The Journal of Liquid Biopsy 2 (2023) 100128

Contents lists available at ScienceDirect

The Journal of Liquid Biopsy

journal homepage: www.sciencedirect.com/journal/the-journal-of-liquid-biopsy

Liquid biopsy for lung cancer: A cross section on the diagnostic routine experience of a referral Italian institution

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ARTICLE INFO

Keywords:
Liquid biopsy
Lung cancer
Predictive biomarkers

ABSTRACT

Background: Lung cancer is the leading cause of cancer death worldwide. Over the last decade, molecular testing of a growing number of predictive biomarkers has become mandatory in the management of NSCLC patients. However, a major obstacle in routine clinical practice is the scant quantity of available tissue specimens obtainable from advanced stage of NSCLC patients. Thus, liquid biopsy, mostly involving blood sampling, has now been integrated in routine diagnostic practice. However, although liquid biopsies constitute a versatile, compliant, and dynamic source of nucleic acids, many of the current testing approaches pose few technical challenges. Here, to validate the feasibility of implementing NGS-based liquid biopsy approaches in routine diagnostic practice, we overviewed the NGS molecular data generated by our in-house narrow gene panel on plasma samples from real-world NSCLC patients.

Methods: Our Institution received testing request on liquid biopsy samples from peripheral institutions not equipped to internally analyze liquid biopsy samples. Molecular data from NSCLC patients following oncological requested for clinically approved plasma-based biomarkers evaluated in a diagnostic routine setting from January 2020 to September 2022 were retrieved from our institutional archive. A customized NGS panel integrated with an optimized bioinformatic pipeline was adopted.

Results: Overall, a total of n = 185 cases were retrieved. Of note, 103 (55.7 %) and 82 (44.3 %) patients were analyzed at basal setting and after resistance to first line TKI administration, respectively. Moreover, 31 out of 185 (16.7 %) cases reveled *EGFR* clinically relevant alterations. In particular, 6 out of 31 (19.3 %) and 25 out 31 (80.7 %) *EGFR* mutated patients were tested in basal setting and after first or second line TKIs progression. In addition, exon 20 p.T790 M mutation was also detected in 12 out of 25 (48.0 %) *EGFR* concomitant mutated cases. Moreover, *KRAS* hot spot mutations were found in 24 out of 185 (13.0 %) cases. Among them, exon 2 p.G12C clinically relevant mutations were observed in 8 out 24 cases (33.3 %).

Conclusions: This review highlights the technical suitability of an NGS-based liquid biopsy system for the analysis of clinically relevant mutations in NSCLC patients.

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https://doi.org/10.1016/j.jlb.2023.100128
Received 17 July 2023; Received in revised form 7 November 2023; Accepted 7 November 2023

1. Introduction

Lung cancer still remains one of the most severe causes of cancer death worldwide [1]. Over the past decade, a plethora of predictive biomarkers have emerged to guide therapeutic decisions in non-small cell lung cancer (NSCLC) [2]. In this scenario, international societies have established a panel of "mandatory testing genes" including epidermal growth factor receptor (*EGFR*), v-Raf murine sarcoma viral oncogene homolog B (*BRAF*) hot-spot mutations, aberrant fusion transcripts in anaplastic lymphoma kinase (*ALK*), ROS proto-oncogene 1 receptor tyrosine kinase (*ROS1*), Neurotrophic Receptor Tyrosine Kinase (*NTRK*), REarranged during Transfection (*RET*) genes, MET Proto-Oncogene Receptor Tyrosine Kinase (*MET*) exon 14 skipping mutations, and programmed death-ligand 1 (PD-L1). Recently, a single arm phase 2 trial has modified the paradigm for Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*) testing in lung cancer [3–13]. In particular, *KRAS* exon 2 p.G12C mutation has shown to have a positive predictive value in the clinical stratification of NSCLC patients [14–16]. In this heterogeneous scenario, however, a comprehensive molecular analysis of all potentially druggable biomarkers is often hindered in a substantial number of patients (20–30 %) owing to scant diagnostic material [17]. In an effort to address the issue of low quality and quantity of nucleic acid yields from scant tissue samples, many institutions have now adopted liquid biopsy as an integrative biological source for DNA and RNA mutation profiling [18]. Indeed, circulating-free nucleic acids may be detected in several types of liquid biopsies including blood, urine, saliva, effusions, and cerebrospinal fluids. However, as of today, only circulating-free DNA (cfDNA) from blood has been approved to assess the molecular heterogeneity of *EGFR* mutations in a diagnostic setting of NSCLC patients [19].

A major advantage of liquid biopsy samples is that they can help monitor patients' response to treatment noninvasively. Indeed, a simple blood draw can actually reveal acquired hotspot resistance mutations (*EGFR* exon 20 p.T790 M) in NSCLC patients after first or second line TKIs progression [18,19]. Remarkably, circulating tumor DNA (ctDNA) constitutes the main target of the molecular analysis. However, because they represent only a small fraction of cfDNA- [20] very sensitive technical approaches are required to identify clinically relevant *EGFR* molecular alterations in NSCLC patients. Commonly, most molecular diagnostics laboratories utilize real-time PCR (RT-PCR) or digital droplet PCR (ddPCR) to analyze liquid biopsy samples in routine practice [21]. However, these technologies, classified as single-gene testing platforms have two major limitations in view of tumor heterogeneity. One is that they can only identify single molecular alterations; the other is that having a low reference range, they cannot identify rare but clinically relevant alterations [19–21].

With the advent of high-throughput sequencing technologies, namely NGS, some of these technical hurdled have been overcome. Indeed, unlike conventional approaches, NGS platforms can simultaneously detect multiple molecular alterations in cancer-related genes [22]. In this scenario, the Molecular Predictive Pathology Laboratory of the Department of Public Health at the University of Naples Federico II is now a referral institution for the molecular identification of predictive biomarkers able to clinically stratify patients with solid tumor. The aim of this study was to validate the feasibility of applying NGS-based liquid biopsy for clinical selection of NSCLC patients. To this aim, we examined our NGS molecular data on clinical requests from oncologists in clinical practice for NSCLC liquid biopsy samples collected during our routine diagnostic practice from January 2019 to September 2022.

2. Materials and methods

2.1. Sample management

Our Institution collected liquid biopsy samples from peripheral centers not equipped for molecular analysis of plasma-based biomarkers. All these samples were tested for RNA-based clinically approved biomarkers on tissue specimens, when available, in peripheral institutions by using immunohistochemistry (IHC) approach. Due to the lack of diagnostic material to successfully carry out molecular analysis of DNA-based biomarkers on liquid biopsy samples, mandatory testing of *EGFR, KRAS* and *BRAF* hot spot mutations was demanded. We collected a retrospective series of n = 185 archival liquid biopsy samples from NSCLC patients tested for diagnostic purposes with NGS. Data were retrieved from our institutional archives. In detail, cfDNA was purified from 1.2 ml of plasma previously collected in Vacutainer tubes (BD, Plymouth, UK). Briefly, plasma was centrifuged twice at 2300 r.p.m. for 10 min to isolate cfDNA supernatant from cell debris. Afterward, the supernatant was stored at −80C°; next, cfDNA was purified with the automatized system QIAsymphony robot (Qiagen) and the QIAsymphony DSPVirus/Pathogen Midi Kit (Qiagen) [22]. In particular, cfDNA was automatically extracted from 1.2 ml of plasma; however, when the plasma volume was insufficient, 1.0–1.2 ml of PBS was added to reach the starting volume required by the manufacturer. Then, cfDNA was eluted in 60 µl of not DEPHC-treated water and immediately stored at −80C° for long-term periods or at −20C° for short-term periods until NGS analysis.

2.2. Molecular analysis

Molecular analysis was performed with a customized seven gene NGS panel (SiRe™). This panel enables to identify 568 solid tumor-related hotspot mutations simultaneously [16]. In detail, 15 µl of purified cfDNA was amplified on an automatized platform (Ion Chef™ system, Thermofisher Scientifics) together with the Ion AmpliSeq DL8 kit (Thermofisher Scientifics). Optimized thermal conditions were set for library construction (22 cycles) and library reamplification post barcoding (6 cycles). A set of n = 8 samples was processed simultaneously on the automatized platform (Ion Chef™). Moreover, two sets of amplified libraries were diluted at 60 pM and pooled together before loading on the Ion Chef™ platform for template preparation in accordance to the manufacturer's instructions for the S5, 520, and 530 Kit-Chef (ThermoFisher Scientifics) platforms. Finally, templates were automatically put into Ion 520 chip and sequenced on the S5 GS platform (ThermoFisher Scientifics). Data analysis was carried out by inspecting signal processing and base calling from default base-caller parameters on Torrent Suite [v.5.0.2] by adopting customized SiRe designed bed files (v.5.0.2.0). Variants were automatically called by adopting variant caller plug-in (v.5.0.2.1) with dedicated SiRe™ panel parameters. In particular, for variants harboring ≥5X allele coverage, an X20 quality score and an amplicon coverage of 1000X alleles were selected. In addition, BAM files were manually inspected on the Golden Helix Genome Browser v.2.0.7 (Bozeman,MT, USA) analysis software Fig. 1.

3. Results

Overall, n = 185 samples of NSCLC patients were included in this study. In particular, n = 106 (57.3 %) were male. The median age was 68.3 years (range 23–97 years). All patients had adenocarcinoma (ADC) (Table 1). Of note, 103 (55.7 %) and 82 (44.3 %) patients were analyzed at basal setting and after resistance to first line TKI administration, respectively. NGS analysis was successfully carried out in all instances. Regarding the sequencing coverage, NGS yielded an average of 620643.5 reads (ranging from 13973.0 to 12429697.0), an average read length of 129.5 bp (ranging from 112 to 155 bp), an average of 612549.6 mapped reads (ranging from 13960.0 to 12206922.0), an average of 94.25 % on-target reads (ranging from 60.63 % to 98.78 %), an average of 12206.8 reads per amplicon (ranging from 323.1 to 181056.0), and **a** 99.0 % coverage uniformity (ranging from 25.3 % to 100.0 %) (Supplementary Table 1). Regarding the molecular assessment, 31 out of 185 (16.7 %) cases highlighted *EGFR* clinically relevant alterations. More specifically, 6 out of 31 (19.3 %) and 25 out 31 (80.7 %) *EGFR* mutated patients were tested at basal setting and after first or second line TKIs progression,

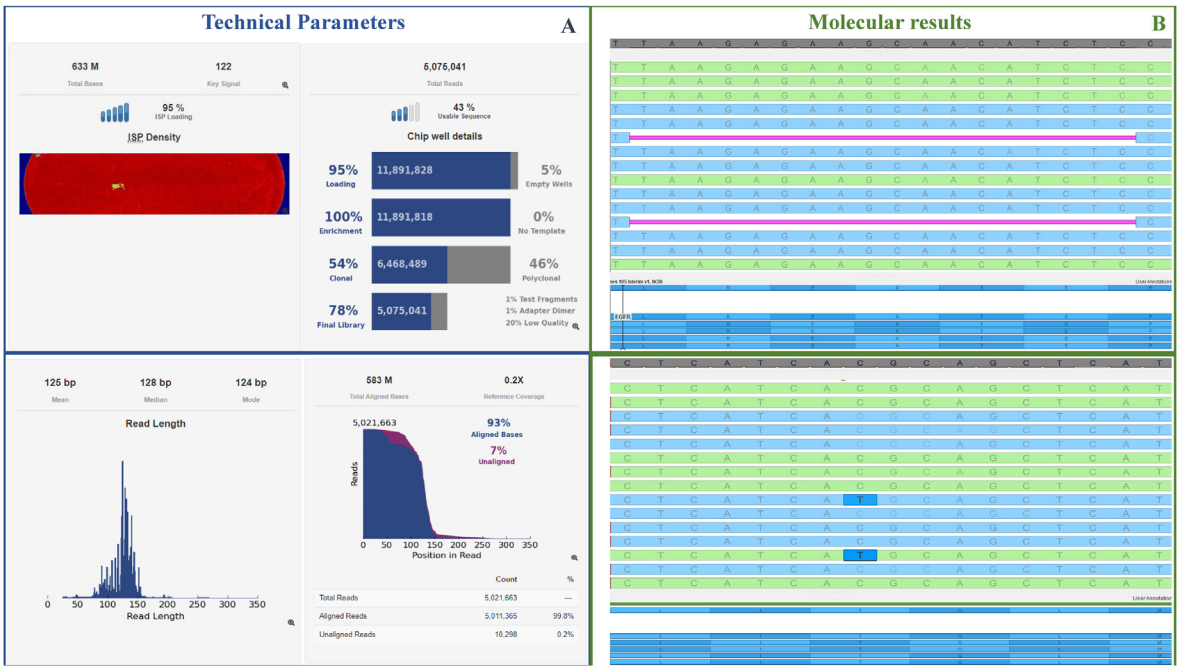

Fig. 1. Exemplificative case of NGS technical and molecular results on liquid biopsy sample. A) An overview of the technical parameters inspected to accept molecular data from NGS analysis. B) Second level data analysis by using Golden Helix Genome Browser v.2.0.7 (Bozeman,MT, USA) software. This case shows EGFR concomitant mutation: exon 19 deletion (purple bar) plus exon 20 p.T790M mutation (blue box).

Table 1
Clinical data of NSCLC patients from January 2020 to September 2022.

Sex (n)	Total	n = 185
	Male	n = 106
	Female	n = 79
Age (range)	Total	23 to 97 (±68.29)
	Male	23 to 97 (±68.85)
	Female	36 to 92 (±67.53)
Stage (n)	**Basal** (total)	n = 103
	Male	n = 70
	Female	n = 33
	Progression (total)	n = 82
	Male	n = 36
	Female	n = 46
Diagnosis (n)	Total	n = 185
	ADC	n = 183
	NSCLC-NOS	n = 2

Abbreviations: *ADC* (Adenocarcinoma), *NSCLC-NOS* (Non-Small Cell Lung Cancer-Not Otherwise Specified).

Table 2
Representation of detected alterations and relative exons distributions in NSCLC patients.

Basal setting	EGFR (total)	n = 6
	EGFR Exon 19	n = 6
	KRAS (total)	n = 17
	KRAS Exon 2	n = 17
Progression setting	**EGFR (total)**	n = 37[b]
	EGFR Exon 18	n = 2
	EGFR Exon 19	n = 16
	EGFR Exon 20	n = 13[a]
	EGFR Exon 21	n = 6
	PIK3CA (total)	n = 1
	PIK3CA Exon 20	n = 1
	KRAS (total)	n = 7
	KRAS Exon 2	n = 6
	KRAS Exon 3	n = 1

Abbreviations: EGFR (Epidermal Growth Factor Receptor), KRAS (Kirsten Rat Sarcoma virus), NSCLC (Non-Small Cell Lung Cancer), PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), TKI (Tyrosine Kinase Inhibitor).

[a] n = 12/13 EGFR Exon 20 p.T790M mutations were detected.

[b] n = 12/25 patients in progression setting showed a concomitant EGFR p.T790M and sensitive EGFR hotspot mutation to first-second generation TKIs.

respectively. The entire basal group harbored an *EGFR* exon 19 deletion. Instead, in the progression cohort, 2 out 25 (8.0 %), 16 out 25 (64.0 %), 1 out 25 (4.0 %), and 6 out 25 (24.0 %) carried exons 18, 19, 20, and 21 first/second generation TKI-sensitive molecular alterations. (Table 2). In addition, 12 out of 25 (48.0 %) *EGFR* concomitant mutated cases harbored exon 20 p.T790 M hotspot resistance mutation. Moreover, 1 out of 12 (8.3 %), 6 out of 12 (50.0 %), and 5 out of 12 (41.7 %) exon 18, 19, and 21 EGFR concomitant mutated patients harbored exon 20 p.T790 M mutations. Remarkably, 24 out of 185 (13.0 %) harbored *KRAS* hotspot mutations. In addition, 8 (n = 5 basal and n = 3 progression cases) out of 24 (33.3 %) *KRAS*-mutated cases harbored exon 2 p.G12C hotspot mutations, Finally, only one patient (0.5 %) harbored *PIK3CA* hotspot molecular alteration (Table 3).

4. Discussion

The growing number of predictive biomarkers comes with the urgent need to streamline molecular profiling in routine clinical practice, rendering it accessible to all patients with solid tumors. This study shows that liquid biopsy samples can successfully be used in clinical practice to identify *EGFR* clinically relevant hotspot mutations in NSCLC patients and to stratify treatment selection accordingly. As of today, while tissue samples are still widely regarded as the gold standard for molecular testing, their use is oftentimes limited by insufficient diagnostic material. In fact, in 20–25 % of NSCLC cases, tissue samples are either totally absent because surgically unreachable or barely sufficient for diagnosis, let alone molecular testing [17]. Moreover, scant tissue availability may not fully reflect the actual molecular heterogeneity of individual tumors [17]. In this scenario, our referral institution and others have integrated liquid biopsy in the management of NSCLC patients as a valuable biological source of nucleic acids to detect druggable hotspot molecular alterations, indeed, circulating nucleic acids have been shown to play a

Table 3
List of detected mutation from NSCLC cohort.

Sample ID	Mutations
3	KRAS p.G12V (c.35C > T)
5	KRAS p.G12D (c.35G > A)
7	KRAS p.G12C (c.34G > T)
10	KRAS p.G12C (c.34G > T)
11	EGFR p.L861Q (c.2582T > A)
14	KRAS p.G12A (c.35G > C)
25	EGFR p.L858R (c.2573T > G); EGFR p.T790 M (c.2369C > T)
26	EGFR p.E746_S752delinsV (c.2236_2254del)
28	KRAS p.G12S (c.34G > A)
31	EGFR p.L747_P753delinsS (c.2240_2257del); EGFR p.T790 M (c.2369C > T)
32	EGFR p.G719S (c.2155G > A); EGFR p.T790 M (c.2369C > T)
35	KRAS p.G12A (c.35G > C)
36	EGFR p.E746_A750del (c.2235_2249del)
47	KRAS p.G12S (c.34G > A)
48	EGFR p.G719S (c.2155G > A)
50	KRAS p.G12D (c.35G > A)
54	EGFR p.E746_A750del (c.2235_2249del)
57	KRAS p.G12C (c.34G > T)
61	EGFR p.E746_A750del (c.2235_2249del); EGFR p.T790 M (c.2369C > T)
62	EGFR p.E746_S752delinsV (c.2236_2253del)
67	EGFR p.E746_A750del (c.2235_2249del); EGFR p.T790 M (c.2369C > T)
70	KRAS p.G12C (c.34G > T)
74	KRAS p.G12V (c.35C > T)
81	EGFR p.L858R (c.2573T > G); EGFR p.T790 M (c.2369C > T)
83	KRAS p.G12V (c.35C > T)
88	KRAS p.G12C (c.34G > T)
91	EGFR p.E746_A750del (c.2235_2249del); EGFR p.T790 M (c.2369C > T)
92	EGFR p.S768I (c.2303G > T)
94	KRAS p.G12S (c.34G > A)
96	KRAS p.G12D (c.35G > A)
100	KRAS p.G13C (c.37G > T)
109	KRAS p.G12C (c.34G > T)
112	KRAS p.G12C (c.34G > T)
113	KRAS p.G12C (c.34G > T)
121	EGFR p.V738_I744ins (c.2214_2232insTTAAAATTCCCGTCGCTA)
125	KRAS p.G12A (c.35G > C)
129	KRAS p.G12A (c.35G > C)
130	EGFR p.L747_A750delinsP (c.2239_2248delinsC)
132	EGFR p.E746_A750del (c.2235_2249del)
133	EGFR p.E746_A750del (c.2235_2249del)
134	EGFR p.E746_A750del (c.2235_2249del)
135	EGFR p.E746_A750del (c.2235_2249del)
137	EGFR p.L747_T752del (c.2239_2256del); KRAS p.Q22K (c.64C > A)
144	EGFR p.E746_R748del (c.2236_2244del)
146	PIK3CA p.G1049R (c.3145G > C)
159	EGFR p.E746_A750del (c.2235_2249del)
165	EGFR p.E746_A750del (c.2235_2249del)
166	EGFR p.E746_A750del (c.2235_2249del); EGFR p.T790 M (c.2369C > T)
167	EGFR p.E746_A750del (c.2235_2249del)
168	KRAS p.G12V (c.35C > T)
169	EGFR p.L861Q (c.2582T > A)/EGFR p.T790 M (c.2369C > T)
170	EGFR p.E746_T751delinsA (c.2235_2251delinsAG)
175	EGFR p.L858R (c.2573T > G); EGFR p.T790 M (c.2369C > T)
179	EGFR p.L858R (c.2573T > G); EGFR p.T790 M (c.2369C > T)
182	EGFR p.E746_T751delinsA (c.2235_2251delinsAG); EGFR p.T790 M (c.2369C > T)

Abbreviations: *EGFR* (Epidermal Growth Factor Receptor), *KRAS* (Kirsten Rat Sarcoma virus), *PIK3CA* (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha).

pivotal role in detecting *EGFR* p.T790 M alterations, thus providing clinicians an integrated tool for the clinical administration of targeted therapies, namely, TKIs [18,19]. Due to methodological critical issues, high expertise is required to adequately manage blood samples in pre-analytical, analytical and molecular data interpretation setting. With the approval of implementing liquid biopsy for the detection of *EGFR* sensitive mutations, the application of high-sensitive technology has become instrumental in detecting very low frequency and clinically relevant alterations in small amounts of circulating nucleic acids [18,20]. As the matter of fact, our institution has become a referral institution for molecular testing on liquid biopsy samples. Particularly, we provide a support in molecular analysis of clinically approved biomarkers in liquid biopsy samples derived from institutions that have not yet implemented a robust diagnostic workflow tailored on liquid biopsy critical points. In this report, our molecular data demonstrated that our customized NGS panel is able to successfully cover clinically relevant hotspot mutations in different solid tumors, including *EGFR* in lung cancer patients. Actually, the present research was prompted by a previous study in which we demonstrated that our in-house SiRe™ panel was able to properly analyze 98.4 % (63 out of 64) diagnostic routine liquid biopsy samples [23]. Moreover, the efficiency of our SiRe™ panel was also evidenced by the consistency between our, technical parameters and mutation rates in pivotal genes for TKIs administration and those reported in the literature. Similarly, our present findings further corroborate previous data on the technical feasibility of applying the SiRe™ panel to liquid biopsy samples in routine practice. Indeed, we detected *EGFR* sensitive mutations in 31 out of 185 (16.7 %) cases. In particular, we found that most of the *EGFR* clinically relevant molecular alterations were present in exon 19 (71.0 %), as widely observed by other research groups [24,25]. In addition, we identified p.T790 M mutation in 12 out of 25 (48.0 %) patients who experienced relapse after first or second generation TKIs administration. This finding may have very important clinical implications given that exon 20 p.T790 M hotspot mutations are the most recurrent mechanisms behind *EGFR*-TKI resistance [5,19]. Indeed, the identification of this driver alteration is allowing clinicians to adopt third generation TKIs [19]. Moreover, our in-house panel was also able to detect another relevant hotspot mutation in n = 8 patients, namely, *KRAS* p.G12C. This was also an encouraging result given that recently approved small molecules that inhibit *KRAS* p.G12C hotspot mutations have proven to be clinically beneficial for *KRAS* p.G12C positive NSCLC patients undergoing TKI therapy [14]. Moreover, *BRAF* exon 15 p.V6003 clinically relevant alterations were not found in this series. In view of this promising scenario, the implementation of NGS panels able to distinguish several hotspot mutations in scant diagnostic specimens is crucial to optimize the clinical stratification of NSCLCA patients. However, for many clinical laboratories, adopting NGS assays able to thoroughly cover all the possible molecular alterations in predictive biomarkers still remains challenging. Accordingly, our findings support the idea that designing customized NGS panels tailored according to specific diagnostic routine practices may help to streamline the use and workflow of this amazing technology in routine clinical practice. Indeed, our team and others have fully demonstrated that this strategy significantly improves the success rates of the molecular analysis of liquid biopsy samples [21,22].

In conclusion, our NGS molecular data highlights the technical feasibility of our customized SiRe™ panel to detect clinically relevant hotspot molecular alterations in diagnostic routine liquid biopsy samples from NSCLC patients. This approach enabled us to detect very low frequency alterations starting from very low concentrations of nucleic acids, thus optimizing the clinical stratification of NSCLC patients.

Finally, in view of the ever-growing number of emerging biomarkers, further studies with larger cohorts are warranted to fully validate the reliability and clinical applicability of NGS-based liquid biopsy assays in solid tumors.

Declaration of competing interest

Giancarlo Troncone received personal fees (as a bureau speaker and/or advisor) from Roche, MSD, Pfizer and Bayer, for work unrelated to the current paper.

The other authors declare no potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jlb.2023.100128.

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