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Review Article

# Blood-borne Biomarkers: CT-DNA Ushers in a New Era of Cancer Detection

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ABSTRACT

Circulating tumor DNA, also called ctDNA, is gaining popularity as a valuable tool for monitoring cancer through non-invasive methods. This review seeks to offer a comprehensive overview of ctDNA, encompassing its biological basis, detection technologies, clinical applications, and challenges. ctDNA originates from tumor cells that undergo apoptosis and necrosis and contain cancer-specific genomic and epigenomic alterations. Sensitive methodologies leveraging sequencing, digital PCR, and enzymatic assays enable the analysis of mutations, methylation patterns, and copy number variations. ctDNA facilitates early diagnosis, minimal residual disease tracking, therapeutic monitoring, detection of emergent resistance mutations, and prognostic estimates across diverse cancer types. Ongoing trials aim to validate ctDNA's clinical utility and determine whether ctDNA-guided early interventions enhance outcomes. Detection using ctDNA analysis faces some challenges, including specificity, sensitivity, and result interpretation. Research is needed to advance the detection technology, development of standards, and establish clinical validity. Overall, the analysis of plasma ctDNA provides a powerful and minimally invasive avenue for understanding tumor dynamics in real time to enable personalized therapeutic approaches, monitoring, and potentially early cancer detection.

## Introduction of CT-DNA

In the search for non-invasive methods for cancer diagnosis, evaluating therapeutic response, molecular profiling, and tumor growth, detecting circulating tumor DNA (ctDNA) and analyzing it has emerged as a promising approach. ctDNA consists of single or double-stranded DNA fragments that have been shed by tumor cells in the bloodstream, carrying mutations from the primary tumor.[1]

Freely circulating DNA is present in the bloodstream of not only sick, but healthy individuals as well. Mandel and Métais first demonstrated the presence of extracellular genetic material in human plasma in their 1948 study, where they identified it among patients with SLE, or systemic lupus erythematosus. This groundbreaking discovery opened the door for further research by Thierry *et al*. in 2016 into the presence of freely circulating DNA in the bloodstreams of both healthy and ill individuals. Three decades later, Leon *et al*. (1977) identified increased concentrations of cell-free DNA (cfDNA) in the blood of cancer patients, distinguishing them quantitatively from healthy individuals. Subsequently, in 1989, Stroun, along with some colleagues postulated the existence of ctDNA that originated from tumor cells in plasma and serum samples in 1989.[2 -5]

Further evidence substantiated the tumor origin of these circulating DNA fragments. Sorenson *et al*. (1994) identified point mutations in a gene within the plasma

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(N-Ras gene), which originated from the bone marrow tumor cells. Fig. 1 describes a brief history of the discovery of ctDNA. Simultaneously, Vasioukhin and colleagues (1994) identified mutated K-Ras sequences within the plasma of pancreatic cancer patients, further supporting the conclusion that these mutated DNA sequences were indeed originating from cancerous cells. Despite the ubiquitous presence of DNA in bodily fluids, the precise molecular origins remain inadequately elucidated, with multiple potential sources speculated. Circulating DNA (CirDNA), also called cfDNA refers to any extracellular DNA, irrespective of its structural association with extracellular vesicles or protein complexes.[2-5]

Neoplastic cells discharge circulating tumor DNA (ctDNA) in the bloodstream *via* biological processes like programmed cell death, necrosis, or active excretion.[6] When malignant cells undergo cell death, they liberate ctDNA fragments into the circulatory system, which can subsequently be detected and analyzed as a biomarker for neoplastic diseases.[7] These ctDNA fragments can be isolated from the plasma, which is derived from the blood of cancer patients.[8] Plasma contains not only ctDNA from tumorigenic origin but also genetic material originating from normal cells or clonal hematopoietic progenitors. The ctDNA released by tumor cells has a relatively short circulatory half-life, ranging from approximately 16 minutes to 2.5 hours.[9,10] This rapid clearance from the bloodstream renders ctDNA a dynamic and contemporaneous biomarker for monitoring tumor progression and therapeutic response.

## Biological Basis of ctDNA

Numerous sources of cell-free DNA (cfDNA), have been found to date, encompassing endogenous as well as exogenous origins, such as necrosis, apoptosis, and viral and bacterial genetic material.[2] Necrosis and apoptosis are the most important factors resulting in the formation of ctDNA. Macrophages normally phagocytose necrotic and apoptotic cells, releasing digested DNA fragments into the surrounding tissue microenvironment.[11] Several investigations, however, have shown that cfDNA can be produced by an active cellular release process that is not dependent on cell death. The sequential process of ctDNA release and circulation is depicted in Fig. 2. Furthermore,

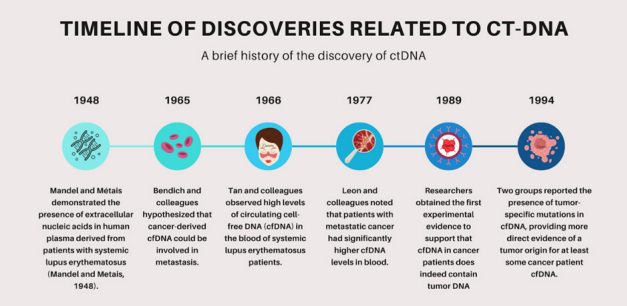


**Fig. 1:** Timeline of discoveries related to ctDNA. Information adapted from references[25]

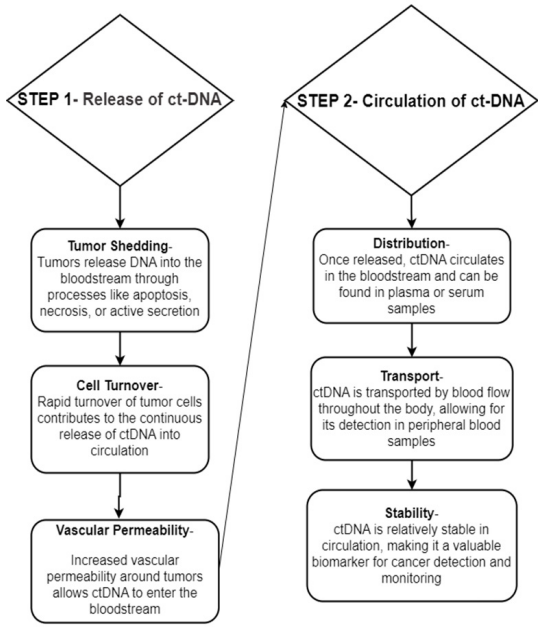


**Fig. 2:** Biological basis of ctDNA[14-17]

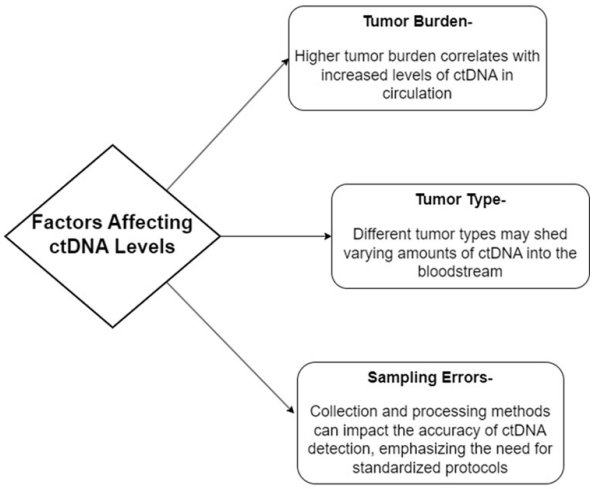


**Fig. 3:** Factors affecting ctDNA levels[14-17]

another source of DNA in the body fluids has been found to be erythroid cells, which release DNA during terminal differentiation.[12,13]

Various mechanisms enable the transfer of DNA from intracellular to extracellular compartments, ensuring the molecule's structural stability. Two key theories have been suggested to explain the primary origins of cfDNA: (I) processes related to cellular breakdown and (II) mechanisms involving active DNA release. Fig. 3 outlines the factors influencing ctDNA levels.[14-17]

## Detection Methods and Technologies

The detection of ctDNA involves a wide range of techniques (as shown in Fig. 4), including tumor-informed and tumor-naive assays, hybridization-based capture, multiplex PCR, and sequencing-based approaches for detection.[18,19]

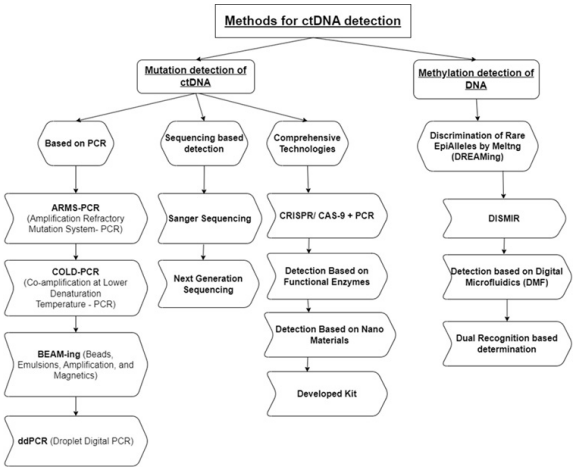


**Fig. 4:** Methods for ctDNA detection[18]

Tumor-informed assays, like multiplex PCR designed to identify tumor-specific structural variants (SVs), have shown excellent sensitivity in identifying ctDNA even at minimal concentrations. The approach with the highest sensitivity is Hybrid capture sequencing, which focuses on a broad range of tumor-specific mutations with high depth, allowing for the detection of ctDNA at extremely low allele frequencies. [19]

Another technique, known as hybridization- and tag-based error-corrected sequencing (HYTEC-seq), utilizes both hybridization-based capture and molecular tagging, demonstrating significant sensitivity for ctDNA detection. Additionally, sequencing-based techniques, such as high-throughput sequencing, have been commonly employed for the analysis of ctDNA mutations and methylation patterns. Furthermore, innovative methods, including the utilization of the CRISPR/Cas system and graphene, have been explored for ctDNA detection. Table 1 compares the most common ctDNA detection techniques based on various parameters. Together, each of these techniques offers a diverse approach towards precise and sensitive detection of ctDNA throughout many stages of cancer development.[19]

Gene mutations along with DNA methylation are pivotal factors in the detection of ctDNA, as alterations in driving genes can promote tumor formation, and DNA methylation can impede transcription; consequently, various effective detection methods have been developed based on PCR principles. [18, 20]

## Clinical Applications

Applications of ctDNA analysis in cancer diagnosis, treatment, and monitoring are outlined in Table 2.

### *Enhancing precision in tracking treatment response and detecting minimal residual disease*

The detection of minimal residual disease (MRD) through ctDNA frequently utilizes next generation sequencing (NGS), a method renowned for its capacity to identify tumor-specific genomic changes with high throughput. However, the error rate of NGS can range from 1 to 0.01%, depending on the specific sequencer used. Two key personalized ctDNA detection methods are tumor-customized panels, which sequence biopsies to create custom panels targeting tumor mutations, and custom PCR assays. While effective at detecting low-frequency variants, these methods depend on biopsy quality and face challenges due to tumor heterogeneity.

Droplet digital PCR (ddPCR) is highly specific for detecting predetermined genomic variants with variant allele frequencies (VAF) as low as 0.01%, particularly useful for hematological neoplasia and solid tumors with known driver mutations. Non-personalized approaches, like NGS gene panels and PCR, identify mutations in various tumors. In summary, ctDNA-based MRD detection includes personalized methods, which offer detailed tumor insights, and non-personalized approaches, which have broader applicability but face sensitivity challenges. Advances in sequencing technologies continue to improve the precision of MRD detection and treatment response monitoring.[36]

### *Predicting treatment success with ctDNA: unraveling its prognostic power*

In a prospective study involving early-stage colorectal cancer patients, the presence of detectable ctDNA at the initial follow-up after surgery was associated with a 100% relapse rate within three years, whereas the relapse rate was 10% in patients who were ctDNA-negative. CtDNA monitoring demonstrates potential for assessing tumor resistance and treatment effectiveness, providing a less invasive option compared to traditional biopsies and allowing for more frequent updates on tumor genetics. FDA-approved "liquid biopsies" for EGFR mutation testing highlight its clinical value. CtDNA's ability to detect resistance before clinical symptoms represents a

**Table 1:** Comparison between various methods of ctDNA detection[20-22]

| *Assay category* | *Technology* | *Target size* | *Advantages* | *Limitations* |
|---|---|---|---|---|
| PCR-based assays | PCR | Single mutations, small panels | High specificity, low cost | Low sensitivity, limited scope |
| Next-generation sequencing (NGS) | Sequencing | Whole genome, targeted panels | High sensitivity, broad scope | High cost, potential for false positives |
| Enzyme-based assays | Enzymes | Specific DNA sequences | High specificity, enrichment for mutant DNA | Limited target range, under development |

**Table 2:** Applications of ctDNA

| *Application of ctDNA* | *Explanation* | *References* |
|---|---|---|
| Diagnosis | ctDNA analysis plays a crucial role in early cancer detection. By detecting cancer-specific mutations and methylation changes in the bloodstream, it enables diagnosis even before clinical symptoms become evident.<br>Characterizing tumor molecular profiles is essential for personalized medicine. ctDNA analysis helps achieve this by identifying unique genetic alterations specific to the tumor.<br>Regular monitoring of ctDNA levels provides a dynamic view of disease progression. | [15,23,24] |
| Minimal residual disease (MRD) monitoring | MRD identification: ctDNA analysis detects residual cancer cells post-treatment, aiding in assessing disease recurrence risk.<br>Survival estimates: ctDNA-guided treatment decisions enhance overall survival rates for patients with minimal residual disease (MRD).<br>Reduced false positives: Compared to tissue biopsy, ctDNA analysis offers a less invasive option with fewer complications and false positives | [15,25,26] |
| Therapy monitoring | ctDNA analysis identifies emerging resistance mutations promptly, enabling timely intervention and treatment adjustments.<br>ctDNA levels predict treatment outcomes and survival rates. | [27,28] |
| Screening | ctDNA-based screening tests exhibit high specificity (>99%) but moderate sensitivity (around 50 to 70%).<br>High negative predictive value (NPV): ctDNA tests effectively exclude cancer in asymptomatic individuals. | [15] |
| Personalized medicine | Tailoring treatment decisions by analyzing the tumor's molecular profile. | [29,30] |
| Adjuvant setting | ctDNA-guided approaches can decrease adjuvant chemotherapy utilization in specific cancers, like stage II colorectal cancer, while maintaining recurrence-free survival rates. | [31] |
| Non-invasive sampling | Eliminating the requirement of invasive procedures for instance, biopsies | [32,33] |
| Companion diagnostics | Used alongside traditional diagnostic methods, providing additional insights into cancer progression and treatment response | [34] |
| Clinical trials | Through the utilization of ctDNA, clinical trials have the capability to identify individuals who could gain advantages from early interventions, thereby possibly reducing sample sizes and improving statistical power.<br>The dynamics or elimination of ctDNA can serve as an alternative endpoint within clinical trials, expediting new therapeutic approvals and offering early insights into treatment efficacy. | [35] |

**Table 3:** Actionable gene variations and therapeutic responses in various cancer types[39-42]

| *Cancer type* | *Actionable gene* | *Response/Resistance* | *Therapy* |
|---|---|---|---|
| Melanoma | BRAF mutation | Results in therapeutic response | Vemurafenib + Trametinib |
| Colorectal | K-RAS mutation | Associated with resistance | Panitumumab |
| Breast | PIK3CA mutation | Induces therapeutic response | Alpelisib (typically used in combination with fulvestrant)[c] |
| Breast | HER2 mutation | Elicits therapeutic response | Neratinib |
| Breast | AKT mutation | Results in therapeutic response | Capivasertib |
| NSCLC | EGFR mutation (ex 19 del, L858R) | Leads to therapeutic response | Erlotinib, gefitinib |
| NSCLC | EGFR mutation (T790M) | Associated with Resistance | Erlotinib, gefitinib |
| NSCLC | EGFR mutation (T790M)[b] | Induces therapeutic response | Osimertinib |
| NSCLC | Translocated ALK | Elicits therapeutic response | Alectinib, lorlatinib |
| Multiple | Tumor mutational burden (TMB) | Induces therapeutic response | Immunotherapy |
| Multiple | Microsatellite instability (MSI) | Elicits therapeutic response | Immunotherapy |

significant advancement, with escalating ctDNA levels in breast cancer indicating imminent relapse more effectively than imaging. Personalized ctDNA panels for early NSCLC patients can detect tumors an average of 70 days before radiological evidence. Elevated ctDNA levels correlate with greater disease burden and larger tumor size, showing a 100-fold increase in stage IV disease compared to stage I. However, the effectiveness of ctDNA in detecting small cancers in asymptomatic individuals has not yet been established.[37]

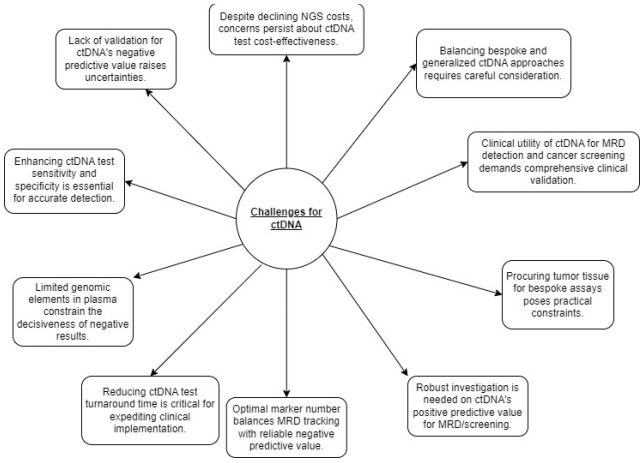


**Fig. 5:** Challenges in detection of ctDNA[43]

- *Advantages of ctDNA-based liquid biopsies*

Include being non-invasive for continuous tumor monitoring, enabling frequent assessments for timely treatment decisions, and identifying specific mutations for personalized treatment strategies.

- *Limitations of ctDNA-based liquid biopsies*

Includes sensitivity issues in detecting small tumors, sampling errors from limited blood samples, specificity challenges due to mutations in non-cancer individuals, and high costs and complexity hindering widespread use.[37]

## Impact of CtDNA Analysis on Therapeutic Decision-Making

CtDNA analysis has revolutionized oncology by enabling precise, tailored treatment strategies through the identification of actionable gene variations in various cancers, as summarized in Table 3.[39-42]

## Challenges

The use of ctDNA as a crucial tool in guiding cancer prevention and therapy faces a range of challenges that require thorough consideration and resolution to effectively integrate it into clinical practice.

Proving the clinical utility of ctDNA, especially in detecting minimal residual disease (MRD) and in primary cancer screening, necessitates extensive clinical validation to establish its predictive value and effectiveness in enabling significant interventions. Reducing the turnaround time for ctDNA-based tests is critical to ensuring timely clinical decision-making.

Despite the decreasing costs of NGS, the cost-effectiveness of ctDNA tests remains an issue, requiring further reductions to enhance accessibility. Improving the sensitivity and specificity of ctDNA tests is crucial for increasing their accuracy in detecting disease presence. Furthermore, the limited data on the positive predictive value of ctDNA in MRD detection and screening highlights the need for thorough investigation.

Similarly, the lack of validation of its negative predictive value raises uncertainties about its ability to definitively rule out cancer. The balance between bespoke and generalized ctDNA approaches presents another challenge, as personalized assays, while offering potentially higher accuracy, face practical limitations such as inadequate tissue samples and time constraints. Additionally, the limited number of genomic elements found in plasma samples affects the reliability of negative results, necessitating strategies to increase the number of mutations or methylated DNA detected for MRD monitoring. However, these approaches require thorough validation to improve both positive and negative predictive values. Challenges also persist in obtaining sufficient tumor tissue for bespoke assays, complicating the practicality of these personalized approaches in clinical settings. Fig. 5 shows the challenges involved in the detection of ctDNA.[43]

Finally, identifying the optimal number of markers for effective MRD tracking is essential. While increasing the number of markers may improve the positive predictive value, their effect on the negative predictive value needs further investigation to ensure the clinical utility of ctDNA tests in accurately identifying patients who are truly cured of their disease. [43]

### *Ongoing research aims to enhance ctDNA detection and interpretation in several ways*

Efforts to advance ctDNA assays are concentrated on several key areas: enhancing sensitivity and specificity through innovative technologies and bioinformatics tools to identify low-frequency mutations while reducing false positives and negatives; standardizing methods for ctDNA sampling, preservation, and analysis to ensure consistent results and enable cross-study comparisons; and broadening detection capabilities to encompass chromosomal rearrangements, copy number variations, methylation patterns, and gene expression profiles.

Additional initiatives involve evaluating mitochondrial DNA as an alternative ctDNA source when nuclear DNA shedding is limited, integrating ctDNA with other biomarkers and imaging modalities to enhance diagnostic and treatment accuracy, and analyzing ctDNA dynamics and clearance to determine if these changes can serve as alternative endpoints in clinical trials and predict treatment responses. Collectively, these efforts aim to establish ctDNA as a dependable and widely applicable tool in personalized medicine, early cancer detection, and monitoring.

## Regulatory Guidelines Related for CT-DNA Testing

Regulatory directives governing the use of ctDNA in clinical practice and trials encompass guidelines for patient selection, assay considerations, and the use of investigational devices. In adjuvant treatment, ctDNA is employed for patient selection based on genetic or epigenetic alterations, with a thorough evaluation of assay sensitivity recommended.[45]

CtDNA acts as a marker for MRD following surgery or (neo)adjuvant therapy, helping to refine patient selection and guide treatment strategies, including escalation or de-escalation, within clinical trials. For response evaluation, ctDNA helps identify drug activity, and its role as a benchmark in trials is being explored, though more data is needed to establish its predictive value for long-term outcomes.

When designing MRD panels, tumor-informed, tumor-naïve or tumor-agnostic options should be considered, with an emphasis on their strengths and limitations. Tumor characteristics and the timing of ctDNA testing impact assay results, so standardized protocols for sample collection, storage, and processing are critical. Baseline pre-treatment samples are essential for accurate assay performance. Analytical validation studies, which assess sensitivity, specificity, accuracy, and precision, are essential for marketing applications and encompass the entire assay process, from sample collection to result interpretation. High sensitivity and specificity are especially critical for supporting treatment decisions.

Investigational ctDNA devices used in trials are subject to the FDA's investigational device exemption (IDE) regulations, which differentiate between significant risk (SR) and non-significant risk (NSR) devices, requiring clear delineation.[46]

## Conclusion and Future Scope

In summary, this review encompasses a comprehensive analysis of ctDNA, spanning its biological and technological underpinnings to diverse clinical applications and ethical considerations. CtDNA analysis has rapidly gained traction as a minimally invasive tool with immense promise in advancing personalized oncology, albeit with continuing challenges that necessitate careful consideration. Ongoing and future research efforts, integrating bioinformatic, statistical, and technological innovations, are requisite to push this swiftly evolving field toward more refined and validated clinical applications. While obstacles persist, the emergence of ctDNA signifies a novel paradigm in furnishing molecular insights with remarkable potential and versatility to transform diverse facets of the cancer care continuum ranging from screening to molecular diagnostics, therapeutic decisions, monitoring, longitudinal tracking of residual disease, detection of emergent resistance, and recurrence predictions.

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