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Beyond DNA Mutations in Liquid Biopsy: From a Primarily Genotypic Tool to a Functional Plasma Biomarker

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A B S T R A C T

Liquid biopsy is considered an innovative revolutionary technology in precision medicine and offers non-invasive and real-time molecular profiling of individual patients. Over the past decade, the analysis of circulating tumor DNA (ctDNA) has become nearly synonymous with the term of "liquid biopsy". The discovery of somatic mutations, copy number variations, and epigenetic signatures in cell-free(cf)DNA from peripheral blood has revolutionized oncological diagnostics enabling genotyping from a simple venipuncture, tracking of clonal evolution, and detection of minimal residual disease with remarkable sensitivity. Yet a conceptual limitation has quietly accompanied these remarkable progresses. Most current liquid biopsy methods answer one essential question: What genetic alterations are present? However, they do not directly answer a question equally central to clinical pathophysiology: What is the disease actively doing in real time?

Keywords: Liquid biopsy; pathophysiology; exosomes; circulating tumor DNA; cell-free mitochondria; laboratory medicine; functional biomarkers

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From a Primarily Genotypic Tool to a Functional Pathophysiological Assay

Liquid biopsy is considered a novel revolutionary technology in precision medicine and offers non-invasive and real-time molecular profiling of individual patients (1). There are two main perspectives regarding liquid biopsy (2, 3) paradigm, from a primarily genotypic tool to a functional pathophysiological assay:

Clinical perspective: Consider two patients with metastatic non-small cell lung cancer, both possessing an epidermal growth factor receptor (EGFR) mutation detectable using ctDNA. Patient A has low exosomal programmed death-ligand 1 (PD-L1) and no circulating tissue factor (TF). Exosomes play a central role in cell-cell communication and are closely related to the pathogenesis of most human malignancies and actually they have been acknowledged as significant biomarkers in liquid biopsy (1). On the contrary, patient B has high exosomal PD-L1 and elevated TF. Although, they are identical genotypically, however, they are profoundly different from the pathophysiological aspect in real time, as patient B is at higher risk for immunotherapy resistance and venous thromboembolism. Current ctDNA-only liquid biopsy methods cannot distinguish these two scenarios from each other. A functional assay could and might change management, prompting earlier anticoagulation therapy or a different immunotherapy strategy.

Methods perspective: A mutation bestows the malignant cells a potential to the capacity for aberrant signaling, immune evasion, or metastatic spread. But the realization of that this potential depends on functional molecules that execute pathophysiological processes in real time, as exosomes shuttle pro-inflammatory cargo, circulating TF triggers coagulation, cell-free mitochondria act as damage-associated molecular patterns, and dynamic epigenetic marks reflects ongoing transcriptional regulation. Measuring these analytes requires essentially different pre-analytical and analytical approaches than ctDNA sequencing. This distinction is not merely semantic and it has direct implications for laboratory medicine and for the future of liquid biopsy as a discipline. The main concepts regarding the functional blind spot of genocentric liquid biopsy has been presented in Figure 1.

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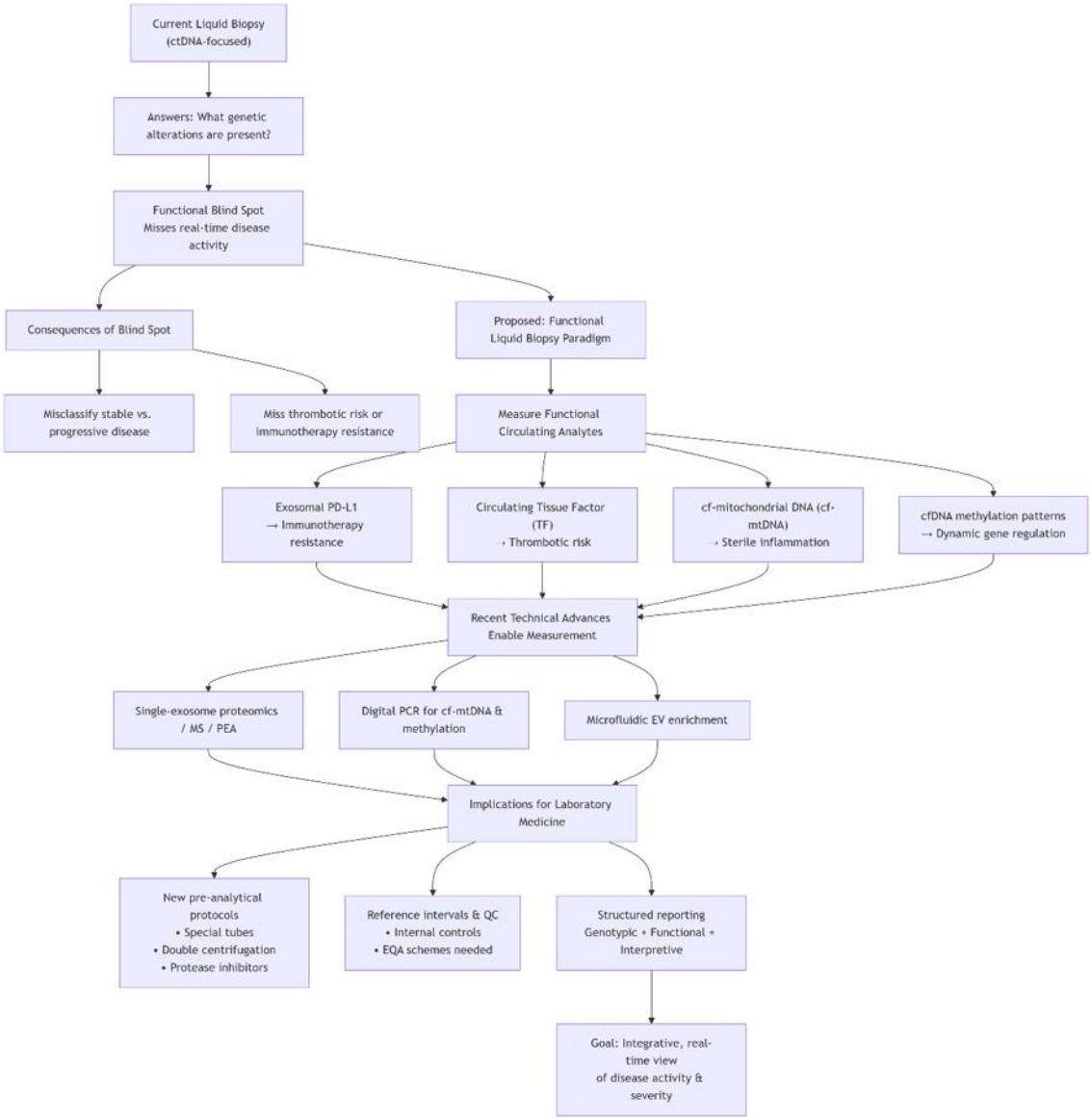

Figure 1. The main concepts regarding the functional blind spot of genocentric liquid biopsy.

The Functional Blind Spot of Genocentric Liquid Biopsy

The dominance of ctDNA analysis has been driven by technical accessibility as cell-free DNA is relatively stable, amplifiable, and amenable to high-throughput sequencing. In contrast, functional circulating analytes have historically presented greater pre-analytical and analytical challenges. Exosomes require careful handling to preserve integrity and circulating proteins such as TF is labile. Meanwhile, cell-free mitochondrial DNA is easily contaminated by cellular debris. Moreover, epigenetic marks demand specialized detection methods such as methylation-specific digital PCR or bisulfite sequencing.

Clinical consequences of the blind spot

This technical convenience has come at a clinical cost. A patient with progressive cancer on immunotherapy may have undetectable ctDNA (due to low tumor shedding) yet rising exosomal PD-L1.

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Under a ctDNA-only paradigm, the clinician might erroneously conclude that the disease is stable. Conversely, a patient with high ctDNA allele frequency but low functional activity might receive unnecessary therapy. Several studies have documented discordance between ctDNA mutations and functional protein activity in some cases, depending on tumor type and treatment context (4-6). This discordance represents not noise, but biologically meaningful signal that is neglected by the current liquid biopsy workflows. Fortunately, recent advances in laboratory techniques have begun to overcome these blind spots and barriers.

Recent advances in laboratory techniques

- Single-exosome proteomics using high-resolution mass spectrometry or proximity extension assays now allows quantitative profiling of exosomal surface and cargo proteins from as little as 100–200 µL of plasma, with low coefficients of variation in the optimized workflows (7, 8).

- High-sensitivity detection of circulating histones and mitochondrial DNA is achievable using digital PCR (dPCR) with primers targeting mitochondrial-encoded genes (e.g., mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 1/ abbreviated as MT-ND1) and nuclear-encoded reference genes (e.g., beta-2-microglobulin/ abbreviated as B2M), enabling absolute quantification without standard curves with very low detection limits in plasma samples (9, 10).

- Digital PCR-based methylation assays for loci such as septin-9 (SEPT9), RASSF1A (Ras association domain family member 1 isoform A), or SHOX2 (Short stature homeobox 2) provide quantitative methylation indices with single-molecule resolution, circumventing the need for bisulfite conversion bias inherent to next-generation sequencing approaches (11, 12).

- Microfluidic enrichment strategies (e.g., acoustic trapping, immunoaffinity capture) enable isolation of intact extracellular vesicles directly from whole blood or plasma under 30 minutes, preserving native conformation of surface proteins (1).

These methods are no longer confined to specialized research laboratories; but they are increasingly transferable to routine diagnostic settings, with several commercial platforms (ExoDx, ExoView, qEV columns) receiving regulatory clearance for specific applications.

A Proposed Framework: Functional Liquid Biopsy

Here, I propose a conceptual expansion of the liquid biopsy paradigm i.e. from a primarily genotypic tool to a functional pathophysiological assay. In this framework, the most informative analyte is not necessarily the mutation with the highest allele frequency, but rather the functional molecule that best correlates with disease activity and clinical outcomes. Several illustrative examples may clarify this principle:

- Thrombotic risk: Circulating TF, often carried on tumor-derived extracellular vesicles, activates coagulation factor VII and triggers the extrinsic pathway. In pancreatic and colorectal cancers, elevated plasma TF-positive exosome levels correlate with a 4- to 6-fold increased risk of venous thromboembolism within 3-6 months, independently of Khorana score or ctDNA mutation status (13-16). From a methods standpoint, TF activity can be measured using factor Xa generation assays following immunocapture of TF-positive vesicles, or by flow cytometry using fluorescently labeled anti-TF antibodies and size-calibrated beads for absolute counting. Pre-analytically, samples must be processed within 2 hours of collection or treated with protease inhibitors and platelet-poor plasma prepared by double centrifugation to avoid artifactual TF release.

- Immunotherapy resistance: Exosomal PD-L1 protein has been shown to correlate with anti-PD-1 response failure(17, 18), potentially offering real-time functional readouts that complement static

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measures of tumor mutational burden or PD-L1 expression by immunohistochemistry (IHC). Clinically, a rising exosomal PD-L1 level during the first two cycles of pembrolizumab may identify patients who will progress by 12 weeks, allowing early treatment switch. Methodologically, exosomal PD-L1 is quantified by enzyme-linked immunosorbent assay (ELISA) using validated antibody pairs recognizing native conformation epitopes, or by single-vesicle flow cytometry (nanoscale flow cytometry) with fluorescence triggering. Critical pre-analytical steps include ultracentrifugation at 100,000×g to pellet exosomes, resuspension in defined buffer, and storage at −80°C with avoidance of freeze-thaw cycles beyond one.

- Sterile inflammation: Cell-free mitochondrial DNA (cf-mtDNA), acting as a damage-associated molecular pattern (DAMP), triggers innate immune responses via Toll-like receptor 9 (TLR9), leading to neutrophil activation, cytokine release (IL-1β, IL-6, TNF-α), and organ injury. Its elevation may serve as a dynamic biomarker of tissue injury and systemic inflammation across oncological and non-oncological conditions, including sepsis, trauma, and autoimmune disease (19, 20). Clinically, plasma cf-mtDNA levels rise within 6-12 hours of acute myocardial infarction or traumatic brain injury and predict intensive care unit mortality (21). Methodologically, cf-mtDNA is quantified by real-time PCR or dPCR using primers targeting mitochondrial genes (e.g., Cytochrome c oxidase subunit I/ abbreviated as MT-CO1 and MT-ND1) with results expressed as copy number per µL of plasma (22). Absolute quantification requires a standard curve from purified mitochondrial DNA or plasmid standards. Critically, blood must be collected in cell-stabilizing tubes (e.g., Streck BCT) or processed within 4 hours to prevent release of mitochondrial DNA from leukocytes during storage.

- Dynamic gene regulation: Methylation patterns on the circulating DNA fragments, particularly at promoter regions of genes involved in immune modulation or metastasis, can change rapidly in response to therapy, offering a pathophysiological readout that fixed mutation analysis cannot provide (23, 24). Clinically, demethylation of the melanoma-associated antigen A3 (MAGE-A3) promoter in plasma DNA within 14 days of chemotherapy predicts eventual response in melanoma patients, with a negative predictive value of 92% in a recent prospective study (25). Methodologically, methylation-specific dPCR uses bisulfite-converted DNA and probes discriminating methylated from unmethylated alleles. Multiplexing allows simultaneous assessment of up to 4-6 loci from 1 mL of plasma. Digital platforms (Bio-Rad QX200, Thermo Fisher QuantStudio 3D) provide absolute quantification of methylated copies without reference to housekeeping genes. Pre-analytical considerations include efficient bisulfite conversion (≥99% conversion efficiency verified by unconverted control sequences) and use of carrier RNA to minimize loss of fragmented DNA.

Implications for Laboratory Medicine

Adopting a functional liquid biopsy paradigm carries practical implications for laboratory practice at multiple levels.

Pre-analytical protocols: New pre-analytical protocols are required to standardize the collection, processing, and storage of samples for functional analytes:

- For exosomes: Use of citrate or EDTA tubes (not serum, due to coagulation-induced vesicle release), centrifugation at 2,500×g for 15 minutes within 1 hour of collection, followed by a second centrifugation at 10,000-20,000×g to remove residual platelets and large vesicles. Addition of protease inhibitor cocktail is recommended.

- For cf-mtDNA: Use of cell-stabilizing tubes to prevent leukocyte lysis, or immediate plasma separation by double centrifugation (first at 1,600×g, second at 16,000×g).

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• For circulating TF: Avoidance of prolonged tourniquet application and venipuncture trauma, which can activate platelets and release TF. Samples should be processed to platelet-poor plasma (platelet count <10,000/μL) within 2 hours.

Reference intervals and quality control: Reference intervals and quality control procedures must be established for each functional biomarker, analogous to those for conventional laboratory parameters. For example, plasma exosomal PD-L1 (concentration), and for cf-mtDNA, healthy reference intervals (copies per μL) of plasma, regarding to age, physical activity, and inflammation status must be determined.

Quality control should include internal controls, external quality assessment schemes and batch-to-batch calibration. For internal controls, synthetic exosome standards (spike-in) and exogenous DNA fragments (e.g., pUC19) processed identically to clinical samples and for external quality assessment schemes, currently available for cfDNA (e.g., IFCC, UK NEQAS) but not yet for exosomal proteins or cf-mtDNA; thus, their development is necessary. In batch-to-batch calibration, the use of pooled plasma reference material stored in aliquots at −80°C is necessary.

Reporting conventions: Reporting conventions may need to evolve. A laboratory report might include not only variant allele frequencies but also quantitative measures of exosomal protein cargo, circulating TF activity, or cf-mDNA copy number, accompanied by pathophysiological interpretation. Structured reporting with three tiers can be suggested as follow:

1. Genotypic section: ctDNA mutations, variant allele frequency, clonality.

2. Functional section: Exosomal PD-L1 (ng/mL), TF activity (relative fluorescence units or mU/mL), cf-mtDNA (copies/μL).

3. Interpretive summary: e.g., "Elevated exosomal PD-L1 suggests possible immune evasion; elevated cf-mtDNA indicates ongoing sterile inflammation."

For any functional assay to enter routine use, analytical validation must demonstrate:

Limit of blank (LOB), limit of detection (LOD), limit of quantification (LOQ): Defined according to Clinical and Laboratory Standards Institute (CLSI) EP17-A2 guidelines.

Precision: Within-run CV <10%, between-run CV <20% at clinically relevant concentrations.

Linearity: Across the expected clinical range (typically 2-3 logs).

Interference: No significant effect from hemolysis (hemoglobin <500 mg/dL), lipemia (triglycerides <1,000 mg/dL), or icterus (bilirubin <20 mg/dL).

Sample stability: Documented for at least 24 hours at 4°C and 6 months at -80°C.

Conclusion

The original promise of the liquid biopsy was to capture tumor heterogeneity and dynamic evolution without invasive tissue sampling. That promise remains unfulfilled in one crucial respect: most current approaches capture the genome of the disease, but not its pathophysiological state. By extending the liquid biopsy concept to include functional molecules that actively drive pathophysiology, our field may move closer to a truly integrative, real-time view of disease activity and severity. From a clinical standpoint, this expansion means better risk stratification (thrombosis, immunotherapy failure, organ injury), earlier detection of treatment resistance, and the possibility of dynamic monitoring with therapeutic decisions guided not only by what mutations are present but by what the disease is actively doing. From a laboratory medicine standpoint, it requires new pre-analytical rigor, novel analytical platforms, reference interval establishment, and quality assurance programs- all achievable but not yet routine.

The blood contains not only DNA fragments but also exosomes, mitochondria, active enzymes, and

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epigenetic marks, a circulating repository of pathophysiological information that remains largely untapped. It is time to read that information systematically, rigorously, and with the full armamentarium of modern laboratory medicine.

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Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Software, Data Curation, Writing–Original Draft Preparation, Writing–Review & Editing, Visualization, Supervision, Project Administration: ANZ

Declaration of Generative AI and AI-assisted technologies in the writing process

No AI writing assistance was utilized in the production of this manuscript.

Conflict of interest

The authors have no competing interests to disclose.

Ethical considerations

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Data availability statement

There are no associated data with this article

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