

Original Research

Aspyre Lung enables robust variant calling in samples that fail next generation sequencing quality control

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ABSTRACT

Molecular testing in non-small cell lung cancer (NSCLC) identifies patients likely to respond to targeted therapeutics. Panel-based testing often employs next-generation sequencing, but challenges include high sample failure rates, quality control issues, high tissue requirements and long turnaround times. Significant proportions of patients do not receive appropriate targeted therapy, with inferior clinical outcomes. Aspyre Lung is a targeted genomic profiling assay for 114 actionable or prognostic genomic variants across 11 genes with a two-day turnaround time of specimen to result. We profiled 198 NSCLC patient tissue samples using Aspyre Lung and a next-generation sequencing- (NGS)-based assay. Cohort A comprised 107 samples that failed to inform due to NGS quality checks, and Cohort B 91 samples that underwent successful NGS testing. Results were compared, and discrepancies resolved by orthogonal methods. For Cohort A (NGS fails), 103 (96 %) passed Aspyre Lung quality control, successfully yielding genomic results, including 48 (47 %) samples with \geq one variant. In Cohort B (NGS pass), all samples passed Aspyre quality control with 97 % concordance to NGS-based testing. Notably, 80 % *EGFR* variant-positive samples were stages I and II. Aspyre Lung profiled 96 % of samples where NGS-based methods failed, and uncovered variants in samples successfully tested by NGS and deemed negative. Aspyre Lung detected *ALK* and *EGFR* variants from patients with early-stage disease, demonstrating utility as a rapid screening assay prior to neoadjuvant immuno-chemotherapy consideration.

Introduction

Lung cancer is the leading cause of cancer mortality worldwide and the third most common cancer in the United States, with around 238,340 new cases and 120,790 deaths in 2023 [1], 80–85 % are non-small cell lung cancer (NSCLC). Genomic biomarker-driven therapy, or ‘targeted therapy’ is now an integral part of therapeutic management; rapid, actionable results based on DNA and RNA biomarker testing are critical for first-line treatment planning in both early

(eNSCLC) and advanced stage (aNSCLC) disease. In resectable eNSCLC (stages IB–IIIA, IIIB [T3, N2]), cancer treatment guidelines now recommend *EGFR*, *ALK*, and PD-L1 testing to exclude positive patients from neoadjuvant immunotherapy, while in aNSCLC, guidelines recommend broad molecular testing for 12 genes (*EGFR*, *ALK*, *ROS1*, *BRAF*, *NTRK* 1/2/3, *MET*, *RET*, *ERBB2*, *NRG1* and *KRAS*) that have associated therapeutics (e.g. [2]). Survival outcomes of empirically treated (chemotherapy or chemotherapy/ immunotherapy combination) aNSCLC patients in a recent United States-based study were inferior to patients

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who waited for genomic test results before initiating therapy [3], supporting these recommendations.

Patient eligibility for targeted therapy is determined through testing for actionable mutations by tumor genomic profiling. Identification of biomarkers using hybrid-capture or amplicon-based next-generation sequencing (NGS) has become common clinical practice. However, NGS does not always successfully inform results, as patient samples may not always meet the required assay specifications, preventing tests from being performed or completed. While investigating the clinical practice gaps affecting appropriate biomarker testing, a claims-based analysis of >38,000 newly diagnosed aNSCLC patients in the United States, Sadik et al. found for every 1000 patients in the study, 49.7 % were lost to precision oncology because of factors associated with obtaining biomarker test results [4], and a further 29.2 % who did receive results did not receive appropriate targeted treatments. Overall, 64.4 % of potentially eligible patients did not benefit from appropriate precision oncology therapies for multiple reasons, many of which relate directly to characteristics of NGS, such as long turnaround time, significant nucleic acid input and high-quality nucleic acid requirements [5,6], together resulting in inferior outcomes for patients.

Solid tumor molecular profiling is typically performed on formalin-fixed, paraffin-embedded (FFPE) tissue. FFPE-derived nucleic acid varies in quality, due to multiple factors during the fixation process and subsequent storage conditions and duration, especially RNA (for examples see [7–9]). Additionally, nucleic acid extraction requires reversal of formalin-formed cross-linking, resulting in fragmentation and impacting sample quality [10]. 5.1 to 25 % of tissue samples fail NGS-based testing due to quality control (QC) parameters contributing to inadequate patient care [4,11,12]. These are failures of sample quality, rather than tumor cell content or cellularity; this also does not include samples unsuitable for NGS testing due to small biopsy specimen size (scant tissue); for example, a recent study demonstrated that 14.6 % of samples had insufficient tissue or tumor cell content, inhibiting biomarker testing and accuracy [5]. Small and pauci-cellular biopsies are typical for eNSCLC, with rapid results required for eligibility for neoadjuvant chemo-immunotherapy and planning for subsequent adjuvant therapy. Alternative testing methods to NGS robust enough to process poor quality and low input samples are required for both aNSCLC and eNSCLC patients.

Allele-Specific Pyrophosphorolysis REaction (Aspyre) is a novel method for molecular testing of DNA and RNA biomarkers [13,14] that relies on highly specific enzymatic degradation (pyrophosphorolysis) of probes hybridized with perfect complementarity to target strands. The assay is adoptable by most molecular diagnostics laboratories as it does not require specialized or expensive equipment [15,16]. Importantly, the sample-to-result process is completed in just two days with a straightforward, stepwise workflow that integrates easily into existing laboratory processes. Built on this technology, Aspyre Lung is a targeted multi-gene profiling assay that detects 114 actionable genomic biomarkers in 11 genes (*ALK*, *BRAF*, *EGFR*, *ERBB2*, *KRAS*, *RET*, *ROS1*, *MET* & *NTRK1/2/3*) [15,17] that have well-established clinical utility in NSCLC with FDA-approved targeted therapeutics, and recommendations for testing by current practice guidelines [2]. A recent study compared the performance of Aspyre Lung runs conducted across three sites on the same samples and found high levels of reproducibility and robustness between different users [16]. Overall, with 97.0 % of samples processed from sample to results at Biofidelity's CAP/CLIA laboratory within two working days (data from Jan 1 to Dec 31, 2024), the simple Aspyre Lung workflow offers the potential for increased and faster access to biomarker testing for patients. The assay permits lower tumor cell content compared to many NGS and single gene assays, has fewer steps and reduced bioinformatics requirements than NGS-based testing.

In this study, performed after Aspyre Lung underwent formal assay validation [15], we investigated a predominantly NSCLC sample set that deliberately included a large number that failed NGS QC to determine whether Aspyre Lung could rescue these samples. Samples were divided

into two cohorts: Cohort A comprising those that failed NGS QC, and Cohort B comprising samples that underwent successful NGS-based analysis, and was included for concordance testing.

Aspyre Lung performed with a very high assay success rate compared to NGS, rescuing nearly all samples that failed NGS QC and had no results. Aspyre Lung detected one or more targetable mutations in 47 % of rescued samples; in a clinical setting, these patients would be candidates for personalized targeted therapy. Additionally, Aspyre Lung provided highly concordant data compared to NGS in samples that passed NGS QC. Aspyre Lung is a rapid testing solution that delivers high-fidelity data while focussing on NCCN-recommended genes for first-line treatment decisions in NSCLC. Unlike NGS, which interrogates a broad range of genes - including some without clear clinical significance - Aspyre Lung provides the critical insights needed for rapid clinical decision-making, and can rescue a significant number of patient samples that fail NGS, ensuring more patients have access to targeted therapies.

Materials and methods

Ethical approval

This was a retrospective research study and no clinical management decisions were made on the basis of any assay results generated herein.

Prospective collection: Institutional Review Board (IRB) or equivalent approval was obtained for sample use in diagnostics development through participating collection sites. All patients provided written informed consent and data were de-identified so no patients could be identified by study personnel outside of the clinical trial.

Precision for Medicine biobanked samples: IRB approval was obtained for the use of remnant biospecimens (Advarra IRB CR00535011).

Sample selection

Prospective collection: FFPE lung tissue blocks were collected from eligible patients via commercial biobanks (Geneticist, Tissue Solutions, Reproc, BocaBio, Cureline, VitroVivo). All patients were treatment-naïve.

Precision for Medicine biobanked samples: FFPE resection tissue blocks were selected from the archive of Precision for Medicine that had under 20 % necrosis, and had enough tissue to yield multiple sections for performing multiple tests. Pathology reports and H&E slides were reviewed internally by qualified personnel.

Clinical characteristics

Demographic data of patients in this study are in Table 1. Eleven non-NSCLC subjects were sent in error but were appropriately processed through Aspyre Lung and orthogonal testing assays and classified in the table as "Other". Samples in this category were diagnosed as carcinoid (two samples), extramedullary plasmacytoma (one sample), spindle cell carcinoma (one sample), neuroendocrine lung carcinoma (two samples), small cell lung carcinoma (two samples), solitary fibrous tumor (one sample), thymic carcinoma (one sample) and colorectal adenocarcinoma (one sample).

Sample processing

Prospective collection: FFPE blocks were manually sectioned into 12 µm scrolls (Shandon Finesse, ThermoFisher), at Biofidelity (Cambridge, UK). All samples had tumor content in excess of the minimum requirement for Aspyre Lung (≥ 10 %). No samples were macrodissected or further treated. DNA and RNA were extracted in parallel using the Quick-DNA/RNA™ FFPE miniprep kit (Zymo Research). Nucleic acid concentration was determined by Qubit™ 1x dsDNA or RNA high sensitivity kit (ThermoFisher). The same extraction was used for testing via Aspyre Lung and the Roche Avenio Targeted assay.

Table 1
Demographic and clinical data for samples used in this study.

Variable	No. Patients (%) (n = 198)
Age (years)	
Mean (SD)	63.03 (SD: 10.3)
Median	65
Range	72 (20–92)
Sex	
Female	62 (31.3)
Male	136 (68.7)
Histology dx	
Adenocarcinoma NSCLC	111 (56)
Squamous NSCLC	55 (27.8)
Large Cell NSCLC	9 (4.5)
Adenosquamous NSCLC	2 (1)
Mixed Adenocarcinoma and large cell neuroendocrine	1 (0.5)
Lung Cancer, not otherwise specified	9 (4.5)
Other (non-NSCLC)	11 (5.6)
Stage of disease	
IA	30 (15)
IB	28 (14)
II (not specified)	2 (1)
IIA	30 (15)
IIB	28 (13)
III (not specified)	3 (1.5)
IIIA	25 (12.6)
IIIB	10 (5)
IIIC	1 (0.5)
IV (not specified)	26 (13)
IVA	2 (1)
IVB	2 (1)
Staging Unknown	11 (5.6)
Sample biopsy date	
2004–2008	27 (13.6)
2009–2013	52 (26.3)
2014–2018	7 (3.5)
2019–2022	112 (56.6)
Smoking status	
Previous smoker	41 (20.7)
Has Never Smoked	46 (23.2)
Unknown	111 (56.2)

Demographic characteristics of patients with samples used in this study reflect the wider United States NSCLC patient population in age and pathology diagnosis, but tend towards earlier stages.

Precision for Medicine biobanked samples: FFPE blocks were manually sectioned into 5 μ m scrolls (EpreDia). All samples had tumor content in excess of 30 %. No samples were macrodissected or further treated. Nucleic acids were extracted using a KingFisher Sample Purification System instrument (ThermoScientific) with the MagMAX™ FFPE DNA/RNA Ultra Kit (ThermoScientific). The same extraction was used for testing via Aspyre Lung and orthogonal testing.

Aspyre Lung

The workflow of Aspyre Lung and the variants detected are described in [15]. Most Aspyre Lung assay runs were performed at Biofidelity Inc. (BFI) in Morrisville, USA, a CAP/CLIA site, with sixteen samples run at Biofidelity Ltd in Cambridge, UK. Samples were tested by Aspyre Lung LDT (BFI) or Aspyre Lung RUO reagents (Cambridge, UK). All assay runs used 20 ng DNA and 6 ng RNA inputs except for seven samples: six with DNA quantity below the minimum required (4.25 to 14 ng DNA), and one with a concentration too low to measure (Supplementary Table 1). All 107 samples from Cohort A that failed NGS assay QC were run through Aspyre Lung without any further QC gating. Data were downloaded from QuantStudio™ 5 Real-Time PCR System instruments (ThermoFisher) running Design and Analysis 2 software. Raw Data CSV produced by this software were analyzed using custom Aspyre Lab v1.1.1 software (<https://analysis.biofidelity.com>). This cloud-based web application takes the Raw Data CSV as input and provides variant

calls and control statuses as output. All variant calling was blinded to results from orthogonal analyses.

NGS-based orthogonal testing

Prospective collection: DNA extracts were analyzed through targeted enrichment (Roche Avenio Targeted Assay) and sequencing (NextSeq 500, Illumina) assay by Glasgow Polyomics (University of Glasgow, UK), according to the manufacturer's guidelines. Analysis was performed by the Roche Sequencing Solutions team (Mannheim, Germany).

Precision for Medicine biobanked samples: DNA and RNA extracts were characterized in-house using the Illumina TruSight Oncology 500 High Throughput (TSO500) DNA/RNA Kit and sequencing (NovSeq 6000).

Calculations

Basic calculations of mean, median, standard deviation, and percentages were calculated using inbuilt functions of Google Sheets. Concordance calculations were based on genes and loci shared by tests being compared.

Results

To test whether Aspyre Lung could analyze samples sent for NGS-based testing, we evaluated two independent collections of predominantly NSCLC samples (Table 1), all of which were tested with Aspyre Lung and an NGS-based comparator assay. The first 122 samples included 96 deliberately selected because they failed one or more QC steps of the Illumina TruSight Oncology 500 High Throughput (TSO500) assay; the remaining 26 passed all QC steps. The second set of 76 came from a prospective collection from 146 patients (the 70 excluded samples were analyzed in [15] and [16]) and were sequenced with the Roche Avenio Tumor Tissue Targeted kit (Avenio); 11 of the 76 failed Avenio QC and sequencing. The Avenio assay uses DNA as input, whereas TSO500 and Aspyre Lung use DNA and RNA. Fig. 1 shows sample groupings and QC results. In Cohort A, 103 of 107 samples (96 %) that failed NGS QC passed Aspyre Lung QC. In Cohort B, all 91 (100 %) samples passed NGS and Aspyre Lung QC.

Cohort A – failed NGS QC samples

107 samples were assessed by Aspyre Lung. 96 of these had failed QC control procedures for the TSO500, and 11 had failed QC control procedures for the Avenio assay. These were samples with adequate tissue levels post-extraction (i.e. not scant samples) but inadequate quality, resulting in a failure to meet post-sequencing NGS analysis requirements (Supplementary Table 2), but with sufficient material for further testing. Of these, 61 failed in part (DNA fail, 54 samples; RNA fail, seven samples) or entirely (DNA and RNA fail, 46 samples). 103 out of the 107 (96 %) passed Aspyre Lung QC for both DNA and RNA. These 103 samples included 48 with at least one variant call by Aspyre Lung; six samples had multiple calls. Out of these 48, 42 had no NGS results due to failure of QC by DNA or RNA. Five samples had positive calls from NGS DNA but failed NGS RNA. These five cases passed Aspyre Lung QC for both DNA and RNA with concordant results to NGS DNA, all patients with a KRAS exon 2 p.G12 mutation. One sample failed DNA NGS QC but passed RNA NGS QC and was ALK-fusion positive, concordant with the result from Aspyre Lung.

Fifty-five samples produced valid Aspyre Lung results but did not identify any actionable variant calls. Of these, two had valid positive calls from NGS for which the relevant nucleic acid passed QC: EGFR exon 20 p.T790M and KRAS exon 2 p.G12S. The EGFR p.T790M variant call was from a sample with a variant allele fraction (VAF) of <3 %, below the published limit of detection of TSO500 and close to that of Aspyre Lung [15,18]). A second extract of this sample was negative by both

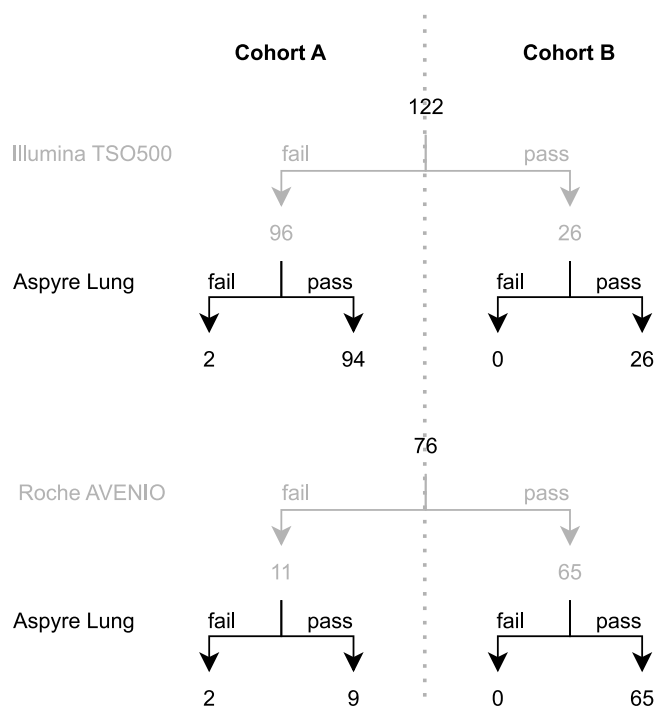


Fig. 1. Cumulative quality control Pass/Fail Results for TSO500, Roche Avenio and Aspyre Lung. Cohort A includes samples that failed part or all the NGS QC stages: DNA and/or RNA components for TSO500, and DNA for the Avenio. Cohort B includes samples that passed all NGS QC stages for the TSO500 or Avenio. The number of samples that passed Aspyre Lung QC is shown for each sub-group. Failure modes for all assays are shown in Supplementary Table 2.

assays. At these levels there is a statistical chance that either assay would not call this variant as positive as the probability of a sample fraction containing an amplifiable variant molecule is dependent on Poisson sampling [13]. For the *KRAS* exon 2 p.G12S-positive by NGS, the calculated VAF was 27.0 %, considerably above the detection threshold of Aspyre Lung. This indicated a discrepancy not due to a sensitivity issue. Orthogonal testing using qPCR was performed on the sample (Supplementary Figure 1 and Supplementary methods), and this indicated a negative result. Due to the uncertainty and the possibility of a sample swap, it was removed from further analyses.

Forty-two samples that failed NGS QC did not yield variant calls DNA or RNA by NGS but produced positive calls with Aspyre Lung. Although these samples failed NGS QC, some samples had sequencing read data still available for review, ranging from 21 to 857 DNA reads at the site of the pertinent biomarker (guided by the Aspyre Lung result), with a mean of 261, and a median of 210 (Supplementary Methods and Supplementary Table 3). These 42 samples had 49 Aspyre Lung positive variant calls in total (Supplementary Table 3) and 36 calls could be manually inspected in this way (Supplementary Figure 2). A remaining thirteen calls could not be manually reviewed; nine samples had insufficient DNA and RNA reads, three variants were *ROS1* or *ALK* rearrangements, and one was *MET* exon 14 skipping-positive. These last four samples were analyzed by two independent methods. First reverse-transcriptase digital polymerase chain reaction (RT-dPCR) was performed, which confirmed all calls (Supplementary Figure 3). To assess the three samples with a gene fusion in more detail, the initial amplification step of Aspyre Lung was carried out, followed by purification and Sanger sequencing of amplicons. These confirmed Aspyre Lung results for two samples, one fusion of *CD74* exon 6 to *ROS1* exon 34, and one fusion of *EML4* exon 13 to *ALK* exon 20 (Supplementary Figure 4). Amplification from a second *ROS1*-positive sample failed to yield specific products. To further analyze the *MET* exon 14 skipping call, the few NGS sequencing

reads available from RNA were examined (no reads were available at all from the DNA). Fifteen reads mapped to within exon 14 from the RNA. A single read mapping to exons 13 and 15 that skipped exon 14 was present out of three that crossed exon boundaries (a second bridged exons 13–14, and the third exons 14–15). Additional support for the Aspyre Lung result included a c.3082+1G>A variant seen in both reads covering the junction of exon 14 and intron 14, which has previously been linked to exon skipping [19], but due to the low numbers, it was not possible to definitively confirm production of *MET* reads with exon 14 skipped at levels that might be compatible with cancer compared to physiological [20,21].

Six samples that failed NGS QC had two or more variants detected by Aspyre Lung. Combinations included four patients with two concurrent variants in *EGFR*, and two with a *ROS1* gene fusion and another independent driver variant. All of these combinations have been reported previously [22–28].

Four samples failed both NGS and Aspyre Lung QC (Supplementary Table 4). The failure modes for the NGS assays for these four samples were no different from others that had been rescued by Aspyre thus it is not possible to isolate a cause of the failures. Several factors may have contributed, including low nucleic acid concentration (for one sample) or quality (high levels of fragmentation or cross-linking), possibly related to the age of the FFPE blocks (Supplementary Tables 4 and 5).

The overall performance of Aspyre Lung runs on Cohort A samples is summarized in Table 2 against ground truth (defined as testing independent of Aspyre Lung: guided inspection of NGS reads or orthogonal testing methods), and concordance was 98.9 %.

Cohort B - NGS pass samples

All 91 samples that passed NGS comparator assay QC passed Aspyre Lung QC, with variants identified shown in Table 3. There were 36 samples with variants identified by either assay, including one sample with two variant calls. Of these 36, 33 samples gave concordant results, one had discordant results, and two were negative by NGS but positive by Aspyre Lung. 22 of the 36 patients had actionable mutations; the remaining 14 had mutations in *KRAS* outside of *KRAS* exon 2 p.G12C.

Three samples had different results between Aspyre Lung and the NGS-based assay. Two were NGS-negative but Aspyre Lung-positive (one *EGFR* exon 19 deletion and one *MET* exon 14 skipping). One sample had discordant positive results in *KRAS* (exon 2 p.G12C and p.G12V by NGS, *KRAS* exon 2 p.G12F by Aspyre Lung). Samples with *EGFR* and *KRAS* variants were investigated by sequencing read inspection (Supplementary Methods). The *EGFR* exon 19 deletion call from Aspyre Lung was supported by NGS read level analysis (Supplementary Table 3). The discordant results for the *KRAS*-positive sample were similarly inspected: reads with c.34_35delinsTT (p.G12F) at a variant allele fraction of approximately 80 % were seen. *KRAS* p.G12C or p.G12V would be c.34G>T or c.35G>T respectively, therefore the result from Aspyre Lung is supported (Supplementary Table 6 and Supplementary Figure 5). This phenomenon of a false *KRAS* p.G12C

Table 2

Comparison of Aspyre Lung results from NGS QC-failed samples to another method.

Cohort A NGS partial or full QC fail	Aspyre Lung- positive	Aspyre Lung- negative	Total
True-positive	34 samples/38 calls	1 sample/1 call	35
True-negative		54 samples/54 calls	54
Unconfirmed	10 samples/12 calls		9
Total	43 samples	55 samples/55 calls	98

Concordance: $(34 + 54) / 89 = 98.9\%$. Ground truth was gained either from inspection of NGS reads or orthogonal testing methods. One sample with two variant calls falls in two categories with a True-positive (*ROS1*) call and Unconfirmed (*KRAS* p.G12) calls and has not been double-counted in the sample totals. Concordance was determined using supported variants only.

Table 3

Reportable variant calls by Aspyre Lung and NGS-based methods in samples which passed all QC checks.

Variant class	Number of samples (n = 91)	NGS result	Aspyre Lung result	Concordance (by class), number (%)
Negative (none)	55	Negative	Negative	55 (100)
SNV	11	<i>KRAS</i> exon 2 p.G12C substitution	<i>KRAS</i> exon 2 p.G12C substitution	28 (100)
	10	<i>KRAS</i> exon 2 p.G12(other) substitution	<i>KRAS</i> exon 2 p.G12(other) substitution	
	3	<i>KRAS</i> exon 2 p.G13 substitution	<i>KRAS</i> exon 2 p.G13 substitution	
	4	<i>EGFR</i> exon 21 substitution	<i>EGFR</i> exon 21 substitution	
	1	<i>KRAS</i> exon 2 p.G12C & p.G12V	<i>KRAS</i> exon 2 p.G12F	0 (0)
Complex	1	<i>EGFR</i> exon 19 deletion	<i>EGFR</i> exon 19 deletion	4 (80)
	1	Negative	<i>EGFR</i> exon 19 deletion	
Indel	3	<i>ERBB2</i> exon 20 insertion	<i>ERBB2</i> exon 20 insertion	
	1	<i>EGFR</i> exon 18 substitution	<i>EGFR</i> exon 18 substitution	1 (100)
	1	<i>EGFR</i> exon 21 substitution	<i>EGFR</i> exon 21 substitution	
Multi-variant	1	Negative	<i>MET</i> exon 14 skipping	0 (0)
Exon skipping	1	Negative	<i>MET</i> exon 14 skipping	0 (0)
Totals	91	34 positive samples, 36 mutations	36 positive samples, 37 mutations	

91 samples were analysed, 36 variants were reported for 34 samples by NGS, and 37 variants for 36 samples by Aspyre Lung.

variant call instead of p.G12F has been reported previously for molecular tests [29,30]. Both these samples were considered to be true positive by Aspyre Lung. The final sample (*MET* exon 14 skipping variant-positive by Aspyre Lung) only had associated NGS DNA reads (Aspyre Lung input for this variant is RNA). Substitutions at the 3' end of exon 14 previously confirmed to cause exon 14 skipping in *MET* transcripts (e.g. c.3028G>T) [19] were present in the DNA, albeit at low levels. Analysis using RT-dPCR confirmed high levels of transcripts with exon 14 of *MET* skipped (Supplementary Figure 4). For our analysis, we considered this sample as *MET* exon 14 skipping variant-positive.

The overall concordance to NGS at the patient level was 97 % (88/91), with a positive percent agreement of 100 % (34/34) and a negative percent agreement of 96 % (55/57) (Table 4). Overall, NGS resulted in one false positive and one false negative result while Aspyre Lung had no false positives or false negatives out of 91 samples (Table 5).

EGFR/ALK early-stage samples

For patients with eNSCLC (IB-IIIa), exclusion of those with *EGFR* variants and *ALK* rearrangements is required for consideration for neoadjuvant immuno-chemotherapy per NCCN guidelines [2] hence access to highly sensitive tests is crucial. Most samples used in this study were resections, therefore early-stage samples were well represented. Some samples from the prospective collection that passed NGS QC and were *ALK* fusion-positive were used in previous studies and therefore are not re-presented here [15,16], however, 80 % of samples in this study that were *EGFR*-positive by Aspyre Lung were at stages I and II, showing excellent detection rates even at these early stages (Fig. 2).

Discussion

Today, patients diagnosed with aNSCLC have effective therapeutic options, but these are only available to the ~50 % of patients who successfully undergo biomarker testing [4] due to multiple gaps in the genomic testing landscape. Several testing-related factors contribute to these gaps, including cost, the practical need to batch samples leading to

Table 4

Concordance of Aspyre Lung with comparator test results at the patient (per sample) level.

	Aspyre Lung-positive	Aspyre Lung-negative	Total
NGS-positive	34	0	34
NGS-negative	2	55	57
Total	36	55	91
Overall Concordance: 88/91 = 96.7 %			
Positive Percent Agreement (PPA): 34/34 = 100 %			
Negative Percent Agreement (NPA): 55/57 = 96.5 %			

NGS results (Roche Avenio or TSO500) were compared to Aspyre results per patient.

Table 5

Comparison to ground truth, defined as testing independent of Aspyre Lung.

	Aspyre-positive	Aspyre-negative	Total
True-positive	37 calls	0	37
True-negative	0	55	55
Total	37 calls	55	91 samples (92 calls)
Sensitivity of Aspyre per call: 37/37 = 100 %			
Sensitivity of NGS per call: 34/37 = 91.9 %			

In this analysis, each call from a sample is considered independently (one sample had more than one variant call).

prolonged turnaround times, and high sample quality and tissue level requirements leading to many samples being rejected before processing has even started [4]. Aspyre Lung addresses these gaps by providing targeted genomic profiling of 114 actionable or prognostic genomic variants across 11 genes, encompassing all guideline-recommended biomarkers for first line NSCLC treatment management, with a turnaround time of two days [31]. This study evaluated the performance of Aspyre Lung on two independent cohorts of NSCLC patient samples: one cohort that failed NGS QC and another that successfully underwent NGS-based testing.

The results are impressive (Fig. 3). In Cohort A (samples that failed NGS QC), Aspyre-Lung successfully rescued 96 % of cases, generating valid results including for 42 samples with driver variants. These were samples deemed acceptable for NGS processing by input quantity, but that failed quality procedures during sequencing preparation. Orthogonal testing or manual inspection of NGS reads was viable for a subset of cases and supported Aspyre Lung results. From Cohort A overall, Aspyre Lung showed a concordance of 98.9 % compared to ground truth. From Cohort B, Aspyre Lung and NGS-based methods had a concordance of 96.7 %, with discordant results resolved in favor of Aspyre Lung. Taken together, these data demonstrate Aspyre Lung's capability to deliver clinically important data from samples otherwise deemed unfit for analysis by NGS, thereby addressing a critical gap in biomarker testing and enabling more patients with cancer to benefit from targeted therapies.

While these samples were non-randomly selected to include a high proportion of NGS failures, most of the demographic characteristics (Table 1) reflect the wider NSCLC patient population in the United States which has a mean and median age of 71 at diagnosis, and sub-diagnoses of adenocarcinoma and squamous cell carcinoma in 51 % and 24 % of patients (with 26 % other or unspecified) [1]. Samples in this study are less representative of the wider patient population in terms of cancer stage, as a higher proportion of samples tested from the general population are at Stages III and IV (28 % and 38 % respectively) compared to our cohort (20 % and 15 %), and there were more Stage II samples in our cohort (8 % nationally compared to 29 %) [32]. Additionally, 39.9 % of

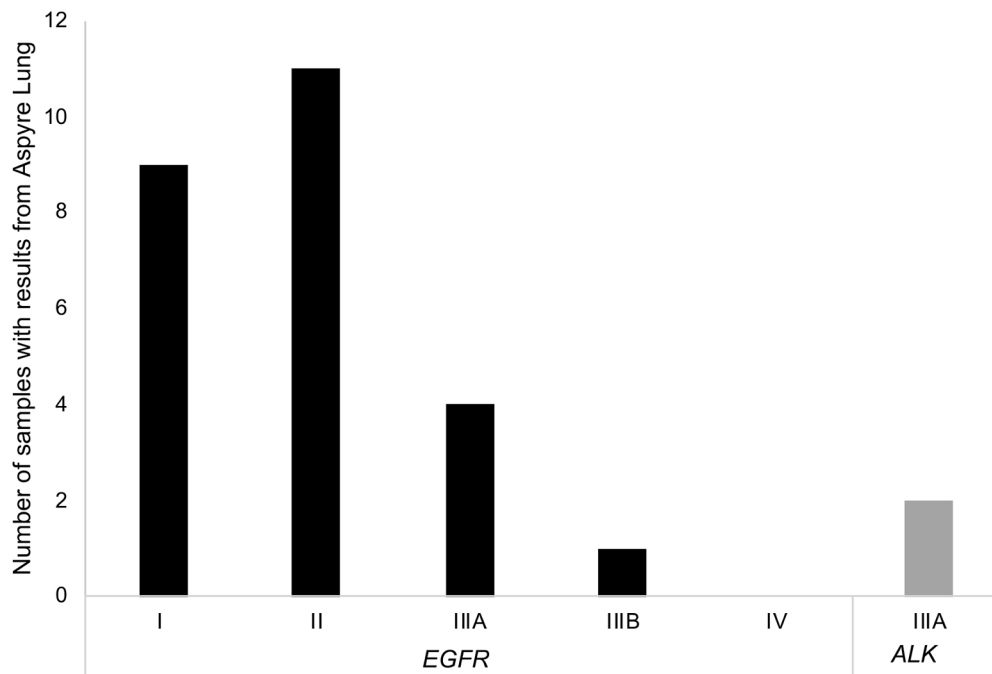


Fig. 2. Breakdown of sample classification by stage for *EGFR*/*ALK* variant-positive samples. Black: samples with an *EGFR* call; grey: samples with an *ALK* call.

our FFPE samples were over ten years old. Historically, it has been observed that NGS QC failure rises with increasing age of FFPE tissue, characterized by lower average read depth and reduced coverage, particularly for samples older than ten years [7,33]; in this study, older blocks demonstrated a high failure rate with the TSO500 (Supplementary Table 5). Conversely, the Aspyre Lung assay yielded valid results for nearly all blocks, including identifying targetable and driver variants (Supplementary Table 5), with over 30 blocks aged thirteen years or older. This feature of the assay may be valuable for biobanks with NSCLC FFPE block stocks previously considered uncharacterizable due to age [7,33].

Even when samples do pass NGS QC stages and valid results are reported, issues with bioinformatic complexities associated with the detection of insertions, deletions and compound substitutions from NGS reads mean that these classes of variants may be missed [34–36]. This is illustrated by examples in this study such as a p.L747_A750delinsP *EGFR* exon 19 indel call from Aspyre Lung (Table 3). When the NGS reads were manually examined, clear evidence of the indel was found. Another illustrative example is the discordant call of *KRAS* p.G12C and p.G12V from NGS, which was correctly identified as p.G12F by Aspyre Lung (Table 3, Supplementary Figure 5 and Supplementary Table 6). This error could have significant implications for patient care, as a false positive result could lead to ineffective *KRAS* p.G12C targeted therapy, potentially causing patient harm.

In eNSCLC, rapid testing for *EGFR* and *ALK* is essential. A recent real-world study by Muthusamy et al. showed that patients who underwent molecular profiling before recurrence and harbored a targetable *EGFR*, *ALK*, *RET*, or *ROS1* driver mutation were able to start first-line therapy sooner (19 days) compared to patients with testing ordered on an existing specimen after recurrence (47 days; $p < 0.001$) [37]. The current United States national coverage determination for NGS (NCD 90.2) does not cover testing for early-stage cancers and/or repeat testing using NGS due to the high cost, and in this clinical setting most of the samples are fine needle aspirates or core biopsies [4]. Early-stage testing of key genes in NSCLC using Aspyre Lung is strategic as it is fast, can utilize small biopsy samples, and our data has shown high sensitivity and concordance to NGS, accelerating the start of guideline-recommended first-line therapy in patients.

Aspyre Lung is designed to interrogate the most prevalent alterations in the 11 genes covered and has limitations. It does not detect all low-prevalence alterations, whereas NGS is in theory capable of identifying all alterations. In a clinical scenario, Aspyre Lung, with its fast turnaround time of two days [31] and high sensitivity and specificity [15,38], could be used as a first line test, with reflex to NGS after a negative result to identify any rare prevalence alterations, as well as alterations outside of NCCN guideline genes for potential inclusion in clinical trials.

Aspyre is a unique and novel technology that leverages the exquisite discrimination of pyrophosphorolysis to power a rapid assay with the simple equipment requirements of PCR, but with the sensitivity of dPCR or NGS, and high robustness across laboratories [15,17,31]. While other technologies that perform mutation detection are also based on multiplex PCR, the use of pyrophosphorolysis to reduce the proportion of wild-type amplicons is unique. The Aspyre Lung panel means that simplified genomic profiling can be performed on 114 different biomarkers covering all first-line recommended genes, beyond the capability of standard PCR, qPCR or dPCR assays. It was highlighted over a decade ago that ‘tissue is the issue’ [39] which remains true today, despite the number of panel assays on offer, partially due to high sample input requirements of many [40,41], and partially due to growing additional requirements for tissue (for example, mandated cellular-based tests for markers such as PD-L1). Recent work has validated lower inputs of down to 5 ng DNA and 1.5 ng RNA in Aspyre Lung while still maintaining high sensitivity [38], optimizing tissue conservation. For some patients liquid biopsies are a more appropriate option, and the recent validation of Aspyre Lung Blood facilitates compliance with recommendations for complementary testing of ctDNA and tissue to reduce turnaround time and increase the likelihood of detection of targetable variants [2,17,42]. Plasma and tissue samples can be run in parallel on the same plate, rationalising and simplifying clinical and laboratory workflows.

In conclusion, Aspyre Lung offers a notable advancement in the genomic testing landscape for NSCLC. The ability to rescue samples that failed NGS analysis and provide rapid and accurate results position it as a transformative tool in cancer diagnostics. By improving access to broad molecular profiling, Aspyre Lung can help ensure that more

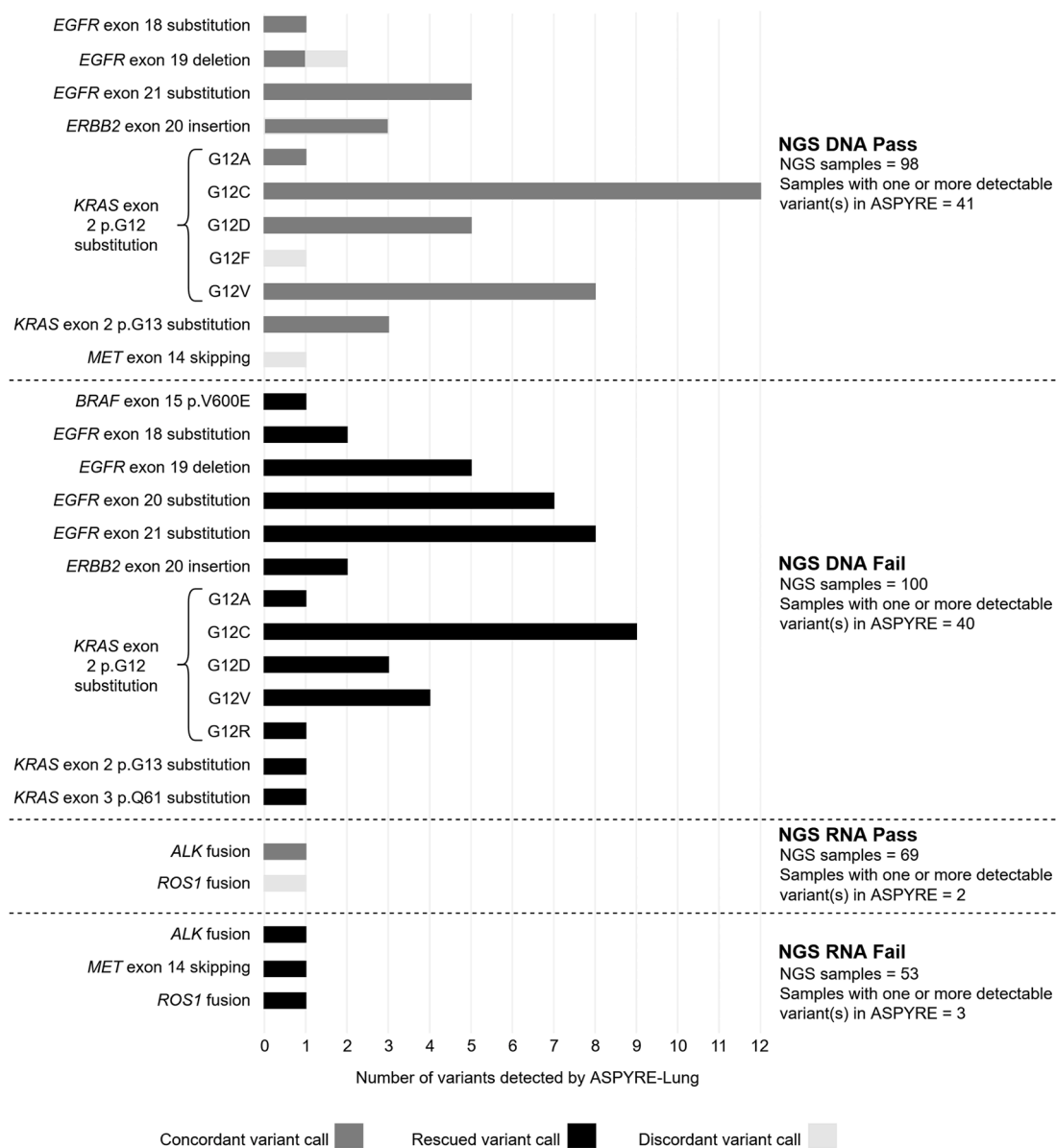


Fig. 3. Reportable positive variant calls by Aspyre Lung in all samples (Cohort A + B). Samples are categorized by NGS QC status and either passed all NGS QC stages or failed one or more stages for DNA (TSO500/Roche Avenio). NGS RNA Pass/Fail status is only available for TSO500 results. “Concordant variant call” and “Discordant variant call” describe the Aspyre Lung call relative to passing NGS results. “Rescued variant call” corresponds to variant calls made by Aspyre Lung in samples that previously failed NGS.

NSCLC patients benefit from effective and personalized treatment strategies, ultimately improving survival outcomes.

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Data and material availability

The dataset supporting the conclusions of this article is available in the Short Read Archive repository, <https://submit.ncbi.nlm.nih.gov/subs/sra/SUB14579449/overview>. The Aspyre data analysis process can be accessed at <https://analysis.biofidelity.com>. Aspyre Lung Reagents are available from Biofidelity Ltd (www.biofidelity.com).

CRedit authorship contribution statement

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The following authors are or were employees or consultants of Biofidelity Ltd or Biofidelity Inc and may have financial interests including stocks, stock options, intellectual property and paid travel expenses: ERG, RTE, EGZ, KEK, KvB, JNB, CKing, CKiser, MBR, RJO, JG, BWB, WJL and ES. The following authors are employees of Precision for Medicine Inc and may have financial interests including stocks, stock options, intellectual property and paid travel expenses: DD, MLT, JC, JY, CT and RWS.

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Supplementary materials

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