REVIEW

When Tissue is an Issue the Liquid Biopsy is Nonissue: A Review

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ABSTRACT

Precision medicine has impacted the field of medical oncology by introducing personalized therapies, improving all measurable outcomes. This field, in turn, has expanded to obtaining and analyzing a vast and ever-increasing amount of genomic information. One technique currently applied is the liquid biopsy, which consists of detecting and isolating DNA and exosomes in cancer patients. Newly developed techniques have made it possible to use the liquid biopsy in a wide range of settings. However, challenges regarding the validation of its clinical utility exist because of a lack of standardization across different techniques and tumor types, confounder genomic information, lack of appropriate clinical trial designs, and a non-measured, and therefore not estimated, economic impact on population health. Nowadays, liquid biopsy is not routinely used, but ongoing research is increasing its popularity, and a new era in oncology is developing. Therefore, it is essential to have an in-depth understanding of the liquid biopsy technique. In this review, we summarize the leading techniques and liquid biopsy applications in cancer.

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Key Summary Points
Precision medicine has renewed the field of medical oncology, allowing a more personalized treatment.
This field in turn has expanded to obtaining and analyzing a vast and every-increasing amount of genomic information.
The liquid biopsy technique consists of detecting and isolating circulating tumor DNA and exosomes.
Many newly developed techniques and next-generation sequencing analyses allow a broad application of liquid biopsy in a wide range of settings.
Some difficulties have been identified in carrying out this procedure, including lack of standardization, presence of confounder genomic information, and lack of appropriate clinical trial designs.
This review summarizes the main techniques and applications of the liquid biopsy technique in cancer.

DIGITAL FEATURES

This article is published with digital features, including a summary slide, to facilitate understanding of the article. To view digital features for this article, go to https://doi.org/10.6084/m9.figshare.13855316.

INTRODUCTION

The liquid biopsy (LB) technique has become highly relevant in the clinical field, and the acquisition of cell free DNA (cfDNA), including circulating tumor DNA (ctDNA), has become recognized as an emerging biomarker and a tool for the research, diagnosis, and prognosis of solid tumors [1]. Different studies have confirmed that this type of DNA contains information on specific tumor-related alterations, such as mutations, methylations, and copy number variants/variation (CNVs) [2]. The most striking advantage of LB is its ability to isolate ctDNA through a minimally invasive technique [1, 3].

cfDNA is released into the bloodstream as a consequence of cellular processes, such as apoptosis, pyroptosis, mitotic catastrophe, autophagy, phagocytosis, or a regulated form of neutrophil cell death referred to as NETosis [4]. The levels of cfDNA are higher in patients with cancer and, consequently, structural and epigenetic changes in the DNA sequence can be observed, which in turn reflect the disease process [5, 6]. LB also provides real-time information on the tumor, which is relevant because such information is temporary and spatially heterogeneous. The detection of these variations positively impacts timely treatment modifications that could be beneficial for the patient [5, 7]. Notably, the analysis of nucleic acids in tumor tissue from traditional biopsies provides information on the predominant cells in the tumor while, in contrast, tumor analysis based on cfDNA reveals information on all tumor sites. As such, LB can be used to monitor a patient's disease burden and progression more accurately and in real-time, thus detecting information on the heterogeneity of different tumors [8].

Another significant advantage of LB is that the cfDNA released by the tumor into the bloodstream has the same variants as those of the tumor cells. Thus, information can be obtained at different times of the disease and, given the ease of sampling, repeated sampling is possible without further inconvenience to the patient [1]. This has led to significant technological advances, including the isolation of cancer cells and the collection of DNA derived from them, that provide accurate and timely information through the use of LB of oncogene-related variants that can be taken into account when designing targeted therapies [9]. In this context, the LB technique is a powerful resource

for determining the appearance of additional genetic changes, such as the drug resistance mutations that appear with the use of certain targeted therapies. The possibility of being able to take repeated samples allows for adjustment of the therapy to respond to the evolution of the tumor [6]. It is also possible to obtain ctDNA in several body fluids, such as urine, feces, cerebrospinal fluid, and saliva [10, 11]. Thus, advances in this field will surely change how the treatment of a patient with cancer is selected and monitored.

The development of precision medicine (PM) represents a breakthrough in cancer management [12]. The application of individualized treatments that emphasize tumor biology and predictive responses has significantly improved clinical outcomes. Hence, the clinical applications of PM are broad, encompassing screening, diagnosis, prognosis, prediction of treatment response and resistance, early detection of recurrence/metastasis, and biological cancer stratification [13]. However, challenges related to early diagnosis and real-time treatment monitoring remain. Some of these issues are due to previously described tumor biopsies, including limited tissue availability, the continuously evolving tumor genetic landscape in response to treatment, the emergence of resistant subclones, and the lack of knowledge on spatial and temporal heterogeneity of tumoral cells [14].

The completion of the Human Genome Project (HGP) in 2001 opened the floodgates to a deeper understanding of the diseases and their potential treatment [15]. Therapeutic advances in genome-guided precision oncology rely upon the prospective molecular identification of oncogenic alterations and resistance mechanisms to guide accurate treatments [16]. Technological advances in the genetic sequencing of cfDNA in plasma have increased the advantages of the LB technique so that it now has the potential to identify actionable alterations in tumor-derived DNA present in blood and other fluids and to capture intra-tumoral heterogeneity not addressed by tumor tissue biopsy (that only captures a single site), potentially avoiding the need for invasive measures that consume time and generate unnecessary risks.

In this same context, ctDNA is often present in patients without detectable circulating tumor cells, suggesting that these two biomarkers represent distinct entities. At large academic cancer centers, the failure rates from diagnostic tests derived from tissue biopsy-based next-generation sequencing (NGS) are approximately 14%; therefore, ctDNA analysis may be useful in guiding treatment selection in patients for whom tissue-based NGS is not an option [17].

This article is based on previously conducted studies and does not contain any new studies with human participants or animals performed by any of the authors.

BASIC CONCEPTS OF LB

Liquid biopsy is an alternative technique that allows the analysis of different tumor biomarkers, mainly from blood. It is a non-invasive, safe, and effective method with the potential for applications in the clinical diagnosis and treatment management in patients with cancer [18]. The main components released by primary or metastatic tumors in a LB are circulating tumor cells (CTC), cfDNA, ctDNA, microRNA (miRNA), tumor-educated platelets (TEP), and exosomes, among others; the first three components are the most studied [19]. Exosomes are small membrane vesicles released by the cell under both normal and pathological conditions. Their concentrations in the blood correlate with tumor progression, suppression of the immune response, angiogenesis, and metastasis. They are detectable in patients with various types of cancers at concentrations of < 109 vesicles/ml. These biomarkers can be used for diagnosis, evaluation of tumor treatment, and prognosis, even in the setting of drug resistance [20, 21]. TEP are involved in systemic and local responses to tumor growth because tumor cells can alter the mRNA profile of platelets. TEP can splice the circulating mRNA released by the tumor cells and sequester solubilized proteins associated with the tumor. These interactions between platelets and tumor cells may have a significant potential for cancer diagnosis and monitoring of tumor progression. miRNAs are non-coding endogenous RNAs that

regulate the expression of approximately 30% of protein-coding genes and function as oncogenes or tumor suppressors under certain conditions; they can also regulate different cellular pathways and they play an essential role in tumor growth and treatment resistance. miRNAs can be transported by exosomes, TEP, apoptotic bodies, or miRNA protein complexes. They have been proposed as possible biomarkers for the diagnosis, prognosis, and prediction of various types of cancers [19].

Circulating tumor cells (CTCs) are cells that have broken away from a primary tumor and/or metastatic lesion and travel through the bloodstream throughout the body. They can be isolated as individual cells or in groups. They are present in the blood at a low concentration (10 cells/ml of blood), even in metastatic environments [19]. CTCs are useful for determining tumor heterogeneity, identifying specific gene alterations on target, and evaluating the presence of treatment-associated markers (such as the programmed cell death ligand 1 [PD-L1]) for immunotherapies. The concentration of cfDNA is higher in advanced stage cancers and also correlates with tumor burden. Although the amount of cfDNA is higher in serum, plasma is preferred for the detection of ctDNA since the tumor DNA fraction is smaller and more variable in serum [19–21].

Elevated ctDNA levels were first reported in the serum of patients with cancer in 1977 [22]. More recently, a recent large-scale study reported that ctDNA was detectable in > 75% of patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers, but in < 50% of primary brain, renal, prostate, or thyroid cancers [23]. In the last two decades ctDNA has been explored both as a prognostic or predictive marker, as well as a cancer diagnostic tool [24–26]. The introduction of digital PCR (dPCR) enabled the accurate identification and absolute quantification of diverse mutant fragments. Furthermore, a modification of this technique, using beads in emulsions and flow cytometry, has enabled the quantification of the mutant allele fraction of cancer mutations in the plasma of patients at various stages of colorectal cancer [27–29]. In 2008, Diehl et al. indicated that ctDNA is a highly specific tumor dynamic marker that may reflect the total systemic tumor burden and residual disease [29]. In parallel, allele-specific PCR and other methods were conceived and tested for their ability to identify epidermal growth factor receptor (EGFR) mutations in the serum or plasma of lung cancer patients, following the elucidation of the role of such mutations in predicting response to targeted therapy [30, 31]. An explanation of the differences and potential of LB versus tumor tissue biopsy is provided in Fig. 1 .

CFDNA TECHNOLOGIES AND APPLICABILITY IN THE CLINICAL SETTING

In early disease stages, specific tumor-derived DNA (i.e., cfDNA) is around 0.01% and identifying such a small quantity is very difficult [32–34]. Two main techniques are used to identify cfDNA. One technique identifies tumoral mutations from the primary tumor, providing the possibility of monitoring minimal residual disease but requiring a priori characterization using traditional strategies on tissue-based analyses. This technique is fast and has high specificity, and it dectects extremely low allele frequencies [35, 36]. The second technique detects aberrations or point mutations without prior tumoral profiling [37–39]. The methodologies used are NGS, dPCR, realtime PCR (qPCR), mass spectrometry, and detection of hypermethylation.

Non-specific methods based on the study of DNA methylation can be classified into site-specific methylation detection and genome-wide methylation detection. After some previous processes, such as bisulfite conversion and methylations of enriched complementary DNA (cDNA), hypermethylation can be detected using different techniques, such as conventional methylation-specific PCR (MSP), quantitative multiplexed specific methylation PCR (QM-PCR), and a modified version of PCR known as bead methylation (BOM), which involves the integration of three different processes in one tube: DNA extraction, bisulfite
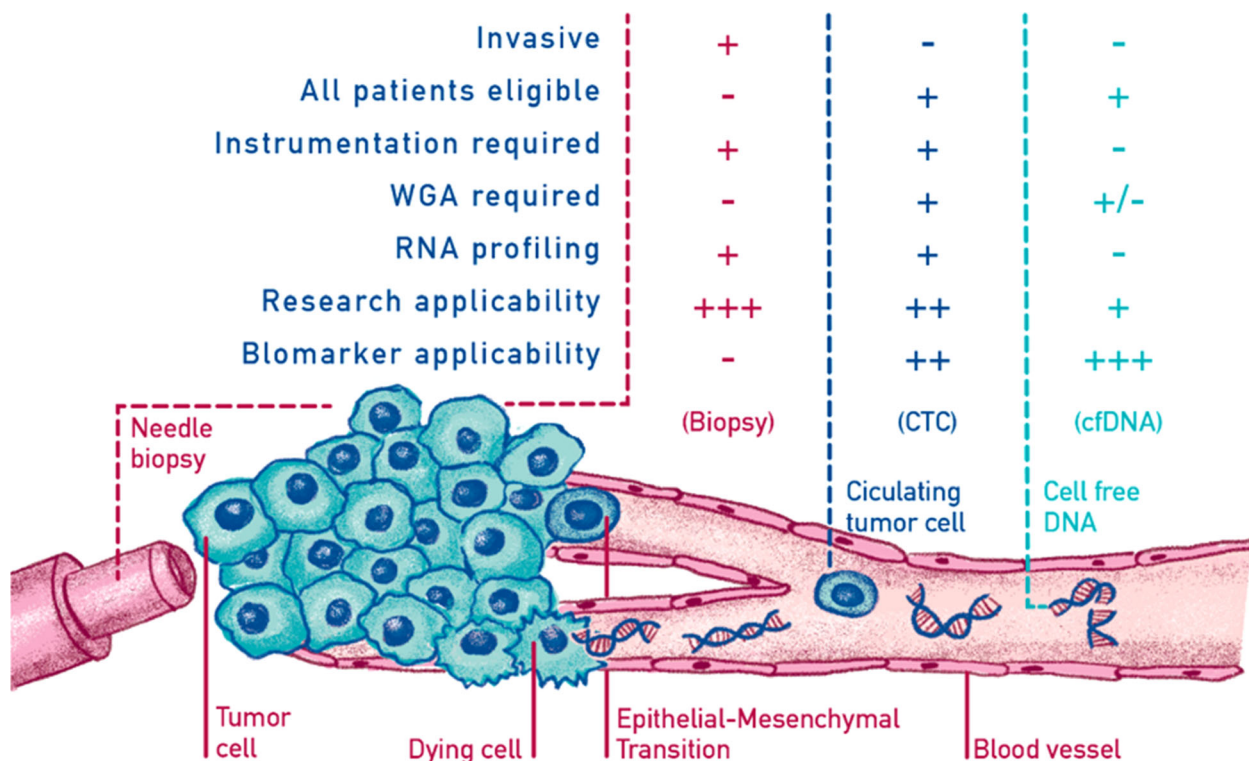
Fig. 1 The molecular characterization of neoplasms allows the correct application of precision medicine, maximizing the results and reducing the treatment's toxicity. Tissue biopsy is the most widely used method for categorizing tumors and detecting biomarkers. However, it has several limitations: it is an invasive method; it is not always feasible or repeatable; and it provides information limited to a single point in space and time, therefore failing to capture the complexity of cancer. Today it is clear that cancer is an incredibly dynamic disease. During disease, cancers generally become more heterogeneous, with the results that the tumor might include a diverse collection of cells harboring distinct molecular signatures with differential sensitivity levels to treatment. This heterogeneity might result in a non-uniform distribution of genetically distinct tumor-cell subpopulations across and within disease sites (spatial variation) or temporal variations in cancer cells' molecular makeup (temporal heterogeneity). For this reason, liquid biopsies can help achieve a correct and early detection of various neoplasm diseases, stratify patients to optimize treatment, and monitor the response and resistance mechanisms in the tumor. The non-invasive nature of the liquid biopsy allows systematically repeating of the sampling to monitor changes related to the dynamics of clonal and subclonal variation of the disease.

conversion and conventional PCR using DNA carriers, such as superparamagnetic iron oxide nanoparticles within silica microparticles [40].

Next-generation, deep sequencing has a specificity of around 80% and sensitivity close to 100%, and it detects low allele frequencies. Additionally, it is advantageous in detecting copy number variation with deficient quantities of cfDNA [41–43]. The highest sensitivity for identifying point mutations is offered with tagged amplicon deep sequencing (Tam-Seq),

Safe-Sequencing System (Safe-SeqS), Cancer Personalized Profiling by deep Sequencing (CAPP-Seq), Bias Corrected Targeted NGS, and Multiplex PCR NGS, reaching a value close to 98–99%. Safe-SeqS amplifies a tagged region into families and sequences by a prespecified DNA template [43]. Tam-Seq implements an efficient library preparation and algorithms to identify hotspot regions and selected coding regions [33]. To detect low concentrations of cfDNA, CAPP-Seq sequencing involves certain

regions previously hybridized by cDNA oly-gonucleotides and obtains an evaluation of mutations, rearrangements, and alteration in copy number. With further optimization of bioinformatics algorithms, a theoretical detection rate of 0.00025% allele frequency can be achieved [44, 45]. Bias Corrected Targeted NGS results in a high number of zero false positives because it uses markers that bind to small capture probes that are then amplified [46].

Two dPCR techniques can be used to conduct LB, namely, droplet PCR (ddPCR) and beads, emulsion, amplification, magnetics dPCR (BEAMing). The first technique, ddPCR, evaluates specific amplicons with flow cytometry by using specific fluorescent probes in an oil–water emulsion [38, 47]. BEAMing characterizes specific strand termination at divergent positions by flow cytometry. It is highly effective, but the cost and complex workflow make it an option that is complicated to implement [48].

Multiplex PCR NGS is the combination of high-throughput PCR amplification and sequencing and reaches a 99% sensitivity for the detection of mutations in target regions [49]. However, ot is relatively expansive to implement compared to dPCR. Conventional somatic mutation detection methods, such as quantitative PCR, only analyze ctDNA at high concentrations because of their low sensitivity. For that reason, more sensitive and specific methods using a combination of pyrophos-phorolysis-activated polymerization and allele-specific amplification during PCR have been used [19]. Another recently developed method is dPCR, which seeks to detect and quantify mutations by analyzing individual molecules; 0.01% of ctDNA can be detected with this method [20, 50].

Finally, qPCR identifies only common mutations, at > 10% allele frequency, by blocking amplification at the oligo 3'-end, and amplifies only the mutant allele or enriches only variant alleles [46]. To overcome the limits of PCR, mass spectrometry is used, which detects up to 40 targets with low DNA input by signal emission from laser excitation or biotin markers that bind to specific regions [51–53].

CLONAL HEMATOPOIESIS AS A CONFOUNDING FACTOR

When a hematopoietic stem cell expands and produces recurrent, specific, and disruptive genetic variants, a patient without hematological malignancy is known to have clonal hematopoiesis (CH) [54, 55]. When CH is present, somatic mutations in genes recurrently mutate in hematologic malignancies with a variant allele frequency (VAF) $\geq$ 2%; in the absence of known hematologic malignancy or other clonal disorders, it is defined clonal hematopoiesis of indeterminate potential (CHIP). The most frequent somatic mutations involve the genes *DNMT3A*, *ASXL1*, and *TET2* [36, 38]. Similar to monoclonal gammopathy of undetermined significance (MGUS), individuals with CHIP have an increased risk of progression to hematologic neoplasia (0.5–1% per year) and all-cause mortality [56]. CH seems more common with aging (approx. 10% of individuals > 65 years, but it is relatively rare in subjects aged < 50 years) [57, 58]. Since cfDNA is a complex mixture of DNA from many sources (including germline, fetal, infectious, and malignant cells), CH may be a potential source of false positives in LB.

CH false positives represent a challenge for minimal residual disease (MRD) profiling and early cancer detection. It can also potentially cause discordances between tumor genotyping and plasma cfDNA genotyping in non-small-cell lung cancer (NSCLC) [59, 60]. Nowadays, the use of highly sensitive plasma NGS increases false positive results due to CH [61]. A comparison of four different NGS platforms to matched tumor–normal tissue pairs revealed that the conventional NGS approach (with both sensitivity and specificity of 95%) could not effectively distinguish between a cfDNA mutation with low VAF (< 0.1%) of hematopoietic origin and a tumor-derived mutation [39, 51]. Recently, a novel approach that incorporates whole blood cell sequencing into cfDNA analysis to filter potential contamination from somatic mutations attributable to CH has been described [62]. This study demonstrated that ultra-deep NGS of plasma cfDNA with CH

filtering can accurately detect a wide variety of oncogenic drivers and resistance mechanisms in patients with advanced lung cancers, with a detection sensitivity comparable to that of established dPCR methods [62].

APPLICABILITY OF LB IN SOLID TUMORS

Over 8.2 million people die of cancer each year due to inaccessibility to appropriate detection procedures and treatments [63]. In this scenario, LBs have been recently gaining attention as an alternative to traditional tissue biopsies, given their minimal invasive nature and the vast amount of information provided [64]. The most commonly used techniques are the quantification of ctDNA and CTCs [65, 66]. Detection of specific genetic abnormalities based on mutation identification and DNA methylation analyses, both genome-wide and site-specific, have also been gaining relevance [67]. Furthermore, ctDNA carries some genomic and epigenomic alterations concordant to the tumor mutational spectrum, such as point mutations, degree of integrity, rearranged genomic sequences, CNV, microsatellite instability (MSI), loss of heterozygosity (LOH), and DNA methylation [68]. These biological characteristics discriminate ctDNA from normal cfDNA and reinforce the latter as a specific biomarker that provides personalized information to detect residual disease or monitor tumor progression during therapy [69]. Under normal circumstances, in healthy individuals, apoptotic and necrotic cells are cleared up by infiltrating phagocytes, and cfDNA levels are relatively low. However, this mechanism does not act effectively with tumoral masses. In solid tumors, ctDNA can also be released through necrosis, autophagy, and other physiological events induced by microenvironmental stress and treatment pressure [7]. The size of most fragments ranges between 180 and 200 bp, suggesting that apoptosis is the predominant source of ctDNA [69].

Advances in LB as a prognostic and predictive tool are mostly associated with lung, colon, and breast cancer (BC). Interestingly, each histology outcome has been preferably associated with a special type of LB. While research in lung or colon cancer has been linked to ctDNA analyses, for BC, the combination of ctDNA and CTCs are more widely used [70]. Advancements have been mainly achieved in the metastatic setting, as the amount of CTCs and ctDNA, which increase with tumoral burden, is the greatest. This, in turn, offers the best disease model for monitoring and assigning new therapies [44].

Specifically in the case of lung cancer, many multidisciplinary efforts have been made to advance the clinical application of LB. In this regard, the standardized isolation methods, such as solid-phase extraction techniques, have been used to isolate ctDNA, and many sequencing or mutation analysis methods have been developed to study and understand the clinical implications [71]. Furthermore, with the current development of high-throughput techniques for NGS, the evaluation of gene panels is expected to be included as a routine analysis, especially for the screening of high-risk individuals. In terms of treatment management, ctDNA has been used in NSCLC for targeted therapy selection, treatment monitoring, and detection of resistance mechanisms through the identification of somatic mutations in the EGFR gene. The Cobas® EGFRMutation Test v2 (Roche Diagnostics, F. Hoffmann-La Roche AG, Basel, Switzerland) was the first LB test approved by the U.S. Food and Drug Administration (FDA) in June 2016 for the detection of EGFR exon 19 deletions or exon 21 (L858R) substitution mutations in patients with NSCLC [72]. This approval represented major progress in establishing the clinical utility of ctDNAs.

In the case of colorectal cancer (CRC), detection of ctDNA in plasma has been associated with worse survival outcomes compared to undetectable levels [73]. Additionally, a strong association between ctDNA positivity and recurrence-free survival (RFS), and overall survival (OS) in patients with CRC irrespective of tumor stage, study size, tumor markers, detection methods, and sample type, is also worth noting [74, 75]. The utility of ctDNA as a prognostic parameter and alternative modality for mutation detection before treatment in

metastatic CRC (mCRC) was demonstrated in the randomized CORRECT phase III trial. The progression-free survival (PFS) of patients with KRAS mutations detected in plasma treated with regorafenib was shorter than in those without mutations [76]; this finding established the prognostic value of KRAS mutations in plasma and eliminated the need for tissular confirmation [77]. In turn, it allowed the development of several ctDNA mutation assays and platforms for KRAS mutation screening and treatment selection, such as the Idylla™ (Biocartis, Mechelen, Belgium) and Cobas® platforms, both FDA approved.

BC was one of the first tumor models in which different types of LBs were studied. The prognostic value of ctDNA in BC has been documented in numerous studies, showing the clinical value of detecting of HER2 amplification and the PIK3CA and ESR1 mutations in ctDNA [78].

In the recent years, ctDNA analyses have been reviewed in other different neoplasia, such as liver, pancreas, ovarian, or prostate cancer [79]. In the case of pancreatic cancer (PC), one of the most aggressive and devastating of all malignancies, the absence of a reliable methodology for early identification and the limited therapeutic options for advanced disease set the stage for a possible broad-scale LB implementation [67]. In this context, a study conducted by Shapiro et al. reported that the presence of ctDNA is markedly elevated in patients with PC in comparison to healthy controls, leading these authors to conclude that ctDNA may serve as a useful diagnostic and prognostic biomarker [80]. Similar studies show that patients with PC with notable ctDNA levels have worse survival and greater risk of a diagnosis of an advanced disease stage [72]. Tjensvoll et al. also noted that during chemotherapy, changes in mutant KRAS circulation levels corresponded to radiological response assessments and cancer antigen 19-9 (CA19-9) values, suggesting the utility of ctDNA for monitoring treatment efficacy and tumor progression [81]. These studies demonstrate the potential clinical utility of ctDNA as a prognostic marker in PC.

In the case of ovarian cancer, historically there has been a lack of reliable screening strategies; however, the clinical value of ctDNA-based assays have since been demonstrated [82]. The first study on the application of LB for screening ovarian cancer, conducted by Cohen et al., was based on the detection of CNV in cfDNA and obtained satisfactory results [83]. Similar studies are being developed for other solid tumors, including hepatocellular carcinoma, head and neck cancer, and prostate cancer [84–86]. However, in other tumor types, ctDNA studies are still underexplored. Current standard clinical tools for the diagnosis and monitoring of central nervous system (CNS) tumors have several important limitations [87], of which the most important are the requirement of invasive tissue sampling, with its accompanying higher risk of complications relative to other locations, and the decreased feasibility of repetitive sampling. As a consequence, the use of LB in patients with CNS malignancies is particularly attractive. However, further studies are still necessary [88].

In conclusion, clinical evidence confirms that there are increased levels of ctDNA, particularly in metastatic disease, in patients with any of the solid tumors analyzed to date. In addition, ctDNA levels in cancer patients likely correlate with tumor burden, stage, vascularity, cellular turnover, and response to therapy. However, not all metastatic tumors seem to have the same impact on ctDNA levels, and more research is required since critical clinical standards (including uniform pre-analytic and analytical phase) are scarce and need to be established [17]. The harmonization of studies and comparative studies is crucial to provide clear evidence of the clinical utility of ctDNA in metastatic solid tumors.

DESIGN OF CLINICAL TRIALS TO ASSESS THE USEFULNESS OF LB IN CANCER

The design of clinical trials that evaluate the effectiveness of LB are under development. In the context of evaluating the LB technique, multiple objectives can be assessed: evaluating resistance pathways, pharmacodynamics, treatment efficacy, and prognostic and predictive

markers. A very comprehensive review of studies assessing the effectiveness of LB in all these settings has been published [11]. Traditionally, LB implementation has been carried out parallel to treatment trials assessing the outcomes mentioned above. However, an interesting approach would be to assess LB as an allocation strategy between the treatment arms of clinical trials. Clinical trials assessing immune checkpoint inhibitors could potentially take advantage of this. PD-L1 expression correlates with responses to pembrolizumab in urothelial carcinoma, NSCLC, head and neck cancer, renal carcinoma, and melanoma [21, 89–91]. The currently running study Targeted Agent and Profiling Utilization Registry (TAPUR) conducted by the American Society of Clinical Oncology (ASCO) and several participating centers in the USA (ClinicalTrials.gov Identifier NCT02693535) recruits patients with advanced cancers with evidence of a potentially targetable genomic alteration who are not candidates for standard treatment. Patients with a LB genomic assessment also candidates for inclusion in the study. A specialized genomic tumoral board defines treatment, and a corresponding medication is administered. Results are expected in the upcoming years. This study is the closest design possible to a master protocol of patient recruitment based on a serum sample test.

Within the traditional trial design, it is difficult to conduct several studies for each possible tumor that carries a specific genomic driver. One strategy by which to address this issue is to implement master protocol trials that run multiple sub-studies in parallel. The three types of master protocol trials are basket, umbrella, and platform trials. Several definitions for each of these trial types are not consistent, but a review published by Hirakawa et al. comprehensively explores each definition and provides caveats [92]. The underlying notion for this avant-garde approach would be to utilize LB assays as a recruitment tool in large prospective clinical trials. Another design is the liquid dynamic medicine model. The basis for this proposal comes from the dynamic variation of molecular profiles in cancer: taking into account that resistance mechanisms arise in virtually all patients receiving targeted therapies, serial evaluation after disease progression could help elucidate the cause of set resistance. Although this concept is not new, the idea would be to administer sequential treatments, i.e., second and further lines, based on LB results, leading to the extreme personalization of care. Difficulties with this approach present in the form of the other lines: as the patient's disease progresses, there remains fewer possible comparators of efficacy. One possible solution would be to compare the patient against her/himself in a previous treatment line in terms of time to disease progression. This novel strategy is referred to as the 'N of 1 trial' [4].

LB has also raised questions regarding the design and conducting of clinical trials. As more evidence on the usage of these assays is gathered, clinical trials should advance in order to provide optimal evidence-based strategies to implement these diagnostic modality.

USAGE OF LB IN CURRENT CLINICAL PRACTICE

The use of LB in the clinical field has been increasing in recent years, not only due to the ease of obtaining samples from the patient but also because of the possibility of obtaining information at different stages of the disease and of early detection and prognosis [93]. The characteristics of cfDNA are different in cancer patients than in healthy patients [94]. Low levels of cell-free DNA have been reported in healthy patients at levels typically not exceeding 25 ng/ml plasma, while higher levels have been reported in cancer patients, ranging from < 0.1 to approximately 10% of DNA molecules, in association with tumor burden, stage, cell turnover, and accessibility to the bloodstream [95]. Similarly, it has been established that wild-type cfDNA is less fragmented than mutated cfDNA, with a size range of 250–320 and 90–150 bp, respectively [11]. Therefore, LB provides information that is useful for early detection, high-risk determination, choice, response to treatment, and early relapse detection in cancer patients. (Fig. 2). In fact, studies have reported that radiological imaging
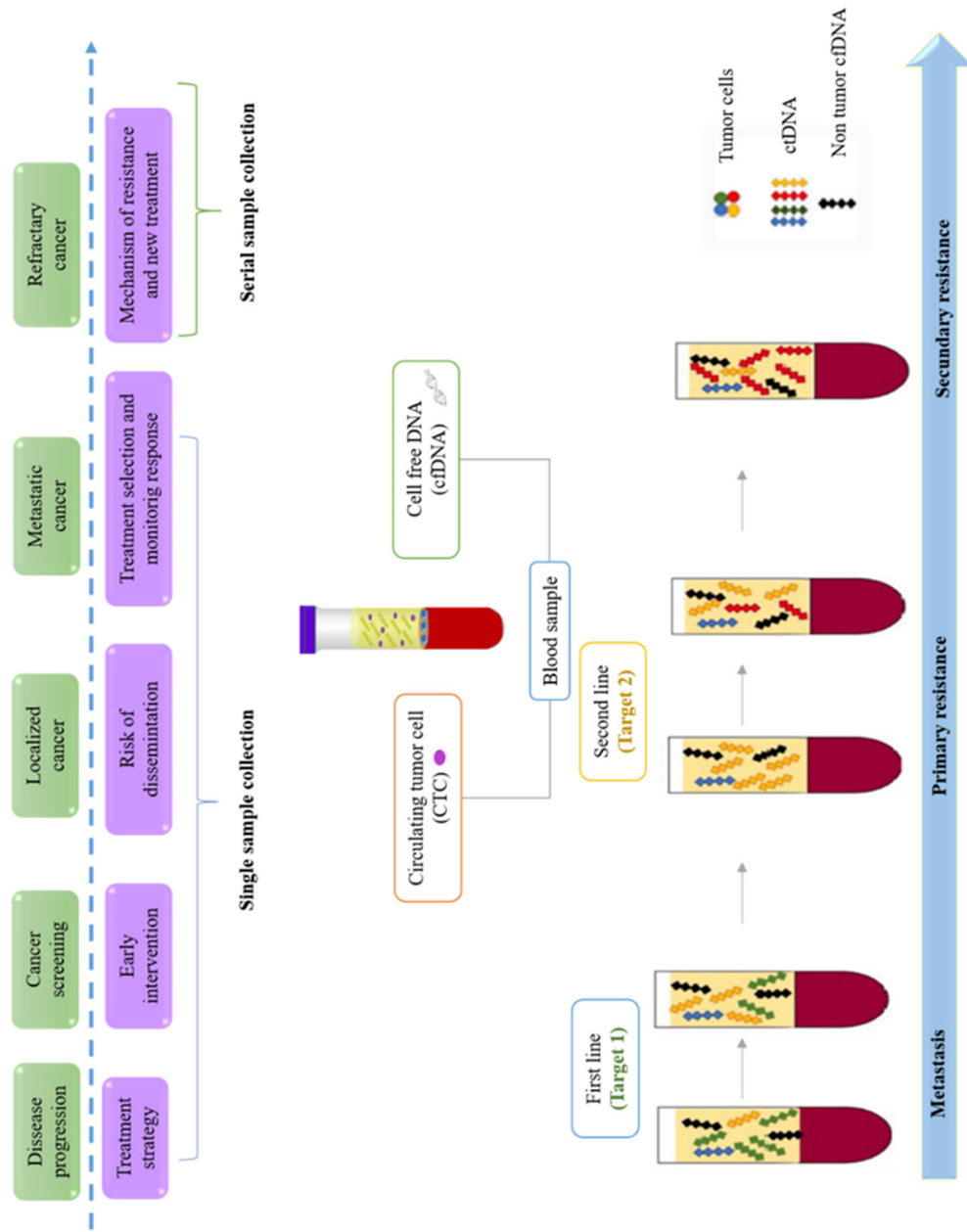
Fig. 2 Reported usages of liquid biopsy in cancer patients. The level of cfDNA in cancer patients has been used for the early detection of various types of cancer and patient monitoring at different stages of the disease. This is achieved through the information obtained from the analysis of circulating ctDNA, which provides information on the diagnosis, stage, response to treatment, and mechanisms of resistance that develop after treatment and predict the minimal residual disease and recurrence after surgery or therapy (Taken and adapted from Bronkhorst et al. [6]; Haber et al. [50], and Vymetalkova et al. [2])

can only detect tumors when these are about 7–10 mm in size and contain about 1 billion cells [96, 97]. In this context, there is undoubtedly a potential for the use of LB in the early diagnosis of cancer, at a stage in which it can be curable [98].

It also has been suggested that the cfDNA found in different body fluid samples, particularly in plasma, could represent a promising biomarker in patients with cancer because the relative levels of cfDNA are related to tumor burden as well as response to therapy. Additionally, more aggressive chemotherapy regimens have also been reported to be more effective in patients whose metastatic cancer is associated with high levels of circulating tumor cells [99, 100]. There is a correlation between cfDNA levels and cancer before treatment begins; as well, persistent and high levels of this type of DNA may indicate relapse and are likely a sign of poor prognosis [101]. Furthermore, for patients with localized cancer, the presence of circulating tumor cells or high levels of ctDNA could contribute towards the identification of patients at a higher risk of recurrence, in which adjuvant therapy could be considered [50]. Also, the high levels of ctDNA favor the determination of specific mutations in the cancer-conducting genes, such as those coding for EGFR, KRAS, BRAF, and PIK3CA, which are predictive of the response to different inhibitors. Another use that can be given is to find personalized biomarkers that are subsequently used to detect residual disease or monitor tumor levels during therapy [102].

Another use of LB in clinical practice is the detection of acquired resistance to targeted therapies. Taking into account that tumors acquire resistance to targeted therapies through the appearance of resistance mutations, which in turn lead to the activation of alternative signaling pathways, increased expression of anti-apoptotic genes, or reduced drug binding, LB is a useful tool in patient relapse, the potential need for continuous follow-upm and the appropriate selection of a second effective line of response for the patient [103].

USEFULNESS OF LB FOR SCREENING AND EARLY DETECTION

Identifying oncogenic driver mutations in asymptomatic or early-stage neoplasms could have a tremendous impact in cancer control and sequels. Breast and lung cancer are among the most prevalent candidates for inclusion in screening programs. An study of a large cohort of patients with BC analyzed promoter methylation in cfDNA in *SFN*, *P16*, *hMLH1*, *HOXD13*, *PCDHGB7*, and *RASSFI*, and a sensitivity and specificity close to 80% was obtained in terms of BC detection. Similar results were obtained with the comparison of benign breast lesions and infiltrating ones [7, 104, 105]. However, larger clinical trials are needed to search for potential markers for BC screening.

Similar conclusions can be drawn from studies on the early detection of lung cancer. Several techniques have provided exciting results. Homologous to the findings for BC, the methylation profiles in cfDNA in *APC*, *CDH13*, *KLK10*, *DLEC1*, and *RASSF1A* revealed a higher hypermethylation profile in patients with lung cancer than in healthy controls, achieving a sensitivity close to 84% and specificity of 74% [106]. mRNA analysis, proteomics, transcriptomics, metabolomics, microbiome evaluation, and other techniques have been examined [107]. Of the methodologies previously mentioned, the use of miRNAs has revealed promising results; specifically, the measurements of miR7, mi126, and mi145 in the sputum of 30 healthy controls and patients with lung cancer achieved a high diagnostic performance [108]. Proteomics in a similar setting with 139 cancer patients and 49 controls offered an area under the ROC curve (AUC) of 0.94 with the identification of five proteomic markers [109]. Although encouraging, several disparities exist in the standardization of techniques and studies, yielding a vast amount of results that differ from the reported ones, although similar methodologies were used. Standardization should be addressed in the planning of further studies in the screening and early detection of lung cancer [107]. In conclusion, although LBs

are potentially an effective diagnostic tool in this setting, they need further development and characterization of markers.

BIOLOGICAL FLUIDS, COLLECTION AND MANAGEMENT OF LB SAMPLES

The feasibility of conducting LB in any fluid with a sufficient ctDNA concentration opens the door to the possibility of characterizing other compartments beyond the circulation. Taking into consideration that pleural effusions, cerebrospinal fluid (CSF), and urine can be populated with malignant cells in much higher concentrations than the circulation, direct analysis of these fluids could be feasible. Detection of *EGFR* mutations in patients who have lung cancer-related malignant pleural effusions have opened up the possibility to characterize fluids and offer both a diagnostic and prognostic strategy based solely on effusion analysis [110]. Also, detecting ctDNA in CSF for the assessment of primary brain tumors is possible for most patients. Contrary to other tumors, the lack of a widespread validation of targetable molecular targets limits this test's applicability. On the other hand, metastatic tumors with seeding in the central nervous system could potentially be evaluated for treatment mechanisms as well as molecular disease profiling [111]. Urine has been gaining attention as a reliable source of LB material as it is easy to obtain and does not require any invasive procedure. To an extent, ctDNA is filtered through the kidneys from the blood which also re-collects cellular debris from the urinary tract. Although validated for urological tract neoplasms, the use of LB for non-urological tumors is a matter of controversy. Using both PCR and ddPCR, several studies have published different results across pathologies, using different sample sizes and specific mutation evaluation [112]. Notably, these techniques are not the best for detecting small quantities of DNA, opening the possibility for further studies employing low and extra low allele frequency detection, such as deep sequencing.

Considering that sample processing could also positively influence LB performance, a few points are worth exploring. cfDNA concentration per se is considered to be very low. Therefore, sample extraction and isolation should be efficient. Modern solutions employ columns that have improved extracted quantities compared to the older methods. However, fragments < 100 bp are still lost during the purification process [112]. Blood centrifugation, for example, leads to a release of DNA from cells present in the sample, introducing confounding material into the analysis. Newer techniques, such as dielectrophoresis, nanochip, and nanowire methods, have yielded interesting results that can potentially increase ctDNA detection ocess [113].

Other relevant sources of cfDNA include saliva and pleural fluids, among others. Salivary DNA has been used to detect germline mutations in breast and brain cancers because it provides good quality genomic DNA. Studies have shown that ctDNA isolated from saliva and blood can serve as a biomarker for head and neck squamous cell carcinoma (HNSCC) [114]. Tumor DNA detected in sputum may be useful for the early detection of lung cancer. Although the detection of *EGFR* mutations in pleural effusion samples is feasible, it is difficult due to the small number of tumor cells. However, studies have shown that the presence of ctDNA in pleural fluid indicate the existence of these mutations in patients with NSCLC [116]. Some studies have also been done to investigate the diagnostic, prognosis, or predictive value of miRNAs in pleural effusion fluid in patients with NSCLC, although more research is needed to validate the usefulness of these biomarkers in this type of sample [114].

SAMPLING AND HANDLING OF SAMPLES

The most common source of ctDNA is plasma. Certain sample processing recommendations should be followed to ensure maximal performance; these are summarized in Fig. 3. A method designed for a selective extraction of small fragments of cfDNA (i.e., 166 bp) or a size
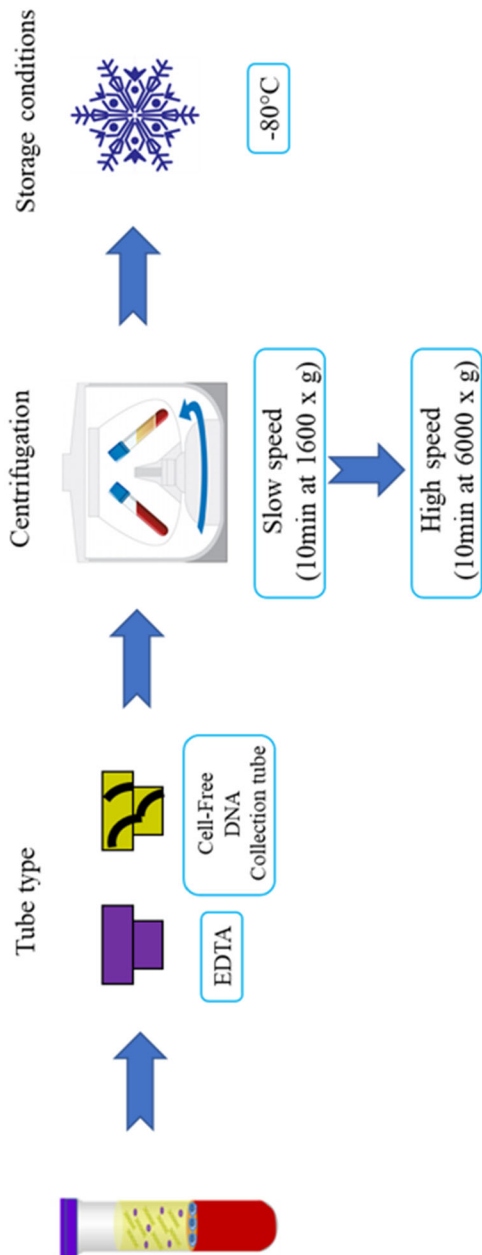
Fig. 3 Recommendations for the processing of plasma samples. Blood should be collected in EDTA-containing tubes (e.g., EDTAK3) as heparin and citrate could inhibit PCR. To prevent lysis of peripheral blood cells and the release of germline DNA, blood samples must be processed immediately after venipuncture by two rounds of refrigerated centrifugation, first at a slow speed to remove cells, followed by high-speed centrifugation of the carefully collected supernatant. Processing can be delayed to a maximum of 4–6 h. Following processing, the samples can be aliquoted and stored at − 80 °C [9] (Taken and adapted from Bronkhorst et al. [6])

selection for short DNA fragments should be performed [115].

ECONOMIC IMPACT OF LIQUID BIOPSIES

An in vitro diagnosis assay requires different levels of investment in each phase of the study. Regarding the LB technique, we are in an initial phase, with the test having been demonstrated to be capable of identifying the presence of certain cancers. Clinical utility, such as clinical decision-making, including treatment regime change, can be taken based on LB results [72]. Positive assessments in areas are essential for a new LB assay to be approved by regulatory agencies, which will also require proof of an overall positive impact on healthcare providers' systems, as this will enable a high return on investments. One of the main concerns of healthcare providers is the allocation of financial resources; consequently, assessing the impact of a single intervention on the whole system is a crucial task, but also it is not an easy one. To show the full positive economic impact on general health, several impact models have been proposed. One field which can be used as a mirror to develop LB economic impact models is personalized medicine (PM) and within it, economic models developed for genetic testing [116, 117]. As mentioned above, there is no single model to evaluate the economic impact of any clinical intervention; rather, there are many different strategies, of which cost-effective analysis (CEA) and cost–benefit analysis (CBA) are the most widely applied. These two systems differentiate on the normative paradigms which support them, extra-welfarism and welfare, respectively [118]. Extra-welfarism, which supports CEA, focuses on obtaining the maximum overall health status, and welfarism, which supports CBA, looks at individual utilities as its primary outcome. While both have their own individual issues, CEA is the more appropriate in the context of the utility of LBs as it quantifies the impact of the intervention on overall health using parameters such as quality-adjusted life years (QALY) and life-years gained (LYG), both of which measure clinical effectiveness.

While CEA should be a must in developing LB essays, there are not many publications that report such an analysis. One example of a LB assay which has demonstrated its positive economic impact is SelectMDx (MDXHealth, Irvine, CA, USA), an assay based on circulating epigenetic markers for prostate cancer. A CEA in four different countries (Germany, France, Spain, and Italy) reported that, on average, LB intervention results in a savings of €665 and a gain of 0.025 QALY per patient compared to using the PSA alone [119].

Due to this uncertainty and lack of CEA of the different LB assays, it is challenging to calculate the future size of the market for LBs. Also, the full sets of clinical utilities are still to be fully defined. Currently, LB is being used mainly for cancer prognosis and therapy selection in metastatic cancers. However, there are already studies that have proposed that LBs be used for early diagnosis and non-tumoral applications [120–122]. These new LB applications have still to go through the full cycle of regulatory approval and clinical efficacy defined above. The CEA on these applications will determine the full scale of the economic impact of LBs on health systems.

Hence, a broad range of forecasted figures, ranging from $2.4 billion to $100 billion in 2030, can be found. Combining their potential clinical utilities and the broad market that could be created has made investors think it is an opportunity too good to be missed. In the meantime, most LB assays are currently available as laboratory developed tests (LDT) or FDA-approved products and offered as an out-of-pocket test to patients.

LIQUID BIOPSIES FOR POPULATION WELLBEING

According to the World Health Organization, "a screening program should respond to a recognized need, and its objectives should be defined at the outset. There should be a defined target population and scientific evidence of the screening program's effectiveness. The program

should integrate education, testing, clinical services, and program management, along with quality assurance and mechanisms to minimize potential risks of screening. It should ensure informed choice, confidentiality, and respect for autonomy, and promote equity and access to screening for the entire target population. The evaluation should be planned from the outset, and the overall benefits of screening should outweigh the harm" [123]. In this context, the screening programs for healthy and high-risk individuals for cancer is a crucial application of LB approaches, independently of whether the high-risk population is based, or not, in a developed country [124].

According to the National Cancer Institute (NCI) dictionary of cancer terms, liquid biopsy is "a test done on a sample of blood to look for cancer cells from a tumor that are circulating in the blood or for pieces of DNA from tumor cells that are in the blood" [125]. However, from our point of view, this definition is rather imperfect, since this terminology cannot be applied to screening as it does not include: circulating DNAs that are analyzed in the field of prenatal diagnosis, severe/acute inflammation (sepsis), and transplantation, or its application for evaluating pre-tumor or cancer interception (chronic obstructive pulmonary disease [COPD], asthma, or liver disease) [126]. Therefore, all previous concepts should be integrated in the definition, including the concept of cancer interception. This concept could help to develop tools for early cancer detection because it studies the events associated with cancer initiation and progression [127].

Nowadays, many groups are developing cancer-screening tests based on LBs. The majority of these studies are focused on CRC and lung cancer. In CRC, many markers have been identified, such as the methylated Septin9, which tend to be higher in CRC patients than in patients without the disease [128, 129]. These results include the development of a specific test named Epi proColon® 2.0 CE (Epigenomics AG, Berlin, Germany). This test discriminated patients with CRC and healthy controls with high clinical sensitivity and specificity in pivotal case–control studies [130]. Other studies have been conducted with the aim to also

evaluate different hypermethylated DNA promoter regions and genes specific to CRC that are present in the plasma [137]. In addition, an age-adjusted panel of four cell-free nucleosomes was developed by Volition (Volition SPRI, Namur, Belgium) for the discrimination between CRC patients and healthy controls. The results of this study showed that the panel had a high sensitivity for early stages (75 and 86% for stages I and II, respectively) at 90% specificity [131]. Regarding lung cancer, the increased levels of cfDNA were also analyzed in a recent study by Esposito et al., where the cfDNA levels from cancer patients were compared to those of healthy individuals [132]; the results of this work demonstrated an increase of cfDNA levels in patients with early LC compared to the cfDNA levels in healthy donors. Similar results were observed for BC with 78% sensitivity and 83% specificity, and ovarian cancer with 70% sensitivity and 90% specificity [133, 134]. Other types of LBs have been used for screening both CCR and lung cancer [129, 135]. However, large studies are still necessary to demonstrate their usefulness as early diagnostic markers.

The patient experience has been recognized as one of the three pillars of quality health care, alongside with clinical quality and patient safety [136]. Patient experience is the most effective measurement of the patient-approach, defined as providing care that is respectful of and responsive to patient preferences, needs, and values. Thus, patients' groups have become involved in scientific and therapeutic activism to achieve positive feedback for technological development. In the case of LBs and, in particular, in the use of LB as a screening marker, the implication of patients' associations is especially important to spread knowledge about these new screening methodologies. Note that the potential use of LBs as cancer-screening tests involves the analyses of large populations. Patients' organizations help promote research and develop processes and methodologies to ensure that their members are fully prepared to get involved in research and clinical trials, and ensure that patients can participate wherever needed. These associations can promote initiatives to bring together patient organizations from different countries and to help others

engage as experts in their disease (e.g. EU-funded initiatives) [137]. In addition, patients' associations could help to involve health centers, encouraging primary care practitioners to engage in early intervention through LB programs, since only a mere blood extraction is needed.

Also, patients' organizations can play a key role in obtaining funding for LB research, thereby facilitating more research and obtaining money to support early screening programs. However, despite patients' associations and academic and clinical research in LB programs, implementation of LBs can only become possible if pharmaceutical companies are involved and technology companies provide advice. Based on LB, the screening programs involve the need to work with highly sensitive and specific technologies. In addition, pharmaceutical companies have a double purpose in this context. Their participation promotes research and the dissemination of knowledge on LB, but they are also responsible for the development of "therapeutic responses" that these high-risk populations could need.

CONCLUSIONS

Liquid biopsy is an umbrella term that encompasses several techniques for evaluating genomic information that is simple to obtain but complex to perform and analyze. Although several methodologies exist that have been clinically validated, the continuous developmental effort and standardization will lead in the future to further indications for LB that could impact outcomes in cancer patients specifically and population health in general. The implementation of clinical studies with alternate designs will facilitate the evaluation and final measurement of the impact of this novel diagnostic tool.

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