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REVIEW

The molecular profiling of solid tumors by liquid biopsy: a position paper of the AIOM—SIAPEC-IAP—SIBioC—SIC—SIF Italian Scientific Societies☆

A. Russo[1*†], L. Incorvaia[2†], M. Del Re[3], U. Malapelle[4], E. Capoluongo[5,6], V. Gristina[1], M. Castiglia[2], R. Danesi[3], M. Fassan[7,8], G. Giuffrè[9], S. Gori[10], A. Marchetti[11], N. Normanno[12], C. Pinto[13], G. Rossi[14], D. Santini[15], A. Sartore-Bianchi[16], N. Silvestris[17,18], P. Tagliaferri[19], G. Troncone[4], S. Cinieri[20‡] & G. D. Beretta[21‡]

Departments of [1]Surgical, Oncological and Oral Sciences; [2]Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), Section of Medical Oncology, University of Palermo, Palermo; [3]Unit of Clinical Pharmacology and Pharmacogenetics, Department of Clinical and Experimental Medicine, University Hospital of Pisa, Pisa; Departments of [4]Public Health; [5]Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Naples; [6]CEINGE, Biotecnologie Avanzate, Naples; [7]Department of Medicine (DIMED), Surgical Pathology Unit, University of Padua, Padua; [8]Veneto Institute of Oncology (IOV-IRCCS), Padua; [9]Department of Human Pathology in Adult and Developmental Age 'Gaetano Barresi', Section of Pathology, University of Messina, Messina; [10]Department of Oncology, IRCCS Ospedale Sacro Cuore Don Calabria, Negrar di Valpolicella, Verona; [11]Center of Predictive Molecular Medicine, University-Foundation, CeSI Biotech Chieti, Chieti; [12]Cell Biology and Biotherapy Unit, Istituto Nazionale Tumori-IRCCS-Fondazione G. Pascale, Naples; [13]Medical Oncology Unit, Clinical Cancer Centre, IRCCS-AUSL di Reggio Emilia, Reggio Emilia; [14]Pathology Unit, Ospedale Santa Maria Delle Croci, Ravenna; [15]Department of Medical Oncology, University Campus Biomedico, Rome; [16]Department of Oncology and Hemato-Oncology, Università degli Studi di Milano, Milan; [17]Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Istituto Tumori 'Giovanni Paolo II' of Bari, Bari; [18]Department of Biomedical Sciences and Human Oncology, Aldo Moro University of Bari, Bari; [19]Medical and Translational Oncology Unit, Department of Experimental and Clinical Medicine, Magna Graecia University, Catanzaro; [20]Medical Oncology Division and Breast Unit, Senatore Antonio Perrino Hospital, ASL Brindisi, Brindisi; [21]Department of Oncology, Humanitas Gavazzeni, Bergamo, Italy

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The term liquid biopsy (LB) refers to the use of various biological fluids as a surrogate for neoplastic tissue to achieve information for diagnostic, prognostic and predictive purposes. In the current clinical practice, LB is used for the identification of driver mutations in circulating tumor DNA derived from both tumor tissue and circulating neoplastic cells. As suggested by a growing body of evidence, however, there are several clinical settings where biological samples other than tissue could be used in the routine practice to identify potentially predictive biomarkers of either response or resistance to targeted treatments. New applications are emerging as useful clinical tools, and other blood derivatives, such as circulating tumor cells, circulating tumor RNA, microRNAs, platelets, extracellular vesicles, as well as other biofluids such as urine and cerebrospinal fluid, may be adopted in the near future. Despite the evident advantages compared with tissue biopsy, LB still presents some limitations due to both biological and technological issues. In this context, the absence of harmonized procedures corresponds to an unmet clinical need, ultimately affecting the rapid implementation of LB in clinical practice. In this position paper, based on experts' opinions, the AIOM—SIAPEC-IAP—SIBIOC—SIF Italian Scientific Societies critically discuss the most relevant technical issues of LB, the current and emerging evidences, with the aim to optimizing the applications of LB in the clinical setting.

Key words: cfDNA, circulating cell-free DNA, circulating tumor DNA, ctDNA, digital PCR, liquid biopsy, next-generation sequencing, real-time PCR

INTRODUCTION

The term liquid biopsy (LB) refers to the use of biological fluids as a surrogate for neoplastic tissue to achieve information for diagnostic, prognostic and predictive purposes. Circulating tumor DNA (ctDNA), a fraction of circulating cell-free DNA (cfDNA) extracted from plasma, represents the only approved analyte in clinical practice. However, other blood derivatives, such as circulating tumor cells (CTCs), circulating tumor RNA (ctRNA), microRNAs (miRNAs), platelets, extracellular vesicles, as well as other biofluids such as urine and cerebrospinal fluid (CSF), may be

*Correspondence to: Prof. Antonio Russo, Section of Medical Oncology, Department of Surgical, Oncological and Oral Sciences, University of Palermo, Via del Vespro 129, 90127 Palermo, Italy. Tel: +39-091-23893207; Fax: +39-347-5797863

E-mail: antonio.russo@usa.net (A. Russo).

☆ Note: AIOM (Associazione Italiana Oncologia Medica); SIAPEC/IAP (Società Italiana di Anatomia Patologica); SIBioC (Società Italiana Biochimica Clinica e Biologia Molecolare Clinica); SIC (Società Italiana Cancerologia); SIF (Società Italiana Farmacologia).

† These authors contributed equally to this work.

‡ These authors should be considered equally co-last authors.

validated in the near future,[1] and new applications are emerging as useful clinical tools.

In this position paper, based on experts' opinions, the AIOM—SIAPEC-IAP—SIBIOC—SIF Italian Scientific Societies revised the most relevant technical issues of LB, the current and emerging evidence, to optimize the applications of LB in the clinical setting.

LB: CHALLENGES AND ADVANCES

In current clinical practice, LB is used for the identification of driver mutations carried by the ctDNA deriving from both tumor and circulating neoplastic cells. The release of cfDNA/ctDNA into the bloodstream is influenced by several factors and may vary according to the patient's clinical condition and sampling time.[1]

LB provides some evident advantages with respect to tissue biopsy. It is minimally invasive and free of complications, it can be repeated over time to monitor the molecular evolution of the disease and modulate the therapeutic choice, and it comprehensively represents tumor heterogeneity, as it potentially contains DNA deriving from different areas of the same tumor and different disease sites.[2]

However, LB shows some limitations that can be related to both biological and technological issues. Regarding the biological matter, one of the main problems is the risk of 'false-negative' results that can be due to an extremely limited amount of ctDNA in the context of cfDNA. Several factors, such as volume and disease location, seem to affect the concentration of ctDNA, being the metastatic setting associated with higher ctDNA shedding into the bloodstream compared with early-stage disease.[3] Unsurprisingly, LB results are sometimes discordant with those obtained on tissue specimens, mostly due to the tumor heterogeneity that should be considered for proper data interpretation.[4] Concerning the technological issues, several aspects need to be taken into account, including sample collection, processing and DNA storage. Several methodological options, along with a wide range of constantly updated commercial tests, are currently available. As a result of such variables, using widely applicable standards is crucial, particularly when considering that a consensus on the optimal pre-analytical procedures has not yet been reached in this regard. Therefore, the harmonization of this phase of the LB assay is still an unmet need that generates critical specific issues, such as the random reporting of cfDNA quantity and its qualitative evaluation, limitations in inter-individual and inter-study comparisons together with difficulty in data interpretation and reproducibility. Thus, such determinants appear to significantly hamper the systematic optimization of the procedures, eventually affecting the rapid implementation of cfDNA analysis in clinical practice.

TECHNICAL ASPECTS

Pre-analytical issues: from blood sampling to cfDNA

Almost all human cells release fragments of their genome into body fluids and circulation, following cell apoptosis and necrosis. These cfDNA molecules are stable and maintain the distinctive genetic characteristics of the cells from which they originate. The cfDNA released by apoptosis is much shorter (166-498 kb) than that released by necrosis (>10 kb).[5] The most recommended and used procedure is cfDNA extraction from plasma. The concentration of ctDNA approximately ranges from 1 to 10 ng/ml and depends on several factors, including disease burden, mutation extent in primary tumor cells and cf/ctDNA shedding into the bloodstream. It is important to point out that not all circulating DNA is tumor DNA; indeed, inflammatory processes in healthy tissue surrounding tumor mass can lead to an increase of cfDNA, but not of ctDNA.[6] For all these reasons, the pre-analytical phase must be carefully controlled. Sampling procedure could affect sample quality, as it might lead to hemolysis during phlebotomy; it is therefore strongly recommended that blood withdrawal is carried out by highly qualified personnel. cfDNA can be isolated from both serum and plasma. However, several studies have shown that the use of plasma is preferable to serum.[7-10] 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.

Standard K2- or K3-EDTA tubes can be used for sample collection; however, there are specific preservative tubes containing special fixatives able to stabilize blood and cfDNA for several days. Published studies clearly showed that after 3 h from sample drawing, leukocyte lysis can occur with consequent release of germline DNA, which dilutes tumor DNA. Therefore, 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 prevent leukocyte lysis. Tubes containing specific preservatives should be used whenever it is not possible to process the sample within 3 h from collection.[11,12]

To eliminate cell residues, plasma is obtained by two centrifugation steps: a first low-speed centrifugation (1200-1600 *g*) to avoid leukocyte lysis and a second, high-speed centrifugation of the supernatant (≥3000 *g*) to remove all contaminants. Centrifugations must be carried out without brake. 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[11] (Figure 1).

Extraction, quantification and cfDNA storage

cfDNA extraction should assure the highest yield of cfDNA in order not to compromise the result of the analysis.

cfDNA concentration in plasma correlates with tumor burden. Therefore, ctDNA tests used for early cancer detection purposes should be highly sensitive: however, highly sensitive tests are always expensive, making large-scale

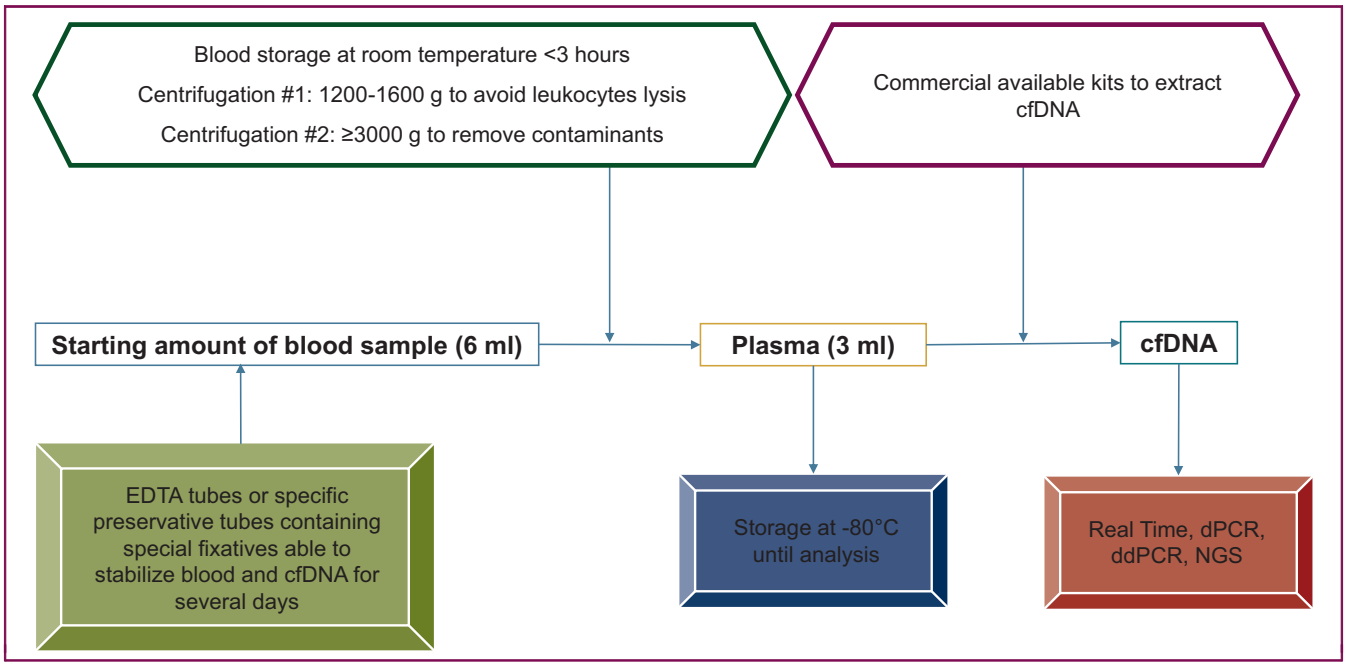

Figure 1. Technical and analytical aspects for liquid biopsy.
cfDNA, cell-free DNA; ddPCR, droplet digital PCR; dPCR, digital PCR; NGS, next-generation sequencing.

practical applications unrealistic. There is always a trade-off between sensitivity and cost. Various methods have been proposed to reduce costs, background noise and errors induced in the amplification phase.[13]

Currently, several commercial kits enable the extraction and purification of cfDNA from plasma, based on the use of columns equipped with silica membranes, in association with a vacuum pump, or with the use of magnetic beads, for the capture of nucleic acids[14,15] (Figure 1).

Technologies for the analysis

Real-time PCR. Real-time PCR is currently the gold standard for the analysis of point mutations and/or small insertions/deletions on ctDNA; this method is considerably available in molecular diagnostic laboratories as it assures proper sensitivity and turnaround time (TAT) at low costs. Real-time PCR relies on the use of a probe that can be modified in order to improve diagnostic sensitivity. This is the case of amplification refractory mutation system (ARMS/Scorpion) technology which increases sensitivity by simultaneous amplification of one or more mutated alleles of the gene of interest and an endogenous control gene.[16] Furthermore, a specific mixture of control oligonucleotides allows the evaluation of DNA quality and quantity. With this specific technology it is possible to reach a limit of detection (LOD) of 0.5%; therefore, this method is suitable to detect low percentages of mutated alleles among high quantities of wild-type genomic DNA as in the case of ctDNA.[17]

Digital PCR. Digital PCR (dPCR) is a technological advancement of the classic PCR[18]; this innovative approach is intended to transform the exponential, analog nature of PCR into a linear, digital (or binary) signal.

There are three types of dPCR platforms: (i) droplet dPCR (ddPCR), (ii) solid digital PCR (sdPCR) and (iii) beam, emulsion, amplification, magnetics (BEAMing) dPCR. In the ddPCR system, partitions are represented by ∼20 000 homogeneous droplets in an oil-water emulsion.[19] In the sdPCR system the bioreactors are represented by ∼20 000-12 000 partitions spotted on a solid support (chip), thus avoiding an emulsion procedure and droplets breaking, which can lead to decrease in analysis performances.[20] In both systems single DNA molecules are spread out inside the bioreactors (droplets or wells) according to Poisson distribution.[18] After the PCR amplification steps, the analysis is based on fluorescence detection. By partitioning the amplification reactions, it is possible to obtain both qualitative and quantitative information of even small numbers of mutated in a background of wild-type alleles. Noteworthy, dPCR is more sensitive than real-time PCR, reaching a sensitivity limit of 0.1%-0.01% with high precision and reproducibility.[19] In the BEAMing dPCR, a standard PCR amplification step is requested before carrying out the analysis.[21] Afterwards, the amplification products are distributed in thousands of homogeneous droplets generated with an oil-water emulsion together with magnetic microspheres, which will bind the PCR products. The beads are subsequently isolated by centrifugation or by a magnet. Finally, by means of an optical scan or flow cytometry it is possible to quantify the DNA bound to the microspheres, with an LOD equal to 0.01%.[18] 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.[22,23] Despite its highly sensitive and specific performances, its

workflow is complicated and expensive to apply in routine clinical settings.[24]

One main limitation of all digital PCR methods, compared with sequencing-based methods, is the potential to detect only known mutations, thus impairing the identification of new alterations.[25]

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.[26]

In case of a result of difficult interpretation, if pre-analytical and analytical issues are excluded, it is always recommended to evaluate the clinical parameters.[27] Low disease burden, brain or bone progressions are generally characterized by low ctDNA shedding, which may cause results misinterpretation.[27]

Next-generation sequencing. Compared with dPCR methods, next-generation sequencing (NGS) offers a great opportunity to investigate multiple genes and multiple known and unknown alterations [single-nucleotide variant (SNV), in/dels, rearrangements] simultaneously. The development of new and more sensitive NGS applications allowed a sensitivity $<1\%$ (0.1%-0.01%) to be reached, that perfectly fits with the needs of ctDNA testing.[28] These applications are based on targeted sequencing, including: the tagged-amplicon (*TAm-seq*) and its more advanced version the *eTAmSeq*™; the safe-sequencing system (*Safe-SeqS*); the CAncer Personalized Profiling (*CAPP-seq*); the *AmpliSeq*. Therefore, there are several NGS panels dedicated to ctDNA analysis and some of these are able to investigate both circulating DNA and RNA. Indeed, RNA is preferred to DNA for the detection of fusion genes and other tricky alterations (i.e. *MET* amplification).[29-31]

The concordance between NGS analysis carried out on primary tissue and ctDNA can be low, whereas a concordance of 97% between metastasis and ctDNA has been reported.[32] This discrepancy may be due to tumor heterogeneity or to clonal hematopoiesis, which is known to increase with age.[33] Notwithstanding, the clonal hematopoiesis that frequently occurs in genes responsible for myelodysplastic syndrome and/or in leuko-emogenesis, other genes, such as the Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*) can be involved.[34] In order to discriminate the mutation determined by clonal hematopoiesis from those in ctDNA, it is suggested to isolate and store the fraction of mononuclear cells (peripheral blood leukocytes): in this way we can establish the origin of a variant as soon as both ctDNA and genomic DNA analyses are carried out.

Report of LB results

The reporting phase is an integral part of the diagnostic procedure; each report should contain 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 must be completed on a pre-established model, dated and signed (possibly digitally) by the laboratory manager. Considering the impact of the test for therapeutic strategy, reporting time should not exceed five working days from the request. Given the overall diagnostic sensitivity of LB ($\sim$87%), negative results for mutation should not be identified as 'wild-type', as a false negative is always possible. Therefore, in case of a negative result and whenever it is technically feasible, the use of a tissue biopsy or eventually a second LB withdrawal is recommended.

LB IN CLINICAL SETTINGS: CURRENT AND EMERGING APPLICATIONS

Non-small-cell lung cancer

LB is currently recommended in clinical practice for the molecular determination of the *EGFR* mutational status in advanced non-small-cell lung cancer (NSCLC) patients.[35] In this setting, the pre-analytic phase evaluating adequate sampling represents a crucial turning point for the assessment of predictive biomarkers of response to targeted therapies.[36] Considering the increasing number of biomarkers to be evaluated for both diagnostic and therapeutic purposes, the management of biological material results is very critical.[37,38] In the light of the acceptable concordance between ctDNA and tissue for *EGFR* evaluation,[27] LB is currently recommended as a viable option to tissue analysis in two important clinical scenarios: (i) patients at the time of the initial diagnosis of advanced NSCLC, before receiving any first-line treatment ('treatment-naive'), when the quantity or quality of the available tissue is not adequate for molecular testing or when molecular assay of tissue is not deemed satisfactory; (ii) in *EGFR*-mutated patients progressing during standard first-line tyrosine kinase inhibitors (TKIs) in order to detect the *EGFR* exon 20 T790M resistance mutation and to offer a targeted treatment based on third-generation TKIs.[39]

Even if limited prospective cohorts have been evaluated to assess the reliability of plasma ctDNA in the identification of other genetic variants, especially concerning the detection of rearrangements,[40] supportive data are emerging for ctDNA analysis to assess ALK rearrangements and other genomic alterations.[41] Accordingly, using ctDNA to evaluate the status of other genomic alterations, conferring sensitivity and/or resistance to targeted treatments, should be

Table 1. Principal indications for liquid biopsy

Tumor type	Indications	References
Non-small-cell lung cancer	- Initial molecular assessment, if tissue not adequate - In EGFR-mutated patients progressing during standard first-line TKIs to detect T790M mutation	22,41,123-125
Breast cancer	Identification of PI3K mutations in ER+, HER2-negative, metastatic breast cancer patients	51,53,58
Colorectal cancer	- Performing RAS and BRAF test as substitute for analysis on tumor tissue in stage IV metastatic CRC - Analysis of RAS mutations for rechallenge in patients resistant to first line anti-EGFR therapies	65,66
Melanoma	Identification of biomarkers predicting response/resistance to targeted therapy (BRAF, NRAS), and longitudinally monitoring of treatment response to targeted therapy and immunotherapy	74,78,84

CRC, colorectal cancer; EGRF, epidermal growth factor receptor; TKIs, tyrosine kinase inhibitors.

considered according to the clinical scenario, within the context of clinical trials. However, given the amount of scientific literature providing evidence to initiate a targeted treatment in case of a positive finding of actionable mutation in ctDNA, selected clinical cases should be discussed within multidisciplinary groups and eventually considered for ctDNA analysis on plasma using validated assays, according to appropriately identified clinical needs[41] (Table 1).

Treatment-naive advanced NSCLC patients. The identification of driver genomic alterations has been a breakthrough in the treatment of NSCLC patients over the last few years.[42] According to the current guidelines, molecular profiling should be offered to all advanced or metastatic NSCLC patients.[39]

The analysis of plasma ctDNA could be considered a viable option to cytohistological sample analysis for the assessment of *EGFR* status in advanced NSCLC patients with unavailable or limited quantity and/or poor quality of tissue samples for molecular purposes, or when the molecular profiling on tissues is inadequate. Nonetheless, in light of the high rate of false negatives, a negative finding of an actionable mutation in ctDNA should be repeated or followed up with a biopsy sampling, unless technical unfeasibility or in the case of patient's refusal[43] (Figure 2, Table 1). If a tissue re-biopsy is not feasible, the therapeutic strategy should be considered according to the presence or absence of actionable genomic alterations.

Although PCR-based technologies, such as real-time PCR and ddPCR, demonstrated acceptable sensitivity and optimal TAT, they can detect only known mutations by specific probes, eventually not identifying less common but potentially clinically relevant mutations.[44] Such limitations could be overcome by NGS panels which retain the advantage of reliably covering a broader spectrum of genomic alterations, despite the longer TAT and the need for consolidated expertise that make this technique not yet widespread.[37]

Advanced NSCLC patients progressing during TKIs. All the EGFR-mutated advanced NSCLC patients progressing on first- or second-generation EGFR TKIs (gefitinib, erlotinib or afatinib) should undergo molecular profiling for the identification of exon 20 T790M *EGFR* mutations, which is the most common mechanism of resistance in this cancer.[43] Given the quantity and quality of studies demonstrating the diagnostic accuracy of ctDNA analysis, it is reasonable to investigate the detection of T790M from ctDNA using ddPCR or real-time PCR.[43] In the case of a positive result, the third-generation EGFR TKI osimertinib should be considered; conversely, a negative result for EGFR T790M should be further investigated using ddPCR (or NGS) on DNA from a tumor tissue re-biopsy, if clinically feasible and accepted by the patient. More recently, in light of the improved overall survival rates in the FLAURA trial, osimertinib has also been approved by the Italian regulatory agency (AIFA) in the first line for advanced NSCLC patients harboring EGFR activating mutations.[45] Given the inhibitory activity of osimertinib either on such common mutations or T790M, testing of the EGFR T790M mutation on liquid or tissue biopsy for patients progressing to osimertinib is not indicated.[43]

Other oncogenic drivers or resistance mechanisms to standard first-line TKIs (such as *EGFR* secondary mutations, *MET* or *HER-2* alterations, *ALK* point mutations, phosphoinositide 3-kinase (*PI3K*) or *RAS/MAPK* alterations, new genomic rearrangements) could be effectively evaluated in liquid and/or tissue biopsy and treated based on the biological mechanism responsible for the development of the resistance within a clinical trial or extended access program[3,46-49] (Figure 3, Table 1).

Breast cancer

Breast cancer (BC) is characterized by a high degree of molecular heterogeneity, which has a crucial role in driving cell growth and proliferation. Selected cell clones are sensitive to specific treatments, allowing tumor response; however, under the selective pressure of treatments, minor resistant cell subpopulations take growth advantages, developing resistant sub-clones that induce tumor progression.[50]

Several studies demonstrated the clinical utility of LB for BC patients, to identify predictive biomarkers of response/resistance to treatment, and to follow up patients' response during treatment.[51-55]

In particular, the assay on LB of genes frequently mutated in BC [i.e. estrogen receptor 1 (ESR1), PI3K, tumor protein p53] has been correlated to the tumor burden: it can work as a useful strategy to monitor residual disease in patients undergoing surgery[56] and as a significant prognostic biomarker.[57]

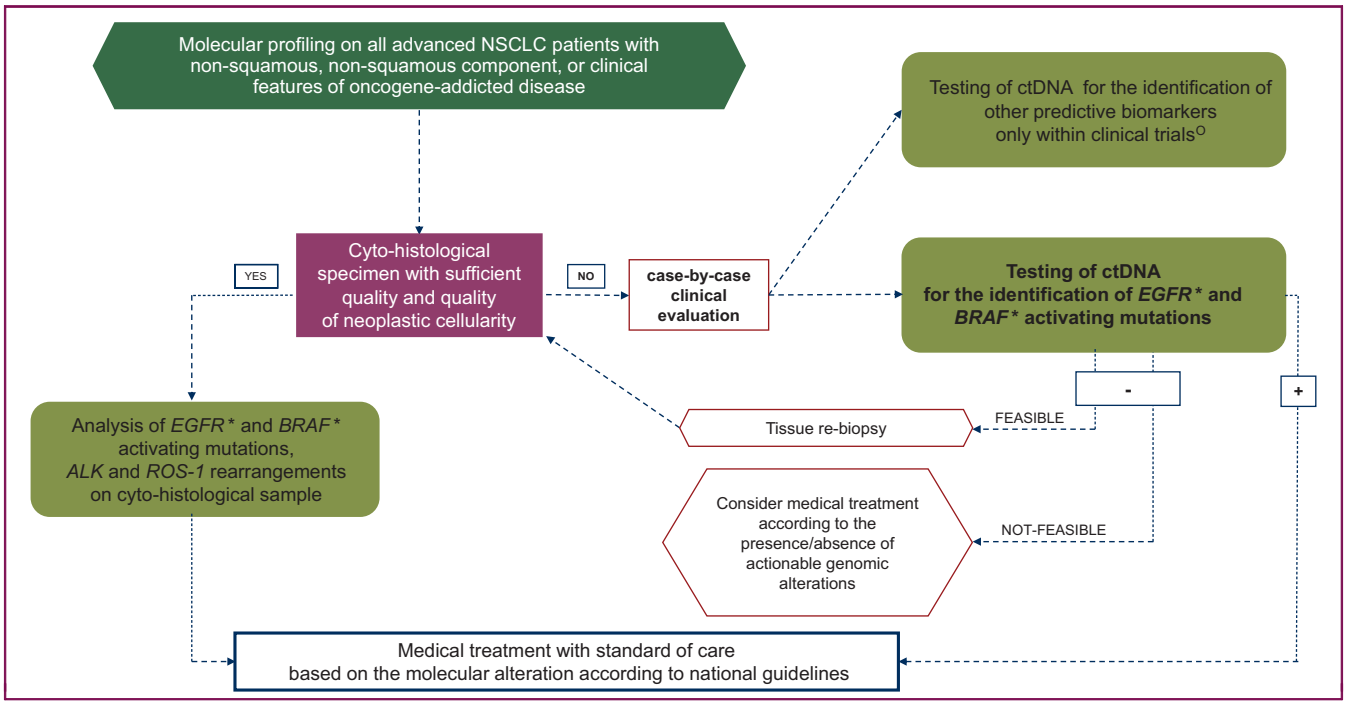

Figure 2. Flow diagram algorithm depicting the role of ctDNA analysis in treatment-naive advanced NSCLC patients.
ctDNA, circulating tumor DNA; EGFR, epidermal growth factor receptor; NSCLC, non-small-cell lung cancer.
[a] *EGFR* exon 18 point mutation, exon 19 deletions, exon 20-21 point mutations; *BRAF* V600 point mutations.
[b] *ALK*, *ROS-1*, *RET*, and *NTRK* rearrangements; *MET* amplification and exon 14 skipping mutation, *HER*-2 amplification and point mutation; *KRAS* G12C point mutation (next-generation sequencing is preferred).

It has recently been shown that mutations in the ESR1 gene may predict the resistance to treatment with aromatase and cyclin-dependent kinase 4/6 (CDK4/6) inhibitors. In particular, ESR1 mutations were analyzed in the ctDNA of 1017 patients with metastatic BC before and after 1 month of first-line treatment with palbociclib in combination with an aromatase inhibitor, showing an inverse correlation with the presence of ESR1 mutations, corresponding to a reduced progression-free survival (PFS). Furthermore, in the group of mutated patients, the clearance of ESR1 mutations in ctDNA after 1 month of treatment was predictive of a longer survival, compared with the group of patients who maintained detectable amounts of mutations in circulation. These preliminary results were presented during the ASCO 2020 congress, and the final results will show whether the screening for mutations of the ESR1 gene may have a clinical validity (PADA-1 trial—NCT03079011).

Alpelisib, a PIK3CA inhibitor, was approved by the Food and Drug Administration (FDA) in 2019 for the treatment of patients with metastatic, PIK3CA-mutated BC. The SOLAR-1 study showed that the addition of alpelisib to fulvestrant treatment significantly improved PFS in PIK3CA-mutated, hormone receptor-positive (HR+), human epidermal growth factor receptor 2-negative (HER2−) advanced ABC.[58] Alpelisib was approved by the FDA on the basis of the presence of PIK3CA mutations both on tissue (if available) and on LB.[58] Moreover, some studies have shown that the onset of mutations in the PIK3CA gene may be one of the mechanisms of acquired resistance to hormone or CDK4/6 inhibitor treatments[59-61] (Figure 4).

Based on available evidence, the use of LB in BC can be a valuable option for the detection of PK3CA mutations in patient candidates for alpelisib treatment (Table 1).

Colorectal cancer

The applications of LB for the detection of ctDNA in colorectal cancer (CRC) are an emerging field of research and are mainly focused on: (i) early-stage disease for a prognostic evaluation and adjuvant therapy selection, and (ii) advanced disease for the analysis of RAS and BRAF mutations and monitoring of molecular targeted therapies.

The ability to use ctDNA in stage I-III disease as a marker of minimal residual disease (MRD), either by searching for specific tissue somatic mutations or to evaluate methylation markers, are emerging as fields of clinical research for localized CRC. In this context, a correlation has already been observed between the presence of ctDNA after surgical excision of the primary tumor and the relapse of the disease, both in stage II and III,[62-64] highlighting that the searching for more than one variant and using serial sampling increases the accuracy in predicting the presence of MRD.[64] Unfortunately, currently available data come from a heterogeneous case series, with a limited number of patients. Therefore, this application of LB for early-stage disease is still under investigation.

By contrast, a variety of studies has demonstrated the feasibility of carrying out the RAS test on LB as a potential substitute for analysis on tumor tissue in stage IV metastatic

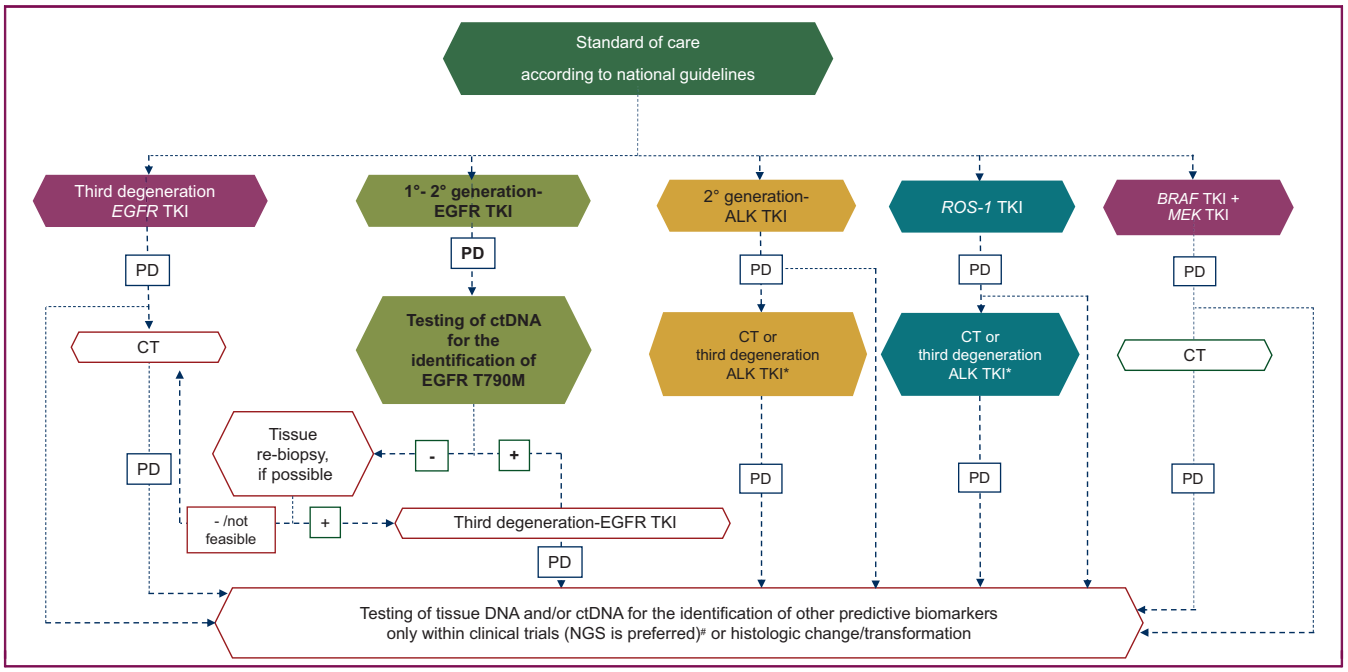

Figure 3. Flow diagram algorithm describing the role of ctDNA analysis in advanced oncogene-addicted NSCLC patients progressing during first-line TKIs.
ALK, anaplastic lymphoma kinase; CT, platinum-based chemotherapy; ctDNA, circulating tumor DNA; EGFR, epidermal growth factor receptor; NGS, next-generation sequencing; NSCLC, non-small-cell lung cancer; PD, progressive disease; TKI, tyrosine kinase inhibitors.
[a] Currently not approved by the Italian Medicines Agency: use only within a clinical trial or extended access program.
[b] *EGFR* secondary mutations, *MET* or *HER-2* alterations, *ALK* point mutations, *PI3K* or *RAS/MAPK* alterations, new genomic rearrangements.

CRC (mRCR).[65] The concordance between tissue versus LB varies from 60% to 80%[5]: we underline that tumor and peripheral blood are two distinct tissues and discrepancies observed in terms of specificity—taking tumor tissue as a reference—are justified by the fact that LB is able to overcome the spatial and temporal heterogeneity that limits tissue analysis. Undoubtedly, LB offers the advantages of a relatively noninvasive and more flexible approach, both for the possibility of making the determination of the mutational status more easily (based on the exact time of therapeutic intervention with anti-EGFR) and for the reduced TAT. Moreover, published data also reported a key role for LB in the evaluation of RAS mutational status on ctDNA in patients with RAS wild-type mCRC with acquired resistance to anti-EGFR therapies for a rechallenge strategy.[66] Therefore, given the amount of scientific evidence reported in the literature to support the analysis of alterations in the ctDNA in addition to what is already available in regard to tissue-deriving DNA and to monitor patients for rechallenge, in selected cases and after approval by multidisciplinary groups, it is possible to propose the above procedures (Figure 5).

Melanoma

Numerous studies have demonstrated a possible clinical usefulness of LB in patients with melanoma, both for the identification of BRAF and NRAS mutations to set up treatment (if tissue is not available), and for quantitative monitoring of ctDNA during treatment[67-71] (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2021.100164).

In particular, BRAF and/or NRAS gene ctDNA mutations, evaluated by real-time PCR or ddPCR methods, have been associated with: (i) tumor burden analysis,[72] (ii) identification of MMR in patients undergoing radical surgery,[73] and (iii) as a significant prognostic factor in patients with stage II/III[74-76] or with metastatic disease.[72] In addition, ctDNA analysis was proposed as a useful biomarker of response to therapy with kinase inhibitors or immunotherapy, and of the early appearance of resistance to treatment.[77-80]

NGS-based multigenic panels have been recently introduced for the study of ctDNA.[81] This approach makes it possible to extend the analysis of the LB also to cases not carrying mutations in BRAF/NRAS. Furthermore, this method allows a better study of clonal heterogeneity in metastatic disease and for noninvasive evaluation of the molecular evolution during the clinical follow-up. In addition to the study of cfDNA, other biomarkers in LB have been proposed in patients with melanoma. Among these, the evaluation of the exosomal expression of programmed death-ligand 1 (PD-L1) was found to be a predictive marker of response to immunotherapy.[82]

It should be emphasized that BRAF mutations have been identified in the cfDNA of 1.4% of patients in dermatological screenings[83]: therefore, further studies are required to validate its possible diagnostic implications in order to avoid false negatives, due to the low disease burden, which leads to a minimal release of ctDNA. In fact, the concordance between ctDNA and tissue increases proportionally to the stage of the disease, rising to about 25%-40% in stages II/III, up to about 70% in stage IV.[74,76,84]

In summary, the use of ctDNA in metastatic melanoma has been proven as a useful tool for the identification of

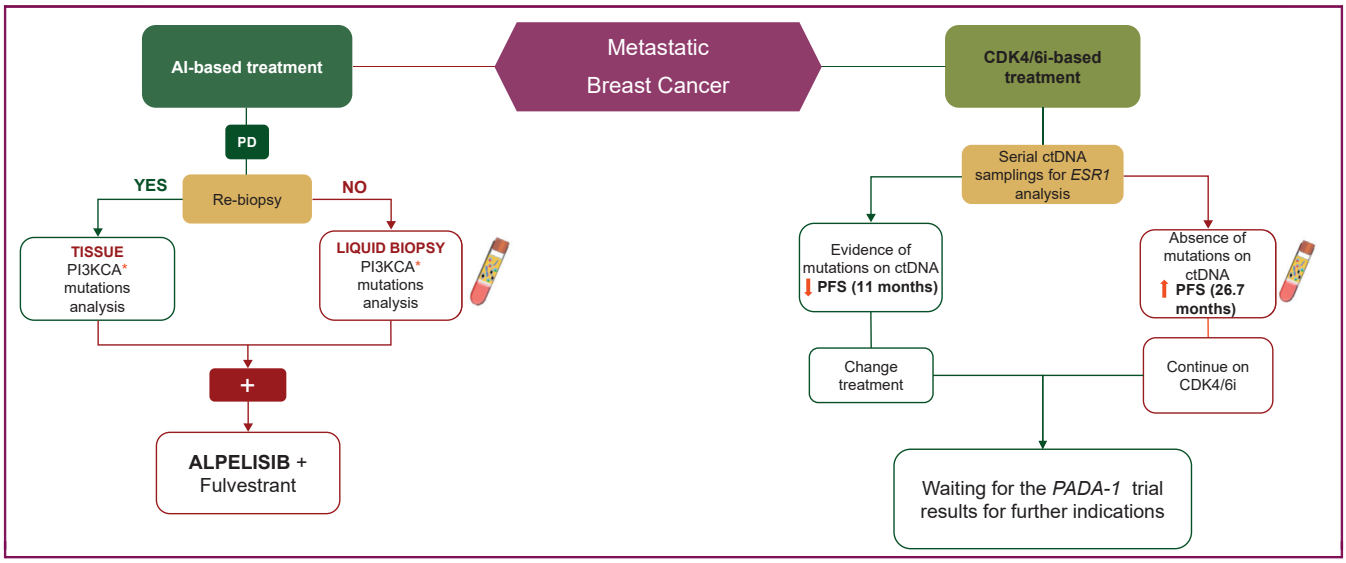

Figure 4. Emerging applications of LB in breast cancer.
AI, aromatase inhibitors; CDK4/6I, cyclin-dependent kinase 4/6 inhibitors; ctDNA, circulating tumor DNA; PFS, progression-free survival; PIK3CA, phospho-inositide 3-kinase.
[a] Analysis of the following mutations: p.C420R, p.E542K, p.E545A, p.E545D, p.E545G, p.E545K, p.Q546E, p.Q546R, p.H1047L, p.H1047R, p.H1047Y.

biomarkers predicting response/resistance to targeted therapy (BRAF, NRAS), and the longitudinal monitoring of treatment response to targeted therapy and immunotherapy.[74,78,84] However, given the amount of scientific evidence reported in the literature to support the analysis of alterations in ctDNA in addition to tissue biopsy, in selected cases discussed within multidisciplinary groups, it is possible to suggest the above procedures.

UPCOMING AND FUTURE APPLICATIONS OF LB

Monitoring of therapeutic response

Besides the application of LB for predictive purposes in order to give targeted therapies, other important fields of investigation are related to the possibility to investigate MRD, to monitor the outcome and for the rapid identification of resistance mechanisms.[85-87] MRD refers to the presence of occult micrometastases without clinical and/or radiological evidence of disease after curative treatments.[88] In this setting, the adoption of LB testing may be a useful tool for the identification of MRD.[89,90] A preliminary experience carried out in a cohort of 18 CRC patients demonstrated that the identification of ctDNA in the bloodstream after surgical resection was predictive of disease recurrence.[85] In another study in stage II CRC patients, the post-operative risk of recurrence was higher (>10-fold) in those with detectable ctDNA, compared to individuals in whom ctDNA was undetectable.[86] LB for MRD analysis was also adopted for lung cancer patients. In the experience of Chaudhuri et al.,[87] in 40 stage I-III lung cancers treated with radiation and/or surgery, a significant risk of recurrence was demonstrated when detectable post-treatment ctDNA was present. Similar results were also reported for other cancers, including BC patients, and when CTCs were evaluated.[91-93] Noteworthy, ctDNA levels decrease after surgery and/or chemotherapy. Dawson et al.[94] demonstrated that in metastatic BC patients, the increase in ctDNA levels may predict disease progression beforehand (at least 5 months) with respect to radiological procedures and standard serum markers. Regarding systemic treatments, LB may be used to monitor response. Tie et al.[86] reported that early changes (within 2 weeks) in ctDNA concentration may predict radiologic responses. Similar results have also been obtained when targeted treatments were considered. As an example, Mok et al.[95] highlighted that NSCLC patients with ctDNA *EGFR* sensitizing mutations at baseline showed dynamic changes after EGFR TKIs which correlated to treatment outcome.

Analysis of other biological fluids

The term 'LB' includes not only blood samples, but also other body fluids, such as urine, saliva, CSF and effusions.[96] In addition, supernatants, usually discarded after cytology, may be adopted as a valuable source of nucleic acids released from tumors.[97] Due to the low size of ctDNA, these small DNA fragments can cross the glomerular membrane enabling its presence in urine.[98] A significant major advantage of urine is represented by the noninvasive collection.[97] The major disadvantage is represented by the high risk of nucleic acid degradation due to the activity of nucleic acid hydrolyzing enzymes.[99,100] Reckamp et al.[101] underlined the complementary role of urine, plasma and tissue as sources for the detection *EGFR* p.T790M mutation. Urine samples were even used for analysis of other solid tumors, including *KRAS* detection in patients with stage IV pancreatic cancer, and in stage III-IV CRC patients.[102]

It has been reported that saliva samples contain different proteins, nucleic acids, electrolytes and hormones, derived from different organs.[103] Streckfus et al.[104] were able to detect c-erbB-2 in saliva specimens of BC patients. Wang

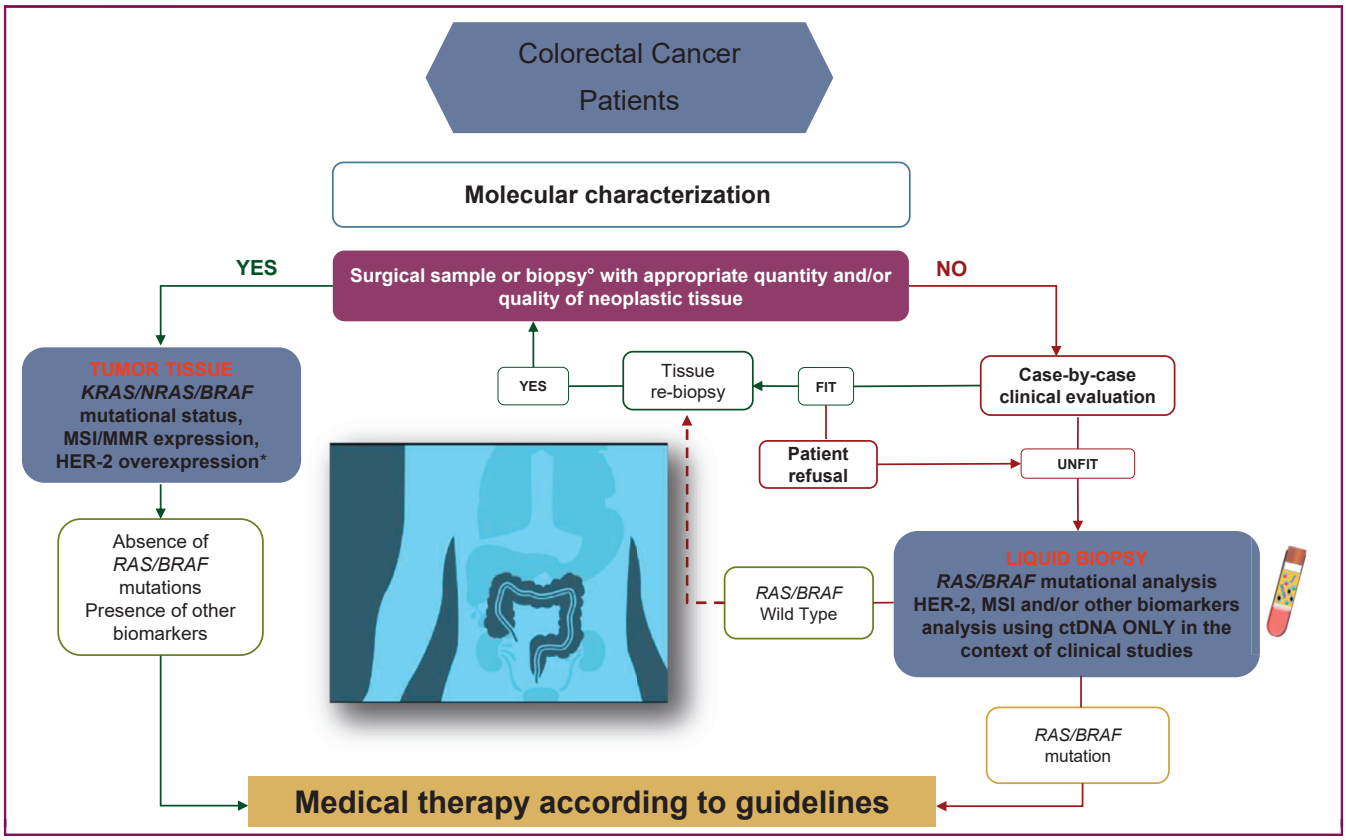

Figure 5. Future perspectives in the clinical applications of liquid biopsy in patients with colorectal cancer.
ctDNA, circulating tumor DNA.
[a] RAS and BRAF mutational status: carried out either on primitive tumors or liver metastases.
Discordance rate of 25%: primary tumor versus lymph node and lung metastases.
[b] Optional.

et al.[105] successfully adopted saliva to evaluate human papillomavirus genes or somatic mutations in genes involved in head and neck squamous cell carcinoma. In addition, saliva may even be used in lung cancer patients.[106]

Although lumbar puncture is an invasive procedure, CSF may be a valid source of cfDNA derived from cancer cells present within the central nervous system (CNS, primary or metastatic tumors).[96] De Mattos-Arruda et al.[107] highlighted a strong relation among tumor type, localization and presence of tumor-derived cfDNA in the CSF of brain tumor patients, underlining the higher presence of cfDNA in CSF with respect to matched plasma samples. In addition, it has been demonstrated that cfDNA extracted from CSF may be more informative than that extracted from plasma, even in the case of single metastasis to the CNS.[108]

Pleural effusions may be optimal for cfDNA analysis in the case of localized metastasis.[108] Although thoracentesis is an invasive procedure, it is fundamental for diagnostic, therapeutic and molecular purposes.[109] Kimura et al.[110] reported for the first time the possibility of identifying *EGFR* sensitizing mutations by analyzing the cfDNA extracted from pleural effusion.

However, even if promising and appropriate in some particular conditions, this source of nucleic acids still needs to be validated in analytical and clinical settings.

Potential application in immunotherapy

Considering the important results obtained in patients treated with targeted therapy, numerous studies are evaluating the potential use of cfDNA/ctDNA, CTCs and other dynamic biomarkers for immunotherapy. Recent studies evaluated the expression of plasma or serum levels of PD-L1, programmed cell death protein 1 (PD-1) and other immune checkpoints.[111-115] In these studies, elevated levels of the soluble form of PD-L1 (sPD-L1) were mainly associated with poor prognosis and worse clinical outcome.[116-118] Exosome PD-L1 has been also studied, showing how elevated concentrations of exosome PD-L1 in melanoma patients treated with immune checkpoint inhibitors (ICIs) were associated with worse prognosis.[119,120]

Recently, literature data showed that the quantitative evaluation of cfDNA, the 'genomic instability number' (GIN),[121] and the blood tumor mutational burden (bTMB),[122] are promising predictive biomarkers for ICIs. Finally, the experimental data concerning the functional study of T-cell receptors (TCR) of patients treated with immunotherapy, are currently under investigation for patient stratification.

Therefore, the use of LB in immunotherapy represents a field of activity research which has the potential to provide 'dynamic' biomarkers in the near future.

CONCLUSIONS

LB represents a promising, noninvasive tool to guide therapeutic choices in solid tumors. The potential of LB is significant, to predict the primary and acquired resistance to treatments early and to monitor the molecular evolution of the disease, modulating the therapeutic choice. Applications of LB in tumors other than NCSLC are presently emerging, and other blood derivatives, together with other biofluids, are an active field of research and may be adopted in the near future. The Molecular Tumor Board appears to be the critical tool to provide the required multidisciplinary expertise, and to translate the molecular information for personalized treatment indication for each patient. Biological and technical issues, as well as the standardization of the procedures, need to be addressed to ensure the widespread implementation in clinical practice. Collaboration between clinical and laboratory scientific societies is further encouraged in this regard.

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