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Liquid Biopsy from research to clinical practice: focus on Non-Small Cell Lung Cancer

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Abstract

Introduction: In the current era of personalized medicine, liquid biopsy has acquired a relevant importance in patient management of advanced stage non-small cell lung cancer (NSCLC). As a matter of fact, liquid biopsy may supplant the problem of inadequate tissue for molecular testing. The term "liquid biopsy" refers to a number of different biological fluids, but is most clearly associated with plasma-related platforms. It must be taken into account that pre-analytical processing and the selection of the appropriate technology according to the clinical context may condition the results obtained. In addition, novel clinical applications beyond the evaluation of the molecular status of predictive biomarkers are currently under investigation.

Areas covered: This review summarizes the available evidence on pre-analytical issues and different clinical applications of liquid biopsies in NSCLC patients.

Expert opinion: Liquid biopsy should be considered not only as a valid alternative but as complementary to tissue-based molecular approaches. Careful attention should be paid to the optimization and standardization of all phases of liquid biopsy samples management in order to determine a significant improvement in either sensitivity or specificity, while significant reducing the number of "false negative" or "false positive" molecular results.

Keywords: liquid biopsy, NSCLC, ctDNA, CTC, biomarkers.

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Article Highlights

- Although tumor tissue samples, histological or cytological, still arguably represent the gold standard starting material for the molecular assessment of clinical relevant biomarkers, a percentage of advanced stage cancer patients, in particular non-small cell lung cancer (NSCLC) patients, do not have tissue availability to meet the requirements for adequate molecular testing purposes.
- In this setting, liquid biopsy plays a central role allowing molecular analysis and ensuring the best treatment options for advanced stage cancer patients.
- Beyond blood, other body fluids or supernatants usually discarded after cytological preparations may be a valid source of nucleic acids for molecular analysis.
- Careful attention should be paid to pre-analytical (collection, centrifugation, extraction and storage), analytical and post-analytical issues in liquid biopsy samples management.
- Liquid biopsy has demonstrated its complementarity with tissue based analysis for different clinical purposes, including early diagnosis, predictive and prognostic purposes and to monitor minimal residual disease and efficacy of target therapies in NSCLC patients.

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1. Introduction

In the current era of personalized medicine, molecular testing for several clinically relevant biomarkers play a crucial role in the management of cancer patients. It is widely accepted that targeted therapies, including monoclonal antibodies and tyrosine kinase inhibitors (TKIs), result in improved efficacy and safety over that of standard non-targeted standard therapeutic regimens, such as radiotherapy and/or chemotherapy, in patients harboring a specific molecular alteration.[1] Although tumor tissue samples, histological or cytological, still arguably represent the gold standard starting material for the molecular assessment of these biomarkers, a percentage of advanced stage cancer patients, in particular non-small cell lung cancer (NSCLC) patients, do not have tissue availability to meet the requirements for adequate molecular testing purposes.[2] In this setting, liquid biopsy plays a central role allowing molecular analysis and ensuring the best treatment options for advanced stage cancer patients.[3] Blood samples are not only interesting for ctDNA analysis. Other potential analytes may be extracted and analyzed in blood samples. The latter include, among others, circulating tumor cells (CTCs), circulating tumor RNA (ctRNA), extracellular vesicles (EVs), platelet RNA, and others (Figure 1).[4]

Beyond blood samples, the concept of “liquid biopsy” can be extended to other body fluids, such as cerebro-spinal fluid [CSF], effusions, urine, saliva.[5] In addition, another interesting source of tumoral DNA under investigation is represented by the supernatant fluids obtained after cytological preparations. This material, usually discarded, may be a viable source of high-quality nucleic acids (Figure 1). [6-12]

To date, only circulating tumor DNA (ctDNA) extracted from plasma samples has obtained Food and Drug Administration (FDA) approval in clinical practice in two different settings. The first is represented by advanced stage NSCLC patients at diagnosis or acquired resistance settings for predictive purposes. In particular, in advanced stage NSCLC patients naïve to any treatment, the Epidermal Growth Factor Receptor (*EGFR*) gene mutational status analysis can be performed on ctDNA extracted from plasma when tissue samples are not available or are inadequate for molecular

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analysis, in order to administer EGFR tyrosine kinase inhibitors (TKIs).[13] In patients with advanced stage NSCLC who develop resistance against a first or second generation EGFR TKI treatment, ctDNA extracted from plasma analysis is recommended as the initial step to detect the *EGFR* exon 20 p.T790M resistance point mutation, in order to switch to third generation EGFR TKI osimertinib.[13] In addition, ctDNA extracted from plasma has obtained the approval for the evaluation of the methylation status of Septin9 (*SEPT9*) gene in asymptomatic average risk colorectal cancer (CRC) individuals who are unwilling or unable to be screened by conventional colonoscopy.[14]

However, due to the short half-life (about 15 minutes) and concentration (<0.5% of the total circulating free DNA [cfDNA]), there remain several issues regarding clinical application of ctDNA.[15] For these reasons, an International Association for the Study of Lung Cancer (IASLC) statement paper described the importance of details specific to sample collection, extraction and analysis of this complex analyte.[4]

Finally, the importance of differences in technology cannot be overstated. Several methodologies have been adopted for analysis of circulating nucleic acids. Real-time polymerase chain reaction (RT-PCR) or digital droplet PCR (ddPCR) approaches are commonly adopted in molecular predictive pathology laboratories due to short turnaround time (TAT), low costs, FDA approval and high sensitivity for known mutations (Figure 1). [16-18] However, these "targeted methods" suffer from an important limitation related to the use of specific probes able to detect only a limited number of known mutations. [19, 20] These issues may be overcome by next generation sequencing (NGS) and epigenetic analysis (Figure 1). The rapid emergence of this technology relates to its ability to broadly test for a large number of potential targetable abnormalities simultaneously.[21] As with any new technology, careful attention should be paid to the process of validation, the employment of dedicated technicians, and the implementation of sophisticated bioinformatics tools before the adoption in clinical practice.[22]

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Here, we review the main elements in liquid biopsy sample management from the collection to the clinical applications and the minimal requirements of a modern molecular testing laboratory.

2. Liquid Biopsy: blood and beyond

As previously discussed, the term "liquid biopsy" comprises not only blood samples but also other bodily (CSF, urine, saliva, bile, effusions) fluids as well as those obtained from cytological preparations. Blood samples (CTCs, plasma and serum) have been adopted in several clinical trials to overcome the limitations of tissue-based molecular approach, including risks for patients, discomfort, and limited amount of starting material. The first clinical trial focusing the attention on the possibility to determine *EGFR* molecular status on blood samples was the IPASS clinical trial. In this phase III randomized clinical trials, *EGFR* molecular status assessment was evaluated on ctDNA extracted from serum samples of advanced stage NSCLC patients. Results from ctDNA specimens were compared with those obtained on tissue samples as the "gold standard". Overall, by adopting a RT-PCR based approach, despite a specificity of 100.0% (no false positive results were reported among ctDNA samples), a limited sensitivity was reached (43.1%).[23] The low sensitivity of ctDNA extracted from serum was further confirmed in the LUX-Lung 3 clinical trial (28.6%) by using the same molecular approach.[24] Conversely, a higher sensitivity was reported when serum specimens were replaced by plasma samples, as reported in the IFUM and LUX-Lung 6 clinical trials (65.7% and 60.5%, respectively).[24-26] An additional increase in sensitivity, was also reported by Karachaliou *et al* when plasma and serum samples were both employed for each advanced stage NSCLC patient. In the EURTAC clinical trial the authors reported a specificity of 100.0% and a sensitivity of 78.4% using a high sensitivity RT-PCR approach.[16] A further improvement in sensitivity (90.5%) with respect to RT-PCR based approaches was described by Malapelle *et al* using a custom next generation sequencing (NGS) panel (SiRe®) on ctDNA extracted from both plasma and serum samples.[27]

Urine samples represent natural ultra-centrifuged components of blood through the glomerular filtration process. Due to small size and the capacity to pass through the glomerular membrane,

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tumor nucleic acids may be recovered in urine samples. [8, 28, 29] The increasing interest in this alternative source of tumor nucleic acids is related to its non-invasiveness. A major limitation to the adoption of urine in clinical practice is associated with a high risk of nucleic acid degradation due to the activity of DNA and RNA hydrolyzing enzymes.[30, 31] The TIGER-X clinical trial was the first study to explore the feasibility of performing high sensitivity mutation enrichment PCR coupled with NGS on urine samples in the setting of EGFR TKI administration in advanced stage NSCLC patients.[32] Comparing the results obtained on urine samples (90-100 mL) with those obtained on tissue samples ("gold standard"), the authors reported a sensitivity of 93%, 80% and 83%, and a specificity of 96%, 100% and 94% for the detection of *EGFR* exon 20 p.T790M, exon 21 p.L858R and exon 19 deletions, respectively.[32]

Saliva specimens represent another source of nucleic acids and other elements.[33] For example, Streckfus *et al* reported the presence of soluble c-erbB-2 in saliva samples of breast cancer patients.[34] Nevertheless, discordant evidence has been reported in the literature. By adopting a NGS approach, Wu *et al* reported an overall concordance rate between saliva and tissue samples of 74% in patients with advanced stage NSCLC. This concordance rate was higher than those observed between urine and tissue samples (70%).[35] Hubers *et al*, by using different molecular approaches, were able to reach a 100% specificity with a low sensitivity (50%) in saliva samples of advanced stage NSCLC patients .[36]

Other body fluids, such as CSF, bile and effusions, may be useful sources of tumor nucleic acids in metastatic cases. In those cases, it has been widely demonstrated that fluids obtained more closely related to the metastatic site show a higher sensitivity than blood in detection of clinically relevant mutations for targeted treatments.[37] Despite the invasiveness, lumbar puncture is a valid procedure not only for morphological diagnosis but also a suitable source of cell free nucleic acids in advanced stage NSCLC patients with brain metastases.[8, 37-39] Of note, different studies highlight the higher detection rate of mutations, including the *EGFR* exon 20 p.T790M, in CSF by

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comparison to plasma samples in advanced stage NSCLC patients harboring brain metastases.[40-44]

Pleural effusions are a frequent occurrence in advanced stage NSCLC patients.[45] Similar to lumbar puncture, obtaining pleural fluid thoracocentesis is an invasive procedure, but is very useful, not only for diagnostic and therapeutic purposes, but also for molecular testing.[46-48] As early as 2006, Kimura *et al* reported for the first time the detection of *EGFR* sensitizing mutations in DNA extracted from pleural effusions of advanced stage NSCLC patients.[49] Comparing the results of ctDNA extracted from pleural effusions with tissue specimens the "gold standard", Lin *et al* highlighted a sensitivity and specificity of 92.3% and 100.0%, respectively.[50]

Supernatant fluids obtained after cytological preparations may be a valuable option to avoid sacrifice of tissue specimens for molecular analysis and the necessity of re-biopsy patients with lack of tissue or inadequate results.[7] Guibert *et al* demonstrated the feasibility of fine needle aspiration (FNA) supernatant as a source of fresh tumor DNA, suitable for rapid genotyping in advanced stage NSCLC patients at diagnosis and after drug resistance occurs.[8] Similar results in terms of predictive utility of FNA supernatant in lung cancer were reported by Hannigan *et al*.[10, 12] and Bellevicine *et al*.[51]

3. Sample management: circulating free Nucleic Acids (cfNAs)

Circulating free nucleic acids (cfNAs) should be handled with care.[6] In particular, careful attention should be paid to four crucial pre-analytical phases: collection, centrifugation, extraction and storage.[4]

Different collection tubes are commercially available for blood sample collection. Ethylenediaminetetra-acetic acid (EDTA)-containing tubes (Vacutainer, BD, Plymouth, UK) feature the advantages of a lower cost than preservative tubes, and the capacity to avoid clotting. However, when considering the adoption of these collection tubes, a rapid management of blood samples (within 1-2 hours) is required to avoid cfNAs degradation.[52] This collection tube

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approach is preferable when blood withdrawal and cfNAs analysis is carried out within the same Institution. A dedicated nurse performing blood sample collection in a room more close to the laboratory where nucleic acid extraction occurs may be a valid solution ensuring a short turnaround time (TAT).[27] In cases of outsourcing of blood samples in centralized laboratories for cfNAs analysis, PAXgene Blood DNA tubes (Qiagen, Hilden, Germany) or Cell-Free DNA BCT tubes (Streck, La Vista, NE, USA) are preferred. Due to a low stabilization of cell free RNA (cfRNA), cell-free DNA BCT® tubes should be replaced by cell-free RNA BCT® tubes.[53] Overall, these tubes ensure that the time between blood collection and cfNAs analysis has no major influence on the quality of the results.[54] The presence within the tubes of formaldehyde-free preservative agents avoids leucocytes lysis significantly reduces cfNAs degradation.[55] Of note, Cell-Free DNA BCT tubes seem to perform better than PAXgene Blood DNA tubes in avoiding cell lysis.[56] In this regard, the IASLC consensus statement paper recommend that the optimal time between blood withdrawal to cfNAs extraction for an adequate analysis should not go beyond two hours for EDTA tubes and three days for preservative tubes.[4] In addition, at least two tubes each containing 10 mL of blood should be collected.[4]

The second crucial step is represented by centrifugation, which is used to remove non-neoplastic blood formed elements, that carry genomic non-neoplastic nucleic acids, in order to better purify blood samples.[52] Malapelle *et al* adopted a protocol based on two centrifugation steps (each of 2300 rpm for 10 min at room temperature) to obtain 3-4 mL of purified plasma from 10 mL of blood.[27] Page *et al* carried out three centrifugation steps (the first at 1000 rpm for 10 min at 4° C, the second at 1000 rpm, or 2000 rpm, or 10000 rpm for 10 min at 4° C, and the third, after thawing, at 1000 rpm for 5 min at room temperature) showing that a third centrifugation was useful to remove any remaining cells, platelets and cellular debris.[57] Sorber *et al* compared five centrifugation protocols and highlighted that the platelet-generating protocol (first step 120 rpm for 20 min, second step 360 rpm for 20 min, and third step 360 rpm for 5 min with the washing of platelets in the third centrifugation with PBS; at room temperature) enabled the recovery of high

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quality cfDNA and cfRNA in EDTA tubes.[58] The adapted European Committee for Standardization (CEN) protocol (two-step, 1900 rpm for 10 minutes and then 16000 rpm for 10 minutes at room temperature), able to generate high quality cfDNA should be preferred when cfDNA analysis alone is carried out.[58] A basic protocol (first step 400 rpm for 10 min, and second step max speed for 1 min at room temperature) should be performed before cfNAs storage at -80°C.[58] As far as Cell-free DNA BCT® (Streck) tubes cfDNA analysis is concerned, both Streck (first step 1600 rpm for 10 min, and second step 6000 rpm for 10 min, at room temperature) were able to obtain high quality cfDNA.[58] Despite the different centrifuge protocols, the IASLC statement paper recommends at least two centrifugation steps (the first time in the original tube and then, a further centrifugation in a second tube).[4]

The third crucial step is represented by cfDNAs extraction. A plethora of different kits are commercially available. Mauger *et al* compared 11 different commercial kits for cfDNA extraction, showing that the highest accuracy and reproducibility can be obtained by the QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany), the Norgen Plasma/Serum Circulating DNA Purification Mini Kit, and the Norgen Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen, Thorold, ON, Canada).[59] In another study, Sorber *et al* confirmed the high efficacy of the QIAamp circulating nucleic acid kit.[60] In addition, the authors underlined that better results in terms of efficacy may be obtained by adopting the Maxwell RSC cfDNA Plasma Kit (Promega, Leiden, Netherlands), which featured the further advantage of a magnetic beads–based fully automated protocol.[60] Similar results in terms of high efficacy of magnetic beads isolation has been reported in the experience by Kerachian *et al*.[61] Despite the efficacy of these kits, an important issue is related to the high costs. For this reason, other methods may be considered. In this setting, the QIA symphony DSP Virus/Pathogen Midi Kit on the QIA symphony automatic platform may be a useful tool to adequately replace the QIAamp circulating nucleic acid kit, enabling also the recovery of high quality cfRNA.[27]

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ctRNA suffers from a higher instability than ctDNA, and this issue may negatively influence the possibility to adopt this analyte for molecular testing.[62] An important source of ctRNA is represented by tumor-educated blood platelets (TEPs).[63, 64] The ability of platelets to directly ingest (spliced) circulating mRNA determine a highly dynamic mRNA repertoire for TEPs, which may be useful for cancer diagnostics.[65, 66] By sequencing mRNA obtained from TEPs, Best *et al* were able to distinguish among 283 individuals (n = 228 with local or advanced cancer patients and n = 55 healthy people) with an overall accuracy of 96%.[63] An overall accuracy of 71% was reported in distinguishing between six cancer types (NSCLC, colorectal cancer, glioblastoma, pancreatic cancer, hepatobiliary cancer, and breast cancer).[63] In another study, Best *et al* adopted RNA sequencing on TEPs mRNA to detect late and early stage NSCLC patients. Overall, a high accuracy was reached (88% and 81% in the late and early-stage validation cohorts, respectively).[67] Using streck tubes, Raez *et al* [68, 69] have been able to stabilize and isolate cfRNA and follow clinical outcomes in more than 130 patients with lung, breast and colon cancers correlating changes in the cfRNA with overall response rate. They were also able to measure PD-L1 RNA by RT-PCR to monitor clinical responses to check point inhibitors in patients with NSCLC.

Finally, another crucial pre-analytical step is represented by cfNAs storage. As a general rule, -20°C refrigerators represent a valid option to store samples for no more than three months, whereas -80°C freezers are useful for longer storage periods (exceeding three months but no more than 12 months).[70, 71]

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4. Epigenetic analysis in liquid biopsy.

Epigenetic modifications such as DNA methylation, microRNA expression, and histone modifications, have emerged as novel and promising key areas of cancer research in a liquid biopsy with broad potential applications in early cancer detection, risk assessment, prognosis, and prediction of response to therapy.

Epigenetic changes are considered to be among the earliest genomic aberrations occurring during carcinogenesis before somatic mutations and histopathological changes can be detected. [72, 73]

DNA methylation is one of the most common and well-established epigenetic markers that regulates gene expression.[74] It involves the covalent transfer of a methyl group to the 5' carbon of cytosine base by DNA methyltransferases. The resulting 5-methylcytosine (5mC) is preferentially found in the context of a cytosine base linked by the DNA phosphate-backbone to guanosine, termed a CpG site. [75, 76]

Carcinogenesis is characterized by a globally hypomethylated genome with focal hypermethylation of CpG islands which are also detectable in plasma and serum.[72, 73] The loss of methylation increases during the development of a neoplasm and occurs predominantly at repetitive DNA sequences and coding regions and introns, causing chromosomal instability, reactivation of transposable elements, and loss of imprinting.[72, 73] Hypermethylation of CpG islands occurs at different stages during carcinogenesis and can drive the silencing of key tumor suppressors or regulatory regions leading to dysregulation of cell growth.[75, 77]

cfDNA methylation profiling provides several advantages over methods based on genetic somatic mutations. DNA methylation is a tissue-specific epigenetic mark, conserved among cells of the same type in the same individual and among individuals.[74] Therefore, the plasma DNA methylation data allows one to determine the tissue of origin. In addition, each tumor type has a specific and defined methylome profile of hypermethylation of the CpG island in tumor-suppressor genes which distinguishes cancer tissue from their normal counterparts. [78-81] Also, the epigenetic modifications are constant in cancer compared with somatically acquired genetic alterations which are highly variable between patients and across tumor types, resulting in improved sensitivity.[82]

Multiple studies have been performed using DNA methylation markers in liquid biopsies in various cancer types, either at a genome-wide scale or a locus-specific level. The principal technologies to assess methylation DNA include methylation-sensitive restriction enzyme based methods (HpaII tiny fragment enrichment by ligation-mediated PCR assay, HELP [83]; Methylation-sensitive

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Restriction Enzyme digestion followed by sequencing, MRE-seq [84]), sodium bisulfite conversion (whole-genome bisulfite sequencing, WGBS [85]; Reduced Representation Bisulfite Sequencing, RRBS [86]; MethylC-sequencing, MethylC-seq [87, 88] and Bisulfite sequencing, BS-seq [89]), and enrichment-based methods (Methylated DNA immunoprecipitation sequencing, MeDIP-seq [84, 90]; Cell-free methylated DNA immunoprecipitation-sequencing, cfMeDIP-seq[91, 92]; Methyl-CpG-binding domain sequencing, MBD-seq[84] and methyltransferase-directed transfer of activated groups sequencing, mTAG-seq).[83] These methods yield largely concordant results, but differ significantly in the extent of genomic CpG coverage, resolution, quantitative accuracy, and cost.[93, 94] (Table 1)

Methylation detection at specific sites is mainly achieved through the recognition of specific methylation sites in the genome by methylation-sensitive restriction enzyme proteins, such as HpaII and Mspl that target CCGG for digestion.[84] Although this method is very simple, its application is limited by the enzyme recognition sites and not suitable for complex genetic samples.

Genome-wide methylation is detected mainly by measuring the content of 5mC in the genome. [85, 95] Almost all of the cfDNA methylation analysis methods depend on bisulfite sequencing, which transforms non-methylated cytosines to uracils after sodium bisulfite treatment while not altering methylated cytosines.[85] Following DNA sequencing, DNA methylation sites can be detected. Sodium bisulfite treatment would cause a degree of DNA degradation which may lead to loss of some critical information.[96] For example, WGBS-based methods produce the most comprehensive and high-resolution DNA methylome maps, but typically require sequencing to 30× coverage which is still expensive for routine analysis. Additionally, optimized approaches named single-cell reduced-representation bisulfite sequencing (scRRBS) and Methylated CpG tandems amplification and sequencing (MCTA-seq) aim at capturing CpG-enriched cfDNA fragments, which would lead to loss of some critical DNA methylation sites, reducing the sensitivity of the methods.

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The development of monoclonal antibodies specific to 5-methylcytosines revolutionized the analyses of DNA methylation, providing an unprecedented opportunity to map and compare complete DNA CpG methylomes. [91, 97] The limitation of the methods based on affinity enrichment, including methylated DNA immunoprecipitation sequencing (MeDIP-seq) assay, was that large amount of cfDNA were needed. The new method called cell-free methylated DNA immunoprecipitation and high-throughput sequencing (cfMeDIP-Seq) showed a detection of methylated ctDNA with high sensitivity and specificity, even at low sequence depth and using 1-10ng of cfDNA. [91, 92]

Until now, there have been only a few published examples of genome-wide DNA methylome analysis in the context of liquid biopsies for the diagnosis or monitoring of malignancies.[82, 92, 98-101] Successful examples include the Circulating Cell-Free Genome Atlas Study (CCGA) and STRIVE studies, where more than 6000 participants, including cancer patients and non-cancer patients, were subjected to WGBS analysis.[101] This study was able to identify more than 50 types of cancers in different stages with a specificity of 99.3% and a specificity ranging from 43.9% in the stages I-III to 92% in stage IV.[101]

In another study, a new bisulfite-based method called PannSeer test interrogated 477 cancer-specific methylation regions known to have different patterns of methylation in different solid cancers. The test detected approximately 95% of cancers among patients who still were asymptomatic 4 years before a radiological detection study.[100]

Finally, the CfMeDIP-seq seems to be the most promising sensitive and cost-effective methodology used for genome-wide methylation analysis of plasma cfDNA without sodium bisulfite.[91, 92] CfMeDIP-seq was able to detect and classify early stage cancers from seven disease sites (lung, pancreatic, colorectal, breast, leukemia, bladder, and kidney) with samples from healthy donors, and analyzed the methylation patterns of circulating cfDNA in plasma to track the origin and type of cancer.[91, 92] The immunoprecipitation-based approach has recently independently validated to

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the detection of renal cancer in blood and urine samples [82, 99] and in the detection and discrimination of common primary intracranial tumor.[98]

A lot of challenges for cfDNA methylation analysis still remain and several important aspects need to be considered, such as the correlation with gender and ethnicity, the epigenetic plasticity in normal noncancerous cells, the flexibility of the epigenome when exposed to external and internal factors that have a great impact on the epigenetic code.[102-104] Nevertheless, epigenetics will continue be a dynamically evolving field in liquid biopsy in the next few years with the availability of sensitive tests that can be quickly adopted by routine laboratories.

5. Extracellular vesicles (EVs) cargo NGS analysis

Extracellular vesicles (EVs) are small double membrane, spherical, cell-derived vesicles (diameter ranging between 20 to 2000 nm) that are generated by the inward budding of the plasma membrane (microvesicles) or multivesicular bodies of late endosome (exosomes) and released by exocytosis.[105-109] Although EVs being released by different cell types in physiological and pathological conditions, it has been reported that neoplastic cells generate a higher proportion by comparison with normal cells.[107-112] As far as EV function is concerned, these vesicles play a crucial role in cell-to-cell communication and are able to deliver DNA in addition to proteins, lipids, messenger RNAs (mRNAs), and micro RNAs (miRNAs) to the target cells.[113] In particular, EVs are involved in tumor progression, immune escape, and modulation of the tumor microenvironment.[114] So far, no standard protocols are currently available for exosome collection and isolation.

Plasma is usually preferred for EV studies due to additional EVs released during coagulation in serum.[115] For EV collection from plasma, common EDTA tubes are recommended, however other anti-coagulant tubes such as citrate are also used.[116]

Regarding EV isolation protocols, several technologies are available and there is no gold-standard procedure that can be used in all conditions with high recovery and high specificity. For example,

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several commercial polymer-based precipitation kits report a fast and high recovery of EVs, however they report an associated low specificity due to the high free protein, nucleic acids, and non-vesicular material that are co-precipitated.[117] On the other hand, other techniques such as serial centrifugation or size-exclusion chromatography are commonly used and have demonstrated intermediate recovery and specificity. Moreover, their combination with density gradient centrifugations, or other affinity isolation methods show increased sensitivity, but also decreased recovery. However, in comparison with precipitation kits, these methods are more laborious and require longer procedures, which hinders their implementation in routine clinical-practice.[118] Therefore, for proteomic analysis, ultracentrifugation has shown better results than precipitation kits.[119, 120] On the other hand, for RNA analysis, RNA quality was similar across all compared methods including ultracentrifugation, Total Exosomes Isolation kit Invitrogen (Carlsbad, CA, USA), PureExo isolation kit 101Bio (Mountain View, CA, USA), MagCapture exosomes isolation kit Wako Life Sciences (Richmond, VA,USA), and qEV size-exclusion column from iZON sciences (Cambridge, MA USA).[120]

In particular, few studies have performed NGS evaluation of nucleic acids in EVs, most of them focused on the analysis of miRNA and as previously reported for EV isolation, there are several methodologies used for the analysis of DNA or RNA contained in EVs. One particularly interesting study compared different isolation and RNA extraction kits, showing that ultracentrifugation, sedimentation and membrane affinity methods resulted in higher number of mapped miRNAs while stringent size selection by size exclusion chromatography led to lower mapping rates and abundance of short RNA fragments. Authors of this study also postulated that EV isolation presented a greater impact on the RNA analysis that the RNA extraction method used be it the miRCURY RNA Isolation Kit – Biofluids (Exiqon, Vedbaek, Denmark) or exoRNeasy Serum/Plasma Midi Kit (Qiagen, Hilden, Germany).[121]

Other of the most popular assays for nucleic acid isolation of EVs is miRNeasy micro kit (Qiagen) that was used for the NGS analysis of EV RNA in serum of early-stage gastric patients and as a

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result of this study, a signature of 4 miRNAs was found able to improve the diagnostic power of CEA levels.[122] Other technologies such as the MagAttract HMW DNA Kit (Qiagen) have been used for EV DNA purification. Of particular interest, a study performed the analysis of EV DNA and RNA by whole genome, exome, and transcriptome sequencing of EV nucleic acids purified with this kit after ultracentrifugation of pleural effusions in pancreatobiliary cancers. As a result, EV DNA extraction shows high molecular weight double-stranded DNA fragments (>10 kb in size) and alterations in genes such as *NOTCH1* and *BRCA2* were observed.[116] Moreover, several studies have shown good results with other methodological combinations such as the Total Exosome Isolation kit and Total Exosome RNA and Protein Isolation kit (Life Technologies, Inc., Carlsbad, CA, USA) in plasma and serum [123] or Total Exosome RNA and Protein Isolation kit (Life Technologies, Inc.) after ultracentrifugation methods.[124]

Other kits such as the Ambion mir Vana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, USA) have shown good results in serum EV samples.[125]

Interestingly, Möhrmann *et al* analyzed plasma-cell free nucleic acids as well as nucleic acids in EVs from plasma from advanced cancers using the ExoLution Plus Isolation Kit (Exosome Diagnostics, Waltham, MA, USA). They showed that the NGS analysis of EV nucleic acids for common *BRAF*, *KRAS*, and *EGFR* mutations presented higher sensitivity than archival tumor and plasma cfDNA. Additionally, a lower median amount of mutations in EV nucleic acids prior to therapy could predict those patients who exhibit a partial response or stable disease after systemic therapy. This result highlights the promising future of EV NGS analysis in the clinical practice for biomarker discovery.[126] Castellanos *et al* developed a qPCR-based test (ExoDx EGFR) that interrogates mutations within *EGFR* using Exosomal RNA/DNA and cfDNA (ExoNA) derived from plasma in a cohort of 110 NSCLC patients. The performance of the assay yielded an overall sensitivity of 90% for p.L858R, 83% for p.T790M and 73% for exon 19 indels with specificities of 100%, 100%, and 96% respectively. In a subcohort of patients with extrathoracic disease (M1b and

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MX) the sensitivities were 92% (p.L858R), 95% (T790M), and 86% (exon 19 indels) with specificity of 100%, 100% and 94% respectively.[127]

6. Clinical applications and future perspectives

Since the FDA approval in pre-treatment and resistance settings, the analysis of ctDNA extracted from plasma has acquired a relevant importance in advanced stage NSCLC patients.[4, 13] In fact, as previously stated, due to its limited invasiveness the analysis of ctDNA extracted from plasma plays a central role in the algorithm for the treatment decision in advanced stage NSCLC patients.[4, 13] The growing attention on ctDNA is also associated with the implementation of high sensitivity analysis approaches, such as NGS.[128, 129] As an example, by using a very high sensitivity NGS approach, named short footprint mutation enrichment NGS assay, Reckamp *et al* were able to reach, in advanced stage NSCLC patients setting, a sensitivity of 93.0%, 100.0%, and 87.0% and a specificity of 94.0%, 100.0%, and 96.0% for *EGFR* exon 20 p.T790M, exon 21 p.L858R point mutations, and exon 19 deletions, respectively.[32] The adoption of ctDNA extracted from plasma analysis in newly diagnosed advanced stage NSCLC patients as a valid alternative to tissue was recognized by Leighl *et al* and Aggarwal *et al*.[130, 131] To save precious tissue material for the assessment of tissue-based biomarkers (such as PD-L1 expression) and to further investigate negative results on ctDNA analysis, a liquid biopsy first approach has been proposed.[130, 131] A "blood-first" approach should be considered also in resistance setting as proposed by Oxnard *et al*.[132] To improve the sensitivity, the adoption of narrow NGS gene panels should be taken into account. Malapelle *et al*, adopting a six gene NGS panel, were able to reach a sensitivity of 90.5% and a specificity of 100.0%.[27] Beyond ctDNA, NAs extracted from CTCs and EVs may play a pivotal role for predictive purposes in NSCLC patients.[133] In particular, Maheswaran *et al* and Sundaresan *et al* reported the possibility to adopt CTCs for *EGFR* mutational status assessment and monitoring target therapy response.[134, 135] Interestingly, EVs may be an useful tool to recover high quality ctRNA for gene fusion analysis as reported by Reclusa

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et al.[136] Another field of investigation for CTCs is represented by the possibility to be adopted for gene fusions and copy number variations detection and to evaluate the expression of PD-L1 due to the possibility to be subjected to immunohistochemistry and fluorescence in situ hybridization.[137-140] Despite lack of clinical approval, liquid biopsy analysis may also be adopted as a screening tool for early cancer detection of asymptomatic individuals, cancer interception and monitoring the minimal residual disease. [133, -141, 142] In particular, it has been demonstrated that liquid biopsy may represent a valid non-invasive tool to intercept lung cancer development and progression in chronic obstructive pulmonary disease (COPD) patients.[143] Ilie *et al* and Marquette *et al* demonstrated that the identification of CTCs in the bloodstream of COPD patients may be a valuable option for early lung cancer diagnosis.[144, 145] Beyond the screening utility, Romero-Palacios *et al* highlighted that the isolation of CTCs into the bloodstream of COPD patients may be correlated with a poor prognosis.[146] Another field of investigation is represented by the adoption of miRNAs in lung cancer early detection. In particular, Sozzi *et al* underlined the predictive, diagnostic, and prognostic value of a plasma miRNA signature classifier (MSC).[147] ctDNA analysis has been successfully adopted by Ye *et al* in solitary pulmonary nodules to predict malignancy and the necessity of surgical treatment.[148] Phallen *et al*, by using targeted error correction sequencing (TEC-Seq), underlined the utility of ctDNA analysis as a non-invasive screening advice for the identification of early stage lung cancer patients.[149] DNA hypermetilation analysis in several genes and epigenetic changes in the expression patterns of histone acetyltransferases (HATs) and Histone deacetylases (HDACs) may play a role in early may cancer detection.[150] Liquid biopsy may be also adopted to monitor minimal residual disease and the risk of relapse as reported in the TRACERx study,[151] and to evaluate evaluating tumor genomic heterogeneity.[152, 153]

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7. Laboratory requirements

Liquid biopsy represents a challenge for molecular laboratories all over the world. Thus, due to the limitations that may significantly interfere with the implementation of liquid biopsy into the clinical routine diagnostic practice, laboratories should implement innovative and standardized approaches able to solve these issues.

First of all, it is pivotal to scale the laboratory to meet the needs of the users. This point is crucial for the implementation of the different platforms enabling liquid biopsy specimen analysis. As a matter of the facts, an adequate minimum number of dedicated rooms (at least three), in which each of the following activities should be carried out: (i) liquid biopsy specimen administration; (ii) extraction; (iii) analysis. The size of these rooms should be evaluated taking into account different aspects, such as the number and qualification of laboratory users (such as researchers, graduate or undergraduate students) and dedicated laboratory equipment (including centrifuges, analytical platforms). Outside the "working area", a dedicated room for the storage of the collected specimens and laboratory materials should be considered. The adoption of diagrams and plans to develop an efficient use of spaces while ensuring safety is warranted.

Second, it is necessary to identify "control areas" designed to satisfy safety requirements. These areas have to be adopted for the storage of dangerous reagents. The identification of this area should be defined according to Environment, Health & Safety (EH&S) in the early schematic design phase.

Third, for laboratory safety, containment, and pressurization purposes, the fume hoods and the Heating, Ventilation and Air Conditioning (HVAC) control should be periodically inspected. This activity should determine an adequate air change rate.

Finally, another crucial point is the identification of laboratory equipment. This aspect plays a pivotal role for the analysis of liquid biopsy specimens by the implementation of a robust workflow. Overall, laboratory equipment should be adopted in accordance with manufacturer instructions or following laboratory developed test (LDT) procedures. In particular, the localization and implementation of each platform should be performed according to its role in the analytical

workflow. Briefly, centrifuges should be employed for the pre analytical management of liquid biopsy specimens taking into account the characteristics of ctDNA (concentrations, stability, half-life). Subsequently, ctDNA isolation and purification may be performed manually or automatically. Finally, different analytical platforms, such as NGS or ultra-deep real time qPCR platforms, should be adopted. A dedicated storage area with -20°C refrigerators, for the routine use of ctDNA samples (no more than three months), and -80°C freezers, for the long period storage of ctDNA samples (exceeding three months but no more than 12 months), should be implemented. In addition, analyzed data generated from experimental procedures should be stored by using back-up devices able to maintain them for at least 10 years.[154]

8. Conclusions

Liquid biopsy is a very rapid evolving field in different solid tumors.[155-157] Besides, the approval of ctDNA in both treatment naive and acquired resistance settings for advanced stage NSCLC for the evaluation of *EGFR* gene molecular assessment for the administration of target therapies, novel interesting approaches are being under investigation, in particular in the field of cancer interception, early detection and monitoring the responsiveness to therapies. Beyond *EGFR* analysis, in NSCLC a variety of other targetable genes have emerged as actionable targets assessable by liquid biopsy. [158-160] A novel field of investigation is represented by the analysis of blood tumor mutational burden (bTMB) for the administration of immune-checkpoint inhibitors (ICIs). In this setting, the feasibility and clinical utility of using ctDNA extracted from plasma to assist in treatment decision-making for administration of ICIs has been demonstrated.[161]

Despite the incredible advantages offered by liquid biopsy approach (Table 2, refs. 8, 32, 35, 37, 49, 162, 163), we caution that is in other technologies (Table 3), careful attention should be paid to specimen processing and the specific molecular technique employed in order to reduce the percentage of false negative results while maintaining high specificity. In this setting, newer approaches, such as NGS, may help in overcoming these issues.[128, 164, 165]

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9. Expert opinion

The clinical management of lung cancer patients has dramatically evolved during the last decades. In order to avoid to leave any patient behind, liquid biopsy has acquired a pivotal role in patient treatment decision making. As previously described, liquid biopsy should be considered not only as a valid alternative but as complementary to tissue-based molecular approaches. It has been underlined that with a "blood-first" molecular testing approach an increasing number of patients could access to target treatment while contemporary saving precious tissue material to test tissue-based biomarkers, such as the immunohistochemical or immunocytochemical evaluation of the PD-L1 expression, and to confirm negative molecular results on liquid biopsy samples. However, despite the evidences strongly supporting the role of liquid biopsy in clinical routine diagnostic practice, careful attention should be paid to pre-, post- and analytical phases. It has been widely demonstrated that the optimization and standardization of these crucial phases may determine a significant improvement in either sensitivity or specificity, significant reducing the number of "false negative" or "false positive" molecular results. In this setting, several efforts have been spent and reported into international guidelines and statement papers. As a matter of fact, the implementation of novel molecular approaches, such as NGS, is strongly recommended and encouraged when considering to work with liquid biopsy samples. This technology may play a relevant role to overcome the limitation of PCR-based approach, including RT-PCR and dPCR. In particular, NGS enabling the identification of known and unknown alterations for several genes in different patients, simultaneously. In addition, it should be taken into account that the term "liquid biopsy" refers not only to blood samples, but also to other different liquid sources of cell free tumoral nucleic acids, such as effusions, urine, saliva, CSF, bile. These may be a valid source of cell free tumoral nucleic acids in particular metastatic cases. Beyond body fluid samples, even supernatant fluids, obtained after cytological preparations and usually discarded, have demonstrated to be high-quality nucleic acid sources. In addition, liquid biopsy demonstrated its usefulness not only in advanced stage setting of disease for the administration of targeted treatments but also in other settings, such as

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monitoring the minimal residual disease and the efficacy of therapies, cancer interception and early cancer detection. In these cases, liquid biopsy may be “an arrow in the quiver” of molecular pathologists and oncologists to evaluate, in a minimal invasive way, the carcinogenesis process and the earliest phases of cancer development in high risk individuals and to identify resistance to any treatment and cancer progression before the radiological evidences. However, further studies are warranted in these settings in order to improve the clinical applications of the minimal invasive liquid biopsy approach. Finally, the development of molecular tumor boards is strongly encouraged. In this view, liquid biopsy role will play a central role in cancer patients management, from diagnosis to treatment. However, it should be borne in mind that the liquid biopsy should be understood not as a substitute but as an integrative and complementary specimens to the tissue-based analysis, in order to improve cancer patients’ clinical outcomes.

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Figure legend

Figure 1. Beyond blood samples, the concept of "liquid biopsy" can be extended to other body fluids, such as cerebro-spinal fluid, effusions, urine, saliva, bile. In addition, another source of tumoral DNA is represented by the supernatant fluids obtained after cytological preparations. The potential analytes that may be extracted and analyzed in liquid biopsy samples include, among others, circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), circulating tumor RNA (ctRNA), extracellular vesicles (EVs), platelet RNA, and others. Different molecular approaches may be adopted in this setting, to evaluate different genomic and epigenetic alterations.

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Table 1. Summary of the principal advantages and disadvantages of enzyme digestion, bisulfite conversion and enrichment-based methods.

Method	Advantages	Disadvantages
Enzyme digestion methods	- High sensitive methylation detection and cost effective - Gene-specific analysis	- Loss of information due to the use of semi-random primers for amplification - False positive results caused by incomplete digestion - Time consuming - Not suitable for complex genetic samples
Bisulfite conversion methods	- Genome-wide information at single-base resolution - High specificity	- High degree of DNA degradation - Loss of information due to the DNA digestion - High amount of input DNA (50ng - 5ug) - Very expensive
Enrichment-based methods	- Genome-wide information at single-base resolution - Cost-effective - Low amount of input DNA (5ng- 10 ng)	- The results of methylation status are affected by the pull-down effciency

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Table 2. Brief overview on the main advantage of different biofluids.

Biofluid [reference]	Main advantage
Blood[162]	Can be adopted for diagnosis, prognosis, early detection, disease recurrence, predictive purposes and as surrogates for traditional biopsies.
Pleural effusion[49]	Tumor DNA extracted from pleural effusions can be adopted to *EGFR* molecular as a predictor of the response to tyrosine kinase inhibitors.
Urine[32]	DNA derived from lung cancer can be identified in urine and adopted for predictive purposes.
Bile[163]	Bile-derived cell free DNA may be a valid alternative if tissue sampling and is useful for disease monitoring.
Ascites[37]	Fluids close to metastatic sites are superior to blood for predictive purposes.
Saliva[35]	Tumor DNA extracted from saliva can be considered as a valid tool for predictive and diagnostic purposes.
Cerebro-spinal fluid[37]	Fluids close to metastatic sites are superior to blood for predictive purposes.
Supernatant from cytological preparations[8]	Supernatants are a rich source of fresh tumor DNA

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Table 3. Summary of the different methodologies used for cfNAs analysis with advantages and disadvantages.

Methodology	Advantages	Disadvantages
RT-PCR	- low TAT; - limited costs; - widely adopted.	- low limit of detection; - detection of only known alterations; - limited multiplexing power.
dPCR	- low TAT; - limited costs; - high sensitivity; - quantitative.	- detection of only known alterations; - limited multiplexing power.
NGS	- high sensitivity; - quantitative; - broad reference range; - high multiplexing power.	- careful validation; - bioinformatics support; - high trained personnel.

Abbreviations: cfNAs: circulating free nucleic acids; dPCR: digital polymerase chain reaction; NGS: next generation sequencing; RT-PCR: real time polymerase chain reaction; TAT: turnaround time.

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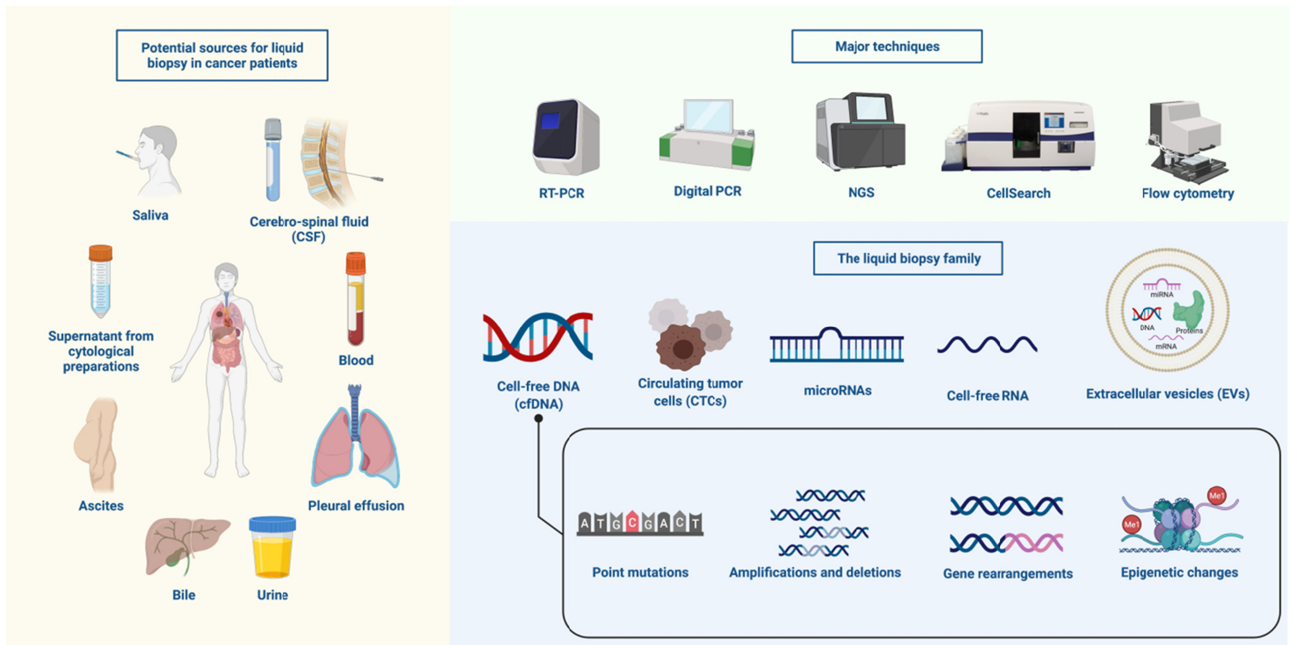

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