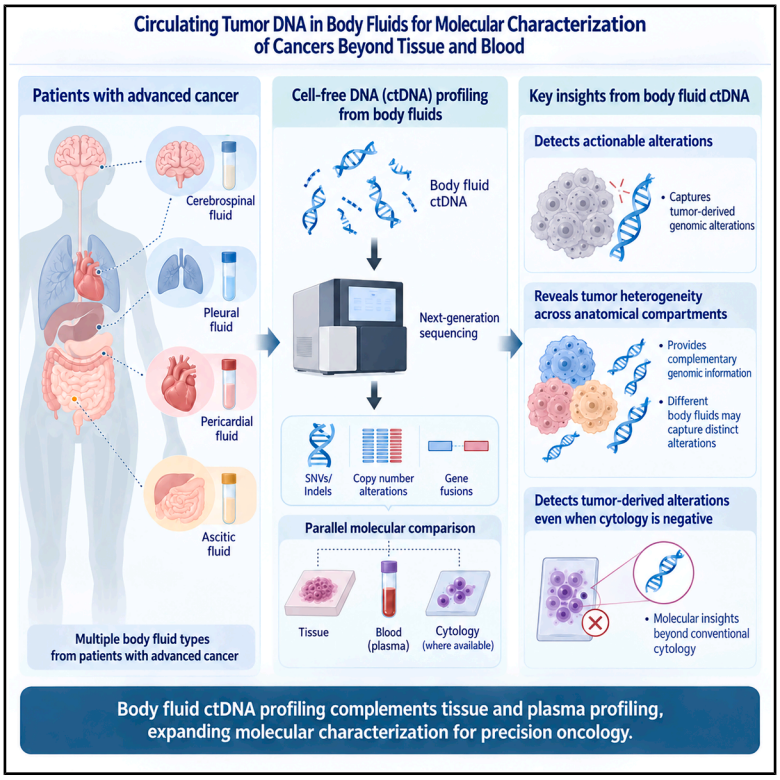
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Article

Circulating tumor DNA in body fluids for molecular characterization of cancers beyond tissue and blood

Graphical abstract



Highlights

- ctDNA was found in 87% of body fluid samples from patients with advanced cancer
- 13.3% of body fluid ctDNA revealed unique mutations missed in tissue or blood
- *TP53*, *EGFR*, and *KRAS* were the most frequently mutated genes detected
- 31% of cases had ESCAT tier IA actionable mutations for therapy

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In brief

Molecular biology; Genomics

Shreenivas et al., 2026, iScience *29*, 117893
November 20, 2026 © 2026 The Authors. Published by Elsevier Inc.
https://doi.org/10.1016/j.isci.2026.117893

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Circulating tumor DNA in body fluids for molecular characterization of cancers beyond tissue and blood

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https://doi.org/10.1016/j.isci.2026.117893

SUMMARY

Liquid biopsy using circulating tumor DNA (ctDNA) is increasingly used for cancer profiling and monitoring, but blood-based analysis may not capture tumor heterogeneity across anatomical compartments. We evaluated cell-free DNA from cerebrospinal (n = 7), pleural (n = 11), pericardial (n = 1), and ascitic (n = 26) fluids from 45 patients with advanced cancer, comparing molecular findings with matched tissue, blood, and cytology where available. Oncogenic alterations were detected in 87% of body fluid samples, with a median variant allele frequency of 3.15%. *TP53* (49%), *EGFR* (18%), and *KRAS* (18%) were the most frequently altered genes, and ESCAT tier IA alterations occurred in 31.1% of patients. In six patients (13.3%), potentially targetable alterations were detected in body fluids but not in blood and/or tissue. Among five cytology-negative samples, four were ctDNA positive. These findings support body fluid ctDNA profiling as a complementary approach to tissue, plasma, and cytology, thereby warranting prospective validation.

INTRODUCTION

Body fluids in patients with cancer, including pleural, peritoneal, pericardial effusions, and cerebrospinal fluid (CSF), represent important clinical challenges, often serving as markers of advanced-stage disease, progression, or recurrence.[1–5] The etiological spectrum of effusions spans both benign and, frequently, malignant conditions. Given this diversity of potential causes, accurate differentiation between malignant and benign involvement of these body compartments is critical for clinical decision-making in oncology practice. Conventional cytological examination has long represented the cornerstone diagnostic approach for evaluating malignant involvement of effusion fluids and CSF. Cytopathological analysis is minimally invasive, technically simple, and cost-effective, relying on well-established diagnostic criteria.[6] However, despite its widespread application, body fluid cytology is marred by several diagnostic challenges. The cytomorphological features of malignant and reactive cells can exhibit a substantial overlap, thereby creating interpretative difficulties. CSF cytology analysis can be particularly challenging, because of low cellular yield and difficulty in reliably differentiating between reactive lymphocytes and malignant cells. Additionally, technical factors such as delayed fixation and sampling variability further complicate cytological assessment.[7]

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Diagnostic limitations of conventional cytopathology are reflected in its suboptimal sensitivity across various body fluids. Studies have documented false-negative rates of up to 30% in effusion fluid cytology[8] and up to 50% in CSF analysis for leptomeningeal disease, particularly in solid tumors.[9] False negatives commonly result from pauci-cellular specimens, rapid cellular degeneration, obscuring elements such as blood or inflammatory cells, or morphologically subtle malignant populations.[10] Conversely, while less common, false-positive results also occur from the misinterpretation of highly reactive mesothelial cells in effusions or activated lymphocytes and ependymal cells in CSF that mimic malignant features during inflammatory or infectious processes.[11] The diagnostic assessment of body fluids thus represents one of the most challenging areas in cytopathology, with significant potential for errors despite best practices.[12]

The emergence of plasma-based liquid biopsy techniques has transformed the landscape of diagnostic oncology, offering a complementary approach to traditional tissue biopsy. Through analysis of circulating tumor DNA (ctDNA), RNA, and other tumor-derived materials, high-throughput liquid biopsy technologies provide insights into tumor genetics, evolution, and heterogeneity.[13,14] Beyond its well-established role in identifying predictive alterations for targeted therapy selection, liquid biopsy has demonstrated growing utility in monitoring treatment response and detecting minimal residual disease (MRD), enabling the possibility of early intervention for recurrent disease before clinical or radiological manifestation.[15–21]

The application of liquid biopsy principles to body fluids beyond plasma represents a natural extension of this technology, with distinct advantages.[22–24] Body fluids such as pleural fluid in lung malignancies may offer superior sensitivity for detecting tumor-specific alterations, owing to two key advantages: their direct contact with tumor sites and a lower background of contaminating DNA from peripheral blood mononuclear cells compared to peripheral blood.[20,25] Similarly, liquid biopsy analysis for ctDNA through plasma may not be very robust in cases of leptomeningeal disease or brain metastases, due to the protective effect of the blood-brain barrier, low ctDNA shedding, and signal dilution in peripheral blood. ctDNA assessment through CSF could be a more reliable biomarker in this context.[26,27] These compartmentalized body fluids may capture region-specific tumor clones and resistance mechanisms that may be diluted or absent in plasma sampling, providing crucial insights into spatial tumor heterogeneity and differential treatment responses across metastatic sites.[28–30] Discordance between body fluid and plasma genomic profiles has been observed in prior studies, highlighting the importance of sampling the most relevant body compartment for genomic profiling.

Body fluid ctDNA-based molecular analysis may reveal resistance mechanisms and therapeutically relevant alterations that inform treatment selection when conventional approaches have failed.[19] The detection of acquired resistance mutations in real-time could facilitate rapid therapeutic adaptation, potentially extending survival.[31–33] It may also serve as a complementary diagnostic tool when conventional cytology yields inconclusive results, potentially reducing the need for multiple invasive diagnostic procedures and accelerating time to treatment initiation.

Despite this compelling rationale, the systematic molecular characterization of body fluids for biomarker detection and malignancy assessment remains underdeveloped compared to plasma-based approaches. This represents a significant missed opportunity in precision oncology, particularly for patients with advanced malignancies and limited therapeutic options. The ability to comprehensively profile effusions at the molecular level could revolutionize diagnostic approaches, therapeutic decision-making, and response assessment for patients with malignant effusions.[34]

To our knowledge, most studies examining fluid-based genomic alterations have traditionally focused on single-organ, single-body fluid evaluation. This study expands the scope of body fluid analysis by evaluating the molecular landscape of ctDNA derived from multiple body fluids across patients with varied solid-organ malignancies. In addition to evaluating genomic alterations with established therapeutic relevance, we focus on two relatively less explored potential uses of body fluid molecular analysis: enhancing diagnostic sensitivity for the malignant involvement of body fluids beyond conventional cytology and capturing tumor heterogeneity through comparative analysis of body fluids with matched tissue and plasma samples. Although such comparisons were feasible only in a subset of patients, this integrative approach provides insights into the complementary value of body fluid molecular analysis and highlights its potential clinical relevance.

RESULTS

Patient characteristics

Data on a total of 45 patients were available for analysis, comprising 18 men and 27 women. The median age was 52, with a range of 13–102. Fluids collected included CSF (n = 7), pleural fluid (n = 11), pericardial fluid (n = 1), and ascitic fluid (n = 26) (Figure 1A). As shown in Figure 1A, ascitic fluid samples were mainly derived from ovarian cancer (34.6%), followed by breast (15.4%), lung (11.5%), pancreatic (11.5%), and smaller proportions of upper gastrointestinal and other solid malignancies. Pleural fluid samples were predominantly represented by patients with lung cancer (54.5%), followed by breast (18.2%) and smaller contributions from cervix, pancreas, and gastrointestinal stromal tumors (GISTs). CSF samples largely reflected central nervous system (CNS) involvement, with cases arising from breast (42.9%), lung (28.6%), gastroesophageal junction (14.3%), and primary CNS tumors (14.3%). The single pericardial fluid sample was obtained from a lung cancer case. This distribution reflects the clinical contexts in which fluid sampling is typically performed in advanced or metastatic diseases. The study included a diverse range of cancer specimens, representing multiple solid tumors with varying biological characteristics and clinical significance (Figure 1B).

Among the most prevalent cancers, lung cancer was the most represented in the study cohort, with 12 (26.7%) patients. Lung cancer was predominantly distributed in pleural fluid (6/12, 50%), which aligns with its direct anatomical proximity to the pleural cavity and frequent association with malignant pleural effusions. Involvement of CSF was seen (2/12, 16.7%), highlighting its known propensity for CNS metastases, often manifesting as

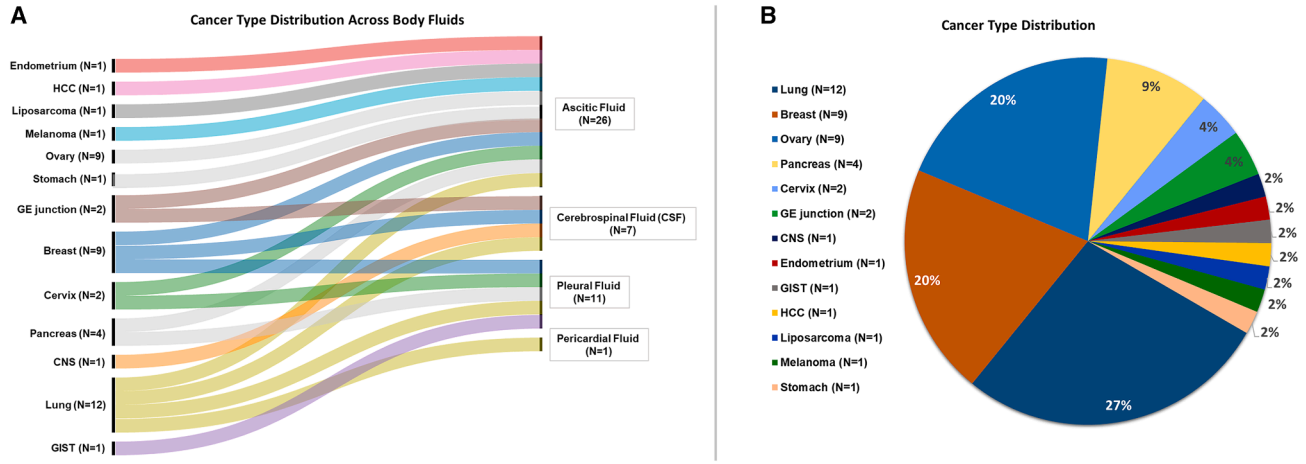


Figure 1. Cancer types examined for body fluid molecular analysis (*N* = 45)

(A) Alluvial (Sankey) diagram showing the distribution of cancer types across body fluids *n* = 45). The flow diagram illustrates how individual cancer types contribute to each fluid category: ascitic fluid (*n* = 26), pleural fluid (*n* = 11), Cerebrospinal fluid (CSF) (*n* = 7), and pericardial fluid (*n* = 1). The width of each connecting flow is proportional to the number of cases represented. Ovarian carcinoma was predominantly represented in ascitic fluid, lung carcinoma was most frequent in pleural fluid, and breast carcinoma was the most common malignancy in CSF. Pericardial fluid contained a single case of lung carcinoma.

(B) Distribution of cancer types in the study cohort (*n* = 45). The pie chart illustrates the distribution of cancer types in the study cohort. Lung cancer was the most prevalent, followed by breast and ovarian cancers. Pancreatic cancer constituted 9% of the cases, while cervical and gastroesophageal junction cancers each accounted for 4%. The remaining cancer types including central nervous system tumor, endometrial, gastrointestinal stromal tumor, liver, liposarcoma, melanoma, and stomach cancer—were each represented by a single case.

CNS, central nervous system; GE junction, gastroesophageal junction; GIST, gastrointestinal stromal tumor; HCC, hepatocellular carcinoma.

leptomeningeal disease, a challenging complication in late-stage lung cancer. The only pericardial fluid sample in the entire cohort was from a patient with lung cancer, emphasizing the known link between lung malignancies and malignant pericardial effusions, which often arise from direct tumor invasion or hematogenous dissemination. Though involvement of ascitic fluid is less common in lung cancer, peritoneal metastases can be observed, particularly in advanced-stage cases where transcoelomic spread may occur. In the study cohort, ascitic fluid was sent for analysis in three patients with lung cancer (3/12, 25%).

Breast cancer was represented by a total of nine cases (9/45, 20%) in this study, with its presence detected across multiple fluid types, thereby reflecting its diverse metastatic pathways. In patients with breast cancer, the common site of fluid involvement was ascitic fluid (4/9, 44.4%), as peritoneal carcinomatosis is seen in advanced breast cancer. CSF was the second most frequently affected fluid in the breast cancer cohort, with 3 of 9 cases (33.3%), followed by pleural fluid in two patients (2/9, 22.2%). Leptomeningeal metastases remain a significant clinical challenge in metastatic breast cancer, often leading to neurological symptoms that necessitate specialized management, including intrathecal therapy or radiation.

For all patients with ovarian cancers (9/45, 20%), we received ascitic fluid for analysis. Pancreatic cancer, known for its aggressive nature and poor prognosis, was represented by four specimens. Cervical cancer and gastroesophageal junction cancer were included with two specimens each. The study also encompassed a broad spectrum of rarer cancers, each represented by a single specimen, including primary CNS tumors, melanoma, stomach cancer, endometrial cancer, GISTs, liposarcoma, and hepatocellular carcinoma (HCC).

ctDNA positivity in body fluids

In this study, the overall ctDNA detection rate was 87% (*n* = 45), demonstrating the effectiveness of capturing tumor-derived DNA from various fluid types (Figure 2). Detection rates were high in all fluid types analyzed, ranging from 71.4% in CSF to 100% in pericardial fluid (albeit with only one analyzed sample in the latter). Of interest, ascitic fluid had a high detection rate of 96% (*n* = 26). Pleural fluid, which is commonly analyzed in cases of lung cancer, mesothelioma, and metastatic disease, exhibited a positive detection rate of 72.7% across 11 samples. Given the sample size and heterogeneity, these detection rates should be interpreted as descriptive observations rather than as definitive performance estimates.

Oncogenic alterations in fluids

The Cancertrack platform can detect multiple classes of genomic alterations, including point mutations, copy number alterations including amplifications, truncations, splice variants, indels, and gene fusions. This profiling of the fluid samples revealed a diverse spectrum of genetic alterations with significant implications for tumor biology, potential therapeutic targets, and prognostic assessment (Figures 3A and 3B).

The analysis identified *TP53* (49%) as the most frequently altered gene. All the detected alterations were single nucleotide variants (SNVs), with no copy number variations (CNVs) or gene fusions observed. Mutations in *EGFR*, a receptor tyrosine kinase, are commonly associated with lung adenocarcinomas,

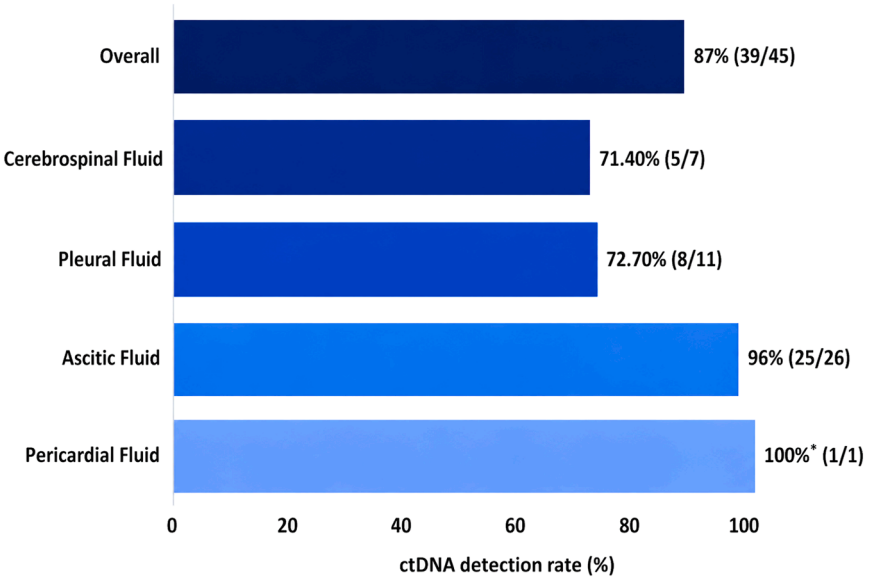


Figure 2. ctDNA detection rate across body fluids (n = 45 patients)

The bar chart presents ctDNA detection rates across various body fluids in a cohort of 45 samples, with an overall detection rate of 87%. Detection rates were highest in ascitic fluid (96%), followed by pleural fluid (72.7%) and cerebrospinal fluid (71.4%). *Pericardial fluid showed ctDNA positivity in the single analyzed sample (1/1, 100%); however, this observation based on one case should be interpreted with caution. ctDNA, circulating tumor DNA.

suggesting the potential benefit of targeted therapies like tyrosine kinase inhibitors (TKIs). *EGFR* alterations were seen in 18% of patients (n = 8), primarily as SNVs and indels. Co-existing amplifications in *EGFR* were detected in three cases with sensitizing *EGFR* mutations.

KRAS mutations were also present in 18% of patients (n = 8), all of which were SNVs. *KRAS* is a pivotal oncogene frequently altered in gastrointestinal, pancreatic, and lung cancers. These point mutations, especially in codons 12, 13, and 61, are critical markers for prognosis and therapeutic resistance. Its presence often drives aggressive tumor behavior, further supporting the complexity of signaling pathways at play.

Other frequently mutated genes include *PIK3CA* (9%), *FGFR3* (9%), *GNAS* (7%), *MYC* (7%), and *CTNNB1* (4%). *PIK3CA* alterations indicate aberrant *PI3K/AKT/mTOR* signaling, a critical pathway in cancer proliferation and survival and commonly altered in breast cancer. *PIK3CA* was altered in 9% of patients (n = 4), with 7% (n = 3) showing SNVs and one case having amplification. This gene encodes a subunit of the PI3K enzyme complex, which is involved in cell growth and survival pathways. Mutations here are often associated with resistance to certain therapies and can be therapeutically targetable with PI3K inhibitors. *FGFR3* alterations were detected in 9% of patients (n = 4), comprising equal proportions of SNVs (4%, n = 2) and amplification (4%, n = 2). *GNAS* was mutated in 7% of patients (n = 3), with SNVs observed in 2% (n = 1) and amplification in 4% (n = 2). *GNAS* encodes a G-protein subunit, and its mutations are classically seen in gastrointestinal tumors such as pancreatic intraductal papillary mucinous neoplasms (IPMNs) and can influence cyclic AMP-mediated signaling.

MYC alterations, found in 7% of patients (n = 3), were exclusively amplifications. *MYC* is a well-known oncogene associated with aggressive tumor behavior and poor prognosis, and its amplification can contribute to increased proliferation and metabolic reprogramming in cancer cells. *CTNNB1* mutations were seen in 4% of cases (n = 2). This gene encodes β-catenin, a key component of the Wnt signaling pathway. Mutations in *CTNNB1* often lead to pathway activation and have been observed in liver, colorectal, and endometrial cancers. *ESR1* was altered in 4% of patients (n = 2). *ESR1* encodes the estrogen receptor alpha and is a major biomarker in breast cancer. Mutations in *ESR1* may influence hormone therapy resistance.

All the samples were negative for fusions/rearrangements.

Variant allele frequency of the oncogenic alterations in body fluids

The variant allele frequency (VAF) distribution across different fluid types revealed notable variations among liquid biopsy samples (Figure 3C). CSF demonstrated a high median VAF of 23.2% (mean: 37.56%), with a range from 0% to 95.52%, highlighting its sensitivity for detecting mutations in CNS involvement. Pleural and ascitic fluids showed median VAFs of 6.5% (mean: 21.33%; range: 0%–95.7%) and 1.23% (mean: 15.53%; range: 0%–92.3%). Across all fluid types, the median VAF was 3.15% (mean: 21.52%; range 0%–95.7%), reflecting body fluids as an enriched source of ctDNA. Pericardial fluid, represented by a single sample, harbored a mutation with a VAF of 70%.

Clinical relevance of the molecular alterations

To demonstrate how the identified molecular alterations impact treatment selection, each alteration was categorized using the ESCAT (European Society for Medical Oncology [ESMO] Scale for Clinical Actionability of Molecular Targets) framework (see Table 1). ESCAT is a structured system that ranks genomic alterations by the amount of clinical evidence supporting their use as treatment targets. If a patient had more than one alteration in the same ESCAT tier, we counted the classification only once per patient, since the percentage was calculated per patient, not per alteration.

Tier IA mutations, which represent the most clinically relevant alterations with approved targeted therapies in the specific tumor type, were observed in 31.1% of cases (14 unique patients). All but one of the cases with tier IA alterations were breast or lung cancer, emphasizing these two as the leading tumor types with clinically relevant therapeutic targets currently. Tier IIIA includes

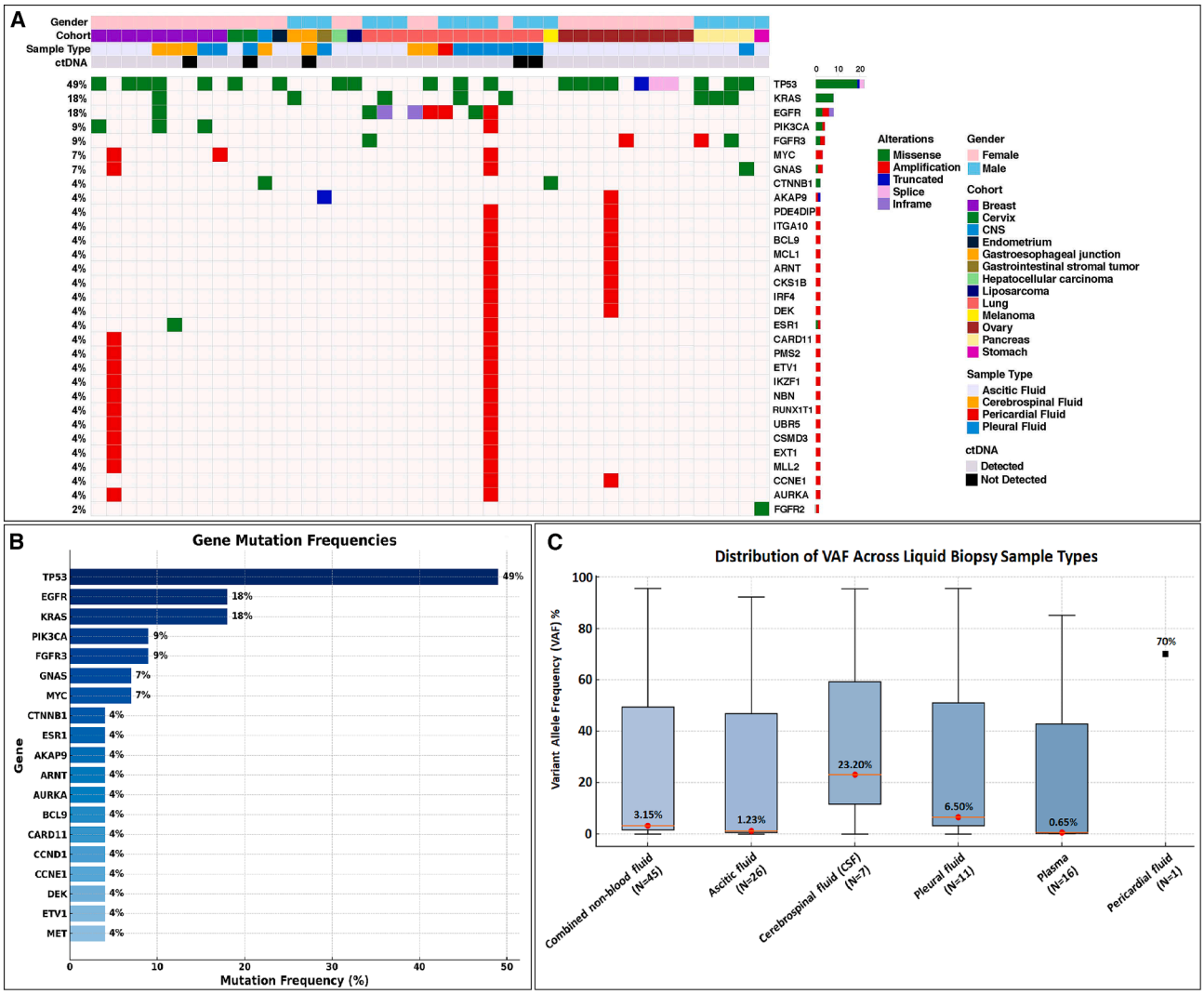


Figure 3. Molecular alterations in body fluids

(A) This Oncoprint illustrates genomic alterations identified through ctDNA analysis in body fluids from 45 patients across a range of cancer types. The top annotations indicate gender, tumor type (cohort), and sample type, with the sidebar reflecting mutation frequency across the cohort. Each column represents an individual sample, and each row depicts an altered gene. Alterations include missense mutations, amplifications, truncations, splice site changes, and in-frame variants. Fusions/rearrangements were analyzed but found negative in this cohort. ctDNA was successfully detected in various fluid types—ascitic, pleural, cerebrospinal, and pericardial—highlighting the utility of liquid biopsy beyond plasma for comprehensive genomic profiling and disease monitoring.

(B) This image shows gene alteration frequencies in these samples, with *TP53* being the most frequent, followed by *EGFR* and *KRAS*. The depicted frequencies include missense mutations, amplifications, truncations, splice site changes, and in-frame variants. Fusions were analyzed but were negative in the cohort. Only oncogenic and likely oncogenic variants were considered, excluding VUS and benign variants. Notable mutations include *PIK3CA*, *FGFR3*, *GNAS*, and *MYC*, while certain genes with low frequencies (≤4%) are not shown. Amplifications were seen in 20% of patients, most frequently in *EGFR* and *MYC* (7% each), with *CCND1* and *FGFR3* at 4%.

(C) The boxplot illustrates the distribution of VAFs across different types of body fluid and blood liquid biopsy samples. The central line in each box represents the median VAF for the respective sample type. The box represents the IQR. The whiskers represent the range of the data within 1.5 times the IQR from the first and third quartiles, showing the spread of most of the data while excluding outliers. Cerebrospinal fluid shows a high median VAF of 23.2%, pleural fluid has median VAF of 6.5%, and ascitic fluid has the lower median VAF at 1.23%. Pericardial fluid had a single sample with an *EGFR* mutation at 70% VAF. Plasma shows lowest median VAF at 0.65%. The high median VAF observed in cerebrospinal fluid, compared to other fluid types, underscores its relative purity and minimal contamination from peripheral blood-derived genomic DNA. These findings highlight the relatively higher VAF in body fluid liquid biopsy.

ctDNA, circulating tumor DNA; CNS, central nervous system; IQR, interquartile range; VAF, variant allele frequency; VUS, variants of unknown significance.

clinically relevant alerations for which drugs were previously shown to benefit in the molecularly defined subset in another tumor type, was the most prevalent category, occurring in 35.6% of cases (16 patients). Tier IVA mutations, associated with drug sensitivity evidence in preclinical *in vitro* or *in vivo* models, were identified in 53.3% of cases (24 patients), highlighting the need for clinical trial enrollment. Tiers IIIB and IVB, representing mutations with emerging but limited therapeutic evidence,

Table 1. ESCAT categorization—Tier-level distribution of oncogenic variants in body fluid samples (excluding blood and tissue)

ESCAT tier	Incidence	Unique patients
IA	31.1%	14
IIA	0.0%	0
IIIA	35.6%	16
IIIB	4.4%	2
IVA	53.3%	24
IVB	2.2%	1
X	20.0%	9

https://www.esmo.org/scales-and-tools/esmo-scale-for-clinical-actionability-of-molecular-targets-escat.
ESCAT, European Society for Medical Oncology.

accounted for 4.4% and 2.2% of cases, respectively. Notably, tier X alterations, which currently lack evidence for therapeutic targeting, were present in 20% of cases (9 patients).

Among the 45 patients, six had therapeutically relevant alterations detected in body fluids—linked to approved or investigational agents—that were not identified in blood and/or tissue (Table 2). The alterations included *KRAS* mutations for which several experimental agents are in clinical trials, as well as an ESR1 resistance mutation that might, however, be sensitive to fulvestrant and elacestrant. In addition, there was a KIT mutation and *FGFR3* amplification, and perhaps, most importantly, one patient with breast cancer had *ERBB2* amplification in ascitic fluid, while it was not found in blood or tissue; *ERBB2* amplification in breast cancer can be targeted by multiple FDA-approved agents. The presence of certain therapeutically relevant alterations only in body fluids reflects tumor heterogeneity and possible clonal evolution, a phenomenon commonly reported in advanced solid tumors. Tumors exhibit spatial heterogeneity across different areas of the same tumor or across different anatomical sites, and a single tissue biopsy or liquid sample often does not fully represent the complete genomic landscape, especially in recurrent relapsing advanced cancers. Differences in the timing of sample draws, particularly after intervening therapies, may also contribute to temporal heterogeneity due to clonal evolution under therapeutic duress. Additionally, localized shedding of ctDNA into the adjacent body cavity may lead to enrichment of certain alterations in body fluids that are not detectable in archived tissue samples or may not be as sensitively detected in plasma.

Cross-sample concordance of tumor-associated alterations

We evaluated tissue and plasma assays for genomic overlap and technical comparability before analyzing concordance to reduce bias. Comparisons focused on shared genomic regions of the NGS panels, including only variants with sufficient sequencing depth and platform-specific sensitivity. This approach was designed to minimize technical variability and ensure that concordance or discordance mainly reflected biological differences, not assay design or panel size. The comparative analysis of simultaneous body fluid with blood and/or tissue biopsies in a cohort of 20 patients highlights the concordance of oncogenic alterations across different sample types (Table S1). Among 16 patients undergoing both body fluid and blood biopsies, 8 patients (50%) exhibited at least one concordant oncogenic alteration, while 6 patients showed no concordant alteration. In the comparison between body fluid and tissue biopsies, 11 samples were assessed, 7 patients had at least one shared oncogenic alteration in both sample types, and 2 patients showed no concordant findings. The most comprehensive comparison, incorporating simultaneous body fluid, tissue, and blood biopsies, involved 7 samples, where only 3 patients displayed concordant oncogenic alterations across all three sample types, while another 3 patients had no shared alterations in any of the analyzed samples. All the samples were negative for fusions/rearrangements. Notably, a small subset of patients (1 or 2 cases per group) exhibited no oncogenic alterations in one sample type, while an additional sample type captured a relevant alteration, underscoring the potential limitations of individual approaches. These findings suggest that while non-blood liquid biopsies can capture a subset of unique oncogenic alterations (as given in Table 2), paired analyses remain crucial for comprehensive genomic profiling, to address tumor heterogeneity and molecular evolution.

Cytology correlation

We evaluated ctDNA status in samples where fluid cytology data were available (Table 3). Out of 25 samples with available comparative data, 17 samples showed positivity for both cytology and ctDNA alterations. Three cytologically positive samples tested negative on NGS. In the remaining 5 cytology-negative samples, 4 were positive for ctDNA, demonstrating that molecular profiling of body fluids may be useful in cases where conventional cytology fails to identify malignancy.

DISCUSSION

Our study highlights the feasibility and clinical relevance of molecular profiling in non-blood body fluids across a broad spectrum of solid tumors. Lung, breast, and ovarian cancers were the most frequently represented among the tumors interrogated. Fluids included pleural and pericardial effusions, ascites, and CSF. ctDNA was detected in 87% (39/45) of all samples, with the highest detection rate observed in ascitic fluid (96%). This finding indicates that peritoneal malignancies shed substantial amounts of ctDNA into the peritoneal cavity. This high detection rate supports the potential clinical relevance of ctDNA analysis in body fluids, particularly in cases where obtaining a tissue biopsy is not feasible. Variability in ctDNA detection across fluid types may reflect both biological and technical factors. Biological contributors include tumor burden at the time of sampling, vascular permeability, and tumor shedding dynamics. Technical variables such as DNA yield, input quantity, sequencing depth, and assay sensitivity thresholds may also influence detection rates. The overall detection rate of 87% observed in this cohort appears comparable to prior reports evaluating ctDNA in similar clinical contexts, recognizing that detection rates are influenced by tumor biology and technical factors.

Table 2. Examples of oncogenic alterations found in non-blood fluids but not in tissue or blood ctDNA (ESCAT tiers IA and IIIA included)

Patient	Cancer type	Non-blood fluid type	Body fluid alteration	Tissue result	Blood result	ESCAT tier
#1	breast	ascites	*ERBB2* amplification	wild type	wild type	tier IA
#2	breast	CSF	*ESR1* p.D538G	wild type	wild type	tier IA
#3	GIST	pleural	*KIT* p.V560E	–[a]	wild type	tier IA
#4	ovary	ascites	*FGFR3* amplification	wild type	wild type	tier IIIA
#5	pancreas	ascites	*KRAS* p.G12V	–[a]	wild type	tier IIIA
#6	pancreas	ascites	*KRAS* p.G12V and *FGFR3* amplification	–[a]	wild type	tier IIIA

CSF, cerebrospinal fluid; ESCAT, European Society for Medical Oncology; GIST, gastrointestinal stromal tumor; ctDNA, circulating tumor DNA.
[a]Not analyzed.

The Cancertrack platform has the ability to detect multiple classes of genomic alterations, including point mutations, copy number alterations including amplifications, truncations, splice variants, indels, and gene fusions. This profiling of the fluid samples revealed a diverse spectrum of genetic alterations with significant implications for tumor biology, potential therapeutic targets, and prognostic assessment. Indeed, molecular profiling revealed therapeutically relevant alterations, with 31.1% of cases harboring ESCAT tier IA mutations. *TP53* (49%), *EGFR* (18%), and *KRAS* (18%) were the most frequently altered genes. Notably, among 45 patients, six had alterations detected in body fluids that were potentially targetable with approved or investigational agents but were not identified in blood and/or tissue. These included *ESR1* D538G (breast cancer CSF—targetable with elacestrant,[35] fulvestrant,[36] lasofoxifene,[37] and camizestrant[38]); *KIT* (GIST pleural fluid—other testing wild-type *KIT*; targetable by imatinib[39]); *ERBB2* amplification (breast ascites; targetable by multiple *ERBB2*/*HER2* agents); and *RAS* mutations (targetable by several experimental drugs) as well as *FGFR3* amplification.[40–42] Previous studies have reported analogous results. For instance, Husain and colleagues found *EGFR* amplification in the ascites fluid of a patient with lung cancer who had no *EGFR* alteration on tissue.[23] Ovarian cancer-related ascites has also been suggested as an important source of ctDNA-based genomic information.[42,43] Several mechanisms can explain the discordant findings between tissue and body fluid samples. Spatial tumor heterogeneity may result in subclonal alterations being underrepresented or absent in the sampled tissue specimen. Temporal differences due to clonal evolution between tissue biopsy and fluid collection may also contribute, particularly in the setting of disease progression or treatment exposure leading to acquired resistance.

The VAF distribution across different fluid types revealed notable variations. For instance, CSF demonstrated a high median VAF of 23.2% (range: 0%–95.52%), highlighting its potential sensitivity for detecting alterations in the presence of CNS involvement. Pericardial fluid, represented by a single sample, harbored a mutation with a VAF of 70%. Pleural and ascitic fluids showed median ctDNA VAFs of 6.5% and 1.23%, respectively. These results are consistent with the concept that body fluids are an important source of ctDNA. High VAFs are not unexpected, as fluids such as ascites, pleural fluid, CSF, and pericardial fluid are often in direct contact with tumor cells or are infiltrated by malignant cells. This proximity may account for the high concentrations of tumor-derived DNA observed in these compartments.

Table 3. Fluid cytology and ctDNA comparison (*n*= 25 samples)

Fluid cytology	ctDNA positive	ctDNA negative
Positive (*n* = 20)	17	3
Negative (*n* = 5)	4	1

ctDNA, circulating tumor DNA.

Concordance analysis highlighted partial overlap with blood and tissue biopsies, emphasizing tumor heterogeneity and the need for sampling multiple body sources. Importantly, ctDNA profiling proved valuable even in cytology-negative samples; indeed, in four patients in whom cytology performed on body fluids was negative, ctDNA was positive, thereby suggesting malignancy. The opposite was also seen; of 20 body fluid samples positive for cytology, three were negative for ctDNA. These results reinforce the role of body fluid ctDNA testing as a complementary diagnostic tool and suggest that, while non-blood liquid biopsies can identify a subset of unique deleterious alterations, paired analyses remain critical for comprehensive genomic profiling, to address tumor heterogeneity and molecular evolution. Clinically, cytological evaluation also plays a pivotal role in staging cancers.[43] For instance, in ovarian cancer, positive ascitic fluid cytology contributes to upstaging the disease to FIGO stage III. Similarly, in gastric and colorectal cancers, it serves as a critical tool for confirming peritoneal metastases. Positive findings also signal a more aggressive disease, increased chemotherapy resistance, and higher recurrence risk. Our results suggest that ctDNA analysis of body fluids may be an important complement to cytology.

Detection and characterization of tumor-related alterations are the cornerstone of oncology management. Traditional diagnostic tools have their limitations. A tissue biopsy alone may not be sufficient for diagnosis and staging of cancer. Moreover, in some cases, obtaining a tissue biopsy may not be feasible if the tumor is in inaccessible anatomical areas or if comorbidities and poor health condition of the patient are contraindications for the procedure. Additionally, tissue biopsies may not capture the heterogeneity of the tumor or the dynamic changes it undergoes during treatment.[44] Body fluid-derived ctDNA analysis may be particularly valuable in clinical settings where tissue biopsy is infeasible, insufficient, or associated with procedural risk.

Identification of clinically relevant alterations exclusively in body fluids could expand therapeutic options, guide optimal therapy selection, and expand the enrollment in biomarker-driven clinical trials. While prospective validation is required, these findings suggest a potential complementary role for fluid-based molecular profiling in precision oncology workflows.

Tumor-derived fragmented DNA, known as ctDNA, is released into the bloodstream as a result of apoptosis or necrosis of tumor cells and serves as an ideal biomarker for real-time monitoring of tumor spread.[45,46] By capturing the genetic diversity of a tumor, ctDNA analysis provides a real-time snapshot of the tumor's genetic landscape. ctDNA can be detected and analyzed, providing detailed insights into tumor genetics, thereby enabling the identification of predictive molecular alterations, associated with cancer progression and therapeutic resistance.[47] Liquid biopsy using blood has become vital in cancer management but may be associated with false negatives, as well as false positives attributable to clonal hematopoiesis of indeterminate potential variants.[48]

Traditionally, body fluids have been used to diagnose malignant spread or seeding into body cavities. The gold standard method to diagnose malignancy is fluid cytology. However, these methods are marred by the consistent challenge of false-negative or false-positive results. This is often due to reactive cells, which can mimic malignant cells, thereby leading to erroneous or inconclusive results. At times, the tumor may have low shedding, making detection challenging and leading to a false-negative result. While fluid cytology remains valuable, its limitations cannot be ignored. Molecular profiling of body fluids offers a path forward, supplementing cytology and, in some scenarios, even providing an alternative when cytology fails. Consistent with this, the NCCN guidelines for CNS cancers recommend that, when available, in patients with possible leptomeningeal disease, assessment of CSF-tDNA should be considered, as it increases the sensitivity of tumor cell detection and enables better assessment of response to treatment.[49–52]

Applying blood liquid biopsy methods for molecular profiling of effusions has the potential to expand the utility of this valuable sample type.[50–53] Analyzing ctDNA from body fluids does not share the same challenges as the interrogation of blood samples. For instance, false-positive CHIP variants are less likely in body fluids. The higher DNA concentrations in these samples may overcome some analytical limitations of current sequencing platforms, potentially enabling the detection of lower frequency mutations or structural variants that are challenging to identify in plasma.

The identification of therapeutically relevant mutations and the discordance observed between fluid and tissue analyses highlight tumor heterogeneity and the importance of multi-compartment sampling, which is crucial for developing personalized treatment strategies, guided interventions, and monitoring treatment response. The detection of unique variants in body fluids, compared to tissue and blood samples, emphasizes the possible benefits of integrating fluid-based diagnostics as an additional strategy in cancer treatment, subject to further validation.

There are several limitations to the current study. The relatively small sample size, particularly for less common fluid types such as pericardial fluid, and the range of tumor histologies limited our ability to perform an in-depth analysis of individual tumor types or fluid types but may speak to the generalizability of our findings. Prior studies have suggested that urine may also be a source of ctDNA, though there may be technical challenges due to fragmentation by the renal system.[22] Matched urine samples were not collected in our study. Additionally, non-uniform timing of sample collection across tissue, plasma, and body fluids may have contributed to discordant findings due to temporal tumor evolution.

Since this was a retrospective look at the data and was not designed to establish clinical utility or predictive value for treatment outcomes, our data should not be used to make firm claims about therapeutic benefits or how this stacks up against traditional diagnostics. We view these results as purely exploratory and descriptive. Our study group was relatively small and quite a mix, covering different cancers and clinical situations. This variety, combined with the fact that we did not always have matching tissue, plasma, or cytology for every patient, meant our concordance analysis had to focus on a smaller subset. Detection rates and concordance metrics should be considered preliminary rather than definitive performance estimates. Some fluid types were barely represented; for instance, our findings on pericardial fluid come from just one case and warrant cautious interpretation. Accordingly, detection rates across fluid types should be viewed as preliminary observations rather than definitive performance estimates. Though genomic overlap and technical comparability were factored in before analyzing concordance across sample types, observations may have been influenced by differences in assay characteristics, including panel design, genomic breadth, sequencing depth, and analytical sensitivity; therefore, a minimal contribution of platform-related variability to discordant findings cannot be entirely excluded. Then there are the real-world factors influencing the data: how the samples were handled, the specific tumor burden, DNA yields, and when the sample was drawn in relation to the patient's treatment. While finding unique changes in one sample type over another might indicate biological differences, the way this study was set up does not prove those mechanisms.

We used ESCAT to keep our evidence labeling consistent. It is important to remember that ESCAT helps link drugs to mutations; it does not automatically mean a patient responded to therapy. Because this was an exploratory, descriptive study involving a relatively small and heterogeneous cohort, with limited sample numbers in several body fluid categories, formal statistical hypothesis testing was not performed. Accordingly, the reported detection rates and concordance analyses should be interpreted as descriptive observations rather than as inferential comparisons. We hope these findings lay the groundwork for larger studies that can truly define the clinical readiness of body fluid ctDNA.

In conclusion, our study demonstrates that body fluid ctDNA analysis represents a potentially valuable addition to the precision oncology toolkit, offering sensitivity for genomic alteration detection and the potential to uncover clinically relevant genomic alterations, including those missed by conventional sampling approaches. The high detection rates, including the detection of ctDNA in cytology-negative fluids, and the

identification of unique therapeutic targets in the form of oncogenic molecular alterations, including those not discerned in tissue or blood, support further prospective evaluation of body fluid molecular profiling as a complementary component of precision oncology workflows. As the field of oncology continues to evolve toward more sophisticated molecular characterization, body fluid analysis provides a practical approach to addressing tumor heterogeneity and capturing the full spectrum of therapeutic vulnerabilities.

Limitations of the study

The findings of this study should be interpreted in the context of several limitations. The relatively small and heterogeneous cohort, comprising multiple tumor types and body fluid categories, particularly limited the ability to perform disease-specific or fluid-specific analyses, with minimal representation of less frequently sampled fluids such as pericardial fluid. The retrospective and exploratory nature of the study also precludes definitive conclusions regarding the clinical utility, diagnostic performance, predictive value, or treatment benefit of body fluid ctDNA analysis; the reported detection and concordance rates should therefore be considered descriptive. Furthermore, matched tissue, plasma, and cytology data were not available for all patients, resulting in smaller subsets for comparative analyses, while differences in sample collection timing relative to tissue biopsy, plasma collection, and treatment may have contributed to molecular discordance through temporal tumor evolution. Variations in assay design, genomic coverage, sequencing depth, analytical sensitivity, and pre-analytical factors such as sample handling, tumor burden, and DNA yield may also have influenced the observed results. Although ESCAT classification was used to contextualize the potential clinical actionability of detected alterations, it does not establish treatment response or clinical benefit. Larger prospective studies with disease-specific cohorts, standardized sampling time points, comprehensive matched-sample analysis, and clinical outcome assessment are warranted to validate these findings and further define the role of body fluid ctDNA in precision oncology.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Aditya Shreenivas (ashreenivas@coh.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- De-identified genomic data supporting the findings of this study are available from the lead contact upon reasonable request, subject to institutional and ethical regulations. No datasets were deposited in public repositories due to patient privacy constraints; access may be granted subject to institutional approvals.
- This study did not generate original code. All bioinformatic analyses were performed using commercially available software (Torrent Suite v.5.16) as described in the STAR Methods section.
- Any additional information required to re-analyze the data reported in this study is available from the lead contact upon reasonable request.

ACKNOWLEDGMENTS

We sincerely thank all the patients and their families for their participation in this study. We acknowledge the clinical teams for their support in patient management and sample coordination. The molecular analysis and data processing for this study were performed at Datar Cancer Genetics using institutional resources. We gratefully acknowledge the laboratory team for their technical expertise and meticulous efforts. This research did not receive any specific external grant or other dedicated funding.

AUTHOR CONTRIBUTIONS

A.S., conceptualization, investigation, formal analysis, data curation, writing – original draft, writing – review and editing, and supervision. S.L., conceptualization, investigation, formal analysis, resources, data curation, writing – original draft, writing – review and editing, and supervision. M.C., writing – review and editing. M.L., writing – review and editing. A.M.G., resources, writing – review and editing. T.C., resources, writing – review and editing. P.L., writing – review and editing. K.M., writing – review and editing. D.S.-N., resources, writing – review and editing. H.O.A.-S., writing – review and editing. D.P., conceptualization, methodology, investigation, formal analysis, resources, data curation, writing – original draft, writing – review and editing, and supervision. R.D., conceptualization, methodology, investigation, resources, writing – review and editing, and supervision. R.K., conceptualization, investigation, formal analysis, data curation, writing – original draft, writing – review and editing, and supervision.

DECLARATION OF INTERESTS

R.K. has received research funding from Boehringer Ingelheim, Debiopharm, Foundation Medicine, Genentech, Grifols, Guardant, Incyte, Konica Minolta, Medimmune, Merck Serono, Omniseq, Pfizer, Sequenom, Sysmex, Takeda, and TopAlliance and from the NCI; has received consultant and/or speaker fees and/or advisory board/consultant relationships with Actuate Therapeutics, AstraZeneca, Bicara Therapeutics, Inc., Biological Dynamics, Caris, Daiichi, EISAI, EMD Serono, EOM Pharmaceuticals, Iylon, Jackson Laboratories, LabCorp, Lanauria Therapeutics, Merck, NeoGenomics, Neomed, Pfizer, Precirix, Prosperdtx, Quanta Therapeutics, Recordati, Regeneron, Roche, TD2/Volastra, Turning Point Therapeutics, and X-Biotech; has an equity interest in CureMatch Inc.; serves on the Board of CureMatch, CureMetrix, and XZOM; and is a co-founder of CureMatch. A.S. serves on the advisory boards of Merus, Taiho, and Eli Lilly. He also serves as a steering committee member of a Bayer-sponsored study. S.L., M.C., A.M.G., T.C., and K.M. serve as external advisors to Datar Cancer Genetics. D.P. is a full-time employee of Datar Cancer Genetics. R.D. is the Founder and Chairman of Datar Cancer Genetics. No direct funding was provided by Datar Cancer Genetics for this research. Molecular analysis and data processing were performed at Datar Cancer Genetics using institutional resources.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT to assist with language editing. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2026.117893.

Received: August 15, 2025
Revised: March 8, 2026
Accepted: September 29, 2026

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Cancertrack™ assay	Datar Cancer Genetics	CLIA ID: 99D2171983
TaqMan™ RNase P assay	Thermo Fisher Scientific	Cat#4316831
Oncomine™ Pan-Cancer Cell-Free Assay	Thermo Fisher Scientific	Cat#A37664
Oncomine™ Comprehensive Assay Plus	Thermo Fisher Scientific	Cat#A48577
Deposited data		
1000 Genomes Project Database	1000 Genomes Project	RRID: SCR_006828; https://www.internationalgenome.org/
Exome Aggregation Consortium (ExAC)	Broad Institute	RRID: SCR_004068; https://exac.broadinstitute.org/
Genome Aggregation Database (gnomAD)	Broad Institute	RRID: SCR_017984; https://gnomad.broadinstitute.org/
Software and algorithms		
Torrent Suite Software, version 5.16	Thermo Fisher Scientific	Version 5.16; https://www.thermofisher.com/
Integrative Genomics Viewer (IGV)	Broad Institute	RRID: SCR_011793; https://www.broadinstitute.org/scientific-community/software/integrative-genomics-viewer
Other		
Streck Cell-Free DNA BCT® tubes	Streck, Omaha, NE, USA	Cat#218992; RRID: SCR_019465
MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit	Thermo Fisher Scientific	Cat#A36716
Qubit® 2.0 Fluorometer	Thermo Fisher Scientific	Cat#Q32866; RRID: SCR_020553
QIAamp® DNA FFPE Advanced UNG Kit	QIAGEN	Cat#56704
RecoverAll™ Total Nucleic Acid Isolation Kit	Thermo Fisher Scientific	Cat#AM1975
Ion Chef™ System	Thermo Fisher Scientific	Cat#4484177
Ion GeneStudio™ S5 Prime System	Thermo Fisher Scientific	Cat#A38196; RRID: SCR_017984
Ion 550™ Chip Kit	Thermo Fisher Scientific	Cat#A34538
SuperScript™ VILO™ Master Mix	Thermo Fisher Scientific	Cat#11754050

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human participants

This retrospective study included patients whose body fluid samples (cerebrospinal fluid, pleural fluid, pericardial fluid, and ascitic fluid) were submitted for clinical molecular analysis at Datar Cancer Genetics (CLIA ID: 99D2171983). Where available, matched plasma (n = 16) and tissue samples (n = 11) were also analyzed. Fluid cytology status was available for 25 patients. The median age of the study cohort was 52 years (range: 13–102 years). The cohort included 18 male and 27 female patients.

Data on gender identity, ancestry/race/ethnicity, and socioeconomic status were not systematically collected as part of this retrospective dataset. The absence of these variables limits the ability to assess potential demographic influences on molecular alteration profiles and may reduce the generalizability of the findings across diverse populations.

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the Indian Council of Medical Research (ICMR) guidelines for biomedical research involving human participants. Written informed consent was obtained from all participants at the time of sample collection to allow the use of de-identified clinical data for research purposes. The study protocol was reviewed and approved by the Independent Ethics Committee of Datar Cancer Genetics Ltd., registered with the Central Drugs Standard Control Organization (CDSCO), Government of India (Registration No.: ECR/231/Indt/MH/2015/RR-25; File No.: EC/25/000020).

Participants were allocated to analytical groups (fluid-only, fluid + plasma, fluid + tissue) based on the availability of matched samples rather than by experimental randomization, consistent with the retrospective design of the study. Analyses were performed on de-identified datasets; no investigator blinding was required due to the retrospective nature of the study.

METHOD DETAILS

Study design

This retrospective cohort study examined body fluid samples submitted for molecular analysis at Datar Cancer Genetics (https://datarpgx.com/, CLIA ID: 99D2171983) to characterize genomic alterations and assess concordance with matched plasma or tissue specimens. Sample types included cerebrospinal fluid, pleural fluid, pericardial fluid, and ascitic fluid. Concordance analysis was restricted to oncogenic or likely oncogenic alterations. Concordant tissue molecular analysis was available in 11 patients and concordant plasma molecular analysis was available in 16 patients. Fluid cytology status was recorded for 25 patients.

Sample collection

Body fluid and peripheral blood samples were collected into Streck Cell-Free DNA BCT® tubes (Streck, Omaha, NE, USA; Cat. No. 218992; RRID: SCR_019465). All specimens were stored and transported at 2°C–8°C prior to processing. Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were used for tissue-based molecular analysis.

Sample processing

Nucleic acid extraction and quantification

Body fluid and blood samples collected in Streck Cell-Free DNA BCT® tubes were processed using Datar Cancer Genetics' CancerTrack™ assay (CLIA ID: 99D2171983).

For fluid samples, centrifugation was performed at 3,000 × g for 20 min, and the clarified supernatant was transferred to 1.5 mL microcentrifuge tubes. For blood samples, centrifugation was performed at 3,000 × g for 20 min to separate plasma, followed by a second centrifugation at 16,000 × g for 10 min to pellet residual cellular debris. The resulting supernatant was transferred to fresh 1.5 mL microcentrifuge tubes.

Cell-free total nucleic acid (cfTNA) was extracted from plasma and fluid supernatants using the MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific; Cat. No. A36716) according to the manufacturer's instructions. cfTNA concentration was quantified using the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific; Cat. No. Q32866; RRID: SCR_020553).

Tissue DNA was extracted from FFPE sections (5–10 μm thickness) with a minimum tumor content of >10% using the QIAamp® DNA FFPE Advanced UNG Kit (QIAGEN; Cat. No. 56704) following manufacturer's instructions after deparaffinization. Tissue RNA was extracted from FFPE sections with a minimum tumor content of >25% using the RecoverAll™ Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific; Cat. No. AM1975). Amplifiable DNA was quantified by qPCR using the TaqMan RNase P assay (Thermo Fisher Scientific; Cat. No. 4316831); RNA was quantified using the Qubit® 2.0 Fluorometer.

Library preparation

For liquid biopsy (fluid and plasma) samples, 20 ng of cfTNA was used as input for library preparation with the Oncomine™ Pan-Cancer Cell-Free Assay (Thermo Fisher Scientific; Cat. No. A37664), targeting somatic alterations across 52 genes. cfTNA was reverse-transcribed to cDNA using SuperScript™ VILO™ Master Mix (Thermo Fisher Scientific; Cat. No. 11754050). cDNA targets were amplified using dual-pool PCR, and amplicons were purified with magnetic bead-based purification. Purified amplicons were further amplified with barcode-adaptor primers using cfDNA Library PCR Master Mix, primer P1, and Ion Tag sequencing barcodes.

For tissue DNA and RNA, libraries were prepared using the Oncomine™ Comprehensive Assay Plus (Thermo Fisher Scientific; Cat. No. A48577), targeting 511 genes, with approximately 20 ng input. PCR amplicons were treated with FuPa Reagent to partially digest primer sequences and phosphorylate amplicons, followed by ligation of multiplex barcodes. Libraries were purified using magnetic beads and amplified with amplification master mix per the manufacturer's instructions. All enzymatic reactions were performed under manufacturer-recommended thermal cycling conditions.

Barcoded libraries were pooled to a final concentration of 100 pM. Template beads were generated and enriched using the Ion Chef System (Thermo Fisher Scientific; Cat. No. 4484177) per manufacturer's recommendations. Sequencing was performed on the Ion GeneStudio™ S5 Prime System (Thermo Fisher Scientific; Cat. No. A38196; RRID: SCR_017984) using the Ion 550™ Chip Kit (Thermo Fisher Scientific; Cat. No. A34538).

Bioinformatic data analysis

Raw sequencing data were processed using Torrent Suite Software v5.16 (Thermo Fisher Scientific), which performs base calling, quality filtering, and alignment to the human reference genome (GRCh37/hg19). The mean sequencing depth achieved was approximately 17,000× for the liquid biopsy panel and 1,000× for the tissue-based panel.

For the liquid biopsy panel, default Torrent Suite parameter settings were applied. Somatic SNV/indel calls required a minimum molecular coverage of six unique molecules and a minimum variant allele frequency (VAF) of 0.1%. Copy number variant (CNV) detection required a median absolute pairwise difference (MAPD) below 0.4 and a CNV ratio above the assay-defined threshold for

amplifications. Fusion transcripts were called when molecular counts exceeded the assay-defined threshold. For the tissue-based panel, a minimum VAF of 5% was applied.

Read alignments and variant calls were reviewed using the Integrative Genomics Viewer (IGV; Broad Institute; RRID: SCR_011793) to evaluate strand bias and assess sequencing error. Variants with population allele frequencies >0.1% in the 1000 Genomes Project database (RRID: SCR_006828), the Exome Aggregation Consortium (ExAC; RRID: SCR_004068), or the Genome Aggregation Database (gnomAD; RRID: SCR_017984) were classified as likely germline and excluded from downstream oncogenic analysis.

Concordance analysis

Concordance between body fluid molecular profiles and those from matched plasma or tissue specimens was assessed based on the presence or absence of oncogenic or likely oncogenic alterations in each matched sample pair. Concordant alterations were defined as variants detected in both the body fluid and the comparator sample type. Discordant alterations were separately categorized as fluid-only or comparator-only findings.

QUANTIFICATION AND STATISTICAL ANALYSIS

All analyses in this study were exploratory and hypothesis-generating in nature. Statistical parameters are reported in the main text, figures, and tables of the manuscript.

Concordance analyses were performed descriptively, based on the presence or absence of oncogenic or likely oncogenic alterations across matched sample types. The number of concordant and discordant alterations is reported as counts and proportions where applicable. The exact value of n for each comparison is specified in the relevant figures and tables: n refers to the number of individual patient specimens analyzed per comparison.

Continuous variables were summarized using descriptive statistics (median, range). No formal statistical hypothesis testing was performed, and no adjustments for multiple comparisons were applied, given the limited and unequal numbers of matched sample pairs available.

No methods were used to formally assess whether the data met the assumptions of any inferential statistical approach, as no such tests were performed.