Book Chapter

Liquid Biopsy in Non-Small Cell Lung Cancer (NSCLC): State of the Art and the Next Opportunity

Carl KHAWLY

Cancer Research Center of Toulouse (CRCT), France

***Corresponding Author:** Carl KHAWLY, Cancer Research Center of Toulouse (CRCT), France

Published **December 13, 2022**

How to cite this book chapter: Carl KHAWLY. Liquid Biopsy in Non-Small Cell Lung Cancer (NSCLC): State of the Art and the Next Opportunity. In: Hussein Fayyad Kazan, editor. Immunology and Cancer Biology. Hyderabad, India: Vide Leaf. 2022.

© The Author(s) 2022. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

With lung cancer maintaining alarming mortality rates, the clinic calls for sensitive cancer screening tools. This need is dire for the nine-out-of-ten patients experiencing the non-small cell variant (NSCLC) that deploys high rates of drug resistance. The concept of liquid biopsy is nothing short of a breakthrough in precision oncology. Not only did recent advancements demonstrate the competence of liquid biopsy technologies but also showed that this new paradigm is here to stay. Based on peer-reviewed journals and publicly available information from the industry leaders, this chapter depicts the state of the art of liquid biopsy in

NSCLC patient monitoring, the different clinical and academic uses, an objective view of the field, and the advancements we can expect to see in the coming years that will push the boundaries of precision medicine.

Introduction

Lung cancer leads cancer lethality rates causing nearly two out of every ten cancer deaths [2]. Non-small-cell lung cancer (NSCLC) presents exceptional cellular heterogeneity and a broad spectrum of resistance mechanisms [3]. An added problem is that the tumor does not relinquish reliable biomarkers that facilitate detection or gauge treatment efficiency. A few parameters can make or break patient outcomes, including detection sensitivity and the ability to obtain a tumor profile. But lung cancer poses problems in the clinic as well. The difficulty of performing tissue biopsy for lung cancer patients and the percentage of tumors that could go below the radar of imaging techniques are worrying. Meanwhile, doctors would still be pressed for a decision – even amid such uncertainty. This is when the fifteen minutes of liquid biopsy started. The need for biomarkers, the difficulty of obtaining tissue biopsy, and the inferior sensitivity of CT scans [4] all primed liquid biopsy to be the next gold standard. Liquid biopsy has the potential to coin the term ‘precision medicine.’ It has the power to transform the clinical workflow by circumventing the hurdles of traditional protocols, all the while improving patient outcomes, alleviating the stress and uncertainty of decision-making, and improving the quality of patient care. The past decade witnessed technical advancements in PCR and sequencing methods and trial output. This enhanced the reliability of liquid biopsy as the preferred method of analysis for some clinical settings and a complement to tissue analysis in others. But the march to the clinic is not over, and we are yet to see the best of liquid biopsy. This chapter depicts the recent advancements in liquid biopsy for NSCLC and highlights opportunities to push the boundaries of the field.

Liquid biopsy is the analysis of body fluids for information about the pathological state (Figure 1). It is used in cancer as an alternative to tissue (solid) sampling to diagnose or follow

disease progression using tumor components present in body fluids – mainly blood. Liquid biopsy relies on the isolation and enrichment of tumor components from the rest of the blood, like isolating ctDNA from the pool of cell-free DNA (cfDNA). Despite tissue sampling being the gold standard for tumor profiling, and CT scans being the standard for detection, liquid biopsy has clear advantages. Compared to tissue sampling, liquid biopsy is non-to-minimally invasive, repeatable, requires a small sample volume, and provides information from all locations as opposed to a single locus provided by tissue biopsy. Compared to CT scans, it is radiation-free and more sensitive [4]. A growing list of tumor components can be isolated from patient blood, including circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), platelets, vesicles, and RNA. Different isolation, enrichment, and analysis techniques are developed, and different outputs are drawn from each.

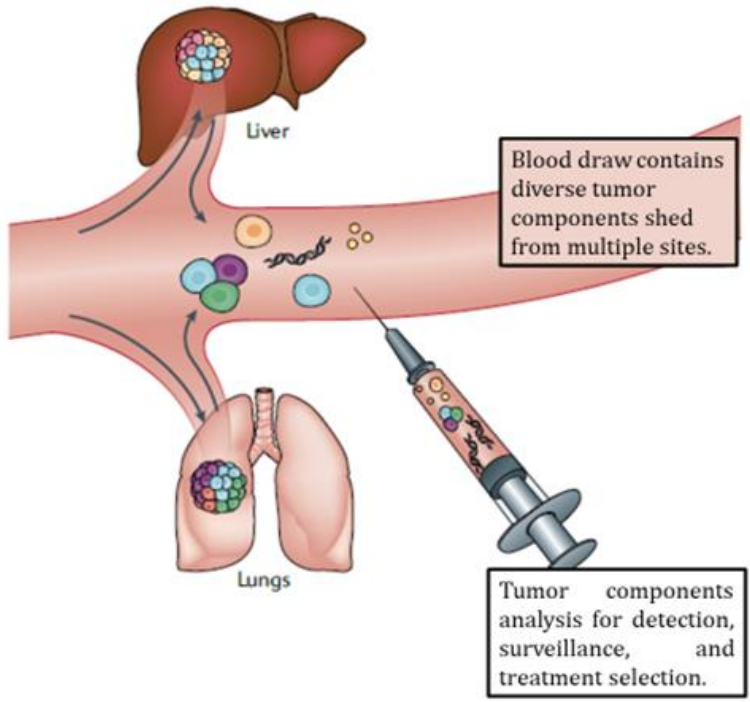

Figure 1: Liquid biopsy illustration, adapted from Ignatiadis and colleagues [1].

Circulating Tumor DNA (ctDNA)

CtDNA analysis is the flagship product of liquid biopsy. Being the most established component of the tumor, sequencing and PCR-based assays are witnessing improvement and a high number of ongoing trials to demonstrate the competence of this parameter. Tumor DNA represents a fraction of the pool of cell-free DNA (cfDNA) in plasma, often referred to as mutant-allele fraction (MAF). This fraction strikingly ranges between 0.01 and 90% [5]. Although this proportion is parallel to the tumor load, patients with the same cancer type can show different MAF [6]. The half-life of ctDNA is just shy of two hours, making it a real-time snapshot of the tumor profile. CtDNA is analyzed using one of two methodologies: polymerase chain reaction (PCR) or next generation sequencing (NGS) – each having its merits. PCR-based technologies (ddPCR, BEAMing) partition the sample and allow high sensitivity of detection that can reach 0.01% MAF [7,8]. However, PCR can only detect known mutations and is not suitable for high-throughput analyses nor for detecting new mutations. Contrarily, NGS sacrifices sensitivity for a more comprehensive tumor profile. NGS allows the discovery of new mutations in the blood of patients and high throughput screening of samples. Recent advancements in NGS technology allowed it to compete in sensitivity with PCR [24]. However, price is still an issue. NGS runs cost anywhere in the four-digit ballpark. Both approaches have clinical employment, and the choice depends on the clinical application. The best-case scenario would be having a highly sensitive, cost-effective, and fast NGS platform that would be one fit for all.

Circulating Tumor Cells CTCs

CTCs hold a versatile edge over ctDNA. With tumor cells at hand, both genetic and biological assessments are possible. The ability to capture CTCs opened more possibilities, like CTC-derived xenografts, by expanding CTCs from patients to immune-compromised mice [9]. Immunohistochemical staining and FISH can be done on CTCs [10]. NSCLC is also known for the ALK rearrangements that are difficult to detect via ctDNA, making CTCs staining an efficient alternative [10]. Monitoring

patients' CTCs is a booming field with plenty of room for imagination. This is evident from the capital pouring in and the innovation in detection, capture, enrichment, and analyses performed on tumor cells. Peculiarities of CTCs can be combined with ctDNA profiling – potentially giving birth to a comprehensive approach for patient monitoring, i.e., eradicating the need for validation using tissue biopsy. Tumor DNA and cell analysis holds promise for the future but is facing technical hurdles on its way to the clinic. Meanwhile, new players are emerging with the potential to complement the shortcomings of DNA and circulating cells.

Tumor-Educated Platelets TEPs

Platelets serve as a warehouse of high-quality tumor RNA molecules. They give direct insight into the exome of the tumor. Platelets are the second-most abundant cell type in blood. Robust isolation and purification protocols are already established. The tumor 'educates' blood platelets by an influx of information via vesicles that contain RNA [11]. Platelets are also proven to collude with CTCs: information exchange, protecting CTCs from the shearing forces in the blood circulation, and immune protection by transferring MHC I molecules to the surface of CTCs allowing their escape from NK cell recognition [11]. The relationship with tumors positions platelets as subjects for clinical investigation and further investment.

Extracellular vesicles (Exosomes)

Another way tumors thrive – and give up information – is by exosomal secretion. By carrying a plethora of tumor cargo (DNA, RNA, and proteins), vesicles provide genomic and metabolomic insight into tumor biology. Exosome secretion is exaggerated in tumors [12]. They are involved in angiogenesis, EMT, invasion, metastasis, immune escape, drug resistance, and other aspects of resistance and survival of tumors and are hot prospects in liquid biopsy [12,13]. Exosomes shelter their components and allow for supplementary ctDNA and RNA/miRNA analyses. Accumulating evidence in the literature

backs exosomes for being diagnostic, prognostic, and predictive biomarkers and another potential versatile tool in the clinic [34].

RNA Tumor Components

Amid the genetic mesh, what makes the difference inside tumors is what is being expressed – or what is not. RNA adds this dimension to the clinical tumor analysis. Aside from mRNA, non-coding RNA molecules are slowly taking the limelight. New players include miRNA, circRNA, and lncRNA. MicroRNA (miRNA) interferes in gene expression by binding mRNA and preventing it from being translated into protein. Tumors avail miRNAs that target tumor-suppressor genes [14]. Detecting miRNA fragments in plasma depicts epigenetic regulation inside the tumor cell. Circular RNA (circRNA) audits miRNA activity by acting as a sponge against miRNA. Pro-tumor circRNAs are up-regulated in cancers to dampen miRNAs that target oncogenic proteins [15]. Long non-coding RNAs (lncRNAs) are also regulatory molecules that act on nucleic-acid binding proteins (e.g., histones) [16]. All three non-coding forms of RNA are proven to be biomarker material for lung cancers as well as therapeutic vehicles (circRNA and lncRNA) [17]. The performance of RNA molecules hinted at possible future employment in the early detection of lung cancers [18].

Post-enrichment sample analysis uses a variety of multidisciplinary technologies and platforms. For ctDNA and RNA, analysis requires either polymerase chain reaction or sequencing technologies. CTCs, platelets, and vesicles are versatile and can be utilized for genomic (DNA, RNA) analyses, morphological (shape and size), metabolomic (protein composition), and biological (functionality) ones [19].

Employment of liquid biopsy spans all stages of disease development. It can be used for the detection of the disease, following progression upon treatment (longitudinal follow-up), testing for minimal residual disease (MRD), and screening for resistance mechanisms and alternative drug targets [20]. Numerous tests and kits for ctDNA and CTCs monitoring have been approved by the FDA for certain cancer types. Those

endorsements include: Cobas, Guardant360 CDx, FoundationOne CDx (ctDNA), CancerSEEK (ctDNA and protein biomarkers), CellSearch (CTCs), and more! [21].

NSCLC Cooperativeness

To detect tumor components in blood samples, they must break free from the tumor and enter the circulation. What constitutes 'cooperativeness' is how efficiently the tumor debris makes it to the blood stream. Wide discrepancies in MAF are observed among patients [22]. This phenomenon is governed by biological parameters including the location of the tumor relative to the bloodstream, the rate of cell death, and ctDNA suppression by treatment. A pan-cancer study combining samples from 10,000 patients demonstrated that both variants of lung cancer (NSCLC and SCLC) and pancreatic cancer showed relatively high detectability of ctDNA in patient blood samples [23]. Contrary to what was seen in more introverted tumors like renal, breast, and brain. Another study in a smaller cohort showed a promising 100% detectability of ctDNA across NSCLC patients in stages II-IV. The sensitivity of NSCLC ctDNA detection was shown to be correlated to tumor size. NSCLC also exhibits detectability, although variable, in earlier tumor stages [24]. Concordance between liquid and tissue biopsy is commonly used as a reference [23]. The literature suggests that NGS results between plasma and tumor are reproducible. Therefore, discrepancies predominantly stem from additional information ctDNA uncovers – which gives plasma analysis an edge over tissue samples, as mentioned earlier. Gauging such numbers, especially in earlier stages, is crucial before pushing the diverse areas of employment for liquid biopsy approaches like early detection and minimal residual disease follow-up.

State of the Art

Early Detection

Nothing holds more promise for the future of cancer diagnosis than a robust early detection system. Catching NSCLC in the 'controllable' stages is key to improving patient outcomes. A 2018 study used a multi-analyte test called Cancer SEEK on a

cohort of 1,005 patients of various cancer types. Cancer SEEK attempted detection of resectable cancers using both ctDNA and protein biomarkers. The scientists at Johns Hopkins were able to detect cancers with an overall 99% specificity and a 69-98% sensitivity. Cancer SEEK is an FDA-approved test as of 2019 [25]. But perhaps the most promising results in NSCLC were delivered at Stanford University using Cancer Personalized Profiling by deep Sequencing (CAPP-Seq), where 100% of NSCLC tumors in stages II-IV were detected [24]. Like the Cancer SEEK blood test, combinatorial approaches add a sense of certainty to the test, which manifests in higher sensitivities. CTC analysis had a 100% concordance with tissue biopsy when analyzed for mutations using PCR, cell count, and single-cell-level gene expression for molecular characterization. A combination of genomic and expression level analyses on CTCs holds the potential for circumventing the lack of biomarkers in NSCLC. Positioning platelets and miRNAs as predictive biomarkers in NSCLC is showing encouraging returns. The best result was obtained in a cohort of 283 patients. Platelet-derived RNA-Seq was able to distinguish the 55 healthy individuals from the 228 patients with 96% accuracy [26]. Two other studies on NSCLC cohorts attempted to detect early-stage disease using miRNAs. The studies showed sensitivities and specificities in the 80-90% ballpark, seemingly outperforming ctDNA in the early stages [27,28]. In the two ctDNA studies, NSCLC stage I tumors went undetected in nearly half the cases (50-60% sensitivity). Judging by results from two different approaches, this talks more about the biology of NSCLC tumors in early stages – Stage I NSCLC could be invisible to plasma analysis. This stimulates the search for other tumor components that improve detectability. But even when early detection falls short, longitudinal monitoring constantly reports significantly better prognosis and survival in patients with undetected ctDNA in the early stages compared with those who flash signs of the disease early [29]. Such correlation adds value to liquid biopsy by positioning tumor components as prognostic factors.

Prognosis

Physicians encounter a plethora of drug resistance mechanisms, relapse in nearly 100% of patients, and an overwhelming number of options and combinations of therapeutics [30]. Predictive and prognostic values are capable of alleviating the stress of decision-making. Collecting data to establish the best therapeutic options has been one area of employment for liquid biopsy. This is achieved by demonstrating the outcomes of therapeutic choices and establishing a consensus for each case. All ctDNA assessments report better prognosis and higher survival for patients with undetected ctDNA. This can extend to establishing the predictive value of mutations. A study attempted to map the effect of somatic copy number variation (SCNV) on the therapeutic outcome using ddPCR and shallow NGS. It was proven that SCNV in resistance-related genes had significantly lower progression-free survival, overall survival, and response rate to Osimertinib [31]. Additionally, the EURTAC intended to compare the efficiency of erlotinib to chemotherapy as a first-line treatment in NSCLC. By analyzing the cfDNA of participants using PCR, the trial pointed out that the presence of the L858R mutation marked a reduction of overall survival by half (13.7 vs. 27.7 months) [32]. In a cohort of NSCLC patients stages III to IV, the presence of fewer than 5 CTCs per blood sample (7.5 ml) correlated to a 2-3-fold increase in progression-free and overall survival. CTCs were also the strongest predictor of overall survival in multivariate analysis [33]. Besides ctDNA and CTCs, smaller tumor components carry a predictive value of their own. Exosome-derived miRNA has been proven to induce resistance to cisplatin (DDP) in A549 cell lines (a lung adenocarcinoma cell line). A different miRNA molecule was shown to desensitize cells to the same chemotherapy [34]. Choosing chemotherapy is a tough call to make. This showcases an opportunity to test for either plasma exosomes or miRNA as prognostic factors prior to chemotherapy treatment. Extrapolation of such *in vitro* studies on patient cohorts could prove valuable for the prognosis of this option.

Tracking Changes in Tumor Profile

Longitudinal follow-up is one of the most widely – and rapidly – adopted uses of liquid biopsy. Endorsement of plasma analysis is accelerating, especially in lung cancer. A liquid biopsy allows for baseline measurement of the tumor mutational status. This occurs during diagnosis and before any treatment is administered. One approved use of NGS is screening for EGFR mutations which select patients for first-line TKI treatment. Initial screening allows for the selection of drugs according to the tumor profile provided by NGS. However, this does not guarantee a relapse-free journey. As mentioned earlier, NSCLC tumors switch between resistance mechanisms to survive the pressure of treatment, and relapse is almost inevitable in NSCLC – rendering the first line of treatment inefficient. A successful selection of the second-line treatment warrants tracking ctDNA mutations and anticipating resistance mechanisms early by a repetitive follow-up of the tumor profile. But taking repetitive tissue biopsies to analyze how the tumor evolves is not feasible for lung cancer, and CT scans provide no genetic and molecular information. Meanwhile, the repeatability of liquid biopsies allows for constant patient surveillance. The residual disease is detectable in ctDNA months before light patches are visible on the X-ray [4,35,36]. Liquid biopsy has demonstrated its superiority to CT imaging in terms of sensitivity. Plasma analysis is now the preferred choice for clinicians for longitudinal follow-up of patients. Longitudinal follow-up not only anticipates relapse but also depicts the tumor profile and which mutations will amplify after the first line of treatment. Another example of how liquid biopsy can hedge against guesswork and aid decision-making is in the case of ALK rearrangements. Similar to EGFR, ALK is another tyrosine kinase driver of NSCLC. But unlike EGFR, ALK can be activated in two ways: an activating mutation or a rearrangement in the receptor. The problem with ALK rearrangements is that there are no solid criteria to select the proper TKI treatment for ALK-positive patients. Using NGS can identify and quantify ALK rearrangements and mutations and narrow down the options for a better selection of therapeutics

[37]. Approval for using NGS for ALK-positive NSCLC has been granted [38].

Platelets are another prominent element in NSCLC research. Monitoring plasma mutations using RT-qPCR on platelet-derived RNA isolated from advanced NSCLC patients surpassed the gold-standard FISH technique in terms of sensitivity, specificity, and accuracy in detecting ALK rearrangements [39].

Beyond mutations, ctDNA analysis in NSCLC can also assess gene changes such as loss of heterozygosity, microsatellite instability, and gene methylation [40-42]. All pivotal intra-tumor events inform clinicians about cancer behavior and allow for other therapy options. But perhaps the most appealing use of NGS is uncovering new druggable targets. This benefit was demonstrated in 53 patients with difficulty in tissue sampling who were assessed using Guradant360 NGS genotyping. Actionable mutations appeared in 12 patients who then received corresponding therapies and had significant clinical benefits [43]. As time passes, repetitive monitoring of patients by liquid biopsy adds value to precision oncology by default. One hidden benefit that liquid biopsy trials are accumulating is uncovering molecular patterns of resistance. Depending on the patient's mutational status at baseline, the physician might prescribe Osimertinib as a first-line treatment or leave it to a possible second line (in case resistance emerges). One striking phenomenon observed in NSCLC is that frequencies of resistance mechanisms are different when Osimertinib is chosen for first-line therapy instead of a second-line – despite being the same drug [44]. Evaluating the molecular profile allows for observing a pattern in resistance mechanisms resulting from each choice. One example is the AURA3 trial. Researchers utilized the Guardant360 NGS platform to track mutational profile evolution as a function of Osimertinib treatment. 15% of the cohort in the trial acquired EGFR mutations (mainly C797S), while 19% showed MET receptor amplification [44]. Another study used ddPCR to find that the ratio of the amount of the T790M mutation to the EGFR activating mutation correlated with the response to Osimertinib [45].

Without a doubt, liquid biopsy clears the fog and narrows down imprecision on different fronts. The field promises to cover tumor biology blind spots, enhance patient outcomes, and improve the quality of life for cancer patients. But the race to the clinic is far from over. Plenty of building blocks still need to be laid – and plenty of opportunity still exists.

The Next Opportunity

Both academia and the industry report an influx of innovation in the field, meaning liquid biopsy is shaping up by the day. However, the uses mentioned in Section IV are only seen in research and trials and not yet in the clinic. New technology needs more than a proof of concept to make it to the clinical routine. Despite being approved for several uses, especially in the niche of longitudinal follow-up, liquid biopsy clinical use is currently restricted to only a few specific cases. The IASLC surveyed 2537 health professionals from Asia, Europe, the US and Canada, Latin America, and the rest of the world [46]. The survey showed that cost is the main barrier to the adoption of ctDNA analysis (Figure 2). Money is one of many concerns of thought leaders in the field as liquid biopsy's journey to the clinic warrants multidisciplinary efforts – and a wealth of opportunity. Here are some aspects of liquid biopsy that will be groomed in the coming years.

Technology Transfer

For a new paradigm to leap to the clinic, it will be molded and polished into a more practical form that fits the day-to-day clinical work. A process termed technology transfer. Technology transfer guarantees (1) a user-friendly interface that shortcuts the complexities of the academic bench work, (2) reproducibility of the results observed in the academic literature in the absence of academic expertise, (3) agile modes of operation with minimal training required, and (4) standard pre-analytical and analytical procedures. The leap to the clinic allows all user interface and commercialization experts in the life sciences industries to help push this forward. However, a prerequisite for technology

transfer is consensus about the protocols and workflow followed in the field, which is another box to check.

Standardization of Procedures

Discrepancies between platforms are a cause for concern. Two commercially available NGS platforms could yield different results for the same population of patients [47]. This could be attributed to the sample preparation process that alters the sample. For liquid biopsy to be reliable, it has to be reproducible. Hence the importance of agreement on the optimal conditions and best practices in all phases of sample and data processing. Modern healthcare still suffers from a high percentage of pre-analytical errors to the total laboratory testing errors (62-68%) [48]. Blood samples are susceptible to bad storage, DNA degradation, and blood cells lysis diluting the pool ctDNA with more unwanted healthy genetic material. Such hurdles pushed standardizing sample handling and processing procedures to the top of the agenda of thought leaders. BloodPAC is an American initiative specialized in information transfer among stakeholders: academia, industry, regulatory authorities, and the public media. BloodPAC aims at galvanizing liquid biopsy assay adoption for improved patient outcomes. SPIDIA4P and SPIDIA European consortia address handling samples before *in vitro* diagnosis involving 10 European countries. CANCER-ID consortium specializes in standardizing protocols for blood-based biomarkers and involves partners from 13 European countries. The international association for the study of lung cancer (IASLC) is also placing liquid biopsy high on its agenda. In 2021 the IASLC released a consensus statement with a list of the recommended practices for sample treatment prior to cfDNA analysis [49]. The practices include the recommended volume of the sample, favoring plasma over serum, and rapid blood treatment after extraction. Such attempts to standardize the practice are crucial. Performing optimization and quality control experiments would complement those efforts and boost the regulatory process. As analysis techniques increase by the day, quality control needs to catch up by providing the optimal conditions for each workflow and each tumor component.

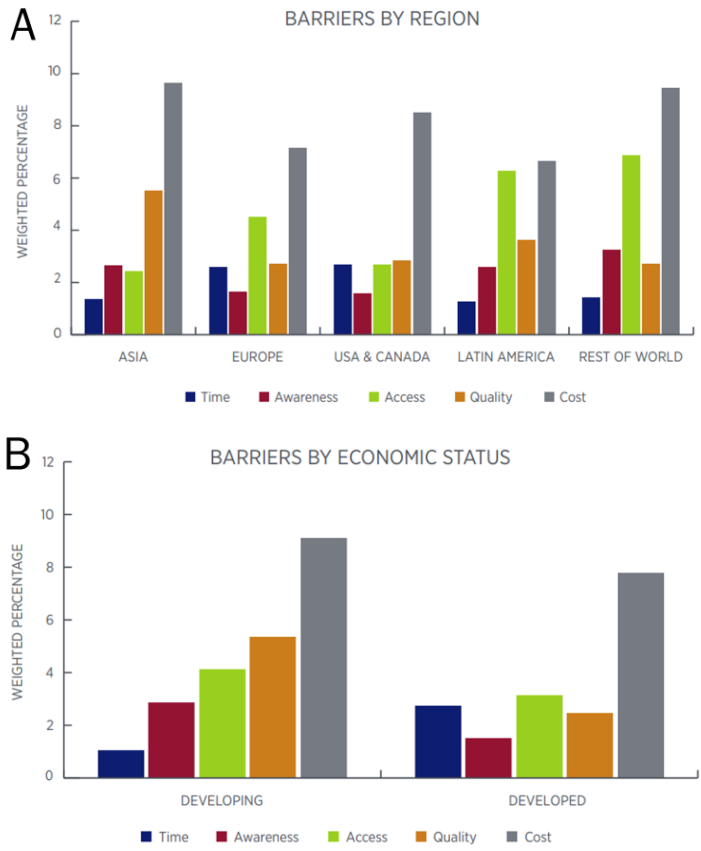

Figure 2: Most frequently reported barriers to molecular testing by (A) region of the world and (B) the economic status of the respondents' countries. Adopted from the IASLC global survey on molecular testing in lung cancer [46]. The survey clearly shows that cost is the major barrier regardless of the economic status of the population.

Clinical Validation

Liquid biopsy detects earlier, is more sensitive, and is more convenient. But is it tangibly helping? Clinical validation is simply demonstrating the added value of liquid biopsy, i.e., will the adoption of liquid biopsy indeed lead to statistical improvements in clinical outcomes and precision healthcare? One example is the additional mutations observed in plasma analysis. That could be justified by the fact that plasma

represents the mutations from different areas in the body and not just a single location like in the case of a tumor, which is more comprehensive and accurate. However, it is unclear whether acting on those mutations is of added value to the patient. The clinical utility of plasma analysis is to be demonstrated. Another example is improved sensitivity. Liquid biopsy can detect changes months before they appear on CT scans [4,35,36]. One of the next outlooks for thought leaders is capitalizing on this superpower for early detection and MRD, even in the absence of previous pathological diagnoses. But this presents another area where clinical utility needs to be showcased: will early detection lead to improved patient survival statistics? Will tumors be more 'controllable' if detected earlier? Lead time bias is one of the arguments against the benefit of earlier detection (Figure 3). Lead time bias refers to when patients appear to have longer survival only because detection was done earlier [50]. Finally, plasma DNA analysis allows us to deduce the density of unique mutations inside the cancer cell's coding genome - a parameter called tumor mutational burden (TMB). TMB measures the tumor's neo antigenic and neoplastic power [51]. A tumor with a higher frequency of mutations tends to grow more aggressively and express more surface proteins. TMB can be obtained from both tissue (tTMB) and blood (bTMB) samples. Because of such a correlation to the antigenic profile of a tumor cell, one could reason about a predictive role of TMB in patient response to immune checkpoint inhibitors (ICI) that assist the immune system in recognizing the tumor. Retrospective analysis of bTMB has shown that bTMB indeed predicted the outcome of patients treated with ICIs and combination treatments [52]. The technical difficulty in extracting tTMB could be circumvented shortly by the predictive power of bTMB, and exploiting this biological parameter definitely will have its place in the clinic with the surge in ICIs. bTMB "harmonization" experiments to assess reproducibility between different methodologies are expected soon [51-54]. Clinical utility is demonstrated by clinical trials using liquid biopsy techniques that go hand in hand with discovering new therapeutic molecules and technological improvement in analytical power.

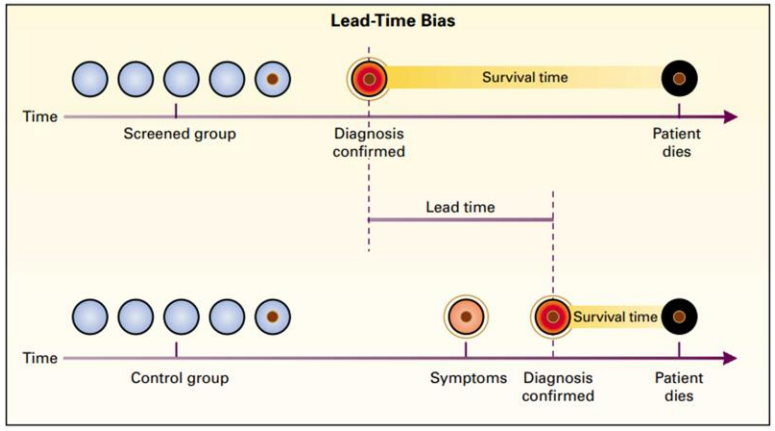

Figure 3: Lead time bias as illustrated by Patz and colleagues [50]. Disease diagnosis is done earlier in the screened group, resulting in an apparent increase in survival time (lead-time bias), although the time of death is the same in both groups.

Technical Hurdles

The most obvious need for improvement can be found in the technicalities of pre- and analytical procedures. CTCs, TEPs, exosomes, and RNAs are extremely interesting components. Each has its advantage over ctDNA analysis. However, complicated purification, capture, enrichment, and analysis protocols call for expertise on the bench and delay their arrival in the clinic. The biggest hurdle in CTCs is enrichment. The number of CTCs per ml of patient blood is a single digit. More sensitive enrichment methods are needed to more efficiently sort out and count CTCs and draw out more conclusions about tumors. TEPs and Exosomes face the same problems. Exosomes are showing the potential for high versatility in terms of analysis. However, their richness in the plasma is the bottleneck. Cost and efficiency are also limiting factors to be addressed. TEPs' promising attributes have their bottlenecks. TEPs adoption into the clinical routine demands validation using studies on larger cohorts of patients to demonstrate the potency of analyzing TEPs. Additionally, a round of simplifying the pre-analytical procedures is necessary for clinical incorporation. MicroRNAs have shown appealing results, except for normalization and

specificity. miRNA lacks a reliable normal to which we refer when analyzing [55]. Additionally, miRNA conservation among different tumors diminishes their biomarker status because they are not specific to tumor types. What can be improved for RNAs is validating the reliability of the pre-analytical procedures [55,56]. Despite being the most developed component, ctDNA analysis presents room for improvement. The spectrum of ctDNA analysis technologies ranges from PCR with high sensitivity but low throughput to NGS with high throughput and high cost. Finding alternatives that would bring costs down would revolutionize and field and spark the adoption of NGS technologies. Another dent in the reliability of ctDNA analysis is a phenomenon called clonal hematopoiesis of indeterminate potential (CHIP). Mutations detected in the pool of cfDNA could originate from this natural event that occurs in blood cells and are not of tumor origin. This spikes the pool of ctDNA and presents indistinguishable false positives [57]. The CHIP problem was mitigated in a large pan-cancer study by separately sequencing WBC [23]. This approach might rule out some biases. However, our best bet is to cross-validate ctDNA results with other parameters – like sequencing CTCs. Finally, it is notable that all ctDNA analysis techniques favor specificity over sensitivity. Therefore, the rate of false negatives trumps false positives [49]. Then when a negative result comes up, this result has to be validated by a tissue biopsy, as the negative predictive value of ctDNA is higher. Another window of opportunity is establishing complementarity between ctDNA, and CTCs analyses independent of tissue biopsy. That would circumvent the CHIP bias (as CTCs do not bear hematopoietic mutations), double-check the negative results of ctDNA, and drop the need for tissue biopsy to validate results.

A.I.

Machine learning is where countless opportunities hide. And because liquid biopsy is a relatively new approach, artificial intelligence did not have the time to fully catch up. One can see plenty of employment opportunities for artificial intelligence. Raw data coming from blood samples is noisy and hard to interpret manually. Machines would lift the weight of

interpreting, storing, and retrieving data. In addition, deducing patterns is complementary to mere data storage. With large volumes of trial results and longitudinal follow-ups, computers are good at drawing out tumor behavior patterns for the different types of therapy. Such patterns include the frequency by which mutations and resistance mechanisms arise in response to each drug. This data will be of high value once established, as it would point out patterns of emergence of mutations or resistance mechanisms. Finally, selecting drugs based on data can be daunting for the physician. Artificial intelligence can boost decision-making by narrowing down therapy options as well as present outcomes of previous trials, giving clinicians a landscape of scenarios of what to expect after every decision. Armed with data and outcomes of each drug option and biopsy results, A.I. would help deliver the right drug to the right patient, at the right time.

A Word to Academia

The contribution of fundamental research will be valuable in layering the rationale and credibility for using liquid biopsy. Many questions regarding the biology of tumors, shedding, and interaction between tumors and tumor components remain unanswered. The biology of TEPs' interaction with the tumor is not well established. This gap makes it natural to argue against the 'representativeness' of platelets to the tumor's actual exome. The interest in platelets calls for a clearer understanding of how the tumor exchanges information with those components and how reliable this mechanism is in TEPs as biomarkers. More clinical trials, proof of utility and mechanistic studies could place TEPs high in the clinical hierarchy. Exosomes and their miRNA cargo showed pivotal roles in tumor survival, communication, and overall biology [58,59]. Mechanistic insight into these roles would solidify such tumor derivatives as biomarker candidates. Investigating the tumor-tumor component relationship will undoubtedly be of added value to our reliance on plasma analysis. Finally, the resilience of NSCLC and the plethora of resistance mechanisms make it hard to ignore. Numerous explanations have surfaced on how the tumor develops resistance and whether it follows Darwinian selection or a more

Lamarckian model (Figure 4). A 2022 review highlighted the role of drug-tolerant persistent (DTP) cells that act as reservoirs of resistance mechanisms that are induced according to the stress in the medium [30]. The presence of such engines of resistance spikes interest in targeting them therapeutically or utilizing them as biomarkers. DTPCs present an opportunity for a leap in our understanding of cancer biology.

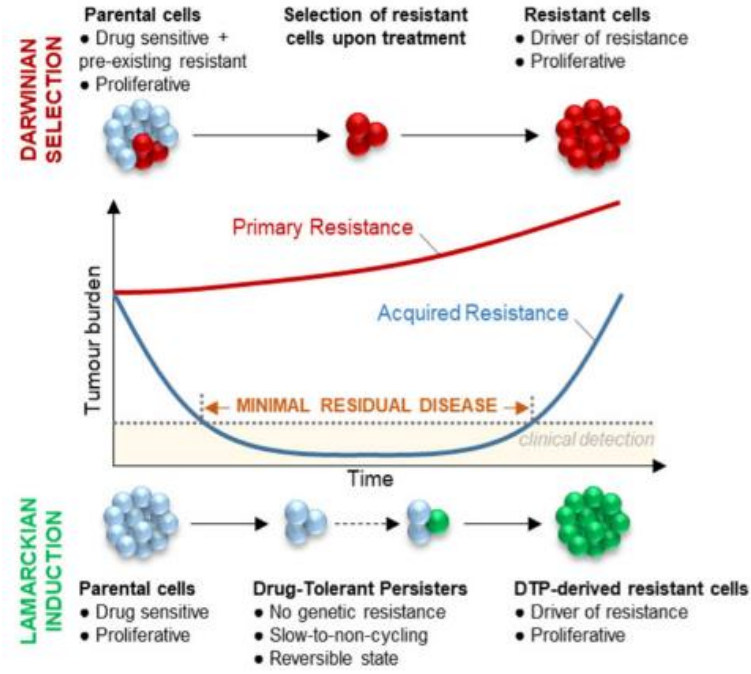

Figure 4: The two different models of clinical response to treatment in NSCLC. Primary resistance that resembles the Darwinian selection (red) exhibits very little initial response as the resistant clone is amplified. Meanwhile, the Lamarckian-like, DTP-derived resistant clones (green) are induced after a strong response to therapy and clearance of the tumor. Adapted from Delahaye et al [30].

Conclusion

What the market needs could turn out to be radically different from the initial purpose of the technology. Historically, before endorsing a new technology on a full scale, it has to go through

an ‘amorphous’ and uncertain phase. This phase lasts until the technology is molded to match the market needs and its most convenient uses are demonstrated. Liquid biopsy gave birth to a diversity of uses. It is not yet the gold standard for diagnosis and monitoring but is certainly shaping out of the uncertain introduction phase. A few years ago, the literature presented data with a dose of constraint. Today, we see a more optimistic presentation of trial results and a broader endorsement of new technologies. We see a clear trend in the scientific literature: liquid biopsy has already started to take shape. According to the director of the Thoracic Medical Oncology and the Early Clinical Trials Departments at the University of Maryland, Professor Christian Rolfo, there are two major obstacles: first is the price of liquid biopsy – especially for NGS assays that falls in the $1,000-$7,000 ballpark per run. The second big obstacle is false negative results for non-shedding patients, despite such assays being awaited eagerly. Until the field improves and achieves higher sensitivity, institutes should strive to respect standards, and boards should keep the use of NGS in check to avoid misinterpretation of NGS results. Efforts on the academic end are anticipated. High trial output, proof of clinical utility, and studies on tumor biology will clear out uncertainties and give the clinic a confidence interval to operate within. Standardizing workflows, sensitivity, and false negatives are still to be handled. One solution is to improve those aspects of the technology. But a more creative approach would be designing a new workflow that paves the way around these obstacles. Emerging new technologies are circumventing the pre-analytical hurdles. Electric field-induced release and measurement – or EFIRM – strikingly detected two major lung tumor mutations from the saliva of NSCLC patients with little to no sample preparation procedure [60]. EFIRM is still far from the market, but it promises to skip the pre-analytical impediment and provide a 100% non-invasive approach. The new ‘fragmentomics’ approach developed by Delfi Diagnostics is an example of novel technologies that made it to the market. By discerning ctDNA from healthy cfDNA using its differential fragmentation pattern, Delfi promises to skip the sequencing bottleneck and reduce the cost of ctDNA analysis compared to NGS by nearly 10-fold [61]. A few boxes need to be checked before we see liquid

biopsy as the gold standard for NSCLC patient care. But despite the rough patches, the clinical value of liquid biopsy is undeniable. Innovation is picking up, and capital is pouring into the startups. What we have at hand shows glimpses of its power. Papers present their 'Discussion' section with a tone of encouragement. We can see a continual influx of new trends and ideas – we can even predict upcoming ways the clinic will avail liquid biopsy. Such tendencies can only tell a few things: liquid biopsy is here to stay, and an accelerated endorsement is expected.

References

1. Ignatiadis M, Sledge GW, Jeffrey SS. Liquid biopsy enters the clinic - implementation issues and future challenges. Nature reviews. Clinical oncology. 2021; 18: 297–312.
2. World Health Organization (WHO). Available online at: who.int/news-room/fact-sheets/detail/cancer
3. Morgillo F, Della Corte CM, Fasano M, Ciardiello F. Mechanisms of resistance to EGFR-targeted drugs: lung cancer. ESMO open. 2016; 1: e000060.
4. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. The New England journal of medicine. 2013; 368: 1199–1209.
5. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, et al. Circulating mutant DNA to assess tumor dynamics. Nature medicine. 2008; 14: 985–990.
6. Shen S, Wei Y, Zhang R, Du M, Duan W, et al. Mutant-allele fraction heterogeneity is associated with non-small cell lung cancer patient survival. Oncology letters. 2018; 15: 795–802.
7. Li H, Jing C, Wu J, Ni J, Sha H, et al. Circulating tumor DNA detection: A potential tool for colorectal cancer management. Oncology letters. 2019; 17: 1409–1416.
8. Huang A, Zhang X, Zhou SL, Cao Y, Huang XW, et al. Detecting Circulating Tumor DNA in Hepatocellular Carcinoma Patients Using Droplet Digital PCR Is Feasible and Reflects Intratumoral Heterogeneity. Journal of Cancer. 2016; 7: 1907–1914.

9. Vasseur A, Kiavue N, Bidard FC, Pierga JY, Cabel L. Clinical utility of circulating tumor cells: an update. Molecular oncology. 2021; 15: 1647–1666.
10. Kulasinghe A, Lim Y, Kapeleris J, Warkiani M, O'Byrne K, et al. The Use of Three-Dimensional DNA Fluorescent In Situ Hybridization (3D DNA FISH) for the Detection of Anaplastic Lymphoma Kinase (ALK) in Non-Small Cell Lung Cancer (NSCLC) Circulating Tumor Cells. Cells. 2020; 9: 1465.
11. Varkey J, Nicolaides T. Tumor-Educated Platelets: A Review of Current and Potential Applications in Solid Tumors. Cureus. 2021; 13: e19189.
12. Whiteside TL. Tumor-Derived Exosomes and Their Role in Cancer Progression. Advances in clinical chemistry. 2016; 74: 103–141.
13. Osaki M, Okada F. Exosomes and Their Role in Cancer Progression. Yonago acta medica. 2019; 62: 182–190.
14. Peng Y, Croce CM. The role of MicroRNAs in human cancer. Signal transduction and targeted therapy. 2016; 1: 15004.
15. Chen L, Shan G. CircRNA in cancer: Fundamental mechanism and clinical potential. Cancer letters. 2021; 505: 49–57.
16. Hu D, Lou X, Meng N, Li Z, Teng Y, et al. Peripheral Blood-Based DNA Methylation of Long Non-Coding RNA H19 and Metastasis-Associated Lung Adenocarcinoma Transcript 1 Promoters are Potential Non-Invasive Biomarkers for Gastric Cancer Detection. Cancer control: journal of the Moffitt Cancer Center. 2021; 28: 10732748211043667.
17. Zhang L, Li C, Su X. Emerging impact of the long noncoding RNA MIR22HG on proliferation and apoptosis in multiple human cancers. Journal of experimental & clinical cancer research: CR. 2020; 39: 271.
18. Li W, Liu JB, Hou LK, Yu F, Zhang J, et al. Liquid biopsy in lung cancer: significance in diagnostics, prediction, and treatment monitoring. Molecular cancer. 2022; 21: 25.
19. Lim M, Park J, Lowe AC, Jeong HO, Lee S, et al. A lab-on-a-disc platform enables serial monitoring of individual CTCs associated with tumor progression during EGFR-targeted

therapy for patients with NSCLC. Theranostics. 2020; 10: 5181–5194.
20. Adashek JJ, Janku F, Kurzrock R. Signed in Blood: Circulating Tumor DNA in Cancer Diagnosis, Treatment and Screening. Cancers. 2021; 13: 3600.
21. Available online at: Food and Drug Adminitration: fda.gov/medical-devices/in-vitro-diagnostics/list-cleared-or-approved-companion-diagnostic-devices-in-vitro-and-imaging-tools.
22. Shen S, Wei Y, Zhang R, Du M, Duan W, et al. Mutant-allele fraction heterogeneity is associated with non-small cell lung cancer patient survival. Oncology letters. 2018; 15: 795–802.
23. Zhang Y, Yao Y, Xu Y, Li L, Gong Y, et al. Pan-cancer circulating tumor DNA detection in over 10,000 Chinese patients. Nature communications. 2021; 12: 11.
24. Newman AM, Bratman SV, To J, Wynne JF, Eclov NC, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nature medicine. 2014; 20: 548–554.
25. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science (New York, N.Y.). 2018; 359: 926–930.
26. Best MG, Sol N, Kooi I, Tannous J, Westerman BA, et al. RNA-Seq of Tumor-Educated Platelets Enables Blood-Based Pan-Cancer, Multiclass, and Molecular Pathway Cancer Diagnostics. Cancer cell. 2015; 28: 666–676.
27. Shen J, Todd NW, Zhang H, Yu L, Lingxiao X, et al. Plasma microRNAs as potential biomarkers for non-small-cell lung cancer. Laboratory investigation; a journal of technical methods and pathology. 2011; 91: 579–587.
28. Sozzi G, Boeri M, Rossi M, Verri C, Suatoni P, et al. Clinical utility of a plasma-based miRNA signature classifier within computed tomography lung cancer screening: a correlative MILD trial study. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2014; 32: 768–773.
29. Seremet T, Jansen Y, Planken S, Njimi H, Delaunoy M, et al. Undetectable circulating tumor DNA (ctDNA) levels

correlate with favorable outcome in metastatic melanoma patients treated with anti-PD1 therapy. Journal of translational medicine. 2019; 17: 303.
30. Delahaye C, Figurearol S, Pradines A, Favre G, Mazieres J, et al. Early Steps of Resistance to Targeted Therapies in Non-Small-Cell Lung Cancer. Cancers. 2022; 14: 2613.
31. Buder A, Heitzer E, Waldispühl-Geigl J, Weber S, Moser T, et al. Somatic Copy-Number Alterations in Plasma Circulating Tumor DNA from Advanced EGFR-Mutated Lung Adenocarcinoma Patients. Biomolecules. 2021; 11: 618.
32. Karachaliou N, Mayo-de las Casas C, Queralt C, de Aguirre I, Melloni B, et al. Association of EGFR L858R Mutation in Circulating Free DNA With Survival in the EURTAC Trial. JAMA oncology. 2015; 1: 149–157.
33. Krebs MG, Sloane R, Priest L, Lancashire L, Hou JM, et al. Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2011; 29: 1556–1563.
34. Wu H, Mu X, Liu L, Wu H, Hu X, et al. Bone marrow mesenchymal stem cells-derived exosomal microRNA-193a reduces cisplatin resistance of non-small cell lung cancer cells via targeting LRRC1. Cell death & disease. 2020; 11: 801.
35. Campos-Carrillo A, Weitzel JN, Sahoo P, Rockne R, Mokhnatkin JV, et al. Circulating tumor DNA as an early cancer detection tool. Pharmacology & therapeutics. 2020; 207: 107458.
36. Diaz LA, Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2014; 32: 579–586.
37. NGS for ALK rearrangements and mutations: Dagogo-Jack I, Shaw AT. Screening for ALK Rearrangements in Lung Cancer: Time for a New Generation of Diagnostics? The oncologist. 2016; 21: 662–663.
38. Available online at: NGS approval granted by FDA: https://www.fda.gov/drugs/resources-information-approved-

drugs/fda-approves-liquid-biopsy-ngs-companion-diagnostic-test-multiple-cancers-and-biomarkers.
39. Park CK, Kim JE, Kim MS, Kho BG, Park HY, et al. Feasibility of liquid biopsy using plasma and platelets for detection of anaplastic lymphoma kinase rearrangements in non-small cell lung cancer. Journal of cancer research and clinical oncology. 2019; 145: 2071–2082.
40. Palanca-Ballester C, Rodriguez-Casanova A, Torres S, Calabuig-Fariñas S, Exposito F, et al. Cancer Epigenetic Biomarkers in Liquid Biopsy for High Incidence Malignancies. Cancers. 2021; 13: 3016.
41. Boldrin E, Nardo G, Zulato E, Bonanno L, Polo V, et al. Detection of Loss of Heterozygosity in cfDNA of Advanced EGFR- or KRAS-Mutated Non-Small-Cell Lung Cancer Patients. International journal of molecular sciences. 2019; 21: 66.
42. Tieng F, Abu N, Lee LH, Ab Mutalib NS. Microsatellite Instability in Colorectal Cancer Liquid Biopsy-Current Updates on Its Potential in Non-Invasive Detection, Prognosis and as a Predictive Marker. Diagnostics (Basel, Switzerland). 2021; 11: 544.
43. Zugazagoitia J, Ramos I, Trigo JM, Palka M, Gómez-Rueda A, et al. Clinical utility of plasma-based digital next-generation sequencing in patients with advance-stage lung adenocarcinomas with insufficient tumor samples for tissue genotyping. Annals of oncology : official journal of the European Society for Medical Oncology. 2019; 30: 290–296.
44. Papadimitrakopoulou VA, Han JY, Ahn MJ, Ramalingam SS, Delmonte A, et al. Epidermal growth factor receptor mutation analysis in tissue and plasma from the AURA3 trial: Osimertinib versus platinum-pemetrexed for T790M mutation-positive advanced non-small cell lung cancer. Cancer. 2020; 126: 373–380.
45. Ariyasu R, Nishikawa S, Uchibori K, Oh-Hara T, Yoshizawa T, et al. High ratio of T790M to EGFR activating mutations correlate with the osimertinib response in non-small-cell lung cancer. Lung cancer (Amsterdam, Netherlands). 2018; 117: 1–6.
46. Smeltzer MP, Wynes MW, Lantuejoul S, Soo R, Ramalingam SS, et al. The International Association for the

Study of Lung Cancer Global Survey on Molecular Testing in Lung Cancer. Journal of thoracic oncology: official publication of the International Association for the Study of Lung Cancer. 2020; 15: 1434–1448.
47. Kuderer NM, Burton KA, Blau S, Rose AL, Parker S, et al. Comparison of 2 Commercially Available Next-Generation Sequencing Platforms in Oncology. JAMA oncology. 2017; 3: 996–998.
48. Mrazek C, Lippi G, Keppel MH, Felder TK, Oberkofler H, et al. Errors within the total laboratory testing process, from test selection to medical decision-making - A review of causes, consequences, surveillance and solutions. Biochemia medica. 2020; 30: 020502.
49. Rolfo C, Mack P, Scagliotti GV, Aggarwal C, Arcila ME, et al. Liquid Biopsy for Advanced NSCLC: A Consensus Statement From the International Association for the Study of Lung Cancer. Journal of thoracic oncology: official publication of the International Association for the Study of Lung Cancer. 2021; 16: 1647–1662.
50. Patz EF, Jr, Goodman PC, Bepler G. Screening for lung cancer. The New England journal of medicine. 2000; 343: 1627–1633.
51. Samstein RM, Lee CH, Shoushtari AN, Hellmann MD, Shen R, et al. Tumor mutational load predicts survival after immunotherapy across multiple cancer types. Nature genetics. 2019; 51: 202–206.
52. Bonanno L, Dal Maso A, Pavan A, Zulato E, Calvetti L, et al. Liquid biopsy and non-small cell lung cancer: are we looking at the tip of the iceberg?. British journal of cancer. 2022; 127: 383–393.
53. Carbone DP, Reck M, Paz-Ares L, Creelan B, Horn L, et al. First-Line Nivolumab in Stage IV or Recurrent Non-Small-Cell Lung Cancer. The New England journal of medicine. 2016; 376: 2415–2426.
54. Brueckl WM, Ficker JH, Zeitler G. Clinically relevant prognostic and predictive markers for immune-checkpoint-inhibitor (ICI) therapy in non-small cell lung cancer (NSCLC). BMC cancer. 2020; 20: 1185.
55. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. Accurate normalization of real-time quantitative

RT-PCR data by geometric averaging of multiple internal control genes. 2002; 3.
56. Farazi TA, Hoell JI, Morozov P, Tuschl T. MicroRNAs in human cancer. Advances in experimental medicine and biology. 2013; 774: 1–20.
57. Yaung SJ, Fuhlbrück F, Peterson M, Zou W, Palma JF, et al. Clonal Hematopoiesis in Late-Stage Non-Small-Cell Lung Cancer and Its Impact on Targeted Panel Next-Generation Sequencing. JCO precision oncology. 2002; 4: 1271–1279.
58. Padda J, Khalid K, Khedr A, Patel V, Al-Ewaidat OA, et al. Exosome-Derived microRNA: Efficacy in Cancer. Cureus. 2021; 13: e17441.
59. Li C, Zhou T, Chen J, Li R, Chen H, et al. The role of Exosomal miRNAs in cancer. Journal of translational medicine. 2002; 20: 6.
60. Tu M, Wei F, Yang J, Wong D. Detection of exosomal biomarker by electric field-induced release and measurement (EFIRM). Journal of visualized experiments: JoVE. 2015; 95: 52439.
61. Cristiano S, Leal A, Phallen J, Fiksel J, Adleff V, et al. Genome-wide cell-free DNA fragmentation in patients with cancer. Nature. 2019; 570: 385–389.