AVVISO PUBBLICO, PER TITOLI E COLLOQUIO, PER L'ASSUNZIONE A TEMPO DETERMINATO DI N. 1 POSTO DI COLLABORATORE PROFESSIONALE DI RICERCA SANITARIA – CATEGORIA D – CON LAUREA TRIENNALE IN BIOLOGIA O IN BIOTECNOLOGIE, NELL'AMBITO DEL PROGETTO CODICE PNC-E3-2022-23683266 PNC-HLS-DA, CUP H83C22000910001, DAL TITOLO "ITALIAN NETWORK OF EXCELLENCE FOR ADVANCED DIAGNOSIS (INNOVA), FINANZIATO DAL MINISTERO DELLA SALUTE NELL'AMBITO DEL PIANO NAZIONALE COMPLEMENTARE ECOSISTEMA INNOVATIVO DELLA SALUTE, CODICE UNIVOCO INVESTIMENTO: PNC-E.3 DEL PIANO COMPLEMENTARE AL PNRR , DA ASSEGNARE AI LABORATORI DI RICERCA SPERIMENTALE E TRASLAZIONALE DELL'ISTITUTO REGINA ELENA (IRE), P.I. PROF. GENNARO CILIBERTO.

1) Descrivere le diverse tecnologie di NGS (next generation sequencing), che prevedono differenti approcci di preparazione delle librerie e differenti metodiche di sequenziamento.
2) Biopsia liquida in oncologia descrivere le principali applicazioni cliniche, il materiale biologico che può essere analizzato e i principali analiti
3) Biopsia liquida in oncologia descrivere le principali applicazioni cliniche, vantaggi e limiti del test
4) Definizione di DNA libero circolante e di DNA tumorale circolante e descrizione delle principali metodiche che possono essere utilizzate per individuare mutazioni tumore specifiche
5) Descrivere i passaggi della fase preanalitica relativi all'isolamento del DNA tumorale circolante da un fluido biologico
6) Descrizione delle principali metodiche che vengo utilizzate in biopsia liquida in oncologia in particolare: real time PCR, digital PCR, NGS (vantaggi e svantaggi)
7) Ruolo dei microRNA circolanti in oncologia
8) Tecnologia di next generation sequencing (NGS): descrivere brevemente di cosa si tratta e quali sono i principali passaggi nell'esecuzione della metodica con un approccio di "ibridazione e cattura"
9) Tecnologia di next generation sequencing (NGS): descrivere brevemente di cosa si tratta e quali sono i principali passaggi nell'esecuzione della metodica con un approccio "amplicon based"
10) Descrivere le differenze tra whole exome sequencing, targeted next generation sequencing e RNA-sequencing

AVVISO PUBBLICO, PER TITOLI E COLLOQUIO, PER L'ASSUNZIONE A TEMPO DETERMINATO DI N. 1 POSTO DI COLLABORATORE PROFESSIONALE DI RICERCA SANITARIA – CATEGORIA D – CON LAUREA TRIENNALE IN BIOLOGIA O IN BIOTECNOLOGIE, NELL'AMBITO DEL PROGETTO CODICE PNC-E3-2022-23683266 PNC-HLS-DA, CUP H83C22000910001, DAL TITOLO "ITALIAN NETWORK OF EXCELLENCE FOR ADVANCED DIAGNOSIS (INNOVA), FINANZIATO DAL MINISTERO DELLA SALUTE NELL'AMBITO DEL PIANO NAZIONALE COMPLEMENTARE ECOSISTEMA INNOVATIVO DELLA SALUTE, CODICE UNIVOCO INVESTIMENTO: PNC-E.3 DEL PIANO COMPLEMENTARE AL PNRR , DA ASSEGNARE AI LABORATORI DI RICERCA SPERIMENTALE E TRASLAZIONALE DELL'ISTITUTO REGINA ELENA (IRE), P.I. PROF. GENNARO CILIBERTO

Prova colloquio 4 dicembre 2023

Domande concorso:

1) Cos'è il provider?

2) Cosa significa Login?

3) Cos'è word?

4) Cos'è un database?

5) Che cos'è il back-up?

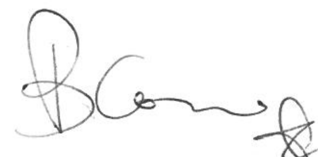
6) A cosa serve Power Point?

7) Definizione di motore di ricerca:

8) A cosa serve Access?

9) Definizione di Browser

10) Cosa è la PEC



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The evolving role of liquid biopsy in lung cancer

Umberto Malapelle [a,*], Pasquale Pisapia [a], Francesco Pepe [a], Gianluca Russo [a], Mauro Buono [a], Alessandro Russo [b], Jorge Gomez [c], Ola Khorshid [d], Philip C. Mack [c], Christian Rolfo [c], Giancarlo Troncone [a]

[a] Department of Public Health, University of Naples Federico II, Naples, Italy
[b] Medical Oncology Unit, A.O. Papardo, Messina, Italy
[c] Center for Thoracic Oncology, Tisch Cancer Institute, Mount Sinai Medical System & Icahn School of Medicine, New York, NY, USA
[d] National Cancer Institute, Cairo University, Cairo, Egypt

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ABSTRACT

Liquid biopsy has revolutionized the management of cancer patients. In particular, liquid biopsy-based testing has proven to be highly beneficial for identifying actionable cancer markers, especially when solid tissue biopsies are insufficient or unattainable. Beyond the predictive role, liquid biopsy may be a useful tool for comprehensive tumor genotyping, identification of emergent resistance mechanisms, monitoring of minimal residual disease, early detection, and cancer interception. The application of next generation sequencing to liquid biopsy has led to the "quantum leap" of predictive molecular pathology. Here, we review the evolving role of liquid biopsy in lung cancer.

1. Introduction

Liquid biopsy refers to the analysis of different biofluid-derived analytes [1,2]. In the liquid biopsy landscape, blood constitutes the principal biofluid and, in cancer patients, plasma-derived circulating tumour DNA (ctDNA) is the most commonly analyzed derivative [3–5]. First identified in 1977, ctDNA consists of a small fraction of cell-free DNA (ctDNA) that is released from tumor cells into biofluids through either passive (apoptosis or necrosis) or active mechanisms [1]. In the last decades, with the advent of personalized medicine and the increasingly growing number of predictive biomarkers, liquid biopsy has acquired a pivotal role in treatment decision-making [6–8].

1 — In particular, liquid biopsy-based testing has proven to be highly beneficial for identifying actionable cancer markers, especially when solid tissue biopsies are insufficient or unattainable [9]. Advanced non-small cell lung cancer (NSCLC), or more specifically, lung adenocarcinoma, is a case in point: Food and Drug Administration (FDA) approval of liquid biopsy for the detection of epidermal growth factor receptor (*EGFR*) mutations in 2016, ctDNA analyses have evolved from being a research oriented alternative to tumor tissue for *EGFR* mutational testing to being an optimal source for a wide variety of emerging clinical applications [10]. Among these are, comprehensive tumor genotyping, identification of emergent resistance mechanisms, monitoring of minimal residual disease (MRD), early detection, and cancer interception [11–13]. 2 — This revolution has been hastened by the terrific advances in molecular technologies, such as next generation sequencing (NGS). Indeed, unlike conventional molecular biology assays (Sanger Sequencing and Real-Time PCR (RT-qPCR), multiplexed sequencing platforms have a much higher sensitivity and specificity for the characterization of ctDNA, with its high bandwidth for multiple markers leading to tissue preservation for additional assays [14].

The application of NGS to liquid biopsy has thus led to the "quantum leap" of predictive molecular pathology, hence opening the door to the "dynamic era" of precision oncology. In addition, as we showed in our previous work, the "marriage" of biological and mathematical sciences has generated new ways of understanding and implementing liquid biopsy in clinical trials and in routine settings [15]. Taking into account all the advancements herein discussed, we will review the evolving role of liquid biopsy in lung cancer.

2. Current applications of liquid biopsy in NSCLC

The first FDA approval of a liquid biopsy ctDNA-based test occurred rather recently in 2016 [10,16]. The approval was granted soon after the ENSURE study reported a high concordance rate between the plasma and tissue-based tests, particularly 76.7 % in tissue positive patients and

* Corresponding author at: Department of Public Health, University of Naples Federico II, Naples, Italy.
E-mail address: umbertomalapelle@gmail.com (U. Malapelle).

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98.2 % in tissue negative patients [17]. Marketed as cobas® *EGFR* mutation test (cobas® EGFR mutation test v2, Roche Molecular Systems, Inc.) this plasma test was designed as a companion diagnostic test to detect *EGFR* exon 19 deletions or exon 21p.L858R mutation in NSCLC patients potentially eligible for erlotinib treatment [10,16]. Since then, numerous prospective and retrospective studies have followed, consistently confirming the high degree of sensitivity and specificity of these tests for druggable biomarkers. Today, liquid biopsy is considered an easily available complement to tissue biomarkers testing to select patients not only for first-line targeted treatments by identifying targetable genomic alterations (e.g., *EGFR* mutations) but also for second-line targeted therapy by identifying specific emergent resistance mutations [18] ctDNA analysis in these two settings has been validated in many clinical trials, with newer, more innovating concepts still underway. One major goal of these trials has been to confirm its ability to serve as a companion diagnostic test to detect *EGFR* exon 19 deletions or exon 21p. L858R mutation alongside RT-qPCR approaches.

Indeed, the approval of liquid biopsy-based tests has been a crucial game changer for advanced NSCLC patients. Prior to its advent, many metastatic patients were potentially undertreated either because their tumor specimens for conventional *EGFR* testing were insufficient or unattainable, or because they presented with comorbidities [19]. For many patients, this scenario completely changed after the FDA approval of a liquid biopsy alternative.

Optimal isolation of ctDNA was a subject of intensive study in the early days of the field. For instance, Kimura *et al.* evaluated serum as a non-invasive integrated source of nucleic acids for NSCLC clinical management [20]. In brief, they assessed *EGFR* mutations in 27 NSCLC patients by applying a refractory amplification system on serum specimens before first line-TKI administration. Overall, *EGFR* sensitive mutations were detected in 13/27 patients (48.1 %). Moreover, the clinical outcomes showed a statically significant association with the predictive value of serum-based *EGFR* molecular alterations. At that time, *EGFR* testing was not required for gefitinib treatment, thus it was remarkable that the median PFS was higher in NSCLC patients with *EGFR* mutations detected in serum samples than in *EGFR* mutation negative patients (200 versus 46 days; P = 0.005, log-rank test). In addition, to further confirm their data, the authors carried out a retrospective analysis of the *EGFR* status of n = 11 corresponding tissue specimens. Gratifyingly, the molecular results indicated a strong concordance between tissue and serum specimens in 8/11 (72.7 %) patients [20]. Kimura *et al.* also performed a second clinical trial involving a cohort of Japanese NSCLC patients (n = 42) to determine the predictive value of serum specimens in the clinical stratification of tumor patients under gefitinib administration. For this purpose, they compared the sensitivity and specificity of patient tumor and serum specimens for *EGFR* [21]. Even in this case, they observed a high concordance rate (39/42; 92.9 %) for *EGFR* molecular profiling between tissue and serum specimens (P < 0.001). Remarkably, the clinical outcomes also confirmed the robustness of the molecular data on serum specimens. Like in their previous study, the PFS of patients with *EGFR* sensitive mutations was higher that of *EGFR* wild-type NSCLC patients (194 vs 55 days, P = 0.016, in tumor samples; 174 vs 58 days, P = 0.044, in serum samples) [21]. In contrast to serum, Mack et al investigated plasma as a source of ctDNA, demonstrating that patients treated on an erlotinib-docetaxel regimen has significantly improved response rates and PFS when *EGFR* mutations were detected in plasma [22]. Subsequently, double clarified plasma has become the standard for blood-based ctDNA isolation [4,23].

The use and reliability of liquid biopsy as an integrative source for *EGFR* molecular testing in clinical settings has also been investigated in other international clinical trials involving much larger cohorts. One of these is the IGNITE trial [24]. The trial enrolled n = 2,581 NSCLC patients. Molecular analysis, as well as data interpretation, was successfully carried out in all patients by using different techniques from each participating center. In brief, molecular analyses on plasma specimens showed a technical sensitivity < 50 % in comparison with gold standard tissue analyses. In addition, a cohort of 1,162 treatment-naïve NSCLC patients was evaluated by comparing plasma and matched tissue specimens. Even in this case, molecular data highlighted a concordance rate of 89.0 % in matched tissue and plasma specimens with a sensitivity and specificity of 46.0 % and 97.0 %, respectively. Interestingly, the overall clinical response of a subset of n = 25 patients with *EGFR* wild-type NSCLC positive tissue specimens was consistent with that of patients with *EGFR*-mutation positive plasma specimens [25].

All these data strongly underline the predictive value of liquid biopsy as an integrated source in the molecular assessment of *EGFR* in the clinical management of NSCLC patients. However, despite these promising results, a lack of harmonization between the different technical approaches conventionally used for molecular analysis still remains an open challenge [25].

This problem, however, has been largely solved by the recent application of NGS to plasma specimens. Indeed, NGS, while revolutionizing tissue testing, has emerged as a powerful tool when applied to the liquid biopsy arena. Today, several commercially available platforms have been developed to detect multiple genomic alterations across dozen to hundreds of cancer-informative genes, including mutations, small insertions and deletions, gene amplification events and gene fusions. The clinical use of these platforms is robustly supported by numerous molecular data indicating a high degree of agreement between plasma and tissue NGS [23,26–28].

As the great advantage of NGS-based platforms is the ability to evaluate multiple genomic alterations simultaneously, it has paved the way for the upfront use of plasma NGS to perform thorough genotyping of treatment-naïve advanced/metastatic patients. Equally important, this approach has drastically reduced the turnaround time from sampling to initiation of treatment [29], thereby allowing an increased percentage of patients to receive timely targeted therapies [28,29].

On the basis of these results, proponents of liquid biopsies have advocated the use of a "blood first" approach in advanced NSCLC patients as a useful diagnostic strategy either concurrently or sequentially to histologic review when tumor tissue is either scant or insufficient for comprehensive tumor genotyping [4,30]. An important aspect highlighted in the study by Rolfo et al. is the handiness of this approach especially during global health crises such as the recent COVID-19 pandemic. In effect, during the recent COVID-19 pandemic, invasive diagnostic procedures were either delayed or totally suspended to reduce the risks of contagion [31,32]. Such safety measures resulted in the denial of some patient access to hospitals and created health care backlogs in all medical fields, not the least of which was cancer care. The authors argue that the use of liquid biopsy in such grim scenarios constitutes an important diagnostic tool for reducing the potential risks of contagion for patients with cancer by limiting clinic visits or hospitalizations [32]. Moreover, owing to the short turnaround time of test results, we speculate that it could also be fully exploited to reduce the backlogs and waiting lists caused by the pandemic. Supporting the usefulness and efficiency of liquid biopsy in cancer care is a very recent study highlighting how a blood-first ctDNA-NGS analysis in subjects with suspected advanced-stage lung cancer increases the breadth and rapidity of detection of actionable variants compared to tissue analysis, leading to timely treatment decisions [33].

The variable structures of ctDNA are not fully appreciated and are still under scrutiny, as suggested by the vast literature available so far. Generally, ctDNA consists of degraded DNA fragments, usually of 167 base-pairs in length, that are released into the bloodstream [34–36]. The specific length likely results from the action of a caspase-dependent endonucleases that cleaves DNA after a core histone and its linker. Interestingly, however, recent studies have shown a distinct nuclear fragmentation pattern, with variable fragment lengths of ctDNA, depending on the origin of tissues [37–41]. Several different types of ctDNA have been described in the bloodstream, including double-stranded (ds) and single-stranded (ss) DNA particles. In humans, although ctDNA originates from all cells, the vast majority stems from

hematopoietic tissues [42–44]. Within the 'classical' ctDNA, more specific subclasses can be distinguished according to their site of origin or mechanisms of release. Among these are mitochondrial DNA (mtDNA) [45], cell-free fetal DNA (cffDNA) [46,47], extrachromosomal circular DNA(48), and microbial DNA (mbDNA) [48–50].

Decade-long efforts have been dedicated to investigating ctDNA. The first record of ctDNA dates back to 1948 when Mandel and colleagues detected for the first time extracellular DNA in serum and plasma [50]. However, it was not until 30 years later that it was associated with cancerous tissues. Indeed, a 1977 study reported the presence of higher levels of ctDNA in patients with pancreatic cancer than in healthy controls[51].

6

Such finding led to the concept that tumors released DNA fragments in the bloodstream. Inspired by this intriguing hypothesis, the next decades witnessed burgeoning research in this field. For example, Shapiro and colleagues in 1983 confirmed a correlation between benign versus malignant tumors and ctDNA concentration [52]. A few years later, Strounet al. (54)provided compelling evidence that some of these DNA fragments were of tumor origin because of their genomic instability. Still, in the early 1990 s, two independent studies noted the presence of specific *KRAS* proto-oncogene GTPase (*KRAS*) and *NRAS* proto-oncogene GTPase (*NRAS*) mutations in ctDNA from pancreatic adenocarcinoma [54] and acute myelogenous leukemia [54,55]. This fraction of ctDNA was later termed as circulating tumor DNA (ctDNA).

In detail, ctDNA refers to ctDNA fragments that are released into the bloodstream from primary tumor sites or metastatic cells and display tumor-specific point mutations, chromosomal rearrangements, copy-number variation (CNV), and DNA methylation [56–58]. Importantly, ctDNA is tipically fragmented than ctDNA, resulting in a much higher < 100 bp fraction in plasma [59]. The 10-bp periodicity observed for fragments smaller than 167 bp [36,60] corresponds to a turn of the DNA helix wrapped around the histone. Studies suggest that this might protect one part of the DNA from the nucleases present in blood. This specific fragmentation pattern suggests that apoptosis may be a major source of ctDNA and that histones may be the key protein complex associated with DNA in the blood. On the other hand, the release of longer ctDNA fragments from tumor cells has been associated with necrotic cell death and occurs in living cells via active processes [44,61,62];

ctDNA presents several relevant characteristics that support its integration in oncology practice. For example, the ctDNA fragments that are released into the bloodstream mirror the tumor status, its evolution, and the genomic alterations present in primary and/or metastatic tumors [44]. In a pan-cancer study involving 640 patients, Bettegowda et al., [63] demonstrated that ctDNA analysis might allow clinicians to monitor the therapeutic response by tracking drug resistance, and, in some cases, early detection of localized malignancies. They also pointed out a correlation between tumor burden and the stages of the disease. Indeed, they saw significant differences between ctDNA levels depending on the cancer types, and a median ctDNA concentration that was 100-fold higher in stage IV patients than in stage I patients [63].

Another important characteristic in terms of clinical utility is that ctDNA has a notably short half-life in the bloodstream [64]. This means that it can be of great help both to analyze the dynamics of mutations and the tumor burden after surgery and to track patient responses to systemic treatments throughout the course of the disease. Thus, ctDNA analysis may be used to monitor real-time tumor dynamics for early prediction and assessment of drug response, as well as to determine early intervention independently of imaging examinations or clinical symptoms [58,65].

Recent studies are also investigating whether ctDNA could be exploited to detect early stage cancers before symptoms appear. In this regard, in 2021, a research group built an intriguing mathematical model to predict the shedding rate of early-stage NSCLC [66]. The model estimated an average of only 1.7 genome copies of ctDNA in 15 mL of blood for lung tumors with a volume of 1 cm^3, thereby suggesting that highly sensitive sequencing techniques are needed to harness the diagnostic potential of ctDNA [37]. In practice, the detection of ctDNA requires the presence of known tumor mutations that can be readily detected by simple sequencing techniques. With the advent of comprehensive cancer genomics it is well understood that all cancers harbor somatic alterations. Hence, some researchers argue that since cancer somatic mutations occur at minor frequencies in normal cell populations, ctDNA can provide impeccably specific diagnostic biomarkers from a biological perspective [67].

Besides being exploited for diagnostic purposes, ctDNA detection could also be used to monitor drug response. In this regard, some historic studies provide temporal analyses of the total tumour burden and of specific mutations that appear during therapy [61,68–73]. Identification of somatic mutations within white blood cells might be a recurring source of discordance between tumor and total ctDNA genotyping. This phenomenon, called clonal hematopoiesis (CH), is an aging-related phenomenon, whereby non-malignant hematopoietic stem and progenitor cells acquire somatic alterations that confer a selective advantage [74]. CH-derived mutations present in a similar fashion to tumor-derived mutations when detected in plasma. Thus, a plasma ctDNA measurement and may involve both canonical CH genes—such DNA methyltransferase 3 alpha (*DNMT3A*), methylcytosine dioxygenase 2 (*TET2*), *ASXL* transcriptional regulator 1 (*ASXL1*), and Janus kinase (*JAK*)—and cancer driver mutations—such as, *KRAS*, phosphatidylinositol-4,5-bisphosphate3-kinase catalytic subunit alpha (*PIK3CA*), and epidermal growth factor receptor (*EGFR*) mutations [75–78]. Because hematopoietic cells are the primary source of ctDNA [79] and contribute somatic variants to the ctDNA pool [76,77], some studies have proposed several approaches to distinguish mutation-derived CH from their tumor-derived counterparts [75,77,80]. One often-cited way to discriminate between CH and tumor ctDNA is to examine the relative differences in mutant allele frequencies (MAFs), but this can be precarious and misleading proposition, as tumor metastatic lesions and heterogeneous sub-clones often result in MAF variances. Some investigators have indicated that CH and tumor ctDNA have fragments of a different size and distribution that might also help to distinguish between the two [81–83].

Chan et al., [84] elegantly elaborate on further clinical implications of the CH phenomenon. In addition to considering CH before implementing ctDNA analysis in the clinic strict guidelines and standard operating procedures need to be formulated. Factors like pre-analytical standardization need to be well optimized. Various attempts have been made to achieve this end. For instance, the CANCER-ID consortium, funded by both public and private sector units, was created to establish standard protocols for and clinical validation of ctDNA- and circulating tumour cell (CTC)-based biomarkers. Based on the efforts of the CANCER-ID consortium, Lampignano et al., [85] highlighted the importance of harmonizing the overall pre-analytical and analytical workflow of ctDNA purification, quantification, and characterization by comparing the preanalytical and analytical workflows of ctDNA-based techniques in multiple diagnostic centers. In an earlier study, Grolz et al., [86] focused specifically on the standardization of specimen preservation. In this regard, the authors underlined the importance of standardizing blood product preservation procedures after blood drawn for use as liquid biopsies and reviewed the most valid preservation solutions that are currently available. Thus, by virtue of entities like the CANCER-ID or the International Liquid Biopsy Standardization Alliance (ILSA) [87], it is now apparent that it is paramount to validate and optimize the use of this highly valuable and cost-effective approach in order to implement global use of liquid biopsy in oncology practice.

As previously mentioned, the growing implementation of ctDNA-based biomarker assessment in NSCLC advanced stage clinical practice as a complement to tissue-based testing is mainly driven by the difficulty in obtaining sufficient tumor material via invasive tissue biopsy. One reason for this hindrance is that most lung cancer patients present with advanced-stage NSCLC and are not eligible for curative surgical

treatment. This contributes to a high prevalence of small-specimen biopsies. Not surprisingly, tissue biopsy failure/inadequacy is estimated to affect up to 43 % of patients with NSCLC, limiting optimal first-line treatment selection or subsequent treatments following disease progression. In this scenario, liquid biopsy could serve as an integral solution for molecular diagnosis in NSCLC patients with limited or inadequate tissue biopsies.

7

Despite the undisputable advantages of biomarker testing, its full implementation in Europe is still lagging because of several barriers, including access, reimbursement, and technical limitations; such limitations have all been thoroughly reviewed in previous research [42]. However, in this article, we explore one other key reason: tissue unavailability (Fig. 1). In a recent review, we highlighted that biomarker testing is often not fully exploited because not all patients with NSCLC undergo tissue biopsy. For example, in one study it appears that only 7 % of patients with NSCLC in Italy and 15 % of patients with NSCLC in Spain do not undergo a tissue biopsy [42]. Consequently, biomarker testing in NSCLC is drastically underutilized for the very reason that tissue samples are either quantitatively insufficient or qualitatively inadequate [41,46,54]. Similar conclusions were drawn in the IASLC global survey on molecular testing in lung cancer[46]. Indeed, the survey revealed that molecular diagnosis failed either because of insufficient tumour cells (93 % of respondents) or because of inadequate tissue quality (55 %). Several authors have highlighted that the acquisition of adequate tissue biopsies in NSCLC can be particularly challenging for two main reasons: the first is that tumor sites in patients with advanced NSCLC are often difficult to access; the second is that invasive biopsies are associated with bleeding and pneumothorax complications [58,59]. For example, the incidence of pneumothorax complications in patients undergoing transthoracic needle biopsy is approximately 20 % (ranging from 9 to 54 %) [60]. Moreover, even when technically feasible, not all biopsies provide enough tissue for molecular diagnosis. Similar study results [59,61–63] have indicated that tissue biopsy may not be feasible in approximately 20 % of patients, and even when biopsy is feasible, samples are inadequate for testing (molecular diagnoses and/or histological diagnosis) in up to a quarter of cases. Overall biopsy failure rates seem to vary considerably, ranging from 8 % to 43 %. The proportion of patients with initially unsuccessful tissue biopsies that have been salvaged via re-biopsy have not yet been reported for European cohorts.

Diagnostic tests do not always produce uniform yields (i.e., the proportion of biopsies permitting a successful pathological diagnosis) but vary from one biopsy technique to another, thereby underlying the problem of "disharmonization" mentioned above. For example, endobronchial ultrasonography (EBUS) ranges from 58 to 70 %, CT-guided transthoracic needle aspiration from 80 to 96 %, EBUS transbronchial needle aspiration (TBNA) up to 94 %, and thoracoscopy from 93 to 94 % [68–72]. In an attempt to understand the discrepant molecular results obtained by different biopsy techniques, the ongoing German, prospective, and randomised PROFILER study is currently investigating how biopsy techniques can affect molecular genetic tumor characterization in NSCLC [73]. As a guiding principle, the authors recommend that the least invasive biopsy techniques that provide maximum tissue yields be used to establish molecular diagnoses and minimize the risk of bleeding and other complications. Following this recommendation, many more European institutions should strongly advocate the use of liquid biopsy in routine practice to guarantee the largest possible number of patients the clinical benefits of a test as simple and harmless as a blood draw. Indeed, if one considers that 40,000 NSCLC patients do not receive biomarker testing annually and that the inability to do so in 40 % of these cases is due to unfeasible/inadequate tissue biopsy, it should not be surprising that if liquid biopsy coupled with NGS were introduced in routine clinical practice, 16,000 patients in Europe would be able to obtain a molecular diagnosis every year.

Several European clinical guidelines/position papers recommend the use of liquid biopsy for patients with NSCLC in certain settings. For instance, whereas the ESMO NSCLC guidelines recommended it for detecting p.T790M mutations in the context of EGFR TKI therapy (36) the ESMO PMWG and the IASLC statement papers on liquid biopsy recommend it in all advanced NSCLC regardless of the driver mutation [43,74]. In particular, the ESMO PMWG report recommends that a tumor or plasma sample from a patient with advanced non-squamous NSCLC be profiled using NGS technology to detect ESCAT level I alterations [43]. On the other hand, the IASLC statement paper recommends that frontline liquid biopsy testing be considered in all patients who require molecular tumor profiling, particularly when tissue is scarce or unavailable, and when tumor biopsies are expected to be significantly delayed [74]. Fig. 2 summarizes specific recommendations for treatment-naïve patients and patients who progress, either clinically or radiologically, during treatment with first- or second-generation EGFR TKIs [75]. In both populations, although a positive finding would provide sufficient evidence to initiate appropriate targeted treatment, a negative liquid biopsy result should be considered inconclusive and

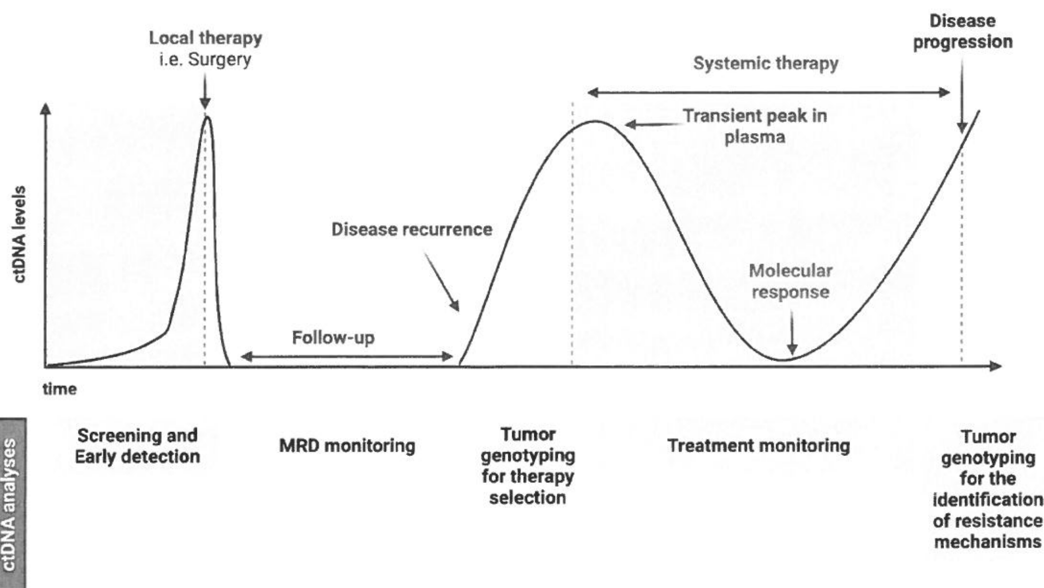

Fig. 1. Potential applications of ctDNA analyses during the course of lung cancer patient journey. Credit: Created with BioRender.com.

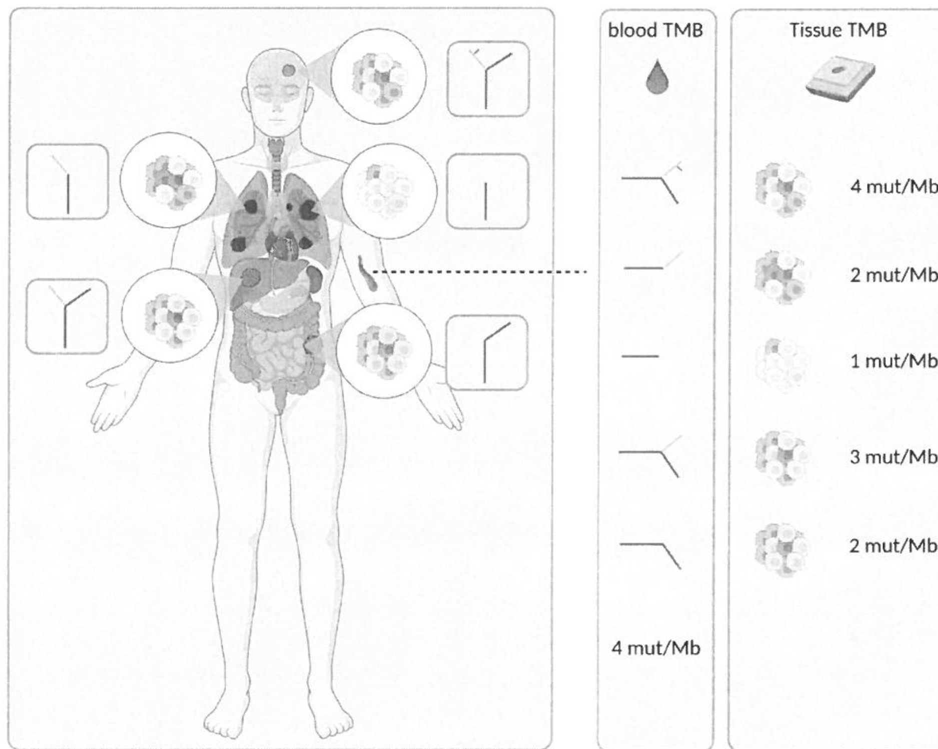

Fig. 2. Potential pitfalls in the overall evaluation of Tumor Mutational Burden (TMB) on ctDNA versus tissue-based analysis of primary tumor and metastatic sites. The simultaneous detection of all non-synonymous mutations in ctDNA is not always representative of the tissue private mutations Credit: Created with BioRender.com.

should require secondary testing. For this reason, the IASLC recommendations highlight the complementary nature of tissue and liquid biopsies in the NSCLC setting.

The liquid biopsy testing landscape is rapidly changing in other countries. For instance, whereas single gene liquid biopsies have been approved for several years in the USA, pan-tumor, NGS-based liquid biopsy companion diagnostics (Guardant360® CDx and FoundationOne® Liquid CDx (Foundation Medicine, Cambridge, MA, USA) was only recently approved by the FDA in 2020 [53,88–90]. Furthermore, the updated molecular testing guidelines from the College of American Pathologists (CAP), IASLC, and the Association for Molecular Pathology (AMP) now recommend the use of multiplexed genetic sequencing panels is preferred over multiple single-gene tests to identify other treatment options beyond *EGFR*, *ALK*, and *ROS1*" [90]. Finally, in Japan, FoundationOne® Liquid CDx was approved in 2021 by the Ministry of Health, Labour and Welfare, and Guardant360® CDx is still waiting for regulatory approval [91,92].

3. Emerging application of liquid biopsy in NSCLC

- Monitoring of treatment response and Minimal Residual Disease

In the new era of personalized medicine, targeted therapy has emerged as a valuable approach for the management of lung cancer patients [93]. Although an increasing number of therapeutic strategies are routinely employed in today's clinical practice, clinical outcomes still remain poor for advanced lung cancer patients, particularly those in the latest stages of the disease [94]. In this scenario, a small fraction of scant representative analytes derived from tumor cells (ctDNA, ctRNA, CTC, EVs) may be used to signal tumor relapse in torrent blood [94]. A major advantage of this approach is that when standardized functional imaging is unable to measure minimal residual disease (MRD), the identification of these analytes becomes a promising alternative strategy to treat lung cancer patients quickly and effectively with targeted therapies [95]. This strategy has been borrowed from the management of hematologic malignancies, where MRD analysis is routinely performed with both flow cytometry and molecular techniques to identify relapsed circulating tumor cells and druggable molecular alterations, and to determine a prognostic classification of tumor patients [96,97].

Similarly, ctDNA has been recommended as a reliable biomarker to evaluate MRD in solid tumor patients. In this context, biological parameters, such as very low concentrations of ctDNA in peripheral blood, together with tumor burden and clinical stage, have drastically elicited the diffusion of these biomarkers in clinical practice [1,98]. This has also been made possible by the adoption of ultra-deep technical approaches (ddPCR, NGS). Being highly sensitive to these very low frequency targetable biomarkers, they have improved therapeutic decision-making for patients with solid tumors patients [99]. For example, De Cock et al. designed a highly sensitive ddPCR pentaplex assay able to identify the five most recurrent *EGFR* TKI-sensitizing mutations (exon 19p. E746_A750del, exon 21p.L858R and L861Q, exon 18p.G719S and p. S768I mutation) simultaneously. In addition, a ddPCR triplex assay, highly sensitive for *EGFR* resistance mutations, namely, exon 20p. T790M and p.C797S, was combined with each specific sensitive *EGFR* mutation to monitor molecular residual disease on the basis of the clinical outcomes of n = 6 NSCLC patients. The approach was able to estimate up to 0.25 % of allelic fractions. Moreover, ddPCR revealed acquired resistant alterations (exon 20p.T790M point mutation) in all patients with TKI-sensitive *EGFR* mutations when progressive disease (PD) occurred [100]. In another experience, an ultra-deep NGS approach (CAPP-seq) was applied to evaluate MRD after surgical treatment of localized lung cancer patients. This approach proved to be equally effective in predicting clinical response and in guiding therapeutic decision making in early-stage settings [101]. In particular, plasma samples were collected from a set of n = 40 early stage (IB-III) lung cancer patients at follow-up visits occurring every-two to six

months after initiation of curative-intent first line treatment; surveillance CT or PET/CT scans were also administered. For each sample, a CAPP-seq approach was able to analyze 128 recurrently mutated genes in lung cancer. Moreover, the molecular analysis revealed a high pretreatment ctDNA detection rate ranging from 89.0 to 100 % compared with the histological classification and metabolic tumor volume (Pearson r = 0.55, P = 0.0004). Then, to further investigate the efficiency of CAPP-seq of ctDNA for disease surveillance, a longitudinal plasma analysis was carried out in n = 37 enrolled patients with detectable pretreatment ctDNA. Remarkably, ctDNA was detected in 20/37 (54.0 %) patients with a comparable distribution between driver and passenger alterations [101]. Patients positive for ctDNA analysis at any post-treatment time point showed a statistically significant lower PFS than negative ctDNA patients, with a median of 5.2 months before radiographic criteria [101].

ctDNA clearance after neoadjuvant chemo-immunotherapy has also been recently demonstrated to predict more favorable outcomes[102]. In addition, ctDNA evidence after surgery and/or adjuvant therapies has been proposed as a potential biomarker for patients requiring post-operative adjuvant immunotherapy. This hypothesis is currently being tested in two clinical trials with adjuvant durvalumab (MERMAID-1 & −2). Furthermore, studies show that the use of ctDNA clearance in patients undergoing immune checkpoint blockage is a potential biomarker not only to select optimal candidates for elective treatment discontinuation after a long-term response [103] but also to facilitate personalized therapeutic strategies during consolidation immunotherapy after chemo-radiotherapy [104].

Despite the many scientifically substantiated advantages, the adoption of ctDNA to detect MRD in clinical practice is hampered by two limitations. The main limitation is the scant amount of target material. As previously mentioned, ultra-deep approaches are needed to ensure a thorough molecular characterization of target genes. To achieve this level of sensitivity, it is essential that rigorous error-proofing methods are incorporated to reduce the chance of assay-based false-positive signals. The secondary limitation is the high probability of confounding findings generated by CH, as discussed previously [105]. In this regard, other integrative solutions are currently being investigated to optimize MRD analysis in advanced tumor patients.

Another exploitable blood-based biomarker is circulating tumor cells (CTCs) Generally, CTCs, which derive from rapid self-renewal tumor processes, may be considered an integrating biological source for monitoring the dynamic changes of clonal tumor cells under specific stimuli [106]. In this setting, in an observational study, Wu et al investigated the potential role of CTCs as an MRD predictive tool by comparing pre- and post-treatment CTC levels in a series of patients with suspicious lung malignancy. Overall, 41 out of 50 patients with a primary lung tumor showed a median cell count of 8.0 (ranging from 0.0 to 74.7 cells/mL) compared with 5/ 51 patients with oligometastatic cancer who showed a median of 7.5 (ranging from 3.0 to 74.5 cells/mL), and with controls who showed a median of 1.0 ranging from 0.0 to 6.0 cells/mL). Interestingly, in all cases, a statistically significant decrease in CTC levels was observed 3 days after surgical treatment (2.3 cells/mL/ day in cancer group) with an appreciable difference between 30 out of 41 recurrences (s 2.5 cells/mL/day) and 11 out of 41 non recurrences (1.4 cells/mL/day) [107].

Finally, a promising approach for evaluating MRD in solid tumor patients is the analysis of the methylation status or other epigenetic markers as an integrative tool to track somatic mutations. In this scenario, the use of the Guardant Reveal assay, which detects ctDNA to assess MRD, has proven to be a technically feasible tool for the enrichment of somatic-naïve alterations (epigenetic changes and somatic mutations) in a cohort of colon rectal patients [108,109].

- Lung cancer screening and Cancer Interception

Recent years have witnessed great strides in cancer research and in finding ways to detect malignant lesions at an early stage of the disease. In particular, thanks to the development of molecularly-driven detection methods, alongside conventional cancer diagnostics, an increasingly number of patients can now benefit from curative therapies based on targeted drugs or immunotherapeutic agents [110–112]. Despite the constant burgeoning of technical strategies, liquid biopsy tests seem to be the most promising tool in this field [110,112,113]. However, the use of liquid biopsy does raise some scepticism among specialists in the field. For instance, the call of only molecular alterations residing in evolutionary tumor cells require technical assays with a high diagnostic sensitivity and specificity for the somatic mutations carried by ctDNA [68,114]. Moreover, the possibility of false positive results makes follow up examinations with expensive high-cost imaging technologies necessary [115]. Lastly, diagnostic sensitivity of liquid biopsy-based tests still remains a challenge owing to the very low abundance of somatic molecular alterations in early-stage cancer patients: [116,117]. This explains why recent research on liquid biopsy has focused primarily on advanced stage cancer patients. In an effort to address some of these issues, Cohen *et al.* have designed and validated the CancerSEEK assay. This assay consists of a wide integrating DNA/protein panel able to detect eight of the most recurrent mutations in the early stages of adult solid tumors [117]. In brief, DNA analysis is performed with a customized multiplex-PCR assay that allows to reduce amplification duplicates in the sequencing phase, while a plasma aliquoting strategy for NGS analysis increases the detection rate for somatic alterations carried by ctDNA (from 41 % to 95 % of most cancer related somatic mutations). Moreover, this protein-based analysis features multiple immunoassays of n = 41 cancer-related proteins involved in cancer detection (sensitivities > 10.0 % and specificities > 99.0 %)[117]. To test the validity of this panel, the authors evaluated a set of 1005 stages I, II, and III patients and 812 healthy controls. Remarkably, the assay showed a median sensitivity of 70.0 % (ranging from 30.0 to 88.0 %) and specificity > 99.0 %. Moreover, a high median sensitivity was also reached in all staging groups (ranging from 43.0 % for stage I to 78.0 % for stage III). To further validate their results, they analyzed tissues from 153 patients. Of note, they achieved an overall concordance rate of 90.0 %. Overall, the CancerSEEK study elucidated that although it is technically possible to integrate a plasma-based approach for early diagnosis clinical validation of molecular results still remains technically challenging [117]. As a result, several efforts are being made to identify new blood-based analytes that could integrate ctDNA molecular evaluation [118]. For example, current research is exploring the possibility of using large scale epigenetic modifications as reliable biomarkers while improving the technical sensitivity and specificity of new devices for early-stage lung cancer diagnosis [118]. In this regard, Shen *et al.* developed a highly sensitive immunoprecipitation-based approach able to detect tumor methylation profiles from scant ctDNA input [118]. This system accurately identified tumor-related methylation profiles in different types of tumors [118]. Building on this research aimed at improving the landscape of molecular biomarkers for early-stage cancer detection, one study explored the feasibility of using fragmentomics (ctDNA fragment size distribution), as a method of identifying the molecular fingerprinting of tumor cells[119]. Based on the simultaneous and integrative analysis of genetic and epigenetic modifications (DELFI), this approach was tested on a wide perspective cohort of suspicious lung cancer patients (including benign and malignant lesions) and healthy donors by adopting DELFI to discriminate between patients with lung cancer malignant lesions from those with benign lesions and control [119]. Interestingly, this approach accurately distinguished the non-cancer group (DELFI score 0.16–0.21) from the cancer group (DELFI score 0.35–0.99 according to tumour stage). The performance of this model was also evaluated in combination with the predictive powerful conventional serum-based biomarkers (CEA and other secreted proteins). Similarly, this multivariate approach produced encouraging results (AUC > 80 %) in cancer detection, as evidenced by the lack of any significant differences according to cancer grading and staging [119].

On the basis of these results, a clinical trial (NCT02889978) called Circulating Cell-free Genome Atlas Study (CCGA) was promoted a few years ago [120]. The primary goal of this ongoing trial is to explore and validate the possibility of combining DNA and methylation profile analysis with a machine learning approach from a single liquid biopsy specimen to detect cancer patients in the early-stage setting [120]. So far, the study has demonstrated an increasing stage related sensitivity (ranging from 39.0 to 92.0 %) in the identification of cancer patients compared to conventional strategies. Interestingly, the trial has also demonstrated that this approach can successfully evaluate the dynamic changes in tumor fragmentation profiles as opposed to traditional approaches. This suggests that the clinical outcome is strongly associated with modifications in the fragmentome profile. Indeed, they have observed a decreased overall survival in patients with a higher DELFI score [119].

10

Nowadays, novel screening criteria are being discussed in view of recent cancer prevention programs approved by various international agencies [121]. Not surprisingly, the last decade has witnessed a growing interest in the identification of molecular signatures to diagnose early-stage cancer patients [122]. The new frontier of cancer prevention is thus based on the identification of biological hallmarks and de-regulated pathways that increase individual cancer risk in healthy people (cancer interception) [123]. In this scenario, what better way to guarantee patients with fast and accurate early cancer screening than by adopting a liquid biopsy approach. Indeed, compared with conventional diagnostic approaches, liquid biopsy is barely invasive. A sample can be obtained by a simple blood draw and can reveal traces of multiple analytes that collectively contribute to cancer development and progression [4]. Starting from a mere liquid biopsy sample, several groups have compared differentially expressed miRNA in lung cancer and control patients to establish tumor-related miRNA panels able to detect cancer at an early stage [124,125]. In addition, other research teams have retrospectively evaluated the analytical performance of miRNA signature classification (MSC) in high-risk subjects [126]. Overall, in this study, MSC yielded a diagnostic sensitivity of 87.0 % in lung cancer patients and 81.0 % in healthy individuals (PPN: 99.0 % and 99.8 %). Similarly, in another large scale validation study, another miRNA panel (miR-Test) was assessed in high risk lung cancer patients [127]. Consistent with the previous results, MSC was highly accurate in identifying high risk patients (overall accuracy, sensitivity, and specificity of 74.9 %, 77.8 %, and 74.8 %, respectively). Interestingly, a direct comparison between these two distinct molecular signatures is being investigated in an ongoing clinical trials. (NCT02247453). So far, the trial has evidenced a reduction in false-positive cases in lung cancer detection in heavy-smoker volunteers subjected to MSC-based first-line screening.

Other studies, instead, have evaluated cancer risk by detecting conventional biomarkers (DNA, RNA) [128], In detail, a cohort of patients with bronchial premalignant lesions was analysed using RNA-seq; results showed four distinct molecular phenotypes according to the biological pathway and clinical features. Recent development in this area of research has demonstrated that CTCs may also play a key role in improving early detection in lung cancer [12]. Interestingly, Sequist et al. have developed and validated a CTC-based chip as a tool for lung cancer interception [12].

3.1. Liquid biopsy and immunotherapy

Immunotherapy has significantly revolutionized the way cancer patients, particularly NSCLC lacking actionable driver mutations, are clinically managed [129–131]; To date, evaluation of high levels of programmed death-ligand 1 (PD-L1) expression on tumor cell surfaces through immunohistochemistry (IHC) or immunocytochemistry (ICC) remains the most widely used approach to select patients for immune checkpoint inhibitor (ICI) therapy [132,133]. However, recent studies have indicated that patients expressing low levels of PD-L1(>1%) may also benefit from ICI treatments.:[134,135]. Beyond PD-L1, other biomarkers are currently under investigation. Among these, careful attention is being paid to tumor mutational burden (TMB), that is, the total number of somatic, coding, base substitutions, and short insertions/deletions (indels) per tumor genome [136]. In particular, high TMB (TMB-H) has been correlated with better outcomes for advanced stage NSCLC patients treated with ICIs. In addition, the analysis of TMB in association with PD-L1 may allow the selection of a higher number of patients for ICI administration [137]. However, the analysis of these biomarkers is often hampered by scarce tissue material from advanced stage NSCLC patients [138]. Thus, liquid biopsy emerges once again, as in previous but different settings, a game changer in providing NSCLC patients the best possible treatment option [4,139]. Recent experiences have indeed documented the possibility of analyzing TMB in blood samples, the so called bTMB [140]. Gandara *et al.* demonstrated the utility of this biomarker as a predictor of drug response by using a retrospective analysis of POPLAR and OAK clinical trials as test and validation studies. bTMB was determined by using an FDA approved 394-gene plasma NGS platform FoundationOne Liquid CDx. High bTMB levels, assessed as $\geq$ 16 mutations/Mb, were detected, indicating the potential use of this biomarker as a clinically actionable biomarker for atezolizumab in NSCLC [141]. In the wake of these encouraging results, a research team adopted the same assay and cut-off values in the prospective BF1RST (NCT02848651) clinical trial. The aim of this trial was to evaluate the role of bTMB $\geq$ 16 mutations/Mb in the administration of atezolizumab, regardless of PD-L1 expression. The trial results showed clinical benefit in terms of progression free survival (PFS), objective response rate (ORR), and overall survival (OS). Similar promising data have been reported in the phase III MYSTIC trial by comparing durvalumab $\pm$ tremelimumab with platinum-based chemotherapy. In this clinical trial, the 500-gene panel Guardant OMNI was employed. Overall, by using a bTMB $\geq$ 20 mutations/Mb, clinically meaningful benefits were reported in the overall PFS, ORR, and OS of patients receiving ICI combination therapies compared to chemotherapy regimens. Interestingly, a slightly higher efficacy was reported with durvalumab alone compared to chemotherapy [142].

The assessment of PD-L1 expression in circulating tumor cells (CTCs) has also been investigated [143]. However, contradictory results have been reported. In particular, Guibert *et al* demonstrated that although the evaluation of PD-L1 expression in CTCs is feasible, it does not predict the response to nivolumab administration [144]. Conversely, Ilie *et al* showed that CTC assessment of PD-L1 may be a useful noninvasive tool to evaluate PD-L1 expression in patients with advanced-stage NSCLC [145]. Finally, another surrogate of tissue biopsies is the assessment of PD-L1 expression levels on exosomes extracted from plasma samples to predict ICI response in NSCLC patients [146].

3.2. Liquid biopsy and inclusion in clinical trials

Liquid biopsy has significantly modified the way clinical trials are carried out. A number of different clinical trials have adopted liquid biopsy as an inclusion criterion to select advanced stage NSCLC patients [147,148]. These studies include early detection, diagnosis, treatments, and evaluation of minimal residual disease (MRD) of advanced stage NSCLC patients. The wide diffusion of liquid biopsy within clinical trials derives from the minimally invasiveness and reproducibility of liquid biopsy sampling, which enables a dynamic evaluation of the evolution of NSCLC [149]. For example, one study evaluated ctDNA in a subset of patients in a phase 2 clinical trial study of tepotinib in advanced stage NSCLC patients harboring *MET* exon 14 skipping mutations [150]. In an ongoing phase II clinical trial, the MELROSE study, ctDNA analysis is being used to study the mechanisms of resistance to osimertinib in non-pretreated advanced *EGFR* mutated NSCLC patients [151]. In several other clinical trials, ctDNA analysis is being widely adopted for early detection and monitoring of NSCLC evolution [101,114,151]. In this scenario, a paradigm shift from traditional "*tissue biopsy-based*" trials

toward "*liquid biopsy-based*" trials has been crucial to minimize the absence of histological specimens as a barrier to the enrollment of advanced stage NSCLC patients.

4. Future perspectives

The three-dimensional nature of Liquid Biopsy: DNA, RNA and proteins.

Nowadays the use of a simple blood test is rapidly emerging as an excellent screening approach in preventive, predictive, and personalized medicine. As stated in the NILE study, its growing popularity is due to its ability to provide multifunctional biomarkers through a simple blood draw without the need to sacrifice any precious and scant tissue material. Undoubtedly, tissue samples are still the gold standard material to evaluate gene fusions and PD-L1 expression in clinical practice [29]. However, critical issues related to tissue scarce availability in certain types of cancers advocate major changes to improve cancer diagnosis and management. Among these is the use of circulating- tumour RNA (ctRNA) extracted from exosomes. In a preliminary study, Reclusa et al. demonstrated the feasibility of ctRNA extracted from extracellular vesicles in the analysis of *ALK* gene rearrangements [152]. Additionally, Raez et al. used cfRNA analysis to define the expression profile of PD-L1 to predict clinical responses to ICIs. (http://dx.https://doi.org/10.20517/cdr.2021.78) Similarly, other studies are investigating the possibility of evaluating PD-L1 expression in circulating tumour cells [143] and in extracellular vesicles [153].

4.1. TNM(b)

To date, Generally, lung cancer patients are diagnosed according to the TNM classification system of malignant tumors—a widely adopted criteria for staging metastasized lung cancers [154]. In brief, the classification stages lung cancer patients into four groups (I, II, III, and IV) to for diagnostic, prognostic, and selective treatment purposes and treatment. Patients who fall in the stage IV group have distant metastases. However, as technological advances continue to deepen our understanding of the molecular hallmarks of cancer, and as the application of new cancer screening approaches, like liquid biopsy, continues to gain more momentum in clinical practice, [155] the current histological-based categorization system needs to be modified to keep up with recent advances [156]. In this regard, recent research has proposed the feasibility of adopting a blood TNM (bTNM) system as an integrative approach for the prognostic and therapeutic implications of ctDNA in lung cancer settings. According to this classification, patients are classified either as, "B0", which indicates the absence of ctDNA, or as "B1", which indicates the presence of detectable levels of ctDNA [63,157,158]. This "blood-biomarker" based classification system may prove to be a useful guide for the comprehensive management of lung cancer patients.

4.2. Artificial intelligence in liquid biopsy

Another highly promising field of investigation is to combine liquid biopsy and artificial intelligence to improve lung cancer early detection, as previously discussed [38].

In another experience, a machine-learning tool, namely 'lung cancer likelihood in plasma' (Lung-CLiP), has shown a great ability to discriminate early-stage lung cancer patients from risk-matched controls. (82).

5. Conclusion

Liquid Biopsy is dramatically transformin lung cancer research and practice. The landscape is rapidly evolving, shifting from established applications, like ctDNA mutational analysis in metastatic settings to the simultaneous characterization of different analytes (such as ctDNA mutations, methylation status, protein levels) [159–161]. The rationale behind these changes is the quest toward intercepting tumor growth before it metastasizes. However, to fully exploit this elegant and powerful tool in clinical practice, concerted efforts should be made to standardize all the phases of liquid biopsy analysis. Moreover, educational programs should be organized in an attempt to overturn the linger skepticism surrounding the remarkable advantages of this valuable tool for cancer care.

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CRediT authorship contribution statement

Umberto Malapelle: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Supervision, Project administration, Writing – original draft, Writing – review & editing. **Pasquale Pisapia:** Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Methodology, Writing – original draft, Writing – review & editing. **Francesco Pepe:** Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Gianluca Russo:** Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Writing – review & editing. **Mauro Buono:** Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization,Writing – review & editing. **Alessandro Russo:** Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Writing – review & editing. **Jorge Gomez:** Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Writing – review & editing. **Ola Khorshid:** Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Writing – review & editing. **Philip C. Mack:** Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Writing – review & editing. **Christian Rolfo:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Visualization, Supervision, Writing – review & editing. **Giancarlo Troncone:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Umberto Malapelle has received personal fees (as consultant and/or speaker bureau) from Boehringer Ingelheim, Roche, MSD, Amgen, Thermo Fisher Scientifics, Eli Lilly, Diaceutics, GSK, Merck and AstraZeneca, Janssen, Diatech, Novartis and Hedera unrelated to the current work. Pasquale Pisapia has received personal fees as speaker bureau from Novartis, for work performed outside of the current study. Alessandro Russo reports advisory board role/consultancy for AstraZeneca, Novartis, Pfizer and MSD, unrelated to the current work. Christian Rolfo reports grants from Pfizer and MSD, consulting fees from Archer, Inivata, BMS, Novartis, Boston Pharmaceuticals, MD Serono; other from AstraZeneca, Roche, GuardantHealth, and MSD; safety monitoring board for MD Serono; leadership roles with ISLB, IASLC, ESO and ESMO; other support from GuardantHealth, unrelated to the current work. Giancarlo Troncone reports personal fees (as speaker bureau or advisor) from Roche, MSD, Pfizer, Boehringer Ingelheim, Eli Lilly, BMS, GSK, Menarini, AstraZeneca, Amgen and Bayer, unrelated to the current work. The other Authors have nothing to disclose.

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References

[1] E. Crowley, F. di Nicolantonio, F. Loupakis, A. Bardelli, Liquid biopsy: monitoring cancer-genetics in the blood, Nat Rev Clin Oncol. 10 (8) (2013 Aug) 472–484.
[2] G. Siravegna, S. Marsoni, S. Siena, A. Bardelli, Integrating liquid biopsies into the management of cancer, Nat Rev Clin Oncol. 14 (9) (2017 Sep) 531–548.
[3] C. Rolfo, P.C. Mack, G. Scagliotti, et al., Liquid biopsy for advanced non-small cell lung cancer (NSCLC): A statement paper from the IASLC, J Thorac Oncol. 13 (9) (2018) 1248–1268.
[4] C. Rolfo, P. Mack, G. Scagliotti, et al., Liquid biopsy for advanced NSCLC: A consensus statement from the international association for the study of lung cancer, J Thorac Oncol. 16 (10) (2021) 1647–1662.
[5] U. Malapelle, P. Pisapia, A. Addeo, O. Arrieta, B. Bellosillo, A.F. Cardona, et al., Liquid biopsy from research to clinical practice: focus on non-small cell lung cancer, Expert Rev Mol Diagn. 21 (11) (2021 Nov) 1165–1178.
[6] D. Fernández-Lázaro, J.L. García Hernández, A.C. García, A. Córdova Martínez, J. Mielgo-Ayuso, J.J. Cruz-Hernández, Liquid biopsy as novel tool in precision medicine: origins, properties, Identification and Clinical Perspective of Cancer's Biomarkers, Diagnostics (Basel) (2020). Apr 13;10(4.
[7] O.A. Zill, K.C. Banks, S.R. Fairclough, S.A. Mortimer, J. Vowles, v, Mokhtari R., et al., The landscape of actionable genomic alterations in cell-free circulating tumor DNA from 21,807 advanced cancer patients, Clin Cancer Res. 24 (15) (2018) 3528–3538.
[8] P.C. Mack, K.C. Banks, C.R. Espenschied, R.A. Burich, O.A. Zill, C.E. Lee, et al., Spectrum of driver mutations and clinical impact of circulating tumor DNA analysis in non-small cell lung cancer: Analysis of over 8000 cases, Cancer 126 (14) (2020) 3219–3228.
[9] J. Rodríguez, J. Avila, C. Rolfo, A. Ruíz-Patiño, A. Russo, L. Ricaurte, et al., When tissue is an issue the liquid biopsy is nonissue: A review, Oncol Ther. 9 (1) (2021 Jun) 89–110.
[10] D. Kwapisz, The first liquid biopsy test approved. Is it a new era of mutation testing for non-small cell lung cancer? Ann Transl Med. 5(3):46 (2017 Feb).
[11] A. Russo, P.D. de Miguel, M. Gunasekaran, K. Scilla, R. Lapidus, B. Cooper, et al., Liquid biopsy tracking of lung tumor evolutions over time, Expert Rev Mol Diagn. 19 (12) (2019) 1099–1108.
[12] M.J. Serrano, M.C. Garrido-Navas, J.J. Diaz Mochon, M. Cristofanilli, I. Gil-Bazo, P. Pauwels, et al., Precision prevention and cancer interception: the new challenges of liquid biopsy, Cancer Discov. 10 (11) (2020) 1635–1644.
[13] C. Freitas, C. Sousa, F. Machado, M. Serino, V. Santos, N. Cruz-Martins, et al., The role of liquid biopsy in early diagnosis of lung cancer, Front Oncol. 11 (2021), 634316.
[14] C. Rolfo, A.F. Cardona, M. Cristofanilli, L. Paz-Ares, J.J. Diaz Mochon, I. Duran, et al., Challenges and opportunities of ctDNA analysis implementation in clinical practice: Perspective of the International Society of Liquid Biopsy (ISLB), Crit Rev Oncol Hematol. 151 (2020 Jul), 102978.
[15] U. Malapelle, M. Buono, P. Pisapia, G. Russo, R. Tufano, F. Pepe, et al., Circulating tumor DNA in cancer: Predictive molecular pathology meets mathematics, Crit Rev Oncol Hematol. 163 (2021 Jul), 103394.
[16] U. Malapelle, R. Sirera, E. Jantus-Lewintre, P. Reclusa, S. Calabuig-Fariñas, A. Blasco, et al., Profile of the Roche cobas® EGFR mutation test v2 for non-small cell lung cancer, Expert Rev Mol Diagn. 17 (3) (2017) 209–215.
[17] Y.L. Wu, C. Zhou, C.K. Liam, G. Wu, X. Liu, Z. Zhong, et al., First-line erlotinib versus gemcitabine/cisplatin in patients with advanced EGFR mutation-positive non-small-cell lung cancer: analyses from the phase III, randomized, open-label, ENSURE study, Ann Oncol. 26 (9) (2015 Sep) 1883–1889.
[18] L. Bonanno, A. Dal Maso, A. Pavan, E. Zulato, L. Calvetti, G. Pasello, et al., Liquid biopsy and non-small cell lung cancer: are we looking at the tip of the iceberg? Br J Cancer (2022 Mar 9).
[19] F.R. Hirsch, M.W. Wynes, D.R. Gandara, P.A. Bunn, The tissue is the issue: personalized medicine for non-small cell lung cancer, Clin Cancer Res. 16 (20) (2010 Oct 15) 4909–4911.
[20] H. Kimura, K. Kasahara, M. Kawaishi, H. Kunitoh, T. Tamura, B. Holloway, et al., Detection of epidermal growth factor receptor mutations in serum as a predictor of the response to gefitinib in patients with non-small-cell lung cancer, Clin Cancer Res. 12 (13) (2006 Jul 1) 3915–3921.
[21] H. Kimura, M. Suminoe, K. Kasahara, T. Sone, T. Araya, S. Tamori, et al., Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA), Br J Cancer. 97 (6) (2007 Sep 17) 778–784.
[22] P.C. Mack, W.S. Holland, R.A. Burich, R. Sangha, L.J. Solis, Y. Li, et al., EGFR mutations detected in plasma are associated with patient outcomes in erlotinib plus docetaxel-treated non-small cell lung cancer, J Thorac Oncol. 4 (12) (2009 Dec) 1466–1472.
[23] J.I. Odegaard, J.J. Vincent, S. Mortimer, J. Vowles, v, Ulrich BC, Banks KC., et al., Validation of a plasma-based comprehensive cancer genotyping assay utilizing orthogonal tissue- and plasma-based methodologies, Clin Cancer Res. 24 (15) (2018) 3539–3549.
[24] B. Han, S. Tjulandin, K. Hagiwara, N. Normanno, L. Wulandari, K. Laktionov, et al., EGFR mutation prevalence in Asia-Pacific and Russian patients with advanced NSCLC of adenocarcinoma and non-adenocarcinoma histology: The IGNITE study, Lung Cancer. 113 (2017) 37–44.
[25] M. Reck, K. Hagiwara, B. Han, S. Tjulandin, C. Grohé, T. Yokoi, et al., ctDNA determination of EGFR mutation status in european and japanese patients with advanced NSCLC: The ASSESS study, J Thorac Oncol. 11 (10) (2016 Oct) 1682–1689.
[26] A.B. Schrock, A. Welsh, J.H. Chung, D. Pavlick, E.H. Bernicker, B.C. Creelan, et al., Hybrid capture-based genomic profiling of circulating tumor DNA from patients with advanced non-small cell lung cancer, J Thorac Oncol. 14 (2) (2019) 255–264.
[27] M.A. Pritchett, D.R. Camidge, M. Patel, J. Khatri, S. Boniol, E.K. Friedman, et al., Prospective clinical validation of the invisionfirst-lung circulating tumor DNA assay for molecular profiling of patients with advanced nonsquamous non-small-cell lung cancer, JCO Precis Oncol. 3 (2019).
[28] J.K. Sabari, M. Offin, D. Stephens, A. Ni, A. Lee, N. Pavlakis, et al., A Prospective study of circulating tumor DNA to guide matched targeted therapy in lung cancers, J Natl Cancer Inst. 111 (6) (2019 Jun 1) 575–583.
[29] N.B. Leighl, R.D. Page, V.M. Raymond, D.B. Daniel, S.G. Divers, K.L. Reckamp, et al., Clinical utility of comprehensive cell-free DNA analysis to identify genomic biomarkers in patients with newly diagnosed metastatic non-small cell lung cancer, Clin Cancer Res. 25 (15) (2019 Aug 1) 4691–4700.
[30] C. Aggarwal, C.D. Rolfo, G.R. Oxnard, J.E. Gray, L.M. Sholl, D.R. Gandara, Strategies for the successful implementation of plasma-based NSCLC genotyping in clinical practice, Nat Rev Clin Oncol. 18 (1) (2021) 56–62.
[31] C. Rolfo, N. Meshulami, A. Russo, F. Krammer, A. García-Sastre, P.C. Mack, et al., Lung cancer and severe acute respiratory syndrome coronavirus 2 infection: identifying important knowledge gaps for investigation, J Thorac Oncol. 17 (2) (2022) 214–227.
[32] C. Rolfo, A. Russo, D. de Miguel-Pérez, Speeding tumor genotyping during the SARS-CoV-2 outbreak through liquid biopsy, Cancer 126 (18) (2020) 4089–4091.
[33] W. Cui, C. Milner-Watts, T.P. McVeigh, A. Minchom, J. Bholse, M. Davidson, et al., A pilot of Blood-First diagnostic cell free DNA (ctDNA) next generation sequencing (NGS) in patients with suspected advanced lung cancer, Lung Cancer. 20 (165) (2022 Jan) 34–42.
[34] M. Alcaide, M. Cheung, J. Hillman, S.R. Rassekh, R.J. Deyell, G. Batist, et al., Evaluating the quantity, quality and size distribution of cell-free DNA by multiplex droplet digital PCR, Sci Rep. 10 (1) (2020) 12564.
[35] F. Diehl, M. Li, D. Dressman, Y. He, D. Shen, S. Szabo, et al., Detection and quantification of mutations in the plasma of patients with colorectal tumors, Proc Natl Acad Sci U S A. 102 (45) (2005 Nov 8) 16368–16373.
[36] Y.M.D. Lo, K.C.A. Chan, H. Sun, E.Z. Chen, P. Jiang, F.M.F. Lun, et al., Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus, Sci Transl Med. (2010). Dec 8;2(61):61ra91.
[37] M. Filipska, R. Rosell, Mutated circulating tumor DNA as a liquid biopsy in lung cancer detection and treatment, Mol Oncol. 15 (6) (2021) 1667–1682.
[38] S. Cristiano, A. Leal, J. Phallen, J. Fiksel, V. Adleff, D.C. Bruhm, et al., Genome-wide cell-free DNA fragmentation in patients with cancer, Nature 570 (7761) (2019) 385–389.
[39] M.W. Snyder, M. Kircher, A.J. Hill, R.M. Daza, J. Shendure, Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin, Cell 164 (1–2) (2016 Jan 14) 57–68.
[40] K. Sun, P. Jiang, S.H. Cheng, T.H.T. Cheng, J. Wong, V.W.S. Wong, et al., Orientation-aware plasma cell-free DNA fragmentation analysis in open chromatin regions informs tissue of origin, Genome Res. 29 (3) (2019) 418–427.
[41] P. Ulz, G.G. Thallinger, M. Auer, R. Graf, K. Kashofer, S.W. Jahn, et al., Inferring expressed genes by whole-genome sequencing of plasma DNA, Nat Genet. 48 (10) (2016) 1273–1278.
[42] W.K.J. Lam, W. Gai, K. Sun, R.S.M. Wong, R.W.Y. Chan, P. Jiang, et al., DNA of erythroid origin is present in human plasma and informs the types of anemia, Clin Chem. 63 (10) (2017 Oct) 1614–1623.
[43] J. Moss, J. Magenheim, D. Neiman, H. Zemmour, N. Loyfer, A. Korach, et al., Comprehensive human cell-type methylation atlas reveals origins of circulating cell-free DNA in health and disease, Nat Commun. 9 (1) (2018) 5068.
[44] A.R. Thierry, S. el Messaoudi, P.B. Gahan, P. Anker, M. Stroun, Origins, structures, and functions of circulating DNA in oncology, Cancer Metastasis Rev. 35 (3) (2016 Sep) 347–376.
[45] J. Fernandes, V. Michel, M. Camorlinga-Ponce, A. Gomez, C. Maldonado, H. de Reuse, et al., Circulating mitochondrial DNA level, a noninvasive biomarker for the early detection of gastric cancer, Cancer Epidemiol Biomarkers Prev. 23 (11) (2014 Nov) 2430–2438.
[46] M. Alberry, D. Maddocks, M. Jones, M. Abdel Hadi, S. Abdel-Fattah, N. Avent, et al., Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast, Prenat Diagn. 27 (5) (2007 May) 415–418.
[47] P. Kumar, L.W. Dillon, Y. Shibata, A.A. Jazaeri, D.R. Jones, A. Dutta, Normal and cancerous tissues release extrachromosomal circular DNA (eccDNA) into the circulation, Mol Cancer Res. 15 (9) (2017) 1197–1205.
[48] S.K. Li, R.K.K. Leung, H.X. Guo, J.F. Wei, J.H. Wang, K.T. Kwong, et al., Detection and identification of plasma bacterial and viral elements in HIV/AIDS patients in comparison to healthy adults, Clin Microbiol Infect. 18 (11) (2012 Nov) 1126–1133.
[49] A. Moustafa, C. Xie, E. Kirkness, W. Biggs, E. Wong, Y. Turpaz, et al., The blood DNA virome in 8,000 humans, PLoS Pathog. 13 (3) (2017) e1006292.
[50] G.D. Poore, E. Kopylova, Q. Zhu, C. Carpenter, S. Fraraccio, S. Wandro, et al., Microbiome analyses of blood and tissues suggest cancer diagnostic approach, Nature 579 (7800) (2020) 567–574.

[51] S.J. Schraa, K.L. van Rooijen, M. Koopman, G.R. Vink, R.J.A. Fijneman, Cell-free circulating (Tumor) DNA before surgery as a prognostic factor in non-metastatic colorectal cancer, A Systematic Review. Cancers (Basel) (2022). Apr 29;14(9.
[52] B. Shapiro, M. Chakrabarty, E.M. Cohn, S.A. Leon, Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease, Cancer 51 (11) (1983 Jun 1) 2116–2120.
[53] M. Stroun, P. Anker, P. Maurice, J. Lyautey, C. Lederrey, M. Beljanski, Neoplastic characteristics of the DNA found in the plasma of cancer patients, Oncology. 46 (5) (1989) 318–322.
[54] **Sorenson GD, Pribish DM, Valone FH, Memoli VA, Bzik DJ, Yao SL. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomarkers Prev. 3(1):67–71.**
[55] V. Vasioukhin, P. Anker, P. Maurice, J. Lyautey, C. Lederrey, M. Stroun, Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia, Br J Haematol. 86 (4) (1994 Apr) 774–779.
[56] R.B. Corcoran, B.A. Chabner, Application of cell-free DNA analysis to cancer treatment, N Engl J Med. 379 (18) (2018 Nov 1) 1754–1765.
[57] E. Heitzer, P. Ulz, J.B. Geigl, Circulating tumor DNA as a liquid biopsy for cancer, Clin Chem. 61 (1) (2015 Jan) 112–123.
[58] K.C.S. Oliveira, I.B. Ramos, J.M.C. Silva, W.F. Barra, G.J. Riggins, V. Palande, et al., Current perspectives on circulating tumor DNA, precision medicine, and personalized clinical management of cancer, Mol Cancer Res. 18 (4) (2020) 517–528.
[59] F. Mouliere, B. Robert, E. Arnau Peyrotte, M. del Rio, M. Ychou, F. Molina, et al., High fragmentation characterizes tumour-derived circulating DNA, PLoS ONE 6 (9) (2011) e23418.
[60] P. Jiang, C.W.M. Chan, K.C.A. Chan, S.H. Cheng, J. Wong, V.W.S. Wong, et al., Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients, Proc Natl Acad Sci U S A. 112 (11) (2015 Mar 17) E1317–E1325.
[61] F. Diehl, K. Schmidt, M.A. Choti, K. Romans, S. Goodman, M. Li, et al., Circulating mutant DNA to assess tumor dynamics, Nat Med. 14 (9) (2008 Sep) 985–990.
[62] M. Stroun, J. Lyautey, C. Lederrey, A. Olson-Sand, P. Anker, About the possible origin and mechanism of circulating DNA apoptosis and active DNA release, Clin Chim Acta. 313 (1–2) (2001 Nov) 139–142.
[63] C. Bettegowda, M. Sausen, R.J. Leary, I. Kinde, Y. Wang, N. Agrawal, et al., Detection of circulating tumor DNA in early- and late-stage human malignancies, Sci Transl Med. (2014). Feb 19;6(224):224ra24.
[64] A. Kustanovich, R. Schwartz, T. Peretz, A. Grinshpun, Life and death of circulating cell-free DNA, Cancer Biol Ther. 20 (8) (2019) 1057–1067.
[65] P.C. Mack, J. Miao, M.W. Redman, J. Moon, S.B. Goldberg, R.S. Herbst, et al., Circulating tumor DNA (ctDNA) kinetics predict progression-free and overall survival in EGFR TKI-treated patients with EGFR-mutant NSCLC (SWOG S1403), Clin Cancer Res (2022 Jun 17).
[66] S. Avanzini, D.M. Kurtz, J.J. Chabon, E.J. Moding, S.S. Hori, S.S. Gambhir, et al., A mathematical model of ctDNA shedding predicts tumor detection size, Sci Adv. 6 (50) (2020).
[67] B. Vogelstein, N. Papadopoulos, V.E. Velculescu, S. Zhou, L.A. Diaz, K.W. Kinzler, Cancer genome landscapes, Science 339 (6127) (2013 Mar 29) 1546–1558.
[68] S.J. Dawson, D.W.Y. Tsui, M. Murtaza, H. Biggs, O.M. Rueda, S.F. Chin, et al., Analysis of circulating tumor DNA to monitor metastatic breast cancer, N Engl J Med. 368 (13) (2013 Mar 28) 1199–1209.
[69] L.A. Diaz, M. Sausen, G.A. Fisher, V.E. Velculescu, Insights into therapeutic resistance from whole-genome analyses of circulating tumor DNA, Oncotarget. 4 (10) (2013 Oct) 1856–1857.
[70] L.A. Diaz, R.T. Williams, J. Wu, I. Kinde, J.R. Hecht, J. Berlin, et al., The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers, Nature 486 (7404) (2012 Jun 28) 537–540.
[71] M. Holdhoff, K. Schmidt, R. Donehower, L.A. Diaz, Analysis of circulating tumor DNA to confirm somatic KRAS mutations, J Natl Cancer Inst. 101 (18) (2009 Sep 16) 1284–1285.
[72] R.J. Leary, M. Sausen, I. Kinde, N. Papadopoulos, J.D. Carpten, D. Craig, et al., Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing, Sci Transl Med. (2012). Nov 28;4(162):162ra154.
[73] M. Murtaza, S.J. Dawson, D.W.Y. Tsui, D. Gale, T. Forshew, A.M. Piskorz, et al., Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA, Nature 497 (7447) (2013 May 2) 108–112.
[74] D.P. Steensma, R. Bejar, S. Jaiswal, R.C. Lindsley, M.A. Sekeres, R.P. Hasserjian, et al., Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes, Blood 126 (1) (2015 Jul 2) 9–16.
[75] Y. Hu, B.C. Ulrich, J. Supplee, Y. Kuang, P.H. Lizotte, N.B. Feeney, et al., False-Positive Plasma Genotyping Due to Clonal Hematopoiesis, Clin Cancer Res. 24 (18) (2018) 4437–4443.
[76] J. Liu, X. Chen, J. Wang, S. Zhou, C.L. Wang, M.Z. Ye, et al., Biological background of the genomic variations of cf-DNA in healthy individuals, Ann Oncol. 30 (3) (2019) 464–470.
[77] P. Razavi, B.T. Li, D.N. Brown, B. Jung, E. Hubbell, R. Shen, et al., High-intensity sequencing reveals the sources of plasma circulating cell-free DNA variants, Nat Med. 25 (12) (2019) 1928–1937.
[78] L. Xia, Z. Li, B. Zhou, G. Tian, L. Zeng, H. Dai, et al., Statistical analysis of mutant allele frequency level of circulating cell-free DNA and blood cells in healthy individuals, Sci Rep. 7 (1) (2017) 7526.
[79] Y.Y.N. Lui, K.W. Chik, R.W.K. Chiu, C.Y. Ho, C.W.K. Lam, Y.M.D. Lo, Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation, Clin Chem. 48 (3) (2002 Mar) 421–427.
[80] B.T. Li, F. Janku, B. Jung, C. Hou, K. Madwani, R. Alden, et al., Ultra-deep next-generation sequencing of plasma cell-free DNA in patients with advanced lung cancers: results from the Actionable Genome Consortium, Ann Oncol. 30 (4) (2019) 597–603.
[81] J.J. Chabon, E.G. Hamilton, D.M. Kurtz, M.S. Esfahani, E.J. Moding, H. Stehr, et al., Integrating genomic features for non-invasive early lung cancer detection, Nature 580 (7802) (2020) 245–251.
[82] A. Leal, N.C.T. van Grieken, D.N. Palsgrove, J. Phallen, J.E. Medina, C. Hruban, et al., White blood cell and cell-free DNA analyses for detection of residual disease in gastric cancer, Nat Commun. 11 (1) (2020 Jan 27) 525.
[83] F. Marass, D. Stephens, R. Ptashkin, A. Zehir, M.F. Berger, D.B. Solit, et al., Fragment Size Analysis May Distinguish Clonal Hematopoiesis from Tumor-Derived Mutations in Cell-Free DNA, Clin Chem. 66 (4) (2020) 616–618.
[84] H.T. Chan, Y.M. Chin, Y. Nakamura, S.K. Low, Clonal Hematopoiesis in Liquid Biopsy: From Biological Noise to Valuable Clinical Implications, Cancers (Basel) (2020). Aug 14;12(8.
[85] R. Lampignano, M.H.D. Neumann, S. Weber, V. Kloten, A. Herdean, T. Voss, et al., Multicenter Evaluation of Circulating Cell-Free DNA Extraction and Downstream Analyses for the Development of Standardized (Pre)analytical Work Flows, Clin Chem. 66 (1) (2020) 149–160.
[86] D. Grölz, S. Hauch, M. Schlumpberger, K. Guenther, T. Voss, M. Sprenger-Haussels, et al., Liquid Biopsy Preservation Solutions for Standardized Pre-Analytical Workflows-Venous Whole Blood and Plasma, Curr Pathobiol Rep. 6 (4) (2018) 275–286.
[87] D. Connors, J. Allen, J.D. Alvarez, J. Boyle, M. Cristofanilli, C. Hiller, et al., International liquid biopsy standardization alliance white paper, Crit Rev Oncol Hematol. 156 (2020 Dec), 103112.
[88] J.J.K. Lee, S. Park, H. Park, S. Kim, J. Lee, J. Lee, et al., Tracing Oncogene Rearrangements in the Mutational History of Lung Adenocarcinoma, Cell 177 (7) (2019) 1842–1857.e21.
[89] J.D. Cohen, L. Li, Y. Wang, C. Thoburn, B. Afsari, L. Danilova, et al., Detection and localization of surgically resectable cancers with a multi-analyte blood test, Science 359 (6378) (2018 Feb 23) 926–930.
[90] A.M. Newman, A.F. Lovejoy, D.M. Klass, D.M. Kurtz, J.J. Chabon, F. Scherer, et al., Integrated digital error suppression for improved detection of circulating tumor DNA, Nat Biotechnol. 34 (5) (2016) 547–555.
[91] N. Howlader, G. Forjaz, M.J. Mooradian, R. Meza, C.Y. Kong, K.A. Cronin, et al., The Effect of Advances in Lung-Cancer Treatment on Population Mortality, N Engl J Med. 383 (7) (2020) 640–649.
[92] M.G. Kris, B.E. Johnson, L.D. Berry, D.J. Kwiatkowski, A.J. Iafrate, I.I. Wistuba, et al., Using multiplexed assays of oncogenic drivers in lung cancers to select targeted drugs, JAMA 311 (19) (2014 May 21) 1998–2006.
[93] R.L. Siegel, K.D. Miller, H.E. Fuchs, A. Jemal, Cancer Statistics, 2021, CA Cancer J Clin. 71 (1) (2021) 7–33.
[94] M.R. Luskin, M.A. Murakami, S.R. Manalis, D.M. Weinstock, Targeting minimal residual disease: a path to cure? Nat Rev Cancer. 18 (4) (2018) 255–263.
[95] E.T. Goddard, I. Bozic, S.R. Riddell, C.M. Ghajar, Dormant tumour cells, their niches and the influence of immunity, Nat Cell Biol. 20 (11) (2018) 1240–1249.
[96] R. Pieters, H. de Groot-Kruseman, V. van der Velden, M. Fiocco, H. van den Berg, E. de Bont, et al., Successful Therapy Reduction and Intensification for Childhood Acute Lymphoblastic Leukemia Based on Minimal Residual Disease Monitoring: Study ALL10 From the Dutch Childhood Oncology Group, J Clin Oncol. 34 (22) (2016) 2591–2601.
[97] C.S. Hourigan, J.E. Karp, Minimal residual disease in acute myeloid leukaemia, Nat Rev Clin Oncol. 10 (8) (2013 Aug) 460–471.
[98] H. Schwarzenbach, D.S.B. Hoon, K. Pantel, Cell-free nucleic acids as biomarkers in cancer patients, Nat Rev Cancer. 11 (6) (2011 Jun) 426–437.
[99] C. Abbosh, N.J. Birkbak, C. Swanton, Early stage NSCLC - challenges to implementing ctDNA-based screening and MRD detection, Nat Rev Clin Oncol. 15 (9) (2018) 577–586.
[100] R. de Kock, B. van den Borne, M. Youssef-El Soud, H. Belderbos, L. Brunsveld, V. Scharnhorst, et al., Therapy Monitoring of EGFR-Positive Non-Small-Cell Lung Cancer Patients Using ddPCR Multiplex Assays, J Mol Diagn. 23 (4) (2021) 495–505.
[101] A.A. Chaudhuri, J.J. Chabon, A.F. Lovejoy, A.M. Newman, H. Stehr, T.D. Azad, et al., Early Detection of Molecular Residual Disease in Localized Lung Cancer by Circulating Tumor DNA Profiling, Cancer Discov. 7 (12) (2017) 1394–1403.
[102] J.E. Reuss, V. Anagnostou, T.R. Cottrell, K.N. Smith, F. Verde, M. Zahurak, et al., Neoadjuvant nivolumab plus ipilimumab in resectable non-small cell lung cancer, J Immunother Cancer. 8 (2) (2020).
[103] M.D. Hellmann, B.Y. Nabet, H. Rizvi, A.A. Chaudhuri, D.K. Wells, M.P.S. Dunphy, et al., Circulating Tumor DNA Analysis to Assess Risk of Progression after Long-term Response to PD-(L)1 Blockade in NSCLC, Clin Cancer Res. 26 (12) (2020) 2849–2858.
[104] E.J. Moding, Y. Liu, B.Y. Nabet, J.J. Chabon, A.A. Chaudhuri, A.B. Hui, et al., Circulating Tumor DNA Dynamics Predict Benefit from Consolidation Immunotherapy in Locally Advanced Non-Small Cell Lung Cancer, Nat Cancer. 1 (2) (2020) 176–183.
[105] E.J. Moding, B.Y. Nabet, A.A. Alizadeh, M. Diehn, Detecting Liquid Remnants of Solid Tumors: Circulating Tumor DNA Minimal Residual Disease, Cancer Discov. 11 (12) (2021) 2968–2986.
[106] A. Tognela, K.J. Spring, T. Becker, N.J. Caixeiro, V.J. Bray, P.Y. Yip, et al., Predictive and prognostic value of circulating tumor cell detection in lung cancer: a clinician's perspective, Crit Rev Oncol Hematol. 93 (2) (2015 Feb) 90–102.

[107] C.Y. Wu, C.L. Lee, C.F. Wu, J.Y. Fu, C.T. Yang, C.T. Wen, et al., Circulating Tumor Cells as a Tool of Minimal Residual Disease Can Predict Lung Cancer Recurrence: A longitudinal, Prospective Trial. Diagnostics (Basel) (2020). Mar 6;10(3.

[108] A.R. Parikh, E.E. van Seventer, G. Siravegna, A. Hartwig, v, Jaimovich A, He Y,, et al., Minimal Residual Disease Detection using a Plasma-only Circulating Tumor DNA Assay in Patients with Colorectal Cancer, Clin Cancer Res. 27 (20) (2021) 5586–5594.

[109] X. Chen, Z. Dong, E. Hubbell, K.N. Kurtzman, G.R. Oxnard, O. Venn, et al., Prognostic Significance of Blood-Based Multi-cancer Detection in Plasma Cell-Free DNA, Clin Cancer Res. 27 (15) (2021) 4221–4229.

[110] I. Bozic, J.G. Reiter, B. Allen, T. Antal, K. Chatterjee, P. Shah, et al., Evolutionary dynamics of cancer in response to targeted combination therapy, Elife. 25 (2) (2013 Jun) e00747.

[111] T.J. Semrad, A.R. Fahrni, I.Y. Gong, V.P. Khatri, Integrating Chemotherapy into the Management of Oligometastatic Colorectal Cancer: Evidence-Based Approach Using Clinical Trial Findings, Ann Surg Oncol. 22 (Suppl 3) (2015 Dec) S855–S862.

[112] A.C. Huang, M.A. Postow, R.J. Orlowski, R. Mick, B. Bengsch, S. Manne, et al., T-cell invigoration to tumour burden ratio associated with anti-PD-1 response, Nature 545 (7652) (2017) 60–65.

[113] C.G. Moertel, T.R. Fleming, J.S. Macdonald, D.G. Haller, J.A. Laurie, C.M. Tangen, et al., Fluorouracil plus levamisole as effective adjuvant therapy after resection of stage III colon carcinoma: a final report, Ann Intern Med. 122 (5) (1995 Mar 1) 321–326.

[114] C. Abbosh, N.J. Birkbak, G.A. Wilson, M. Jamal-Hanjani, T. Constantin, R. Salari, et al., Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution, Nature 545 (7655) (2017) 446–451.

[115] I.A. Cree, L. Uttley, H. Buckley Woods, H. Kikuchi, A. Reiman, S. Harnan, et al., The evidence base for circulating tumour DNA blood-based biomarkers for the early detection of cancer: a systematic mapping review, BMC Cancer. 17 (1) (2017 Oct 23) 697.

[116] M. Scimia, J. Du, F. Pepe, M.A. Bianco, S. Russo Spena, F. Patell-Socha, et al., Evaluation of a novel liquid biopsy-based ColoScape assay for mutational analysis of colorectal neoplasia and triage of FIT+ patients: a pilot study, J Clin Pathol. 71 (12) (2018 Dec) 1123–1126.

[117] J.D. Cohen, A.A. Javed, C. Thoburn, F. Wong, J. Tie, P. Gibbs, et al., Combined circulating tumor DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers, Proc Natl Acad Sci U S A. 114 (38) (2017) 10202–10207.

[118] S.Y. Shen, R. Singhania, G. Fehringer, A. Chakravarthy, M.H.A. Roehrl, D. Chadwick, et al., Sensitive tumour detection and classification using plasma cell-free DNA methylomes, Nature 563 (7732) (2018) 579–583.

[119] D. Mathios, J.S. Johansen, S. Cristiano, J.E. Medina, J. Phallen, K.R. Larsen, et al., Detection and characterization of lung cancer using cell-free DNA fragmentomes, Nat Commun. 12 (1) (2021) 5060.

[120] Liu MC, Oxnard GR, Klein EA, Swanton C, Seiden M v, CCGA Consortium, Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA, Ann Oncol. 31 (6) (2020) 745–759.

[121] A. Andermann, I. Blancquaert, S. Beauchamp, V. Déry, Revisiting Wilson and Jungner in the genomic age: a review of screening criteria over the past 40 years, Bull World Health Organ. 86 (4) (2008 Apr) 317–319.

[122] P.S. Steeg, Targeting metastasis, Nat Rev Cancer. 16 (4) (2016 Apr) 201–218.

[123] J. Beane, J.D. Campbell, J. Lel, J. Vick, A. Spira, Genomic approaches to accelerate cancer interception, Lancet Oncol. 18 (8) (2017) e494–e502.

[124] M.B. Wozniak, G. Scelo, D.C. Muller, A. Mukeria, D. Zaridze, P. Brennan, Circulating MicroRNAs as Non-Invasive Biomarkers for Early Detection of Non-Small-Cell Lung Cancer, PLoS ONE 10 (5) (2015) e0125026.

[125] T. Powrózek, P. Krawczyk, D.M. Kowalski, K. Winiarczyk, M. Olszyna-Serementa, J. Milanowski, Plasma circulating microRNA-944 and microRNA-3662 as potential histologic type-specific early lung cancer biomarkers, Transl Res. 166 (4) (2015 Oct) 315–323.

[126] G. Sozzi, M. Boeri, M. Rossi, C. Verri, P. Suatoni, F. Bravi, et al., Clinical utility of a plasma-based miRNA signature classifier within computed tomography lung cancer screening: a correlative MILD trial study, J Clin Oncol. 32 (8) (2014 Mar 10) 768–773.

[127] F. Montani, M.J. Marzi, F. Dezi, E. Dama, R.M. Carletti, G. Bonizzi, et al., miR-Test: a blood test for lung cancer early detection, J Natl Cancer Inst. 107(6): djv063 (2015 Jun).

[128] J.E. Beane, S.A. Mazzilli, J.D. Campbell, G. Duclos, K. Krysan, C. Moy, et al., Molecular subtyping reveals immune alterations associated with progression of bronchial premalignant lesions, Nat Commun. 10 (1) (2019) 1856.

[129] M. Reck, D. Rodríguez-Abreu, A.G. Robinson, R. Hui, T. Csőszi, A. Fülöp, et al., Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer, N Engl J Med. 375 (19) (2016) 1823–1833.

[130] T.S.K. Mok, Y.L. Wu, I. Kudaba, D.M. Kowalski, B.C. Cho, H.Z. Turna, et al., Pembrolizumab versus chemotherapy for previously untreated, PD-L1-expressing, locally advanced or metastatic non-small-cell lung cancer (KEYNOTE-042): a randomised, open-label, controlled, phase 3 trial, Lancet 393 (10183) (2019) 1819–1830.

[131] Y. Zhang, Z. Zhang, The history and advances in cancer immunotherapy: understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications, Cell Mol Immunol. 17 (8) (2020) 807–821.

[132] F.R. Hirsch, A. McElhinny, D. Stanforth, J. Ranger-Moore, M. Jansson, K. Kulangara, et al., PD-L1 Immunohistochemistry Assays for Lung Cancer: Results from Phase 1 of the Blueprint PD-L1 IHC Assay Comparison Project, J Thorac Oncol. 12 (2) (2017) 208–222.

[133] M.S. Tsao, K.M. Kerr, M. Kockx, M.B. Beasley, A.C. Borczuk, J. Botling, et al., PD-L1 Immunohistochemistry Comparability Study in Real-Life Clinical Samples: Results of Blueprint Phase 2 Project, J Thorac Oncol. 13 (9) (2018) 1302–1311.

[134] Y. Xu, B. Wan, X. Chen, P. Zhan, Y. Zhao, T. Zhang, et al., The association of PD-L1 expression with the efficacy of anti-PD-1/PD-L1 immunotherapy and survival of non-small cell lung cancer patients: a meta-analysis of randomized controlled trials, Transl Lung Cancer Res. 8 (4) (2019 Aug) 413–428.

[135] S. Gadgeel, D. Rodríguez-Abreu, G. Speranza, E. Esteban, E. Felip, M. Dómine, et al., Updated Analysis From KEYNOTE-189: Pembrolizumab or Placebo Plus Pemetrexed and Platinum for Previously Untreated Metastatic Nonsquamous Non-Small-Cell Lung Cancer, J Clin Oncol. 38 (14) (2020) 1505–1517.

[136] Z.R. Chalmers, C.F. Connelly, D. Fabrizio, L. Gay, S.M. Ali, R. Ennis, et al., Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden, Genome Med. 9 (1) (2017) 34.

[137] A. Galvano, V. Gristina, U. Malapelle, P. Pisapia, F. Pepe, N. Barraco, et al., The prognostic impact of tumor mutational burden (TMB) in the first-line management of advanced non-oncogene addicted non-small-cell lung cancer (NSCLC): a systematic review and meta-analysis of randomized controlled trials, ESMO Open. 6 (3) (2021), 100124.

[138] K.M. Kerr, F. Bibeau, E. Thunnissen, J. Botling, A. Ryška, J. Wolf, et al., The evolving landscape of biomarker testing for non-small cell lung cancer in Europe, Lung Cancer. 154 (2021) 161–175.

[139] P. Pisapia, U. Malapelle, G. Troncone, Liquid Biopsy and Lung Cancer, Acta Cytol. 63 (6) (2019) 489–496.

[140] R. Büttner, J.W. Longshore, F. López-Ríos, S. Merkelbach-Bruse, N. Normanno, E. Rouleau, et al., Implementing TMB measurement in clinical practice: considerations on assay requirements, ESMO Open. 4 (1) (2019) e000442.

[141] D.R. Gandara, S.M. Paul, M. Kowanetz, E. Schleifman, W. Zou, Y. Li, et al., Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab, Nat Med. 24 (9) (2018) 1441–1448.

[142] N.A. Rizvi, B.C. Cho, N. Reinmuth, K.H. Lee, A. Luft, M.J. Ahn, et al., Durvalumab With or Without Tremelimumab vs Standard Chemotherapy in First-line Treatment of Metastatic Non-Small Cell Lung Cancer: The MYSTIC Phase 3 Randomized Clinical Trial, JAMA Oncol. 6 (5) (2020) 661–674.

[143] P. Hofman, S. Heeke, C. Alix-Panabieres, K. Pantel, Liquid biopsy in the era of immuno-oncology: is it ready for prime-time use for cancer patients? Ann Oncol. 30 (9) (2019) 1448–1459.

[144] N. Guibert, M. Delaunay, A. Lusque, N. Boubekeur, I. Rouquette, E. Clermont, et al., PD-L1 expression in circulating tumor cells of advanced non-small cell lung cancer patients treated with nivolumab, Lung Cancer. 120 (2018) 108–112.

[145] M. Ilié, E. Szafer-Glusman, V. Hofman, E. Chamorey, S. Lalvée, E. Selva, et al., Detection of PD-L1 in circulating tumor cells and white blood cells from patients with advanced non-small-cell lung cancer, Ann Oncol. 29 (1) (2018) 193–199.

[146] G. Chen, A.C. Huang, W. Zhang, G. Zhang, M. Wu, W. Xu, et al., Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response, Nature 560 (7718) (2018) 382–386.

[147] S. Park, J.C. Lee, C.M. Choi, Clinical Applications of Liquid Biopsy in Non-Small Cell Lung Cancer Patients: Current Status and Recent Advances in Clinical Practice, J Clin Med. (2021). May 21;10(11.

[148] **Rijavec E, Coco S, Genova C, Rossi G, Longo L, Grossi F. Liquid Biopsy in Non-Small Cell Lung Cancer: Highlights and Challenges. Cancers (Basel). 2019 Dec 19; 12(1).**

[149] P. Pisapia, J.L. Costa, F. Pepe, G. Russo, G. Gragnano, A. Russo, et al., Next generation sequencing for liquid biopsy based testing in non-small cell lung cancer in 2021, Crit Rev Oncol Hematol. 161 (2021 May), 103311.

[150] P.K. Paik, E. Felip, R. Veillon, H. Sakai, A.B. Cortot, M.C. Garassino, et al., Tepotinib in Non-Small-Cell Lung Cancer with MET Exon 14 Skipping Mutations, N Engl J Med. 383 (10) (2020) 931–943.

[151] **Bennouna J, Girard N, Audigier-Valette C, le Thuaut A, Gervais R, Masson P, et al. Phase II Study Evaluating the Mechanisms of Resistance on Tumor Tissue and Liquid Biopsy in Patients With EGFR-mutated Non-pretreated Advanced Lung Cancer Receiving Osimertinib Until and Beyond Radiologic Progression: The MELROSE Trial. Clin Lung Cancer. 2020;21(1):e10–4.**

[152] **Reclusa P, Laes JF, Malapelle U, Valentino A, Rocco D, Gil-Bazo I, et al. EML4-ALK translocation identification in RNA exosomal cargo (ExoALK) in NSCLC patients: a novel role for liquid biopsy. Transl Cancer Res. 2019 Jan;8(Suppl 1): S76–8.**

[153] D. de Miguel-Perez, A. Russo, O. Arrieta, M. Ak, F. Barron, M. Gunasekaran, et al., Extracellular vesicle PD-L1 dynamics predict durable response to immune-checkpoint inhibitors and survival in patients with non-small cell lung cancer, J Exp Clin Cancer Res. 41 (1) (2022 Jun 2) 186.

[154] A.G. Nicholson, M.S. Tsao, M.B. Beasley, A.C. Borczuk, E. Brambilla, W.A. Cooper, et al., The 2021 WHO Classification of Lung Tumors: Impact of Advances Since 2015, J Thorac Oncol. 17 (3) (2022) 362–387.

[155] D. Hanahan, Hallmarks of Cancer: New Dimensions, Cancer Discov. 12 (1) (2022) 31–46.

[156] M.K. Gospodarowicz, D. Miller, P.A. Groome, F.L. Greene, P.A. Logan, L.H. Sobin, The process for continuous improvement of the TNM classification, Cancer 100 (1) (2004 Jan 1) 1–5.

[157] M. Schwaederle, R. Chattopadhyay, S. Kato, P.T. Fanta, K.C. Banks, I.S. Choi, et al., Genomic Alterations in Circulating Tumor DNA from Diverse Cancer Patients Identified by Next-Generation Sequencing, Cancer Res. 77 (19) (2017) 5419–5427.

[158] M. Yang, M.E. Forbes, R.L. Bitting, S.S. O'Neill, P.C. Chou, U. Topaloglu, et al., Incorporating blood-based liquid biopsy information into cancer staging: time for a TNMB system? Ann Oncol. 29 (2) (2018) 311–323.

[159] J. Pascual, G. Attard, F.C. Bidard, G. Curigliano, L. de Mattos-Arruda, M. Diehn, et al., ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: a report from the ESMO Precision Medicine Working Group. Ann Oncol (2022 Jun 9).

[160] A. Passaro, N. Leighl, F. Blackhall, S. Popat, K. Kerr, M.J. Ahn, et al., ESMO expert consensus statements on the management of EGFR mutant non-small-cell lung cancer. Ann Oncol. 33 (5) (2022 May) 466–487.

[161] W. Cai, C. Milner-Watts, T.P. McVeigh, A. Minchom, J. Bholse, M. Davidson, et al., A pilot of Blood-First diagnostic cell free DNA (cfDNA) next generation sequencing (NGS) in patients with suspected advanced lung cancer, Lung Cancer. 20 (165) (2022 Jan) 34–42.