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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☆

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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

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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

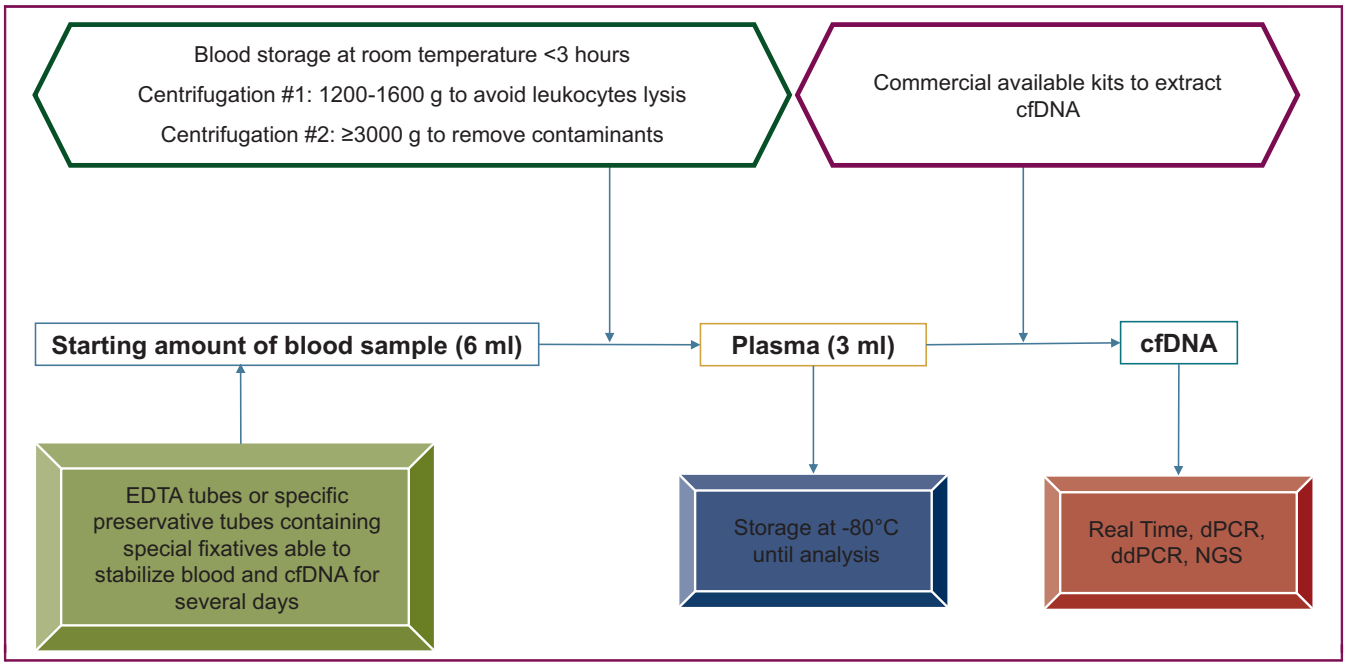

Figure 1. Technical and analytical aspects for liquid biopsy.
cfDNA, cell-free DNA; ddPCR, droplet digital PCR; dPCR, digital PCR; NGS, next-generation sequencing.

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]

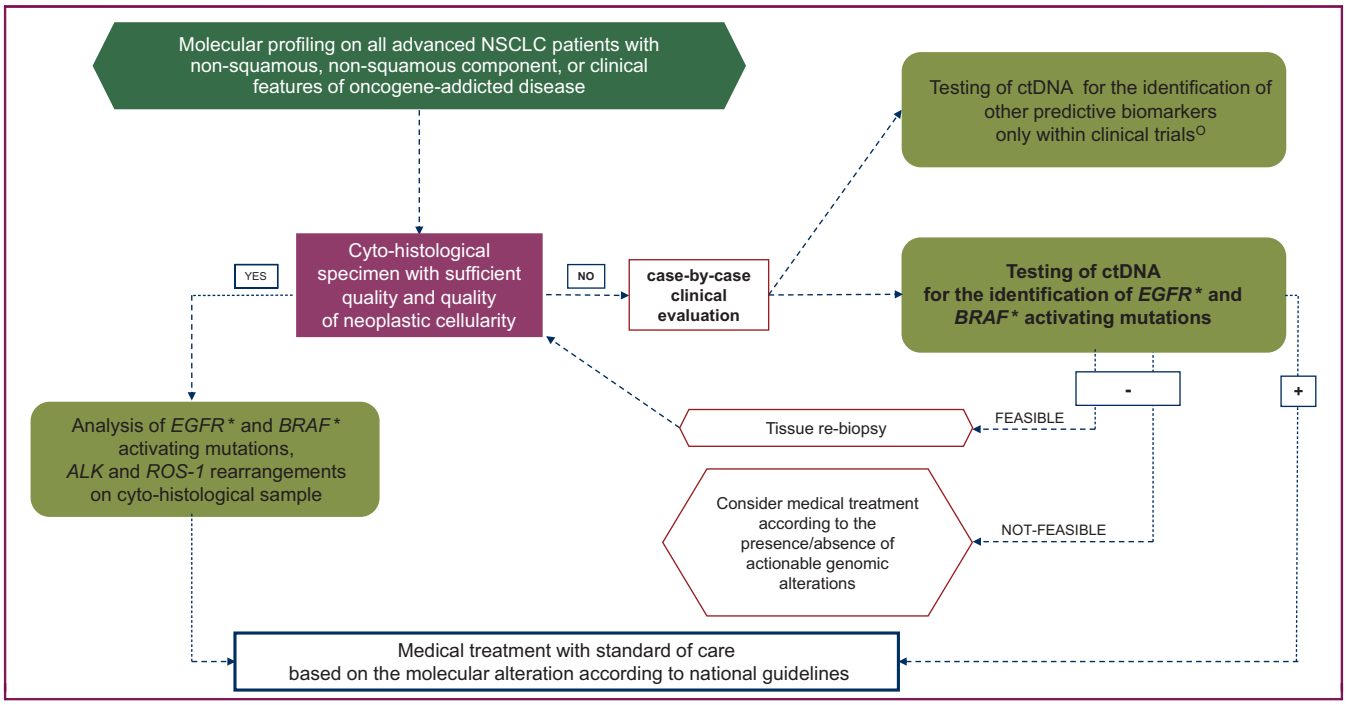

Figure 2. Flow diagram algorithm depicting the role of ctDNA analysis in treatment-naive advanced NSCLC patients.
ctDNA, circulating tumor DNA; EGFR, epidermal growth factor receptor; NSCLC, non-small-cell lung cancer.
[a] *EGFR* exon 18 point mutation, exon 19 deletions, exon 20-21 point mutations; *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

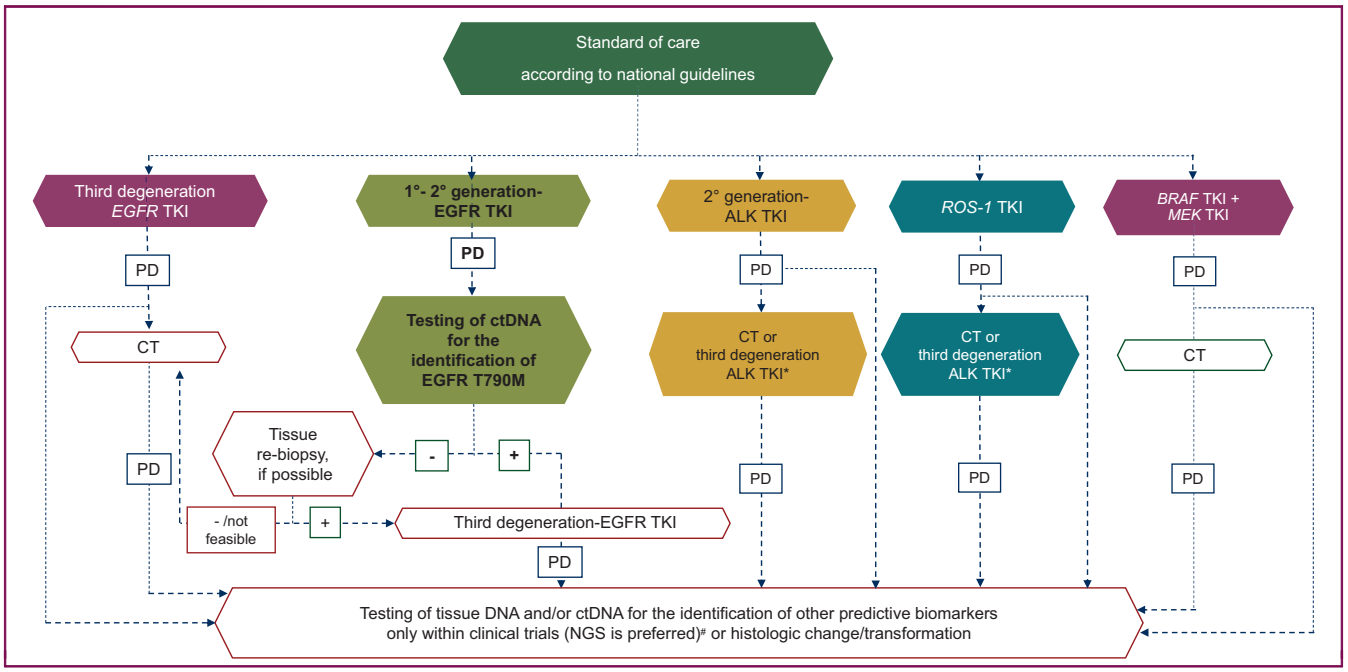

Figure 3. Flow diagram algorithm describing the role of ctDNA analysis in advanced oncogene-addicted NSCLC patients progressing during first-line TKIs.
ALK, anaplastic lymphoma kinase; CT, platinum-based chemotherapy; ctDNA, circulating tumor DNA; EGFR, epidermal growth factor receptor; NGS, next-generation 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

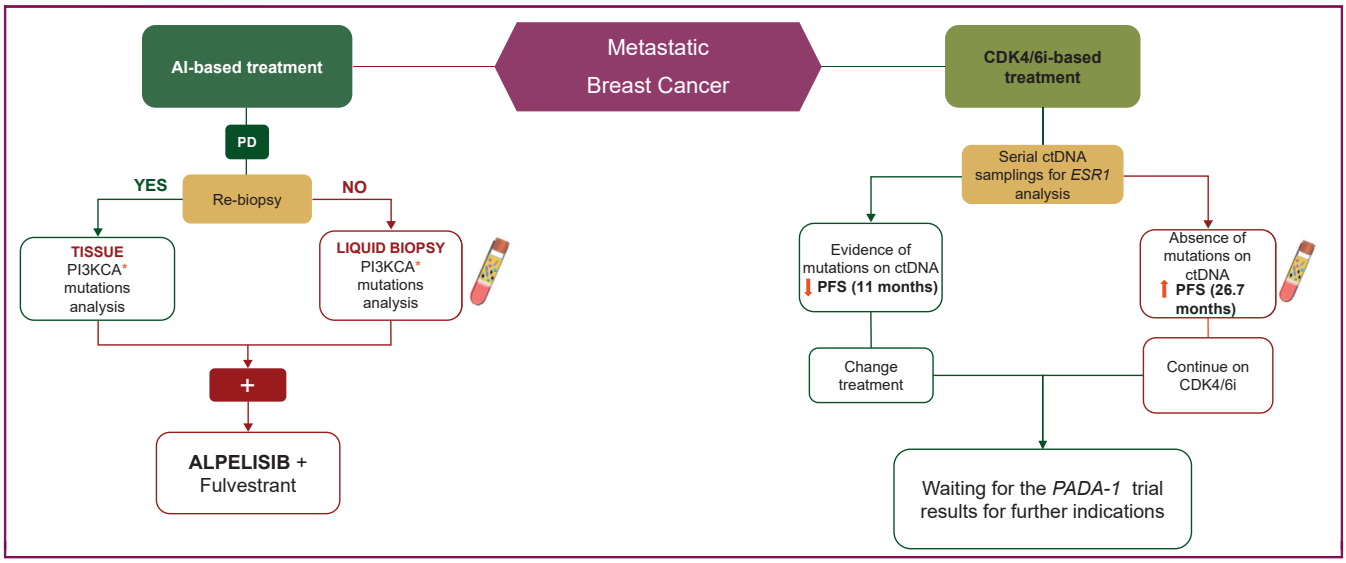

Figure 4. Emerging applications of LB in breast cancer.
AI, aromatase inhibitors; CDK4/6I, cyclin-dependent kinase 4/6 inhibitors; ctDNA, circulating tumor DNA; PFS, progression-free survival; PIK3CA, phospho-inositide 3-kinase.
[a] Analysis of the following mutations: p.C420R, p.E542K, p.E545A, p.E545D,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

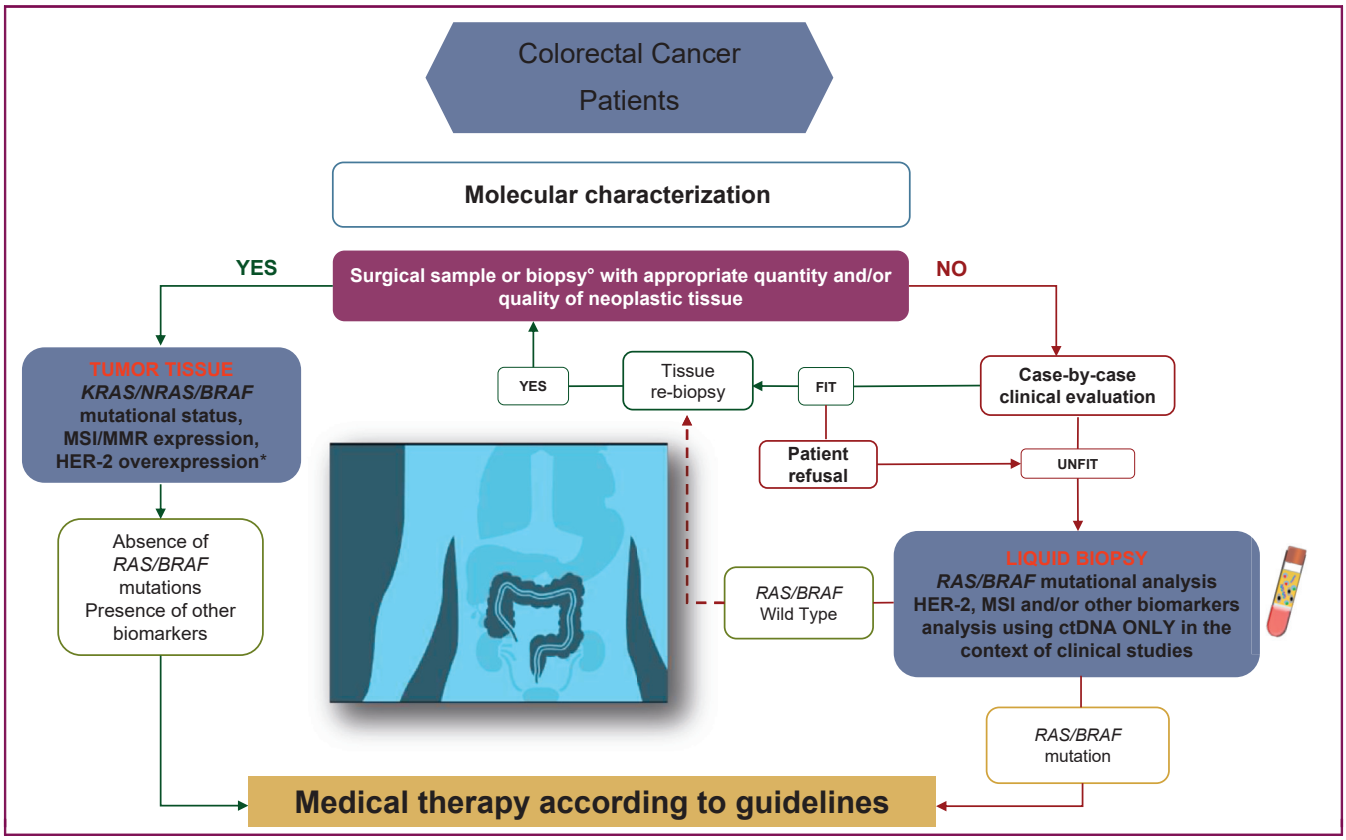

Figure 5. Future perspectives in the clinical applications of liquid biopsy in patients with colorectal cancer.
ctDNA, circulating tumor DNA.
[a] RAS and BRAF mutational status: carried out either on primitive tumors or liver metastases.
Discordance rate of 25%: primary tumor versus lymph node and lung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

1. Said R, Guibert N, Oxnard GR, Tsimberidou AM. Circulating tumor DNA analysis in the era of precision oncology. *Oncotarget*. 2020;11(2):188-211.
2. Fernández-Lázaro D, García Hernández JL, García AC, Córdova Martínez A, Mielgo-Ayuso J, Cruz-Hernández JJ. Liquid biopsy as novel tool in precision medicine: origins, properties, identification and clinical perspective of cancer's biomarkers. *Diagnostics (Basel)*. 2020;10(4):215.
3. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6(224):224ra24.
4. Russano M, Napolitano A, Ribelli G, et al. Liquid biopsy and tumor heterogeneity in metastatic solid tumors: the potentiality of blood samples. *J Exp Clin Cancer Res*. 2020;39(1):95.
5. Kustanovich A, Schwartz R, Peretz T, Grinshpun A. Life and death of circulating cell-free DNA. *Cancer Biol Ther*. 2019;20(8):1057-1067.
6. Meddeb R, Dache ZAA, Thezenas S, et al. Quantifying circulating cell-free DNA in humans. *Sci Rep*. 2019;9(1):5220.
7. Vallée A, Marcq M, Bizieux A, et al. Plasma is a better source of tumor-derived circulating cell-free DNA than serum for the detection of EGFR alterations in lung tumor patients. *Lung Cancer*. 2013;82(2):373-374.
8. Thierry AR, Mouliere F, Gongora C, et al. Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. *Nucleic Acids Res*. 2010;38(18):6159-6175.
9. Cai Z, Chen G, Zeng Y, et al. Comprehensive liquid profiling of circulating tumor DNA and protein biomarkers in long-term follow-up patients with hepatocellular carcinoma. *Clin Cancer Res*. 2019;25(17):5284-5294.
10. Morgan SR, Whiteley J, Donald E, et al. Comparison of KRAS mutation assessment in tumor DNA and circulating free DNA in plasma and serum samples. *Clin Med Insights Pathol*. 2012;5:15-22.
11. Greytak SR, Engel KB, Parpart-Li S, et al. Harmonizing cell-free DNA collection and processing practices through evidence-based guidance. *Clin Cancer Res*. 2020;26(13):3104-3109.
12. Pös Z, Pös O, Styk J, et al. Technical and methodological aspects of cell-free nucleic acids analyzes. *Int J Mol Sci*. 2020;21(22):8634.
13. Ungerer V, Bronkhorst AJ, Holdenrieder S. Preanalytical variables that affect the outcome of cell-free DNA measurements. *Crit Rev Clin Lab Sci*. 2020;57(7):484-507.
14. Sorber L, Zwaenepoel K, Deschoolmeester V, et al. A comparison of cell-free DNA isolation kits: isolation and quantification of cell-free DNA in Plasma. *J Mol Diagn*. 2017;19(1):162-168.
15. Trigg RM, Martinson LJ, Parpart-Li S, Shaw JA. Factors that influence quality and yield of circulating-free DNA: a systematic review of the methodology literature. *Heliyon*. 2018;4(7):e00699.
16. Ye P, Cai P, Xie J, Wei Y. The diagnostic accuracy of digital PCR, ARMS and NGS for detecting KRAS mutation in cell-free DNA of patients with colorectal cancer: a systematic review and meta-analysis. *PLoS One*. 2021;16(3):e0248775.
17. Li C, He Q, Liang H, et al. Diagnostic accuracy of droplet digital PCR and amplification refractory mutation system PCR for detecting EGFR mutation in cell-free DNA of lung cancer: a meta-analysis. *Front Oncol*. 2020;10:290.
18. Vogelstein B, Kinzler KW. Digital PCR. *Proc Natl Acad Sci U S A*. 1999;96(16):9236-9241.
19. Zhang BO, Xu CW, Shao Y, et al. Comparison of droplet digital PCR and conventional quantitative PCR for measuring. *Exp Ther Med*. 2015;9(4):1383-1388.
20. Malapelle U, de Luca C, Vigliar E, et al. EGFR mutation detection on routine cytological smears of non-small cell lung cancer by digital PCR: a validation study. *J Clin Pathol*. 2016;69(5):454-457.
21. Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nat Methods*. 2006;3(7):551-559.
22. Thress KS, Brant R, Carr TH, et al. EGFR mutation detection in ctDNA from NSCLC patient plasma: a cross-platform comparison of leading

technologies to support the clinical development of AZD9291. *Lung Cancer*. 2015;90(3):509-515.
23. Garcia J, Forestier J, Dusserre E, et al. Cross-platform comparison for the detection of RAS mutations in cfDNA (ddPCR Biorad detection assay, BEAMing assay, and NGS strategy). *Oncotarget*. 2018;9(30): 21122-21131.
24. Elazezy M, Joosse SA. Techniques of using circulating tumor DNA as a liquid biopsy component in cancer management. *Comput Struct Biotechnol J*. 2018;16:370-378.
25. Iwama E, Sakai K, Azuma K, et al. Monitoring of somatic mutations in circulating cell-free DNA by digital PCR and next-generation sequencing during afatinib treatment in patients with lung adenocarcinoma positive for EGFR activating mutations. *Ann Oncol*. 2017;28(1):136-141.
26. Pisapia P, Malapelle U, Troncone G. Liquid biopsy and lung cancer. *Acta Cytol*. 2019;63(6):489-496.
27. Passiglia F, Rizzo S, Di Maio M, et al. The diagnostic accuracy of circulating tumor DNA for the detection of EGFR-T790M mutation in NSCLC: a systematic review and meta-analysis. *Sci Rep*. 2018;8(1):13379.
28. Esposito Abate R, Pasquale R, Fenizia F, et al. The role of circulating free DNA in the management of NSCLC. *Expert Rev Anticancer Ther*. 2019;19(1):19-28.
29. Vymetalkova V, Cervena K, Bartu L, Vodicka P. Circulating cell-free DNA and colorectal cancer: a systematic review. *Int J Mol Sci*. 2018;19(11):3356.
30. Bennett CW, Berchem G, Kim YJ, El-Khoury V. Cell-free DNA and next-generation sequencing in the service of personalized medicine for lung cancer. *Oncotarget*. 2016;7(43):71013-71035.
31. Lam SN, Zhou YC, Chan YM, et al. Comparison of target enrichment platforms for circulating tumor DNA detection. *Sci Rep*. 2020;10(1): 4124.
32. Lebofsky R, Decraene C, Bernard V, et al. Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. *Mol Oncol*. 2015;9(4):783-790.
33. Watson CJ, Papula AL, Poon GYP, et al. The evolutionary dynamics and fitness landscape of clonal hematopoiesis. *Science*. 2020;367(6485): 1449-1454.
34. Hu Y, Ulrich BC, Supplee J, et al. False-positive plasma genotyping due to clonal hematopoiesis. *Clin Cancer Res*. 2018;24(18):4437-4443.
35. Lagos GG, Izar B, Rizvi NA. Beyond tumor PD-L1: emerging genomic biomarkers for checkpoint inhibitor immunotherapy. *Am Soc Clin Oncol Educ Book*. 2020;40:1-11.
36. Salvianti F, Gelmini S, Costanza F, et al. The pre-analytical phase of the liquid biopsy. *N Biotechnol*. 2020;55:19-29.
37. Malapelle U, Pisapia P, Rocco D, et al. Next generation sequencing techniques in liquid biopsy: focus on non-small cell lung cancer patients. *Transl Lung Cancer Res*. 2016;5(5):505-510.
38. Passiglia F, Rizzo S, Rolfo C, et al. Metastatic site location influences the diagnostic accuracy of ctDNA EGFR- mutation testing in NSCLC patients: a pooled analysis. *Curr Cancer Drug Targets*. 2018;18(7): 697-705.
39. Passiglia F, Pilotto S, Facchinetti F, et al. Treatment of advanced non-small-cell lung cancer: the 2019 AIOM (Italian Association of Medical Oncology) clinical practice guidelines. *Crit Rev Oncol Hematol*. 2020;146:102858.
40. Guibert N, Pradines A, Favre G, Mazieres J. Current and future applications of liquid biopsy in nonsmall cell lung cancer from early to advanced stages. *Eur Respir Rev*. 2020;29(155):190052.
41. Leighl NB, Page RD, Raymond VM, et al. Clinical utility of comprehensive cell-free DNA analysis to identify genomic biomarkers in patients with newly diagnosed metastatic non-small cell lung cancer. *Clin Cancer Res*. 2019;25(15):4691-4700.
42. Skoulidis F, Heymach JV. Co-occurring genomic alterations in non-small-cell lung cancer biology and therapy. *Nat Rev Cancer*. 2019;19(9):495-509.
43. Rolfo C, Mack PC, Scagliotti GV, et al. Liquid biopsy for advanced non-small cell lung cancer (NSCLC): a statement paper from the IASLC. *J Thorac Oncol*. 2018;13(9):1248-1268.
44. Gristina V, Malapelle U, Galvano A, et al. The significance of epidermal growth factor receptor uncommon mutations in non-small cell lung cancer: a systematic review and critical appraisal. *Cancer Treat Rev*. 2020;85:101994.
45. Ramalingam SS, Vansteenkiste J, Planchard D, et al. Overall survival with osimertinib in untreated. *N Engl J Med*. 2020;382(1):41-50.
46. Pepe F, Pisapia P, Gristina V, et al. Tumor mutational burden on cytological samples: a pilot study. *Cancer Cytopathol*. 2020. https://doi.org/10.1002/cncy.22400.
47. Paweletz CP, Sacher AG, Raymond CK, et al. Bias-corrected targeted next-generation sequencing for rapid, multiplexed detection of actionable alterations in cell-free DNA from advanced lung cancer patients. *Clin Cancer Res*. 2016;22(4):915-922.
48. Dagogo-Jack I, Rooney M, Lin JJ, et al. Treatment with next-generation ALK inhibitors fuels plasma. *Clin Cancer Res*. 2019;25(22):6662-6670.
49. Parikh AR, Corcoran RB. Monitoring resistance through liquid biopsy. *Ann Oncol*. 2018;29(1):8-11.
50. Kalinowski L, Saunus JM, McCart Reed AE, Lakhani SR. Breast cancer heterogeneity in primary and metastatic disease. *Adv Exp Med Biol*. 2019;1152:75-104.
51. O'Leary B, Hrebien S, Morden JP, et al. Early circulating tumor DNA dynamics and clonal selection with palbociclib and fulvestrant for breast cancer. *Nat Commun*. 2018;9(1):896.
52. Alimirzaie S, Bagherzadeh M, Akbari MR. Liquid biopsy in breast cancer: a comprehensive review. *Clin Genet*. 2019;95(6):643-660.
53. Majure M, Logan AC. What the blood knows: interrogating circulating tumor DNA to predict progression of minimal residual disease in early breast cancer. *Ann Transl Med*. 2016;4(24):543.
54. Wang P, Bahreini A, Gyanchandani R, et al. Sensitive detection of mono- and polyclonal ESR1 mutations in primary tumors, metastatic lesions, and cell-free DNA of breast cancer patients. *Clin Cancer Res*. 2016;22(5):1130-1137.
55. Beije N, Sieuwerts AM, Kraan J, et al. Estrogen receptor mutations and splice variants determined in liquid biopsies from metastatic breast cancer patients. *Mol Oncol*. 2018;12(1):48-57.
56. Zhou Y, Xu Y, Gong Y, et al. Clinical factors associated with circulating tumor DNA (ctDNA) in primary breast cancer. *Mol Oncol*. 2019;13(5): 1033-1046.
57. Lee JH, Jeong H, Choi JW, Oh HE, Kim YS. Liquid biopsy prediction of axillary lymph node metastasis, cancer recurrence, and patient survival in breast cancer: a meta-analysis. *Medicine (Baltimore)*. 2018;97(42):e12862.
58. André F, Ciruelos EM, Juric D, et al. Alpelisib plus fulvestrant for PIK3CA-mutated, hormone receptor-positive, human epidermal growth factor receptor-2-negative advanced breast cancer: final overall survival results from SOLAR-1. *Ann Oncol*. 2021;32(2):208-217.
59. Ma CX, Crowder RJ, Ellis MJ. Importance of PI3-kinase pathway in response/resistance to aromatase inhibitors. *Steroids*. 2011;76(8): 750-752.
60. Del Re M, Crucitta S, Lorenzini G, et al. PI3K mutations detected in liquid biopsy are associated to reduced sensitivity to CDK4/6 inhibitors in metastatic breast cancer patients. *Pharmacol Res*. 2021;163:105241.
61. Araki K, Miyoshi Y. Mechanism of resistance to endocrine therapy in breast cancer: the important role of PI3K/Akt/mTOR in estrogen receptor-positive, HER2-negative breast cancer. *Breast Cancer*. 2018;25(4):392-401.
62. Tie J, Wang Y, Tomasetti C, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. *Sci Transl Med*. 2016;8(346):346ra92.
63. Reinert T, Henriksen TV, Christensen E, et al. Analysis of plasma cell-free DNA by ultradeep sequencing in patients with stages I to III colorectal cancer. *JAMA Oncol*. 2019;5:1124-1131.
64. Tarazona N, Gimeno-Valiente F, Gambardella V, et al. Targeted next-generation sequencing of circulating-tumor DNA for tracking minimal residual disease in localized colon cancer. *Ann Oncol*. 2019;30(11):1804-1812.
65. Galvano A, Taverna S, Badalamenti G, et al. Detection of RAS mutations in circulating tumor DNA: a new weapon in an old war against

colorectal cancer. A systematic review of literature and meta-analysis. *Ther Adv Med Oncol*. 2019;11:1758835919874653.
66. Cremolini C, Rossini D, Dell'Aquila E, et al. Rechallenge for patients with RAS and BRAF wild-type metastatic colorectal cancer with acquired resistance to first-line cetuximab and irinotecan: a phase 2 single-arm clinical trial. *JAMA Oncol*. 2019;5(3):343-350.
67. Boyer M, Cayrefourcq L, Dereure O, Meunier L, Becquart O, Alix-Panabières C. Clinical relevance of liquid biopsy in melanoma and merkel cell carcinoma. *Cancers (Basel)*. 2020;12(4):960.
68. Syeda MM, Wiggins JM, Corless B, Spittle C, Karlin-Neumann G, Polsky D. Validation of circulating tumor DNA assays for detection of metastatic melanoma. *Methods Mol Biol*. 2020;2055:155-180.
69. Diefenbach RJ, Lee JH, Rizos H. Monitoring melanoma using circulating free DNA. *Am J Clin Dermatol*. 2019;20(1):1-12.
70. Pinzani P, Salvianti F, Zaccara S, et al. Circulating cell-free DNA in plasma of melanoma patients: qualitative and quantitative considerations. *Clin Chim Acta*. 2011;412(23-24):2141-2145.
71. Herbreteau G, Charpentier S, Vallée A, Denis MG. Use of circulating tumoral DNA to guide treatment for metastatic melanoma. *Pharmacogenomics*. 2019;20(18):1259-1270.
72. Santiago-Walker A, Gagnon R, Mazumdar J, et al. Correlation of BRAF mutation status in circulating-free DNA and tumor and association with clinical outcome across four BRAFi and MEKi clinical trials. *Clin Cancer Res*. 2016;22(3):567-574.
73. Rowe SP, Luber B, Makell M, et al. From validity to clinical utility: the influence of circulating tumor DNA on melanoma patient management in a real-world setting. *Mol Oncol*. 2018;12(10):1661-1672.
74. Lee RJ, Gremel G, Marshall A, et al. Circulating tumor DNA predicts survival in patients with resected high-risk stage II/III melanoma. *Ann Oncol*. 2018;29(2):490-496.
75. Lee JH, Saw RP, Thompson JF, et al. Pre-operative ctDNA predicts survival in high-risk stage III cutaneous melanoma patients. *Ann Oncol*. 2019;30(5):815-822.
76. Tan L, Sandhu S, Lee RJ, et al. Prediction and monitoring of relapse in stage III melanoma using circulating tumor DNA. *Ann Oncol*. 2019;30(5):804-814.
77. Gray ES, Rizos H, Reid AL, et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. *Oncotarget*. 2015;6(39):42008-42018.
78. Cabel L, Riva F, Servois V, et al. Circulating tumor DNA changes for early monitoring of anti-PD1 immunotherapy: a proof-of-concept study. *Ann Oncol*. 2017;28(8):1996-2001.
79. Schreuer M, Meersseman G, Van Den Herrewegen S, et al. Quantitative assessment of BRAF V600 mutant circulating cell-free tumor DNA as a tool for therapeutic monitoring in metastatic melanoma patients treated with BRAF/MEK inhibitors. *J Transl Med*. 2016;14:95.
80. Gonzalez-Cao M, Mayo de Las Casas C, Jordana Ariza N, et al. Early evolution of BRAFV600 status in the blood of melanoma patients correlates with clinical outcome and identifies patients refractory to therapy. *Melanoma Res*. 2018;28(3):195-203.
81. Lin SY, Huang SK, Huynh KT, et al. Multiplex gene profiling of cell-free DNA in patients with metastatic melanoma for monitoring disease. *JCO Precis Oncol*. 2018;2:PO.17.00225.
82. Del Re M, Marconcini R, Pasquini G, et al. PD-L1 mRNA expression in plasma-derived exosomes is associated with response to anti-PD-1 antibodies in melanoma and NSCLC. *Br J Cancer*. 2018;118(6):820-824.
83. Calbet-Llopart N, Potrony M, Tell-Martí G, et al. Detection of cell-free circulating BRAF. *Br J Dermatol*. 2020;182(2):382-389.
84. Knuever J, Weiss J, Persa OD, et al. The use of circulating cell-free tumor DNA in routine diagnostics of metastatic melanoma patients. *Sci Rep*. 2020;10(1):4940.
85. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med*. 2008;14(9):985-990.
86. Tie J, Kinde I, Wang Y, et al. Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. *Ann Oncol*. 2015;26(8):1715-1722.
87. Chaudhuri AA, Chabon JJ, Lovejoy AF, et al. Early detection of molecular residual disease in localized lung cancer by circulating tumor DNA profiling. *Cancer Discov*. 2017;7(12):1394-1403.
88. Russo A, De Miguel Perez D, Gunasekaran M, et al. Liquid biopsy tracking of lung tumor evolutions over time. *Expert Rev Mol Diagn*. 2019;19(12):1099-1108.
89. Abbosh C, Birkbak NJ, Swanton C. Early stage NSCLC — challenges to implementing ctDNA-based screening and MRD detection. *Nat Rev Clin Oncol*. 2018;15(9):577-586.
90. Pantel K, Alix-Panabières C. Liquid biopsy and minimal residual disease — latest advances and implications for cure. *Nat Rev Clin Oncol*. 2019;16(7):409-424.
91. Beaver JA, Jelovac D, Balukrishna S, et al. Detection of cancer DNA in plasma of patients with early-stage breast cancer. *Clin Cancer Res*. 2014;20(10):2643-2650.
92. Bayarri-Lara C, Ortega FG, Cueto Ladrón de Guevara A, et al. Circulating tumor cells identify early recurrence in patients with non-small cell lung cancer undergoing radical resection. *PLoS One*. 2016;11(2):e0148659.
93. Fabisiewicz A, Szostakowska-Rodzos M, Zaczek AJ, Grzybowska EA. Circulating tumor cells in early and advanced breast cancer; biology and prognostic value. *Int J Mol Sci*. 2020;21(5):1671.
94. Dawson SJ, Tsui DW, Murtaza M, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med*. 2013;368(13):1199-1209.
95. Mok T, Wu YL, Lee JS, et al. Detection and dynamic changes of EGFR mutations from circulating tumor DNA as a predictor of survival outcomes in NSCLC patients treated with first-line intercalated erlotinib and chemotherapy. *Clin Cancer Res*. 2015;21(14):3196-3203.
96. Siravegna G, Marsoni S, Siena S, Bardelli A. Integrating liquid biopsies into the management of cancer. *Nat Rev Clin Oncol*. 2017;14(9):531-548.
97. Roy-Chowdhuri S, Mehrotra M, Bolivar AM, et al. Salvaging the supernatant: next generation cytopathology for solid tumor mutation profiling. *Mod Pathol*. 2018;31(7):1036-1045.
98. Su YH, Wang M, Brenner DE, et al. Human urine contains small, 150 to 250 nucleotide-sized, soluble DNA derived from the circulation and may be useful in the detection of colorectal cancer. *J Mol Diagn*. 2004;6(2):101-107.
99. Nadano D, Yasuda T, Kishi K. Measurement of deoxyribonuclease I activity in human tissues and body fluids by a single radial enzyme-diffusion method. *Clin Chem*. 1993;39(3):448-452.
100. Mall C, Rocke DM, Durbin-Johnson B, Weiss RH. Stability of miRNA in human urine supports its biomarker potential. *Biomark Med*. 2013;7(4):623-631.
101. Reckamp KL, Melnikova VO, Karlovich C, et al. A highly sensitive and quantitative test platform for detection of NSCLC EGFR mutations in urine and plasma. *J Thorac Oncol*. 2016;11(10):1690-1700.
102. Botezatu I, Serdyuk O, Potapova G, et al. Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. *Clin Chem*. 2000;46(8 Pt 1):1078-1084.
103. Gao K, Zhou H, Zhang L, et al. Systemic disease-induced salivary biomarker profiles in mouse models of melanoma and non-small cell lung cancer. *PLoS One*. 2009;4(6):e5875.
104. Streckfus C, Bigler L, Dellinger T, Dai X, Kingman A, Thigpen JT. The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. *Clin Cancer Res*. 2000;6(6):2363-2370.
105. Wang Y, Springer S, Mulvey CL, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. *Sci Transl Med*. 2015;7(293):293ra104.
106. Hubers AJ, Heideman DA, Yatabe Y, et al. EGFR mutation analysis in sputum of lung cancer patients: a multitechnique study. *Lung Cancer*. 2013;82(1):38-43.
107. De Mattos-Arruda L, Mayor R, Ng CKY, et al. Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. *Nat Commun*. 2015;6:8839.

108. Villatoro S, Mayo-de-Las-Casas C, Jordana-Ariza N, et al. Prospective detection of mutations in cerebrospinal fluid, pleural effusion, and ascites of advanced cancer patients to guide treatment decisions. *Mol Oncol*. 2019;13(12):2633-2645.
109. Bugalho A, Ferreira D, Dias SS, et al. The diagnostic value of transthoracic ultrasonographic features in predicting malignancy in undiagnosed pleural effusions: a prospective observational study. *Respiration*. 2014;87(4):270-278.
110. Kimura H, Fujiwara Y, Sone T, et al. EGFR mutation status in tumour-derived DNA from pleural effusion fluid is a practical basis for predicting the response to gefitinib. *Br J Cancer*. 2006;95(10):1390-1395.
111. Incorvaia L, Fanale D, Badalamenti G, et al. Baseline plasma levels of soluble PD-1, PD-L1, and BTN3A1 predict response to nivolumab treatment in patients with metastatic renal cell carcinoma: a step toward a biomarker for therapeutic decisions. *Oncoimmunology*. 2020;9(1):1832348.
112. Finkelmeier F, Ö Canli, Tal A, et al. High levels of the soluble programmed death-ligand (sPD-L1) identify hepatocellular carcinoma patients with a poor prognosis. *Eur J Cancer*. 2016;59:152-159.
113. Incorvaia L, Badalamenti G, Rinaldi G, et al. Can the plasma PD-1 levels predict the presence and efficiency of tumor-infiltrating lymphocytes in patients with metastatic melanoma? *Ther Adv Med Oncol*. 2019;11:1758835919848872.
114. Okuma Y, Wakui H, Utsumi H, et al. Soluble programmed cell death ligand 1 as a novel biomarker for nivolumab therapy for non-small-cell lung cancer. *Clin Lung Cancer*. 2018;19(5):410-417.e1.
115. Incorvaia L, Fanale D, Badalamenti G, et al. A "Lymphocyte MicroRNA Signature" as predictive biomarker of immunotherapy response and plasma PD-1/PD-L1 expression levels in patients with metastatic renal cell carcinoma: pointing towards epigenetic reprogramming. *Cancers (Basel)*. 2020;12(11):3396.
116. Bian B, Fanale D, Dusetti N, et al. Prognostic significance of circulating PD-1, PD-L1, pan-BTN3As, BTN3A1 and BTLA in patients with pancreatic adenocarcinoma. *Oncoimmunology*. 2019;8(4):e1561120.
117. Zhou J, Mahoney KM, Giobbie-Hurder A, et al. Soluble PD-L1 as a biomarker in malignant melanoma treated with checkpoint blockade. *Cancer Immunol Res*. 2017;5(6):480-492.
118. Tiako Meyo M, Jouinot A, Giroux-Leprieur E, et al. Predictive value of soluble PD-1, PD-L1, VEGFA, CD40 ligand and CD44 for nivolumab therapy in advanced non-small cell lung cancer: a case-control study. *Cancers (Basel)*. 2020;12(2):473.
119. Chen G, Huang AC, Zhang W, et al. Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. *Nature*. 2018;560(7718):382-386.
120. Cordonnier M, Nardin C, Chanteloup G, et al. Tracking the evolution of circulating exosomal-PD-L1 to monitor melanoma patients. *J Extracell Vesicles*. 2020;9(1):1710899.
121. Larrosa-Garcia M, Baer MR. FLT3 inhibitors in acute myeloid leukemia: current status and future directions. *Mol Cancer Ther*. 2017;16(6):991-1001.
122. Gandara DR, Paul SM, Kowanetz M, et al. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab. *Nat Med*. 2018;24(9):1441-1448.
123. Gadgeel SM, Mok TSK, Peters S, et al. LBA81_PR - Phase II/III blood first assay screening trial (BFAST) in patients (pts) with treatment-naïve NSCLC: initial results from the ALK+ cohort. *Ann Oncol*. 2019;30(Suppl 5):v918.
124. Reck M, Hagiwara K, Han B, et al. ctDNA determination of EGFR mutation status in European and Japanese patients with advanced NSCLC: the ASSESS study. *J Thorac Oncol*. 2016;11(10):1682-1689.
125. Karlovich C, Goldman JW, Sun J-M, et al. Assessment of EGFR mutation status in matched plasma and tumor tissue of NSCLC patients from a phase I study of rociletinib (CO-1686). *Clin Cancer Res*. 2016;22:2386-2395.