAF/EGFR-ECD for pretreated patients if EGFR rechallenge is planned
	EGFR	G719, S768I, L861Q mutations	Afatinib	
	ALK	Fusions	Alectinib, Brigatinib, Ceritinib, Crizotinib, Lorlatinib	
	MET	D1010, exon 14 deletion, exon 14 in-frame deletions, exon 14 splice mutations	Capmatinib, Tepotinib	
	KRAS	G12C	Sotorasib, Adagrasib	
	BRAF	V600E	Dabrafenib + Trametinib, Encorafenib + Binimetinib	
	RET	Fusions	Selpercatinib, Pralsetinib	
	ROS1	Fusions	Crizotinib, Entrectinib, Repotrectinib	
	NTRK1/2/3	Fusions	Entrectinib, Larotrectinib, Repotrectinib	
	NTRK1/2/3	Acquired resistance mutations	Entrectinib, Larotrectinib	
	BRAF	V600E	Encorafenib + Cetuximab	
	MSI-H	Microsatellite instability-high (MSI-H)	Pembrolizumab, Nivolumab, Ipilimumab + Nivolumab	
	NTRK1/2/3	Fusions	Entrectinib, Larotrectinib, Repotrectinib	
	KRAS/NRAS	Exon 2,3,4 mutations	Cetuximab, Panitumumab	
Pancreatic and hepato-cellular cancer	KRAS	G12C	Adagrasib + Cetuximab	When tissue not available
	ERBB2	Amplification	Tucatinib + Trastuzumab	
	EGFR	Mutations in the extracellular domain	Cetuximab, Panitumumab	
	MSI-H	Microsatellite instability-high (MSI-H)	Pembrolizumab	
Gastric cancer	NTRK1/2/3	Fusions	Entrectinib, Larotrectinib, Repotrectinib	When tissue not available or urgent therapeutic decision making
	ERBB2	Amplification	Pembrolizumab + trastuzumab + chemotherapy, trastuzumab + chemotherapy, trastuzumab deruxtecan	
	MSI-H	Microsatellite instability-high (MSI-H)	Pembrolizumab	
Breast cancer	NTRK1/2/3	Fusions	Entrectinib, Larotrectinib, Repotrectinib	ESR1 mutations should preferentially be tested in ctDNA. ERBB2 amplification and NTRK fusions when tissue not available
	PIK3CA	C420R, E542K, E545A, E545D, E545G, E545K, H1047L, H1047R, H1047Y, Q546E, Q546R mutations	Pembrolizumab Trastuzumab + Chemotherapy, Trastuzumab + Chemotherapy, Trastuzumab Deruxtecan	
	ERBB2	Amplification	Ado-trastuzumab entansine, Lapatinib + Capecitabine, Lapatinib + Letrozole, Margetuximab + Chemotherapy, Neratinib, Neratinib + Capecitabine, Trastuzumab, Trastuzumab + Chemotherapy, Trastuzumab + Pertuzumab + Chemotherapy, Trastuzumab + Tucatinib + Capecitabine, Trastuzumab Deruxtecan	
	ESR1	D538 and E380, L469V, L536, S463P, Y537	Elacestrant	
NTRK1/2/3	MSI-H	Microsatellite instability-high (MSI-H)	Pembrolizumab	Entrectinib, Larotrectinib, Repotrectinib
	NTRK1/2/3	Fusions	Entrectinib, Larotrectinib, Repotrectinib	

Table 5: (continued)

Tumor type	Gene	Aberrations	Drugs/therapy ^a	ESMO recommendation for ctDNA analysis [4]
Colangiocarcinoma	<i>IDH1</i>	R132 mutations	Ivosidenib	When tissue not available or urgent therapeutic decision making.
	<i>FGFR2</i>	Fusions	Futibatinib, Pemigatinib	
	<i>MSI-H</i>	Microsatellite instability-high (MSI-H)	Pembrolizumab	
Ovarian cancer	<i>NTRK1/2/3</i>	Fusions	Entrectinib, Larotrectinib, Repotrectinib	In women with no germline pathogenic <i>BRCA1/2</i> variant when tissue not available When tissue not available When tissue not available
	<i>BRCA1/2</i>	Mutations	Olaparib, Olaparib + Bevacizumab, Niraparib, Rucaparib	
	<i>MSI-H</i>	Microsatellite instability-high (MSI-H)	Pembrolizumab	
Endometrial cancer	<i>MSI-H</i>	Microsatellite instability-high (MSI-H)	Pembrolizumab	When tissue not available
Prostate cancer	<i>BRCA1/2</i>	Mutations	Olaparib, Olaparib + Bevacizumab, Niraparib, Rucaparib	When tissue not available. When tissue not available.
	<i>MSI-H</i>	Microsatellite instability-high (MSI-H)	Pembrolizumab	
	<i>FGFR</i>	G370C, R248C, S249C, Y373C mutations	Erdafitinib	
Urothelial cancer	<i>FGFR3</i>	Fusions	Erdafitinib	When tissue not available.
	<i>NTRK1/2/3</i>	Fusions	Entrectinib, Larotrectinib, Repotrectinib	
	<i>BRAF</i>	V600E	Dabrafenib + Trametinib	
Thyroid cancer	<i>RET</i>	Mutations and fusions	Pralsetinib, Selpercatinib	When tissue not available
	<i>NTRK1/2/3</i>	Fusions	Entrectinib, Larotrectinib, Repotrectinib	
	<i>NTRK1/2/3</i>	Fusions	Entrectinib, Larotrectinib, Repotrectinib	

^aFDA-approved related drugs defined in OncoKB [120] in October 2024. Gene symbols are shown in italics for emphasis, following common editorial conventions.

specificity and clinically relevant sensitivity is difficult, particularly because early-stage cancers release low levels of ctDNA [55]. To effectively implement ctDNA assays in clinical practice as validated screening tools, large population studies are needed [4]. In line with this, recent studies are increasing the evidence for using ctDNA for early detection/screening of patients with cancer [5, 122, 123].

Regarding the detection of MRD, the analysis of ctDNA after curative treatment in early-stage cancers predicts a high risk of relapse with high clinical specificity [124]. In recent years, interest in MRD has grown significantly, leading to ctDNA-guided randomized clinical trials in colorectal, lung, and breast cancer, which are yielding promising results for the implementation of ctDNA in MRD assessment. In this context, post-surgical ctDNA monitoring in resectable colorectal cancer has proven useful for identifying patients at high risk of recurrence and/or mortality, who are more likely to benefit from adjuvant chemotherapy [66, 125]. Furthermore, serial ctDNA analysis in patients with colon cancer undergoing adjuvant therapy enables treatment escalation or de-escalation, allowing for a more precise selection of patients who truly benefit from adjuvant therapy compared to the conventional tumor/node/metastasis (TNM) staging system [126]. Notably, a recent study in localized colon cancer demonstrated that MRD prediction accuracy can be enhanced by using NGS panels that track multiple ctDNA gene variants across serial plasma samples [127]. In early-stage non-small cell lung cancer, the detection of residual ctDNA after treatment has shown utility in predicting early relapse [128], and in breast cancer, ctDNA profiling is able to detect the onset of recurrences [129].

The use of ctDNA has also shown promise for monitoring treatment responses and resistance development in patients with cancer [21]. Its short half-life and possibility of real-time sampling, make ctDNA valuable for assessing disease dynamics during therapy [4]. Studies indicate that ctDNA levels correlate with treatment response and can detect changes earlier than traditional clinical methods [21, 130]. However, ctDNA is not yet implemented in clinical settings due to several limitations, such as the need for optimal assay strategies, uncertainties about monitoring frequency, and insufficient evidence of improvements in patient outcomes [4].

Reporting of gene variants detected in ctDNA

Generating reports from molecular testing is essential for translating complex genetic data into useful clinical

information. These reports should have a standardised format, clearly state the date of issue, and include diagnosis details and significant medical information when available. They should be clear and concise, presenting clinically significant information in an easily understandable way. Additionally, reports should be formatted for easy integration with electronic health records. Clinically critical information must be placed at the beginning for quick access, and more complex data should be simplified using graphs, charts, and tables [97].

Genetic alterations should be thoroughly described, including the involved genes, the type of variants or genomic features detected (such as SNVs, indels, CNVs, and fusions), and their predicted impact on protein function. Adopting standardized nomenclature according to Human Genome Variation Society (HGVS) guidelines (<http://varnomen.hgvs.org/>) is essential to avoid confusion and clinical errors [94]. The report should include relevant elements for thorough analysis and longitudinal comparisons, such as genomic coordinates, the genome build, and the transcript reference sequence. Including the VAF in reports, whenever possible with quantitative assays, provides critical insights for evaluating the reliability of detected variants, particularly regarding the risk of false negatives.

Following the AMP-Tier system, it is recommended to report variants classified as Tier I to III in order of their clinical significance. Generally, Tier IV variants, categorized as benign or likely benign, should not be included. Interpretative comments should be provided, particularly for Tiers I and II gene variants. Recommendations should be evidence-based and supported by appropriate literature citations [97]. Clinical actionability annotation is a crucial component of the report, supporting the clinical interpretation of results. Only likely pathogenic or pathogenic oncogenic driver alterations should be assessed for clinical actionability using clinical evidence-based frameworks such as ESCAT, OncoKB classification system, or AMP tier classification [131].

On the other hand, when a gene variant is not detected, it is preferable to use terms such as ‘non-informative’ or ‘not detected’ instead of ‘negative’ [4]. The report should acknowledge the potential for discrepancies with tumor testing, especially when no variant is found in plasma DNA.

The analysis of ctDNA could identify incidental germline variants. In the case of reporting these incidental findings, it would be convenient to clearly differentiate between somatic and putative germline variants, as well as include information about the need to perform confirmatory tests in peripheral blood leukocytes, or in other normal tissue samples [78, 79].

Methodological details and limitations should be included at the end of the report, covering the alterations tested, assay performance characteristics [such as the limit

of detection for each variant type and minimal sequencing depth), and critical quality metrics [86]. Information on any preanalytic, analytic, or postanalytic factors that might influence clinical interpretation should be indicated. It is important to note that assay sensitivity may depend on the amount of input cfDNA. Therefore, when plasma cfDNA is limited, the reported sensitivity may be adjusted or a warning included in the report [86].

Future perspectives of ctDNA

In addition to the study of gene variants, one of the most promising areas of ctDNA research are epigenomics (DNA methylation) and fragmentomics. These fields have demonstrated great promise for the early detection of cancer, the identification of tumor origin, and the evaluation of therapy response [5, 35]. It is also important to highlight that artificial intelligence, particularly through machine learning algorithms, is starting to play a crucial role in the discovery and implementation of new ctDNA biomarkers. In addition to enabling more precise and comprehensive analyses of genomics, epigenomics, and fragmentomics [132], artificial intelligence also facilitates the integration of these omics approaches with clinical data, driving further advancements in personalized cancer care [133].

Despite these advances, several challenges need to be addressed before ctDNA can be widely integrated into clinical practice. One major issue is sensitivity, particularly in early-stage cancer where ctDNA levels are typically low. Improved sensitivity in ctDNA assays, achieved through methods such as NGS and dPCR, represents a key area for future research [3, 134].

The potential for ctDNA to provide real-time insights into tumor heterogeneity is a relevant advantage. By capturing spatial and temporal genomic variations within a patient, ctDNA can offer a more comprehensive picture of tumor evolution than traditional tissue biopsies. This can be particularly beneficial in advanced cancer stages where tumors often exhibit significant heterogeneity, contributing to treatment resistance [4, 124]. However, false positives remain a concern, especially when ctDNA mutations overlap with CHIP [75], underscoring the need for robust assay development and validation.

The clinical utility of ctDNA is also gaining traction in the context of MRD detection. Monitoring ctDNA levels post-treatment could help identify patients at risk of relapse, allowing for timely therapeutic intervention [4]. Ongoing clinical trials are expected to provide critical data on the role

of ctDNA in MRD, tracking tumor evolution, and guiding treatment decisions [66].

To further enhance the diagnostic utility of ctDNA, it is crucial to standardize both preanalytical and analytical procedures. Initiatives such as the BloodPac in the United States and Cancer-ID in Europe are actively working on establishing standard operating procedures for ctDNA analysis [135, 136]. Standardization will not only improve reproducibility but also facilitate the large-scale clinical implementation of ctDNA testing.

Another promising area of research lies in combining ctDNA analysis with other circulating biomarkers, such as circulating tumor cells (CTCs) and extracellular vesicles (EVs). This approach could provide more comprehensive insights into tumor biology, helping to refine diagnosis and guide treatment decisions [1]. On the other hand, the study of ctDNA in biological fluids beyond plasma is gaining significant attention for its potential to improve cancer management in certain types of tumors [46, 52, 137].

Despite these advancements, the translation of ctDNA into clinical practice remains limited due to several technical and economic barriers. Current NGS-based approaches, although highly sensitive, require sophisticated laboratory equipment and are time-consuming, making them difficult to implement on a large scale [138]. Further technological developments, including the creation of more cost-effective and user-friendly assays, will be essential to overcome these limitations [3].

Conclusions

The analysis of ctDNA is paving the way for a more personalized cancer care. Over the past decade, advancements in ctDNA technology have been substantial, with numerous studies highlighting its potential to revolutionize the management of patients with cancer [1]. For patients with advanced cancer, validated and adequately sensitive ctDNA assays have nowadays utility in identifying actionable mutations to direct targeted therapy, and may be used in routine clinical practice, particularly when rapid results are needed or when tissue biopsies are not possible or inappropriate. In addition, ctDNA analysis offers significant potential for cancer diagnostics, detection of MRD, monitoring, and evaluation of therapy response [4].

In summary, ctDNA analysis offers significant potential for advancing early cancer detection and personalized treatment approaches, which will significantly improve patient outcomes. However, widespread clinical implementation will require further validation, standardization,

and cost-reduction strategies. As ongoing trials continue to yield valuable insights, it is likely that ctDNA, combined with other circulating biomarkers, will become a cornerstone of modern clinical laboratories.

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References

1. Alix-Panabières C, Pantel K. Liquid biopsy: from discovery to clinical application. *Cancer Discov* 2021;11:858–73.
2. Sánchez-Herrero E, Provencio M, Romero A. Clinical utility of liquid biopsy for the diagnosis and monitoring of EML4-ALK NSCLC patients. *Adv Lab Med* 2020;1:20190019.
3. Rodríguez-Casanova A, Costa-Fraga N, Bao-Caamano A, López-López R, Muínelo-Romay L, Díaz-Lagares A. Epigenetic landscape of liquid biopsy in colorectal cancer. *Front Cel Dev Biol* 2021;9:622459.
4. Pascual J, Attard G, Bidard F-C, Curigliano G, De Mattos-Arruda L, Diehn M, et al. ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: a report from the ESMO Precision Medicine Working Group. *Ann Oncol Off J Eur Soc Med Oncol* 2022;33:750–68.
5. Ruiz-Bañobre J, Rodríguez-Casanova A, Costa-Fraga N, Bao-Caamano A, Alvarez-Castro A, Carreras-Presas M, et al. Noninvasive early detection of colorectal cancer by hypermethylation of the LINC00473 promoter in plasma cell-free DNA. *Clin Epigenet* 2022;14:86.
6. Mouliere F, Smith CG, Heider K, Su J, van der Pol Y, Thompson M, et al. Fragmentation patterns and personalized sequencing of cell-free DNA in urine and plasma of glioma patients. *EMBO Mol Med* 2021;13:e12881.
7. Sánchez-Herrero E, Serna-Blasco R, Robado de Lope L, González-Rumayor V, Romero A, Provencio M. Circulating tumor DNA as a cancer biomarker: an overview of biological features and factors that may impact on ctDNA analysis. *Front Oncol* 2022;12:943253.
8. Ulz P, Thallinger GG, Auer M, Graf R, Kashofer K, Jahn SW, et al. Inferring expressed genes by whole-genome sequencing of plasma DNA. *Nat Genet* 2016;48:1273–8.
9. Jiang P, Chan CWM, Chan KCA, Cheng SH, Wong J, Wong VW-S, et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. *Proc Natl Acad Sci* 2015;112:E1317–25.

10. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin. *Cell* 2016;164:57–68.
11. Underhill HR, Kitzman JO, Hellwig S, Welker NC, Daza R, Baker DN, et al. Fragment length of circulating tumor DNA. *PLoS Genet* 2016;12: e1006162.
12. Keller L, Belloum Y, Wikman H, Pantel K. Clinical relevance of blood-based ctDNA analysis: mutation detection and beyond. *Br J Cancer* 2021;124:345–58.
13. Grabuschnig S, Bronkhorst AJ, Holdenrieder S, Rosales Rodriguez I, Schliep KP, Schwendenwein D, et al. Putative origins of cell-free DNA in humans: a review of active and passive nucleic acid release mechanisms. *Int J Mol Sci* 2020;21. <https://doi.org/10.3390/ijms21218062>.
14. Underhill HR. Leveraging the fragment length of circulating tumour DNA to improve molecular profiling of solid tumour malignancies with next-generation sequencing: a pathway to advanced non-invasive diagnostics in precision oncology? *Mol Diagn Ther* 2021;25: 389–408.
15. Jiang P, Lo YMD. The long and short of circulating cell-free DNA and the ins and outs of molecular diagnostics. *Trends Genet* 2016;32: 360–71.
16. Thierry AR, El Messaoudi S, Gahan PB, Anker P, Stroun M. Origins, structures, and functions of circulating DNA in oncology. *Cancer Metastasis Rev* 2016;35:347–76.
17. Pös O, Biró O, Szemes T, Nagy B. Circulating cell-free nucleic acids: characteristics and applications. *Eur J Hum Genet* 2018;26:937–45.
18. Jeppesen DK, Fenix AM, Franklin JL, Higginbotham JN, Zhang Q, Zimmerman LJ, et al. Reassessment of exosome composition. *Cell* 2019;177:428–45.e18.
19. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science* 2020;367. <https://doi.org/10.1126/science.aau6977>.
20. Tsering T, Nadeau A, Wu T, Dickinson K, Burnier JV. Extracellular vesicle-associated DNA: ten years since its discovery in human blood. *Cell Death Dis* 2024;15:668.
21. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med* 2008;14: 985–90.
22. van der Pol Y, Mouliere F. Toward the early detection of cancer by decoding the epigenetic and environmental fingerprints of cell-free DNA. *Cancer Cell* 2019;36:350–68.
23. Herbst RS, Giaccone G, de Marinis F, Reinmuth N, Vergnenegre A, Barrios CH, et al. Atezolizumab for first-line treatment of PD-L1-selected patients with NSCLC. *N Engl J Med* 2020;383:1328–39.
24. Al-Showbaki L, Wilson B, Tamimi F, Molto C, Mittal A, Cescon DW, et al. Changes in circulating tumor DNA and outcomes in solid tumors treated with immune checkpoint inhibitors: a systematic review. *J Immunother Cancer* 2023;11:e005854.
25. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* (80–) 2018;359:926–30.
26. Tie J, Cohen JD, Lahouel K, Lo SN, Wang Y, Kosmider S, et al. Circulating tumor DNA analysis guiding adjuvant therapy in stage II colon cancer. *N Engl J Med* 2022;386:2261–72.
27. Nakamura Y, Taniguchi H, Ikeda M, Bando H, Kato K, Morizane C, et al. Clinical utility of circulating tumor DNA sequencing in advanced gastrointestinal cancer: SCRUM-Japan GI-SCREEN and GOZILA studies. *Nat Med* 2020;26:1859–64.
28. Abbosh C, Birkbak NJ, Wilson GA, Jamal-Hanjani M, Constantin T, Salari R, et al. Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. *Nature* 2017;545:446–51.
29. Parikh AR, Leshchiner I, Elagina L, Goyal L, Levovitz C, Siravegna G, et al. Liquid versus tissue biopsy for detecting acquired resistance and tumor heterogeneity in gastrointestinal cancers. *Nat Med* 2019;25: 1415–21.
30. Pereira B, Chen CT, Goyal L, Walmsley C, Pinto CJ, Baiev I, et al. Cell-free DNA captures tumor heterogeneity and driver alterations in rapid autopsies with pre-treated metastatic cancer. *Nat Commun* 2021;12: 3199.
31. Cescon DW, Bratman SV, Chan SM, Siu LL. Circulating tumor DNA and liquid biopsy in oncology. *Nat Cancer* 2020;1:276–90.
32. Cristiano S, Leal A, Phallen J, Fiksel J, Adleff V, Bruhm DC, et al. Genome-wide cell-free DNA fragmentation in patients with cancer. *Nature* 2019;570:385–9.
33. Mouliere F, Chandrananda D, Piskorz AM, Moore EK, Morris J, Ahlborn LB, et al. Enhanced detection of circulating tumor DNA by fragment size analysis. *Sci Transl Med* 2018;10:466. <https://doi.org/10.1126/scitranslmed.aat4921>.
34. Jones PA, Ohtani H, Chakravarthy A, De Carvalho DD. Epigenetic therapy in immune-oncology. *Nat Rev Cancer* 2019;19:151–61.
35. Liu MC, Oxnard GR, Klein EA, Swanton C, Seiden MV, Liu MC, et al. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. *Ann Oncol* 2020;31:745–59.
36. Pérez-Barrios C, Sánchez-Herrero E, García-Simón N, Barquín M, Clemente MB, Provencio M, et al. ctDNA from body fluids is an adequate source for EGFR biomarker testing in advanced lung adenocarcinoma. *Clin Chem Lab Med* 2021;59:1221–9.
37. Tivey A, Church M, Rothwell D, Dive C, Cook N. Circulating tumour DNA – looking beyond the blood. *Nat Rev Clin Oncol* 2022;19: 600–12.
38. Xu Z, Ge G, Guan B, Lei Z, Hao X, Zhou Y, et al. Noninvasive detection and localization of genitourinary cancers using urinary sediment DNA methylomes and copy number profiles. *Eur Urol* 2020;77:288–90.
39. Oshi M, Murthy V, Takahashi H, Huyser M, Okano M, Tokumaru Y, et al. Urine as a source of liquid biopsy for cancer. *Cancers (Basel)* 2021;13: 2652.
40. Li G, Wang Y, Wang Y, Wang B, Liang Y, Wang P, et al. PCaseek: ultraspecific urinary tumor DNA detection using deep learning for prostate cancer diagnosis and Gleason grading. *Cell Discov* 2024;10: 90.
41. Ge G, Peng D, Guan B, Zhou Y, Gong Y, Shi Y, et al. Urothelial carcinoma detection based on copy number profiles of urinary cell-free DNA by shallow whole-genome sequencing. *Clin Chem* 2020;66:188–98.
42. Birkenkamp-Demtröder K, Nordentoft I, Christensen E, Høyer S, Reinert T, Vang S, et al. Genomic alterations in liquid biopsies from patients with bladder cancer. *Eur Urol* 2016;70:75–82.
43. Zhang R, Zang J, Xie F, Zhang Y, Wang Y, Jing Y, et al. Urinary molecular pathology for patients with newly diagnosed urothelial bladder cancer. *J Urol* 2021;206:873–84.
44. Pierconti F, Rossi ED, Cenci T, Carlino A, Fiorentino V, Totaro A, et al. DNA methylation analysis in urinary samples: a useful method to predict the risk of neoplastic recurrence in patients with urothelial carcinoma of the bladder in the high-risk group. *Cancer Cytopathol* 2023;131:158–64.
45. Rapado-González Ó, Brea-Iglesias J, Rodríguez-Casanova A, Bao-Caamano A, López-Cedrún J, Triana-Martínez G, et al. Somatic mutations in tumor and plasma of locoregional recurrent and/or

- metastatic head and neck cancer using a next-generation sequencing panel: a preliminary study. *Cancer Med* 2023;12:6615–22.
46. Rapado-González Ó, Costa-Fraga N, Bao-Caamano A, López-Cedrún JL, Álvarez-Rodríguez R, Crujeiras AB, et al. Genome-wide DNA methylation profiling in tongue squamous cell carcinoma. *Oral Dis* 2024;30:259–71.
 47. Rapado-González Ó, Martínez-Reglero C, Salgado-Barreira Á, Santos MA, López-López R, Díaz-Lagares Á, et al. Salivary DNA methylation as an epigenetic biomarker for head and neck cancer. Part II: a cancer risk meta-analysis. *J Pers Med* 2021;11:696.
 48. Rapado-González Ó, Martínez-Reglero C, Salgado-Barreira Á, Muínelo-Romay L, Muínelo-Lorenzo J, López-López R, et al. Salivary DNA methylation as an epigenetic biomarker for head and neck cancer. Part I: a diagnostic accuracy meta-analysis. *J Pers Med* 2021;11:568.
 49. Wang Y, Springer S, Mulvey CL, Silliman N, Schaefer J, Sausen M, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. *Sci Transl Med* 2015;7:293ra104.
 50. Li YS, Jiang BY, Yang JJ, Zhang XC, Zhang Z, Ye JY, et al. Unique genetic profiles from cerebrospinal fluid cell-free DNA in leptomeningeal metastases of EGFR-mutant non-small-cell lung cancer: a new medium of liquid biopsy. *Ann Oncol* 2018;29:945–52.
 51. Rimelén V, Ahle G, Penciech E, Zinniger N, Deblieux A, Zalmá L, et al. Tumor cell-free DNA detection in CSF for primary CNS lymphoma diagnosis. *Acta Neuropathol Commun* . 2019;7:43. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/30885253>.
 52. Escudero L, Lloret A, Arias A, Díaz-Navarro A, Martínez-Ricarte F, Rubio-Perez C, et al. Circulating tumour DNA from the cerebrospinal fluid allows the characterisation and monitoring of medulloblastoma. *Nat Commun* 2020;11:5376.
 53. Mouliere F, Mair R, Chandrananda D, Marass F, Smith CG, Su J, et al. Detection of cell-free DNA fragmentation and copy number alterations in cerebrospinal fluid from glioma patients. *EMBO Mol Med* 2018;10:e9323.
 54. Zheng M-M, Li Y-S, Tu H-Y, Jiang B-Y, Yang J-J, Zhou Q, et al. Genotyping of cerebrospinal fluid associated with osimertinib response and resistance for leptomeningeal metastases in EGFR-mutated NSCLC. *J Thorac Oncol* 2021;16:250–8.
 55. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 2014;6:224ra24.
 56. Liu APY, Smith KS, Kumar R, Paul L, Bihannic L, Lin T, et al. Serial assessment of measurable residual disease in medulloblastoma liquid biopsies. *Cancer Cell* 2021;39:1519–30.e4.
 57. Tong L, Ding N, Tong X, Li J, Zhang Y, Wang X, et al. Tumor-derived DNA from pleural effusion supernatant as a promising alternative to tumor tissue in genomic profiling of advanced lung cancer. *Theranostics* 2019;9:5532–41.
 58. van't Erve I, Rovers KP, Constantinides A, Bolhuis K, Wassenaar EC, Lurvink RJ, et al. Detection of tumor-derived cell-free DNA from colorectal cancer peritoneal metastases in plasma and peritoneal fluid. *J Pathol Clin Res* 2021;7:203–8.
 59. Nair VS, Hui AB-Y, Chabon JJ, Esfahani MS, Stehr H, Nabat BY, et al. Genomic profiling of bronchoalveolar lavage fluid in lung cancer. *Cancer Res* 2022;82:2838–47.
 60. Folch EE, Pritchett MA, Nead MA, Bowling MR, Murgu SD, Krinsky WS, et al. Electromagnetic navigation bronchoscopy for peripheral pulmonary lesions: one-year results of the prospective, multicenter NAVIGATE study. *J Thorac Oncol* 2019;14:445–58.
 61. Rolfo C, Mack P, Scagliotti GV, Aggarwal C, Arcila ME, Barlesi F, et al. Liquid biopsy for advanced NSCLC: a consensus statement from the international association for the study of lung cancer. *J Thorac Oncol* 2021;16:1647–62.
 62. Arechederra M, Rullán M, Amat I, Oyon D, Zabalza L, Elizalde M, et al. Next-generation sequencing of bile cell-free DNA for the early detection of patients with malignant biliary strictures. *Gut* 2022;71:1141–51.
 63. Saura C, Ortiz C, Matito J, Arenas EJ, Suñol A, Martín Á, et al. Early-stage breast cancer detection in breast milk. *Cancer Discov* 2023;13:2180–91.
 64. Lee J-S, Cho EH, Kim B, Hong J, Kim Y-G, Kim Y, et al. Clinical practice guideline for blood-based circulating tumor DNA assays. *Ann Lab Med* 2024;44:195–209.
 65. Heitzer E, Ulz P, Geigl JB. Circulating tumor DNA as a liquid biopsy for cancer. *Clin Chem* 2015;61:112–23.
 66. Kataoka K, Mori K, Nakamura Y, Watanabe J, Akazawa N, Hirata K, et al. Survival benefit of adjuvant chemotherapy based on molecular residual disease detection in resected colorectal liver metastases: subgroup analysis from CIRCULATE-Japan GALAXY. *Ann Oncol* 2024;35:1015–25.
 67. González Á, Pérez Barrios C, Macher H, Sánchez-Carbayo M, Barco Sánchez A, Fernández Suárez A, et al. Recomendaciones preanalíticas para la obtención y análisis de ADN circulante a partir de sangre periférica. Recomendación (2018). *Rev del Lab Clínico* 2019;12:e40–6.
 68. Diaz IM, Nocon A, Held SAE, Kobilyar M, Skowasch D, Bronkhorst AJ, et al. Pre-analytical evaluation of Streck cell-free DNA blood collection tubes for liquid profiling in oncology. *Diagnostics* 2023;13. <https://doi.org/10.3390/diagnostics13071288>.
 69. Parackal S, Zou D, Day R, Black M, Guilford P. Comparison of Roche cell-free DNA collection tubes to Streck cell-free DNA BCT®s for sample stability using healthy volunteers. *Pract Lab Med* 2019;16:e00125.
 70. Meddeb R, Pisareva E, Thierry AR. Guidelines for the preanalytical conditions for analyzing circulating cell-free DNA. *Clin Chem* 2019;65:623–33.
 71. Nishimura F, Uno N, Chiang P-C, Kaku N, Morinaga Y, Hasegawa H, et al. The effect of in vitro hemolysis on measurement of cell-free DNA. *J Appl Lab Med* 2019;4:235–40.
 72. Shin S, Woo HI, Kim J-W, M.D. YK, Lee K-A. Clinical practice guidelines for pre-analytical procedures of plasma epidermal growth factor receptor variant testing. *Ann Lab Med* 2022;42:141–9.
 73. El Messaoudi S, Rolet F, Mouliere F, Thierry AR. Circulating cell free DNA: preanalytical considerations. *Clin Chim Acta* 2013;424:222–30.
 74. Genovese G, Kähler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med* 2014;371:2477–87.
 75. Acuna-Hidalgo R, Sengul H, Stehouwer M, van de Vorst M, Vermeulen SH, Kiemeny LALM, et al. Ultra-sensitive sequencing identifies high prevalence of clonal hematopoiesis-associated mutations throughout adult life. *Am J Hum Genet* 2017;101:50–64.
 76. Bellosillo B, Montagut C. High-accuracy liquid biopsies. *Nat Med* 2019;25:1820–1.
 77. Razavi P, Li BT, Brown DN, Jung B, Hubbell E, Shen R, et al. High-intensity sequencing reveals the sources of plasma circulating cell-free DNA variants. *Nat Med* 2019;25:1928–37.
 78. Laguna JC, Pastor B, Nalda I, Hijazo-Pechero S, Teixido C, Potrony M, et al. Incidental pathogenic germline alterations detected through

- liquid biopsy in patients with solid tumors: prevalence, clinical utility and implications. *Br J Cancer* 2024;130:1420–31.
79. Slavin TP, Banks KC, Chudova D, Oxnard GR, Odegaard JI, Nagy RJ, et al. Identification of incidental germline mutations in patients with advanced solid tumors who underwent cell-free circulating tumor DNA sequencing. *J Clin Oncol* 2018;36:JCO1800328.
 80. Stout LA, Kassem N, Hunter C, Philips S, Radovich M, Schneider BP. Identification of germline cancer predisposition variants during clinical ctDNA testing. *Sci Rep* 2021;11:13624.
 81. Mandelker D, Donoghue M, Talukdar S, Bandlamudi C, Srinivasan P, Vivek M, et al. Germline-focussed analysis of tumour-only sequencing: recommendations from the ESMO precision medicine working group. *Ann Oncol* 2019;30:1221–31.
 82. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of medical genetics and genomics and the association for molecular Pathology. *Genet Med* 2015;17:405–24.
 83. Rodríguez-Casanova A, Bao-Caamano A, Lago-Lestón RM, Brozos-Vázquez E, Costa-Fraga N, Ferreirós-Vidal I, et al. Evaluation of a targeted next-generation sequencing panel for the non-invasive detection of variants in circulating DNA of colorectal cancer. *J Clin Med* 2021;10:4487.
 84. Fairley JA, Cheetham MH, Patton SJ, Rouleau E, Denis M, Dequeker EMC, et al. Results of a worldwide external quality assessment of cfDNA testing in lung Cancer. *BMC Cancer* 2022;22:759.
 85. Keppens C, Dequeker EMC, Patton SJ, Normanno N, Fenizia F, Butler R, et al. International pilot external quality assessment scheme for analysis and reporting of circulating tumour DNA. *BMC Cancer* 2018;18:804.
 86. Lockwood CM, Borsu L, Cankovic M, Earle JSL, Gocke CD, Hameed M, et al. Recommendations for cell-free DNA assay validations. *J Mol Diagn* 2023;25:876–97.
 87. Godsey JH, Silvestro A, Barrett JC, Bramlett K, Chudova D, Deras I, et al. Generic protocols for the analytical validation of next-generation sequencing-based ctDNA assays: a joint consensus recommendation of the BloodPAC's analytical variables working group. *Clin Chem* 2020;66:1156–66.
 88. Stejskal P, Goodarzi H, Srovnal J, Hajdúch M, van 't Veer LJ, Magbanua MJM. Circulating tumor nucleic acids: biology, release mechanisms, and clinical relevance. *Mol Cancer* 2023;22:15.
 89. Henriksen TV, Reinert T, Christensen E, Sethi H, Birkenkamp-Demtröder K, Gögenur M, et al. The effect of surgical trauma on circulating free DNA levels in cancer patients—implications for studies of circulating tumor DNA. *Mol Oncol* 2020;14:1670–9.
 90. Deveson IW, Gong B, Lai K, LoCoco JS, Richmond TA, Schageman J, et al. Evaluating the analytical validity of circulating tumor DNA sequencing assays for precision oncology. *Nat Biotechnol* 2021;39:1115–28.
 91. Dao J, Conway PJ, Subramani B, Meyyappan D, Russell S, Mahadevan D. Using cfDNA and ctDNA as oncologic markers: a path to clinical validation. *Int J Mol Sci* 2023;24. <https://doi.org/10.3390/ijms241713219>.
 92. Singh RR. Next-generation sequencing in high-sensitive detection of mutations in tumors: challenges, advances, and applications. *J Mol Diagn* 2020;22:994–1007.
 93. Sánchez-Martín V, López-López E, Reguero-Paredes D, Godoy-Ortiz A, Domínguez-Recio ME, Jiménez-Rodríguez B, et al. Comparative study of droplet-digital PCR and absolute Q digital PCR for ctDNA detection in early-stage breast cancer patients. *Clin Chim Acta* 2024;552:117673.
 94. Mehta N, He R, Viswanatha DS. Correspondence on standards for the classification of pathogenicity of somatic variants in cancer (oncogenicity): joint recommendations of clinical genome resource (ClinGen), cancer genomics consortium (CGC), and variant interpretation for cancer consort. *Genet Med* 2022;24:1986–8.
 95. Rolfo C, Mack PC, Scagliotti GV, Baas P, Barlesi F, Bivona TG, et al. Liquid biopsy for advanced non-small cell lung cancer (NSCLC): a statement paper from the IASLC. *J Thorac Oncol* 2018;13:1248–68.
 96. Verzè M, Boscolo Bragadin A, Minari R, Pasello G, Perrone F, Scattolin D, et al. NGS detection of gene rearrangements and METexon14 mutations in liquid biopsy of advanced NSCLC patients: a study of two Italian centers. *J Liq Biopsy* 2024;4:100143.
 97. Li MM, Datto M, Duncavage EJ, Kulkarni S, Lindeman NI, Roy S, et al. Standards and guidelines for the interpretation and reporting of sequence variants in cancer: a joint consensus recommendation of the association for molecular Pathology, American society of clinical oncology, and College of American pathologists. *J Mol Diagn* 2017;19:4–23.
 98. Hasenleithner SO, Speicher MR. A clinician's handbook for using ctDNA throughout the patient journey. *Mol Cancer* 2022;21:81.
 99. Adashke JJ, Kato S, Lippman SM, Kurzrock R. The paradox of cancer genes in non-malignant conditions: implications for precision medicine. *Genome Med* 2020;12:16.
 100. Mateo J, Chakravarty D, Dienstmann R, Jezdic S, Gonzalez-Perez A, Lopez-Bigas N, et al. A framework to rank genomic alterations as targets for cancer precision medicine: the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT). *Ann Oncol Off J Eur Soc Med Oncol* 2018;29:1895–902.
 101. Boos L, Wicki A. The molecular tumor board – a key element of precision oncology. *Memo – Mag Eur Med Oncol* 2024;17:190–3.
 102. Leigh NB, Page RD, Raymond VM, Daniel DB, Divers SG, Reckamp KL, et al. Clinical utility of comprehensive cell-free DNA analysis to identify genomic biomarkers in patients with newly diagnosed metastatic non-small cell lung cancer. *Clin Cancer Res* 2019;25:4691–700.
 103. Turner NC, Kingston B, Kilburn LS, Kernaghan S, Wardley AM, Macpherson IR, et al. Circulating tumour DNA analysis to direct therapy in advanced breast cancer (plasmaMATCH): a multicentre, multicohort, phase 2a, platform trial. *Lancet Oncol* 2020;21:1296–308.
 104. Chakravarty D, Johnson A, Sklar J, Lindeman NI, Moore K, Ganesan S, et al. Somatic genomic testing in patients with metastatic or advanced cancer: ASCO provisional clinical opinion. *J Clin Oncol* 2022;40:1231–58.
 105. Merker JD, Oxnard GR, Compton C, Diehn M, Hurley P, Lazar AJ, et al. Circulating tumor DNA analysis in patients with cancer: American society of clinical oncology and College of American pathologists joint review. *J Clin Oncol* 2018;36:1631–41.
 106. Qiu P, Poehlein CH, Marton MJ, Laterza OF, Levitan D. Measuring tumor mutational burden (TMB) in plasma from mCRPC patients using two commercial NGS assays. *Sci Rep* 2019;9:114.
 107. Reichert ZR, Morgan TM, Li G, Castellanos E, Snow T, Dall'Olio FG, et al. Prognostic value of plasma circulating tumor DNA fraction across four common cancer types: a real-world outcomes study. *Ann Oncol* 2023;34:111–20.
 108. Rothwell DG, Ayub M, Cook N, Thistlethwaite F, Carter L, Dean E, et al. Utility of ctDNA to support patient selection for early phase clinical trials: the TARGET study. *Nat Med* 2019;25:738–43.

109. Tamborero D, Rubio-Perez C, Deu-Pons J, Schroeder MP, Vivancos A, Rovira A, et al. Cancer genome interpreter annotates the biological and clinical relevance of tumor alterations. *Genome Med* 2018;10:25.
110. Chang MT, Bhattarai TS, Schram AM, Bielski CM, Donoghue MTA, Jonsson P, et al. Accelerating discovery of functional mutant alleles in Cancer. *Cancer Discov* 2018;8:174–83.
111. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2012;2:401–4.
112. Griffith M, Spies NC, Krysiak K, McMichael JF, Coffman AC, Danos AM, et al. CIViC is a community knowledgebase for expert crowdsourcing the clinical interpretation of variants in cancer. *Nat Genet* 2017;49:170–4.
113. Patterson SE, Statz CM, Yin T, Mockus SM. Utility of the JAX clinical knowledgebase in capture and assessment of complex genomic cancer data. *NPJ Precis Oncol* 2019;3:2.
114. Landrum MJ, Chitipiralla S, Kaur K, Brown G, Chen C, Hart J, et al. ClinVar: updates to support classifications of both germline and somatic variants. *Nucleic Acids Res* 2025;53:D1313–21.
115. Bindal N, Forbes SA, Beare D, Gunasekaran P, Leung K, Kok CY, et al. COSMIC: the catalogue of somatic mutations in cancer. *Genome Biol* 2011;12:P3.
116. Ainscough BJ, Griffith M, Coffman AC, Wagner AH, Kunisaki J, Choudhary MN, et al. DoCM: a database of curated mutations in cancer. *Nat Methods* 2016;13:806–7.
117. Rodrigues E da S, Griffith S, Martin R, Antonescu C, Posey JE, Coban-Akdemir Z, et al. Variant-level matching for diagnosis and discovery: challenges and opportunities. *Hum Mutat* 2022;43:782–90.
118. Jain N, Mittendorf KF, Holt M, Lenoue-Newton M, Maurer I, Miller C, et al. The My Cancer Genome clinical trial data model and trial curation workflow. *J Am Med Inform Assoc* 2020;27:1057–66.
119. Huang L, Fernandes H, Zia H, Tavassoli P, Rennert H, Pisapia D, et al. The cancer precision medicine knowledge base for structured clinical-grade mutations and interpretations. *J Am Med Inform Assoc* 2017;24:513–9.
120. Chakravarty D, Gao J, Phillips S, Kundra R, Zhang H, Wang J, et al. OncoKB: a precision oncology knowledge base. *JCO Precis Oncol* 2017;2017:1–16.
121. Kopanos C, Tsiolkas V, Kouris A, Chapple CE, Albarca Aguilera M, Meyer R, et al. VarSome: the human genomic variant search engine. *Bioinformatics* 2019;35:1978–80.
122. Chung DC, Gray DM, Singh H, Issaka RB, Raymond VM, Eagle C, et al. A cell-free DNA blood-based test for colorectal cancer screening. *N Engl J Med* 2024;390:973–83.
123. Cohen JD, Javed AA, Thoburn C, Wong F, Tie J, Gibbs P, et al. Combined circulating tumor DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers. *Proc Natl Acad Sci U S A* 2017;114:10202–7.
124. Tie J, Wang Y, Tomasetti C, Li L, Springer S, Kinde I, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. *Sci Transl Med* 2016;8. <https://doi.org/10.1126/scitranslmed.aaf6219>.
125. Nakamura Y, Watanabe J, Akazawa N, Hirata K, Kataoka K, Yokota M, et al. ctDNA-based molecular residual disease and survival in resectable colorectal cancer. *Nat Med* 2024;30:3272–83.
126. Sahin IH, Lin Y, Yothers G, Lucas PC, Deming D, George TJ, et al. Minimal residual disease-directed adjuvant therapy for patients with early-stage colon cancer: CIRCULATE-US. *Oncology* 2022;36:604–8.
127. Tarazona N, Gimeno-Valiente F, Gambardella V, Zuñiga S, Rentero-Garrido P, Huerta M, et al. Targeted next-generation sequencing of circulating-tumor DNA for tracking minimal residual disease in localized colon cancer. *Ann Oncol* 2019;30:1804–12.
128. Gale D, Heider K, Ruiz-Valdepenas A, Hackinger S, Perry M, Marsico G, et al. Residual ctDNA after treatment predicts early relapse in patients with early-stage non-small cell lung cancer. *Ann Oncol* 2022;33:500–10.
129. Coombes RC, Page K, Salari R, Hastings RK, Armstrong A, Ahmed S, et al. Personalized detection of circulating tumor DNA antedates breast cancer metastatic recurrence. *Clin Cancer Res* 2019;25:4255–63.
130. Hrebien S, Citi V, Garcia-Murillas I, Cutts R, Fenwick K, Kozarewa I, et al. Early ctDNA dynamics as a surrogate for progression-free survival in advanced breast cancer in the BEECH trial. *Ann Oncol Off J Eur Soc Med Oncol* 2019;30:945–52.
131. van de Haar J, Roepman P, Andre F, Balmaña J, Castro E, Chakravarty D, et al. ESMO Recommendations on clinical reporting of genomic test results for solid cancers. *Ann Oncol* 2024;35:954–67.
132. Tivey A, Lee RJ, Clipson A, Hill SM, Lorigan P, Rothwell DG, et al. Mining nucleic acid “omics” to boost liquid biopsy in cancer. *Cell Rep Med* 2024;5:101736.
133. Prelaj A, Ganzinelli M, Trovo’ F, Roisman LC, Pedrocchi ALG, Kosta S, et al. The EU-funded I3LUNG project: integrative science, intelligent data platform for individualized LUNG cancer care with immunotherapy. *Clin Lung Cancer* 2023;24:381–7.
134. Henriksen TV, Demuth C, Frydendahl A, Nors J, Nesic M, Rasmussen MH, et al. Unraveling the potential clinical utility of circulating tumor DNA detection in colorectal cancer-evaluation in a nationwide Danish cohort. *Ann Oncol Off J Eur Soc Med Oncol* 2024;35:229–39.
135. Lockwood CM, Merker JD, Bain E, Compton C, Grossman RL, Johann D, et al. Towards preanalytical best practices for liquid biopsy studies: a BLOODPAC landscape analysis. *Clin Pharmacol Ther* 2025;117:28–33.
136. Weber S, Spiegl B, Perakis SO, Ulz CM, Abuja PM, Kashofer K, et al. Technical evaluation of commercial mutation analysis platforms and reference materials for liquid biopsy profiling. *Cancers* 2020;12:1588.
137. Sequeira JP, Salta S, Freitas R, López-López R, Díaz-Lagares Á, Henrique R, et al. Biomarkers for pre-treatment risk stratification of prostate cancer patients: a systematic review. *Cancers* 2024;16:1363.
138. Akkari Y, Smith T, Westfall J, Lupo S. Implementation of cancer next-generation sequencing testing in a community hospital. *Cold Spring Harb Mol Case Stud* 2019;5. <https://doi.org/10.1101/mcs.a003707>.

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