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CELL-FREE DNA & CIRCULATING TUMOR CELLS: THE CORE OF LIQUID BIOPSY IN CANCER

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ABSTRACT

Liquid biopsy has emerged as a transformative, minimally invasive approach for cancer diagnosis, prognosis, and therapeutic monitoring. Among its key analytes, cell-free DNA (cfDNA) and circulating tumor cells (CTCs) represent the most informative biomarkers that reflect the molecular and cellular landscape of tumours in real time. cfDNA, including tumor-derived fragments known as circulating tumor DNA (ctDNA), provides valuable genetic and epigenetic insights into tumor heterogeneity, mutation profiling, and treatment resistance mechanisms. Conversely, CTCs offer cellular-level information, enabling phenotypic characterization, enumeration, and culture for functional assays. Together, cfDNA and CTCs form the core of the liquid biopsy concept, offering a comprehensive view of tumor dynamics through a simple blood draw. Recent technological advancements in next-generation sequencing, digital PCR, and microfluidic-based CTC isolation have significantly improved the sensitivity and specificity of these biomarkers. This review summarizes the biology, detection methodologies, and clinical relevance of cfDNA and CTCs, highlighting their synergistic role in early cancer detection, disease monitoring, and personalized therapy. The integration of cfDNA and CTC analyses holds promise for advancing precision oncology and transforming cancer management into a more predictive, preventive, and patient-centric paradigm.

KEYWORDS: Cell-free DNA, Circulating tumor cells, Liquid biopsy, Cancer biomarkers, Precision oncology.

INTRODUCTION

Overview of Liquid Biopsy: The gold standard for identifying and tracking certain malignant tumours, including colorectal, stomach, and lung cancers, is currently pathological tissue biopsy. This approach makes it possible to quickly evaluate the size and kind of lesions, offers a somewhat good pathological categorization, and makes early detection and diagnosis easier. Furthermore, tissue biopsies performed during treatment can reveal the course of the disease and the effectiveness of the treatment, giving doctors important information to customise treatment regimens.[1] However, using limited or inadequate tissue samples can result in diagnostic bias, which tumour heterogeneity can make worse.[2] Additionally, when the condition worsens, patients may experience discomfort from repeated intrusive operations. A number of these issues have been successfully resolved by the development of liquid biopsy. In order to assess physiological conditions, liquid biopsy uses molecular analysis of liquid (non-tissue) samples.[3] Other body fluids, including cerebrospinal fluid (CSF), saliva, pleural effusion, bile, stomach fluid, and urine, can also be used, though blood samples are the most frequently employed.[4] Analysis of circulating tumour cells (CTCs), circulating tumour DNA (ctDNA), circulating cell-free (cf)RNA, extracellular vesicles, and tumor-inducing platelets are the main objectives of liquid biopsy.[5]

Need for Non-Invasive Cancer Diagnostics: In the last ten years, non-invasive approaches like liquid biopsies have gradually supplanted invasive treatments for cancer

diagnosis and monitoring. Clinical oncology has seen a significant transformation thanks to liquid biopsies, which make it simple to sample tumours, provide ongoing monitoring through frequent sampling, allow for the creation of individualised treatment plans, and screen for resistance to treatment. The process of liquid biopsies involves separating tumor-derived materials from cancer patients' bodily fluids, such as circulating tumour cells, circulating tumour DNA, tumour extracellular vesicles, etc., and then analysing the genomic and proteomic information found there. As the paper explains, techniques for isolating and analysing liquid samples have advanced quickly in recent years, offering more information about tumour features such tumour growth, tumour staging, heterogeneity, clonal evolution, gene mutations, etc. Precision medicine-based cancer treatment, ongoing monitoring, and the identification of therapeutic resistance markers have all been made possible by liquid biopsies taken from cancer patients. Despite ongoing advancements, the non-invasive nature of liquid biopsies holds the potential to usher in new eras in clinical oncology. This study aims to give a broad overview of the current approaches used in liquid biopsies and how they are used to isolate tumour markers for prognosis, detection, and tracking the effectiveness of cancer treatment. About 4% of primary central nervous system (CNS) cancers in immunocompetent patients are caused by the rare disease primary central nervous system lymphoma (PCNSL).[6] Non-Hodgkin lymphomas that are restricted to the brain, eyes, leptomeninges, and, in rare instances, the spinal cord make up the rare subgroup known as PCNSL. The absence of any systemic illness presentation upon diagnosis is one of the characteristics of PCNSL, which must be distinguished from secondary systemic lymphoma disseminated to the central nervous system (secondary CNS lymphoma, SCNSL). SCNSL is therefore characterised as parallel lymphoma involvement both inside and outside the central nervous system. Diffuse large B-cell lymphomas make up around 95% of PCNSLs, with T-cell, marginal zone, Burkitt, and lymphoblastic lymphomas making up the remaining 5%.[7] The typical age range for PCNSL development is 50–70 years old, while the illness can strike at any age.[8] Both inherited and acquired immunodeficiencies are risk factors for PCNSL (especially HIV and post-transplant conditions). PCNSLs have a worse prognosis and a more invasive development pattern than traditional diffuse large B-cell lymphoma (DLBCL).[6] PCNSL reacts well to radiation and chemotherapy, in contrast to other primary brain cancers. However, compared to systemic non-CNS lymphomas, survival is typically lower. Furthermore, some patients continue to have extremely dismal prognoses, such as the elderly or those with recurrent or refractory disorders. Numerous pathogenetic processes, such as dysregulations in the signalling pathways of NF-kB, JAK/STAT, Toll-like receptors, and B-cell receptors, have been identified in PCNSL. Mutations in some genes, such as MYD88 (35–80%), PIM1 (69%), TBL1XR1 (24%), TRDM1 (24%), BTG2 (29%), and PRDM1 (24%), frequently play a role in the pathogenesis of disease.[9] Diagnostic brain imaging should be performed immediately on patients who exhibit neurologic symptoms and impairments. The preferred imaging technique is magnetic resonance imaging (MRI). In this case, 60–70% of PCNSLs manifest as solitary lesions, and less frequently, as multifocal illnesses.[10] The majority of periventricular lymphoma lesions involve the corpus callosum, the basal ganglia, or the white matter of the centrum semi vale. On T1-weighted imaging, PCNSLs are usually iso-hypointense, and on T2-weighted imaging, they are iso-hypointense to grey matter. About 85% of PCNSLs have strong homogenous patterns due to the hypercellularity of the lymphomatous lesions. Usually, there is a modest degree of oedema surrounding the lesions. It should be noted that in typical clinical settings, neurological symptoms and MRI results can mimic high-grade gliomas, neurosarcoidosis, tumefactive demyelinating lesions, a rare form of multiple sclerosis, or infectious and other granulomatous disorders. Neuroimaging patterns are suggestive but not diagnostic of PCNSL, despite significant advancements in radiography methods. Therefore, stereotactic brain biopsy, along with histology and immunohistochemistry staining, is still required to achieve a clear diagnosis. With a reported failure rate of up to 35%, stereotactic biopsy is still difficult and unpleasant.[11] Additionally, around 1% of procedures are reported to have serious side effects, such as hematomas, seizures, and cerebral edoema, in addition to biopsy- related death.[12,13] Our own experience, however, indicates that there is very little morbidity and that over 90% of brain biopsies for CNS cancer are effective. In certain cases, a biopsy cannot be performed because of the lesion's small size or deep location. Interestingly, stereotactic brain biopsies and the histopathologic analyses that follow take a lot of time and can delay diagnosis and treatment. Given that a significant obstacle in the treatment of CNS lymphoma is the delay in diagnosis, this may be pertinent.[14] The use of corticosteroids is another possible barrier to lymphoma diagnosis. Before a definitive pathology diagnosis is made, steroids are frequently used to manage symptoms. In fact, because corticosteroids cause lymphoma cells to undergo fast apoptosis, they hinder a definitive (histopathological) diagnosis.[15]

Standard CSF Diagnostics: Unless lumbar puncture is contraindicated (for example, because of abnormal intracranial pressure, impaired CSF kinetics, cerebral herniation, space- occupying lesions with mass effect in the large posterior fossa, Arnold-Chiari malformation, or a high risk of bleeding), CSF analysis is a mandatory component of the standard diagnostic approach. Although contemporary technologies such as cellular immunophenotyping by flow cytometry, molecular genetics, and proteochemical analyses have been further developed to support the diagnosis of leptomeningeal lymphoma, cytopathologic analysis of the CSF is still the gold standard for the diagnosis of leptomeningeal

malignant disease.[16] However, cellular CSF evaluations like cytopathology and immunophenotyping can only be used to conclusively diagnose CNS lymphoma in a small percentage of patients.

Table 1: Overview of standard diagnostic methods.

Biomarker/ Method	Body Fluid	Number of Patients	Sensitivity (%)	Specificity (%)	References
Cytology	CSF	37	13.30	-	[17]
flow cytometry	CSF	37	23.3	-	[17]

In order to speed up the diagnostic process, there is an unmet need to create more sophisticated diagnostic tests. Even though there have been a lot of studies recently on non- invasive PCNSL diagnoses, none of the novel techniques have yet to become standard clinical procedures. With a clinical focus, we plan to go over the most promising non- invasive tests for PCNSL diagnosis in peripheral blood and cerebrospinal fluid (CSF) in this review.

Free Light Chains

Two investigations have looked at the possible diagnostic use of kappa and lambda free immunoglobulin light chain (FLC) contents and ratios in the CSF.[18,19] FLC ratios and concentrations in the CSF were described by Hildebrandt et al. as promising indicators for leptomeningeal lymphoma detection.[18] 21 patients with PCNSL and SCNSL had their FLC concentrations and ratios examined in our own investigation.[19] 52 percent (11/21) of patients with CNS lymphomas had noticeably higher FLC ratios than control subjects with various neurological conditions, regardless of whether leptomeningeal lymphoma was verified by cytopathology. Interestingly, MRI showed that patients with subependymal lymphoma spread had higher FLC ratios in their CSF.[19]

Significance: The "liquid biopsy" method of analysing blood for CTCs or cell-free nucleic acids has created new opportunities for cancer diagnostics, such as better risk assessment and staging, early tumour diagnosis, relapse detection, and tumour evolution tracking in relation to cancer treatments.

Table 2: Overview of the novel diagnostic approaches.

Biomarker/Method	Number of Patients	Body Fluid	Sensitivity (%)	Specificity (%)	References
FLC concentrations/ratios	21	CSF	52.3	-	[19]
IL-10 (cut-off 9.5 pg/mL)	66	CSF	71	100	[20]
IL-10 (cut-off 4 pg/mL)	119	CSF	81.6	88.9	[21]
IL-10 (cut-off 8.2 pg/mL)	102	CSF	95.5	96.1	[22]
IL-10 (cut-off 8.3 pg/mL)	108	CSF	59	98	[23]
IL-10/IL-6 ratio (cut- off 1.6 pg/mL)	108	CSF	66	91	[23]
IL-10/IL-6 ratio (cut- off 0,72 pg/mL)	102	CSF	95.5	100	[22]
IgH gene rearrangement	32	CSF	54	97	[24]
CXCL13	220	CSF	69.9	92.7	[25]
Combination of CXCL13 and IL-10	77	CSF	76.7	90.9	[26]
MYD88	225	CSF	72	-	[27]
Combination of MYD88 and IL-10	225	CSF	94	98	[27]
MYD88	90	Vitreous	69	100	[28]
microRNA (miR-21, - 19b, and -92)	53	CSF	95.7	96.7	[29]
microRNA (miR-21, - 19b, and -92)	53	CSF	63.3	80.7	[30]
Combination of RNU2-1f and miR-21	119	CSF	91.7	95.7	[31]
miR-222	150	Serum	80	82	[32]

Scope of Cell-Free DNA and Circulating Tumor Cells in liquid biopsy

Circulating tumour DNA (ctDNA): DNA fragments expelled from tumour cells that reveal information on the genetic composition of a tumour. The review should cover the advantages and disadvantages of the current techniques for identifying ctDNA, such as next-generation sequencing (NGS) and droplet digital polymerase chain reaction (ddPCR).[5] Intact cancer cells that have separated from a primary tumour and entered the bloodstream are known as circulating tumour cells, or CTCs.[5] The evaluation ought to cover methods for separating and examining CTCs, such as single-cell multi-omics analysis and enrichment technologies. Exosomes and other small, membrane-bound vesicles known as extracellular vesicles (EVs) are responsible for moving proteins, RNA, and DNA between cells.

Objectives of Cell-Free DNA and Circulating Tumor Cells in liquid biopsy

Additional biomarkers: Review articles may also include other biomarkers that are in circulation, such as

tumor-educated platelets, microRNAs, and cell-free RNA (cfRNA) (TEPs).[10]

Objectives of Cell-Free DNA and Circulating Tumor Cells in liquid biopsy

A liquid biopsy review article's main goals are to give researchers, physicians, and other stakeholders a comprehensive and critical overview of the area. Important goals consist of:

Provide an overview of current status: To provide an overview of the current state of liquid biopsy technology, including the existing detection platforms, the biomarkers used, and the clinical data proving its efficacy.[10]

Assess clinical utility: This involves going beyond analytical validity to consider the proven patient benefit and practical application of liquid biopsies in clinical practice.[5]

Discuss obstacles and constraints: to openly talk about the obstacles preventing liquid biopsies from being widely used.[10] These include clinical challenges like the possibility of false positives, technical constraints such low analyte concentration and standardisation problems, and regulatory considerations.

Emphasize future trends and perspectives: To forecast the course of liquid biopsy technology, including current studies, new uses (such in infectious diseases or organ transplantation), and the possibility of integrating several omics and AI-driven analysis.[5]

Determine areas that require more investigation: to draw attention to current knowledge gaps and open topics that need more research, like enhancing biomarker separation methods, raising sensitivity in early-stage malignancies, and standardising procedures.[10]

BIOLOGICAL BASIS OF LIQUID BIOPSY COMPONENTS

What is Cell-Free DNA (cfDNA)?

The dynamics of genetic and epigenetic changes unique to cancer are conveyed by cfDNA.[33] A correlation between the cfDNA level during therapy and the result was demonstrated.[34,35] According to certain research, cfDNA analysis performed better than technical techniques (such computed tomography), necessitated a less tumour burden, and extended the window for making clinical judgments.[34,36] Clinical uses of cfDNA as a tumour biomarker are the main focus of cfDNA research in cancer. Liquid biopsy employing extracellular DNA is an appealing candidate for a regular test in cancer care because of the correlation between the level of cfDNA and treatment outcome, the low invasiveness of the assay, and the use of high-throughput procedures. However, there is still much work to be done in order to identify trustworthy markers, assess prognostic relevance, standardise tests, and validate results from extensive perspective clinical studies.[21,37,38] Furthermore, there are still a lot of unanswered concerns regarding the nature of cfDNA, its subtypes, its release mechanisms, and its clearance in cancer patients, even with the rise in research highlighting the significance of cfDNA in oncology. Furthermore, it is critical to ascertain the role of cfDNA in the development of cancer, specifically its correlation with the genesis, aggressiveness, and propensity for metastasis of tumours, as well as its correlation with therapeutic response. For the majority of ctDNA assays in advanced and early-stage cancer, for treatment monitoring, or for residual disease detection, there is currently insufficient evidence of clinical validity and utility, according to a joint panel of experts from the American Society and Clinical Oncology and the College of American Pathologists.[39]

Levels of cfDNA: Serum cfDNA levels from cancer patients were first identified 30 years after cfDNA was initially identified in immune complexes obtained from patients with systemic lupus erythematosus in 1948.[41,42,43] It was demonstrated that cancer patients have more circulating DNA overall than healthy individuals.[43,44] Nevertheless, elevated levels of cfDNA were found in pregnant women's plasma and in transplant recipients, indicating that an enhanced cfDNA content was not exclusive to cancers.[45,46] Increased cfDNA may also be a reflection of non-malignant pathological processes, such as inflammation, diabetes, tissue trauma, sepsis, and myocardial infarction, as well as physiological events, such as exercise[47,48], and non-malignant pathological processes,[40] such as inflammation, diabetes, tissue trauma, sepsis, and myocardial infarction.[49,50,51] Blood levels of cfDNA vary greatly; in cancer patients, they can range from 0 to 5 to >1000 ng/ml, while in healthy individuals, they can range from 0 to 100 ng/ml.[35,40] Additionally, blood ctDNA levels vary significantly amongst patients with various tumour types. For instance, patients with initial brain, kidney, and thyroid cancers had a lower prevalence of ctDNA detection than patients with advanced pancreatic, ovarian, colorectal, gastroesophageal, breast, melanoma, and several other cancers.[52,53] Tumor localization may account for this diversity, according to Khier and Lohan's hypothesis.[54] For instance, the blood-brain barrier and the capsules enclosing certain organs may restrict the release of ctDNA into bodily fluids. Compared to individuals with advanced or metastatic tumours of similar size, it was shown that patients with benign lesions or early-stage cancer had lower levels of cfDNA.[54,55] This discovery implied that the amount of ctDNA released by tumours varied depending on the stage of the disease; hence, the amount might represent interactions between the tumour and the surrounding environment or the different metabolic characteristics of developing cancer.[56,57] Consequently, although while ctDNA variability is usually ascribed to tumour burden, it may really represent tumour metabolism.[58,59,60] For instance, ctDNA levels were linked to the metabolic disease volume in

melanoma patients, which was calculated using F-labelled fluorodeoxyglucose positron emission tomography.[61,62] Consequently, rather than being only linked to tumour burden or the quantity of dying cells, the ctDNA level was a complicated reflection of tumour biology. According to this finding, ctDNA measures may be less significant for precancerous lesions and more relevant for advanced stages of the disease. However, combinations of many marker types (such as ctDNA and tumor-related glycoproteins) and the use of multi-analyte tools (like Cancer SEEK) are promising methods for early tumour diagnosis.[63]

Tissue origin of cell free DNA: In cancer and other clinical diseases, the origin of cfDNA is a fascinating subject. Determining the cfDNA origin may help identify the impacted tissues or organs and offer insights into the mechanisms underlying cfDNA) shedding. This subject has been investigated using a variety of methods, such as i) identifying tissue-specific promoter methylation patterns[65,66,67,68] ii) examination of tissue-specific changes to circulating nucleosomes in general[18,19] and iii) the detection of nucleosome occupancy or tissue-specific DNA fragmentation patterns.[66,69,70] All of these methods could help identify the cfDNA tissue of origin in cancer without the need for an initial genetic difference search.[21] One possible explanation is that circulating tumour cells are not the primary source of cfDNA production. In fact, there are significantly fewer circulating tumour cells in blood than there are cfDNA, which is comparable to several thousand genomic equivalents.[53,71,72] Next, non-mutated DNA makes up a sizable portion of total cfDNA. Numerous studies indicate that the fraction of ctDNA makes up between 0.1 and 89 percent of cfDNA.[53,61,73], albeit it may rise as the condition worsens.[72] Therefore, it is possible to hypothesise that the majority of cfDNA comes from cells in the tumour microenvironment, cells that are killed in hypoxic environments, or cells that are part of the antitumor response.[74] In fact, studies of nucleosome occupancy and DNA fragmentation patterns revealed that while the nucleosome footprint in healthy individuals matched haematological lineages, it also matched the cancer type in cancer patients.[75] Remarkably, the majority of cfDNAs were of haematological origin (55 percent white blood cells and 30 percent erythrocyte progenitors), according to whole-genome array cfDNA investigations of tissue-specific methylation patterns in healthy persons.[67,68,76] Furthermore, approximately 10% of cfDNAs from solid tissues were from vascular endothelial cells, 2% from neurons, and 1% from hepatocytes.[67,68] The methylation profiles of cancer patients (n = 4 with lung cancer, n = 4 with metastatic colon cancer, and n = 3 with breast cancer) also revealed that cfDNA levels were higher than those seen in healthy people (>20-fold increase). The majority of the cfDNA found in cancer patients came from the original tumour tissue.[39,64,67]

Circulating Tumor DNA (ctDNA) vs. cfDNA: Although both circulating tumour DNA (ctDNA) and cell-free DNA (cfDNA) are extracellular DNA fragments that are present in the bloodstream, their sources, uses, and clinical significance are very different.[21,68] All DNA fragments released into the bloodstream as a result of regular cellular processes including necrosis and apoptosis are referred to be cfDNA. Both healthy people and patients with different illnesses have it, and it can come from a number of tissues. On the other hand, tumour cells release ctDNA, a particular subset of cfDNA. This tumor-derived DNA is a potent biomarker for cancer detection, prognosis, and therapy monitoring because it contains chromosomal abnormalities, genetic mutations, and other cancer-specific molecular characteristics.[66,67] While cfDNA levels may rise in reaction to inflammation, trauma, or other benign diseases, ctDNA also known as a “liquid biopsy” offers vital information about a tumour’s genetic makeup in a non-invasive way. In oncology, ctDNA is very useful for monitoring tumour dynamics, detecting minimal residual disease, and directing individualised treatment because of its excellent specificity. However, it is difficult to identify and quantify ctDNA since it usually makes up a very small portion of total cfDNA, particularly in early-stage malignancies.[67,69] One of the main goals in the field of molecular diagnostics is to differentiate ctDNA from cfDNA with increased sensitivity and precision as technologies advance. Cell-free DNA (cfDNA), which can be obtained from whole blood, urine, or cerebrospinal fluid, is typically recovered from liquid biopsies. DNA fragments released from both healthy and malignant cells during apoptosis and necrosis make up cfDNA.[77,78] Circulating-tumor DNA (ctDNA) is the portion of cfDNA that comes from tumour tissue, and these DNA fragments have the same genomic changes as the tumour cells.[78] Clinical benefits of ctDNA sequencing include early cancer identification, tracking the development and recurrence of cancer in patients, and identifying biomarkers that may make patients eligible for tailored treatments.[78] Numerous pre-analytic and analytic factors can affect the amount of cfDNA that is measured.[79] Healthy people typically have low levels of cfDNA, and when people are under cellular stress, their levels of cfDNA rise.[79] Elderly patients, patients with cancer, stroke, autoimmune diseases, sepsis, and even after exercise had higher levels of cfDNA.[80] In particular, cfDNA levels in peripheral blood tend to be correlated with metastasis and tumour load in cancer patients.[43] To guarantee that a clinical report employing cfDNA as a specimen type produces sensitive and targeted results, several quality control procedures are required. Sometimes there is not enough cfDNA or ctDNA available for sequencing when using cfDNA from whole blood plasma as a specimen type for comprehensive genomic profiling (CGP), which leads to a failed specimen. Using a large cohort of clinical samples submitted for CGP testing, we assessed the effects of patient age, sex, disease stage, and tumour type on cfDNA yield, ctDNA fraction, and projected ctDNA

quantity.

Circulating Tumor Cells (CTCs): Characterizing CTCs is crucial for determining their invasive potential and obtaining additional evidence of their malignant nature. About 1 in 10,000 CTC can cause a metastasis, according to research in animals.[77] It is obvious that study must be done to determine which CTCs have the most capacity to spread, even though this number may vary in the case of human pathology and will depend on tumour diversity.[76] FISH (fluorescent in situ hybridization).[44,60,62,66] or CGH (comparative genomic hybridization) targeting individual tumour cells or clusters of tumour cells[67] are two methods for CTC genotyping. Following laser microdissection of CTC, oncogene amplification analyses (ex HER2) can be carried out using FISH and/or quantitative PCR.[71] Following laser microdissection, cytopathologically confirmed CTC can be identified for oncogene mutations.[78] By evaluating the expression of tumour markers (such as HER- 2, metalloproteinases, EGFR, uPAR, and alpha-fetoprotein) on enriched cells, immunolabelling is an intriguing method to describe the invasive potential of CTC. It is anticipated that gene-expression profiling investigations of human malignancies will yield new indicators that can be used to guide anti-cancer treatment and investigate the invasive potential of CTC.[78] Nevertheless, assays designed to characterise CTC must be created with stringent specificity requirements and used with the proper controls. Results from FISH must be carefully analysed using strict criteria and compared to those from normal blood cells. There may be a certain amount of non-specific labelling in immunological staining. According to reports, CK staining and TUNEL (TdT-uridine nick end labelling) analysis can identify a significant proportion of epithelial cells found in breast cancer patients' blood as apoptotic cells.[79,80] Apoptotic cell detection is important, but we must consider that the technique employed to prepare the cells for analysis may cause apoptotic cell death in cells that have been rendered fragile by conservative blood storage agents through repeated manipulations and contact with magnetic particles.[70] Cell shrinkage, nuclear condensation, pyknotic nucleus, plasma membrane blebbing, and apoptotic bodies are examples of typical cellular features that characterise cell apoptosis that can be identified at the cytomorphological analysis if cell enrichment is carried out without causing damage to the cell morphology. Additionally, the normal DNA breaks can be shown with the TUNEL assay. To evaluate the pro-apoptotic impact of treatment regimens, it may be very important to identify and quantify circulating apoptotic cells both before and after anticancer treatment. However, it becomes challenging to differentiate between tumorous and non-tumorous apoptotic cells once apoptotic cell death has begun to alter cell shape.

Release mechanism of Circulating tumor nucleic acid: The mechanisms underlying the release of ctNA. Regarding the existence, variations, and properties of ctNAs as well as their possible contributions to tumour resistance and evolution, there are significant knowledge gaps. Understanding the biology of ctDN.[69,71] and ctRNA[21,80] has advanced thanks to recent advancements in detection techniques' sensitivity and specificity.[10,76] Unified themes can be extracted from the supplied data, notwithstanding the possibility that they are disparate, weighed down by preanalytical variables, and devoid of standardisation techniques.[77] To our knowledge, systematic studies of the mechanisms behind active and passive ctNA release have not yet been thoroughly documented.

Passive release mechanisms of circulating tumor nucleic acids and their properties: The most important source of cfDNA in blood has been proposed to be hematopoietic cell turnover, which is mostly associated with apoptosis-induced cell death.[67] Apoptosis or necrosis are two ways that cancer cells can die, releasing ctDNA in the process.[53] Although their precise roles in ctDNA release are uncertain, apoptosis and necrosis are thought to be important contributions.[6,11,27]

One well-known feature of cancer is unchecked proliferation. Local nutritional deprivation, hypoxia, inflammation, oxidative stress, acidosis, and the generation of corresponding tissue-specific transcription factors and signalling molecules that induce death are all consequences of rapid cell proliferation.[28,29] The passive release of cellular material into the extracellular space can be caused by apoptosis and necrosis, two prominent outcomes of hypoxic and metabolic stress.[11,30] In this section, we go over the mechanisms underlying the passive release of ctDNA and provide an overview of what is currently known about passive ctRNA release, a phenomenon that is far less well understood than ctDNA release. Circulating tumor nucleic acids may be released passively from tumor bed cells as free or protein-associated fragments or actively as part of extracellular vesicles and lipoprotein complexes.[80]

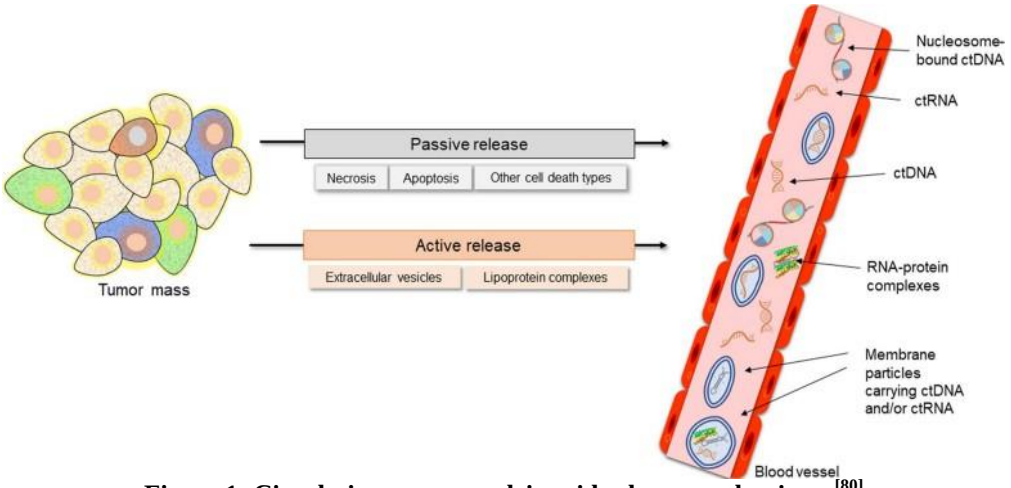

Figure 1: Circulating tumor nucleic acid release mechanisms.[80]

Technologies and Methodologies

Techniques for cfDNA Isolation and Analysis: Isolation and analysis of circulating cell- free DNA (cfDNA) have emerged as crucial methods in liquid biopsy applications, particularly for organ transplant monitoring, prenatal testing, and cancer diagnosis.[54,55,56] Plasma or serum is usually collected first in the process of isolating cfDNA; plasma is preferred since it is less contaminated by lysed blood cells. To prevent deterioration or contamination, blood is drawn in specific tubes (such as EDTA or cfDNA-stabilizing tubes) and processed right away. A low-speed spin is used to separate the plasma, and a high-speed spin is used to eliminate any remaining cells and debris. Then, cfDNA is extracted using a variety of commercial kits that are tailored to recover the small, fragmented DNA molecules (usually 150–200 bp in length) utilising magnetic beads or spin columns based on silicon membranes.[67] After isolation, size distribution analysis by capillary electrophoresis or Bioanalyzer systems, as well as fluorometric tests (such as Qubit), are used to evaluate the quality and quantity of cfDNA. Digital PCR (dPCR) and quantitative PCR (qPCR) enable the sensitive detection of certain mutations or copy number alterations for investigation. Detecting rare mutant alleles among large background quantities of wild-type DNA is made possible by digital PCR's great sensitivity and absolute quantification. On the other hand, next-generation sequencing (NGS) provides a more comprehensive method that makes it possible to identify several mutations, methylation patterns, or fragmentation profiles throughout the genome. End-repair, adapter ligation, and PCR amplification are steps in the library preparation process for NGS, and the procedures are tailored to the low input and fragmented nature of cfDNA. Depending on the goal of the study or treatment, targeted panels, whole-exome sequencing (WES), or whole-genome sequencing (WGS) may be employed. In order to differentiate between background cfDNA from normal cells and tumor-derived ctDNA, bioinformatics techniques are essential for downstream analysis. In order to maintain the integrity and specificity of this important biomarker, cfDNA separation and analysis necessitate meticulously designed methods. DNA fragments released into the bloodstream by necrotic or apoptotic cells, as well as from tumour cells in some situations like cancer, are referred to as circulating cell-free DNA (cfDNA) (known as circulating tumour DNA or ctDNA). Non-invasive diagnostic techniques now heavily rely on the separation and analysis of cfDNA, particularly in the fields of cancer, prenatal diagnostics, and transplant monitoring.[64,76] Sample collection is the first step in the procedure, usually including the drawing of peripheral blood into EDTA tubes or specialised blood collection tubes (such as Streck or Roche Cell-Free DNA BCT) that stabilise cfDNA for a few days at room temperature and inhibit cell lysis. In order to avoid contamination from genomic DNA released by lysed leukocytes, prompt and appropriate management is crucial.[21,43] Following blood collection, plasma is separated using a two-step centrifugation process: first, blood cells are removed using a low speed spin (e.g., 1,600 g for 10–15 minutes), and then any remaining debris is removed using a higher speed spin (e.g., 16,000 g for 10 minutes). To reduce contamination, the buffy coat must not be disturbed during plasma transfer. Commercial kits are commonly used for cfDNA extraction because of their efficiency and standardisation. Magnetic bead-based technologies and silica membrane spin columns (like the QIA amp Circulating Nucleic Acid Kit) are common techniques (e.g., Mag MAX Cell-Free DNA Isolation Kit). Silica columns are prized for their purity and reproducibility, while magnetic beads provide excellent throughput and automation compatibility. These procedures include lysis, cfDNA binding to the matrix or beads, washing steps to eliminate impurities, and elution in a low-salt solution.[44,46] They are best suited for tiny DNA fragments (~150–200 bp). The amount of plasma processed, the illness state, and physiological parameters all have a substantial impact on the overall yield of cfDNA. In healthy people, the concentration usually falls between 0 to 100 ng/mL of plasma, but in cancer patients, it can be significantly higher. Evaluation of both quantity and quality is essential after extraction. Because of its accuracy and specificity at low concentrations, fluorometric assays like

the Qubit dsDNA High Sensitivity test are employed for quantification.[64] In order to verify the distinctive nucleosomal pattern (peaking at 170 bp) and lack of high molecular weight genomic DNA contamination, capillary electrophoresis platforms such as the Agilent Bioanalyzer or Tape Station are commonly used to assess the size distribution of cfDNA. The purpose of the study determines the cfDNA analysis methods. Real-time quantitative PCR (qPCR) is used to detect mutations in known targets, but it is not sensitive enough for low-frequency variants. In a strong background of wild- type DNA, digital PCR (dPCR), including droplet digital PCR (ddPCR), enables the highly sensitive and absolute measurement of mutant alleles, even when they are present at frequencies as low as 0.01 percent.[33] This makes it perfect for tracking minimal residual disease or finding uncommon mutations in early-stage malignancies.[34,56] Next-generation sequencing (NGS) is used for more comprehensive genetic analysis. Because cfDNA is fragmented and low-input, NGS procedures require careful optimization of library preparation (fragment end-repair, adapter ligation, and amplification). Depending on the resolution and breadth needed, sequencing techniques can include whole-exome sequencing (WES), whole-genome sequencing (WGS), or focused panels (such as cancer hotspot panels). Methylation patterns in cfDNA, which can be very tissue- and disease-specific, are studied via bisulfite conversion followed by sequencing for epigenetic and fragmentation pattern analysis. In order to differentiate between cfDNA from various tissues or diseases, such as cancer-derived cfDNA from normal cfDNA, fragmentation pattern analysis, also known as fragment omics, examines the length and end motifs of cfDNA fragments.[21]

Bioinformatics pipelines are crucial for downstream data analysis and interpretation, particularly when it comes to separating low-frequency ctDNA variations from clonal haematopoiesis artefacts or sequencing noise. In order to improve diagnosis and decision- making, advanced algorithms frequently integrate cfDNA data with clinical or other molecular data to perform variant calling, copy number variation analysis, and structural variant detection.

Detection and Enrichment Methods for CTCs: The need for both high sensitivity and good specificity is what makes CTC/CTM detection difficult. Identification and counting of CTC when they are extremely rare (few CTC/CTM per 10 ml of blood, which means few CTC/CTM mixed with approximately 100 million leukocytes and 50 billion erythrocytes) could alert the oncologist about a developing tumour invasion process because invasion can begin very early during tumour development. Another crucial necessity in this discipline is specificity. In actuality, misclassifying "non-tumor cells" (such as epithelial non-tumor cells, for example) as "tumour cells" may result in suboptimal clinical and treatment decisions that negatively affect the quality and/or prognosis of cancer patients. There are a number of recent evaluations available about CTC detection.[1,30,31,34–37]

Numerous techniques have been created, some of which are commercially available. Here, our goal is to objectively evaluate the benefits and drawbacks of the various strategies and offer standards for identifying reference techniques that are anticipated to yield trustworthy clinical data.

Indirect methods: It is not possible to diagnose CTC via indirect approaches. They use organ-specific markers, which identify cells from particular organs but do not show that they are tumorous, or they target epithelial cells.

Indirect immune-mediated methods: Immuno-labelling of cells enriched via various techniques, such as immunomagnetic separation[38] and physical procedures (density gradient, filtration), is how immuno-mediated detection is carried out. Target cell counting is made possible by the 104–105 fold enrichment of CTC obtained using commercially available immunomagnetic methods (MACS systems, macro-iron beads, magnetic beads, ferrofluid (colloidal iron)-based systems), which avoid cell lysis, which is a feature of RT-PCR tests (see below). However, authors have used antibodies specific to epithelial antigens (EpCAM, BerEP4, Cytokeratins (CK)) to isolate CTC because specific antigens that characterise CTC are currently unknown (antigens expressed by all tumour cells from a solid tumour type and not expressed by leukocytes or other circulating non-tumor cells). False positive results can come from epithelial-specific antibodies labelling non-tumor epithelial cells with specific labelling and non-tumor non-epithelial cells with non-specific labelling. In normal controls, the proportion of CK-positive cells varies between 0% and 20%.[34,39] Leukocytes make up the majority of these cells. It has been documented that antibodies against CK or other epithelial-specific antigens bind to macrophages, plasma cells, and progenitors of nucleated hematopoietic cells both specifically and non-specifically. Leukocytes and monocytes that carry the Fc receptor or normal hematopoietic cells that express epithelial antigens illegally are involved in the non-specific binding.[38] It can be challenging to differentiate some of these positive cells from CTC based on their appearance. Peripheral blood from individuals without cancer has been discovered to include varying quantities of epithelial cells[34], which have been linked to inflammation, tissue trauma, benign epithelial proliferative disorders, and semi-surgical and surgical procedures.[39,40] Antibodies to mammaglobin, PSA, CEA, and HER-2 are examples of organ-specific markers that have been utilised to detect CTC. However, because not all tumour cells contain these antigens, false negative results may arise. Moreover, HER-2 and mammaglobin are two examples of these markers that are not totally organ-specific.[41] As a matter

of fact, there are currently no antibodies that are completely specific to any one tumour or tissue.[39] In immuno-magnetic detection, antibodies bound to magnetic particles (beads or ferrofluids) are exposed to whole blood or isolated (via density gradient) mononuclear cells. After that, labelled cells are gathered using a magnetic force, and unlabelled cells are left in the supernatant and disposed of. Some techniques involve a "negative" selection of leucocytes (e.g., with anti-CD 45) in conjunction with a "positive" selection using antibodies specific to epithelial cells (EpCAM, Cytokeratins (CK)) (ex: Cell Search, Veridex)[38], as many leucocytes nevertheless stay trapped with the target cells.[42] The bulk of leukocytes are eliminated during this process, although tumour cells that do not express epithelial antigens are lost and non-malignant epithelial cells are kept. Ferrofluids coated with an EpCAM antibody specific to epithelial cells (directed to a cell membrane antigen) are used in the Cell Search experiment to immunomagnetically enrich epithelial cells. The fluorescent nuclear dye DAPI, a fluorescent antibody to leucocyte-specific CD45, and fluorescent antibodies to intracellular cyto keratins (CK) 8, 18, and 19 are subsequently used to permeabilize, prefix, and label the cells. The Cell-Spotter Analyzer, a four-color, semi-automated fluorescence microscope, analyses the sample by identifying epithelial cells that are negative for the CD45 marker and positive for the CK markers. One of the advantages of the Cell Search assay is that it is semi-automated, less likely to trap leucocytes with epithelial cells, and more sensitive than the Oncoquick approach.[43] Cell counting is also possible. However, antibodies unique to epithelial cells are used for cell isolation and identification (EpCAM, Cytokeratins 8, 18 and 19). As previously indicated, it can be challenging to identify the true amount of tumour cells in a patient with a specific number of circulating epithelial cells (CEpC) since epithelial non-tumor cells can disseminate in the peripheral circulation. This is especially important when CTC counting is used in cancer screening procedures, to evaluate the tumour response to treatment, and to determine the likelihood of a tumour recurrence. In one investigation, Fehm et al.[44] discovered that the blood of one patient with breast cancer had more cytokeratin-negative cells with aneusomy (tumour cells without epithelial antigens) than cytokeratin-positive cells. Additionally, it was discovered that cell lines derived from disseminated tumour cells lacked CK 8, 18, and 19.[13] In 2517 samples of breast cancer, it has been shown that the loss of cyto keratins (CK) and the ectopic expression of vimentin, which indicates EMT, are linked to negative oestrogen/progesterone receptor status, a higher tumour grade, and a mitotic index.[13] Finally, because tumour cell aggregates tend to disintegrate after multiple cell labelling and magnetic particle treatments, CTM cannot be accurately detected using this method.

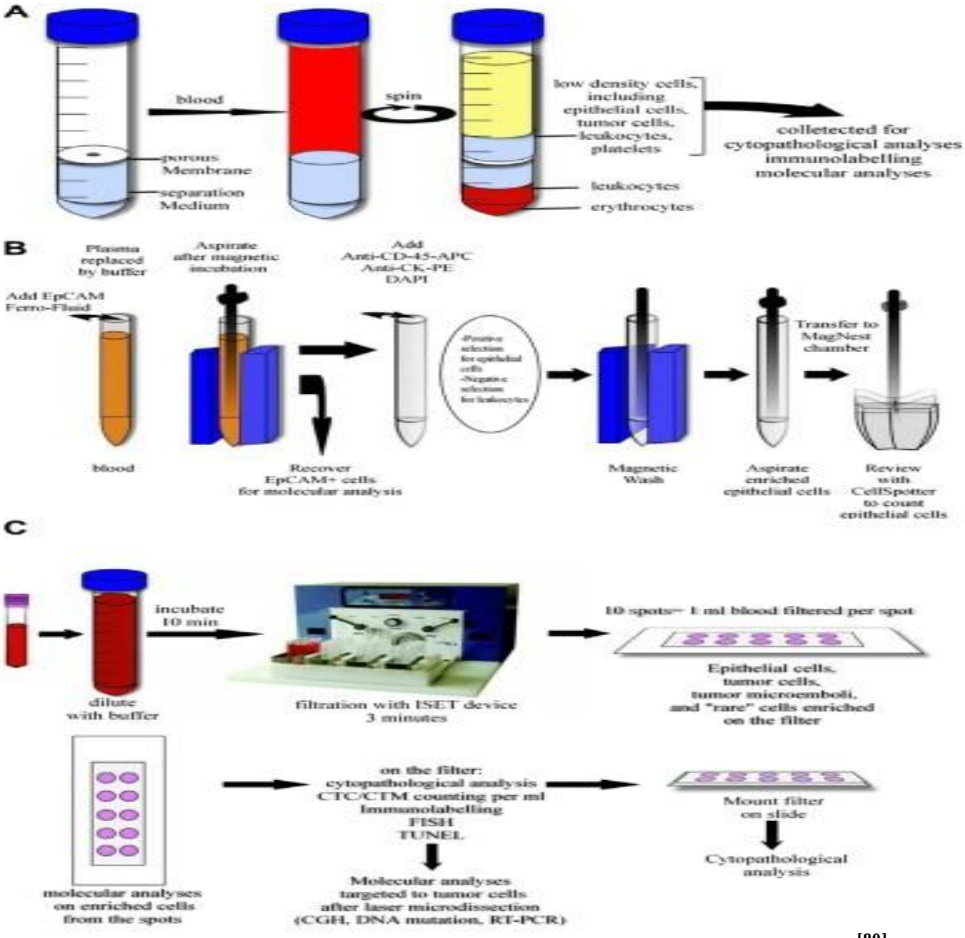

Figure 2: Examples of commercially available methods to isolate CTC.[80]

Indirect molecular methods: The expression of potential genes unique to epithelial cells and/or the normal tissues from which tumour cells originate is examined using RT-PCR- based techniques.[30,3139,46]

This method's primary benefit is its sensitivity, which is thought to be higher than the documented sensitivity of immunocytochemistry and immune-mediated detection.[30] Peripheral blood collection, optional nucleated cell enrichment by physical techniques (density gradient) and/or immune-mediated or immunomagnetic enrichment of epithelial cells, RNA extraction, complementary DNA (cDNA) synthesis, marker gene cDNA amplification, and PCR product analysis are the steps that RTPCR entails (for instance, by gel electrophoresis). One target cell out of 106–107 normal cells, or around one cell in 0.1– 1 ml of blood, can be identified using PCR techniques. One significant drawback of RT-PCR is the destruction of CTC, which prevents their counting or individual analysis. This method also makes CTM undetected as such. Another drawback is the difficulty in selecting the marker RNAs, which are the transcript or transcripts that the test finds and which should show that there are tumour cells in the blood. In peripheral blood leukocytes (PBL) or non-tumorous epithelial cells, the "ideal" marker would be a transcript that is expressed in all tumour cells from a particular tumour but is not expressed at all, not even by illegitimate transcription[47] (low level, non-specific transcription of certain genes, for example, expression of albumin transcripts in lymphocytes).[48] Therefore, it is crucial to carefully identify the transcript and its expression pattern. Last but not least, the risk of PCR products carrying over due to the great sensitivity of RT-PCR testing necessitates stringent negative controls to confirm the positive PCR results. In fact, if the patient has circulating non- tumorous epithelial cells, the signal will be a false positive if the target gene is a normal gene produced in epithelial cells (such as cyto keratins). The blood of 3.7 percent (n = 54) healthy donors, 14.3 percent (n = 28) samples from patients with haematological malignancies, and varying percentages of control persons have all been reported to contain CK19 mRNA. Illegitimate transcription of the CK 19 gene in PBL[49] and/or enhanced cytokine production, which can trigger transcription of tissue-specific genes in PBL[47,50], have been implicated in the detection of CK19 in healthy donors. For CK20, conflicting findings have also been reported. The blood of a variable number of healthy donors and controls contained CK20 mRNA. All prostatic cells express organ-specific marker genes, such as PSA/KLK3, a prostate-specific antigen. As a result, they may produce false-positive results if inflammation[51], invasive diagnostic procedures (such as biopsy), or surgery[40,52] spread non-tumorous prostate cells throughout the blood. The expression of mammaglobin mRNA, a transcript exclusive to the breast, has never been documented in healthy donors[30], although it has been reported that several cytokines can activate it in non-cancerous individuals.[53] It has been frequently demonstrated that leukocytes and blood samples from healthy donors and controls express MUC-1, which is expressed in normal colonic, intestinal, uterine, bronchial, pancreatic, breast, and other glandular cells. According to one study, it was expressed in 73 percent (n = 15) of patients with haematological malignancies and 70 percent (n = 40) of blood samples from healthy participants.[30] Non-tumorous cells may also express tumor- specific markers. For example, CEA transcripts have been found in the blood of healthy donors and in patients with inflammatory bowel disorders[30], and alpha feta-protein is expressed in non-tumorous liver-derived cells.[52] It has been demonstrated that 10.5 percent (n = 38) of healthy donors and 9 percent (n = 22) of people without tumours express EGFR.[54,55] The majority of blood samples from healthy donors and 10% (n = 20) of healthy women have HER-2 mRNA expressed in their blood.[30] Indeed, it has been demonstrated that proliferating peripheral blood leukocytes, but not resting ones, express EGFR, mamma globulin, small breast epithelial mucin, and squamous-cell carcinoma antigen.[56] Although evaluated in a small number of samples, telomerase, a particular polymerase found in about 85% of malignant tumours, has never been shown to express itself in healthy donors or control samples. It can, however, be expressed in certain non-neoplastic tissues and lymphoid cells.[30] The specificity of RT-PCR has been increased through the use of nested real-time RT-PCR[31,46] and quantitative RT-PCR (q-PCR) experiments. The goal of quantitative RT-PCR assays is to determine a cut-off value for a particular transcript marker in relation to a reference marker that is expressed in all cells and would indicate the existence of tumour cells in blood. Because qRT-PCR tests employ internal probes (between primers) that specifically hybridise to the amplified sequence, they have an advantage over traditional RT-PCR assays in terms of PCR specificity. Determining a pertinent quantitative "cutoff" point is challenging, though, because the percentage of tumour cells in blood can vary greatly and the RT step adds a great deal of variability.[46] For example, Schuster R et al.[57] were unable to establish a clear cut-off value to distinguish between tumour cell mRNA and illegitimate transcription in PBL using quantitative real-time PCR. Practically speaking, cut- off levels can be broadly applicable but cannot be tailored to specific circumstances because it is hard to forecast the proportion of tumour and normal cells that express the various transcript types.

Direct methods: Diagnostic identification of CTC is intended to be provided via direct approaches. Only cytopathological examination of the isolated cells[39] and/or genome analysis that provides hints about the tumorous nature of the cell[34,44] can directly diagnose CTC/CTM due to the significant limitations of immune-labelling and RT-PCR techniques. As long as CTC enrichment does not alter cell morphology, cytopathological examination can be performed routinely. On the other hand, for technical reasons,

genome analyses (FISH, CGH, mutation analysis) have not been regularly used for CTC identification; instead, they have been used for their characterization.[42,44,58]

Actually, it can be challenging to interpret the signal, some cells may be tumorous without any discernible aneuploidy, and FISH probes typically do not mark all of the target cells a drawback when evaluating uncommon cells. Mutation studies and comparative genomic hybridization (CGH) are time-consuming and costly processes that need laser microdissection to target individual cells in order to be informative.[58] Moreover, unlike hematologic malignancies, solid tumours are known to have relatively few "marker" mutations or translocations (found in any tumour cell of a certain tumour type). Therefore, there is a compelling case to be made that, as in other oncological diagnostic contexts (such as the PAP-test, cytopathological analysis of tumour biopsies, and aspirates of biological liquids (ascites, urine, and cephalo-rachidien liquid), cytopathological analysis ought to be the standard diagnostic technique and be utilised to detect CTC and CTM. Additionally, CTM, which is the manifestation of "collective migration" and carries a higher risk of metastasis, can be identified through cytopathological analysis of enriched blood cells (see Section 2.1). In order to better describe the malignant nature and invasive potential of CTC/CTM, cytopathological examination might be utilised as a reference basic approach. Additional techniques, such as immunolabelling, FISH, and RNA/DNA analysis, could be applied. Cytopathological study of CTC has previously been carried out using the traditional method of blood smears. This is not possible in a normal way, though, as 100 smears (10 ml per smear) must be analysed in order to detect one CTC in 1 ml of blood. Automatic devices that are frequently used for PBL counting examine 50 ml blood samples; as a result, they are not appropriate for identifying “rare cells”. The physical characteristics of CTC, such as density and size, serve as the foundation for enrichment strategies that seek to separate CTC from their antigens while preventing harm to cell shape. Cytoplasmic and nuclear features can be observed following cytological staining (May-Grunwald Giemsa, Haematoxylin & Eosin, etc.), which enables cytopathological diagnosis of CTC/CTM. Ficoll (Amersham, Upsala, Sweden), Lymphoprep (Nycomed, Oslo, Norway), or other comparable density gradient liquids are used to separate mononucleated cells from blood (including CTC) via a density gradient. On the density gradient, whole blood is directly layered. Erythrocytes, neutrophils, density gradient, mononuclear cells (lymphocytes, monocytes, epithelial cells, and tumour cells), and plasma, the top layer, are detected after centrifugation, from bottom to top. In the plasma fraction, tumour cells may potentially move. However, if whole blood is not centrifuged right away, it quickly begins to mix with the density gradient, preventing the best possible cell separation. The density gradient is contained in 50 ml tubes that are positioned beneath a porous barrier in Oncoquick (Greiner, Frankhauser, Germany). It is intended to separate low-density mononuclear cells and particles from neutrophils and lymphocytes, including low-density leukocytes, epithelial cells, tumour cells, and platelets.

By allowing whole blood (15–35 ml) to be layered on the porous barrier, the tubes prevent the blood from mixing with the density gradient prior to centrifugation. Although Ficoll and Oncoquick have been shown to have comparable tumour cell recovery rates in tumour cell spiking assays, the latter approach yields a higher enrichment of tumour cells from leukocytes, which makes subsequent analyses[59] easier, such as cell staining, immunolabelling, and molecular studies. Oncoquick limiting issue is that rare CTC may be lost during the isolation process because they may migrate in the plasma fraction or become trapped among erythrocytes and neutrophils.[34,59] As a result, the system's sensitivity is extremely low and varies based on factors like temperature, centrifuge time, and tumour cell characteristics. Oncoquick sensitivity in spiking experiments was inferior than Cell Search.[43] Vona et al. initially reported the direct enrichment of epithelial cells using filtration in 2000.[58,60] Because most peripheral blood leukocytes (lymphocytes and neutrophils) are the smallest cells in the body, measuring between 8 and 11 mm in size, the ISET (Isolation by Size of Epithelial Tumor Cells) (Metagenex, Paris, France; www.metagenex.fr) is based on this fact. Thus, blood filtration across a polycarbonate membrane with 8 µm calibrated pores can effectively eradicate them. The simplicity of the assay prevents losing rare cells in numerous phases of separation. After collecting peripheral blood on EDTA, it is diluted with the cell-fixing ISET buffer, allowed to stand for 10 minutes, and then put into the Meta block and filtered by the ISET device for two to three minutes. Filtration occurs through discrete areas on the filter based on the volume of blood; each spot will display the "big" cells that were retained in 1 millilitre of blood prior to filtration. This makes it possible to precisely count the number of CTC per millilitre of blood, regardless of the volume of blood that is being treated. To analyse their antigens, aneuploidy, and rate of apoptotic cells, enriched cells can be described by immunolabelling, FISH, or TUNEL assays, or they can be stained with cytological staining (e.g., May-Grunwald Giemsa, Haematoxylin & Eosin, etc). It's interesting to note that circulating tumour micro emboli (CTM), which are believed to have a high propensity for metastasis, are also reliably countable and sensitively enriched. Following laser CTC/CTM microdissection, molecular investigations can be performed, with a particular focus on tumour cells detected cytopathologically.[58] The viability of investigating HER2 DNA amplification in tumour cells micro dissected following ISET enrichment was shown by Pinzani et al.[61] Enhancement by N.L. Benali and P. Paterlini- Brechot / Cancer Letters 253 (2007) 180–204 197 Since fixed cells greater than 11 mm in at least one

diameter cannot fit through the 8-lm pores, straight filtration is extremely sensitive. Additionally, this direct approach avoids cell damage and numerous stages, both of which increase enrichment sensitivity. The technique has been demonstrated to isolate a single tumour cell introduced by micro pipetting to 1 millilitre of blood in several studies.[60] According to Meng et al.[62], the average tumour cell diameter in breast cancer patients' blood varies between 29.8 and 33.9 mm. Because the pore size is 8 microns, ISET isolates these cells with ease. Additionally, both larger and smaller cells with distinct cytopathological characteristics of CTC are enriched by ISET. The consistent cell morphology makes it simple to differentiate tumour cells from non-tumor epithelial cells using cytological staining and cytopathological analysis, supplemented if necessary by immunolabelling. Although a small percentage of leukocytes are also retained on the filter, they are easily identified without the need for further labeling.[60] Images of CTC/CTM can give oncologists a visual representation of this novel marker and how it changes over time (modification of CTC morphology toward more malignant traits, appearance of CTM, apoptotic cells, etc.)

When entire blood was filtered over a polycarbonate membrane with 8-micron calibrated pores, Zabaglo et al.[63] discovered that 85–100% of MCF7 and T47D breast cancer cells were recovered, with just 0.1 percent of leukocytes left on the filter (approximately 10,000 PBL per millilitre of blood). As a result, this method's enrichment power is lower than ISET's, which keeps between 0.0002 and 0.02% PBL (less than 2000 PBL per millilitre of blood).[60] Automatic examination of CK-positive cells has been linked to Zabaglo et al method, which is less complicated and costly than immunomagnetic techniques. If CK- positive cells are not manually re-stained by H&E and cytopathological investigation, this approach may still provide false positive and false negative results. Multi marker real-time RT-PCR, immunomagnetic separation, and filtration (Zabaglo et al. method) were compared, and the results indicated that RT-PCR is more sensitive than the other two methods in detecting circulating epithelial cells.[64] However, this work has not evaluated a strict detection of circulating tumour cells by cytopathological analyses, which is necessary to precisely count the number of CTC/CTM.

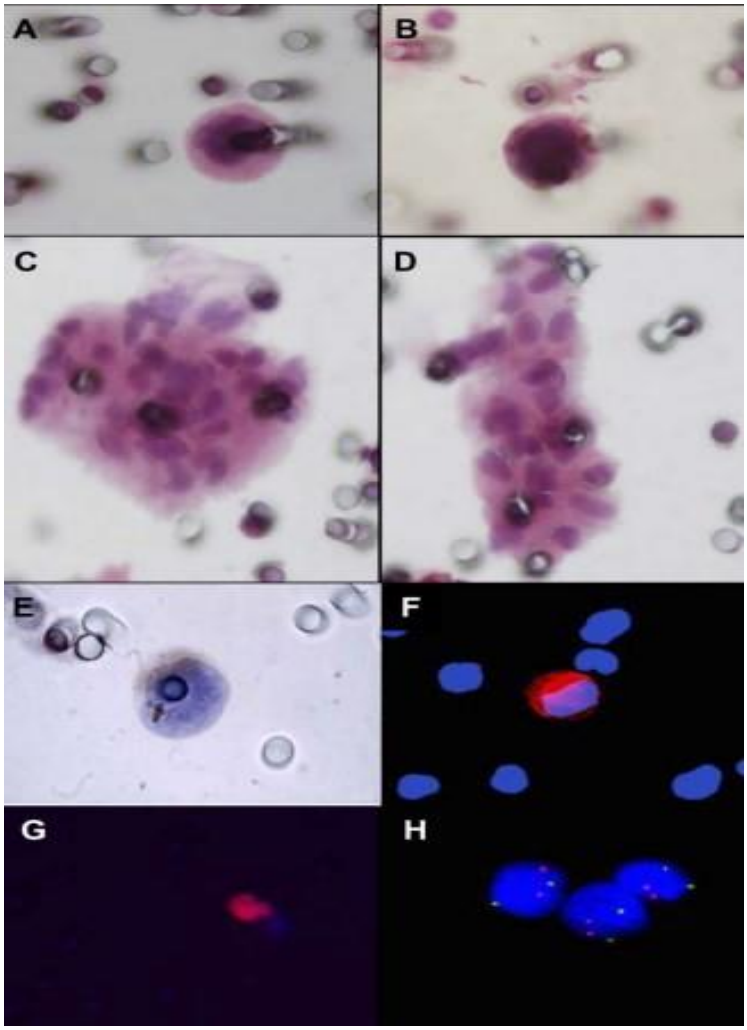

Figure 3: Detection and characterization of circulating tumor cells (CTC) and circulating tumor micro emboli (CTM) enriched by ISET.[80]

Comparative Analysis of cfDNA versus CTCs Detection Sensitivity

Detection sensitivity: One of the most important considerations when comparing circulating tumour cells (CTCs) with cfDNA for cancer monitoring and diagnosis is detection sensitivity, or the capacity to identify tumor-derived biomarkers at extremely low blood levels.[33,46] In general, cfDNA is more sensitive than CTCs, particularly when the tumour burden is minimal or the malignancy is in its early stages. The main reason for this is that cfDNA fragments are more prevalent and evenly dispersed in plasma because tumour cells constantly release them through active secretion, necrosis, or apoptosis. On the other hand, CTCs are exceedingly uncommon and frequently appear at concentrations as low as 1–10 CTCs per 10 ml of blood, which makes them more difficult to extract and identify technically. Particularly in early disease, the scarcity of CTCs adds unpredictability and raises the possibility of false negative results.

Furthermore, the high sensitivity of cfDNA detection techniques like digital PCR and next- generation sequencing (NGS) allows them to identify mutant alleles at rates as low as 0.1 percent, greatly improving early diagnosis and minimal residual disease (MRD) monitoring. However, the sensitivity of CTC detection is reduced because it depends on cell capture technologies (such as size-based filtration or immunomagnetic separation using epithelial markers like EpCAM), which may miss subpopulations of CTCs because of heterogeneity or the epithelial-to-mesenchymal transition (EMT).[67,68] Technical variability is further increased by the fact that CTC isolation frequently necessitates intricate enrichment and imaging procedures, whereas cfDNA isolation and analysis are generally more standardised and repeatable.

When assessing the efficacy of circulating biomarkers in liquid biopsy, detection sensitivity is an essential metric. It alludes to the biomarker's capacity for very low-level detection, which is crucial for recurrence detection, monitoring minimal residual disease (MRD), and early diagnosis.[78] For a variety of biological and technical reasons, circulating cell-free DNA, or cfDNA, typically exhibits higher detection sensitivity in this context than circulating tumour cells, or CTCs.

1. **Biological Availability and Abundance:** The cfDNA fragments are discharged into the bloodstream in comparatively greater amounts, especially ctDNA (circulating tumour DNA), which is the tumor-derived fraction of cfDNA. These fragments occur from apoptosis, necrosis, or active secretion of tumour cells and are routinely secreted even in early disease stages. Particularly when employing high-sensitivity techniques, detectable amounts of ctDNA can be obtained from a few millilitres of plasma. CTCs, on the other hand, are whole tumour cells that separate from the original or secondary location and go into circulation.[21,43,78] They are incredibly uncommon, frequently detected at 1–10 cells per 10 mL of blood, and occasionally completely absent, especially in early-stage malignancies or during remission.

2. **Detection Technologies:** Highly sensitive molecular techniques like;
   - Digital PCR (dPCR) and droplet digital PCR (ddPCR) can be used to analyse cfDNA. These techniques provide quantitative detection of known mutations with allele frequencies as low as 0.01 percent, which makes them perfect for monitoring residual disease or early detection.
   - **Next-Generation Sequencing (NGS):** Comprehensive mutation profiling is made possible by sophisticated NGS procedures with error suppression (such as unique molecular identifiers, or UMIs), which can identify variations with allele frequencies of 0.1–1%, or even lower with deep sequencing.

3. **Temporal and Spatial Representation**
   - A systemic and real-time picture of tumour processes is given by cfDNA. It provides a more thorough depiction of tumour heterogeneity by reflecting DNA released from both primary tumours and metastases.[67,71] cfDNA is a dynamic assessment of disease burden that can alter quickly in response to medication because it is continuously released and removed from circulation (half-life ~16 minutes to a few hours).
   - Conversely, CTCs could not always be found in peripheral blood. They may not accurately reflect all tumour clones due to their sporadic release into circulation, particularly if they are not actively shedding cells or if EMT makes them impossible to identify using conventional capture techniques. As a result, CTCs typically have lesser temporal resolution and consistency of detection.

4. **Pre-analytical and Technical Challenges**
   - **cfDNA:** Simpler to use with commercially available, standardised kits; high-quality data can be obtained with a small volume (a few mL of plasma).
   - **CTCs:** In order to isolate viable cells, complicated sample handling, quick processing, and specialised equipment are needed. Cell deterioration and sensitivity loss may result from processing delays.

5. Clinical Applications and Sensitivity Thresholds

Table 3: Clinical Applications and Sensitivity Thresholds.

Applications	cfDNA Sensitivity	CTC sensitivity
Early cancer detection	High (mutation detection at <0.1% allele frequency possible)	Low (CTCs may be undetectable in early-stage patients)
MRD Monitoring	Very high (tracks tiny residual disease burden)	Moderate to low (rare CTCs make MRD tracking inconsistent)
Recurrence detection	High (can detect recurrence before imaging)	Low to moderate (depends on CTC shedding dynamics)
Longitudinal monitoring	High (serial cfDNA sampling possible)	Low (CTC numbers fluctuate and may be absent)

Role of Next-Generation Sequencing and Digital PCR

Droplet-based digital PCR: Vogelstein and Kinzler used limiting dilutions, as previously described, to find extremely rare sequences in 1999 using a recently invented microtiter plate-based technique.[63,64] Digital PCR, or dPCR, is the name given to this method.[65] Rare events could be identified and measured at the single-molecule level by using dPCR to isolate and test individual target sequences from a complex mixture in different compartments. The number of individual compartments and individual sequences that may be produced and examined, respectively, as well as the false-positive rate of each test, are the primary determinants of dPCR sensitivity. Nevertheless, the limited quantity of 2 M. Postel et al. is one of the technological limitations of this microtiter plate-based technique. Its potential uses in clinical settings have been significantly restricted because to its compartments and huge reaction volume.[4] Thus, based on the substantial reduction of reaction volumes utilising either microchambers or microdroplets[72–74], many techniques have been proposed.[66–71] Platforms that use microchambers have the advantage of being simple to use and offering the ability to automate certain processes, such as sample injection and reaction analysis. However, the number of compartments for the various systems is frequently restricted to a few thousand in order to reduce the chip's dimensions[67], which may reduce the sensitivity of detection. Actually, a number of microchamber-based systems, such as the Clarity™ digital PCR system from JN Medsys, Quant studio™, and Constellation Digital PCR, have been marketed.[4] Similar to these systems, emulsion PCR (ePCR) or ddPCR compartmentalises PCR reactions using aqueous droplets with volumes ranging from a few femtoliters to nano litres dispersed in oil. This allows for an almost infinite number of compartments[75], which significantly improves detection sensitivity. The earliest high throughput ddPCR systems for the identification and counting of genetic variations were Beaming (beads, emulsion, amplification, and magnetics), which is currently marketed by Sysmex Inostics.[76] These systems were initially disclosed in 2003. One mutant DNA molecule in 10,000 wild-type molecules has been reported as the procedure's detection limit.[77] However, routine clinical use necessitates a quite complex and laborious technique.[47,77] Droplet volumes in an emulsion may now be precisely controlled thanks to the combination of ePCR and microfluidic technologies.[73] Raindrop™ digital PCR (Raindance Technologies, recently bought by Bio-Rad), the Bio-Rad QX200TM Droplet Digital™ system (Bio-Rad Laboratories), and the Naica™ System (Stilla Technologies) are some of the ddPCR microfluidic systems that are now being marketed.[4] In these systems, ctDNA samples are separated into separate micro-compartments in the form of water droplets that range in size from nano litres to a few Pico litres. One haploid genome and all the necessary chemicals for the PCR assay, such as certain TaqMan® probes typically one that targets a mutant sequence and the other that is tagged with a distinct fluorophores should ideally be present in each droplet (Figure 1). The identification of mutant sequences inside wild-type ones is subsequently done by counting the droplets with differing fluorescent signals, which has been shown to have a detection sensitivity of less than 0.001 percent.[4] In these systems, multiplex studies have been carried out employing a third fluorescent signal (Stilla Technologies) or based on variations in probe concentrations and/or amplicon size (Raindance Technologies[78], Biorad Laboratories[79,80]). However, the real techniques' ability to accurately identify each tested sequence is restricted to 5–10 combinations.[79] Other multiplex techniques that enable screening for a pool of mutations, such as EGFR exon 19 deletions or RAS/RAF mutations, have been published. If it is necessary to identify the specific mutations, traditional duplex ddPCR must then follow such procedures.

Next-generation sequencing: Millions of ctDNA molecules can be analysed simultaneously using NGS technology, and the sequence data is subsequently compared to a reference genome to detect genetic or epigenetic alterations (Figure 2). However, methods like whole-exome or whole-genome sequencing often produce average sequencing coverage of 30 to 100, which results in too low detection sensitivity to examine uncommon ctDNA mutations within ccfDNA. The detection sensitivity of traditional NGS technologies, including Ion AmpliSeq targeted sequencing, is greater than 2 percent. Because of this, traditional NGS is less flexible when it comes to identifying uncommon ctDNA mutations. Two essential criteria for allowing the use of ctDNA analysis in clinics are detection sensitivity and detection specificity. A low detection sensitivity would make it impossible to detect ctDNA inside ccfDNA, and a false-

positive result could have detrimental psychological effects on patients. The detection sensitivity and specificity of ctDNA within ccfDNA have been significantly improved recently by a variety of improved NGS technologies, including the Safe-Sequencing System (Safe-sequence)[5], Cancer Personalized Profiling by deep sequencing (CAPP-sequence)[6], integrated digital error suppression-enhanced CAPP-sequence (iDES-enhanced CAPP-sequence)[9], and newly developed analysis methods like Base-Position Error Rate (BPER).[8] A predesigned selector probe set hybridises and captures ctDNAs in CAPP-sequence[6], identifying regions with high driver mutation frequencies in the cancer type of interest. Sequencing is then performed to sequence the interest sequence with a significantly higher sequencing coverage (roughly 10,000× coverage). However, sequencing artefacts continue to be an issue for this sequencing strategy.[6] In the meantime, CAPP-sequence achieves a lower background error rate by incorporating optimised bioinformatics analysis methods, which significantly improves the detection sensitivity of ctDNA within ccfDNA to 0.01 percent. Developed from CAPP-sequence, iDES-enhanced CAPP-sequence[9] uses a unique molecular identifier (UID) for every template molecule, just like Safe sequence does. Following amplification of these templates, families of identical UID molecules are produced. In order to differentiate between sequencing errors and actual rare mutations, the majority of molecules in the same family should have true uncommon mutations. The detection sensitivity (0.001 percent) and specificity (96 percent) of iDES-enhanced CAPP-sequence are further boosted for rare mutation detection in ctDNA by means of a computational pipeline that uses barcode- mediated error suppression to maximise molecule retention and suppress background error. Using well-designed algorithms like BPER, it is also possible to increase the sensitivity of uncommon mutation detection in plasma ctDNA to 0.1 percent.[8] This method determines the base location error rates for all probable SNV/indel in a given patient plasma sample as well as for all sequenced bases using control plasma samples. The current mutation frequency in ctDNA is then compared to the corresponding base position error rate, which was earlier computed to detect actual ctDNA mutations, using a binomial test. Finally, compared to traditional focused sequencing, the BPER method's specificity for the discovery of uncommon mutations in ctDNA is increased to 95% since it accounts for the error-rates discovered at all analysed sites for each sample utilising outlier detection.

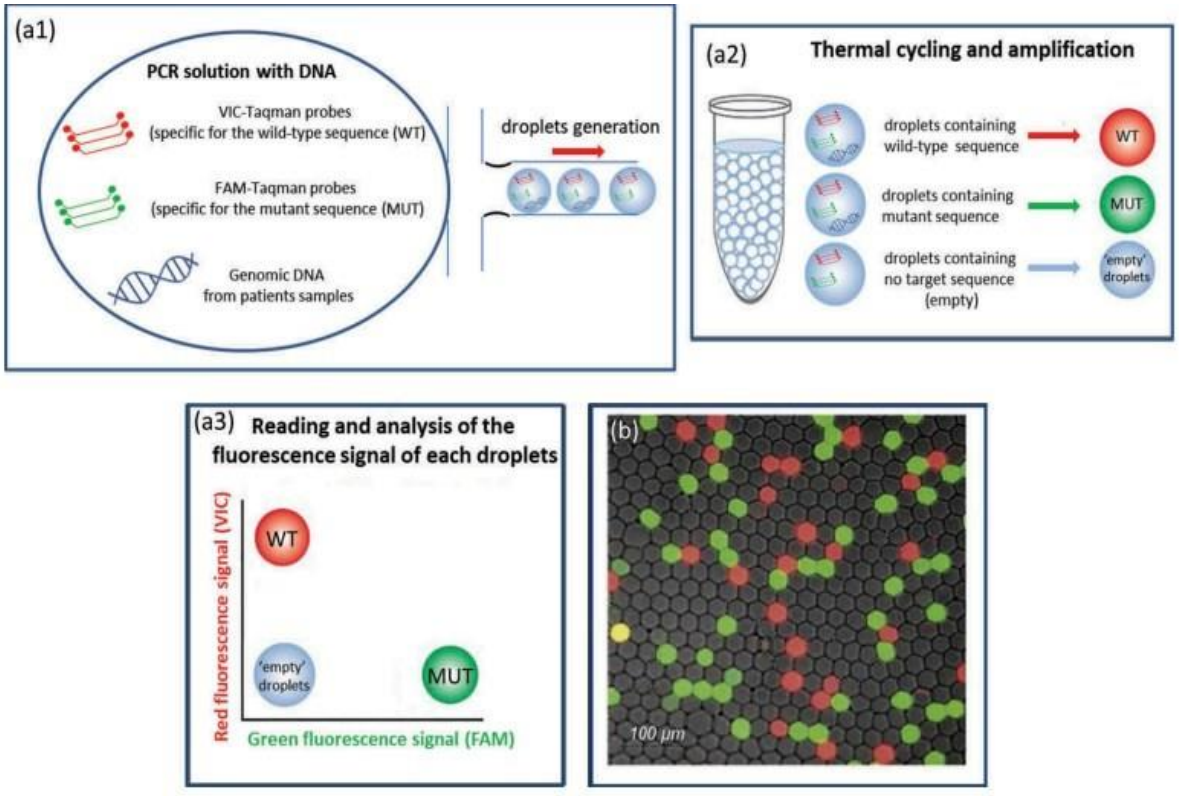

Figure 4: Workflow for picolitre droplet-based digital PCR.[80]

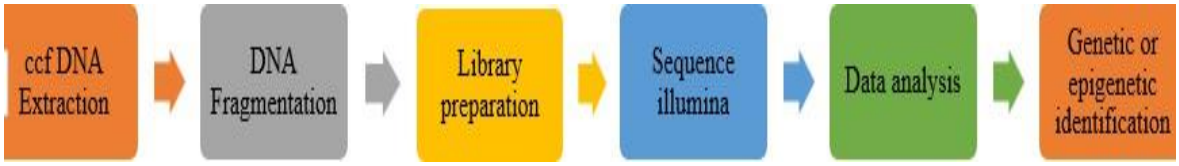

Figure 5: Schematic illustration of optimized highly sensitive next generation sequencing (NGS) procedure.[78]

Table 4: Comparison of several technologies of digital PCR and NGS.

Types	Method	Detection sensitivity	Advantages	Disadvantages
Digital PCR	Beaming PCR ddPCR	0.01% 0.001%	1. ddPCR: ease to use ddPCR: fast Fast	1. Need to know the target in advance
NGS	Safe – seq CAP – seq iDES – CAPP – seq Bper	0.1% 0.01% 0.001% 0.1%	1. Capable of detection of genetic or epigenetic changes without knowing the target in advance; ability of detecting new mutation.	1. Take more time, slow 2. Expensive 3.Necessary of informatics expert support.

Comparison of two highly sensitive technologies: ddPCR and optimized NGS for detecting ctDNA: For the examination of ccfDNA, ddPCR and optimised NGS techniques have their own benefits and drawbacks in addition to the higher detection sensitivity and specificity (Figure 2). The ddPCR studies are simpler to set up, faster, have higher sensitivity, and do not require sophisticated informatics assistance for analysis when compared to NGS. However, it also has limited multiplex powers and requires knowledge of genetic or epigenetic modifications to be recognized.[78,79] To find ctDNA KRAS mutations in plasma from CRC patients, a 5-plex assay was created, and it demonstrated agreement with the duplex ddPCR assay.[78] On the other hand, NGS is time-consuming and needs strong informatic assistance, but it has the potential to detect new genetic or epigenetic changes and has excellent multiplexing capabilities. As a result, numerous approaches have integrated ddPCR and NGS for liquid biopsy analysis.[7,19,54,57]

It has been examined, for instance, employed Figure 2 with BPER NGS. The limit of detection of ctDNA may now be as low as 0.001 percent with great accuracy thanks to recent advancements in ddPCR and improved NGS technology, opening up a wide range of applications for ctDNA in clinical settings. But it's also crucial to note that relatively little ctDNA may be released into the bloodstream in the majority of cases, particularly in early malignancies. The small amount of DNA that can be assayed may frequently be the source of ctDNA's limitations. Therefore, more studies are required to better understand the mechanisms underlying the release of ctDNA and also to identify methods for streamlining sample collection and processing, including the handling of vast volumes of plasma.

Clinical Applications in Cancer Diagnosis

Potential application of ctDNA biomarkers for early cancer diagnosis: The identification of hotspot mutations in ctDNA, such as those in KRAS (colorectal cancer, pancreatic cancer), APC (colorectal cancer)[47], BRAF (melanoma), EGFR (non-small cell lung cancer), HER2 (gastric cancer), PIK3CA (breast cancer), TP53 (pancreatic cancer), or CDKN2A (pancreatic cancer), showed promise for early cancer diagnosis, even if less ctDNA is released at an early stage of the disease. It has been researched and found ctDNA in 48–73 percent of patients with breast, pancreatic, gastroesophageal, and localised (stage I–III) colorectal cancer by examining a panel of hotspot gene mutations.[57] It has been reported that using the Ion AmpliSeq cancer hotspot panel V2 (Life Technologies, USA), ctDNA could be found in over 32.8% of patients with non-small cell lung cancer in its early stages (stages IA, IB, and IIA).[45] Using more sensitive techniques like ddPCR or tailored NGS could significantly increase the percentage of patients with detectable ctDNA. In a similar vein, It has been shown that Beaming can identify mutant APC molecules in over 60% of patients with early and most likely curable colorectal cancer.[47] Using ddPCR, it has been found that 48.3% of patients with early-stage breast cancer (stage I, II, and III) had a PIK3CA mutation in their ctDNA. Additionally, some researches showed that tracking ctDNA mutations could accurately monitor early relapse and minimal residual disease (MRD) in patients with early-stage breast cancer.[77]

Since gene mutations and hotspot codons varied greatly among cancer patients, a wide panel of genes and hotspot codons should be evaluated to enable effective patient follow-up. This clearly makes the detection process more difficult. Using the cBioPortal database, analysis of the 12 most commonly reported CRC mutations[58] in primary tumours of CRC patients shows that 46.2% of the patients tested positive for one of these mutations in tumour tissue. The detection rate would only increase to 54.2% of patients if the number of tested mutations was increased to 30.[78,79] However, a number of recent studies have demonstrated that screening for a substantial number of cancer patients, if not all of them, might be accomplished by analysing a small number of methylation markers.[58,71] Without creating customised assays for every cancer patient, this might obviously reduce the number of tests required, streamline the detection process, and enable the tracking of tumour DNA dynamics.

Using a panel of ctDNA methylation biomarkers, including RASGRF1, CPXM1, HOXA10, and DACH1, researches have shown an 86 percent sensitivity and 83 percent specificity (stages 0–I 84.6 percent, IIA 86.2 percent, IIB-III 81.8 percent) for early breast cancer detection.[17,19] The plasma of 21.4 and 45.4 percent of patients with localised pancreatic cancer had PENK and CDKN2A ctDNA methylation, respectively.[11,21]

Using ddPCR, it has been discovered that methylated ctDNA (MetctDNA) (WIF1 or NPY) was observed in 45% of localised CRC (stage II and III).[58] More surprisingly, a substantial connection between mutant ctDNA (MetctDNA) and MetctDNA was discovered in this investigation.[58] Thus, a breakthrough in the diagnosis of early-stage cancer may be achieved by utilising both ctDNA mutation and methylation biomarkers. Early diagnosis, according to the World Health Organization (WHO), is the process of identifying cancer in patients who exhibit its signs.[31,67] In contrast, cancer screening looks for precancerous lesions or undiagnosed (preclinical) cancer in a target group that appears to be healthy. The creation of novel indicators for use in cancer screening has been the focus of numerous investigations. The Federal Drug Administration (FDA) has authorised a blood Epi pro-colon test for CRC screening based on the methylation of the SEPT9 promoter region for clinical use.[45] This test is recommended to screen people of either sex who have been offered and have a history of not completing CRC screening, and who are 50 years of age or older and considered to be at average risk for the disease.[21,43] When comparing pancreatic cancer to normal pancreas and pancreatitis, it has been reported a panel of methylation biomarkers CD1D, KCNK12, CLEC11A, NDRG4, IKZF1, PKRCB, and KRAS that had a 75% sensitivity and a 95% specificity.[61] To evaluate its accuracy, a bigger clinical trial is currently underway.[71]

Tumour Typing and Genetic Profiling: Our ability to get tumour samples strongly limits the amount of information we can learn about neoplastic traits, which is one of the primary drawbacks of using tumour profiling to guide therapy. Tumor biopsies are frequently obtained for diagnosis in clinical practise; but, depending on the location and accessibility of the tumour, it can be difficult, invasive, and expensive to acquire a sufficiently representative sample, even for patients in good health. Furthermore, biopsies are typically taken from the original tumour, while samples from metastases are frequently infrequent and also disregarded while making treatment recommendations. New methods for identifying tumour products in physiological fluids including blood, urine, or saliva have emerged in the last few decades. Analysing circulating tumour cells (CTCs), circulating tumour nucleic acids (ctDNA and ctRNA), or tumour exosomes are some of the so-called liquid biopsy approaches. Liquid biopsies provide a number of benefits over traditional methods, chief among them being their lower cost and far lower level of invasiveness. First of all, because they are independent of the quality of the biopsy, they offer an objective summary of the tumour molecular characteristics. Second, they can provide information on both primary and secondary tumours. This is particularly pertinent given that the majority of cancer deaths result from the effects of tumour metastases, while current treatment approaches mostly target the original tumour. A change in the way we develop therapeutic approaches to treat cancer patients may result from the ability to get data from the metastases as well. Thirdly, because they can be taken successively over the course of the disease and therapy, they can offer a dynamic perspective of tumour progression and behaviour (i.e. monitoring minimal residual disease). This implies consistent data that can direct treatment and monitoring plans and possibly raise overall survival rates. These significant benefits have begun to take over in clinics, and FDA-validated blood tests to identify EGFR mutations as a first line of treatment for non-small cell lung cancer have now become available.[58] Furthermore, in certain cancer types, studies based on blood biomarker detection have demonstrated the presence of resistance mutations even before relapse was visible by imaging diagnostics.[59,60]

Furthermore, ctDNA sequencing in patients with breast and colorectal cancer can identify HER2 amplifications in patients receiving trastuzumab treatment for gastric cancer or chromosome copy number and structural changes that are therapeutically important.[61,62,63] Additionally, it has been demonstrated that ctDNA sequencing is a useful tool for tracking the development of secondary resistance mutations in KRAS.[64]

Prognostic Value of cfDNA and CTCs: The gold standard for diagnosis at the moment is a tumour biopsy. Tissue biopsies, on the other hand, only provide a single image of the tumour and may be inaccessible or challenging to get. The geographic and temporal tumour heterogeneity commonly observed with tumour tissue biopsy analysis may be overcome by liquid biopsy, which uses biomolecules found in bodily fluids. Compared to conventional imaging-guided or bronchoscopic biopsy techniques, liquid biopsy offers a less intrusive way to evaluate tumour progression, track recurrence, and get a better understanding of metastatic pathways.[4,5] Circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA) are two of the most important biomarkers among the several analytes available for liquid biopsy. A subgroup of tumour cells known as CTCs are released into the bloodstream by primary or metastatic tumours. According to recent research, CTC clusters: two or more CTCs combined: are more resilient and have more potential for metastasis than individual CTCs.[6] In colon, prostate, and breast cancer, CTCs have been employed as predictive biomarkers.[7,8,9,10]

The only CTC enrichment platform that has FDA approval is still Cell Search®. Cell Search® depends on tumour cells expressing EpCAM. EpCAM enrichment may produce false-negative results if a tumour cell has undergone epithelial-mesenchymal transition (EMT). EMT is crucial for metastasis, and CTCs that exhibit an EMT signature have been linked to a lower chance of survival in patients with non-small cell lung cancer.[11,12,13,14] Recent research has demonstrated that cell-surface vimentin (CSV) may be used to identify mesenchymal CTCs.[15,16] In order to determine

whether patients with lung cancer may benefit from Tyrosine Kinase Inhibitor (TKI) treatment, ctDNA (a fraction of cell-free DNA, cfDNA) has been clinically validated to detect EGFR mutations (exon 19 deletion or exon 21 replacement, L858R).[17,18,19]

However, the cellular origin of ctDNA remains unclear (i.e., release of ctDNA into the bloodstream reflects either active secretion from tumour cells or passive release due to cells undergoing apoptosis and/or necrosis). In recent years, ctDNA analysis has demonstrated significant clinical utility in NSCLC. Consequently, it is unclear if ctDNA offers reliable data on tumour dynamics.[20] CTCs, on the other hand, are intact, living cells that can offer insights into the biology of tumours as well as the geographic and temporal heterogeneity of tumours, which the ctDNA mutation signature cannot. It has been proposed that CTCs and ctDNA could be employed in tandem as cancer biomarkers due to their distinct advantages and disadvantages.[21,22]

The speculation of that progression-free survival (PFS) in patients with advanced stage non- small cell lung cancer (NSCLC) may be predicted by CTCs/CTC clusters or cfDNA/ctDNA at study entrance (T0) and three months after therapy (T1). The purpose of this pilot trial was to assess the clinical value and viability of using cfDNA and CTC together as a biomarker to predict PFS in patients with advanced-stage non-small cell lung cancer.

As crucial markers of disease load, responsiveness to treatment, and overall survival, cfDNA and CTCs have both shown considerable predictive utility in oncology. cfDNA is a quantitative and dynamic biomarker, especially its tumor-derived portion (ctDNA). A poor prognosis, increased tumour burden, and advanced-stage malignancies are frequently linked to elevated cfDNA levels. More significantly, certain genetic changes seen in cfDNA, such as TP53, EGFR, or KRAS mutations, copy number changes, or methylation patterns, can offer molecular-level prognostic information that indicates the aggressiveness of the tumour or the possibility of treatment resistance. Persistent ctDNA detection following surgery or chemotherapy, for instance, has been associated with a lower progression-free survival (PFS) and overall survival (OS) for lung and colorectal malignancies, suggesting minimum residual disease (MRD) and imminent recurrence. Additionally, real-time evaluation of therapy effectiveness is made possible by serial cfDNA monitoring, which permits early intervention in the event of molecular relapse.

However, because CTCs are intact, living tumour cells that have the ability to spread, they provide special prognostic information. Across a variety of cancer types, including breast, prostate, and colorectal cancers, high CTC counts have been repeatedly linked to a poor prognosis, decreased survival, and an increased risk of metastasis, especially when evaluated before and after therapy. For instance, individuals with metastatic breast cancer who have more than five CTCs per 7.5 millilitres of blood (as identified by the FDA-approved Cell Search system) had a lower median overall survival than those who have fewer CTCs.

Likewise, baseline CTC counts are a better indicator of overall survival in castration- resistant prostate cancer than PSA levels or imaging results. Furthermore, phenotypic characterisation of CTCs is made possible by the analysis of gene expression, surface markers, and epithelial-to-mesenchymal transition (EMT) status. This characterization might aid in the prediction of therapeutic resistance or the possibility of metastasis.

Crucially, CTCs and cfDNA offer complementary prognostic data. CTCs offer information on tumour biology and metastatic capability at the cellular level, whereas cfDNA accurately captures tumour genetic changes and real-time tumour burden. According to some research, prognosis accuracy is increased when cfDNA and CTC analysis are combined, especially when predicting survival or relapse in advanced malignancies.

The patients who have both detectable ctDNA mutations and high CTC counts, for instance, frequently have worse outcomes than those who have either or both biomarkers raised.

Monitoring Treatment Response and Resistance

ctDNA to Predict Response in Non-small-Cell Lung Cancer Patients to Epithelial Growth Factor Receptor Inhibitors: This article provides a detailed description of the poster child of a therapeutically useful ctDNA predictive biomarker that was created by Roche and AstraZeneca as a companion diagnostic for their epidermal growth factor receptor (EGFR) treatments. Recurrent somatic activating mutations in EGFR are present in around 15% of patients with non-small-cell lung cancer (NSCLC) in the West and 40% of patients in Asia. These are found in exons that encode the kinase domain and typically involve leucine-to-arginine point mutations at position 858 in exon 21 (L858R) and minor in-frame deletions in exon19 (ex19del), which activate EGFR and cause ligand independence.[33]

First-generation EGFR tyrosine kinase inhibitors (TKIs) include erlotinib and gefitinib are EGFR specific, reversible, competitive ATP inhibitors, and a successful standard of therapy in previously untreated patients harbouring these mutations.[35,36,37]

Most patients eventually relapse, usually within 9–14 months, despite the fact that they usually react favourably. The majority of these relapsed patients develop a second EGFR mutation, the T790M gatekeeper, which increases ATP affinity and steric hindrance to block suppression by first-generation TKIs, according to tumour profiling of these patients'

tumours.[37,38] Osimertinib is approved for the treatment of T790M mutant patients who have progressed on EGFR TKI treatment and, more recently, for the first-line treatment of EGFR mutant non-small cell lung cancer (NSCLC). It inhibits both T790M and EGFR-sensitizing mutations.[9,37]

Somatic EGFR mutation testing is now crucial in this patient population due to the development of these targeted treatments. Interest in a liquid biopsy is heightened by the difficulty in getting tumour biopsy samples from patients with advanced non-small cell lung cancer (NSCLC), particularly during recurrence because of poor patient performance status and tumour placement. Using the cobas real-time PCR (RTPCR) instrument (Roche Molecular Systems), studies employing blood samples and matched tumour tissue as a non- reference standard demonstrated high sensitivity (72–87 percent) and specificity (97–98 percent) for L858R and exon19 deletion EGFR mutations.[21,43,67] When erlotinib phase III ENSURE study samples from first-line stage IIIB/IV NSCLC patients were analysed, plasma was found to be positive in 77% of tissue-positive instances and negative in 98% of tissue-negative cases.[67,69]

The US Food and Drug Administration (FDA) approved the cobas EGFR Mutation Test v2 in 2016 as the first authorised ctDNA-based companion diagnostic test for the detection of EGFR ex19del or L858R mutations from plasma as a result of these studies.[44] The T790M resistance mutation in plasma by cobas has a lower sensitivity (61–73 percent) and specificity (67–79 percent) than L858R and ex19del mutations.[44,46,47]

This is probably because T790M is an acquired mutation, meaning it is present at a lower abundance than driver activating mutations. According to later research, individuals with extra-thoracic disease who have a higher baseline tumour burden and who are undergoing later lines of therapy are more likely to have a positive liquid biopsy result, which is consistent with increased ctDNA shedding.[77] However, patients who test positive for plasma T790M also test positive for tumour biopsies, and their clinical response rate to Osimertinib is the same.[21,43] With good platform concordance, additional platforms like as Beaming, droplet digital (ddPCR), and next-generation sequencing (NGS) can also be utilised for ctDNA EGFR mutation detection.[21,43,67]

Although it is generally advised that patients who test positive for an EGFR mutation in their plasma be eligible for an EGFR TKI, a negative plasma result should, whenever feasible, be verified in tumour tissue. These approvals the first for ctDNA testing—brought liquid biopsies into clinical use, making it easier for patients with lung cancer who lack tumour biopsy material to have their EGFR mutations analysed. They also open the door for additional use of predictive biomarkers based on liquid biopsies.

ctDNA to Monitor Treatment Response Using Targeted Approaches: As explained for EGFR mutation detection in NSCLC, ctDNA analysis across a small gene panel can function as a sensitive liquid biopsy in tumour types like BRAF-mutated melanomas[23,24,25] or KRAS mutant pancreatic cancers[44,45], where a small number of recurrent driver mutations are common. These tiny, focused methods typically include analysing one to five genes and use RT-PCR and ddPCR, which evaluate a small number of variations with great sensitivity (variant allele frequencies [VAFs] down to 0.001 percent with sufficient input DNA). This can be used for post-treatment patient monitoring, especially in conjunction with a baseline tumour biopsy to verify the presence of the dominant driver mutations. It was initially shown by the Bardelli lab that cfDNA could be used to monitor these deadly clones while colorectal cancer (CRC) was being treated with EGFR-targeted treatments. Resistance to EGFR blocking was linked to the detection of RAS mutations in cfDNA using ddPCR and BEAMING. Interestingly, mutant RAS clones decreased when therapy was stopped, suggesting that treatment drives dynamic clonal development. This was further illustrated by Parseghian et al. (2019), who confirmed that RAS and EGFR mutant CRC clones had a cumulative half-life of 4.4 months and degrade after anti-EGFR medication cessation, with no growth benefit over other clones. According to these findings, the best time to re-challenge anti-EGFR in CRC may be determined by targeted cfDNA monitoring. The ongoing CACTUS trial in advanced cutaneous melanoma is another instance of using ctDNA to monitor patients across a narrow gene panel. The results of this study's quick turnaround ddPCR ctDNA test, which looks at only three mutations (V600E, V600K, and V600R), are used to guide treatment switching from targeted therapy to immunotherapy.

ctDNA to Monitor Treatment Response with Broad Panels and Genome-wide approaches: When a wider range of clinically significant mutations need to be investigated or when prior knowledge of tumor-associated mutations is unavailable, analysis of bigger gene panels (10–100s of genes) or genome wide techniques are necessary. It is not possible to analyse such extensive panels using ctDNA ddPCR and RT-PCR; instead, NGS techniques are needed. In 2012, it had been initially reported the ability to use cfDNA analysis in conjunction with NGS to identify somatic mutations across several genes in cancer patients (2012). The method, known as TAm-Seq, significantly expanded the area of ctDNA detection by enabling study of over 6,000 genomic bases at VAFs as low as 2 percent.[67,68, 69]

In the same year, the Velculescu lab reported a comparable method for identifying chromosomal changes specific to tumours.[71,72,73]

Serial plasma samples have also shown the clinical value of cfDNA NGS techniques in tracking the genomic

evolution of metastatic tumours in response to therapy in advanced lung, ovarian, and breast malignancies.[79] As previously mentioned, the problems of false- positive (mutations being incorrectly called due to technical error and noise) and false- negative (mutations being missed due to a lack of sensitivity of the assay) variant calling as well as CHIP must be resolved in order to enable the use of ctDNA as a biomarker across more tumour types and disease stages. Significant work has been done to improve the sensitivity of cfDNA NGS, including the use of digital error suppression (iDES) and unique molecular identities (UMI). UMIs reduce false-positive variant calling by enabling the unique identification of each cfDNA fragment and the determination of each molecule's real consensus sequence. With detection of ctDNA down to 4 in 105 cfDNA molecules, the CAPPseq workflow's combination of UMIs and iDES accurately detected EGFR mutations in 66 NSCLC samples with 92% sensitivity and 96% specificity. Using a similar method, TEC-Seq (targeted error correction sequencing), which also incorporates UMIs, found ctDNA across a panel of 58 cancer-associated genes in 200 patients with early-stage cancer.[78,79]

Depending on the type of disease, mutations were found in between 59 and 71 percent of stage I or II patients. Along with the creation of UMIs and enhanced bioinformatic techniques, it has also been noted that ctDNA fragments differ in length from cfDNA from healthy cells; they are usually 90–150 bp or >250 bp as opposed to 166 bp, respectively. This has led to an increase in the sensitivity of ctDNA detection. These distinctions are exploitable from a technological and bioinformatics standpoint.[21,43] It has been showed that a >2-fold median enrichment in tumour DNA detection in >95 percent of 200 pan-cancer patients by using an in vitro and in silico approach to select fragments between 90 and 150 bp. Analysis of size-selected cfDNA revealed somatic mutations in samples that were otherwise undetectable. Although it is technically difficult to physically select low-frequency ctDNA, in silico analysis of variations in fragmentation patterns from shallow whole-genome sequencing (sWGS) also improved the sensitivity of ctDNA detection in 236 patients with seven different types of cancer, with specificities of 98 percent and sensitivities ranging from 57 percent to >99 percent.[33,34,35]

By including tissue- and cancer-specific large-scale epigenetic changes, methylation profiling of cfDNA has the potential to enhance ctDNA detection sensitivity in addition to better mutational analysis. DNA methylation profiles can distinguish between tumour and healthy tissues as cancer methylome mapping advances. Their methylation patterns accurately identified 29 of 30 colorectal cancer metastases from liver biopsies and 19 of 20 breast cancer metastases from normal breast tissue, and four common malignancies were distinguished from normal tissue with >95 percent accuracy.[47] However, the small amounts of material usually associated with a liquid biopsy are incompatible with traditional methylation profiling, which usually requires micrograms of DNA and bisulfite conversion, which destroys non-methylated DNA.[43] A new immunoprecipitation-based methodology was recently utilised to illustrate the possibility of methylation profiling of cfDNA, detecting target cfDNA down to 0.001 percent (as opposed to 0.1 percent for a previously employed hybridization detection approach).[47]

There has also been a description of a similar method for examining transcription start sites where nucleosome occupancy causes distinct read depth coverage patterns for expressed and silent genes. This method discovered that cfDNA analysis from metastatic patients may accurately distinguish expression cancer driver genes in areas with somatic copy number gains. With the possibility of developing predictive biomarkers in the future, this has been further developed to infer accessibility of transcription factor binding sites from cell-free DNA fragmentation patterns, allowing for precise tumour subtype prediction in prostate cancer.[21,43]

Cancer Types and Liquid Biopsy Utility

Gastric Cancer: Gastric cancer (GC) ranks second globally in terms of cancer-related mortality and is the fourth most frequent type of cancer.[19] A total of 9,86,600 new GC cases and 7,38,000 deaths are estimated to have occurred worldwide in 2008, despite recent advancements in diagnostic methods and peri-operative care leading to a rise in GC early identification and a decline in its mortality in recent decades.[19] A number of variables appear to contribute to the insufficient survival rate of GC and to the stringent diagnostic and therapeutic approach used to treat it: (1) a deficiency of effective diagnostic tests for GC early identification; (2) a lack of useful prognostic markers; (3) the lack of efficacy of existing therapies, such as chemotherapy and surgery, for GC patients with advanced stages; and (4) a lack of targeted therapy due to poorly understood mechanisms of tumor growth and treatment resistance. In order to enhance the clinical result for patients with GC, it is crucial to create practical diagnostic and monitoring technologies. Numerous research conducted in the last few decades have shown the potential value of blood-based biomarkers including cell-free nucleic acids (cfNAs) and circulating tumor cells (CTCs).[11,40,29,39]

These promising markers are thought to have a lot of potential and may help with cancer therapy approaches such as early illness identification, prognostic outcome prediction, tumor dynamics monitoring, and the creation of new targeted therapies.

CTC detection in patients with GC and its clinical relevance: Many approaches have been used to try and find circulating tumor cells (CTCs) in gastric cancer (GC), but RT-PCR has been the most popular method because it can identify CTCs in blood at a satisfactory rate even when the concentration of CTCs is very low.

The possibility of false-positive results is a major drawback of this high-sensitivity technique, which is why scientists have created multi-marker mRNA assays to increase specificity and dependability. One well-known example is the work of Wu et al., who created a high-throughput colorimetric membrane array that measures several markers at once, including MUC1, CEA, cytokeratin 19 (CK19), and human telomerase reverse transcriptase (TERT). The combination of these markers is a potent prognostic indicator for both overall survival and recurrence.[49,59,69]

Non-coding RNAs, like miRNAs and Piwi-interacting RNAs (piRNAs), which change specifically in cancer and have potential for CTC detection, have lately come under focus.[41,79]

Nevertheless, a significant difficulty with these methods is the possibility of sample contamination by RNAs from leukocytes in the mononuclear cell layer. This issue also impacts cell-free miRNA analysis and needs to be fixed before clinical use.[51,61]

As a prognostic indication, CTCs are very useful for predicting recurrence, metastasis, and overall survival, which clearly demonstrates their clinical value. Research using the standardized Cell Search System has shown this value time and time again. For example, metastatic GC patients with ≥2 CTCs had a considerably poorer overall survival than those with lower counts, according to Hiraiwa et al. A prospective study by Matsusaka et al. further supported this by demonstrating that CTC counts obtained at 2 and 4 weeks after the start of chemotherapy were highly predictive; patients with ≥4 CTCs had dramatically lower overall and progression-free survival, while the baseline count was not statistically significant, suggesting a strong correlation between CTC status and treatment response.

Although many early studies were constrained by small sample quantities, RT-PCR/qRT- PCR techniques have also been widely employed to establish the predictive capacity of CTC detection in addition to immunomagnetic techniques. Mimori et al. carried out a seminal study to address this constraint, using cDNA microarray analysis to identify membrane type 1 matrix metalloproteinase (MT1-MMP) mRNA as a possible marker. The team next confirmed its applicability in a large-scale qRT-PCR investigation with over 800 GC patients, proving the reliability of PCR-based methods when used on a sizable population and validating MT1-MMP as a significant prognostic marker. The combined results of these Cell Search and PCR-based approaches highlight the importance of CTC discovery in both clinical outcome prediction and possibly therapy efficacy monitoring for patients with gastric cancer.

Detection of circulating DNA in patients with GC and its clinical relevance: The examination of circulating cell-free DNA (cfDNA) in patients with gastric cancer (GC) includes the determination of its concentration using targets such as ALU or beta-action sequences, as well as the well-researched method of identifying methylation DNA specific to tumours. More important targets have been found by thorough microarray analysis since the first report on numerous methylation genes was published by Lee et al. in 2002. One well-known recent example is methylation XAF1 DNA, which Ling et al. shown to be an effective biomarker for diagnosis and prognosis. With a high AUC of 0.909 and a strong connection with a lower disease-free survival, it was found in 69.8% of GC patients but not in healthy controls.

Alongside methylation, detection of genetic changes in cfDNA is being investigated, though this area of GC is still in its early phases. According to Park et al., GC patients had substantially greater plasma levels of the MYC oncogene than healthy controls, and this was connected with the MYC status in tumor tissue. A recent disparity was highlighted by a study on HER2 amplification by Lee et al., which demonstrated no significant correlation between HER2 levels in plasma and the copy number variation in matched tumor tissue. Issues including the use of incorrect reference genes and substantial tumor heterogeneity could be the cause of this discrepancy. In order to settle these contentious concerns and establish the therapeutic usefulness of genetic aberration testing in circulating cfDNA for GC, this field, although promising, needs further thorough research.

Breast Cancer: The most frequent cancer in the world to affect women is breast cancer, which is also the second most common cause of cancer-related deaths among American women, after lung cancer. According to estimates, between 20% and 30% of all cases of breast tumours that have already occurred have metastatic recurrences, while between 6% and 10% of new cases of breast cancer are initially stage IV (de novo metastatic illness). Palliation of symptoms and enhancement of quality of life are still the key objectives for metastatic breast cancer (MBC), with the expectation that successful treatments will also result in objective remission and extension of disease control, which will ultimately affect survival. A mixture of clinical and molecular criteria are taken into account when choosing a systemic treatment approach, with the goal of employing the most efficient and least incapacitating customized method. Endocrine therapy must be the primary option when there is no clinical indication of aggressive illness, especially in patients with hormone-sensitive tumours, since it can offer survivals comparable to those achieved with chemotherapy (although with fewer objective responses).[5,23]

However, long-term endocrine therapy exposure may lead to acquired resistance and eventual disease development. According to recent data, over 30% of patients receiving endocrine therapy have activating mutations in the ligand-binding domain of oestrogen

receptor-a (ESR1), and these genetic anomalies may be the cause of endocrine resistance.

By analysing gene mutations on ctDNA and CTCs, liquid biopsy, a precision medicine technology, offers a real-time evaluation of MBC at baseline and recurrence.

CTC Detection and Its Clinical Relevance in Breast Cancer Patients: Cancerous cells known as circulating tumor cells (CTCs) separate from a primary tumor and enter the bloodstream, where they play a crucial part in the spread of the disease. Their identification offers a minimally invasive "liquid biopsy" for disease monitoring in breast cancer. The most popular method, the FDA-approved Cell Search system, counts CTCs by excluding white blood cells (CD45 negative) and focusing on epithelial markers (EpCAM, cyto keratins). Although it is a standardized clinical tool, it is limited in its ability to capture cells that have undergone epithelial-to-mesenchymal transition (EMT). CTCs have a well-established predictive significance; in metastatic breast cancer (MBC), a count of ≥5 CTCs per 7.5 mL of blood is strongly related with a considerably worse progression-free and overall survival. This predictive capacity is also present in early-stage disease, where CTCs act as an independent biomarker and are associated with a higher risk of early recurrence and a lower survival rate. The molecular characterisation of CTCs provides profound insights into tumor heterogeneity and therapeutic resistance, going beyond basic counting. Single CTCs can have actionable mutations and phenotypic alterations that are not always visible in a traditional tissue biopsy by undergoing genomic and transcriptomic analysis. For example, the identification of HER2 changes in CTCs or ESR1 mutations associated with endocrine resistance might directly inform customized therapy plans. This makes it possible to evaluate tumor progression dynamically and in real time. Monitoring CTC levels in clinical practice can help guide treatment choices; a decreasing count typically signifies a positive response to treatment, whereas steady or increasing counts may indicate treatment failure before scans show it. CTC-guided treatment approaches are being studied in clinical studies such as STIC-CTC, which compare their effectiveness to conventional clinician-driven decisions, especially when choosing between chemotherapy and endocrine therapy. The extraordinary rarity of CTCs in early-stage disease, their phenotypic flexibility, which makes reliable identification more difficult, and technological variability are some of the obstacles that CTC-based diagnostics must overcome despite this potential. Ongoing developments in molecular profiling, high-resolution imaging, and microfluidics, however, are constantly enhancing their collection and interpretation. CTC detection and characterisation, a fundamental aspect of the liquid biopsy field, constitute a potent, real-time instrument for customized oncology. It has great promise to track therapy response, enhance prognostication, and ultimately direct more efficient, individualized care for patients with breast cancer when combined with other indicators.[5,23]

Circulating Cell-Free DNA Detection and Its Clinical Relevance in Breast Cancer Patients: DNA fragments released into the bloodstream, mostly from necrotic and apoptotic cells, make up circulating cell-free DNA (cfDNA). Circulating tumor DNA (ctDNA), a particular subset produced from cancer cells, contains genetic and epigenetic modifications specific to tumours, including point mutations, copy number variations, and methylation changes. A potent, non-invasive "liquid biopsy" to comprehend the molecular profile of breast cancers in real time is made possible by the detection of ctDNA using sensitive technologies such as digital PCR and next-generation sequencing. This method allows for thorough tumor genotyping without the need for repeated invasive tissue biopsies, which is especially helpful when the tumor has spread. Treatment choices can be directly impacted by the clinical detection of actionable mutations in genes such as ESR1, PIK3CA and TP53, using ctDNA testing. For instance, ESR1 mutations may recommend a transition to treatments like fulvestrant and signal resistance to aromatase inhibitors, whereas PIK3CA mutations direct the usage of alpelisib. CtDNA measurement has substantial prognostic value in addition to mutational analysis. As a biomarker for tumor burden and disease aggressiveness, studies have shown that a greater baseline ctDNA level—typically characterized by more than two genomic changes or a mutant allele percentage ≥0.5%—is substantially linked to worse progression-free and overall survival.[126] Dynamic therapy monitoring and early resistance discovery are two crucial uses for ctDNA. More sensitive than imaging, serial ctDNA tests can monitor tumor response; increasing levels frequently occur weeks before radiologic development, offering a critical window for intervention, and the elimination of mutations is associated with therapeutic improvement. Notwithstanding its potential, there are many drawbacks, such as inconsistent sensitivity in early-stage illness, difficulties standardizing tests, and the requirement to distinguish ctDNA from variations in clonal haematopoiesis. However, ctDNA is solidifying its position as an essential tool in precision oncology for breast cancer as validation and technology advance.

Hepatocellular Cancer: Hepatocellular carcinoma (HCC) is the sixth most prevalent malignancy but the second largest cause of cancer-related death worldwide. Even in developed economies, mortality rates are rising, and its poor prognosis endures despite advancements in diagnosis and treatment. Although chronic hepatitis B and C virus infections have historically been connected to HCC, lifestyle variables such as excessive alcohol use, obesity, and type 2 diabetes are also substantially linked to HCC. Determining the target group for screening is a crucial clinical concern as the percentage of HCC cases with non-viral aetiologies rises globally. By finding individuals while more treatment choices are available,

early screening is known to improve survival. Only 30–40% of patients actually receive prompt, effective treatment in practice[10], in part because there are not enough accurate biomarkers. Although they are employed, traditional blood indicators such as des-γ-carboxy prothrombin (DCP), AFP-L3, and alpha-fetoprotein (AFP) are insufficiently sensitive and specific, frequently producing false-positive results. Carcinogenesis is known to be fuelled by accumulating genetic and epigenetic abnormalities, which can be clinically useful in a variety of malignancies. Tissue biopsies have historically provided this information, but because of anatomical limitations, invasiveness, or poor hepatic condition, these procedures are frequently impractical for patients with HCC. Two significant issues arise from this reliance on tissue biopsies: one biopsy provides little information and is unable to capture the dynamic, present state of cancer, including treatment sensitivity. The "liquid biopsy" technique, which analyses blood's circulating tumor cells (CTCs) and circulating cell-free nucleic acids (cfNAs), provides an answer. This method enables more sensitive diagnosis and tailored treatment choices for HCC by enabling frequent, minimally invasive sampling that can capture the tumour's dynamics and features in real time.

CTCs Detection and Its Clinical Relevance in HCC Patients: The Isolation by Size of Epithelial Tumor Cells (ISET) method, initially described, is one of the physical techniques for CTC identification in HCC. This approach is a novel, low-cost substitute for molecular methods, enabling cytopathological diagnosis from blood samples without the need for specialized equipment, and it uses cytomorphologic analysis to demonstrate that CTC circulation reflects tumor growth. However, the inability to easily extract collected CTCs from the membrane is a major drawback of the ISET device, limiting its use in further genetic investigation. The first biological approaches were developed by Matsumura et al., who established CTC positive as a predictor of metastasis and survival in HCC by using RT-PCR to identify alpha-fetoprotein (AFP) mRNA.[51] While following investigations established the clinical value of AFP mRNA[52,53], its prognostic relevance was not always proven, prompting research into alternative markers such as MAGE-1, MAGE-3, glypican-3 (GPC- 3) keratin 19 (K19), CD44[58], and hTERT mRNA.[59] Additionally, ICAM-1, cytokeratin 19, CD133, and CD90 have been analyzed using flow cytometry, proving their predictive relevance. The most popular platform is the EpCAM-based Cell Search™ system, which has been demonstrated in trials to be effective as a therapeutic target, for monitoring treatment response, and for customizing anticancer methods. For easy and effective CTC detection and prognostication in HCC, new platforms such as the aptamer-coated CTC-Chip have been proposed lately.

Circulating Cell-Free DNA Detection & Its Clinical Relevance in HCC Patients: A well- known and possibly novel biomarker for cancer is circulating cell-free DNA (cfDNA), with aberrations generally classified into quantitative and qualitative changes. A higher concentration of total cfDNA is referred to as a quantitative change, whereas particular genetic and epigenetic changes, such as gene mutations, DNA copy number variations, tumor-specific methylation, microsatellite instability (MSI), and loss of heterozygosity, are considered qualitative changes. Analytical techniques are therefore separated into two categories: determining the overall amount of cfDNA and identifying genetic abnormalities unique to a tumor, the latter of which is the method most frequently used in liquid biopsy investigations. Quantitative analysis has been utilized by some researchers to demonstrate that patients with HCC who have higher plasma or serum DNA levels have a worse prognosis. However, this approach is not widely used since it lacks specificity for HCC. Numerous aberrations have been thoroughly examined in the qualitative study of cfDNA, with the "methylation pattern" receiving more attention than single nucleotide mutations or copy number changes. Wong et al. were the first to disclose the clinical value of cfDNA in HCC. They employed methylation-specific PCR (MS-PCR) to identify p15 and p16 methylation in patient blood samples and connected these epigenetic markers to metastasis, recurrence, and tumor tissue methylation. Later research verified that DNA methylation alterations, which are frequently seen in cancers, could be accurately identified in plasma and used as prognostic and diagnostic indicators. For example, Iyer et al. showed that tumor tissue and plasma DNA had a considerable concordance in the methylation of tumor suppressor genes (APC, FHIT, p15, p16, and E-cadherin).[135] While individual genes had little diagnostic value, Huang et al. demonstrated that methylation RASSF1A was an independent prognostic factor and that a combination of four methylated genes (APC, GSTP1, RASSF1A, and SFRP1) reached a high AUC (0.933) for distinguishing HCC. Newer technologies such as genome-wide high-throughput sequencing and droplet digital PCR have evolved for more accurate detection of uncommon and numerous mutations in circulating DNA, but MS-PCR is still a commonly used method due to its simplicity and sensitivity. These cutting-edge methods have demonstrated links between treatment resistance in cancer patients and genetic abnormalities in cfDNA. The complete therapeutic efficacy of cfDNA, including risk assessment, early cancer identification, treatment resistance prediction, and predictive prognosis, must be proven immediately, especially in hepatocellular carcinoma patients.

Pancreatic cancer: Pancreatic cancer (PCa) is the largest cause of cancer-related death globally, with a median survival time of 5-8 months and a 5-year survival rate of less than 10%. The primary explanation of this poor outcome is because the disease is frequently asymptomatic in its early stages, which results in a delayed diagnosis at an advanced stage that is incurable,

with local invasion or distant metastases. Surgical resection is still the only curative treatment, however it is not appropriate for the majority of patients, despite improvements in perioperative therapy and surgical advancements. Therefore, biomarkers that are better than traditional ones like CA19-9 and CEA are desperately needed in order to facilitate prognosis, therapy monitoring, early identification, and precise staging. Traditional techniques for examining tumor tissue from surgical or biopsy specimens are intrusive, difficult to replicate, and do not account for dynamic changes in tumor status and treatment susceptibility. A less intrusive method that enables repeated sampling is the examination of circulating tumor cells (CTCs) and cell-free nucleic acids (cfNAs) using liquid biopsy. This method makes it easier to track the tumour's heterogeneity and changing condition in real time, catching details that a single tissue biopsy could overlook.

Circulating Cell-Free DNA in Plasma/Serum and PCa: Several researchers have so far shown the clinical value of identifying circulating tumor cells (CTCs) in prostate cancer (PCa) using a variety of techniques. RT-PCR was the main method used in early research to target mRNAs relevant to tumours and epithelial cells. CEA mRNA was utilized in the first publication by Funaki et al. and later research revealed high specificities (94.6%-96%) and sensitivities (47.8%-75.0%) for PCa detection. Additional mRNA targets were investigated, including EGFR, CK-19, and CK-20. Although the specificity of these mRNA-based techniques was high, their sensitivities were very modest and varied greatly. The possible contamination from leukocyte-originated RNA when using samples without particular enrichment was a major methodological concern. More recently, Zhou et al. combined the detection of c-Met, h-TERT, CK20, and CEA mRNA following immunomagnetic bead enrichment to obtain 100% sensitivity and specificity. Immunocytochemical methods, particularly the CELLSEARCH system, have also been used, however their detection rates in PCa range from 5% to 42%. Positive CTC results using this approach have been linked to poor prognosis and disease recurrence, indicating clinical usefulness despite this low rate. New technologies have been developed to increase the detection rate, such as size-based isolation and microfluidics, which have produced better outcomes. Overall, even though CTCs appear to be a promising biomarker for PCa, a number of issues need to be resolved before they can be widely used in clinical settings. First, they are not appropriate for early detection screening due to their poor sensitivity, which calls for the creation of new, extremely sensitive technologies. Second, the results of different investigations vary greatly due to the variety and rarity of CTCs as well as the absence of a standardized and constantly changing technique. This makes establishing solid proof and comparing data challenging. Therefore, for the widespread clinical use of CTCs in prostate cancer, a standardized approach and extensive validation trials are needed.

Detecting tumor-specific genetic and epigenetic changes and determining the overall concentration of cell-free DNA are the two primary approaches used in the investigation of circulating cell-free DNA in plasma or serum for pancreatic cancer (PCa). Numerous investigations have confirmed that cancer patients' blood contains these changes, which include methylation, microsatellite instability, point mutations, and allelic abnormalities. Research has moved toward identifying tumor-derived genetic alterations since its initial report in PCa in 1983, which demonstrated that higher serum DNA levels could have diagnostic and prognostic value. Although there are less research on methylation, microsatellite instability, and allelic imbalance, the identification of K-ras mutations has become a common strategy because this mutation is present in more than 90% of pancreatic adenocarcinomas. The therapeutic value of detecting K-ras mutations in circulating DNA for PCa diagnosis, tumor burden assessment, and therapy efficacy monitoring has been extensively studied.[72–80]

The most popular technique for this purpose has been polymerase chain reaction (PCR), but more recent technologies such as genome-wide high-throughput sequencing and droplet digital PCR provide higher accuracy for identifying rare and numerous mutations. It is hoped that cell-free DNA analysis will be similarly successful in PCa for disease detection, minimal residual disease assessment, recurrence prediction, and tracking acquired drug resistance, as these sophisticated techniques have demonstrated promise in other cancers for tracking the emergence of drug resistance.

ADVANTAGES AND LIMITATIONS

Benefits of Non-Invasive Sampling: Non-invasive cancer sampling methods: Such as liquid biopsies derived from blood, urine, or saliva—present considerable advantages compared to traditional tissue biopsies. A key benefit lies in their safety and comfort, as conventional biopsies often involve pain, potential infection, and procedural risks. In some cases, tissue sampling may be impractical due to tumor location or patient condition, whereas non-invasive approaches require minimal intervention, making them more suitable for elderly or critically ill individuals.[21,43]

Another major strength of non-invasive sampling is its ability to support continuous monitoring of disease progression and therapeutic response. Because these methods can be performed repeatedly without endangering the patient, clinicians can observe temporal changes in tumor genetics and biomarker profiles. This facilitates early detection of drug resistance, informed treatment adjustments, and better disease management based on evolving molecular data.[43,44]

Additionally, these techniques enhance the practicality

and reach of cancer screening and early diagnostic efforts. For instance, liquid biopsies can identify circulating tumor DNA (ctDNA) or exosomes even in asymptomatic individuals, allowing cancers to be detected at earlier stages than possible through imaging or clinical signs. Such early detection is crucial for high-risk groups and cancers typically discovered late, such as pancreatic and ovarian malignancies. Finally, non-invasive sampling contributes to a broader understanding of tumor heterogeneity. Since tumours can differ across and within sites, a single tissue biopsy might not capture the full genetic spectrum. Liquid biopsies, however, aggregate genetic data from multiple tumor locations simultaneously, enabling the design of more targeted and effective personalized treatments while advancing the field of precision oncology.[47,48]

Technical and Clinical Challenges: Liquid biopsy, which analyses tumour-derived materials such as circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), exosomes, and microRNAs found in blood and other body fluids, has emerged as a promising method for non-invasive cancer detection. Despite its potential, several technical and clinical obstacles must be overcome before it can be widely used or replace conventional diagnostic approaches.

Low Biomarker Levels and Sensitivity Limitations: A major challenge lies in the very small quantities of tumor-derived material in circulation, particularly at early cancer stages. For example, ctDNA may account for as little as 0.01% of total cell-free DNA, making accurate detection difficult.[45] This low abundance reduces assay sensitivity and increases the likelihood of false negatives. While advanced methods such as digital PCR and next- generation sequencing (NGS) have enhanced detection accuracy, they remain costly and technically complex, limiting their routine clinical use.

Specificity and Biological Interference: Specificity issues also persist due to biological noise. Mutations from non-cancer sources, such as clonal haematopoiesis, can appear similar to tumor-linked mutations, leading to false positives and unnecessary medical follow-up.

Additionally, benign conditions can release comparable extracellular vesicles or RNA fragments into the bloodstream, further complicating data interpretation.[46,47]

Lack of Technical Standardization and Reproducibility: There is still no global consensus on best practices for sample handling, processing, and analysis. Pre-analytical variations—such as differences in blood collection tubes or time delays before processing can significantly influence test accuracy. Inconsistent protocols across laboratories and measurement platforms have hindered reproducibility, making it challenging to standardize liquid biopsy procedures in clinical workflows.

Tumor Heterogeneity and Limited Representation: Liquid biopsies may not fully reflect the complete genetic diversity of tumours. Although they can capture mutations from multiple tumor sites, they might overlook rare or localized clones, especially when biomarker release into circulation is scarce or intermittent. In certain cancers, such as glioblastoma and some sarcomas, ctDNA shedding is minimal, reducing the diagnostic value of liquid biopsy.

Economic, Accessibility, and Time Constraints: Financial and logistical factors remain significant barriers to clinical implementation. Sophisticated sequencing technologies and bioinformatics analysis tools are expensive and often unavailable in low-resource healthcare settings. Although liquid biopsy is promoted as a quicker method, complex analyses and data processing can delay results, limiting its advantages for real-time clinical decisions.[43,57,69]

Clinical Validation and Regulatory Challenges: Many liquid biopsy assays have yet to receive adequate validation across different cancer types and patient groups. Robust, large- scale clinical studies are still needed to demonstrate their accuracy, prognostic relevance, and clinical benefits. Regulatory agencies also require comprehensive evidence before approving such assays, slowing their clinical integration and acceptance.[76]

Standardization and Validation Issues: Liquid biopsy is a promising, non-invasive method for cancer detection and management, analysing tumour-derived components like circulating tumor DNA (ctDNA), circulating tumour cells, and exosomes from blood or other fluids. However, its routine clinical use is hampered by several key standardization and validation issues.

Inconsistent Sample Handling: Methods for collecting, processing, and storing samples are not uniform. Variables like type of blood tube, time to processing, and centrifugation techniques can alter biomarker quality, leading to unreliable results across different types of settings.[21,23,43]

Diverse Testing Platforms: The use of various technologies such as digital PCR and next- generation sequencing (NGS) with differing sensitivities and accuracy makes it difficult to compare results between laboratories. The same sample can yield different outcomes depending on the lab's specific methods.[21,43]

Data Analysis Discrepancies: A lack of standardized computational methods for interpreting data means that the choice of software and algorithms can influence which mutations are identified. This variability increases the risk of both missing real mutations and reporting false ones.[9]

Absence of Benchmark Materials: There are no

universally accepted reference samples to calibrate and validate liquid biopsy tests. This gap makes it challenging to ensure consistent performance and quality control across different labs and platforms.[23]

Inconsistent Clinical Studies: Validation studies often involve small, specific patient groups and use varying definitions of success. This lack of uniformity makes it difficult to generalize findings and confirm the test's real-world effectiveness.[23]

Regulatory Hurdles: The absence of robust, consistent validation data has slowed regulatory approval and accreditation. Agencies require proof of a tests' reliability and clinical benefits, which many current liquid biopsy assays have not yet fully demonstrated.[9,21]

Uncertain Clinical Application: The variability in tests creates confusion for clinicians, who may be unsure how to interpret results or reconcile them with traditional tissue biopsies. The lack of clear reporting standards and action guidelines further hinders its use in daily practice.[9]

Call for Global Standards: Addressing these challenges requires international cooperation to establish unified protocols for sample processing, assay validation, and study design. Initiatives by consortia are working towards these goals, which are essential for making liquid biopsy a trusted and widely used diagnostic tool.[21]

RECENT ADVANCES AND FUTURE PERSPECTIVES

Emerging Biomarkers Beyond cfDNA and CTCs: The capacity of extracellular vesicles (EVs), particularly exosomes, to encapsulate and preserve a variety of molecular cargo, including proteins, lipids, mRNA, and non-coding RNAs, which represent the dynamic state of the tumor microenvironment, has made them interesting biomarkers in liquid biopsy. They are perfect for early detection and long-term tracking of the development of cancer because of their biological stability and abundance. With sensitivities and specificities above 85% even in the early stages, research has shown that EV-derived miRNAs and proteins improve diagnostic accuracy in a variety of malignancies, including pancreatic ductal adenocarcinoma (PDAC).[19] Additionally, current studies highlight the use of EV-based markers in precision immuno-oncology, which allow for the real-time evaluation of therapy response.[21] The sensitivity and repeatability of EV analysis have been further enhanced by technological developments such as in situ nucleic acid tests within intact vesicles and microfluidic-based isolation.[43]

Tumor-educated platelets (TEPs) and circulating RNAs have also gained popularity as potential indicators in liquid biopsy. Tumor-derived RNA molecules are actively sequestered and spliced by platelets, producing distinct transcriptome profiles that can distinguish between malignant and non-cancerous conditions. In more than 1,000 patients, the "ThromboSeq" method showed impressive results, detecting and categorizing tumours with 99% specificity spanning early to late stages.[43]

The clinical promise of TEP-derived mRNA panels as minimally invasive diagnostic tools has been supported by systematic evaluations in lung and colorectal malignancies, which have shown that these panels achieve area under the ROC curve (AUC) values exceeding 0.90.[45] Despite being less durable than DNA by nature, RNA-based biomarkers are now more reliable for practical use thanks to enhanced pre-analytical handling and sequencing procedures. The next generation of liquid biopsy assays is being redefined by fragment omics and epigenomic profiling. These methods examine cfDNA fragmentation patterns, methylation signatures, and nucleosome positioning to infer tumor existence and tissue of origin rather than depending just on mutation detection. Conventional mutation-based assays were outperformed by a thorough multimodal investigation that combined methylation, copy number alteration, and fragment omics, achieving 88.9% sensitivity and 95.2% specificity for multi-cancer identification.[63]

Likewise, Multimodal Epigenetic Sequencing Analysis (MESA) improved the detection accuracy of colorectal cancer by combining methylation with nucleosome occupancy and fragmentation characteristics.[71]

Fragmentomic differences within hypomethylated regions may be reliable early markers of breast cancer, according to another study.[58] All of these results point to the potential of cfDNA fragment omics to greatly enhance early tumor type detection and classification. Future developments in liquid biopsy are moving toward multi-omic integration, where nucleic acid-based biomarkers are complemented by proteomics, metabolomics, and immunophenotyping. Proteomic changes and immunotherapy responses have been linked in recent plasma proteomic studies of non-small cell lung cancer (NSCLC), offering predictive information about treatment outcomes.[9] Metabolomic profiles have also been connected to survival and response to treatment in metastatic melanoma, highlighting their importance in real-time patient monitoring.[10] Additionally, circulating T cell immunophenotypic profiling has demonstrated significant diagnostic accuracy (AUC 0.92–0.94) in differentiating between healthy patients and early-stage lung malignancies.[11] The next significant step toward developing highly sensitive, selective, and dynamic liquid biopsy tests is the combination of these multi-omic techniques with models powered by bioinformatics and artificial intelligence.

Integration with Artificial Intelligence and Bioinformatics: The combination of artificial intelligence (AI) and bioinformatics is fundamentally changing how we use liquid biopsies for cancer. Instead

of just looking for mutations in circulating tumor DNA (ctDNA), the field now analyses multiple components, such as the fragmentation patterns of cell-free DNA (cfDNA), circulating tumor cells, and extracellular vesicles. The immense complexity of this data requires advanced AI, including machine and deep learning, to identify subtle, cancer- related patterns that humans would miss.[9] For example, deep learning can interpret the entire cfDNA fragment profile to detect cancer and even identify where in the body it started, based on the unique "footprint" that tumor cell activity leaves in the blood. A major bioinformatics hurdle is finding these rare cancer signals amidst a vast amount of normal DNA. To solve this, sophisticated computational pipelines are used to process sequencing data, correct errors, and confirm genuine cancer variants. The most significant recent progress involves integrated platforms that examine several data types at once, like combining mutation data with epigenetic markers such as DNA methylation.[11,21,23]

These multi-faceted AI models, which pull together weak signals from various sources, are proving to be much more accurate for diagnosis and prognosis than tests analysing a single factor. This is turning liquid biopsy from a tool for monitoring known cancers into a proactive method for early screening. Future directions are even more transformative, aiming to create “digital twins” highly personalized computer models of a patient's cancer. By constantly updating these models with new data from liquid biopsies, clinical reports, and imaging, AI could simulate how the disease might progress and test potential treatments virtually.[9] This is supported by using ctDNA to track how a tumor evolves over time. Furthermore, the use of "explainable AI" (XAI) will be vital for gaining doctors' trust, as it can clarify the reasoning behind a prediction, moving from a "black box" to an actionable insight for treatment decisions. Despite the promise, challenges remain. Widespread clinical use requires standardizing how samples are processed and data is analyzed to ensure consistent results. Large-scale clinical trials are essential to validate these AI-bioinformatics tools across diverse populations.[21,23]

As the technology matures, the future points to a continuous cycle where liquid biopsies provide real-time data on a tumour’s changes, and AI-powered systems convert this information into dynamic, personalized guidance for treatment, pushing oncology closer to the goal of true precision medicine.

Toward Personalized Cancer Management: The treatment of cancer is shifting away from standardized methods toward highly customized care, with liquid biopsy at the forefront of this change. This technique offers a non-invasive way to monitor cancer by detecting and analysing components released by tumours into the bloodstream, including circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and extracellular vesicles. Technological progress has significantly improved the scope and precision of these tests. For instance, ultra- deep next-generation sequencing can now evaluate hundreds of cancer-related genes from a blood sample, pinpointing treatable mutations and observing how the tumor genetically changes over time, including the development of therapy resistance.[11] Enhanced methods like digital PCR and Beaming are so sensitive they can find extremely rare genetic variants, which is vital for identifying minute traces of cancer left after treatment (minimal residual disease) and for screening in early-stage cancers.[21,23]

These advancements are transforming patient care by allowing treatments to be modified quickly in response to the tumour’s evolving genetic makeup. The applications of liquid biopsy are also expanding from basic genetic testing to a more comprehensive evaluation. For example, examining epigenetic markers on ctDNA, such as methylation signatures and fragmentation patterns, provides a robust method not only for initial cancer detection but also for identifying where in the body the cancer originated a major advantage for cancers of unknown primary.[13] The distinct way tumor DNA breaks down (fragment omics) offers additional diagnostic clues for early cancer identification.[43] Meanwhile, the study of CTCs has progressed beyond simple counting to detailed functional analysis and single-cell genetic sequencing. It is now possible to grow these captured cells in the lab to test drug responses and to analyze their RNA to understand the molecular drivers of metastasis and treatment resistance.[57] By combining these different data sources, liquid biopsy is evolving from a simple diagnostic aid into a powerful tool for investigating cancer biology. Integrating liquid biopsy into standard medical practice is already enhancing personalized cancer care at every stage. For monitoring minimal residual disease, tumor-informed assays such as Signatera™ can predict cancer recurrence in cancers like colorectal, breast, and lung often long before it is visible on scans, creating a potential opportunity for pre-emptive treatment.[69] In advanced cancer, repeated blood tests allow doctors to track how well a therapy is working and to detect emerging resistance mutations (e.g., EGFR T790M in lung cancer or ESR1 in breast cancer), thereby guiding the next line of targeted treatment without resorting to invasive tissue biopsies.[13,77] Perhaps the most significant future impact lies in early detection. Multi-cancer early detection tests, which assess DNA methylation in a standard blood draw, are currently under investigation in large trials and show potential for screening healthy people for numerous cancer types at once.[33] While issues of cost, false positives, and integration into healthcare systems remain, these tests mark a new era in preventive and personalized medicine.

The next phase of liquid biopsy development will be shaped by several key challenges and opportunities. Achieving consistency in sample handling, test

performance, and data interpretation across different technologies is crucial for broad and dependable use. Furthermore, randomized clinical trials are needed to confirm that acting on a positive MRD result actually improves patient outcomes. The complex, multi-layered data from ctDNA, CTCs, and EVs will require sophisticated artificial intelligence algorithms to build accurate predictive models.[78] Finally, making these tests affordable and accessible is essential for global equity. As these obstacles are overcome, the ultimate goal is a future where a routine blood test can perform annual cancer screening, confirm a diagnosis, identify all treatment targets, monitor therapy response, and provide the earliest sign of relapse; all with minimal patient discomfort. In this vision, liquid biopsy acts as the central nervous system for cancer management, enabling a dynamic, responsive, and truly patient-focused approach to overcoming the disease.

CONCLUSION

In conclusion, liquid biopsy, centered on the analysis of circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs), represents a paradigm shift in oncology. This non- invasive approach overcomes the critical limitations of traditional tissue biopsies, including invasiveness, sampling bias due to tumor heterogeneity, and the inability to perform frequent monitoring. By providing a real-time, comprehensive snapshot of tumor genetics from a simple blood draw, liquid biopsy unlocks profound clinical applications. It enables early cancer detection, precise tumor genotyping for targeted therapy, dynamic monitoring of treatment response, and the sensitive identification of minimal residual disease and emergent resistance mechanisms. Despite its transformative potential, the field must address significant challenges, including the standardization of pre-analytical and analytical protocols, validation across diverse cancer types and stages, and integration into clinical workflows. Future advancements lie in the integration of multi-analyte approaches combining ctDNA with extracellular vesicles, tumor-educated platelets, and fragment omics powered by artificial intelligence and sophisticated bioinformatics. These innovations promise to enhance sensitivity and specificity further, paving the way for personalized cancer management. Ultimately, liquid biopsy is poised to become the cornerstone of precision oncology, facilitating a dynamic, patient-centric approach to cancer diagnosis, monitoring, and treatment selection that improves patient outcomes and survival.

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