**Liquid Biopsies-A multimodal cancer diagnostic tool**

**Introduction**

Traditional tissue biopsies, Image guided or not, are considered as a gold standard in cancer diagnostics. They not only facilitate histological diagnosis but also analyse the molecular profiling of the tumour thereby improving the clinical outcome.The molecular profiling of tumors obtained from individual patients has been demonstrated to enhance the selection of personalized cancer treatment therapies, predict patient responses, identify drug resistance, and monitor tumor relapse.(1,2)

However, the technique is invasive,and sometimes it is not easy to obtain it due to its anatomical location or the characteristics of the tumour. Furthermore, the tumours grow, mutate, and become heterogeneous resulting in inconsistencies in the test report(3). These invasive techniques for procuring tissue samples are also linked to a variety of other limitations inherent to the procedure. These include challenges in obtaining tumor samples that meet standards for both quantity and quality, as well as issues such as low sensitivity, high costs associated with sample isolation, and the need for clinical validation.

Furthermore, the process of obtaining biopsy samples through invasive methods throughout the course of treatment, for the purpose of monitoring tumor response and relapse, presents a significant hurdle in comprehensive tumor profiling. This challenge is particularly pronounced in cases of metastasis, where tumors have disseminated and continuously undergo spatial and temporal evolution in response to treatment over time. Consequently, the complex nature of tumor behavior may necessitate multiple biopsies to gain a comprehensive understanding, as it proves difficult to capture a holistic representation of the tumor (4).

Considering the challenges encountered in diagnostic methods related to conventional biopsies, the examination of different biological fluids for components originating from tumors has gained preference over the past decade. This shift from traditional biopsies has been made possible through techniques such as liquid biopsy, empowering onco-pathologists with molecular profiling of tumour at all stage of its development,

The term Liquid Biopsy was Coined by Pantel k and Catherine Alix-Panabières in 2010 to describe blood draws that evaluate circulating tumor cells (CTCs). Since then, the term has evolved to include circulating plasma tumor DNA (5). Biologically, the targets for liquid biopsy can be divided into two categories. One category refers to large or small molecules without cells or without a subcellular structure in the body fluid; these include proteins, nucleic acids, lipids, carbohydrates and other small metabolites and metal ions. The second category includes targets with cellular or subcellular structures, including single or clustered circulating tumor cells (CTCs), circulating cancer-related fibroblasts (CAF), immune cells, tumor-educated platelets (TEP) (6), extracellular vesicles (EVs) and circulating mitochondria (7,8). These sources can be examined to extract insights about both the overall tumor load and the fundamental biology, especially as cancer cells experience immunoediting and encounter evolutionary bottlenecks imposed by therapeutic interventions (9).

The primary benefit of liquid biopsy analysis lies in its minimal invasiveness and the potential for serial repetition, enabling real-time extraction of tumor-related information. Furthermore, the discovery of predictive biomarkers in peripheral blood, capable of monitoring therapy response in real time, presents a significant opportunity for innovative strategies in the therapeutic care of cancer patients.(3)

Liquid biopsy refers to all kinds of procedures that detect or quantitatively measure disease-related biomarkers from a human body fluid.LBs mostly involve blood sampling, although other body fluids like mucosa, pleural effusions, urine, and cerebrospinal fluid (CSF) are also analyzed (10). Liquid biopsy analytes include CTCs, circulating tumor DNA (ctDNA), which is the tumor-derived fraction of cell-free DNA (cfDNA), as well as cell-free RNAs (long non-coding RNAs and microRNAs), extracellular vesicles, tumor-educated platelets, proteins, and metabolites that can be interrogated to derive information about the overall tumor burden as well as the underlying biology as cancer cells undergo immunoediting and therapy-imposed evolutionary bottlenecks.(9)

**Circulating tumor cells (CTCs)**

CTCs are heterogenous population of cancer cells that detach from either the primary tumor or metastatic deposits in the periphery of patients, Initially originating from primary tumors within the tissue, these cells are released, traverse the circulatory system, and play a pivotal role in the formation of metastatic or secondary tumors in distant locations throughout the body (11). They have a short half-life of approximately 1h to 2.4 h.

These cells are very heterogenous, and not only varies in numbers from patient to patient but also vary within the same patient from time to time. In numerical terms, their presence in the bloodstream is relatively low, with approximately one CTC detected per million leukocytes (12). Regarding their morphology, research has indicated that the shape of CTCs varies based on the stage and/or type of tumor.9 Furthermore, CTCs have been observed to aggregate by adhering to cells such as fibroblasts, platelets, and others. These aggregates have been documented to disseminate to more distant locations within the body compared to individual CTCs. This clustering of cells provides them protection against oxidative stress and the immune response in their surroundings (13,14,15).

**Circulating tumor DNA (ctDNA) and Circulating cell free DNA (cfDNA)**

Usually, when cells undergo apoptosis or necrosis, they release nuclear and mitochondrial DNA into the bloodstream as part of the cellular breakdown process. This released DNA is subsequently eliminated from the circulation by phagocytes. Hence the level of these circulating DNA fragments is very low in a healthy individual (16,17).

Circulating tumour cells may release ctDNA in the circulation. Several hypotheses have been put forth to elucidate why viable cancer cells might intentionally release DNA into the bloodstream. One such possibility is that cancer cells release oncogenic DNA with the intention of influencing the transformation of susceptible cells at distant sites (16). It has also been found that effective clearance of these fragments by Phagocytosis may not happen in certain instances like severe inflammation, excessive exercise or molecular changes within a tunoral mass resulting in accumulation of cell debris and DNA fragments which ultimately is released into the circulation (17).

However, CTCs are not the only source of ctDNA. A single human cell contains 6 pg of DNA and there is an average of 17 ng of DNA per ml of plasma in advanced-stage cancers (18,19,20). Therefore, if CTCs were the primary source of ctDNA it would require over 2,000 cells per ml of plasma. In reality, there are, on average, less than 10 CTCs per 7.5 ml blood (21). So,Physiological states such as inflammation or exercise are also known to enhance cfDNA levels, which are not always reflective of underlying malignancy (22,23).

It must be noted that ctDNA accounts for only 0.1–10% of the total circulating cell-free DNA (cfDNA), whose normal plasma levels range from 10–100 ng/ml (24). Both the amount and integrity of circulating cfDNA can be used to distinguish between cancer patients and healthy individuals. Overall levels of cfDNA tend to be higher in cancer patients than in healthy individuals and appear to increase with stage and metastasis (25,26). Recently published research observed the length of ctDNA fraction in patients with cancer to be of 20-50 base pairs which is relatively shorter than the cfDNA (27).

**Extracellular vesicles**

Extracellular vesicles (EVs) are membrane-bound particles generated by all types of cells, both in normal and pathological conditions. They are also produced in response to various stimuli, including proteases, ADP, thrombin, inflammatory cytokines, growth factors, biomechanical shear and stress inducers, as well as apoptotic signals (28).

Depending upon their composition and secretory pathways, these have been divided into microvesicles and exosomes. Extracellular vesicles (EVs), are small, membrane-bound, saucer-shaped vesicles secreted by cells and are usually about 30–100 nm in size. These are found in various body fluids such as plasma, urine, cerebrospinal fluid (CSF), saliva, etc (29).. The mitogen-activated protein kinase pathway, which is up-regulated in many tumor cells, is believed to play a role in the active release of vesicles from cells (30). These vesicles are characterized by specific markers such as CD9, CD63, CD81, ALIX, and heat shock protein 70 (HSP70), which facilitate their isolation and enrichment (31).

Extracellular Vesicles carry diverse biomolecules like DNA, RNA, protein, etc., and play crucial role in intercellular communication. They attains high levels in body fluids as they are secreted profusely by the tumour cells (31). Analysis of EVs helps in analyzing tumor monitoring, prognosis, and therapeutic response (32,33).

Exosomes, with a size between 40–160 nm, are small extracellular vesicles that are released by most cells and play an important role in intercellular communication(34).

Originating from the endosome system, exosomes transfer intercellular information carrying a variety of molecules, including proteins, lipids, nucleic acids (DNA, microRNA, mRNA), and other important information from the cell (35). Various studies suggest that exosomes could be a novel biomarker in liquid biopsy because they are found to exist in almost all body fluids. However, the clinical application has been limited by the lack of elevated standard methods to analyze and separate components.

**miRNA**

MicroRNAs (miRNAs) are a class of non-coding RNAs approximately 18–25 nucleotides in length, serving regulatory roles. Notably, miRNAs govern the expression of diverse oncogenes and tumor-suppressor genes, exerting a pivotal influence on the pathological progression of tumor development (36,37).

Therefore, altered levels of peripheral blood-circulating miRNA are associated with cancer development. Comprehensive studies have demonstrated miRNA in exosomes as a potential non-invasive biomarker for cancer-risk stratification and outcome prediction (38,39,40).

Quantitative real-time PCR (qRT-PCR) represents the gold standard technique for miRNA analysis. However, some disadvantages such as non-absolute quantification, false positives, and expensive equipment limit its clinical application.

**TEPs**

Platelets play a multifaceted role and promote metastasis by various processes. They stimulate tumor angiogenesis and vascular remodeling, protect CTCs from shear forces and evade immune surveillance, and recruit stromal cells.Tumour, on the other hand, educate platelets and induces platelet activation, aggregation, and release of platelet-derived substances in circulation, and promote thrombocytosis via influence megakaryopoiesis in bone marrow.

In the bidirectional interactions between tumors and platelets, platelets exhibit both systemic and localized responses to cancer. They continuously uptake and enhance their content with free proteins, nucleic acids, vesicles, and particles (41,42), consequently causing changes in their RNA and proteomics expression patterns . This phenomenon is referred to as "tumor-educated platelets" (TEPs) (43).

Tumor-educated platelets are defined as functional platelets with a distinct tumor-driven phenotype due to the transfer of tumor-related molecules from cancer cells to platelets (44).. Nonetheless, the process by which platelets undergo education and attain unique RNA and protein profiles remains incompletely understood. The majority of conducted research has concentrated on the RNA content of tumor-educated platelets (TEPs), revealing that distinctive spliced TEP-RNA signatures can furnish specific insights into the existence of cancer cells and contribute to tumor progression (45,46).

**Sampling procedure for Liquid Biopsies**

For Isolating biomarkers from the blood sample, Experienced Phlebotomist should draw the sample to avoid hemolysis. Use of plasma is preferable to serum to isolate cfDNA. Standard K2- or K3-EDTA tubes can be used for sample collection. blood storage at room temperature in EDTA tubes should not exceed 3 h, and plasma collection should be done as soon as possible after blood withdrawal. The storage of whole blood at 4°C does not help as it does not prevent leukocyte lysis.

specific preservative tubes containing special fixatives able to stabilize blood and cfDNA for several days and should be used whenever it is not possible to process the sample within 3 h from collection.There are currently no conclusive indications on the quantity of blood to be used to obtain a sufficient amount of ctDNA, but many diagnostic kits indicate the minimum amount of plasma required for analysis.

Two steps centrifugation must be done to obtain plasma so that no cell residues is left.The first step should be a low speed centrifugation with a speed of 1200-1600 g to avoid leukocyte lysis. the supernatant is separated and again subjected to a second step 0f high-speed centrifugation with a speed of ≥3000 g to remove all contaminants. Centrifugations must be carried out without any pause in between. The use of a refrigerated centrifuge (4°C) is also recommended.

The plasma obtained can be stored at −20°C for short periods (∼1 month). For longer periods, it is recommended to store the plasma at −80°C, to guarantee cfDNA stability, avoiding freezing and thawing cycles that can cause consistent decreased total cfDNA amount (47,48).

For other analytes like mucosa, pleural effusions, urine, and cerebrospinal fluid (CSF), the selection of the appropriate body fluid for a liquid biopsy must be a meticulously considered decision, particularly in cases where the technique is invasive like CSF and where certain fluids might not accurately reflect the tumor's origin. It is always imperative to capitalize on the primary advantage of liquid biopsy, which lies in its ability to procure samples with minimal invasiveness.

It is better to centrifuge the CSF sample in a refrigerated centrifuge at a speed of 400 g for 5 min at 4°C or 2000g for 5 min at 4°C followed by 10000g for 5 min at 4°C. The supernatant can then be divided into 1mL aliquots and stored at -80°C (49).

Pre-analytical variables may hamper the result of the analytes as the molecular characteristics of clinical samples can undergo significant alterations throughout the initial processing steps, encompassing everything from gathering the specimen to extracting the desired molecules like nucleic acids.

This also includes transporting, storing, and archiving the sample, as well as extracting the molecules present in it. These modifications can lead to changes in the intended analytical outcomes. Without a well-defined and carefully followed process, there is a notable risk that the diagnostic tests might not accurately reflect the original analyte composition within the patient's body. Instead, the tests might end up measuring altered levels resulting from the processing steps. Hence for all types of liquid biopsy sample, standardized and validated steps to be performed to achieve reliable diagnostic outcomes (50).

**Technologies for the analysis of Liquid biobsy**

Since the identification of cfDNA, the FDA has granted approval to five tests. These tests encompass the detection of point mutations in cancer-associated genes such as KRAS, EGFR, and PIK3CA. Additionally, they encompass the evaluation of tumor mutation burden (TMB), microsatellite instability, ALK rearrangement, insertions and deletions, and methylation patterns ((51).Liquid biopsy samples can be subjected to various techniques, including real-time PCR, digital PCR, or a multigene NGS-panel. The outcomes typically exhibit strong specificity and a broad spectrum of sensitivity rates. The exact sensitivity levels depend on factors such as the nature of the sample, the clinical attributes of the patients, and the characteristics of the tumor.

**Real-time PCR**

At present, real-time PCR stands as the benchmark for analyzing point mutations and/or minor insertions/deletions within ctDNA. This technique is widely accessible in molecular diagnostic laboratories due to its ability to ensure both sensitivity and a swift turnaround time (TAT) at a cost-effective rate.

Real-time PCR utilizes a specialized probe that can be adapted to enhance diagnostic sensitivity. An example of this is the amplification refractory mutation system (ARMS/Scorpion) technology. This technology enhances sensitivity by concurrently amplifying mutated alleles of the target gene, or genes, of interest along with an internal control gene. Moreover, a specialized blend of control oligonucleotides enables the assessment of both DNA quality and quantity. Using this particular technology, it becomes achievable to attain a limit of detection (LOD) as low as 0.5%. As a result, this approach is well-suited for identifying minor proportions of mutated alleles amidst substantial amounts of wild-type genomic DNA, such as in the context of ctDNA (52).

**Digital PCR**

Digital PCR (dPCR) is a technological advancement of the classic PCR; this innovative approach is intended to transform the exponential, analog nature of PCR into a linear, digital (or binary) signal (53).

There are three types of dPCR platforms:

(i) **Droplet dPCR (ddPCR)**, where Partitions are depicted as approximately 20,000 uniform droplets within an oil-water emulsion. A single DNA molecules are spread out inside the bioreactors (droplets or wells) according to Poisson distribution (54).

(ii) **Solid digital PCR (sdPCR)** where the bioreactors are portrayed through approximately 20,000 to 12,000 partitions that are positioned on a solid support (chip). This approach eliminates the need for an emulsion procedure and mitigates the risk of droplet breakage, which could otherwise result in a reduction in analysis performance (55). DNA molecules are spread similar to the droplet dPCR system.

(iii) **Beam, Emulsion, Amplification, Magnetics (BEAMing) dPCR**.Here, an initial standard PCR amplification step is necessary prior to conducting the analysis (56). Following this, the amplified products are dispersed into numerous uniform droplets formed within an oil-water emulsion. Magnetic microspheres are also introduced, which bind the PCR products. Subsequently, the beads are isolated through either centrifugation or magnetic separation. Ultimately, the DNA bound to the microspheres can be quantified using optical scanning or flow cytometry, achieving a limit of detection (LOD) of 0.01%.18

Once the PCR amplification steps are over , the analysis of the analytes is done by fluorescence detection. Both qualitative and quantitative information of even small numbers of mutated genes in a background of wild-type alleles can be obtained by partitioning the amplification reaction. dPCR is more sensitive than real-time PCR, reaching a sensitivity limit of 0.1%-0.01% with high precision and reproducibility (54).

Both ddPCR and BEAMing have allowed reduction of the detection limit of ctDNA mutations to 0.01%-0.02%, with comparable sensitivity (82%-87%) and specificity (97%) for epidermal growth factor receptor (EGFR)-sensitizing mutations in lung cancer (57,58). Despite its highly sensitive and specific performances, its workflow is complicated and expensive to apply in routine clinical settings (59).

A primary constraint inherent in all digital PCR methods, when contrasted with sequencing-based techniques, is their limitation in detecting solely established mutations. This limitation hinders the ability to identify novel alterations (60).

Despite these limitations, dPCR is a valid confirmatory method and, due to its ability to quantify the mutated alleles and therefore to monitor patients over time, its usefulness as a surrogate biomarker of treatment response is reinforced(60).

When encountering challenging result interpretations, provided that pre-analytical and analytical concerns have been ruled out, it is advisable to assess the clinical parameters. Conditions such as a low disease burden or the progression of disease in the brain or bones typically exhibit minimal ctDNA shedding in the body fluids. This can potentially lead to misinterpretation of results (61).

**Next-generation sequencing**

Multiple genes along with multiple known and unknown alterations such as single-nucleotide variant (SNV), insertions/deletions, rearrangements can be investigated simultaneously with Next Generation Sequencing.

Newer and more sensitive NGS applications based on targeted sequencing allow a sensitivity <1% (0.1%-0.01%) for ctDNA analysis. These targeted sequencing includes the tagged-amplicon (TAm-seq) and its more advanced version the eTAmSeqTM; the safe-sequencing system (Safe-SeqS) and the CAncer Personalized Profiling (CAPP-seq); the AmpliSeq. Some of these panels are able to investigate both circulating DNA and RNAalong with some tricky alterations such as MET amplification (34,35,36).

The concordance between NGS analysis of tumour metastasis and ctDNA has been found to be 97%’as compared to low concordance between primary tissue and ctDNA which is primarily due to tumour heterogeneity or clonal hematopoiesis which increases with age (37,38).

Yi chen et al studied the emerging roles of NGS-based liquid biopsy in non-small cell lung cancer.and concluded that NGS-based ctDNA assay might be applied in identifying actionable genomic alterations, dynamically monitoring response and resistance to targeted agents, prescreening early-stage lung cancer, and tracking spatiotemporal evolution of lung cancer . Nevertheless, complexities associated with discerning clinically significant driver genomic changes, establishing meaningful cutoff frequencies for clinical relevance, difficulties in identifying predominant resistance mechanisms, determining the appropriate course of action upon obtaining positive ctDNA screening outcomes in early-stage non-small cell lung cancer (NSCLC), and evaluating the cost-effectiveness of managing tumor evolution are some of the challenges faced (62).

**Report of LB results**

The integral part of all the diagnostic procedures is the reliable reporting based on which the treatment modalities are decided. Liquid biopsy reports must include the following information:

* unique identification of the patient
* identification of the unit/physician which prescribed the analysis
* material used for the analysis (type and volume) and date of collection
* methods of sample storage
* sample acceptance date and date of reporting
* methods used for analysis
* investigated variants (for targeted assays)
* genes covered (for the untargeted assay)
* test results
* sensitivity, specificity and LOD of the assay
* data interpretation regarding druggability, actionability and resistance profiles

The report needs to be filled out using a predefined template with all the above mentioned requisites, with a date and the signature of the laboratory Specialist.Recognizing the test's significance for treatment planning, the reporting timeframe should not surpass five days from the time of the request. Due to the general diagnostic sensitivity of liquid biopsy (approximately 87%), instances of negative results for mutations should not be categorized as 'wild-type', as the potential for false negatives always exists. Hence, when faced with a negative outcome and when technically possible, it is advisable to consider utilizing a tissue biopsy or potentially conducting a second liquid biopsy procedure.

 **Limitations**

Liquid biopsies hold great promise as a strategy for advancing biomarker research. However, before these biomarkers can find their way into clinical practice, several technical, biological, and clinical challenges must be navigated. Challenges such as the absence of universally standardized assays and the substantial cost associated with liquid biopsies impede the seamless integration of these biomarkers into clinical workflows. Furthermore, the utility of liquid biopsy techniques has often been explored in studies with limited sample sizes, underscoring the necessity for extensive, high-quality research on a larger scale. This rigorous approach is crucial to corroborate earlier findings and establish the foundation for the routine clinical utilization of liquid biomarkers (63).

Special emphasis should be given on precise and validated standard procedures for sample collection and targeted analytes during the pre analytical phase. Specimen collection devices with stabilizing agents for the analytes and kits for isolating nucleic acids should be well checked.duriDiagnostic companies and diagnostic laboratories are increasingly focused on specified and verified generic pre-analytical workflows for different specimen types and analytical targets. Links to state-of-the-art, well-designed, specified, and verified analytical test technologies including related instrument platforms (such as NGS dPCR with verified software and integrated post-analytical steps such as bioinformatics for data analysis and interpretation should be made available. All are increasingly becoming the building blocks and basis for the development of new, safe, and reliable analytical tests.

Though very appealing, clinical application liquid biopsy testing is still limited to primary tumor diagnosis, assessment of treatment response, therapy monitoring, detection of minimal residual disease, evaluation of tumor heterogeneity and emergence of resistances to targeted therapies (64).

In a broader context, the widespread integration of liquid biopsy into clinical practice demands a comprehensive grasp of its complete capabilities and constraints. This comprehensive understanding can only be attained through the meticulous analysis of extensive patient groups spanning various cancer categories and encompassing multiple medical facilities(65). Despite this requisite, the elevated anticipations surrounding liquid biopsy as an innovative instrument for both cancer detection and surveillance are anticipated to galvanize intensified exploration in this domain in the forthcoming years. The overarching aim is to tackle the persisting uncertainties and ultimately determine whether liquid biopsy will stand as a pivotal advancement in the care of individuals afflicted by cancer .

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