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Next generation sequencing for liquid biopsy based testing in non-small cell lung cancer in 2021

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ABSTRACT

Lung cancer is the leading cause of cancer death worldwide, with non-small cell lung cancer (NSCLC) representing its most commonly diagnosed sub-type. Despite the significant improvements in lung cancer biomarkers knowledge, accompanied by substantial technological advances in molecular tumor profiling, a considerable fraction (up to 30 %) of advanced NSCLC patient presents with major testing challenges or tissue unavailability for molecular analysis. In this context, liquid biopsy is on the rise, currently gaining considerable interest within the molecular pathology and oncology community. Molecular profiling of liquid biopsy specimens using next generation molecular biology methodologies is a rapidly evolving field with promising applications not exclusively limited to advanced stages but also more recently expanding to early stages cancer patients. Here, we offer an overview of some of the most consolidated and emerging applications of next generation sequencing technologies for liquid biopsy testing in NSCLC.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide, with non-small cell lung cancer (NSCLC) representing its most commonly diagnosed sub-type (Siegel et al., 2021). Molecularly informed treatment decisions based on molecular pathology analyses play a pivotal role in the correct management of these patients (Matias-Guiu et al., 2020; Malone et al., 2020; El-Deiry et al., 2019; Conway et al., 2019). However, despite the significant improvements in lung cancer biomarkers knowledge accompanied by substantial technological advances in molecular tumor profiling, a considerable fraction (up to 30 %) of advanced NSCLC patient specimens presents with major testing challenges or are inadequate for molecular analysis purposes (Pisapia et al., 2019). With the promise of being an adequate alternative testing option for not assessable tissue samples, thus avoiding to leave any patient with an incomplete biomarker profile, liquid biopsy is on the rise and it is currently gaining considerable interest within the molecular pathology and oncology community (Crowley et al., 2013). Different biological fluids (blood, urine, cerebro-spinal fluid, effusions, saliva, etc.) and multiple analytes (circulating tumor DNA [ctDNA], ctRNA, extracellular vesicles [EVs], circulating tumor cells [CTCs], tumor educated platelets [TEPs], etc.) are under investigation in this novel and fascinating field (Table 1) (Rolfo et al., 2018, 2020; Siravegna et al., 2017; Mader and Pantel, 2017; Hofman and Popper, 2016; Pennell et al., 2019; Taverna et al., 2016; Reclusa et al., 2016; Wu et al., 2020; Shohdy and West, 2020; Neumann et al., 2018; Gorgannezhad et al., 2018; Reclusa et al., 2019; Best et al., 2015a, 2018; De Rubis et al., 2019).

The complete integration of all circulating factors into the bloodstream can be considered as the "tumor circulome." Within the "tumor circulome", ctDNA and CTCs are the only components that have been

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approved by the US Food and Drug Administration (FDA) in clinical practice, whereas EVs, ctRNA, and TEPs are relatively novel "tumor circulome" elements with promising potential at any stage of cancer for an adequate clinical management. Although several efforts are still needed to better define the future role of all circulating factors in cancer diagnosis, monitoring, and prognosis, different evidences highlighted the possibility to adopt these novel elements in the future clinical cancer management (De Rubis et al., 2019).

So far, for advanced stages NSCLC patients, ctDNA extracted from plasma is the only clinically approved FDA cleared multiple tests analyte for the assessment of epidermal growth factor receptor (*EGFR*) gene status (Rolfo et al., 2018, 2020). In regards to *EGFR* analysis, liquid biopsy represents a valid and consolidated alternative to tissue samples molecular testing in newly diagnosed advanced stage NSCLC patients, naïve to any treatment and without tissue availability or with inadequate tissue molecular results (basal setting), for the administration of first or second generation tyrosine kinase inhibitors (TKIs) (Pisapia et al., 2019; Rolfo et al., 2018, 2020). The same applies for advanced stages NSCLC patients who develop resistance after a first or second generation TKI treatment. In these patients, liquid biopsy may be adopted as first approach to investigate the occurrence of *EGFR* exon 20 p.T790 M resistance point mutation, in order to administrate third generation TKIs (Pisapia et al., 2019; Rolfo et al., 2018, 2020). Undoubtedly, compared to tissue sampling, liquid biopsy can minimize patient's discomfort and reduce medical risks along with the possibility to partially overcome issues related to tumor heterogeneity (Sacher et al., 2017; Bai and Zhao, 2018; Jamal-Hanjani et al., 2017). Conversely, several limitations affect ctDNA testing effectiveness, including reduced analyte half-life (about 15 min) and limited concentration into the bloodstream (usually <0.5 % of the total circulating cell free DNA) (Malapelle et al., 2016). Additionally, mounting evidence suggest that ctDNA concentration is significantly influenced by the pathological stage of disease, i.e. ctDNA amount in the bloodstream is higher in the advanced compared to early stages (Fig. 1) (Siravegna et al., 2019; Malapelle et al., 2017a).

While several studies have investigated the potential role and implications of ctDNA profiling in early-stage lung cancer patients (Phallen et al., 2017; Cohen et al., 2018; Chaudhuri et al., 2017; Abbosh et al., 2017), its application in this setting warrant larger and more thoughtful evaluations. Notably, as discussed in detail in the next paragraph, when attempting to analyze circulating tumor nucleic acids at very low concentration, as for in early stages of cancer, it is fundamental to adopt highly sensitive molecular approaches (Chen and Zhao, 2019; Serrano et al., 2020; Beane et al., 2017) (Fig. 1).

Here, we reviewed the most recent advances in the field of liquid biopsy testing via next generation sequencing (NGS) for NSCLC patients.

2. ctDNA from liquid biopsy: notable samples handling considerations

As above mentioned, despite the significant predictive role of ctDNA in the correct management of NSCLC patients, several pre-analytical issues may affect the adoption of this analyte in the clinical routine practice (Rolfo et al., 2018, 2020). In particular, as reported ctDNA suffers from a very low half-life and concentration into the bloodstream (Pisapia et al., 2019; Lam et al., 2004). To overcome these limitations, the International Association for the Study of Lung Cancer (IASLC) recommended in a statement paper how to adequately handle blood samples (Rolfo et al., 2018). Considering pre-analytical phases, careful attention should be paid to three crucial steps: blood collection, centrifugation, and ctDNA extraction (Rolfo et al., 2018). Regarding blood samples collection, different tubes may be employed by laboratories with each holding different advantages and disadvantages. Briefly, ethylenediaminetetra-acetic acid (K2-EDTA)-containing tubes avoid blood clotting and are less expensive respect to preservative tubes (Lam et al., 2004). However, when EDTA-tubes are used, blood samples have to be processes as quickly as possible (within 2−4 h from collection) (Sherwood et al., 2016). It is conceivable to adopt this collection approach when blood withdrawal and ctDNA analysis are performed within the same or nearby physical location, thus minimizing any processing delay. In contrast, preservative tubes, such as PAXgene Blood DNA tubes or Cell-Free DNA BCT tubes, enable to stabilize ctDNA (up to 14 days upon collection depending on storage conditions), thus contributing to ease the sample management and consequent time-pressure to generate results (Medina Diaz et al., 2016; Rothwell et al., 2016). Sample stabilization in achieved by means of formaldehyde-free preservative agents enabling a significant reduction in ctDNA degradation (Schmidt et al., 2017). Some reports suggest that, Cell-Free DNA BCT showed a higher ability to prevent cell lysis compared to PAXgene Blood DNA tubes (Toro et al., 2015). Overall, to optimize ctDNA yield increase testing success rate, IASLC recommendations established that the time between blood sampling and ctDNA extraction should not exceed the two hours for EDTA-tubes and three days for tube containing preservative solutions (Rolfo et al., 2018). Following collection, another essential step is centrifugation. This phase is essential to remove all formed elements of the blood, in particular those containing a nucleus with non-neoplastic genomic DNA (such as white cells), ensuring a proficient plasma samples purification (Sherwood et al., 2016). In this setting, Malapelle et al. proposed a two steps centrifugation-based protocol (both at 2300 G for 10 min at room temperature). After this procedure, the authors were able to obtain 2 mL of clarified plasma from 10 mL of blood sample (Malapelle et al., 2017b). A different approach by Sorber et al. utilizes and adapted protocol from the European Committee for Standardization (CEN) (two-step, 1900 G for 10 min and then 16,000 G for 10 min at room temperature) and has been shown to produce ctDNA of high quality (Sorber et al., 2019). Page

Table 1
Principal analytes identified in blood samples: pros and cons.

Analyte	Pros	Cons
ctDNA	- highly studied - clinical approved for targeted treatments - high specificity (no false positive results)	- short half life - low concentration - low sensitivity (possibility of false negative results)
CTC	- clinical approved for targeted treatments - ensure morph-molecular analysis	- low concentration - low specificity in particular when considering early stage of disease - challenging isolation and extraction
Exosomes	- ensure nucleic acids stabilization - high serum concentration	- lack of standardization - challenging isolation and extraction
ctRNA	- ensure the analysis of gene rearrangements and genomic aberrations	- lack of standardization - short half life - low concentraion
Serum biomarkers	- easy to analyze - less expensive	- very low sensitivity and specificity

Abbreviations: CTC: circulating tumor cell; ctDNA: circulating tumor DNA; ctRNA: circulating tumor RNA.

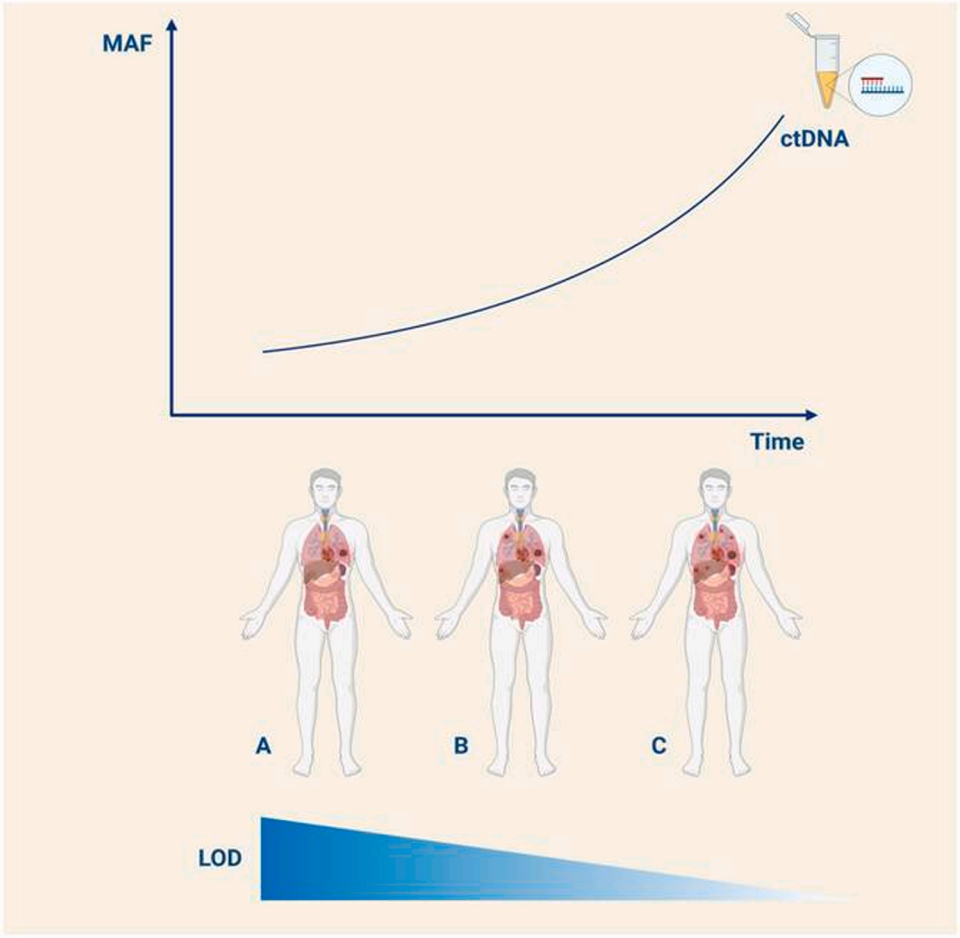

Fig. 1. ctDNA concentration into the bloodstream is usually lower in early stages compared to more advanced stages (from A to C). In this setting, highly sensitive molecular approaches are required. LOD represents the lowest quantity of an analyte that can be distinguished from the absence of that analyte with a stated confidence level (generally 99 %). Abbreviations: ctDNA: circulating tumor DNA; LOD: limit of detection; MAF: mutant allelic fraction. (Credit: Created with Biorender).

et al. suggest instead an additional third centrifugation step (the first at 1000 G for 10 min at 4 °C, the second at 1000 G, or 2000 G, or 10,000 G for 10 min at 4 °C, and the third, performed after thawing, at 1000 G for 5 min at room temperature) to reach high quality and purified plasma samples (Page et al., 2013). Overall, to optimize plasma purification, IASLC recommendations established to perform at least two centrifugation steps. In particular, it is suggested to utilize the original collection tube for the first centrifugation followed by a second tube for the additional centrifugation steps (Rolfo et al., 2018). Last but not least, the final pre-analytical phase is represented by ctDNA extraction. So far, a number of commercial kits are available for this purpose. Overall, 11 different methodologies for ctDNA extraction have been compared in terms of accuracy and reproducibility (Mauger et al., 2015). Each of the tested solutions presents a number of advantages and pitfalls. However overall, the implementation of a magnetic beads–based fully automated protocols represents a reliable and efficient solution (Sorber et al., 2017). Campos et al. adopted a novel microfluidic solid-phase extraction device (μSPE) obtaining a high ctDNA recovery rate for DNA fragment of 100–700 bp and of 50 bp (>90 % and >70 %, respectively), with a limited intrusion of genomic DNA (0.01 %) (Campos et al., 2018). In conclusion, the above described pre-analytical steps are required to obtain a high quality ctDNA and represents a crucial aspect for an appropriate management of liquid biopsy samples. Pre-analytical steps are summarized in Table 2.

3. NGS technologies: enabling the path for cancer detection using liquid biopsy

As previously introduced, early stages lung cancer patients feature a

Table 2
Pre-analytical factors in ctDNA analysis: advantages and disadvantages.

Collection tube	
K2-EDTA-containing tubes	- less expensive - avoid blood clotting - blood samples have to be processes as quickly as possible (within 2 h from collection) - blood withdrawal and ctDNA analysis are necessary performed within the same or nearby physical location
Preservative-containing tubes	- stabilize ctDNA (up to 14 days upon collection depending on storage conditions) - easy sample management (storage and transportation at room temperature) - more expensive
Main centrifugation protocols	
Malapelle et al (Malapelle et al., 2017b)	Two centrifugations at 2300 G for 10 min at room temperature
Sorber et al (Sorber et al., 2019)	Adapted protocol from the European Committee for Standardization (CEN) (two-step, 1900 G for 10 min and then 16,000 G for 10 min at room temperature)
Page et al (Page et al., 2013)	Three centrifugation steps (the first at 1000 G for 10 min at 4 °C, the second at 1000 G, or 2000 G, or 10,000 G for 10 min at 4 °C, and the third, performed after thawing, at 1000 G for 5 min at room temperature)
ctDNA extraction methods	
Manual	- less expensive - necessity of high trained personnel - less accurate (high risk of contamination)
Fully automated	- more accurate (low risk of contamination) - more expensive

Abbreviations: ctDNA: circulating tumor DNA; EDTA: ethylenediaminetetraacetic acid.

limited quantity of ctDNA in their bloodstream (Fig. 1) (Wan et al., 2017). Thus, it is crucial to adopt high sensitive molecular approaches able to adequately detect genomic alterations at extremely low frequency levels (Fig. 2) (Castro-Giner et al., 2018).

NGS technologies have substantially evolved within the last few years and now represent a valid tool to address this need (Rolfo et al., 2020). Notably, among the most relevant advances in the field, the introduction of unique molecular identifiers (UMIs) and unique barcodes, has enabled to improve testing sensitivity while drastically reducing false negative results (Kinde et al., 2011). However, not all NGS approaches are born equal. Among some of most commonly used strategy is worth to mention the Tagged-Amplicon deep sequencing (TAm-seq), the Safe-Sequencing System (Safe-SeqS), the CAncer Personalized Profiling by deep sequencing (CAPP-Seq), the Bias-Corrected Targeted NGS and the Multiplex polymerase chain reaction (PCR) combined with the UMIs approach (Rolfo et al., 2020; Chen and Zhao, 2019). In the TAm-seq, primers are specifically designed to cover specific genomic regions and are adapted to amplify specific sections during the preamplification step. Subsequently, the already pre-amplified regions are selectively re-amplified in an individual (single-plex) PCR, thus determining the elimination of nonspecific products. Finally, adaptors and barcodes are attached to the obtained amplicons in an additional PCR (Forshew et al., 2012). This approach is associated with high sensitivity (>97.0 %) and the ability to detect low frequency alterations (about 2%) (Forshew et al., 2012). The evolution of this approach, optimized for an efficient amplification starting from a lower DNA input, termed enhanced Tam-Seq (eTAm-Seq), displays a sensitivity around 94 % combined with the ability to detect extremely low frequency mutations (about 0.25 %-0.33 %) (Gale et al., 2018). The Safe-SeqS approach uses unique identifiers to avoid sequencing errors (on average at least 70-fold increase to Tam-Seq) (Esposito Abate et al., 2020) and has overall a very high sensitivity, estimated around 98.0 % (Tie et al., 2015). In the CAPP-Seq approach, previously generated copy DNA oligonucleotides are utilized to hybridize specific mutated genomic regions of interest. This preliminary process generates a so called "selector" to detect individual-specific mutations (Newman et al., 2014a, 2016; Chabon et al., 2016). This approach features a sensitivity close to 100.0 % and the ability to identify very low frequency gene alterations (around 0.02 % of allelic frequency) (Newman et al., 2014a). The Bias-Corrected Targeted NGS approach is a novel methodology able to maximize on-target reads and minimize sequencing artifacts. This is possible by using sequence tags that bind small capture probes that are then amplified. This approach allows to reach a sensitivity and specificity ranging between 88.0 % and 100.0 % when testing mutations at 0.1 % allelic frequency, whereas a 100.0 % sensitivity and specificity may be obtained for allelic frequency of 0.4 % or greater (Paweletz et al., 2016a). Lastly, Multiplex-PCR NGS with UMI is characterized by the association of high throughput PCR amplification including UMIs and subsequential ultra-deep sequencing (usually at 25.000X coverage). This approach features a sensitivity up to 99.0 % (Abbosh et al., 2017).

Overall, despite the high sensitivity and specificity reached by all the above mentioned targeted approaches enabling the detection of extremely low frequency point mutations and insertion/deletion (indels), there are some limitations in regards to the gene rearrangement and copy number variant (CNV) assessment (Guan and Sung, 2016). A set of alternative valuable methodologies to tackle this need, may be represented by the personalized analysis of rearranged ends (PARE), the digital Karyotyping and the Fast Aneuploidy Screening Test-Sequencing System (FAST-SeqS) (Chen and Zhao, 2019). PARE approach is a high sensitivity methodology that adopts mate-paired sequence analysis to detect gene rearrangements even at concentrations lower than 0.001 % (Leary et al., 2010). Sensitivity and specificity of 90.0 % and 99.0 % can be usually reached (Leary et al., 2012). Digital karyotyping is able to quantify DNA and detect genome-wide aberrations. This technology requires an enzyme that cuts the DNA into short fragments and another that binds fragments with a tag; this approach is considered useful for the complex aligning process (e.g., highly aberrant cancer genomes) and large DNA abnormalities identification (Wang et al., 2002). Lastly, the FAST-SeqS, this method contemplates adopting a single primer to amplify repeated regions of interest is normally used exclusively for

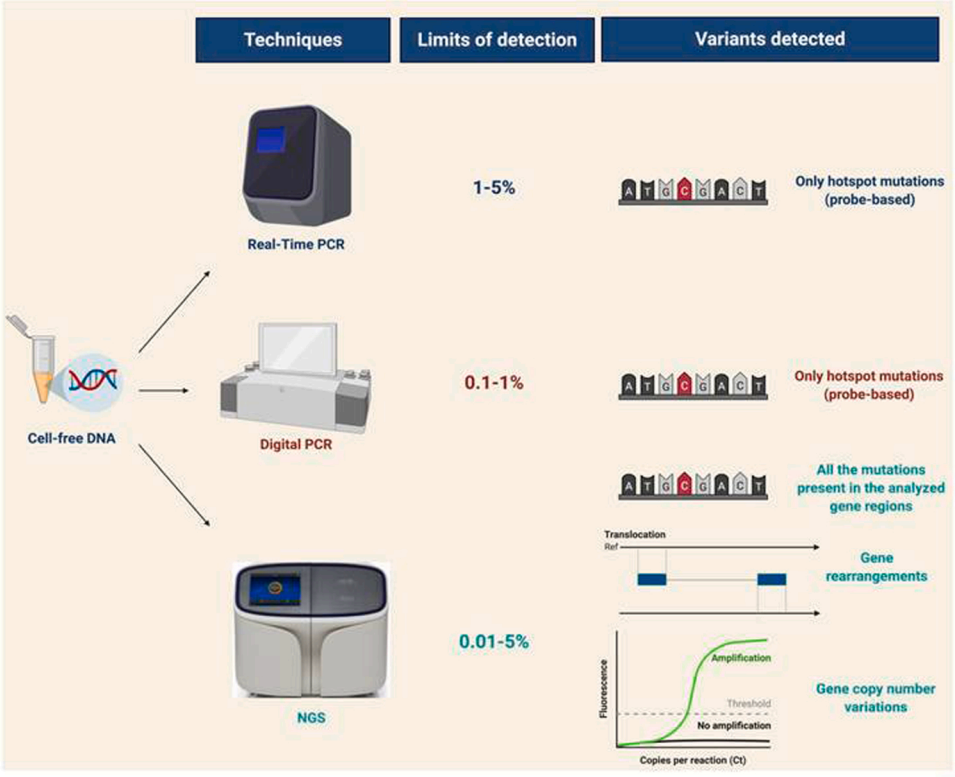

Fig. 2. Different technologies are currently available for cell free DNA analysis. From the top: real time polymerase chain reaction (RT-PCR) and digital PCR are able to detect only hotspot mutations (probe-based). Of note, digital PCR shows a higher sensitivity respect to RT-PCR. Finally, next generation sequencing enables the identification of all the mutations present in the analyzed regions, gene rearrangements and gene copy number variations at high sensitivity. (Credit: Created with Biorender).

CNVs detection (Kinde et al., 2012).

Overall, in this section, we have provided an overview of the most common and reliable approaches used for the detection of low allelic frequency variants. It is clear that while a number of different valid options exist there is no perfect tool currently addressing this question.

4. The role of liquid biopsy in NSCLC: standpoint in metastatic setting

As previously discussed, a great body of literature has now consolidated the knowledge that ctDNA concentration is significantly regulated by the pathological stage of disease. In particular, ctDNA concentration into the bloodstream is typically significantly higher in the advanced compared to earlier stages of cancer progression (Fig. 1) (Siravegna et al., 2019; Malapelle et al., 2017a). Notably, due to severe comorbidities, more invasive (histological) or less invasive (cytological) tissue sampling procedures maybe not feasible in advanced stages NSCLC patients (Rolfo et al., 2018). Liquid biopsy is a valid, clinically useful and well tolerated procedure that adequately meet the molecular diagnostics and clinical needs in the absence of available tissue (Pisapia et al., 2019). In particular, as witnessed by a number of recent FDA approvals, NGS biomarkers analysis on ctDNA extracted from plasma has clearly shown its clinical utility in the context of NSCLC. While is it impossible to appropriately cite all transformative work done in this area, as follow, we report a few significant examples. Reckamp et al. employed a short footprint mutation enrichment NGS assay to analyze ctDNA extracted from plasma samples of advanced stages NSCLC patients. A very high sensitivity was reported for the most relevant actionable *EGFR* mutations (93.0 %, 100.0 %, and 87.0 % for exon 20 p.T790 M, exon 21 p.L858R, and exon 19 deletions, respectively) (Reckamp et al., 2016). Similarly, a significantly high specificity was reported (94.0 %, 100.0 %, and 96.0 % for exon 20 p.T790 M, exon 21 p.L858R, and exon 19 deletions, respectively) (Reckamp et al., 2016). A targeted NGS approach was also adopted by Paweletz et al. who reported in a two-step study a sensitivity of 79.0 % for *EGFR* and Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*) mutations detection in plasma. Interestingly, a specificity of 100 % was reported for both genes (Paweletz et al., 2016b). By using CAPP-Seq to analyze ctDNA extracted from NSCLC plasma samples, Newman et al. reported a diagnostic sensitivity of 50.0 % and 100.0 % for stage I and stage II–IV, respectively. Moreover, the overall sensitivity and specificity were 85.0 % and 96.0 %, respectively, for all stages (Newman et al., 2014b). In our experience at the molecular predictive laboratory of the University of Naples Federico II, we designed, validated, and implemented in the routine practice a narrow, custom NGS panel tailored for advanced solid tumor patients (NSCLC, colo-rectal cancer, melanoma, gastrointestinal stromal tumor). This panel, named SiRe®, is able to cover 568 clinically relevant predictive hotspot mutations across six genes (*EGFR*, *KRAS*, Neuroblastoma RAS Viral Oncogene Homolog (*NRAS*), V-Raf Murine Sarcoma Viral Oncogene Homolog B (*BRAF*), KIT Proto-Oncogene, Receptor Tyrosine Kinase (*KIT*) and Platelet-Derived Growth Factor Alpha (*PDGFRA*)) (Malapelle et al., 2017b). In the validation study, we reported a sensitivity and specificity of 90.5 % and 100.0 %, respectively (Malapelle et al., 2017b). This preliminary experience was further confirmed in a retrospective analysis of our archival plasma samples analysis (Pisapia et al., 2017; Nacchio et al., 2020; Iaccarino et al., 2020). Focusing the attention on the basal setting, we were able to analyze ctDNA extracted from the plasma of advanced stages NSCLC patients without tissue availability. Overall, we observed *EGFR*, *KRAS* and *BRAF* to be mutated in 8.0 %, 18.6 % and 3.1 % of tested cases, respectively and thus in line with the incidence reported for tissue testing in our geographical area (Pisapia et al., 2017; Nacchio et al., 2020; Iaccarino et al., 2020).

To conclude, liquid biopsy testing, more specifically ctDNA analysis, is nowadays an established routine approach for profiling NSCLC patients, not exclusively limited to *EGFR* testing. While it is currently used almost exclusively in specialized diagnostic centers, we envision and advocate more widespread use of this tool for the near to come future, albeit the selection of the most appropriate testing methodology based on the clinical question needs to be addressed.

5. Early detection of NSCLC via liquid biopsy: status quo

Besides the now established role in advanced stages NSCLC patients contributing to define the best treatment strategy, liquid biopsy may play a pivotal role as well in early cancer detection of asymptomatic individuals potentially improving early diagnosis success rate and guiding adequate intervention (Russo et al., 2019a). Here we report a few but highly significant examples. Ye et al., analyzed a cohort of 35 patients with surgery-candidate solitary pulmonary nodules (SPN). ctDNA plasma samples and matched tissue specimens were collected for each subject. By adopting a super-depth NGS approach (>10.000-fold depth), authors observed that only ~6% of malignant nodule patients had driver mutations in ctDNA, with just 3.9 % of the total mutated cases showing same alteration in matched tissue and ctDNA. When considering only confirmed malignant lung nodules, the mutation concordance rate between tissue and corresponding ctDNA samples was only 5.8 %, significantly lower than the one usually observed in the advanced stages of disease. Overall, this strategy achieved a low prediction malignant rate using ctDNA, around 33.3 %. Interestingly, no false positive results on ctDNA were reported (100.0 % specificity), suggesting a possible role for ctDNA to select highly suspected nodules for surgical treatments (Ye et al., 2018).

In a prospective series of 58 NSCLC patients of stages IA, IB, and IIA, Chen et al. evaluated the targeted NGS concordance detection rate for driver mutations in matched tumor DNA and plasma ctDNA. Intriguingly, in the vast majority of cases (52/58; 89.7 %) quantifiable ctDNA was detected, suggesting that this phenomenon may reliably be associated with the presence of cancer in asymptomatic people. Regarding the detected mutations, a similar percentage of *EGFR* mutated cases were reported in tissue and ctDNA samples (39.7 % and 32.8 %, respectively). Overall, in 35 (60.3 %) out of 58 ctDNA samples a mutation was detected with a 50.4 % concordance with tissue samples. Finally, the sensitivity, specificity and positive predictive value (PPV) for ctDNA analysis were 53.8 %, 47.3 %, 53.2 %, respectively (Chen et al., 2016). A higher performance rate for ctDNA analysis was reported by Leung et al. In their study, by comparing results obtained on ctDNA samples, collected before surgery, with those on matched tissue of patients with known or suspected lung cancer, a sensitivity, specificity, PPV, and negative predictive value of 75.0 %, 89.0 %, 98.0 %, and 35.0 %, respectively, were reached (Leung et al., 2020). Another seminal work addressing the high rate of false positive results of low dose computed tomography (LDCT) in SPNs, comes from Liang et al. The authors performed a high-throughput targeted DNA methylation sequencing analysis on ctDNA extracted from plasma samples of malignant (n = 39) and benign solitary pulmonary nodules (n = 27). Interestingly this highly sensitive approach allowed to reach a sensitivity of 79.5 % and a specificity of 85.2 %. The adoption of this approach may be a valuable tool to distinguish malignant from benign lung nodules (Liang et al., 2019). Finally, in a pan-cancer study, Phallen et al. adopted targeted error correction sequencing (TEC-Seq) approach on different stage I and II solid tumors. As far as lung cancer is concerned, the authors were able to detect ctDNA mutations in the 59.0 % of the patients, with a high concordance between liquid and tumor biopsies (Phallen et al., 2017). Overall, the reported examples, while proving a very solid base to build a future that encompasses molecular testing also for early cancer stages, warrant additional investigations to solidify the clinical validity of this approach. If successfully achieved, the ultimate validation of liquid biopsy ctDNA profiling might represent a unique paradigm shift for early stages NSCLC testing and beyond.

6. Liquid biopsy: how to report and the need for molecular tumor board discussions

Appropriate reporting of the detected genomic alterations as identified by NGS analysis is a crucial post-analytical step for every molecular profiling approach, and particularly for liquid biopsy given its early stage of clinical adoption. As a general rule, while reports should include all significant data necessary to enable the most appropriate management of NSCLC patients, due to the lack of knowhow accompanied by limited practical experience in handling such kind of data, misinterpretation might arise, and thus an efficient and exhausting communication is essential between laboratory directors, pathologists and treating physicians. In addition to the classical unique identifier's information, a thoughtful molecular report should conceivably include information regarding the ward of service, the date of shipment, the sample type (including the number of specimens) and the name of the physician who requested the molecular test. Specifically, for liquid biopsy, many laboratories have decided to include in the first part of the report some pre-analytical parameters along with any other info that might have influenced the output of the performed analysis (e.g., fixation problems, artifacts). Subsequently, the mutational profile of the requested and analyzed biomarkers is typically reported (Cree et al., 2014; Li et al., 2017). According to the Association for Molecular Pathology (AMP), American Society of Clinical Oncology (ASCO), and College of American Pathologists (CAP) guidelines, gene alterations can be classified based on a joint consensus recommendation (JCR) 4-tiered system (Li et al., 2017). Tier I includes all clinically relevant variants that have a strong correlation, validated by extensive literature and regulatory bodies certification, with cancer patients development and management. Tier II encompasses variants that may have a potential role in oncogenesis and clinical management. Tier III contains variants associated to cancer via literature but of unknown clinical significance. Finally, within tier IV are variants with known benign or likely benign implications. Consensus in the community has established that only tier IV variants may be avoided in the final report. Overall, the observed variants should be reported following the Human Genome Variation Society (HGVS) indications (den Dunnen et al., 2016). Finally, to close the report, a brief clinical interpretation of the identified gene alterations along with information about the utilized assay, the reference range, the limit of detection (LOD) and the actual NGS run analytics should be compiled (Li et al., 2017). Particularly for liquid biopsy, due to the risk of false negative results (see above sensitivity issue), if investigated alterations are not identified, it should be avoided the utilization of the term "wild type", while it is preferable to use "not detected alterations".

Lastly, difficult cases with complex genomic variants or with challenging interpretations should be discussed in the context of molecular tumor boards (MTBs) (Kato et al., 2020; Koopman et al., 2020). MTBs represent a multidisciplinary board in which different healthcare stakeholders shall discuss together how to approach the examined cases and define a path forward to most appropriately manage the patients (Kato et al., 2020). With an ever-increasing number of clinically actionable variants and biomarkers, MTBs are encouraged to embrace familiarity with tools such as the OncoKB, developed and maintained by Memorial Sloan Kettering Cancer Center (MSK), and the European Society for Medical Oncology Scale for Clinical Actionability of Molecular Targets (ESCAT). While these traditionally represent tools that are far away from a clinician interest, nowadays they constitute necessary items within a modern oncologist's toolbox. Briefly, ESCAT I includes actionable genetic alterations associated with a significant outcome improvement after targeted therapies administration. ESCAT II contains genetic alterations that may be targeted but showing an unknown magnitude of benefit after targeted drugs administration. ESCAT III includes genetic alterations within ESCAT I and II categories in different tumor types. ESCAT IV contains genomic alterations with preclinical evidence of efficacy. ESCAT V includes genetic alterations showing objective responses but without outcome improvement. Finally, ESCAT X includes genetic alterations without actionability (Leichsenring et al., 2019). OncoKB Level 1 includes FDA–recognized biomarkers that are predictive of response to an FDA-approved drug in a specific indication. Conversely, level 2 includes mutations that are not FDA-recognized biomarkers but are standard of care biomarkers (level 2A) or not (level 2B) to an FDA-approved drug for a specific indication. Level 3 includes mutations that are possible candidate predictive biomarkers of drug response but neither the biomarker nor drug is standard of care. Level 4 contains mutations that showed only laboratory data of responsiveness without clinical data. In addition, three levels of resistance are also reported (Chakravarty et al., 2017).

These useful tools, initially created for interpreting NGS results in tissue, were recently evaluated also in the context of plasma NGS (Russo et al., 2019b; Mezquita et al., 2019). Lastly, a number of commercially available solutions are currently being launched in the market with the aim to help the interpretation of the identified alterations, in particular regarding their actionability and their evolution of the follow-up time points (Chakravarty et al., 2017; Mateo et al., 2018), thus being critical for full unleashing liquid biopsy potential.

7. Conclusion

Molecular profiling *via* liquid biopsy is a rapidly evolving field with promising applications not exclusively limited to advanced stages. In particular, due to the limited invasiveness of blood sampling and the high compliance rate, liquid biopsy may represent a valid and complementary tool, in association with other screening methodologies, to intercept cancer development in high-risk individuals or eventually to detect cancer in the earlier phases of development, to ensure a timely surgical treatment if appropriate. However, despite this fascinating scenario, liquid biopsy applicability is still limited by several constraints, especially regarding testing and intercept in the preliminary phase of cancer development. Conversely, a more concrete and nearer to come liquid biopsy use might enable the identification of patients affected by early-stage cancers. Worth to mention, opposite to the metastatic setting, the reduced size of an early cancer lesion is eventually translating into very low amounts of ctDNA in the bloodstream, therefore making detection challenging even with the aforementioned methodologies. The major issue in this scenario is the risk of false negative results. In addition, not all the mutations eventually detected on ctDNA may have a clear pathological significance. In this setting, careful attention should be paid to clonal hematopoiesis (CH) (Bick et al., 2020; Challen and Goodell, 2020; Zink et al., 2017; Bowman et al., 2018; Silver and Jaiswal, 2019; Chan et al., 2020; Champion et al., 1997; Fey et al., 1994; Steensma, 2018). In fact, Genovese et al. demonstrated, by analyzing ctDNA of a high number of individuals ($n = 12,380$) that mutations derived from CH were identified in 10 % of subjects >65 years (Genovese et al., 2014). In particular, mutations in Tumor Protein P53 (*TP53*) and *KRAS* genes may be detected in a not negligible percentage of healthy individuals (Kammesheidt et al., 2016). In addition, beyond ctDNA and CTCs other circulating element, such as TEPs, should be taken into account. In particular, it has been demonstrated that the analysis of platelet mRNA can offer valuable diagnostics information for different cancer patients suggesting that platelets may be adopted as a source to evaluate the molecular assessment of cancer patients (Best et al., 2015b).

In conclusion, careful attention should be paid to the methodology adopted when testing any of the different liquid biopsy analytes, from ctDNA to CTCs, as they are all equally found in low abundance in the bloodstream. While we are pointing in the right direction, the precise path leading to clinically meaningful findings generated via liquid biopsy for cancer testing is still to be determined.

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CRediT authorship contribution statement

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