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Implementation of a 1021-gene liquid biopsy assay for real-world tumor genomic profiling in oncology practice

Chrysiida Florou-Chatzigiannidou[1]✉, Eirini Papadopoulou[1], Vasiliki Metaxa-Mariatou[1], Aikaterini Tsantikidi[1], Stella Maxouri[1], Georgios Tsaousis[1], Dimitris Grigoriadis[1], Nikolaos Touroutoglou[2], Dimitrios Ziogas[3], Eleni Zairi[4], Nektarios Alevizopoulos[5], Jinga Dan Corneliu[6], Iorga Polixenia[7], Mustafa Ozdogan[8], Cemil Bilir[9], Ilhan Hacibekiroglu[10], Sahin Lacin[11], Ramin Ajami[12], Georges El Hachem[13], George Nasioulas[1], Konstantinos Bramis[14], Manousos Konstadoulakis[14] & Christos Papadimitriou[15]

The application of advanced next-generation sequencing (NGS) technologies in the analysis of circulating tumor DNA (ctDNA) enabled the comprehensive evaluation of tumor-derived alterations in the bloodstream. In this study, we evaluated the analytical performance and clinical utility of a large-scale liquid biopsy assay in patients with metastatic cancer. A total of 1,110 unique patients underwent ctDNA NGS testing using a 1021-gene panel; matched tissue biopsy data were available for 145 cases. In 16.18% of the cases, at least one on-label variant was identified. In addition, off-label, clinical trial–related, and resistance-associated findings collectively increased the proportion of clinically actionable results by 40.65%. Importantly, 8.65% of the total population could also benefit from immune checkpoint inhibitors (ICI) therapy, based on the high tumor mutational burden (TMB-H) and/or high microsatellite instability (MSI-H) status. Simultaneous analysis in plasma and white blood cells enabled clonal hematopoiesis-associated variants detection, hence increasing the specificity value for ctDNA analysis and confirming the presence of pathogenic germline variants for 11.26% patients. Of interest, concordance for actionable on-label candidates using tissue and plasma analysis was 90.34%, thereby confirming the reliability of liquid biopsy results. Notably, the combination of liquid biopsy analysis, and tissue-based profiling, increased the total number of actionable biomarkers, facilitating targeted therapy and immunotherapy selection, resistance monitoring, and faster clinical decision-making. The broad genomic coverage of the liquid biopsy NGS assay used enabled clinically meaningful genomic characterization across multiple tumor histological types, although tumor-specific generalization is limited by the heterogeneity of the tumor types analyzed. Overall, this study shows that large-scale LB profiling may increase the number of actionable findings detected beyond guideline-based targets. In addition, it provides a more accurate understanding of tumor biology and ctDNA shedding in circulation, while offering the advantage of parallel germline analysis.

Traditionally, tissue biopsies have served as the cornerstone for diagnosing cancer and understanding its genetic characteristics. While highly informative, tissue acquisition is an invasive, labor-intensive procedure with often

[1]Genekor Medical SA, Athens, Greece. [2]Department of Medical Oncology, Interbalkan Medical Center, Thessaloníki 55535, Greece. [3]General Hospital of Athens LAIKO, Athens 11527, Greece. [4]Department of Medical Oncology and Clinical Trials Unit, European Interbalkan Medical Center, Thessaloniki, Greece. [5]Department of Oncology, Evaggelismos General Hospital, Athens, Greece. [6]Centrul Medical Neolife București, Bucharest, Romania. [7]Spitalul Universitar de Urgență București, Bucharest, Romania. [8]Clinic of Medical Oncology, Memorial Antalya Hospital, Antalya, Turkey. [9]Clinic of Medical Oncology, VM Medical Park Pendik Hospital, Istanbul, Turkey. [10]Faculty of Medicine, department of Medical Oncology Adapazarı, Sakarya University, Sakarya, Turkey. [11]Department of Medical Oncology, Koc University Faculty of Medicine, İstanbul, Turkey. [12]Department of Oncology, The Royal Free Hospital, London NW3 2QG, UK. [13]Department of Hematology and Medical Oncology, Saint George Hospital University Medical Center, Beirut, Lebanon. [14]Surgical Clinic, Aretaieio University Hospital, Athens, Greece. [15]Department of Medical Oncology, Iaso Hospital, Maroussi, Greece. ✉email: c.chatzigiannidou@genekor.com

suboptimal quality and/or quantity of the genetic material. In addition, this material may fail to capture the full extent of tumor heterogeneity. This limitation is particularly evident in metastatic disease, where molecular profiles can vary not only within the primary tumor but also across different metastatic sites. Liquid biopsies (LB) have emerged as a minimally invasive alternative, enabling real-time tumor characterization by analyzing circulating biomarkers in several body fluids, especially blood[1–3].

The main liquid biopsy components detectable in the bloodstream include circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), both gaining significant clinical relevance[4]. Other constituents, such as circulating tumor RNA (ctRNA), exosomes, and tumor-educated platelets, are also under investigation, though their clinical application remains less established[3]. While, CTCs were the first liquid biopsy marker to be clinically employed, they only succeeded in providing prognostic information[5,6].

Nevertheless, ctDNA has since demonstrated greater clinical value. Several studies and clinical trials across cancer types—including lung, colorectal, breast, and pancreatic—have consistently demonstrated ctDNA's utility in treatment selection, therapeutic monitoring, and resistance detection, thereby advancing personalized cancer management[7–9]. Moreover, ctDNA is now recognized as a reliable alternative to tissue biopsy in cases lacking evaluable tumor tissue, with several targeted therapies approved based on ctDNA testing in metastatic patients[3,10].

The application of advanced next-generation sequencing (NGS) technologies in the analysis of ctDNA enabled the comprehensive evaluation of tumor-derived alterations in the bloodstream. Currently, NGS-based panels, ranging from a few genes to several thousand, are increasingly used in routine practice, providing high sensitivity and specificity for the detection of clinically relevant variants. Furthermore, the turnaround periods and costs associated with this type of analysis are consistently decreasing, which enhances patient access to targeted therapies[11–13]. However, ctDNA analysis is also faced with numerous sensitivity challenges because of the limited quantity of ctDNA in the blood. This limitation appears to be both patient- and tumor-specific, and is influenced by various biological and clinical factors, including tumor burden, metastatic sites, and treatment response. It can only be partially addressed through highly sensitive NGS approaches specifically optimized for cell-free DNA (cfDNA) analysis, which improve the lower limit of alteration detection[12].

In addition, specificity challenges arise in distinguishing true tumor-derived variants from clonal hematopoiesis (CH), which may mimic actionable mutations. Clonal hematopoiesis of indeterminate potential (CHIP), a clinically recognized subset of CH, is frequently associated with recurrent mutations in genes such as *DNMT3A*, *TET2*, *ASXL1*, and *JAK2*, which can confound ctDNA analysis if not properly excluded. To address this, parallel sequencing of cfDNA and matched leukocyte genomic DNA is recommended to filter out CH-related variants[6,14].

A major concern in liquid biopsy analysis has traditionally been its degree of concordance between ctDNA and tissue biopsy results. As a result, numerous studies have reported varying degrees of agreement and actionability in the context of concurrent plasma and tissue biopsy biomarker analysis. Discrepancies between the two materials may occasionally occur due to factors such as tumor heterogeneity, clonal evolution, and variations in the analytical sensitivity of the methodologies used[15,16]. However, in the great majority of cases, ctDNA testing could identify clinically significant alterations, and in some cases, even those that were overlooked by tissue biopsy analysis alone[17,18]. This finding indicates that the two approaches may provide complementary information. Moreover, the reliability of liquid biopsy–detected alterations is supported by the comparable response rates observed between patients harboring targetable alterations identified in either tissue or plasma, a finding that has supported the approval of several targeted therapies based on ctDNA testing in metastatic patients[19–25].

While liquid biopsy assays are increasingly used for genomic profiling in metastatic cancer, clinical interpretation can be challenging in real-world settings where ctDNA levels are low, tumor biology is heterogeneous, and canonical actionable alterations may be absent. Broad genomic coverage may increase the probability of detecting non-common somatic alterations that confirm the presence of circulating tumor DNA, thereby supporting the distinction between likely true-negative cases and false-negative results due to absent or insufficient ctDNA[6,15]. In addition, large-scale genomic representation improves the robustness of copy number variation assessment in plasma[26,27]. Finally, the integration of parallel leukocyte sequencing enables the concurrent detection of clinically relevant germline variants, providing an added layer of biological and clinical information that is not routinely captured by many liquid biopsy assays.

In this study, we investigated the performance and clinical utility of liquid biopsy in metastatic cancer patients using an expanded 1021-gene panel with additional assessment of concordance between plasma and tissue profiling. The increased gene representation aimed to increase the likelihood of identifying rare actionable or off-label targets, but also to enhance the biological interpretability of liquid biopsy results.

Patients and methods

Sample collection and DNA extraction

A total of 1110 unique patients with various types of metastatic cancer were included in this study. All patients were referred to our laboratory for molecular profiling in plasma and/or tissue biopsy between October 2023 and May 2025. Detailed clinical records, including line of treatment and treatment status at the time of testing, were not available. According to information obtained from treating physicians, testing was ordered to support therapeutic decision-making, and ongoing treatment was not regarded as a limitation for ordering the assay. This study was carried out in compliance with the principles of the Declaration of Helsinki. All participants provided written informed consent, and the study protocol was reviewed and approved by the Committee on Ethics and Good Practice (CEGP) of the NKUA (National & Kapodistrian University of Athens).

1193 blood samples were collected during routine phlebotomy and 10 mL of whole blood on appropriate conservation tubes, Cell-Free DNA BCT tubes (Streck, La Vista, USA), were collected. Cell-free DNA was

extracted from plasma and genomic DNA was extracted from white blood cells, using QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and MagCore® Genomic DNA Whole Blood Kit (RBC Bioscience, New Taipei City, Taiwan), respectively. Formalin-fixed, paraffin-embedded (FFPE) tissue samples from the most recent surgical procedure were available for 145 cases. DNA extraction from FFPE tissue was performed using the MagMAX™ FFPE DNA/RNA Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Liquid biopsy profiling by targeted deep sequencing

A total of 30 ng of cfDNA was subjected to NGS testing. When matched tumor tissue was available, comparative genomic analysis was performed. Both cfDNA and gDNA libraries were constructed using the Oncology Multi-Gene Variant Assay (GenePlus, Beijing, China), following the manufacturer's protocol as previously described[28]. For tumor DNA analysis, 100 ng of DNA was used for library construction when sufficient material was available. The assay is a qualitative in vitro diagnostic test employing capture probes designed to cover the coding sequences and hotspot exons of 1021 genes frequently mutated in solid tumors, as well as gene rearrangements/fusions in 38 genes. Tumor mutational burden (TMB) and microsatellite instability (MSI) status were also assessed. Sequencing was performed on the MGI DNBSEQ-G400 platform (MGI Tech Co., Ltd., Shenzhen, China). NGS data were analyzed using the corresponding analytical bioinformatics pipeline, and variant interpretation was conducted via the Gene Box platform (GenePlus, Beijing, China).

Single nucleotide variants (SNVs), insertions/deletions (indels), and structural variants (SVs) were considered positive if detected at a frequency of ≥ 1% in both FFPE and plasma samples. Copy number variation (CNV) analysis was performed using the Contra algorithm (http://contra-cnv.sourceforge.net) as previously described[6]. Positivity was defined as a copy number ≥ 3.0 for FFPE and ≥ 2.2 for plasma samples. The MSI-H cutoff values were ≥ 19.5% for FFPE and ≥ 18% for plasma, respectively. Clonal hematopoietic mutations were filtered out by parallel sequencing of cfDNA and gDNA extracted from leukocytes, which served as a control to prevent false-positive results arising from clonal hematopoiesis. This approach, as previously described, enabled the exclusion of mutations in genes with high CH mutation rate, such as *DNMT3A*, *TET2*, *AXSL1*, and *JAK2*, through simultaneous genomic DNA sequencing[29].

Statistical Analysis

Descriptive statistics were used to summarize patient characteristics, biomarker frequencies, and positivity rates across tumor types. Concordance between liquid biopsy and tissue biopsy results was expressed as percentages. Tumor fraction (TF) was estimated using the highest variant allele frequency (VAF) per patient as a proxy.

Results

Genomic alterations and actionable findings identified by LB

A total of 1193 liquid biopsy analysis were performed during the study period. Genetic alterations were assessed for both somatic and germline variants. Among these patients, 77 of the samples tested (6.9%) corresponded to LB samples from patients who had underwent such analysis more than once, with an average interval of 12.9 months between the first and second test (range: 2–27 months). Therefore, our cohort included 1110 unique patients (Supplementary file 1, Supplementary Fig. 1).

The most abundant cases were non-small cell lung cancer cases (19.46%), followed by breast (15.77%), pancreatic (15.14%) and colorectal cases (12.97%). These tumor types collectively represent a substantial proportion of the global cancer burden, accounting for approximately 35–40% of worldwide cancer incidence and around 40% of cancer-related mortality, reflecting their high prevalence in advanced and metastatic disease and their frequent representation among patients undergoing comprehensive genomic profiling in routine oncology practice[30]. In addition, the availability of multiple targeted therapeutic options for these tumor types likely contributes to their overrepresentation in this cohort. In particular, lung and breast cancers are characterized by the frequent emergence of acquired resistance mutations under targeted therapy, and detection of such alterations may support switching to alternative on-label treatment strategies in clinical practice[31,32]. Other relatively common tumor types included sarcoma, prostate cancer, gastric cancer, ovarian cancer, and cholangiocarcinoma, each representing approximately 3–4% of the cohort (Fig. 1).

When considering the presence of either a somatic or a germline alteration in any test, 62.87% LB samples were positive in the entire cohort (including the retested cases). The overall positivity rate for unique patients tested was 65.86%. Somatic alterations were detected in 682 cases (61.44%), while germline alterations were identified in 131 patients (125 unique patients). Among somatic wild-type patients, 49 (7.18%) harbored a germline pathogenic variant.

The vast majority of the oncogenic variants detected (88.00%) were SNVs or small insertion-deletions (indels). Copy number variations (CNVs) accounted for 10.67% of the alterations, while gene fusions accounted for 1.32%. The *TP53* locus was responsible for 7.93% of all detected alterations, with *KRAS* (4.45%), *PIK3CA* (2.64%), *APC* (2.12%), and *LRP1B* (1.61%), following in that order.

In the combined cohort, somatic alterations in the PI3K/AKT/mTOR pathway comprised 6.47% of all detected variants (273/4.221; main genes observed: *PIK3CA*, *PIK3CB*, *PIK3CG*, *PIK3R1*, *PIK3R2*, *AKT1–3*, *MTOR*, *PTEN*, *TSC1*, *TSC2*, *RICTOR*, *RPTOR*, *IRS2*, *PDK1*), while DNA damage repair (DDR) pathway genes accounted for 6.99% (295/4.221; genes observed: *ATM*, *ATR*, *ATRX*, *BAP1*, *BARD1*, *BLM*, *BRCA1*, *BRCA2*, *BRIP1*, *CDK12*, *CHEK1*, *CHEK2*, *C11orf30*, *ERCC1*, *FAM175A*, *FANCA*, *FANCC*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCL*, *FANCM*, *MRE11*, *NBN*, *PALB2*, *RAD50*, *RAD51*, *RAD51B*, *RAD51C*, *RAD51D*, *RAD52*, *RAD54L*, *RECQL*, *RECQL4*, *WRN*).

In terms of findings' actionability, liquid biopsy identified clinically significant variants (both of somatic and germline origin) in more than 50% of the cases. In 16.18% of cases, at least one on-label variant was identified, while in 22.13% of cases, an off-label variant was the most clinically significant finding. The most significant

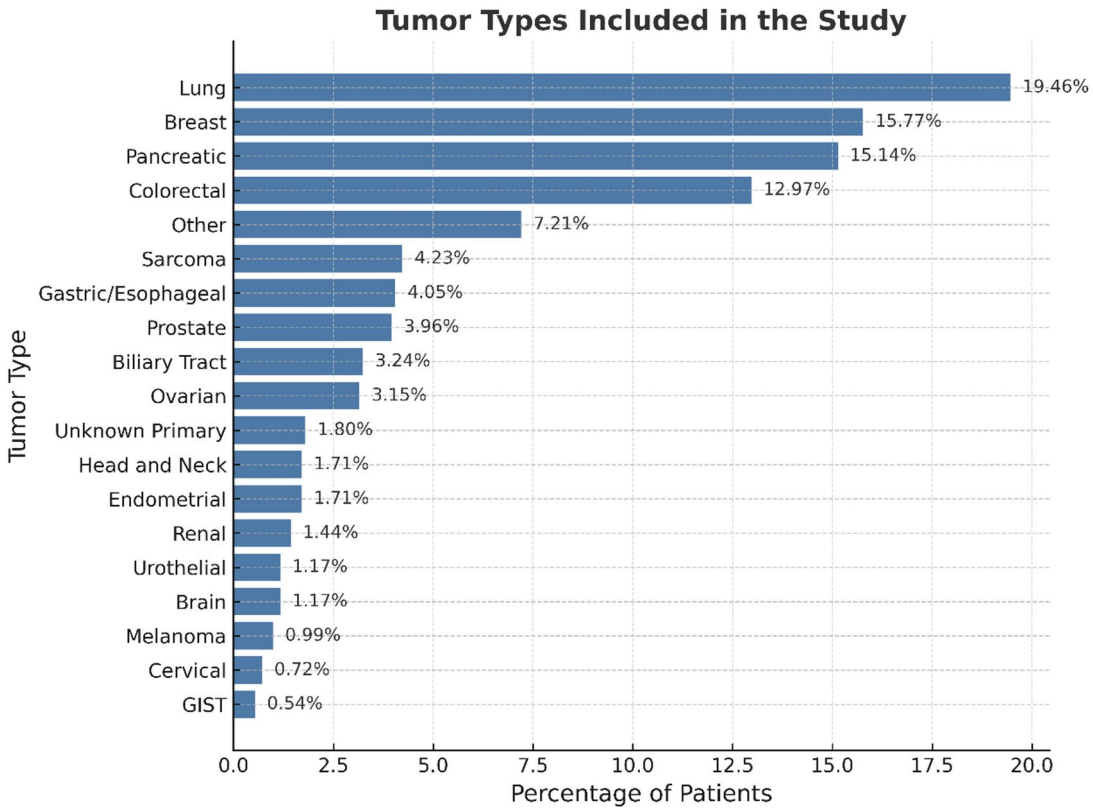


Fig. 1. Tumor types included in the study.

result in 13.91% of patients was variants currently investigated in clinical trials, while resistance mutations were detected in 4.61%. Likely pathogenic or pathogenic variants of unknown actionability were the primary finding in 6.12% of the cases. Variants of uncertain significance (VUS) were common, detected in 484 cases, and represented the only finding in 33 patients (Fig. 2). The most frequently mutated gene with on label indication was *PIK3CA* (detected in 40 breast cancer patients), followed by *ESR1* (28 breast cancer patients), *EGFR* (30 lung cancer patients), *KRAS G12C* (21 lung and colon patients) and *BRAF* (detected in 13 cases). Due to the cancer cohort presented in this study, such prevalence is expected.

In addition, the 1021-gene assay enabled ctDNA-based estimation of tumor mutational burden (TMB) and microsatellite instability (MSI). Although the predictive value of these biomarkers from liquid biopsy has not been firmly established, they represent promising indicators for further investigation of these genomic signatures in tissue re-biopsy[33,34]. In our cohort, 8.65% of patients were identified as potentially eligible for immune checkpoint inhibitor (ICI) therapy, driven by TMB-high status (> 16 mutations/Mb; 7.29%) and/or MSI-H (1.09%). Importantly, when immunotherapy biomarkers were considered alongside targeted therapy markers, the actionability of LB increased substantially: in patients lacking other actionable alterations, an additional 6.29% could, theoretically, benefit from ICI-based strategies (Fig. 2).

Biomarker landscape across tumor types

Moreover, the prevalence and distribution of on-label, off-label, and resistance biomarkers were largely divergent across tumor types (Fig. 3; Table 1).

The highest proportion of patients harboring on-label biomarkers was in breast cancer (44.13%), dominated primarily by *PIK3CA* mutation, *ESR1* alteration, and *ERBB2* amplification. *ESR1* alterations are typical secondary alterations associated with endocrine treatment resistance. Off-label biomarker alterations were detected in 24.58% of the LB screened, predominantly in *NF1*, *ATM*, and *KRAS* genes.

In Colorectal cancer *KRAS/NRAS* mutations are the most frequently detected biomarkers. The absence of such changes is a marker for anti-*EGFR* treatment suitability. Almost half of colorectal cancers (47.37%) had such changes in LB, consistent with identified resistance mechanisms. On-label biomarkers (7.24%) primarily consisted of *BRAF V600E*, *ERBB2* amplification, and *KRAS G12C* alterations. In contrast, off-label biomarkers were identified in nearly a third of the patients (32.89%), with *PIK3CA*, *NF1*, and *RAD51C* being the most prevalent altered genes.

Lung cancer expressed a high percentage of on-label biomarkers (27.20%), with the most common being *EGFR*, *KRAS/G12C*, *ALK* fusions, *BRAF*, and *MET* alterations. There also were off-label events in *STK11*, *PIK3CA*, and *ATM* genes (27.62%). Resistance mutations in *EGFR* (T790M and C797S mutations), *ALK*, *PIK3CA* and *NTRK3* genes, along with *MET* copy-number gain, were also documented.

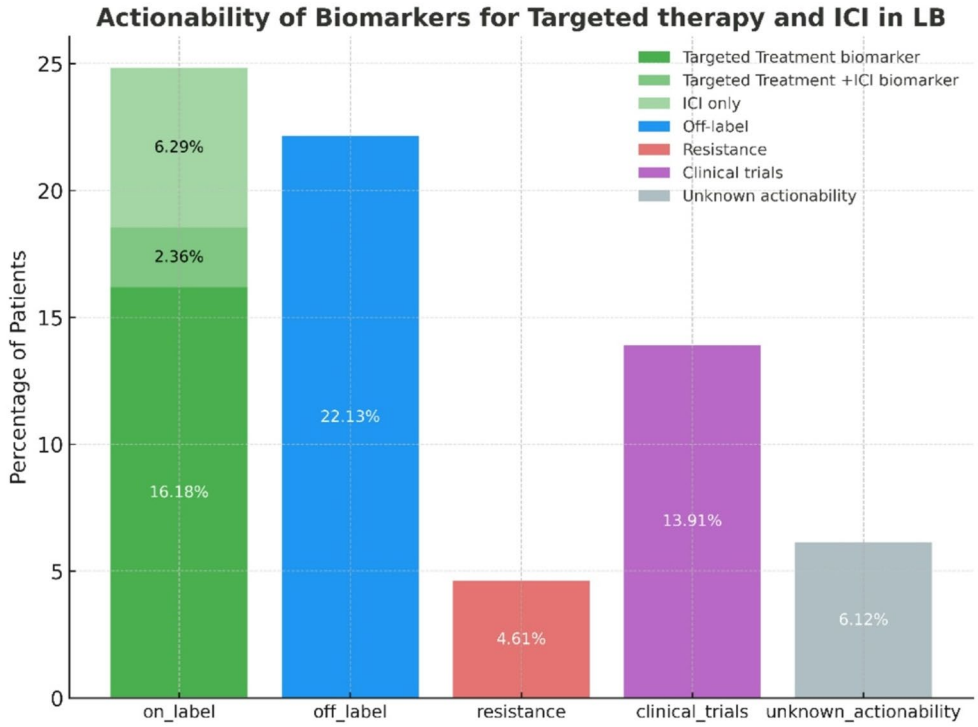


Fig. 2. Actionability of Biomarkers detected in LB.

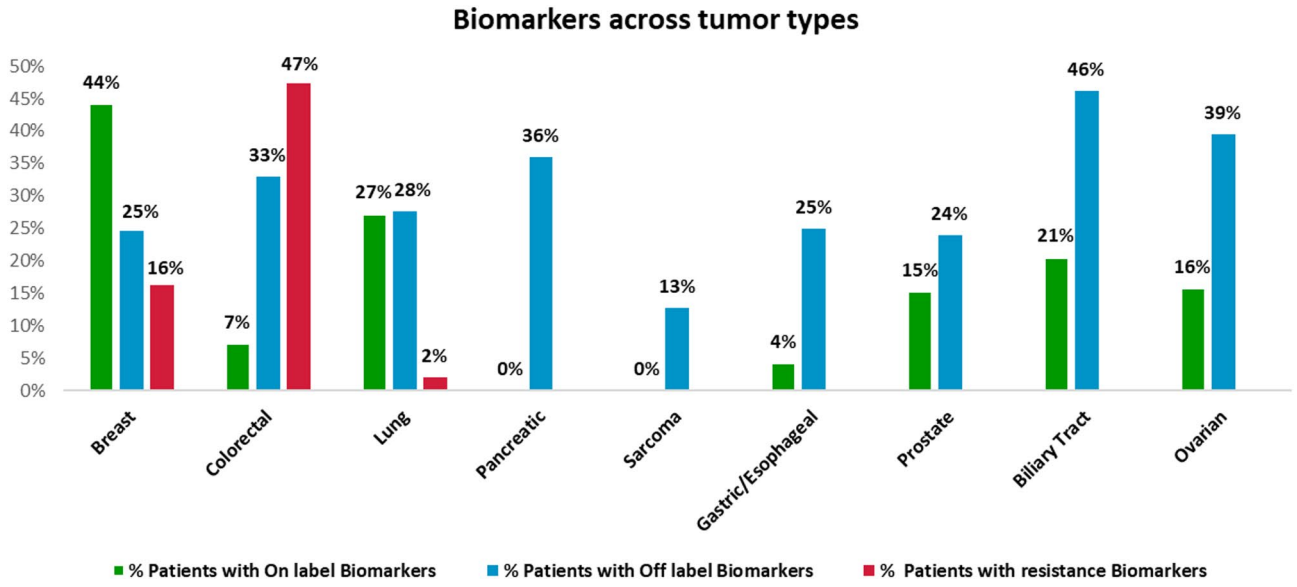


Fig. 3. Biomarkers actionability across tumor types.

In pancreatic cancer, no on-label biomarkers exist, but more than one-third of the patients harbored off-label mutations, most frequently in *KRAS*, *ATM*, and *PIK3CA* genes, offering a potential trial recruitment or an investigational PI3K pathway–targeted therapy.

Sarcomas had few biomarkers representations with no on label variations present. *ERBB2* amplification was the only on label biomarker present in gastric and esophageal cancers (4.7%) but off-label biomarkers were present in a quarter of patients, such as in *PIK3CA*, *BRCA2*, and *KRAS* genes.

Prostate cancer had a significant proportion (15.22%) of on-label alterations in HR genes (*ATM*, *BRCA2*, *CHEK2*), with off-label alterations frequently occurring in other low-intermediate risk HR genes and PI3K/AKT/*PTEN* pathway genes.

TumorType	% Patients with On label Biomarkers	Top 10 on Label genes	% Patients with Off label Biomarkers	Top 10 off-Label genes	% Patients with resistance Biomarkers	Resistance genes
Breast	44.13%	*PIK3CA* (41), *ESR1* (29), *BRCA1* (5), *BRCA2* (5), *PTEN* (5), AKT1 (2), *ERBB2*_copy number gain (2), NTRK3_ fusion (1)	24.58%	*NF1* (6), *ATM* (5), *KRAS* (5), *PALB2* (3), *CHEK2* (2), *ERBB2* (2), FGFR2 (2), MLH1 (2), *STK11* (2), TSC1 (2)	16.2%	*ESR1* (29)
Colorectal	7.24%	*BRAF* (5), *ERBB2*_copy number gain (3), *KRAS* (3)	32.89%	*PIK3CA* (18), *NF1* (4), *RAD51C* (3), *ATM* (2), *BRCA2* (2), *BRIP1* (2), MAP2K1 (2), MTOR (2), *NBN* (2), *PTEN* (2)	47.37%	*KRAS*/*NRAS* (72)
Lung	27.20%	*EGFR* (28), *KRAS* (18), ALK_ fusion (6), *BRAF* (5), MET (3), *MET*(_fusion) (3), *ALK* (1), *ERBB2* (1), *RET*_fusion (1), *NTRK3*_fusion (1)	27.62%	*STK11* (20), *PIK3CA* (9), *ATM* (6), *NF1* (6), *BRCA1* (3), *BRCA2* (3), *CHEK2* (2), *NBN* (2), *PTEN* (2), *CDK12* (1)	2.09%	*EGFR* (2), ALK (2), *PIK3CA* (1), *NTRK3* (1), *MET*_Copy number (1)
Pancreatic	0.00%		36.02%	*KRAS* (55), *ATM* (9), *BRIP1* (2), CDKN2A (2), *CHEK2* (2), *PIK3CA* (2), BARD1 (1), *BRCA2* (1), *ERBB2*_copy number gain (1), *MSH6* (1)		
Sarcoma	0.00%		12.77%	*ATM* (1), *BRCA2* (1), CDKN2A (1), IDH2 (1), *PIK3CA* (1), *STK11*_copy number loss (1), *TSC2* (1)		
Gastric/Esophageal	4.17%	*ERBB2*_copy number gain (2)	25.00%	*PIK3CA* (3), *BRCA2* (2), *KRAS* (2), *BRIP1* (1), *EGFR* copy number gain (1), *MAP2K1* (1), *PALB2* (1), *PTEN* (1), *RAD50* (1), *RAD54*L (1)		
Biliary Tract	20.51%	*FGFR2*_fusion) (6), *IDH1* (4)	46.15%	*PALB2* (2), *PIK3CA* (2), *ATM* (1), *BAP1* (1), *BRCA2* (1), *CHEK2* (1), *IDH2* (1), *KRAS* (1), MAP2K1 (1), MSH2 (1)		
Prostate	15.22%	BRCA2 (3), CHEK2 (2), ATM (1), CDK12 (1), MRE11A (1), PALB2 (1)	23.91%	PTEN (4), BARD1 (2), KRAS (2), PIK3CA (2), PTEN_ copy number loss (2), AR (1), FANCC (1), STK11 (1), TSC2 (1)		
Ovarian	15.79%	*BRCA1* (5), *BRCA2* (1)	39.47%	*PIK3CA* (4), *ATM* (1), *BRIP1* (1), *ERBB2* (1), *FANCA* (1), *FANCG* (1), *FANCM* (1), *NF1* (1), *RAD51C* (1)		

Table 1. Genes associated with on label and off label targeted treatment across main tumor types.

Biliary tract cancers also had some of the highest on-label signals (20.5%), with *FGFR2* fusions and *IDH1* mutations being the most frequent ones, and also exhibited the highest off-label frequency, primarily in HR and PI3K pathway genes (46.2%).

In ovarian cancer, on-label *BRCA1/2* mutations were seen in 15.8% of the patients, but 39.5% had off-label biomarkers, most commonly in *PIK3CA* and the Fanconi pathway genes.

Collectively these results imply that LB analysis is capable of providing a valid option for treatment decision-making by identifying biomarkers that can inform treatment but also identify secondary events that lead to resistance to treatment.

The absence of detectable alterations in liquid biopsy analysis may suggest either a genuine lack of tumor-derived DNA in the circulation or the absence of such alterations in the tumor itself. The most reliable way of conclusively determining the presence of ctDNA in plasma is the presence of oncogenic alterations. Therefore, comprehensive multigene analysis could increase the likelihood of gene alteration detection, even if they are not targetable. This will ensure that ctDNA is present and enhance the credibility of the results. Moreover, the rate of actionable alterations as well as the overall mutation rate shows fluctuations among different tumor types. Consequently, the positivity frequencies of different tumor types in our cohort have fluctuated. The maximum detection rates were observed in gastrointestinal tumors (83.33%), colorectal cancer (75.66%), breast cancer (68.72%), cervical cancer (66.67%), and biliary tract cancer (56.41%), while brain tumors (21.43%) and sarcomas (36.17%) exhibited the lowest rate (Fig. 4).

These differences could be combination of biological and technical factors. Tumor types such as colorectal and breast cancer harbor recurrent, well-characterized driver alterations (e.g., *KRAS*, *BRAF*, *PIK3CA*, *ESR1*) that are frequently represented in targeted gene panels, thereby increasing detection likelihood. Additionally, these cancers often exhibit higher levels of ctDNA due to visceral metastatic spread. In contrast, primary brain tumors are located behind the blood–brain barrier, which limits ctDNA release into the circulation. Differences in cohort composition, including disease stage, and potential biases in gene panel design may further contribute to the observed variability in detection rates across tumor types.

The tumor fraction (TF) in plasma is the most common term used to describe the proportion of ctDNA in the total reservoir of cfDNA in plasma during liquid biopsy. The simplest method frequently employed to estimate TF is to identify the highest variant allele frequency (VAF) among detected somatic alterations for each patient and use this value as a proxy for TF. This method approximates the fraction of tumor DNA in circulation by assuming that the mutation with the highest VAF is heterozygous and present in all tumor-derived fragments[35].

We performed this analysis since it offers information beyond the binary mutation positivity rate. While positivity rate simply measures the proportion of patients with at least one detectable somatic alteration, TF provides a quantitative estimate of tumor DNA burden, which can be biologically and clinically informative. Higher TF values may reflect larger tumor burden, greater vascular access of tumor tissue, or higher shedding rates, whereas low TF values may result from small tumors, low-shedding tumor types, or anatomical barriers such as the blood–brain barrier.

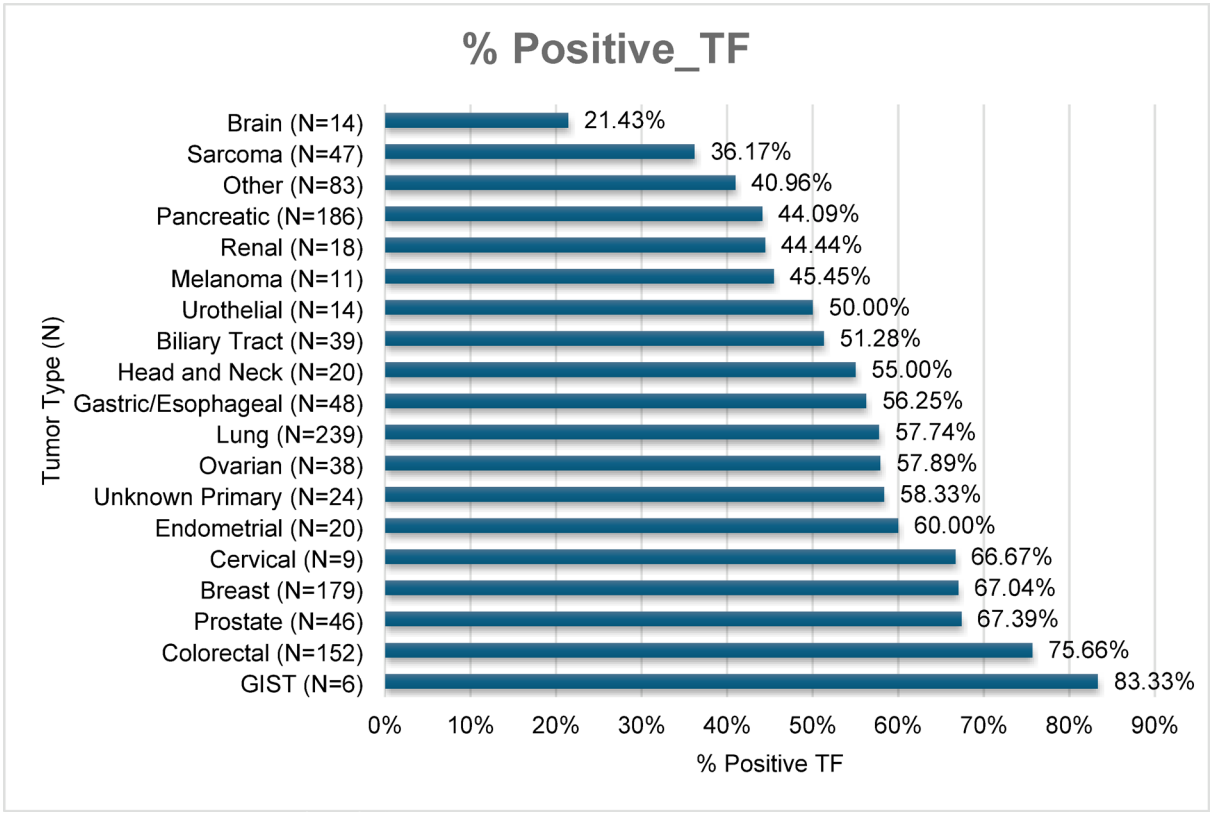


Fig. 4. Positivity rates of LB analysis across the various tumor types.

Nevertheless, the VAF approach used for TF estimation has several limitations. Changes in copy number, subclonality, and technical variability in sequencing may all alter VAF. Therefore, the TF calculation may not accurately reflect the actual tumor burden. In fact, in our cohort 15 patients carried only copy number variation (1.26%) and therefore even though no TF was calculated in these cases the presence of CNV indicates that ctDNA is most probably present. Furthermore, TF obtained from cfDNA only captures shedding at the moment of sampling and may not accurately represent the overall disease burden, especially in tumors that release little or no tumor DNA into circulation.

The median TF in our cohort was considerably low (2.2%), consistent with a heavily pretreated or actively treated population. There was extensive heterogeneity in TF and positive rates across different types of tumors in our findings. Some tumors exhibit both high positivity rates and TF, such as colorectal cancer with 75.66% of ctDNA positive patients and 13.6% median tumor fraction, consistent with the high ctDNA shedding typically observed in this tumor type (Table 2). In prostate cancer, ctDNA was also detectable in a substantial percentage of patients (67.39%) with a median tumor fraction of 14.5%, while the wide interquartile range (96.25) indicates substantial interpatient variability in ctDNA shedding related to disease burden, metastatic pattern, and treatment status. Others such as breast cancer show high positivity rate but moderate TF (4%). This is probably because of the occurrence of highly characterized, high-VAF driver mutations recognizable even at low tumor fraction, along with secondary mutations, such as those in the *ESR1* gene, typically occurring at very low VAF. Brain tumors and sarcomas, however, both exhibit low positive (21.43% and 36.17%) and low TF (0% and 0%, respectively). Sarcoma tumors are known to be frequently driven by chromosomal translocations that create fusion oncogenes, rather than by a large number of point mutations[36], while LB applicability in brain tumors is limited due to the restricted release of ctDNA across the blood–brain barrier[36].

Patients with multiple LB testing

Concerning the 77 patients who underwent repeated testing, 66 had two LB analyses, while 10 underwent three. Among these, 28 patients, who initially were negative for somatic alterations, eventually tested positive. In initially negative patients, conversion to mutation-positive status most often reflected biological increases in ctDNA load, rather than the development of treatment resistance. Twelve patients were somatic-positive in all repeated tests, while 35 remained somatic-negative throughout follow-up (Fig. 5).

Retesting was particularly informative for patients with prior positive results, where changes in the mutational profile could reveal clinically relevant resistance mechanisms. Among repeated LB analysis, true acquired resistance mutations—defined as resistance-associated alterations emerging after a prior positive result—were identified in three patients. Patient 7, who initially harbored two alterations, subsequently exhibited an increase to four alterations, which included the emergence of *MAP2K1* p.G128D (*MEK1*), a recognized MAPK pathway variant that confers resistance to MAPK pathway–targeted therapies, such as BRAF or certain MEK inhibitors.

Tumor type	No. of patients	Median TF (%)	IQR25_TF (%)	IQR75_TF (%)	TF-positive (No.)
GIST	6	3.7	1.45	7.45	5
Colorectal	152	13.6	1	66.3	115
Prostate	46	14.5	0	96.25	31
Breast	179	4	0	23.9	120
Cervical	9	9.6	0	13.4	6
Endometrial	20	3.3	0	18.45	12
Gastric/Esophageal	48	3.6	0	15.85	28
Unknown Primary	24	4	0	19.3	14
Ovarian	38	2	0	12.6	22
Lung	239	2.4	0	11.6	138
Biliary Tract	39	3	0	38.8	22
Head and Neck	20	1.5	0	22.85	11
Urothelial	14	0.8	0	17.45	7
Melanoma	11	0	0	19.4	5
Renal	18	0	0	10.6	8
Pancreatic	186	0	0	5.35	82
Other	83	0	0	10.4	34
Sarcoma	47	0	0	5.5	17
Brain	14	0	0	0	3

Table 2. TF values across major tumor types included in the cohort. IQR25_TF: 25th percentile of TF; IQR75_TF: 75th percentile of TF.

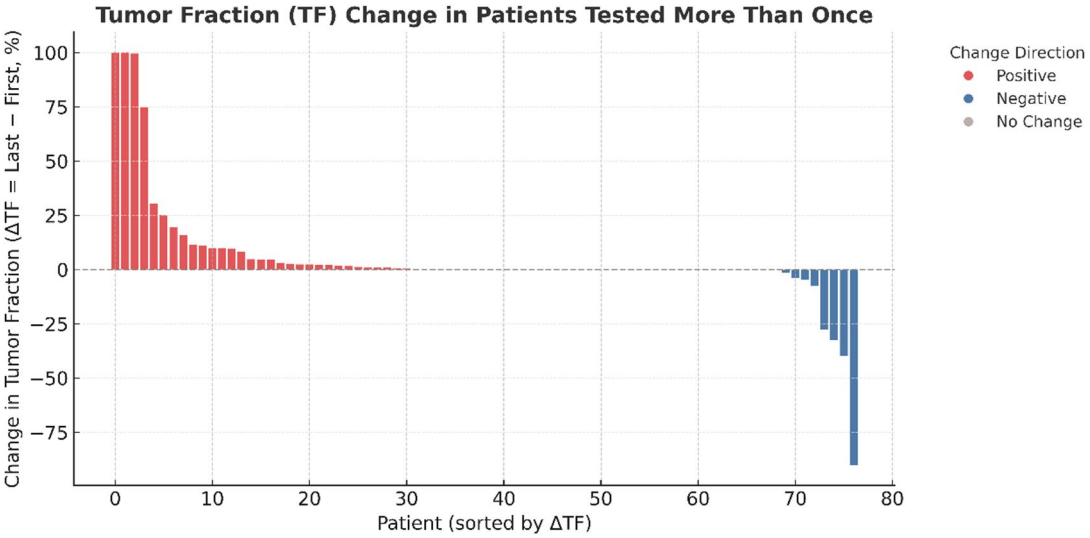


Fig. 5. Tumor Fraction Change in patients who underwent more than one liquid biopsy analysis.

Patient 482 acquired *PIK3CA* p.H1047R during follow-up, a mutation known to confer resistance to targeted endocrine therapies. Patient 903 also exhibited an additional *PIK3CA* p.H1047R alteration after an initially positive test.

Several other patients displayed changes in their somatic mutation profiles between tests; however, these alterations were not classically associated with resistance and more likely reflected ongoing clonal evolution, consistent with tumor progression, rather than therapy escape.

These findings underscore the complementary roles of repeat testing in both resistance monitoring and improved detection sensitivity in the longitudinal management of patients with solid tumors.

Among the 77 patients who underwent more than one liquid biopsy, TF changes between the first and most recent LB test were frequent. TF increased in 29 patients (37.7%), decreased in 8 patients (10.4%), and remained essentially stable in 40 patients (51.9%). Increases often reflect disease progression, rising tumor burden, or emergence of resistance, whereas decreases were consistent, most probably, with treatment response. Stable TF values may indicate disease stability, low tumor shedding, or biological and technical variability. These dynamic changes were seen across tumor types, with high-shedding malignancies such as colorectal and lung

cancer, more frequently showing marked fluctuations. These observations indicate that TF analysis could serve as a valuable biomarker for longitudinal monitoring, enabling earlier detection of progression or resistance to therapy compared to conventional imaging alone.

Prevalence of germline variants per cancer type

Parallel sequencing of white blood cells resulted in the detection of germline pathogenic/likely pathogenic variants in 11.26% of the 1110 unique patients tested. *BRCA1/2* germline pathogenic variants were found in 27 patients. High-risk genes comprised *PALB2*, *RAD51C*, *BRIP1*, *CDKN2A*, *RET*, *BAP1*, *BMPR1*, and MMR genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*, *EpCAM)* (29 total variants). Intermediate-risk gene alterations, comprising *ATM*, *BARD1* mutations, and *CHEK2* splicing or frameshift variants, were detected in 24 patients. Low-risk alterations, comprising *CHEK2* single-nucleotide variants and other genes like *RAD50*, *FAM175A*, *NBN*, *FANCC*, *MUTYH*, *MSH3*, *FANCL*, *NTHL1*, *FANCA*, *RECQL*, *RAD51B*, *FANCM*, *BLM*, *MRE11A*, *RAD51L*, accounted for 57 variants in total. Overall, 137 germline variants in 125 unique patients were detected, in line with the occurrence of more than one germline finding in some patients.

Across the cohort, the prevalence of germline variants differed substantially by tumor type (Fig. 6A). Ovarian and cervical cancers showed the highest proportion of affected patients (>25%). Melanoma, biliary tract, colorectal, breast, pancreatic and tumors of unknown primary, showed intermediate mutation prevalence (10–18%). Other tumor types such as lung, renal, sarcoma, brain, and urothelial cancers were least frequently associated with germline findings. In all tumor types, germline alterations were present at a minimum of 6% of the cases, indicating the importance of such analysis. Additionally, the type of germline alterations was not uniform across cancer types, reflecting the different contributions of hereditary syndromes to tumorigenesis (Fig. 6B). In ovarian, prostate, pancreatic, and breast cancers, a large proportion of variants occur in high-risk genes, frequently driven by *BRCA1/2* and other DNA repair pathway gene alterations. Unexpectedly, *BRCA1/2* alterations also account for a significant portion of germline variants in tumor types that are not typically associated with these genes, such as sarcoma, gastric, and lung cancer. In lung cancer, *BRCA1/2* alterations account for 25% of all detected germline alterations, with 2.31% of patients testing positive. These findings are indicative of the presence of inherited alterations across a variety of tumor types and suggest that high-penetrance genes, such as *BRCA1/2*, may be involved in the development of a broader range of malignancies than was previously recognized. The heterogeneity in prevalence across tumor types highlights both the necessity of this approach in high-prevalence cancers and its potential to yield important findings in those with traditionally lower hereditary risk. This reinforces the argument for implementing germline assessment as a standard adjunct to LB in oncology practice.

Concordance analysis between tissue and liquid biopsy genomic findings

Of the 1193 patients, 145 had concurrent tissue and liquid biopsy testing. Concordance was measured in both negative and positive cases of gene alteration.

Overall, 140 of 145 FFPE samples (96.55%) harbored at least one oncogenic event, whereas 96 of 145 liquid biopsy samples (66.21%) were biomarker-positive. However, the positivity rate for on-label variants was markedly lower — 30 of 145 FFPE samples (20.69%) and 22 of 145 LB samples (15.17%). FFPE analysis detected more off-label variants and more variants considered in clinical trials or of unknown actionability/VUS. Major on-label genes were detected by both assays, as were gene variants associated with potential therapeutic benefit. 131 patients had concordant on-label results (positive or negative) in both tissue and LB. Among those, 114 patients had zero on-label findings, whereas 17 patients carried the same variants in both materials. Among the discordant cases, 9 patients had a tissue-only mutation, and 2 carried additional tissue alterations. On the other hand, 1 patient was ESR1 positive exclusively on LB, and an additional patient carried an extra *ESR1* alteration in LB. Moreover, 1 patient with lung and urothelial cancer had an additional *EML4-ALK* fusion detected only in FFPE and an *FGFR3* alteration detected in both samples. Detection of resistance-associated mutations was similar between the two materials, with 20/145 (13.79%) positive in each, 5 detected only in FFPE, and 5 detected only in LB. These findings suggest that FFPE and LB play complementary roles in identifying resistance mechanisms and that combining both approaches may maximize detection yield.

TMB cut-off in tissue analysis was set at 10muts/Mb according to international guidelines, whereas in LB cut-off was set at 16muts/Mb[37]. 21 patients had a TMB-high score in tissue, whereas TMB was high in only 5 LB cases. Four of the 5 LB TMB-high cases were also TMB-high in tissue, suggesting a reduced sensitivity (19.05%) but increased specificity (99.20%) in TMB detection by the LB assay.

Bar charts showing the comparison of on-label, off-label, and resistance variants between FFPE tissue analysis and liquid biopsy (LB). Each bar represents the percentage of patients in one of four categories: Concordant (same variant status in both FFPE and LB, either identical variants or both negative), Tissue+ / LB (discordant with additional variants detected in FFPE), LB+ / Tissue (discordant with additional variants detected in LB), Discordant-different (same number but different variants detected). Percentages are calculated from the total cohort ($n = 145$).

Discussion

Circulating tumor DNA (ctDNA) analysis using next-generation sequencing (NGS) has emerged as the most clinically useful form of liquid biopsy in routine practice, particularly for patients with metastatic cancer[15,38].

This real-world analysis of 1110 unique metastatic patients undergoing 1193 LB tests provides a comprehensive assessment of LB performance across solid tumors in routine clinical practice.

Using a comprehensive 1021-gene panel, we observed a considerable proportion of actionable alterations spanning targeted therapies and immunotherapy biomarkers. Somatic ctDNA alterations were detected in 61.44% of cases. Including pathogenic germline findings, the overall positivity among unique patients rose to

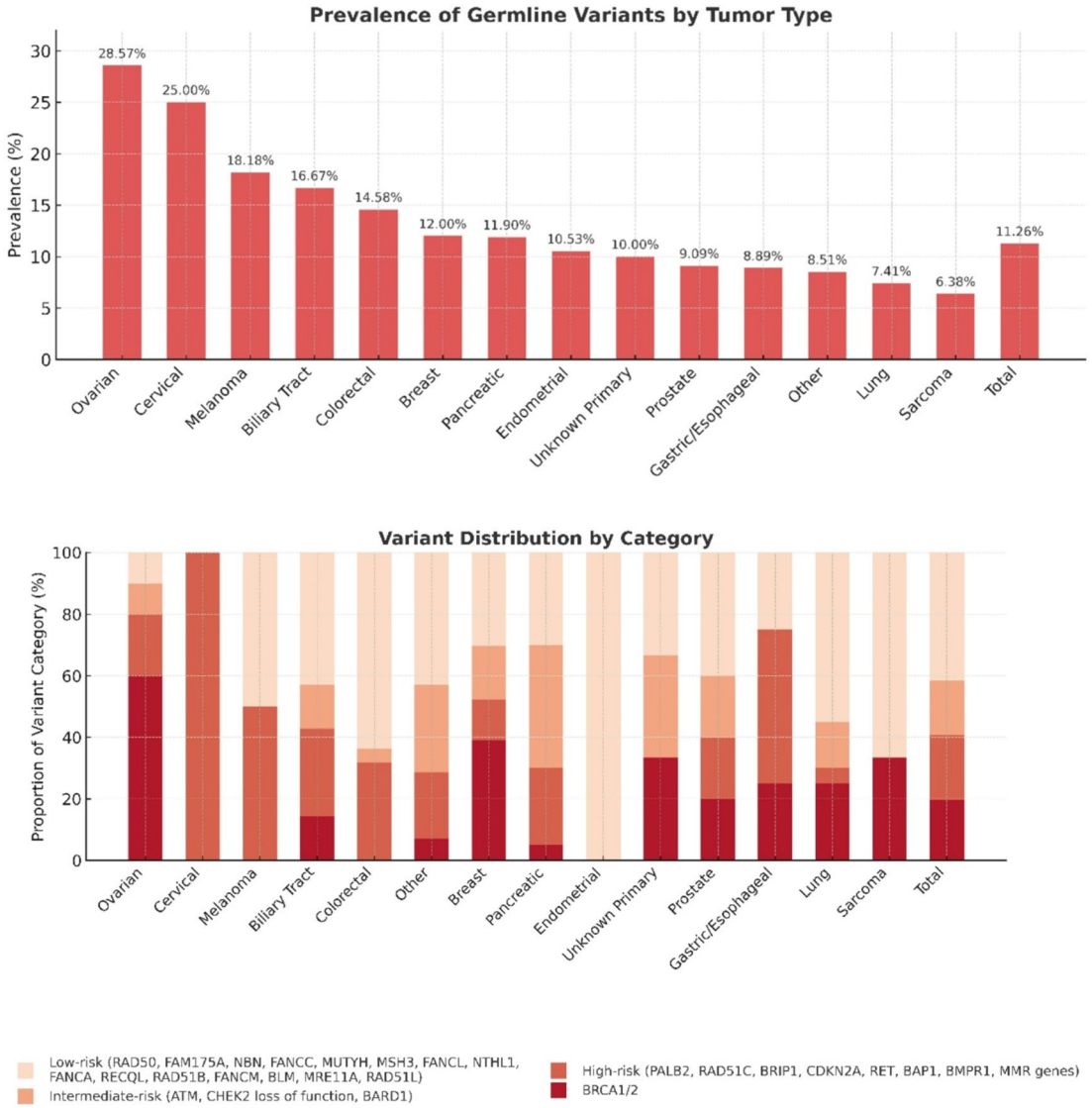


Fig. 6. Prevalence and distribution of germline variants by tumor type. (**A**) Prevalence (%) of germline pathogenic/ likely pathogenic variants across tumor types in the study cohort. Values represent the proportion of patients within each tumor type who harbored at least one germline variant. (**B**) Distribution of germline variants by risk category for each tumor type. Categories include *BRCA1/2* (red), high-risk (*PALB2*, *RAD51C*, *BRIP1*, *CDKN2A*, *RET*, *BAP1*, *BMPR1A*, MMR genes), intermediate-risk (*ATM*, *CHEK2* loss-of-function variants, BARD1), and low-risk (*RAD50*, *FAM175A*, *NBN*, *FANCC*, *MUTYH*, *MSH3*, *FANCL*, *NTHL1*, *FANCA*, *RECQL*, *RAD51B*, *FANCM*, *BLM*, *MRE11A*, *RAD54L*). Percentages within each bar represent the proportion of total variants for that tumor type, noting that individual patients may harbor more than one variant.

65.86%. Actionable alterations were detected in more than 50% of the cases. In 16.18% of cases, at least one on-label variant was identified, while in 22.13% of cases, an off-label variant was the most clinically significant finding. The highest proportion of patients harboring on-label biomarkers was in breast cancer (44.13%), followed by Non-Small Cell Lung Cancer (NSCLC) (27.20%) and prostate cancer (15.22%), while 47.37% of colorectal cancer patients harbored RAS alterations associated with resistance to anti-*EGFR* treatment.

The detection of such genomic alterations via liquid biopsy may not only inform treatment decisions but also be associated with patient prognosis. For example, in prostate cancer, alterations in homologous recombination repair genes, particularly *BRCA1/2* and *ATM* variants detected in ctDNA and common in our cohort, have been associated with aggressive disease biology and may help identify patients with early progression from an oligometastatic state[39].

Moreover, Off-label, clinical trial–related, and resistance-associated findings collectively increased the proportion of clinically actionable results by 40.65% and were predominantly observed in pathways associated with DNA damage repair and PI3K/AKT/mTOR signaling, involving a broad spectrum of genes beyond canonical targets, thereby underscoring the contribution of expanded genomic coverage to clinical interpretation.

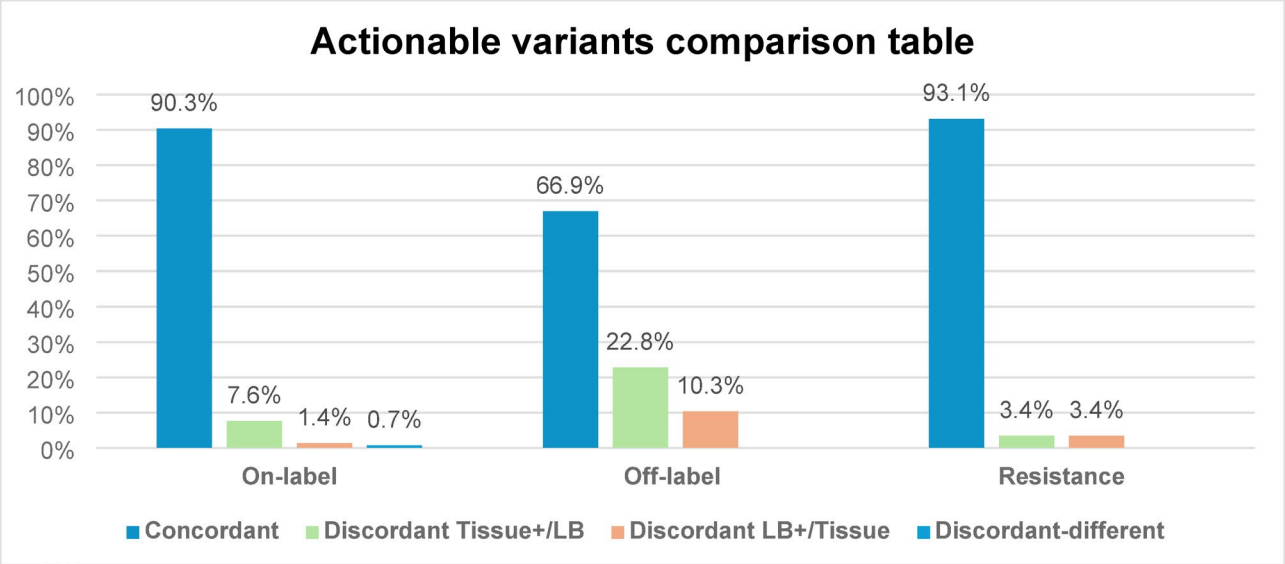


Fig. 7. Concordance analysis of on-label, off-label, and resistance variants detected in matched tissue and liquid biopsy samples from the same patients.

Incorporating blood-based TMB and MSI expanded the potential benefit from immune checkpoint inhibitors by an additional 8.65% (TMB-high 7.29%; MSI-high 1.09%). This aligns with prior real-world and trial datasets showing that combining targeted and immunotherapy biomarkers materially increases therapeutic options derivable from plasma alone. While reported rates vary with tumor mix and assay, our results fit within these ranges and illustrate the complementary role of LB in precision oncology.

The overall positivity rate for unique patients tested was 65.86%. Multicancer series typically report higher baseline detection in stage IV, treatment-naïve settings, frequently > 70–90%[6,40–42]. By contrast, our slightly lower positivity rate likely reflects real-world sampling—many patients were under treatment at the time of testing. Although no large-scale study has directly compared first-line and later-line settings, multiple disease-specific analyses demonstrate that active therapy reduces ctDNA detectability. In metastatic melanoma, only ~ 56% of patients were ctDNA-positive at progression[43]. In breast cancer, neoadjuvant systemic therapy is consistently associated with declining positivity from baseline[44]. In NSCLC, several longitudinal studies confirm that ctDNA levels fall during systemic therapy and that clearance is associated with improved outcomes[45,46]. Likewise, in metastatic colorectal cancer, first-line therapy frequently induces molecular clearance of *RAS* and *BRAF* mutations in plasma[47]. Together, these on-treatment dynamics—absent in most baseline-only series—plausibly account for the lower positivity observed in our cohort compared with pretreatment datasets.

Tumor fraction (TF) further contextualizes these detection patterns. Our overall median TF was considerably low, 2.2%, as expected in patients who were heavily pretreated or receiving ongoing therapy. Highest median TF values were noted in prostate and colorectal cancer (14.5% and 13.6% respectively), while in most tumor types the TF value was < 2–4%. Our lower TF in several entities likely reflects that a substantial portion of sampling occurred after systemic therapy initiation—conditions known to reduce ctDNA levels by decreasing tumor burden[43]. Moreover, TF estimation should be considered when TMB and MSI values are calculated from plasma samples since studies have shown that a more reliable estimation of these biomarkers can be achieved when the circulating tumor DNA fraction is at least ~ 1%, below which mutation detection sensitivity falls, leading to underestimation of TMB/MSI values[48]. Therefore, beyond the detection of guideline-based actionable alterations, the use of such an expanded CGP panel in this study contributed to improved biological interpretation of liquid biopsy results in a real-world metastatic population. Detection of non-common somatic variants served to provide evidence of ctDNA presence in cases lacking canonical biomarkers, supporting the interpretation of negative findings as biologically informative rather than technically inconclusive. This is particularly important for distinguishing true-negative cases from false-negative results caused by low TF or limited shedding[49].

Consistent with prior large datasets demonstrating complementarity of tissue and plasma, our analysis showed that concordance is highest for clinically actionable, truncal alterations (e.g., *KRAS*, *EGFR*, *BRAF*, *PIK3CA*, *ERBB2*, *ESR1*) (Fig. 7), particularly in high-shedding tumors[50–53]. In our series, LB demonstrated high diagnostic performance for these targets, with over 90% sensitivity, whereas subclonal or treatment-emergent mutations were less consistently detected in tissue. Similarly, studies show tumor- and gene-specific variability. In biliary tract cancer, high sensitivity and positive predictive value were achieved for tier 1/2 alterations with LB–tissue pairing[54]. In NSCLC, concordance rises with stage and TF, with CHIP contributing to discordance when not filtered[55,56]. Trials such as CAPItello-291 further show near-perfect agreement for PI3K/AKT/*PTEN* when TF ≥ 1%[57]. Our data is therefore concordant with a broader literature: LB excels for truncal, high-allele-fraction events in high-shedding contexts, while reflex tissue testing remains advisable in low-shedding tumors (e.g., pancreatic, brain) or for suspected secondary resistance alterations.

Moreover, parallel analysis of genomic DNA from peripheral blood cells together with plasma-derived ctDNA, as implemented in this assay, further increases the confidence in the somatic origin of detected variants by enabling the exclusion of clonal hematopoiesis of indeterminate potential (CHIP)–related alterations. CHIP variants, which are not tumor-derived and arise through age-related clonal expansion of hematopoietic progenitors, represent a recognized limitation of plasma-only ctDNA assays; their presence can result in false-positive findings, especially in genes implicated in DNA repair pathways and inaccurate immunotherapy biomarker estimation[6,14]. A key strength is the use of parallel white blood cell (WBC) sequencing, which inevitably lowers apparent "positivity" compared with many prior reports that did not filter non-tumor signals, but more accurately uncover true tumor-derived alterations and the practical performance of LB in the clinic[40,41]. By incorporating matched blood and plasma sequencing, this study mitigates the risk of CHIP-driven misclassification of TMB/MSI status.

Parallel WBC testing in this study provides the additional advantage of enabling the confident identification of germline variants, which may be relevant for hereditary cancer risk assessment. Likely pathogenic/pathogenic germline variants in 11.26% of patients, including 27 *BRCA1/2* carriers and additional high-, intermediate-, and low-penetrance genes across DNA repair and MMR pathways have been identified. Therefore, parallel germline testing in LB offers a dual benefit: optimizing precision oncology decisions for the patient while identifying hereditary cancer syndromes that may impact both patient management and familial risk assessment. The heterogeneity in prevalence across tumor types highlights both the necessity of this approach in high-prevalence cancers and its potential to yield important findings in those with traditionally lower hereditary risk. The capacity to detect hereditary cancer predisposition at the time of LB not only informs immediate treatment planning—for example, PARP inhibitors use in carriers of *BRCA1/2* mutation—but also supplies vital information for long-term screening and prevention strategies. Additionally, detection of germline variants has implications which extend beyond the index patient, enabling cascade testing of at-risk relatives and facilitating early detection or risk-reducing interventions. Since LB is a minimally invasive procedure, parallel germline testing maximizes the value of a single blood draw, simplifying the diagnostic algorithm without incremental procedural risk.

Implications for practice

In aggregate, our real-world results—derived from a large, unselected metastatic population—support LB as a practical complement to tissue-based comprehensive genomic profiling, with particularly strong performance, especially in high-shedding tumor types and truncal, clinically actionable variants. Prospective studies incorporating genome-wide cfDNA methods (such as fragmentomics and methylation) may further improve detection and tissue-of-origin inference in low-shedding cancers[58,59] but our data show that even panel-based LB, may deliver a valuable clinical information in routine oncology practice.

Limitations

This study has several limitations. Although the initial liquid biopsy (LB) cohort is relatively large, tumor types are unevenly represented. As a result, statistical analyses for underrepresented populations should be interpreted as indicative trends rather than definitive conclusions. Furthermore, follow-up was limited to 77 patients who underwent repeated LB testing over a period of 2 to 29 months, with the clinical impact of the results on treatment decisions remaining unclear. Clinical details were also largely unavailable. In most cases, LB testing was performed in heavily pretreated patients or in those receiving active therapy, based on the treating physician's decision. Importantly, key clinical variables that strongly influence LB results—such as tumor burden, anatomical site, time from surgery, exposure to neoadjuvant or adjuvant therapies, treatment type, and treatment duration—were not systematically collected or analyzed.

Conclusions

Liquid comprehensive genomic profiling (CGP) demonstrates high reportability and meaningful clinical utility across tumor types, although ctDNA detectability varies widely. Tumor fraction, shedding dynamics, and tumor biology remain key determinants of assay performance. Beyond guideline-based on-label targets (16.18%), an additional 40.65% of potentially clinically significant findings—including off-label and clinical trial–related alterations as well as resistance-associated variants—were identified, illustrating the added clinical and interpretive value of expanded genomic profiling. The assay successfully detected all major classes of genomic alterations (SNVs, indels, CNVs, and fusions), while parallel ctDNA and gDNA analysis enabled identification of clinically relevant germline variants essential for appropriate patient management.

In addition, ~ 9% of patients were potentially eligible for immune checkpoint inhibitor (ICI) therapy, driven by TMB-high and/or MSI-H status. All this information provided a more complete and trustable biological understanding of tumor characteristics using LB analysis. Among patients with paired tissue and plasma testing, the concordance of on-label variants reached 90%, supporting the reliability of LB in detecting clinically actionable alterations, while additional resistance mutations were uniquely identified in plasma. Together, these findings highlight that integrating liquid biopsy with tissue profiling increases the yield of actionable alterations, supports both targeted and immunotherapy selection, enables resistance monitoring, and shortens time-to-treatment, underscoring its value as a complementary approach to tissue-based testing.

Data availability

NGS raw data can be accessed through *the NCBI Sequence Read Archive (SRA), BioProject ID PRJNA1362977*.

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Author contributions

C.F.C: writing—original draft preparation, experimental proceduresE.P: Writing, conceptualizationV.M.M, A.T, S.M: experimental procedures, validationG.T, D.G: Bioinformatics analysis, figure preparationN.T, D.Z, E.Z, N.A, J.D.C, I.P, M.O, C.B, I.H., A.L, G.E.H: resources, data curationK.B, M.K: supervision, data review, manuscript editingC.P, G.N: project administration, review and editingAll authors reviewed and approved the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to C.F.-C.

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