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Liquid Biopsies of Endobronchial Ultrasound Transbronchial Needle Aspiration Supernatant for the Molecular Characterisation of Non Small Cell Lung Cancer

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Tese para obtenção do Grau de Doutor em
Medicina
(3º ciclo de estudos)

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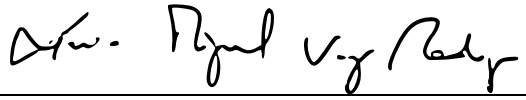

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Dedication

The story is not in the words; it's in the struggle.

Paul Auster, The New York Trilogy, 1987

This work is dedicated to my family, to my parents, for their unwavering support and example, to my wife, for her patience and encouragement, and to my children, who are my greatest source of inspiration and purpose.

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Acknowledgements

I wish to express my deepest gratitude to my three mentors. To **Professor Luís Taborda Barata**, for his constant presence and for being the compass that guided me throughout this doctoral journey. To **Professor Rosa Cordovilla**, for sharing her knowledge and talent, for being a source of inspiration since the genesis of this thesis, for planting the seed that grew into this work, and for her unwavering support that overcame the barriers of distance. To **Professor Vítor Sousa**, for his generous and close support, for guiding me through the complex field of anatomical and molecular pathology, and for helping to build a seamless connection between the clinic and the laboratory.

I also extend my appreciation to the **Department of Pulmonology, IPOC-FG**, including all nurses, technicians, and physicians, with special thanks to my colleagues in interventional pulmonology, **Dr. Michele de Santis, Dr. Paulo Matos** and our dedicated nurses, **Patricia Simões**, **Mónica Moreira** and **Vasco Nunes**, for their continuous assistance during all procedures. My sincere gratitude extends also to **Dra. Lourdes Barradas**, Director of the Department of Pulmonology, for her constant support and for encouraging the pursuit of the research that made this thesis possible.

With sincere appreciation, I acknowledge **Dra. Marta Viegas** and the team at the **Molecular Pathology Laboratory, IPOC-FG**, for their expert collaboration, rigorous guidance, and unfailing support. My gratitude extends to **Dra. Ana Alarcão** and **Dra. Ana Ladeirinha** of the **Molecular Pathology Laboratory, Faculty of Medicine, University of Coimbra**, whose expertise, kindness, and constant availability were invaluable throughout this work.

I am deeply grateful to the **Research Support Unit of IPOC-FG**, especially **Dra. Joana Oliveira** and **Dr. Pedro Mota Veiga**, for their tireless logistical, scientific, and analytical support. I wish to highlight **Dra. Joana Oliveira**, whose expertise and dedication were invaluable in advancing several projects related to this thesis.

Finally, I would like to express my sincere gratitude to **Dra. Ana Pais**, Clinical Director of IPOC-FG, and to the **Board of Directors** for their support and for facilitating, within the institution's possibilities, the resources and conditions that were essential for the successful completion of this work.

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Resumo

Introdução

O diagnóstico e a caracterização molecular do cancro de pulmão de não pequenas células (CPNPC) sofreram uma mudança de paradigma nas últimas décadas, impulsionada pela necessidade de um perfil de biomarcadores abrangente e atempado para orientar terapêuticas de precisão. A punção aspirativa transbronquica guiada por ecobroncoscopia (EBUS-TBNA) tornou-se uma técnica essencial, oferecendo uma abordagem minimamente invasiva que permite o diagnóstico e o estadiamento mediastínico no mesmo procedimento, com elevada rentabilidade. No entanto, persistem desafios na otimização da colheita de tecido, no equilíbrio entre as necessidades morfológicas, imuno-histoquímicas e moleculares, e na redução dos tempos de resposta, de forma a evitar atrasos no início da terapêutica.

O principal objetivo desta tese foi avaliar o papel do EBUS-TBNA no fluxo diagnóstico e molecular do CPNPC avançado, com foco na sua capacidade de fornecer material adequado para testes de biomarcadores e no potencial do seu sobrenadante como fonte de biópsia líquida próxima do tumor. Este objetivo foi explorado em quatro áreas complementares, alinhadas com objetivos secundários que, em conjunto, abordam a questão principal: (i) o posicionamento do EBUS-TBNA nos percursos diagnósticos; (ii) a adequação e limitações das estratégias sequenciais de testagem molecular; (iii) a comparação entre o desempenho da testagem sequencial e da sequenciação de nova geração (*massively parallel-next generation sequencing* [MP-NGS]); e (iv) a viabilidade, concordância e utilidade clínica da fase sobrenadante das amostras de EBUS-TBNA.

Métodos

Este trabalho integra cinco estudos: três análises observacionais retrospetivas, uma revisão sistemática com meta-análise e um estudo prospetivo comparativo. Todos os estudos foram realizados no Serviço de Pneumologia do Instituto Português de Oncologia de Coimbra Francisco Gentil (IPOC-FG), em colaboração com o Laboratório de Patologia Molecular do IPOC-FG e, no estudo prospetivo, com o Laboratório de Anatomia Patológica e Patologia Molecular da Faculdade de Medicina da Universidade de Coimbra. O estudo obteve aprovação ética (ref. 23-2022). O consentimento informado

escrito foi obtido de todos os participantes ou dos seus representantes legais quando aplicável.

A recolha de dados incluiu variáveis demográficas, clínicas, histológicas, e moleculares, bem como os tempos entre a suspeita clínica, os resultados moleculares e o início da terapêutica. Os estudos retrospetivos analisaram processos clínicos eletrónicos e bases de dados de anatomia patológica, enquanto o estudo prospetivo colheu amostras emparelhadas de *pellet* celular e da fase sobrenadante derivados de EBUS-TBNA.

O DNA e RNA foram extraídos, quantificados e analisados por MP-NGS nos Estudos I, III e V. Os Estudos II e III também aplicaram um fluxo sequencial, com análise de *EGFR* por reação em cadeia da polimerase em tempo real e avaliação de rearranjos *ALK*/*ROS1* por hibridização fluorescente *in situ*.

A revisão sistemática e meta-análise (Estudo IV) seguiu as recomendações PRISMA 2020, com protocolo previamente registado (PROSPERO CRD42024600046). As análises estatísticas foram realizadas sob supervisão da unidade de apoio à investigação do IPOC-FG, com recurso ao IBM SPSS v.27.0 (IBM Corp., NY, USA); valores de $p < 0.05$ foram considerados estatisticamente significativos.

Resultados

O **Estudo I** incluiu uma coorte de 205 doentes com CPNPC estádio IV. A mediana de idades foi de 68 anos (intervalo, 38–89), com 59.5% de doentes do sexo masculino. O EBUS-TBNA e o EUS-B representaram mais de metade dos procedimentos diagnósticos iniciais (51.7%), permitindo tempos significativamente mais curtos entre a avaliação clínica e a biópsia quando comparados com a biópsia transtorácica ou abordagens cirúrgicas (medianas de 8 e 5 vs. 20.5 e 24.5 dias; $p < 0{,}001$). O perfil molecular foi tentado em todos os casos, com elevada adequação em todas as modalidades (97.6%). As alterações acionáveis identificadas incluíram mutações *EGFR* (26.3%), *KRAS* (14.1%), *ALK* (6.8%) e outras alterações menos frequentes como *ERBB2* (3.9%), *BRAF* (1%) e *MET* (1%). Atrasos ≥10 dias entre a suspeita clínica e a biópsia foram um preditor independente de mortalidade (HR 1.66; IC95% 1.10–2.50; $p = 0.016$).

O **Estudo II** analisou uma coorte retrospetiva de 59 doentes com diagnóstico de CPNPC que realizaram EBUS-TBNA no seu percurso diagnóstico inicial. Entre estes, 64.4% realizaram a análise molecular de forma sequencial em amostras obtidas a partir deste procedimento. As taxas de adequação foram elevadas, com sucesso na análise de 89.5% das amostras para *EGFR* e 81.3% para *ALK*, confirmando a viabilidade da técnica.

Verificou-se, contudo, uma diminuição progressiva do rendimento entre os diferentes marcadores, sugerindo esgotamento gradual do material disponível ao longo dos testes sequenciais. Adicionalmente, em cerca de um terço dos casos, o estudo molecular foi realizado em amostras biológicas alternativas, apesar de o material obtido por EBUS-TBNA se encontrar disponível.

O **Estudo III** comparou o método de testagem sequencial com o método MP-NGS em 106 doentes com CPNPC em estádio IV avaliados por EBUS-TBNA. A estratégia MP-NGS obteve adequação universal (100%) e detetou uma taxa significativamente mais elevada de mutações acionáveis (40.9% vs. 22.2%; $p = 0.042$), o que se traduziu numa maior taxa de alocação a terapêuticas-alvo (44.3% vs. 22.2%; $p = 0.038$). Estas, por sua vez, podem ter contribuído para a tendência observada para maior sobrevivência global no grupo MP-NGS (mediana de 672 vs. 138 dias), embora a diferença não tenha atingido significância estatística ($p = 0.053$). O tempo até obtenção de resultados moleculares excedeu, em ambas as estratégias, os valores recomendados, sendo ligeiramente inferior com a abordagem sequencial em comparação com MP-NGS, realizado externamente (17 dias vs. 23 dias; $p = 0.076$).

O **Estudo IV**, uma revisão sistemática com meta-análise, sintetizou a evidência de sete estudos (506 doentes). A caracterização molecular do sobrenadante demonstrou elevada viabilidade (87–100%), excelente concordância com as amostras tecidulares emparelhadas (κ=0.947) e tempos de resposta consistentemente mais curtos (até sete dias mais rápidos), apesar da heterogeneidade nos protocolos pré-analíticos. As análises de subgrupo não identificaram impacto significativo do meio preservante ou temperatura de armazenamento no rendimento de DNA, embora a variabilidade dos protocolos limite conclusões definitivas sobre as melhores práticas de processamento.

O **Estudo V**, prospetivo, avaliou amostras emparelhadas de *pellet* celular e do sobrenadante derivado de amostras de EBUS-TBNA de 20 doentes. Os rendimentos de ácidos nucleicos foram superiores no sobrenadante em comparação com o *pellet* celular (DNA: 28.95 ng/µL vs. 9.84 ng/µL, $p = 0.025$; RNA: 37.6 ng/µL vs. 15.95 ng/µL, $p = 0.007$). A concordância molecular entre frações foi de 85%, com três casos discordantes: dois por quantidade insuficiente no sobrenadante e um por discrepância molecular real, com deteção de uma mutação *KRAS* G12C apenas na fase sobrenadante. A simulação de um fluxo diagnóstico paralelo, com análise molecular iniciada no sobrenadante aquando da biópsia, enquanto o *pellet* celular era processado para histologia, reduziu o tempo total de diagnóstico de uma mediana de 21.5 dias para 13 dias ($p < 0.001$).

Discussão e Conclusão

Esta tese demonstra que o EBUS-TBNA é uma ferramenta central no diagnóstico e caracterização molecular do CPNPC avançado, fornecendo material de elevada qualidade para caracterização abrangente e cuja acessibilidade permitiu encurtar os tempos até ao diagnóstico e início de terapêutica, um fator associado de forma independente à sobrevivência. A estratégia de analise molecular sequencial, apesar de viável, mostrou limitações relacionadas com exaustão da amostra e menor deteção de mutações. A metodologia MP-NGS demonstrou desempenho superior, aumentando a identificação de alterações acionáveis e o acesso a terapêuticas dirigidas. A integração da fase sobrenadante, mostrou-se exequível e fiável, com elevada concordância molecular com o percurso convencional de caracterização molecular baseada em tecido e mostrou elevado potencial para acelerar os tempos de resposta, representando um possível avanço significativo no fluxo diagnóstico.

No conjunto, estes resultados suportam um modelo integrado de diagnóstico em que o *pellet* celular é utilizado para histologia e imuno-histoquímica, enquanto o perfil molecular é realizado em paralelo a partir do sobrenadante. Esta abordagem otimiza a utilização do material, assegura a precisão diagnóstica e acelera a disponibilização de resultados moleculares, alinhando os percursos diagnósticos com as exigências da oncologia de precisão. A validação multicêntrica prospetiva e a padronização de protocolos serão essenciais para garantir a reprodutibilidade e facilitar a sua implementação clínica em larga escala.

Palavras-chave

CPNPC; EBUS-TBNA; caracterização molecular; NGS; sobrenadante; oncologia de precisão.

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Abstract

Introduction

The diagnosis and molecular characterisation of non-small cell lung cancer (NSCLC) has undergone a paradigm shift over the last decades, driven by the need for timely and comprehensive biomarker profiling to guide precision therapies. Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) has become a cornerstone technique, providing a minimally invasive approach that allows simultaneous diagnosis and mediastinal staging in a single procedure with high yield. However, challenges remain in optimising tissue acquisition, balancing morphological, immunohistochemical, and molecular needs, and reducing turnaround times to avoid delays in treatment initiation.

The primary objective of this thesis was to evaluate the role of EBUS-TBNA in the diagnostic and molecular workflow of advanced NSCLC, focusing on its ability to provide adequate material for biomarker testing and the potential of its supernatant as a tumour-proximal liquid biopsy source. This aim was explored through four complementary areas, aligned with secondary objectives that collectively address the primary question: (i) the positioning of EBUS-TBNA within real-world diagnostic pathways; (ii) the adequacy and limitations of sequential testing strategies; (iii) the comparative performance of sequential versus massively parallel next-generation sequencing (MP-NGS); and (iv) the feasibility, concordance, and clinical utility of the supernatant phase of EBUS-TBNA derived samples.

Methods

This work integrates five studies: three retrospective observational analyses, one systematic review and meta-analysis, and one prospective comparative study. All studies were conducted primarily at the Pulmonology Department of the Francisco Gentil - Portuguese Oncology Institute of Coimbra (*Instituto Português de Oncologia de Coimbra, Francisco Gentil* – IPOC-FG), in collaboration with the Institute's Molecular Pathology Laboratory, and, for the prospective study, the Institute of Anatomical Pathology and the Molecular Pathology Laboratory of the Faculty of Medicine, University of Coimbra. Ethical approval (ref. 23-2022) was obtained, and written informed consent was secured from all participants, or from their legal representatives when applicable.

Data collection included demographic, clinical, histological, procedural, and molecular variables, as well as timelines from diagnostic suspicion to molecular results and treatment initiation. Retrospective studies analysed electronic medical records and pathology databases, while the prospective study collected paired cell pellet (CP) and supernatant (SP) samples during routine EBUS-TBNA procedures, processed according to standard protocols. DNA and RNA were extracted, quantified, and analysed by NGS in Studies I, III, and V. Studies II and III also applied a sequential workflow, with *EGFR* testing performed by real-time polymerase chain reaction (RT-PCR) and *ALK/ROS1* rearrangements assessed by fluorescence in situ hybridisation (FISH).

The systematic review and meta-analysis (Study IV) followed PRISMA 2020 guidelines with a prospectively registered protocol (PROSPERO CRD42024600046). Statistical analyses were performed under the supervision of a biostatistician from the IPOC-FG Research Support Unit, using IBM SPSS v.27.0 (IBM Corp., NY, USA); a two-sided *p*-value <0.05 was considered statistically significant. Detailed methodology and specific statistical approaches are provided in each study.

Results

Study I involved a real-world cohort of 205 patients with stage IV NSCLC. Median age was 68 years (range, 38–89), with 59.5% male. EBUS-TBNA and EUS-B accounted for over half of the initial diagnostic procedures (51.7%), enabling significantly shorter times from clinical evaluation to biopsy compared with transthoracic biopsy or surgical approaches (median 8 and 5 vs. 20.5 and 24.5 days, respectively; $p < 0.001$). Molecular profiling was attempted in all cases, achieving high adequacy across modalities (97.6%). Actionable alterations identified included *EGFR* mutations (26.3%), *KRAS* (14.1%), *ALK* (6.8%), and less frequent alterations such as *ERBB2* (3.9%), *BRAF* (1%), and *MET* (1%). Delays in diagnostic initiation independently predicted mortality, with delays ≥10 days from clinical suspicion to biopsy associated with higher risk of death (HR 1.66; 95% CI, 1.10–2.50; $p = 0.016$).

Study II analysed a retrospective cohort of 59 patients diagnosed with NSCLC who underwent EBUS-TBNA as part of their initial diagnostic work-up. Among these, molecular testing was performed sequentially on EBUS-TBNA samples in 64.4% of cases. Adequacy rates were high, with successful analysis in 89.5% of samples for *EGFR* and 81.3% for *ALK*, confirming the feasibility of this approach. However, a progressive decline in yield across different biomarkers was observed, suggesting gradual sample exhaustion with multiple sequential tests. Additionally, in approximately one-third of

cases, molecular testing was performed on alternative specimens despite the availability of adequate material obtained through EBUS-TBNA.

Study III compared sequential molecular profiling (SMP) with MP-NGS in 106 patients with stage IV NSCLC. MP-NGS achieved universal adequacy (100%) and detected a significantly higher rate of actionable mutations compared with SMP (40.9% vs. 22.2%; p = 0.042), leading to a greater allocation to targeted therapies (44.3% vs. 22.2%; p = 0.038). This observation may have contributed to the trend toward improved overall survival in the MP-NGS group (median 672 vs. 138 days), although the difference did not reach statistical significance (p = 0.053). Turnaround times were prolonged in both strategies, exceeding recommended benchmarks, but were slightly shorter with SMP compared with externally outsourced MP-NGS (median 17 vs. 23 days; p = 0.076).

Study IV, a systematic review and meta-analysis, synthesised evidence from seven studies including 506 patients. Molecular profiling from the supernatant phase demonstrated high feasibility (87–100%), excellent concordance with matched tissue samples (pooled κ=0.947), and consistently shorter turnaround times (up to seven days faster), despite heterogeneity in pre-analytical processing protocols. Subgroup analyses indicated no significant effects of storage medium or temperature on DNA yield, although protocol variability limited definitive conclusions on optimal processing workflows.

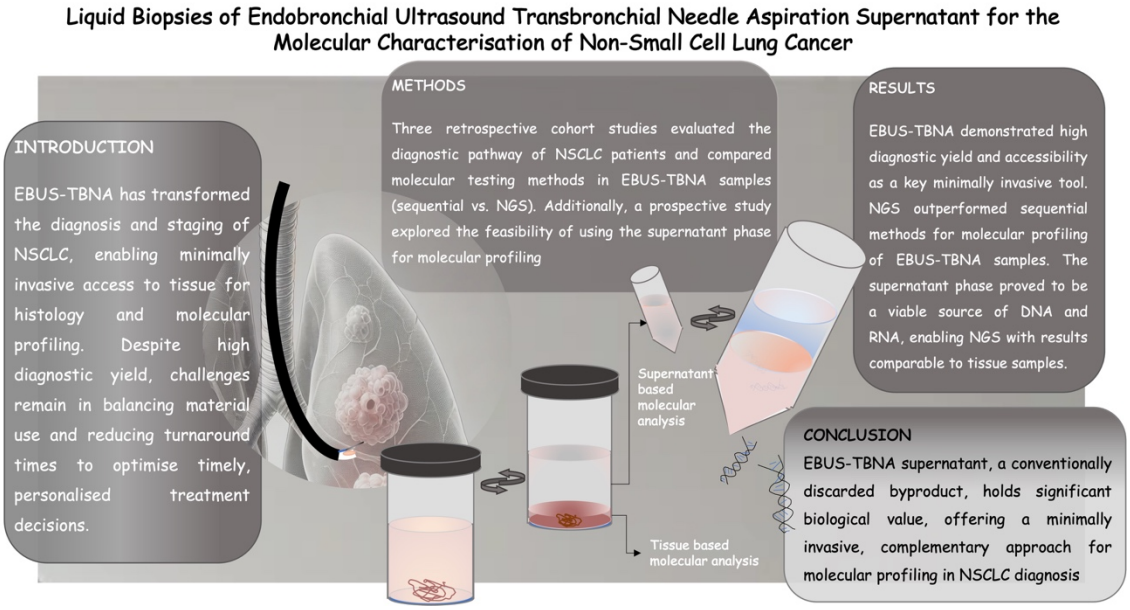
Study V, a prospective study, evaluated paired, EBUS-TBNA derived, cell pellet and supernatant samples from 20 patients. Nucleic acid yields were significantly higher in supernatant compared to cell pellet (DNA: 28.95 ng/µL vs. 9.84 ng/µL, p = 0.025; RNA: 37.6 ng/µL vs. 15.95 ng/µL, p = 0.007). Molecular concordance between fractions was 85%, with three discordant cases: two due to insufficient supernatant material and one true molecular discrepancy, where a *KRAS* G12C mutation was detected exclusively in supernatant. Simulating a parallel diagnostic workflow, in which molecular testing on supernatant was initiated at the time of biopsy while cell pellet underwent standard histological processing, reduced the overall diagnostic time from a median of 21.5 days to 13 days (p < 0.001).

Discussion and Conclusion

This thesis demonstrates that EBUS-TBNA is a pivotal tool in the diagnostic and molecular workflow of advanced NSCLC, providing high-quality material for comprehensive characterisation and enabling shorter timelines to diagnosis and

treatment initiation, a factor independently associated with survival. While sequential molecular strategies proved feasible, they were limited by sample depletion and reduced mutation detection. MP-NGS demonstrated superior performance, improving the identification of actionable mutations and access to targeted therapies. The integration of the supernatant phase, offering valuable nucleic acid yields, strong molecular concordance with conventional tissue based molecular profiling and the potential to accelerate turnaround times, represents a possible key advancement in the diagnostic workflow.

Taken together, these findings support an integrated diagnostic framework where the cellular fraction is used for histology and immunohistochemistry while molecular profiling is performed in parallel from the supernatant phase. This approach optimises tissue use, ensures diagnostic accuracy, and accelerates access to molecular profiling results, aligning workflows with the demands of precision oncology. Validation through multicentre prospective studies and standardised protocols will be crucial to drive widespread clinical implementation.



Graphical Abstract – Visual summary of the thesis workflow and key findings. This diagram illustrates the integration of EBUS-TBNA in the diagnostic and molecular characterisation of advanced NSCLC, highlighting the role of the supernatant phase for parallel molecular testing.

Keywords

NSCLC; EBUS-TBNA; molecular profiling; NGS; supernatant; precision oncology.

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List of Acronyms

Acronym	Definition
ALK	*Anaplastic Lymphoma Kinase*
BAL	Bronchoalveolar Lavage
CP	Cell Pellet
CP-EBUS	Convex Probe – Endobronchial Ultrasound
Cryo-EBUS	Cryobiopsy guided by Endobronchial Ultrasound
CT	Computed Tomography
CT-TTB	Computed Tomography-Guided Transthoracic Biopsy
cfDNA	Cell-free DNA
ctDNA	Circulating Tumour DNA
DNA	Deoxyribonucleic Acid
EBUS	Endobronchial Ultrasound
EBUS-TBNA	Endobronchial Ultrasound–Guided Transbronchial Needle Aspiration
ECOG	Eastern Cooperative Oncology Group
EGFR	*Epidermal Growth Factor Receptor*
EML4–ALK	*Echinoderm Microtubule-Associated Protein-Like 4 - Anaplastic Lymphoma Kinase*
EUS-B	Endoscopic Ultrasound with Bronchoscope
FFPE	Formalin-Fixed Paraffin-Embedded
FISH	Fluorescence In Situ Hybridisation
HR	Hazard Ratio

IHC	Immunohistochemistry
IPOC-FG	*Instituto Português de Oncologia de Coimbra Francisco Gentil*
KRAS	*Kirsten Rat Sarcoma Viral Oncogene Homolog*
LC	Lung Cancer
MP-NGS	Massive Parallel Next-Generation Sequencing
NGS	Next-Generation Sequencing
NSCLC	Non-Small Cell Lung Cancer
PD-1	Programmed cell Death protein 1
PD-L1	Programmed cell Death Ligand 1
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PROSPERO	International Prospective Register of Systematic Reviews
RNA	Ribonucleic Acid
ROS1	*C-Ros Oncogene 1*
RP-EBUS	Radial Probe – Endobronchial Ultrasound
RT-PCR	Real Time - Polymerase Chain Reaction
RS	Reference Standard
SCLC	Small-Cell Lung Cancer
SMP	Sequential Molecular Profiling
SP	Supernatant Phase
STK11	*Serine/Threonine Kinase 11*
TAT	Turnaround Time
TBNA	Transbronchial Needle Aspiration

TP53 *Tumor Protein p53*

TPS Tumour Proportion Score

TTB Transthoracic Biopsy

WHO World Health Organization

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Introduction

1. Lung Cancer: Epidemiological Context

Lung cancer (LC) remains one of the most significant challenges in global health, representing the leading cause of cancer-related mortality worldwide [1,2]. The World Health Organization (WHO), through its Global Cancer Observatory (GLOBOCAN), estimated that approximately 2.48 million new LC cases were diagnosed globally in 2022, accounting for 12.4% of all newly diagnosed malignancies. In the same year, nearly 1.82 million deaths were attributed to LC, corresponding to 18.7% of all cancer-related deaths [3].

In Portugal, national data for 2020 reported 4,737 newly diagnosed LC cases, including 3,289 in men (the second most frequent cancer) and 1,448 in women, ranking fourth among female malignancies [4]. According to the Portuguese National Institute of Statistics, in 2021 there were 3,518 deaths from LC among men and 1,157 among women, making it the leading cause of cancer-related mortality in the country [5].

The close correspondence between incidence and mortality in LC largely reflects its frequently silent clinical course. When symptoms occur, they most commonly include cough, dyspnoea, and chest pain, however, these manifestations are not consistently present, are non-specific and are often attributed to other, more common, respiratory conditions. This non-specificity contributes to substantial diagnostic delays, with reported median intervals of up to 138 days from symptom onset to the initiation of specific treatment [6].

Given its typically silent nature, LC would be expected to benefit substantially from effective screening programs, which have demonstrated mortality reduction in high-risk populations [7,8]. However, the global adoption of such programs has been slow, often limited by logistical, economic, and organisational constraints. In Portugal, the first two pilot projects for LC screening in the National Health Service were announced in July 2025, aiming to reach about 5,000 people (only a small fraction of those eligible) with implementation details still undisclosed at the time of writing this thesis [9]. In the absence of large-scale, systematic screening, a substantial proportion of cases will continue to be diagnosed at advanced stages, when curative surgical options are no longer feasible [10,11].

The predominance of advanced-stage disease at diagnosis has profound prognostic implications. Data from the American Cancer Society [12] indicate that five-year relative survival is approximately 65% when the disease is diagnosed at a localised stage but falls sharply to 37% for regionally spread disease and to just 9% for advanced-stage disease. Mortality patterns in LC are also influenced by histological type [13, 14]. Population-level analyses have demonstrated that, although overall LC mortality has steadily declined in recent years, the magnitude and underlying drivers of this reduction differ between subtypes. In non–small-cell lung cancer (NSCLC), mortality has decreased more rapidly than incidence, a pattern largely attributed to improvements in survival associated with therapeutic advances, particularly the introduction of targeted therapies. By contrast, in small-cell lung cancer (SCLC), mortality reduction has occurred almost exclusively as a result of declining incidence, with little change in survival, reflecting the limited progress in treatment over the same period [13]. This finding has been further explored in a large longitudinal cohort study in France, in which additional stratification by histological subtype revealed marked prognostic variation. In lung adenocarcinoma, median overall survival more than doubled over the past two decades, rising from 8.5 months in 2000 to 20.7 months in 2020. Substantial gains occurred particularly in advanced-stage cases treated with targeted agents or immunotherapy [14]. These observations underscore that histological and molecular classification is fundamental to understand trends in incidence, mortality, and survival, and provides the foundation for more refined prognostic assessment and therapeutic decision-making.

2. Histopathological Classification of LC

LC comprises a heterogeneous group of malignancies. The 2021 WHO classification divides primary lung tumours into four major categories: epithelial tumours, mesenchymal tumours, haematolymphoid (previously lymphohistiocytic) tumours and tumours of ectopic origin (Figure 1) [15,16]. Epithelial tumours are by far the most prevalent category, and within this group, the aggregated sub-group of NSCLC accounts for approximately 85% of all LC diagnoses [16,17,18].

NSCLC encompasses three main histological variants, each arising from distinct epithelial cell types. Adenocarcinoma, currently the most prevalent, represents approximately 40% of all LC cases and typically originates from alveolar epithelial cells lining the small airways. It is characteristically positive for immunohistochemical markers such as thyroid transcription factor 1 (TTF-1) and napsin A. Squamous cell carcinoma accounts for roughly 25% of LC cases and usually arises from the proximal

bronchial epithelium, expressing markers including cytokeratins 5/6 (CK5/6), p40, and desmoglein-3. Large cell carcinoma represents about 5–10% of LC diagnoses and is generally poorly differentiated, composed of large cells with abundant cytoplasm and prominent nucleoli and is considered a diagnosis of exclusion [15,16,17,19].

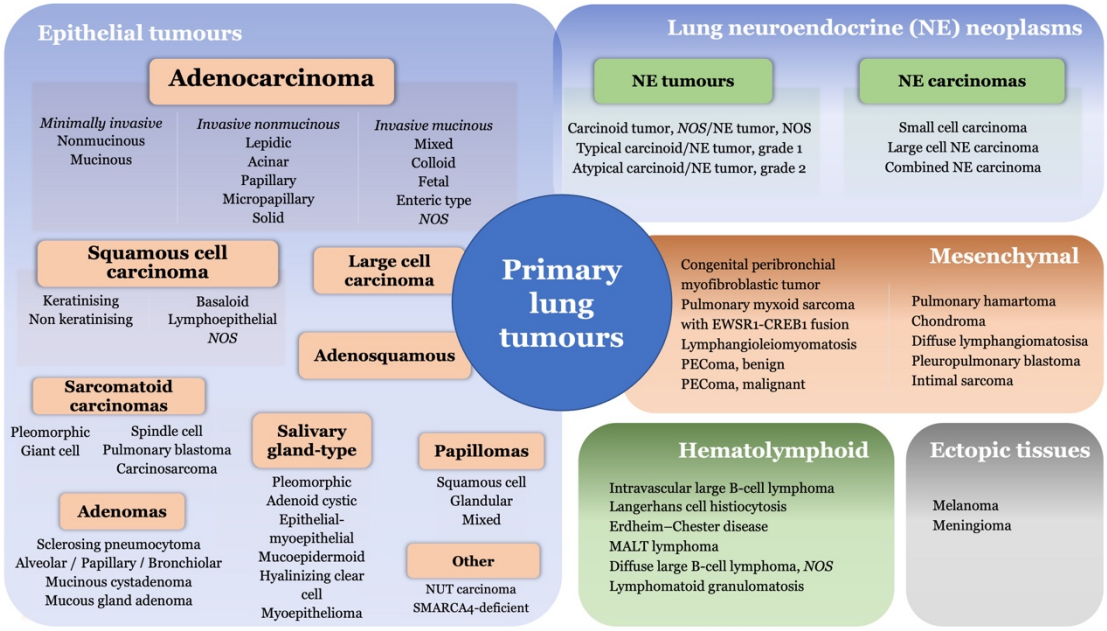

Figure 1 - Summary schematic of the WHO classification of primary lung tumours (2015 and 2021 editions).
The diagram organises primary lung tumours into four major groups: epithelial tumours, mesenchymal tumours, haematolymphoid tumours, and tumours of ectopic origin. In the 2015 classification, neuroendocrine tumours were presented as a subgroup within epithelial tumours; in the 2021 update, they are highlighted as a semi-autonomous group while maintaining their relationship with the epithelial category. The aggregated subgroup of NSCLC (derived from both epithelial and neuroendocrine tumour categories) remains the most prevalent, with adenocarcinoma currently representing the leading histological subtype, accounting for approximately 40% of all new lung cancer diagnoses [15,16,17].

Traditionally, the WHO classification was developed based on large tumour samples, typically obtained from surgical resections [16,17]. However, as discussed earlier, a substantial proportion of LC patients present with advanced-stage disease and are therefore not surgical candidates. In these cases, diagnosis relies on small tissue specimens obtained through minimally invasive procedures such as bronchoscopy, needle, or core biopsies. Recognising this reality, the 2015 WHO classification introduced standardised criteria and terminology for the diagnosis of LC in small biopsies and cytology specimens. These guidelines address the practical need to provide an accurate histopathological classification while preserving tissue for molecular testing, which has become critical for guiding targeted therapy [15,16,17,20].

As small biopsies become increasingly central to the diagnosis of LC, a practical challenge has emerged that can be described as *tissue pressure*. This refers to the need for

specimens of sufficient size and quality to support a comprehensive diagnostic assessment. These samples must provide enough material for accurate morphological classification, while also preserving tissue for an expanding panel of downstream biomarker analyses that are now central to therapeutic decision-making [18, 36, 39]. Meeting both needs places considerable demands on interventional pulmonology teams, responsible for obtaining high-quality specimens through minimally invasive procedures, and on clinical pathologists, who must maximise the diagnostic and molecular yield from often very limited material.

3. Beyond Histopathology: Biomarker-Based Stratification of NSCLC

While histopathological classification remains a cornerstone in the diagnosis of LC, it no longer provides, on its own, the level of biological and clinical detail required for contemporary patient management. Advances in molecular oncology have revealed that tumours sharing the same histological subtype can display markedly different biological behaviours, prognoses, and therapeutic sensitivities [21]. In a large-scale molecular profiling study involving over 1,200 clinically annotated lung tumours, more than 55% carried at least one oncogenic genomic alteration potentially amenable to targeted therapy, including approaches already in clinical use. Notably, genomic characterisation not only revealed substantial heterogeneity within histological categories, but also led to the reassignment of certain cases, such as the reclassification of almost all large cell carcinomas based on molecular features, some with actionable targets. In the same study, a genome-based diagnostic algorithm was prospective applied to more than 5,000 LC patients, enabling molecularly guided diagnoses in 75% of cases. Moreover, this strategy was associated with improved survival in patients harbouring *epidermal growth factor receptor (EGFR)* mutations or *anaplastic lymphoma kinase (ALK)* rearrangements [21]. These findings emphasise the importance of integrating molecular-based stratification into routine clinical practice to refine tumour classification and guide precision LC therapy.

This transition to biomarker stratification has been particularly transformative in NSCLC, where a growing list of genomic drivers has led to the development of targeted therapies with significant clinical benefit. The first and most extensively explored of these genomic alterations were *EGFR* mutations and *ALK* rearrangements.

The *EGFR* gene encodes a transmembrane protein with the same designation that belongs to the erbB family of receptor tyrosine kinases. Upon ligand binding, EGFR undergoes conformational activation and autophosphorylation of its intracellular domain, triggering downstream signalling cascades that promote cell proliferation and survival through inhibition of apoptosis. In its normal state, EGFR activity is tightly controlled by ligand binding. However, specific activating mutations in the *EGFR* gene alter the receptor's structure, leading to constitutive activation of these pathways and driving uncontrolled tumour growth [22,23]. Activating *EGFR* mutations occur in approximately one-third of NSCLC cases (pooled prevalence 32.3%), with marked geographic variability (from 38.4% in China to 14.1% in Europe). They are significantly more frequent in females (43.7% vs. 24.0% in males), in never-smokers (49.3% vs. 21.5% in ever-smokers), and in adenocarcinomas (38.0% vs. 11.7% in non-adenocarcinomas) [24]. *EGFR*-targeted therapies, ranging from first-generation tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib, to third generation osimertinib and regionally approved lazertinib have transformed the management of advanced NSCLC that harbour these mutations. Compared with conventional chemotherapy, these agents significantly improve response rates, prolong progression-free survival, enhance quality of life and have gradually established targeted therapy as the standard first-line approach for this molecular subset [25,26].

Similarly, the *ALK* gene encodes a transmembrane receptor tyrosine kinase of the insulin receptor superfamily. While physiologic activation is ligand-dependent, oncogenic rearrangements, most commonly *echinoderm microtubule-associated protein-like 4 (EML4)–ALK*, place the kinase domain under fusion-partner control, driving constitutive signalling and tumourigenesis in 1–7% of NSCLC, typically in younger, never- or light-smokers with adenocarcinoma [27]. The introduction of *ALK*-targeted therapies, from first-generation TKIs such as crizotinib to more potent second and third-generation agents including ceritinib, alectinib, brigatinib, and lorlatinib, has dramatically improved clinical outcomes in this subgroup, achieving superior response rates, intracranial disease control, and progression-free survival compared with chemotherapy, and establishing targeted inhibition as the standard of care in *ALK*-positive advanced NSCLC [28].

Other recurrent genetic alterations in NSCLC include mutations or rearrangements involving *ROS1*, *RET*, *MET*, *KRAS*, *BRAF*, *HER2*, and *NTRK*, among others, several of which have approved targeted therapies, while others remain under investigation, collectively broadening the scope of precision oncology in this disease (Figure 2) [29].

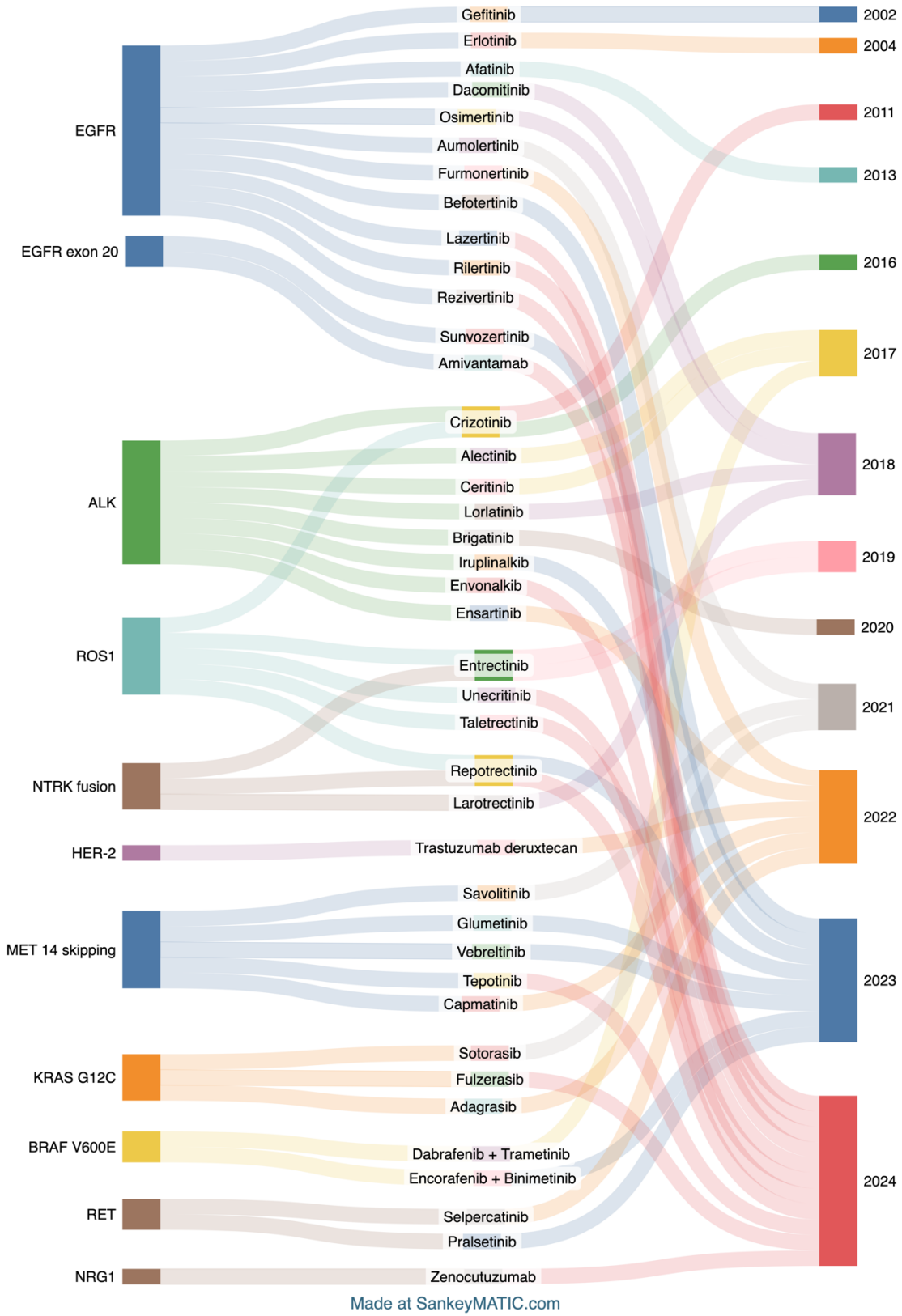

Figure 2 - Sankey diagram illustrating actionable genetic alterations in NSCLC, their corresponding therapeutic targets, and the year of first drug approval.
The diagram depicts the relationship between specific oncogenic drivers and targeted agents, arranged chronologically according to the year of initial regulatory approval. Some approvals are region-specific (e.g., lazertinib), and in certain cases, approval dates have been subsequently revised following changes in indication from later-line use to first-line therapy [18,35,36,37].

In parallel with their role in sustaining proliferative signalling and driving malignant progression, some of the acquired molecular alterations in NSCLC can influence the regulation of the immune system. Such genomic changes, including mutations in genes such as *tumour protein 53* (*TP53*), *KRAS*, and *serine/threonine kinase 11* (*STK11*), can modulate the tumour immune microenvironment by altering cytokine profiles, affecting antigen presentation, and shaping the recruitment and function of immune cell populations [30,31]. Although these alterations are not directly targetable, their impact on immune evasion may be indirectly captured through the assessment of programmed death-ligand 1 (PD-L1) expression, which has emerged as a key biomarker to guide the use of immune checkpoint inhibitors. One study has demonstrated that *TP53* and *KRAS* mutations, especially when co-occurring, are associated with higher PD-L1 expression [32]. Within this framework, the programmed cell death-protein 1 (PD-1)/PD-L1 axis functions as a central inhibitory pathway: PD-1 on activated T cells binds PD-L1 on tumour and immune cells, delivering signals that induce T-cell exhaustion and suppress cytotoxic activity, ultimately allowing tumour cells to evade the immune system [33]. Therapeutic blockade of this interaction with monoclonal antibodies such as pembrolizumab or nivolumab can reinvigorate anti-tumour immunity, yielding durable responses and survival benefits in biomarker-selected patients with advanced NSCLC [34].

Together, these molecular and immunologic parameters now constitute a mandatory component of the diagnostic workup in advanced NSCLC, as reflected in current international guidelines [38,39,40,41]. Consequently, initial tissue sampling must now serve not only for morphologic diagnosis but also for comprehensive biomarker assessment. This includes additional IHC panels with validated clones to determine PD-L1 status, with expression reported either as the absolute percentage of tumour cells showing membranous staining or as the tumour proportion score (TPS), calculated as the proportion of viable PD-L1–positive tumour cells to the total count of viable tumour cells [42].

Additionally, targeted molecular assays can address individual mutations or specific gene rearrangements, for example, allele-specific or RT-PCR for *EGFR* hotspot mutations [43] and break-apart FISH for *ALK* and *ROS1* rearrangements [44]. These targeted strategies are fast, inexpensive, require minimal tissue, and offer high analytical sensitivity/specificity for predefined alterations; however, they interrogate a narrow variant set, may miss rare or atypical mutations, provide little information on co-mutational context, and when performed sequentially, consume tissue and prolong turnaround processing time with potentially negative clinical impact [45,46,47]. Accordingly,

practice is gradually shifting toward broader genomic profiling, preferably with combined DNA and RNA testing using parallel, panel-based next-generation sequencing (NGS) [48]. DNA panels detect single-nucleotide variants, insertions/deletions, copy-number alterations, and selected splice events, while RNA panels enhance the detection of gene fusions and events such as *MET* exon 14 skipping, thereby increasing diagnostic yield and potentially streamlining access to tailored therapeutic approaches [41,47,49].

These developments, however, amplify the previously noted issue of *tissue pressure*. Because most NSCLC diagnoses are established from small biopsies, the success of PD-L1 IHC and combined DNA/RNA NGS depends on obtaining high-quality material and enforcing strict pre-analytical standards to ensure adequate tumour cellularity. In addition, access to more sophisticated, information-rich methodologies such as NGS may be constrained by laboratory infrastructure. Many pathology services cannot implement and sustain these platforms cost-effectively, necessitating external referral, which can introduce additional handling and administrative steps that, in turn, may increase turnaround times and delay treatment decisions [47,50].

4. The Evolving Role of Endobronchial Ultrasound in NSCLC Diagnosis and Molecular Profiling

Endobronchial ultrasound (EBUS) is a diagnostic modality within the realm of interventional pulmonology that integrates flexible bronchoscopy with real-time ultrasonography to simultaneously allow the visualisation of the airway walls and the adjacent mediastinal and hilar structures. The concept of EBUS encompasses two distinct modalities: radial probe (RP)-EBUS and convex probe (CP)-EBUS.

RP-EBUS was the first to be introduced in 1996 [51] and consists of a miniature mechanical rotating 360° radial transducer inserted through the working channel with or without a dedicated guide sheath. It produces a cross-sectional image, typically within the range of 20 MHz and is generally used to help locate peripheral pulmonary lesions. Despite recent developments [52], and anecdotical case reports [53] RP-EBUS does not allow real time sampling of lesions which accounts for its modest yields when used as a solo diagnostic tool [54]. On the other hand, RP-EBUS integrates seamlessly into multimodal workflows, including navigation bronchoscopy, cone-beam CT guidance, and more recently robotic assisted bronchoscopy, where it serves as the confirmation tool at the lesion, increasing overall diagnostic yield compared with any single modality alone [55,56,57].

CP-EBUS was first introduced in 2002 [58] and consists of a dedicated videobronchoscope with a convex probe at the distal tip that enables real-time, image-guided transbronchial needle aspiration (TBNA) of mediastinal/hilar nodes and peritracheal/peribronchial masses. CP-EBUS provides colour Doppler to avoid vessels and the distal probe can be coupled with a saline-filled balloon to improve acoustic contact. Typical transducer settings range from 5 to 12 MHz, balancing resolution and depth for targets within a few centimetres of the airway walls. The same CP-EBUS scope can be passed through the oesophagus, a technique known as endoscopic ultrasound with the bronchoscope (EUS-B). Interventional pulmonologists use this approach to sample posterior and inferior mediastinal nodes (especially stations 8 and 9) and selected upper-abdominal targets (such as the left adrenal gland and left lobe-liver metastases) during the same session as conventional CP-EBUS, enabling complete mediastinal staging and evaluation of disseminated disease [59,60].

Since CP-EBUS is primarily used for performing TBNA, the acronym EBUS-TBNA is the most commonly used in the medical literature and is also the one generally adopted in this thesis as an equivalent to CP-EBUS.

Upon its development, EBUS-TBNA was primarily used for mediastinal staging in LC, aiming to replace more invasive surgical procedures such as videomediastinoscopy. Early studies clearly demonstrated the higher sensitivity of EBUS-TBNA, which was further enhanced when combined with EUS-B. The ASTER study was a pivotal randomised clinical trial that compared endosonography (EBUS-TBNA and EUS-B) with surgical mediastinal staging. The results showed that endosonography, followed by surgical staging, when necessary, had a higher sensitivity for detecting mediastinal metastases (94%) compared to surgical staging alone (79%) leading to fewer unnecessary thoracotomies [61]. The strength and impact of these results, along with their validation in subsequent studies, led to broad consensus among the gastrointestinal, respiratory, and thoracic surgical societies. This collaboration resulted in the development of joint guidelines that continue to position EBUS-TBNA as the frontline approach for invasive mediastinal staging in LC [59].

Over time, the clinical role of EBUS-TBNA has expanded significantly and it is now firmly established as a key diagnostic modality for thoracic malignancies, particularly in patients with centrally located tumours, as demonstrated in several studies, including one to which the author of this thesis contributed [62,63,64].

Importantly, in addition to its diagnostic and staging applications, EBUS-TBNA provides material suitable for predictive biomarker assessment. In advanced NSCLC, formalin-

fixed cell-block preparations from EBUS-TBNA support PD-L1 immunohistochemistry with high adequacy when validated assays are used [65,66], and results show good agreement with matched surgical samples [67,68].

Beyond PD-L1, EBUS-TBNA reliably supports molecular profiling in advanced NSCLC. Meta-analytic estimates and results from moderate to large cohorts (Table 1) report variable adequacy of samples, reflecting differences in the methodologies applied.

Table 1 - EBUS-TBNA for molecular profiling in NSCLC: summary of key studies

Authors	Year	Design	N#	Methods	Adequacy*
Labarca, G. *et al.* [69]	2018	Systematic review and meta-analysis	2,698	SMP[a] (31 studies) NGS (2 studies)	*EGFR*: 94.48% *ALK*: 94.9% *ROS1*: 83.3%
Cicek, T. *et al.* [71]	2019	Retrospective single-centre cohort	114	SMP[a]	*EGFR*: 88.6% *ALK*: 93.8% *ROS1*: 91.8%
Karadzovska-Kotevska, M. *et al.* [70]	2022	Retrospective single-centre cohort	132	SMP[a]	*EGFR*: 69% *ALK*: 49% *ROS1*: 36%
Zhao, J.J. *et al.* [74]	2022	Systematic review and meta-analysis	1,175	NGS (21 studies)	Overall: 86.5%
Zhang, C. *et al.* [75]	2023	Retrospective single-centre cohort	271	NGS[b]	Overall: 92.5%
Rodríguez González, M. *et al.* [73]	2025	Prospective single-centre cohort	120	NGS[c]	Overall: 97%

Footnotes: *Adequacy refers to the proportion of molecular tests that successfully yielded a reportable result, expressed as a percentage of the total number of tests attempted. #N refers to the total number of participants. [a]SMP: sequential molecular profiling, consists of PCR-based methods for *EGFR*; IHC with confirmatory FISH for *ALK* and *ROS1*. [b]NGS: Next generation sequencing assay comprising DNA sequencing of 152 genes (Haloplex, Agilent, Santa Clara, CA, USA) and fusion transcription panel as an RNA sequencing assay of 55 gene rearrangements (ArcherDx, Boulder, CO, USA). [c]Oncomine Precision Assay (Thermo Fisher Scientific, Waltham, MA, USA) detecting hotspot mutations, copy-number variations, and gene fusions across 50 cancer-related genes on the Ion Torrent GX5 chip (Thermo Fisher Scientific, Waltham, MA, USA).

While sequential (non-NGS) methodologies consistently achieve high adequacy for *EGFR* (typically the first target tested), adequacy for *ROS1* (usually the last gene assessed in the sequential workflow) is often lower and more variable [69,70,71]. On the other hand, NGS is being increasingly adopted in line with updated molecular testing guidelines for LC [18,35,41,72] and delivers high success rates, with pooled adequacy around 86.5% and over 90% in some single-centre series [73,74,75]. However, results remain susceptible to heterogeneity, partly due to pre-analytical factors. A recent meta-analysis showed that the number of needle passes is an important modifiable determinant of NGS adequacy ($\beta = 0.495$; $p < 0.001$), with modelled yields of 77.3%, 86.2%, 91.6%, and 94.9% at mean 3, 4, 5, and 6 passes, respectively. These findings support protocolised targets of more than 3 passes per node to maximise molecular yield [74].

Taken together, EBUS-TBNA holds the potential to transform minimally invasive evaluation of LC by allowing staging, diagnosis, and biomarker profiling in a single session [76]. Nevertheless, heterogeneity in sampling strategy, specimen processing, and assay platforms can affect yield, particularly for biomarker testing, as summarised above. As a complementary strategy to improve tissue quantity and quality, recent studies have explored the feasibility of performing mediastinal cryobiopsies, a procedure commonly designated as cryo-EBUS. Briefly, cryo-EBUS combines CP-EBUS guidance with small bore cryoprobes (1.1 to 1.7mm). A short airway tract is created by dilating the TBNA puncture or using dedicated electrocautery knives. This is followed by the insertion of the cryoprobe with brief 3- to 7-second freezes applied and en-block extraction with the EBUS scope to retrieve larger and architecturally preserved specimens [77,78].
According to a recent systematic review of seven studies including 555 patients (about two thirds with malignant nodal disease), cryo-EBUS achieved higher overall diagnostic yield than EBUS-TBNA (92% vs 80%), with advantages in lymphoma, granulomatous disease, and biomarker assessment (genetics and PD-L1) [77], a pattern that is consistent with our published experience [78].

Another important limitation is access to EBUS-TBNA. Although uptake is increasing, direct access is still not universal across interventional pulmonology units. In Portugal, a 2020 national snapshot, co-authored by the present author, reported direct access to EBUS-TBNA in 45.9% of units and to EUS-B in 27.0% [79]. Audits from other health systems show better, though still incomplete, coverage. In the United Kingdom, the third National Lung Cancer Audit found that 77% of acute trusts in England and Wales had an on-site EBUS-TBNA service [80]. In Canada, a recent national survey reported access to EBUS-TBNA/EUS-B in 73% of centres [81].

Variability in tissue adequacy and uneven access to EBUS-TBNA mean that a purely tissue-based pathway will not always deliver complete and timely biomarker profiles. Against this backdrop, liquid biopsy has emerged as a practical adjunct, offering a complementary route to enhance yield without additional invasive procedures.

5. Liquid Biopsy and the Emerging Role of Supernatant Analysis

Liquid biopsy has emerged as a transformative approach in oncology, enabling the detection and molecular characterisation of tumours through minimally invasive

sampling of biological fluids. By analysing circulating tumour DNA (ctDNA), circulating tumour cells, extracellular vesicles, or other tumour-derived analytes, liquid biopsy offers the potential for dynamic monitoring of tumour evolution, early detection of resistance mechanisms, and assessment of minimal residual disease [82]. Compared with conventional tissue biopsy, this strategy allows for repeated sampling over time and better captures the spatial and temporal heterogeneity of cancer [83]. An additional advantage is the potential for shorter turnaround times compared with tissue-based approaches, particularly when rapid molecular profiling is required to guide therapeutic decisions in advanced disease [83,84].

In NSCLC, plasma-derived ctDNA analysis has gained significant clinical relevance, particularly for identifying targetable genomic alterations such as *EGFR* mutations, *ALK* rearrangements, and other actionable drivers, as well as for monitoring response to targeted therapies [85,86].

The clinical utility of plasma liquid biopsy is well established for patients with advanced stages of disease, where ctDNA shedding is higher, facilitating mutation detection. However, its sensitivity is reduced in early-stage tumours or in cases with low tumour burden, limiting its application in these settings [83,85].

These constraints have prompted the exploration of liquid biopsies derived from alternative biological sources. Beyond plasma, other biological fluids collected in closer proximity to the tumour may provide higher concentrations of tumour-derived analytes, potentially improving the sensitivity of liquid biopsy approaches. Bronchoalveolar lavage (BAL) fluid, for example, has been investigated as a source for proteomic and genomic analysis in LC. In a prospective observational study of 90 patients with suspected LC, to which the author contributed as a co-author, mass spectrometry–based proteomic profiling of acellular BAL fluid identified 133 potential biomarkers differentially expressed between LC and non-LC cases, with substantial overlap with those detected in tumour tissue. Notably, individuals who were later diagnosed with LC clustered with LC cases based on their BAL proteomic profile at the time of sampling [87]. Subsequent work from the same research group extended these observations to BAL-derived extracellular vesicles, revealing molecular signatures associated with tumour presence, advanced stage, and poorer prognosis, further reinforcing the potential of proximal fluids for both early detection and comprehensive molecular characterisation [88].

Another example where liquid biopsies have been explored is in pleural effusion, a common clinical manifestation in advanced LC. Pleural fluid contains tumour-derived cells and cell-free nucleic acids, making it a rich source for molecular testing when tissue

acquisition is challenging. Studies have shown that cell-free DNA (cfDNA) extracted from pleural effusion samples can provide high-quality material for detecting actionable mutations, often with higher sensitivity than plasma-derived ctDNA and with strong concordance to tissue genotyping [89,90].

Interestingly, research on pleural effusion has not been limited to molecular testing of the whole sample. Several studies have investigated the potential of by-products generated during routine pre-analytical processing, particularly the supernatant obtained after centrifugation of pleural fluid. This fraction, typically discarded in conventional workflows, may be enriched in tumour-derived cfDNA shed by malignant cells, whereas the cellular pellet often contains, predominantly, mesothelial cells that can dilute tumour-specific signals. A few studies have addressed this question, reporting a strong concordance between the mutational profile of cfDNA from pleural effusion supernatant and that of matched tissue samples [91,92,93,94].

Similar principles have been applied to supernatants from other biological fluids, including those obtained from fine-needle aspiration procedures. Several studies, using heterogeneous protocols, have evaluated these by-products in different solid tumours, such as pancreatic ductal adenocarcinoma [95] and thyroid carcinoma [96], and have shown that they can yield substantial amounts of high-quality ctDNA suitable for genomic profiling, with strong concordance to tissue-based biopsies.

Despite these encouraging results, the use of this approach with EBUS-TBNA specimens remains underexplored. Given that EBUS-TBNA is widely used for the diagnosis and staging of LC, and that the residual supernatant fluid generated during its routine processing is readily available, it represents a promising and minimally invasive source for molecular profiling that could further enhance the diagnostic and therapeutic workflow in NSCLC.

Building on emerging evidence, this thesis provides a comprehensive exploration of the role of EBUS-TBNA in the diagnostic and molecular workflow of advanced NSCLC. It begins with real-world analyses defining its impact on diagnostic pathways, time-to-treatment, and patient survival, followed by studies demonstrating its feasibility for molecular characterisation and the limitations of sequential testing. It then compares conventional sequential approaches with more advanced parallel strategies, highlighting the advantages of NGS in EBUS-TBNA derived samples. The work culminates with an in-depth synthesis of current evidence on EBUS-TBNA supernatant, revealing substantial pre-analytical heterogeneity, followed by a prospective study showing that this fraction yields high quantities of DNA and RNA, with excellent molecular

concordance with tissue samples. Together, these studies add to the growing evidence that positions EBUS-TBNA as a central tool for fast, minimally invasive, and high-yield molecular profiling in NSCLC, while pushing the frontiers through the innovative exploration of its routinely discarded supernatant.

Objectives

The effective management of advanced NSCLC relies critically on timely diagnosis and comprehensive molecular characterisation to guide personalised treatment. EBUS-TBNA has become a cornerstone of minimally invasive diagnosis and staging, yet important questions remain regarding its optimal role in real-world diagnostic pathways, the efficiency of molecular testing strategies applied to its samples, and the potential to maximise its diagnostic yield by exploiting routinely discarded material.

Hypothesis:

The central hypothesis is that EBUS-TBNA, already a high-yield and well-established technique for the diagnosis, staging and biomarker characterisation of NSCLC, can be further strengthened through three complementary approaches: (i) optimising its role within real-world diagnostic pathways, (ii) improving molecular testing strategies to maximise biomarker detection, and (iii) maximising the use of available material by incorporating molecular profiling from the routinely discarded supernatant phase.

Primary outcome:

- The primary outcome of this thesis is to evaluate the role of EBUS-TBNA in the diagnostic and molecular workflow of advanced NSCLC, with particular emphasis on its capacity to provide adequate material for biomarker testing and on the potential utility of its supernatant as a tumour-proximal liquid biopsy source.

Secondary outcomes:

Because the primary aim is multifactorial, the thesis is structured into five studies, each addressing a set of secondary objectives. These studies collectively respond to the central research question by examining complementary aspects of the diagnostic and molecular use of EBUS-TBNA:

- Assessing the positioning and impact of EBUS-TBNA within real-world diagnostic pathways for advanced NSCLC.
- Evaluating the feasibility of sequential molecular testing strategies applied to EBUS-TBNA samples, particularly regarding adequacy and turnaround time.

- Comparing sequential and massively parallel molecular profiling strategies in terms of biomarker yield, turnaround time, and impact on therapeutic allocation and outcomes.
- Consolidating available evidence on the feasibility and accuracy of molecular testing from EBUS-TBNA supernatant through systematic review and meta-analysis.
- Prospectively investigating nucleic acid yield and molecular concordance between paired EBUS-TBNA pellets and supernatants obtained under routine clinical conditions.

Methods

Overall Design

This thesis combines three retrospective observational studies, one systematic review and meta-analysis, and one prospective comparative study. Each study was conceived in close collaboration between the doctoral candidate and the supervisory team, who oversaw all phases of planning, execution, and reporting.

The work was conducted primarily within the Department of Pulmonology of the Portuguese Oncology Institute of Coimbra, Francisco Gentil (*Instituto Português de Oncologia de Coimbra, Francisco Gentil* – IPOC-FG), in close collaboration with the institution's Molecular Pathology Laboratory. Molecular investigations for the prospective study were also developed at the Institute of Anatomical Pathology and the Molecular Pathology Laboratory of the Faculty of Medicine, University of Coimbra.

Retrospective data collection and prospective recruitment of participants were undertaken by the doctoral candidate in conjunction with the co-authors of each publication. Statistical analyses were performed by the doctoral candidate under the guidance of the supervisory team, with specialised input from a biostatistician from the IPOC-FG Research Support Unit. Analyses were performed using IBM SPSS v. 27.0 (IBM Corp., NY, USA), and a two-sided p-value <0.05 was considered statistically significant.

The overall project was approved by the Ethics Committee of IPOC-FG (approval number 23-2022) and authorised by the institution's Board of Directors, with the endorsement of the Direction of the Pulmonology Department. Written informed consent was obtained from all participants, or from their legal representatives when applicable.

1. Study I – Real-World Diagnostic Pathways and Optimisation of Biopsy Techniques for PD-L1 and Molecular Profiling in Advanced NSCLC: a Four-Year Cohort Study

Retrospective cohort study including consecutive patients diagnosed with TNM 8th edition, stage IV NSCLC between January 2020 and December 2023. Eligible patients

were those who underwent a diagnostic biopsy with subsequent molecular and immunohistochemical characterisation as part of their initial diagnostic workup. Data were retrieved from institutional electronic medical records and pathology databases. Variables collected included demographic and clinical characteristics, histological subtype, PD-L1 expression and molecular profile results. Procedural data included the type and site of biopsy, as well as sample adequacy, defined as the availability of sufficient material for the required histopathological and molecular analyses. For each patient, the diagnostic pathway was reconstructed from the first clinical suspicion of LC through to treatment initiation. Key temporal variables were measured, including time from first clinical evaluation to diagnostic procedure, time from procedure to pathological confirmation, time to final molecular results, and time to initiation of systemic therapy. The study also recorded the specifics of therapeutic allocation. Differences in categorical variables were assessed using chi-square or Fisher's exact tests, and continuous variables were compared using Mann–Whitney U or Kruskal–Wallis tests as appropriate. The Fisher-Freeman-Halton exact test assessed diagnostic yield for PD-L1 and molecular profiling. Survival was calculated from the date of pathological diagnosis to death or last follow-up. Kaplan–Meier curves were generated to compare survival across groups, and Cox proportional hazards models were employed to identify independent predictors of overall survival.

2. Study II – Feasibility of EBUS-TBNA for the Molecular Characterisation of NSCLC

This retrospective observational study included patients diagnosed with NSCLC between January 2019 and December 2021. Eligible patients were those with: (i) a histopathological confirmation of NSCLC with referral for molecular testing, and (ii) performance of EBUS-TBNA, either as a stand-alone diagnostic procedure or in combination with other biopsy methods. Clinical and demographic data were extracted from electronic medical records. Procedural data encompassed the type of diagnostic approach performed (EBUS-TBNA alone versus EBUS-TBNA combined with other biopsy procedures). In cases of combined diagnostic approaches, the source of material selected for molecular testing (EBUS-TBNA versus alternative specimen type) was also recorded. EBUS-TBNA procedures were performed under general anaesthesia with a laryngeal mask, using a BF-UC180F bronchoscope (Olympus, Tokyo, Japan) and 21G needles (ViziShot 2, Olympus, Tokyo, Japan). In line with institutional protocol, at least

three passes per lesion were obtained. Neutral buffered 4% formaldehyde was used for fixation, and cell blocks were prepared after centrifugation (400×g, 15 min). Paraffin-embedded sections were processed for histopathological and molecular analyses. Molecular characterisation followed a sequential testing strategy applied after immunohistochemistry and PD-L1 assessment. This strategy involved RT-PCR for *EGFR* mutation analysis using the Cobas® *EGFR* Mutation Test v2 (Roche Diagnostics, Mannheim, Germany). Formalin-fixed paraffin-embedded (FFPE) tumour sections (5 µm) were reviewed by a pathologist, and manual microdissection was performed when tumour cell content was below 10%. DNA extraction was performed with the Cobas® DNA Sample Preparation Kit (Roche Diagnostics, Mannheim, Germany), and amplification/detection was carried out on the Cobas® z 480 analyser (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. *ALK* and *ROS1* rearrangements were evaluated by FISH using 3 µm FFPE tissue sections. Samples with fewer than 100 viable tumour cells were excluded from analysis. Following standard pretreatment, slides were hybridised overnight with dual-colour break-apart probes (SPEC ALK Z-2124 and SPEC ROS1 Z-2144, ZytoVision GmbH, Bremerhaven, Germany). After post-hybridisation washing, slides were analysed under a Leica DMI6000 B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). A descriptive statistical analysis was conducted to assess sample adequacy (as defined in Study I), turnaround time (defined as the interval in days between the diagnostic procedure and the final molecular report), and the proportion of cases in which EBUS-TBNA material was selected for molecular analysis compared with other diagnostic procedures.

3. Study III – Sequential vs Massively Parallel Strategies for the Molecular Characterisation of NSCLC Samples Obtained by EBUS-TBNA

Cross-sectional retrospective study including patients with TNM 8th edition, stage IV NSCLC, diagnosed between January 2020 and December 2023 at IPOC-FG. Eligibility criteria required histopathological confirmation of NSCLC, referral for molecular characterisation, and the use of EBUS-TBNA both as the diagnostic method and as the source for molecular testing.

Patients were divided into two groups according to the molecular profiling strategy. Between January 2020 and December 2021, sequential molecular profiling (SMP) was performed in-house; from January 2022 onwards, MP-NGS was outsourced to an external reference laboratory. EBUS-TBNA procedures were performed as described in Study II. SMP was performed stepwise after immunohistochemistry and PD-L1 assessment, as described in Study II. Briefly SMP included RT-PCR for *EGFR* assessment followed by FISH based assays for *ALK* and *ROS1*. For MP-NGS, FFPE tumour blocks with ≥10% tumour content were selected. Genomic DNA/RNA was extracted using the MagMAX™ FFPE DNA/RNA Ultra Kit (Thermo Fisher Scientific, USA), and nucleic acids were quantified with a Qubit® 3.0 fluorometer. Sequencing was performed on the Genexus platform (Thermo Fisher Scientific, USA) using the Oncomine Precision Assay GX, which detects mutations, copy number variations, and fusion variants across 50 cancer-related genes. The results were interpreted using the Oncomine Reporter to identify associated therapies. Measures of sample adequacy (as defined in Study I), detection rates of actionable mutations, TAT (as defined in Study II), treatment allocation, and overall survival (OS, measured from diagnosis to death or last follow-up) were obtained for all participants. Group comparisons used Chi-square or Fisher's exact test for categorical variables and Mann–Whitney U test for continuous variables. Overall survival was analysed using Kaplan–Meier curves with log-rank tests, and Cox regression models were applied to identify independent prognostic factors.

4. Study IV – Supernatant from EBUS-TBNA Samples for Molecular Profiling in NSCLC: a Systematic Review and Meta-Analysis

This systematic review and meta-analysis was conducted in accordance with the PRISMA 2020 guidelines, with a prospectively registered protocol in PROSPERO (CRD42024600046). The review was structured according to the PICO (Population, Intervention, Comparison, Outcome) framework: (P) patients with suspected or confirmed NSCLC undergoing EBUS-TBNA; (I) molecular profiling of the supernatant phase (SP); (C) comparison with a reference standard (RS) consisting of tissue or cytology-based specimens; (O) outcomes including feasibility, molecular yield, concordance, TAT, and detection of actionable mutations.

A comprehensive search was conducted in PubMed, Embase, Cochrane Library, ClinicalKey, and ConnectedPapers, supplemented by trial registries (ClinicalTrials.gov, controlled-trials.com, anzctr.org.au) and grey literature sources (OpenGrey, OAISter). The search covered studies published from 2000 onward, to include all reports since the introduction of EBUS-TBNA in 2002. No language restrictions were applied, and reference lists of included articles were screened to identify additional studies. Eligible studies were those reporting on NSCLC patients who underwent EBUS-TBNA with molecular analysis performed on SP samples, compared with RS. Observational studies, diagnostic accuracy studies, and randomised clinical trials (RCTs) were considered if they reported outcomes such as molecular yield, concordance, or detection of specific genetic alterations. Exclusion criteria were studies not involving EBUS-TBNA, focusing on non-NSCLC populations, or presenting only narrative data. The quality of included studies was assessed with the Cochrane RoB 2.0 tool for RCTs and the Newcastle-Ottawa Scale for observational studies. Two reviewers independently screened titles, abstracts, and full texts using *Rayyan* [97], extracted data with a standardised form and resolved disagreements by consensus or third-party adjudication. Selection flow and exclusion reasons are detailed in the PRISMA 2022 diagram (Figure 3).

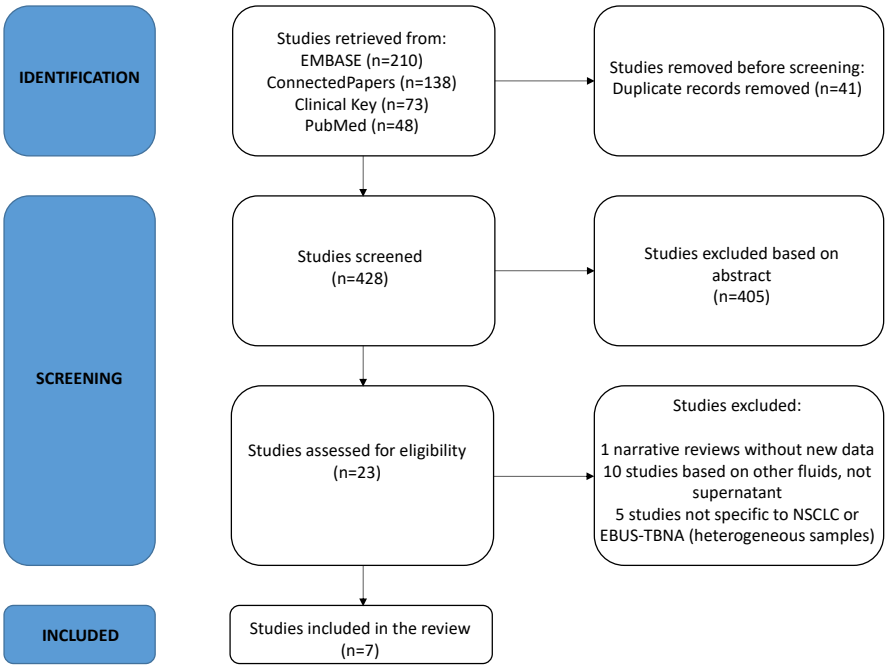

Figure 3 - PRISMA flow diagram of the systematic review process.
The diagram illustrates the selection process of studies included in the systematic review. A total of 469 records were retrieved from four databases (PubMed, EMBASE, ConnectedPapers and Clinical Key). After removing 41 duplicate records, 428 studies were screened based on title and abstract, leading to the exclusion of 405 studies that did not meet the inclusion criteria. Following full-text assessment of 23 studies, an additional 16 were excluded due to irrelevance to the research question, use of non-supernatant samples, or heterogeneous study populations. Ultimately, 7 studies were included in the final review.

Extracted variables included study and participant characteristics, details of EBUS-TBNA and SP processing (needle type, number of passes, preservation medium, storage conditions), nucleic acid yield, type of molecular assays, detection of actionable alterations, concordance with RS, and TAT. The primary outcome was the feasibility of molecular profiling from SP, defined as the ability to obtain DNA/RNA of sufficient quality and quantity for analysis. Secondary outcomes included molecular concordance with RS (percentage agreement and Cohen's kappa), detection of actionable alterations and TAT.

A qualitative synthesis was performed to summarise study characteristics and findings. For quantitative analysis, pooled estimates of feasibility and concordance were calculated using random-effects meta-analysis with the *metafor* package in *R,* version 4.4.1 [98]. Proportions were transformed with the Freeman–Tukey method, and between-study heterogeneity was assessed using the Q statistic, τ^2 (between-study variance), and the I^2 index (percentage of total variation across studies due to heterogeneity). Subgroup analyses by preservative solution and storage temperature were performed, and meta-regression tested the influence of these moderators. Diagnostic accuracy measures (sensitivity, specificity, predictive values) were extracted where possible. Concordance was expressed as the proportion of identical molecular results between SP and RS, with Cohen's kappa coefficients interpreted according to established thresholds.

5. Study V – Feasibility of DNA and RNA Preservation from EBUS-TBNA Supernatant for Molecular Profiling in NSCLC

This prospective study enrolled patients aged ≥18 years with histopathologically confirmed NSCLC referred for EBUS-TBNA and requiring molecular profiling as per clinical indication. Recruitment occurred between December 2023 and December 2024. An interim dataset of 15 patients (December 2023 – August 2024) was submitted for publication (November 2024). In this thesis, both the interim dataset and the complete dataset of 20 patients are presented, analysed with the same methodology to ensure comparability and transparency. Inclusion criteria comprised all patients with advanced adenocarcinoma, as well as squamous cell carcinoma in never-smokers, long-term ex-smokers, or light-smokers (<15 pack-years). Exclusion criteria followed standard contraindications for EBUS-TBNA, including bleeding diathesis (platelets <50,000),

severe respiratory failure (resting arterial partial pressure of oxygen - PaO_2 <50 mmHg despite oxygen supplementation), unstable cardiac conditions, known allergy to anaesthetics, or inability to provide informed consent.

EBUS-TBNA procedures were performed as described in Study II. Samples obtained from EBUS-TBNA were expelled into buffered 4% formaldehyde and centrifuged at 400g for 15 minutes, yielding the cell pellet (CP), which was processed into paraffin-embedded cell blocks. Slides were reviewed by a pathologist, and blocks containing ≥10% tumour content were selected for DNA and RNA extraction. The supernatant phase (SP), which is typically discarded in routine practice, was instead systematically collected for this study. Upon arrival at the laboratory, each sample was registered and visually inspected for macroscopic features (colour, transparency, presence of tissue fragments). The fluid was then centrifuged at 1600g for 10 minutes, the pellet obtained was washed twice with absolute ethanol to remove residual contaminants, air-dried at room temperature, and stored under controlled conditions until DNA and RNA extraction. A schematic representation of the sample processing workflow is provided in Figure 4.

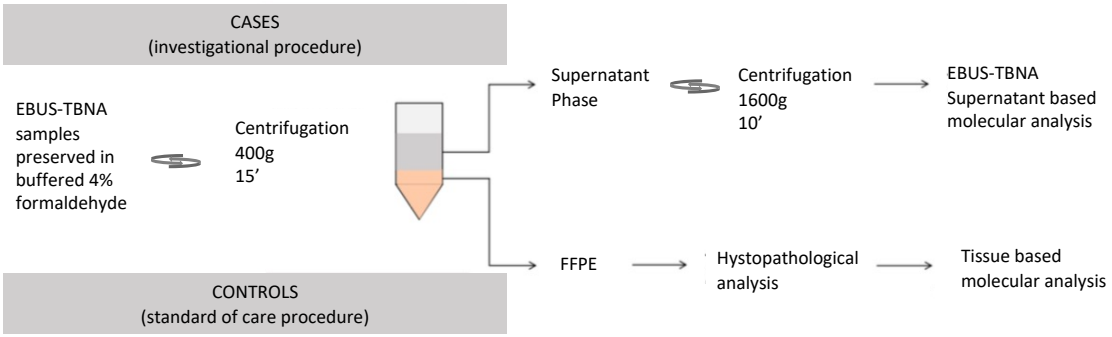

Figure 4 – Workflow of supernatant collection and study methodology.
The samples obtained from EBUS-TBNA were conventionally centrifuged to separate the cell pellet, used for standard histopathology and molecular testing. The supernatant phase, routinely discarded, was systematically collected, processed, and analysed for DNA and RNA extraction and molecular profiling.

DNA and RNA were extracted from both CP and SP using the MagMAX™ FFPE DNA/RNA Ultra Kit (Thermo Fisher Scientific, USA) and quantified with the Qubit® 3.0 fluorometer. Next-generation sequencing (NGS) was performed on the Ion Torrent Genexus platform using the Oncomine Precision Assay GX (Thermo Fisher Scientific, USA), as described in study III.

DNA and RNA concentrations between CP and SP were systematically measured and compared using the Wilcoxon signed-rank test. Concordance of molecular results and allelic frequency was evaluated as both absolute and percentage agreement.

Results

The results are presented in five sections, each corresponding to the individual study conducted within the framework of the thesis. The full text of each individual article, either in its published form or in the final submitted version, is provided in appendix.

1. Study I – Real-World Diagnostic Pathways and Optimisation of Biopsy Techniques for PD-L1 and Molecular Profiling in Advanced NSCLC: a Four-Year Cohort Study

The cohort comprised 205 patients with stage IV NSCLC: 74 (36.1%) IVA and 131 (63.9%) IVB. Median age was 68 years (range, 38–89), 59.5% were male. All had ECOG ≤2 (Table 1). Adenocarcinoma predominated (86.8%). Detailed results concerning participant characteristics are listed in Table 2.

EBUS-TBNA and EUS-B were the most frequent diagnostic modalities (27.3% and 24.4%, respectively), accounting together for 51.7% of initial procedures. Other procedures included transthoracic biopsies (TTB, 19.5%), videobronchoscopy (16.6%), surgical biopsies (7.8%), and pleural procedures (4.4%), as represented in Figure 5.

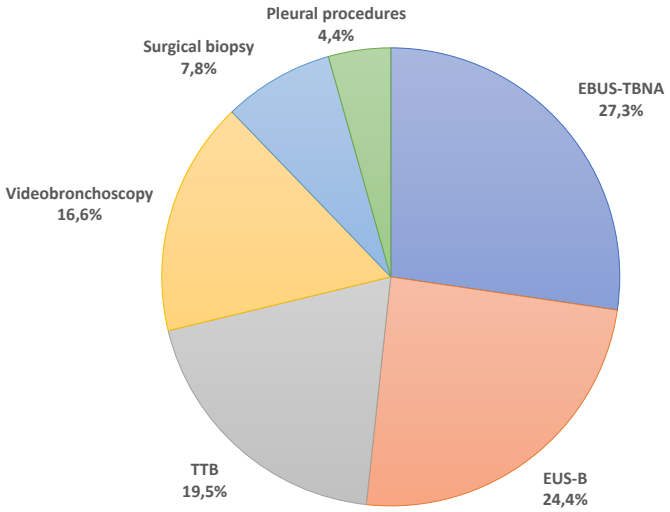

Figure 5 – Diagnostic procedures performed.
The pie chart illustrates the number of diagnostic procedures conducted, categorised by type. Echoendoscopic procedures (EBUS and EUS-B) were the most frequently performed, followed by TTB and videobronchoscopy. Surgical biopsies and pleural procedures were performed less frequently

Molecular profiling was attempted in all cases; five (2.4%) were inconclusive due to insufficient material (2 EBUS-TBNA, 1 EUS-B, 1 TTB, 1 videobronchoscopy). There were no significant differences in molecular success rates across diagnostic modalities (p = 0.968).

Table 2 - Demographic and clinical details of the patients included.

Variable	Count (n=205 patients)
Gender, n (%)	
Male	122 (59.5)
Female	83 (40.5)
Age, median (min; max)	68 (38; 89)
Smoking, history n (%)	
Never smoker	67 (32.7)
Former smoker	67 (32.7)
Current smoker	71 (34.6)
Histology, n (%)	
Adenocarcinoma	178 (86.8)
Adenosquamous carcinoma[#]	11 (5.4)
Squamous cell carcinoma	8 (3.9)
Combined adenocarcinoma and neuroendocrine carcinoma	8 (3.9)
Stage, n (%)	
IVA	74 (36.1)
IVB	131 (63.9)
ECOG performance status, n (%)	
0	91 (44.4)
1	78 (38)
2	36 (17.6)
PD-L1, n (%)	
Positive >=50%	70 (34.15)
Positive <50%	65 (31.7)
Negative	70 (34.15)
Systemic therapies*, n (%)	
Targeted therapy alone	44 (21.5)
Chemotherapy alone	43 (21)
Chemotherapy combined with immunotherapy	24 (11.7)
Immunotherapy alone	18 (8.8)
Chemotherapy followed by targeted therapy	7 (3.4)
Targeted therapy followed by immunotherapy	3 (1.5)

Footnotes: *Aside from the presented therapeutic strategies, 17 additional patients started with some form of palliative radiotherapy and did not progress to further systemic therapies due to clinical deterioration; 49 patients were offered upfront best supportive care, due to clinical deterioration throughout the course of the diagnostic pathway. [#]Among the 11 adenosquamous carcinoma cases, 7 were initially diagnosed by minimally invasive procedures (EBUS-TBNA = 4, EUS-B = 2, TTB = 1), with 6 later confirmed on broader surgical or metastatic site biopsies. The remaining 4 were diagnosed from surgical specimens. All cases were reviewed by an experienced thoracic pathologist.

EGFR mutations were detected in 26.3%, predominantly exon 19 deletions. *KRAS* mutations occurred in 14.1%. Additional drivers included *ALK* (6.8%), *ERBB2* (3.9%), *TP53* (2.9%), and less frequent alterations in *FGFR* (2.4%), *CTNNB1* (2.4%), *ROS1* (1%), *RET* (1%), *BRAF* (1%), *MET* (1%), *CDK4* (1%), and *PIK3CA* (1%). Complex molecular profiles were observed in 4.4% (1% harbouring 3 simultaneous mutations in different genes and 3,4% presenting 3 concomitant mutations in different genes). Detailed results are expressed in Figure 6.

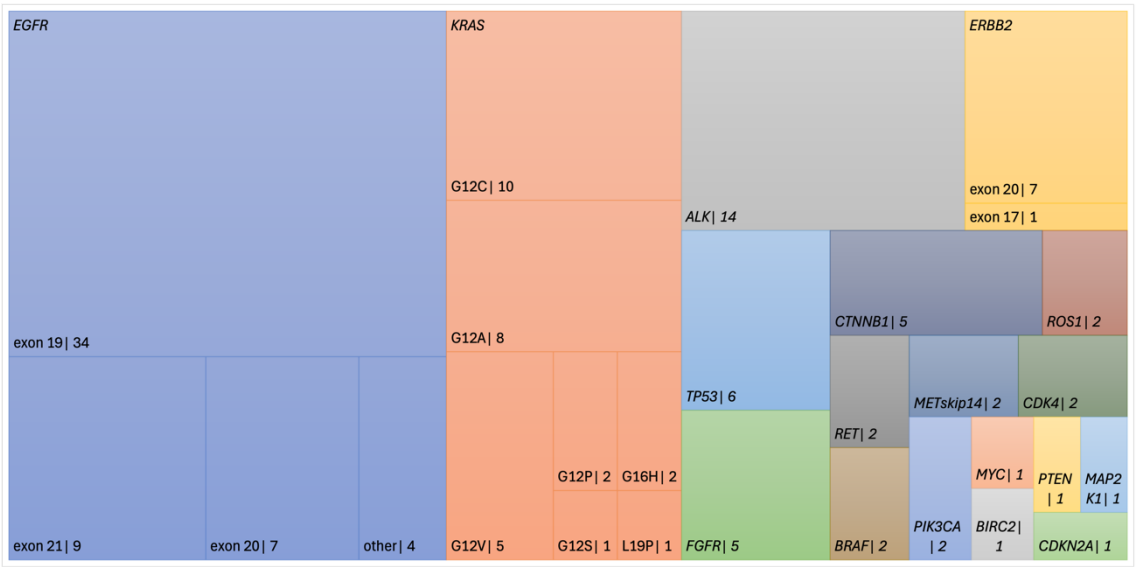

Figure 6 - Distribution of mutations in the study population (n=205).
Each square represents the absolute number of cases with a specific mutation, scaled proportionally. In *EGFR*, "other" stands for mutations in *EGFR* exon 15 and exon 17 in two cases and amplification of *EGFR* in two additional patients. Aside from these results and not represented in the figure are two patients that presented with three simultaneous mutations: *EGFR* exon 21, *EGFR* exon 5, and *TP53* in one case; *EGFR* exon 19, *CDKN2A*, and *PTEN* in another patient. Seven patients exhibited two simultaneous mutations: *EGFR* exon 19 and *CTNNB1*; *EGFR* exon 17 and *TP53*; *EGFR* exon 20 and *ERBB2*; *EGFR* exon 20 and *MAP2K1*; *ROS1* and *KRAS* G12A; *KRAS* G12C and *TP53*; *KRAS* G12C and *FGFR*. Seventy patients had negative molecular findings and were classified as *wild-type* for all tested genes. In five cases, samples were deemed insufficient for molecular analysis (2 EBUS; 1 EUS-B; 1 TTB; 1 Videobronchoscopy).

Timelines showed a median of 9 days (IQR 4–18) from initial clinical evaluation to the first diagnostic procedure, with significant differences by modality (Kruskal–Wallis, $p = 6.14\times10^{-12}$). EUS-B (median 5 days; IQR 1.5–8) and EBUS-TBNA (8; IQR 6–17.5) were faster than TTB (20.5; IQR 12–38.3) and surgical biopsy (24.5; IQR 18–37.8). The interval to histopathology/ immunohistochemistry was 11 days (IQR 8–15.8), without inter-modality differences ($p = 0.840$). Time to final molecular results was 24 days (IQR 16–35), differing by modality ($p = 0.038$): longest for TTB (31.5; IQR 23–58) and surgery (28; IQR 14.5–50.5), intermediate for videobronchoscopy (26.5; IQR 14.3–43), and shorter for EUS-B (20; IQR 14–29) and EBUS-TBNA (24; IQR 15.3–35) - Figure 7).

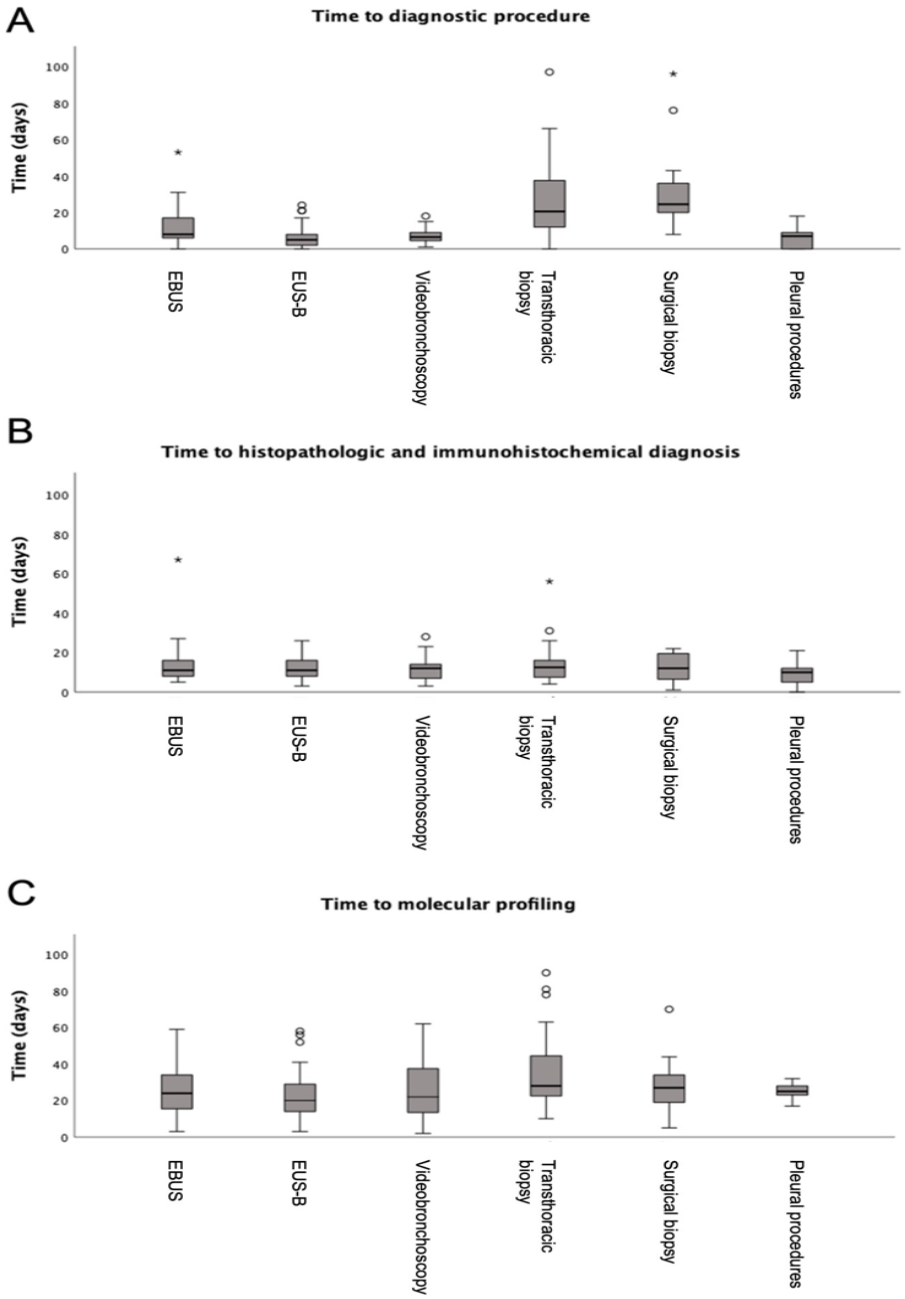

Figure 7 - Timeline analysis stratified by diagnostic modality.
Boxplot illustrating the **time** (in days) **to diagnostic procedure (A)**, **histopathologic and immunohistochemical diagnosis (B)**, and **molecular profiling (C)**, stratified by modality. Endoscopic techniques (EBUS-TBNA, EUS-B, and videobronchoscopy) demonstrated significantly faster access compared to transthoracic and surgical biopsies. Significant differences were observed for time to diagnostic procedure (A) ($p = 6.14 \times 10^{-12}$), while no differences were found for histopathologic diagnosis (B) ($p = 0.840$). Molecular profiling (C) showed variability ($p = 0.038$) with delays for transthoracic and surgical approaches. Moderate and extreme outliers are shown as circles and asterisks, respectively, representing values beyond 1.5 and 3 times the interquartile range.

Most patients received systemic therapy aligned with biomarker status: targeted therapy in 21.5%, chemotherapy alone in 21.0%, chemo-immunotherapy in 11.7%, and immunotherapy alone in 8.8%. Sequential regimens included targeted therapy followed

by immunotherapy (1.5%) and chemotherapy followed by targeted therapy (3.4%). A significant portion of the cohort (23.9%) was allocated to upfront best supportive care. The time from the first clinical evaluation to treatment initiation differed significantly by diagnostic modality (Kruskal–Wallis, $p = 0.011$), being shorter with EUS-B (59 days, IQR 35.5–75.5), videobronchoscopy (60 days, IQR 48.5–72.5), and EBUS-TBNA (61 days, IQR 49–87), compared with surgical biopsies (81 days, IQR 50–121) and TTB (82 days, IQR 55.8–104.8). In contrast, no significant differences were observed when stratified by therapy type ($p = 0.313$). Detailed results are presented in Table 3.

Table 3 - Time to initiation of systemic therapy

Procedure	Median time*	Min	Max	IQR#
EBUS-TBNA	61	22	211	49 - 87
EUS-B	59	6	189	35.5 - 75.5
Videobronchoscopy	60	30	131	48.5 - 72.5
Transthoracic biopsy	82	22	258	55.8 - 104.8
Surgical biopsy	81	41	144	50 - 121
Pleural procedures	62.5	46	111	58.3 - 67

Footnotes: *The median times are expressed in days and reflect the intervals to initiation of systemic therapy stratified by the different diagnostic procedures in the study cohort. # IQR: Interquartile Range

In multivariable Cox analysis, ECOG performance status was the strongest prognostic factor: ECOG 2 (HR 4.24; 95% CI 2.38–7.52; $p = 1\times10^{-7}$) and ECOG 1 (HR 2.29; 95% CI 1.36–3.87; $p = 0.002$) versus ECOG 0. Substage IVB (HR 1.78; 95% CI 1.12–2.82; $p = 0.015$) and male sex (HR 1.92; 95% CI 1.36–3.87; $p = 0.004$) were associated with higher mortality. Actionable mutations (HR 0.56; 95% CI 0.35–0.88; $p = 0.013$) and PD-L1 ≥50% (HR 0.44; 95% CI 0.26–0.75; $p = 0.003$) were associated with improved survival. Diagnostic timeliness independently predicted mortality: each additional day to the first diagnostic procedure increased risk (HR 1.15; 95% CI 1.09–1.28; $p = 0.045$). Using a pragmatic cut-off, delays ≥10 days were associated with higher mortality (HR 1.66; 95% CI 1.10–2.50; $p = 0.016$). Other variables (age, smoking status, diagnostic modality *per se*, time to histology, time to molecular results, and first-line therapy type) were not significant. Kaplan–Meier curves illustrating these associations are shown in Figure 8 and Table 4.

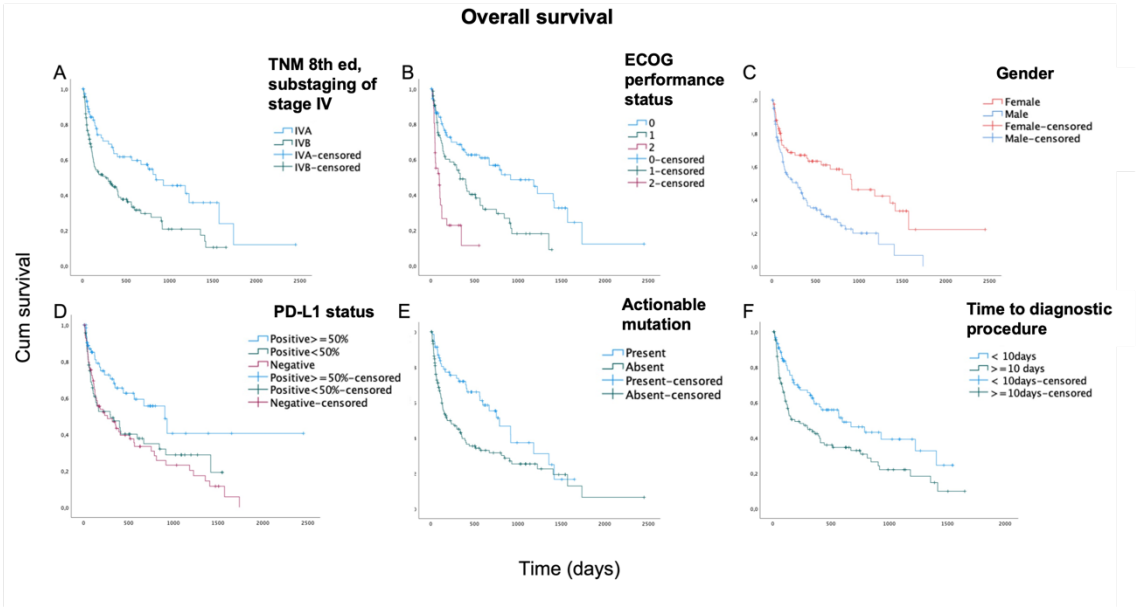

Figure 8 - Kaplan-Meier survival curves illustrating overall survival.
Results concerning overall survival were measured in days and analysed using the Log Rank (Mantel-Cox) test. **A - TNM Sub staging** (8th edition): Median survival was significantly longer for stage IVA compared to IVB (844 vs. 265 days, $p = 4.47x10^{-4}$). **B - ECOG Performance Status**: Median survival decreased with worsening ECOG scores: 917 days (ECOG 0), 337 days (ECOG 1), and 97 days (ECOG 2) ($p = 8.32x10^{-8}$). **C - Gender**: Females had longer median survival than males (917 vs. 295 days, $p = 2.52x10^{-4}$). D - PD-L1 Expression: Patients with high PD-L1 expression (>=50%) had the longest median survival (906 days) compared to low PD-L1 (<50%) or negative expression (299 and 265 days, respectively, $p = 0.008$). **E - Actionable Mutations**: Patients with actionable mutations had significantly longer median survival than those without (789 vs. 188 days, $p = 0.003$). **F - Time to Diagnostic Procedure**: Assess to diagnostic procedure in <10 days was associated with a longer median survival compared to >=10 days (597 vs. 188 days, $p = 0.003$).

Table 4 - Multivariate Cox proportional hazards regression analysis for clinical and molecular predictors of mortality

Variable	Sig.	HR	95%CI
Gender (male vs. female)	0.004	1.92	1.36 – 3.87
ECOG (ECOG2 vs. ECOG0)	$1x10^{-7}$	4.24	2.38 -7.52
ECOG (ECOG1 vs. ECOG0)	0.002	2.29	1.36 -3.87
Sub-staging of stage IV (IVB vs. IVA)	0.015	1.78	1.12 - 2.82
Actionable mutations (yes vs. no)	0.013	0.56	0.35 – 0.88
PD-L1 expression (>=50% vs negative)	0.003	0.44	0.26 – 0.75
Time to diagnostic procedure [a]	0.045	1.15	1.09 – 1.28
Time to diagnostic procedure (>=10 vs <10 days [b])	0.016	1.66	1.10 – 2.50

Footnotes: The table presents the results of the multivariate Cox regression analysis, including hazard ratios (HRs), 95% confidence intervals (CIs), and significance (Sig.) levels for all variables evaluated. For continuous variables, the hazard ratio reflects the risk increase for: [a]each additional day; [s]stratified analysis using a dichotomous approach, comparing groups with <10 days and ≥10 days, based on the pre-assessed median time.

2. Study II – Feasibility of EBUS-TBNA for the Molecular Characterisation of NSCLC

Among a total of 59 patients who underwent both EBUS-TBNA and molecular characterisation, the EBUS-TBNA samples were used for molecular testing in 38 cases (64.4%). Despite the availability of EBUS-TBNA material, alternative specimens were selected in the remaining 21 patients (35.6%), including surgical biopsies (10.2%), forceps biopsies (8.5%), CT-guided TTB (6.8%), and peripheral blood (6.8%).

The 38 patients included were predominantly male (65.7%), with a median age of 67 years (range 40–86). Non-smokers represented the largest group of patients (47.4%), while 31.6% were former smokers and 21.0% were current smokers. Most tumours were adenocarcinomas (86.8%), followed by squamous cell carcinomas (7.9%) and mixed adenosquamous carcinomas (5.3%). All patients presented with advanced disease: stage III (34.2%) or stage IV (65.8%). PD-L1 expression was positive in 44.7%, indeterminate in 5.2%, and negative in 50.0%. A median of two lymph node stations were sampled per patient (range 1–4), with three passes per station (range 3–8). Adequacy rates for molecular characterisation were high: 34 of 38 samples (89.5%) were sufficient for *EGFR* mutation testing and 26 of 32 (81.3%) for *ALK* rearrangement testing. Clinically relevant alterations were identified in 23.7%, comprising mostly *EGFR* mutations (18.4%) and *ALK* rearrangements, observed in 5.3% (Table 5).

Table 5 - Histopathological classification and mutational profiling of patients with NSCLC assessed by EBUS-TBNA

Histopathological classification	Mutational profiling	n (%) *
Adenocarcinoma	Non-mutated	26 (68.4)
	EGFR mutated	5 (13.2)
	ALK rearrangements	2 (5.3)
Squamous cell carcinoma	Non-mutated	1 (2.6)
	EGFR mutated	1 (2.6)
	ALK rearrangements	1 (2.6)
Mixed squamous cell carcinoma[a]	Non-mutated	1 (2.6)
	EGFR mutated	1 (2.6)

Footnotes: *Percentages were calculated considering the total cohort (N = 38). [a]"Mixed squamous cell carcinoma" refers to tumours with combined histological features of adenocarcinoma and squamous cell carcinoma, as assessed by an experienced, dedicated thoracic pathologist.

Therapeutic decisions were directly informed by molecular profiling results. Mutated patients received targeted therapies directed at *EGFR* (n = 6) or *ALK* (n = 2) Among non-mutated patients, immunotherapy was initiated in 15 with PD-L1 positivity, chemotherapy in 15, and best supportive care in 2 due to ECOG decline.

Although sample size precluded multivariate analysis, insufficient cases (n=4) were associated with limited sampling (median 1–2 nodal stations, with 3–8 passes per station).

3. Study III – Sequential vs Massively Parallel Strategies for the Molecular Characterisation of NSCLC samples obtained by EBUS-TBNA

A total of 106 patients with stage IV NSCLC underwent molecular characterisation using EBUS-TBNA specimens during the study period. Forty-five were tested using a sequential molecular profiling (SMP) approach, and 61 with massively parallel next-generation sequencing (MP-NGS).

Baseline characteristics were comparable between groups. Patients were predominantly male (SMP: 62.2%; MP-NGS: 60.7%), with median ages of 67 and 69 years, respectively. Adenocarcinoma was the dominant histological subtype (91.1% vs. 88.5%), and most patients presented with stage IVB disease (68.9% vs. 65.6%). No statistically significant clinicopathological differences were observed between patients in SMP and MP-NGS groups, as detailed in Table 6.

Regarding molecular profiling performance, SMP yielded adequate samples for *EGFR* testing in 93.3% of cases, for *ALK* in 78.4%, and for *ROS1* in 75%, corresponding to an overall success rate of 62.2%. Actionable mutations were detected in 22.2% of patients (*EGFR*: 15.6%; *ALK*: 6.7%), with no *ROS1* rearrangements identified. In contrast, MP-NGS achieved universal adequacy (100%) and significantly outperformed SMP both in analytical success ($p = 2 \times 10^{-5}$) and actionable mutation detection (40.9% vs. 22.2%, $p = 0.042$). Additional clinically relevant alterations were identified in the MP-NGS group, including *HER2* (8.2%), *RET* (1.6%), and *BRAF* (1.6%), while *KRAS* mutations were detected in 21.3% (with G12C variant present in 8.2%). The distribution of molecular profiling results in both methods is presented in Figure 9.

Table 6 - Epidemiological and clinical-pathological characteristics of included patients

Variable	SMP (n=45)	MP-NGS (n=61)	*p value*
Gender, n (%)			
Male	28 (62.2)	37 (60.7)	
Female	17 (37.8)	24 (39.3)	*0.870**
Age, median (min; max)	67 (38; 84)	69 (42; 86)	*0.933#*
Smoking, history n (%)			
Never smoker	11 (24.4)	21 (34.4)	
Former smoker	15 (33.3)	19 (31.1)	
Current smoker	19 (42.2)	21 (34.4)	*0.519**
ECOG performance status			
0	17 (37.8)	34 (55.7)	
1	18 (40)	19 (31.1)	
2	7 (15.5)	7 (11.5)	
3	3 (6.7)	1 (1.6)	*0.223**
Diagnostic procedure			
EBUS alone	28 (62.2)	40 (65.6)	
EBUS and EUS-b	17 (37.8)	21 (34.4)	*0.722**
Type of sample			
Lymph node	32 (71.1)	39 (63.9)	
Tumour	11 (24.4)	20 (32.8)	
Left adrenal gland	2 (4.4)	2 (3.3)	*0.635**
Histology, n (%)			
Adenocarcinoma	41 (91.1)	54 (88.5)	
Adenosquamous carcinoma†	2 (4.4)	4 (6.6)	
Combined adenocarcinoma and NE carcinoma†	1 (2.2)	3 (4.9)	
Squamous cell carcinoma	1 (2.2)	0	*0.556**
Stage, n (%)			
IVA	14 (31.1)	21(34.4)	
IVB	31 (68.9)	40 (65.6)	*0.720**

Footnotes: Comparative analysis of participants assigned to Sequential Molecular Profiling (SMP) and Massively Parallel – NSG (MP-NGS). ECOG: Eastern Cooperative Oncology Group; NE: neuroendocrine; *Pearson Chi-square test; #Mann-Whitney U test. †In cases classified as adenosquamous carcinoma (n = 6) and combined adenocarcinoma with neuroendocrine features (n = 4), the diagnosis was suggested based on morphology and immunohistochemistry performed on FFPE cell blocks obtained by EBUS-TBNA. In five of these cases (3 adenosquamous, 2 combined adenocarcinoma/NE carcinoma), the diagnosis was later confirmed on surgical biopsies from the primary tumour (n = 2) or metastatic sites (pleura, n = 1; subcutaneous tissue, n = 2).

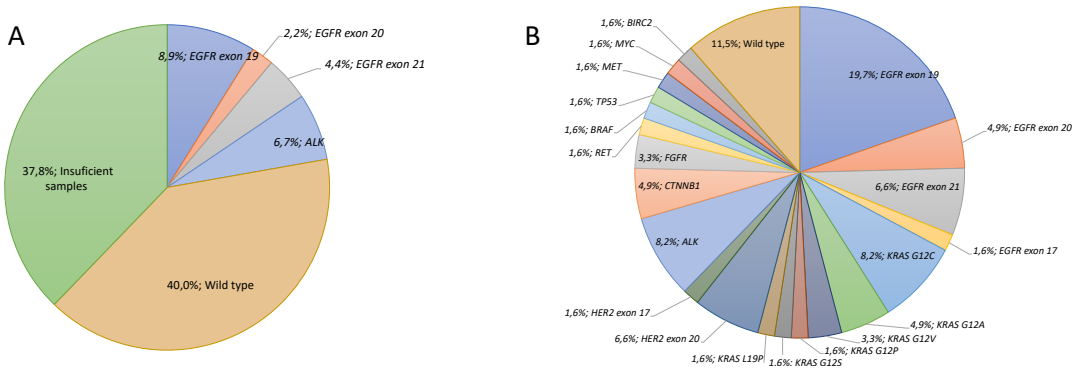

Figure 9 - Pie charts illustrating the molecular profiling results in stage IV NSCLC samples using (A) SMP and (B) MP-NGS.
Aside from the data presented in the charts, 9 patients (14,7%) from the MP-NGS group had complex molecular patterns: 1 patient harboured three mutations (*EGFR* exon 19, *CDKN2A* and *PTEN*); 4 patients combined *EGFR* mutations with a second mutation (2 with *CTNNB1;* 1 with *TP53*; 1 with *PIK3CA*); 4 combined *KRAS* mutations with a second mutation (2 with *FGFR*; 1 with *TP53*; 1 with *BRAF*). The data highlight the superior discriminative power of MP-NGS, the absence of insufficient samples with this method, and a reduced proportion of cases classified as wild-type.

Median turnaround time (TAT) for positive results was shorter with SMP than with MP-NGS (11 vs. 24 days, p = 0.002). The overall TAT also tended to favour SMP (17 vs. 23 days), although the difference did not reach statistical significance (p = 0.076) - Table 7.

Table 7 - Comparison of molecular profiling techniques: SMP vs MP-NGS

Variable	SMP step 1 RT-PCR (*EGFR*)	SMP step 2 FISH (*ALK*)	SMP step 3 FISH (*ROS1*)	SMP Overall results	MP-NGS Overall results	*p value*
Patients tested, n (%)	45 (100)	37 (82.2)	24 (53.3)	45 (100)	61 (100)	*NA*
Adequate samples, n (%)	42 (93.3)	29 (78.4)	18 (75)	28[a] (62.2)	61 (100)	*2x10^{-5}**
Actionable mutations[b], n (%)	7 (15.6)	3 (6.7)	0	10 (22.2)	25 (41)	*0.042*$*
Time to positive result[c], median (max; min)	8 (3; 34)	15 (9; 33)	NA	11 (3; 34)	24 (3; 57)	*0.002#*
Time to final molecular result[c], median (max; min)	15 (9; 33)	17 (3; 58)	23 (3; 58)	17 (3; 58)	23 (3; 57)	*0.076#*

Footnotes: [a]**Overall SMP** combines the positive results of *EGFR* (deemed complete, not requiring further profiling) and the 18 additional cases where both *ALK* and *ROS1* could be tested); [b]**Actionable Mutations**: mutations assessed by both diagnostic methods (*EGFR*, *ALK*, and *ROS1*). [c]**Time to Result**: Interval (days), from the completion of histopathological and PD-L1 evaluation to the final result of the molecular study. *Pearson's Chi square test; *$ Fisher's Exact test; # Mann-Whitney U test. SMP: Sequential molecular profiling; MP-NGS: Massive parallel – Next generation sequencing. NA: not applicable.

Therapeutic allocation differed substantially between groups. Targeted therapy was administered in 44.3% of patients profiled by MP-NGS compared with 22.2% of those tested with SMP, while best supportive care was more frequent in the SMP group (37.8% vs. 13.1%). Both differences were statistically significant (p = 0.026 and p = 0.019, respectively). Treatment distribution and the correspondence between detected alterations and therapeutic decisions are illustrated in Figure 10.

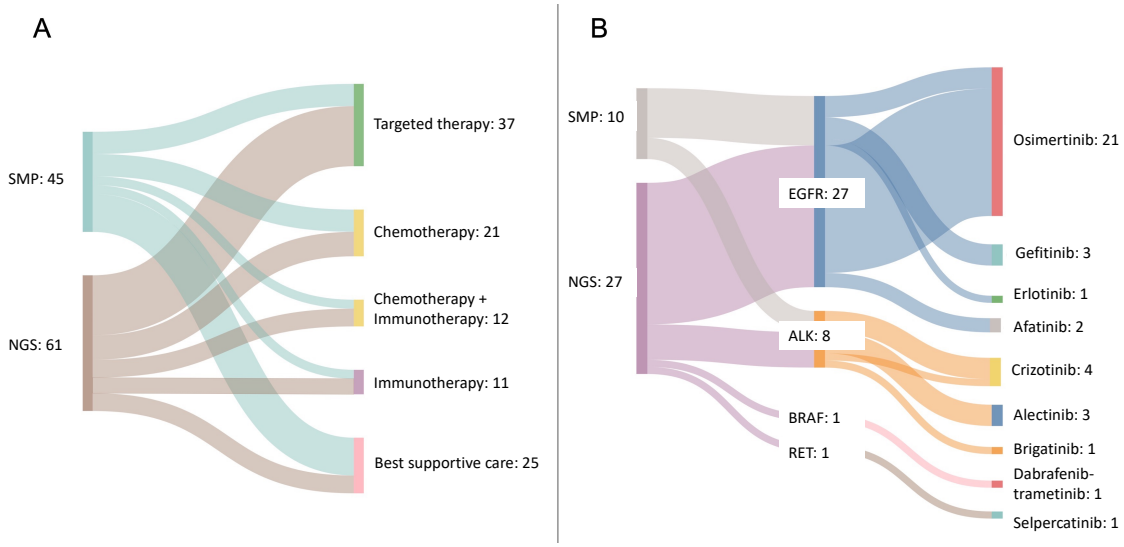

Figure 10 - Relationship between molecular profiling strategies and first line therapeutic choices with detailed targeted therapy selection.
(A) Sankey diagram illustrating the **distribution of first-line therapeutic strategies based on the molecular diagnostic method**. The figure highlights a significant increase in the use of targeted therapies with MP-NGS compared to SMP (44.3% vs. 22.2%, p = 0.026, Pearson's Chi-square test) and a notable reduction in the use of best supportive care strategies with MP-NGS compared to SMP (13.1% vs. 37.8%, p = 0.019, Pearson's Chi-square test). **(B)** Sankey diagram detailing the **targetable mutations identified by each method and their corresponding therapies.** This panel underscores the superior discriminatory capacity of MP-NGS, which identified more actionable mutations and facilitated greater use of targeted therapies.

Survival analyses further underscored the clinical relevance of molecular profiling. Patients harbouring actionable mutations had a markedly prolonged median overall survival (1128 vs. 138 days, log-rank p = 0.002). First-line targeted therapy was associated with the longest survival (median OS: 1128 days), whereas best supportive care corresponded to the shortest (46 days; log-rank p < 0.001). Patients in the MP-NGS group also exhibited a trend toward improved survival compared to SMP (median overall survival: 672 vs. 138 days, log-rank p = 0.053). In the Cox proportional hazards model, the presence of actionable mutations emerged as an independent predictor of survival (HR: 0.48, 95% CI: 0.25–0.96, p = 0.027), while the diagnostic method (MP-NGS vs.

SMP) and first-line therapy type were not statistically significant. Detailed results are presented in Table 8 and differences in survival are illustrated in Figure 11.

Table 8 - Cox regression results for survival analysis

Variable	Sig.	HR	95% CI
Diagnostic Method (MP-NGS vs SMP)	0.674	0.992	0.541 - 1.818
Actionable Mutation (Yes vs No)	0.027	0.488	0.252 - 0.960
First line therapy[1]	0.588	1.125	0.738 - 1.720
ECOG Performance Status (0,1,2,3)	$1x10^{-6}$	1.865	1.334 - 2.602
Age (per year)	0.635	1.003	0.986 - 1.020
Smoking Status[2]	0.794	1.044	0.741 - 1.469

Footnotes: Cox regression analysis assessing the impact of various factors on overall survival in the cohort of 106 patients with stage IV NSCLC. Variables considered included diagnostic method (MP-NGS vs SMP), presence of actionable mutations, ECOG performance status, first line therapy ([1]targeted therapy, chemotherapy, combined chemotherapy and immunotherapy, immunotherapy, best supportive care), smoking status ([2]current smoker, former smoker, never smoker), age, and first-line therapy. Hazard ratios (HR) and the respective 95% confidence interval (CI) indicate the relative risk of survival based on these variables, with significance (Sig.) level set at <0.05.

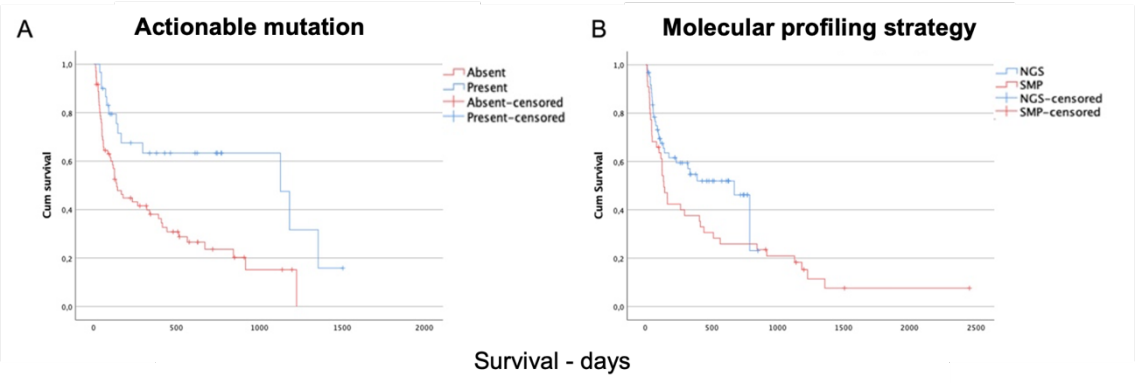

Figure 11 - Kaplan-Meier Survival Curves by Actionable Mutations (A) and molecular profiling method (B).
(A) Kaplan-Meier survival curves comparing overall survival based on the **presence or absence of actionable mutations.** Patients with actionable mutations had a significantly longer median survival time (1,128 days) compared to those without (138 days), with a log-rank test indicating a significant difference (Chi-Square=9.364, df=1, p = 0.002). **(B)** Kaplan-Meier survival curves based on the **diagnostic method, comparing the MP-NGS and SMP groups**. The MP-NGS group had a significantly longer median survival (672 days) compared to the SMP group (138 days), with a marginally significant difference observed (Chi-Square = 3.752, df = 1, p = 0.053).

4. Study IV – Supernatant from EBUS-TBNA Samples for Molecular Profiling in NSCLC: a Systematic Review and Meta-Analysis

From a total of 469 record screened, 7 studies met the inclusion criteria (Figure 3, Methods section). Risk of bias assessment using the Newcastle-Ottawa Scale (NOS) classified six studies as low risk and one as moderate risk; full details are provided in Table 9.

Table 9 - Risk of bias assessment - Newcastle-Ottawa Scale

Study	Selection (Max 4)	Comparability (Max 2)	Outcome (Max 3)	Total (Max 9)	Risk of Bias
Guibert N., *et al.* [114]	4	1	3	8	Low
Hanningan, B., *et al.* [118]	2	1	3	6	Moderate
Janaki, N., *et al.* [121]	4	1	3	8	Low
Gokozan, H., *et al.* [117]	4	1	3	8	Low
Wu, W. *et al.* [120]	4	2	3	9	Low
Tafoya, M., *et al.* [116]	3	1	3	7	Low
Jager, L. *et al.* [119]	4	1	3	8	Low

Footnotes: The table summarises the risk of bias assessment for each included study using the Newcastle-Ottawa Scale (NOS), which evaluates study quality based on three domains: Selection (maximum score of 4), Comparability (maximum score of 2), and Outcome (maximum score of 3). The total score ranges from 0 to 9, with higher scores indicating lower risk of bias. Studies were classified as Low Risk (≥7 points), Moderate Risk (5–6 points), or High Risk (<5 points).

The included studies were published between 2018 and 2024, mostly as prospective observational cohorts, six conducted in the United States and one in China. Reference standards varied, including cellblocks, core biopsies, cytology smears, or surgical specimens. Key methodological features are summarised in Table 10.

The seven studies evaluated a total of 506 patients, with median ages of 63–72 years and a male proportion of 37 to 60%. Histological classification varied, with adenocarcinoma being the most common diagnosis. EBUS-TBNA was the primary diagnostic method, often combined with CT-guided TTB or other bronchoscopy techniques. All studies applied NGS, either alone or in combination with digital droplet PCR or with amplification refractory mutation system (ARMS)-PCR

Table 10 - Characteristics of included studies, study population and diagnostic performance

Author, Year and Country	Population (count)	Type of paper and Research Design	Age in years, median (range)	Gender, n (%)	Diagnostic methods (type and count)	Histological classification (type and count)	Needle Gauge (EBUS)	ROSE	Molecular method; RS	Feasibility of SP	Agreement (SP vs. RS)
Guibert N., *et al.* 2018 U.S.A. [114]	17	Short communication; Prospective	NS	NS	EBUS (16) CT-TTB (1)	ADC(12); SQCC (1); SCC (1); Non-malignant (3)	NS	NS	NGS and ddPCR. Smears and cellblock	100%	100%
Hanningan, B., *et al.* 2019 U.S.A. [118]	150	Original Article; Prospective	68 (31-92)	Male: 64 (43%) Female: 86 (57%)	EBUS and CT-TTB (NS)	ADC (97); SQCC (25); NSCC NOS (6); Poorly diff. c. (8); Sarc. carc. (1); Carcinoid (3); SCC (10)	20-25G	Yes	NGS and ddPCR. Core-biopsy and cfDNA	90%	97%[a] 89%[b] 83%[c] 100[d]
Janaki, N., *et al.* 2019 U.S.A. [121]	30	Original Article; Prospective	71 (48-82)	Male: 18 (60%) Female: 12 (40%)	EBUS	ADC (24); SQCC (2); Mx melanoma (2); Mx breast (2)	20-25G	Yes	NGS; Cellblock or previous cx bx	100%	100%
Gokozan, H., *et al.* 2020 U.S.A. [117]	62	Original Article; Retrospective	68 (39-91)	Male: 24 (40%) Female: 37 (60%)	EBUS	ADC (37); NSCC NOS (19); SQCC (5); NEC (1)	20-25G	Yes	NGS; Cellblock	100%	NS
Wu, W. *et al.* 2020 China [120]	214	Original Article; Prospective	63 (38-90)	Male: 120 (56%) Female: 94 (44%)	EBUS and CT-TTB (NS)	ADC (184); SQCC (9); NSCC NOS (21)	20-25G	Yes	NGS and ARMS-PCR; Cellblock	100%	97%
Tafoya, M., *et al* 2021 U.S.A. [116]	14	Original Article; Prospective	NS	NS	EBUS (16); Bronch. washings (3); Bronch brushing (3); BAL (1)	NSCLC (23) NOS	NS	Yes	NGS; Cellblock or FFPE biopsy	87%	100%
Jager, L. *et al.* 2024 U.S.A. [119]	19	Original Article; Prospective	65 (50-95)	Male: 9 (47%) Female: 10 (53%)	EBUS	ADC (11): SQCC (3); Mx breast (2); Mx prostate (1); Mx renal (1); Poorly diff. c. (1)	19-22G	Yes	NGS; Cellblock	100%	100%

Footnotes: Summary of included studies. ROSE: Rapid on-site evaluation; RS: Reference Standard; SP: Supernatant; ADC: Adenocarcinoma; SQCC: Squamous Cell Carcinoma; SCC: Small Cell Carcinoma; NSCC-NOS: Non-Small Cell Carcinoma Not Otherwise Specified; Mx: Metastatic; NEC: Neuroendocrine Carcinoma; ddPCR: droplet digital PCR; ARMS-PCR: Amplification Refractory Mutation System-PCR; NS: Not Specified. [a]same site/day; [b]different site/same day; [c]different site/day; [d]cfDNA/different day.

Table 11 - Sample handling and DNA yield from EBUS-TBNA Supernatant: centrifugation protocols, storage conditions, and preserving solutions

Author	Sample handling					SP DNA yield (ng/µL)	
	Preserving solution	Storage temperature	Storage time (days): median (range)	1st centrifugation	2nd centrifugation	Median	Range
Guibert N., *et al.* [114]	CytoLyt or Physiologic saline	-80°C	NS	400g duration NS	1500g duration NS	4.8	0.3 - 79
Hannigan, B., *et al.* [118]	Needle rinse in RPMI	4°C	NS	600g for 10'	NS	6.8[a]	0.2 - 107[a]
Janaki, N., *et al.* [121]	CytoLyt	4°C	14 (7 – 29)	1200g for 5'	10000g for 5'	6.6[b]	0.4 - 56.4[b]
Wu, W. *et al.* [120]	CytoLyt	-20°C	NS	3000rpm for 5'	10000rpm for 10'	0.77	<0.05 to 120
Tafoya, M., *et al.* [116]	ThinPrep	5°C	6 (3 – 41)	NS	NS	85.5	<1 to 699
Jager, L. *et al.* [119]	Physiologic saline	4°C	<1[d]	2000rpm for 10'	16000rpm for 10'	4.7[c]	0.1 - 28.6[c]

Footnotes: [a]DNA concentration (ng/µL) was calculated by dividing the total extracted DNA (ng) by the reported elution volume (60 µL), as stated in the original study. [b]DNA concentration (ng/µL) was calculated using the elution volume (50 µL) specified in the Maxwell® RSC Nucleic Acid Purification Kit, as referenced in the original study. [c]DNA concentration (ng/µL) was calculated by dividing the total extracted DNA (ng) by the elution volume (15 µL), based on the MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit protocol. [d]Molecular testing of the supernatant was initiated within 24h of sample collection. SP: Supernatant; NS: Not Specified.

Feasibility of SP for molecular testing was consistently high, ranging from 87% to 100%, despite heterogeneity in processing protocols (storage at −80°C to +5°C; centrifugation 400g to 16,000 rpm; preservation in CytoLyt, RPMI, saline, or ThinPrep).

DNA yield was reported in six studies, with wide variability (median 0.77–85.5 ng/µL). High-speed centrifugation protocols were generally associated with lower yields. Pooled analysis across six studies yielded an overall mean of 40.1 ng/µL (95% CI: 1.25–78.94), though heterogeneity was high (I^2 = 99.5%). Subgroup analyses by preservation medium and temperature showed no significant differences. Detailed results are presented in Table 11 (page 39) and illustrated in Figure 12.

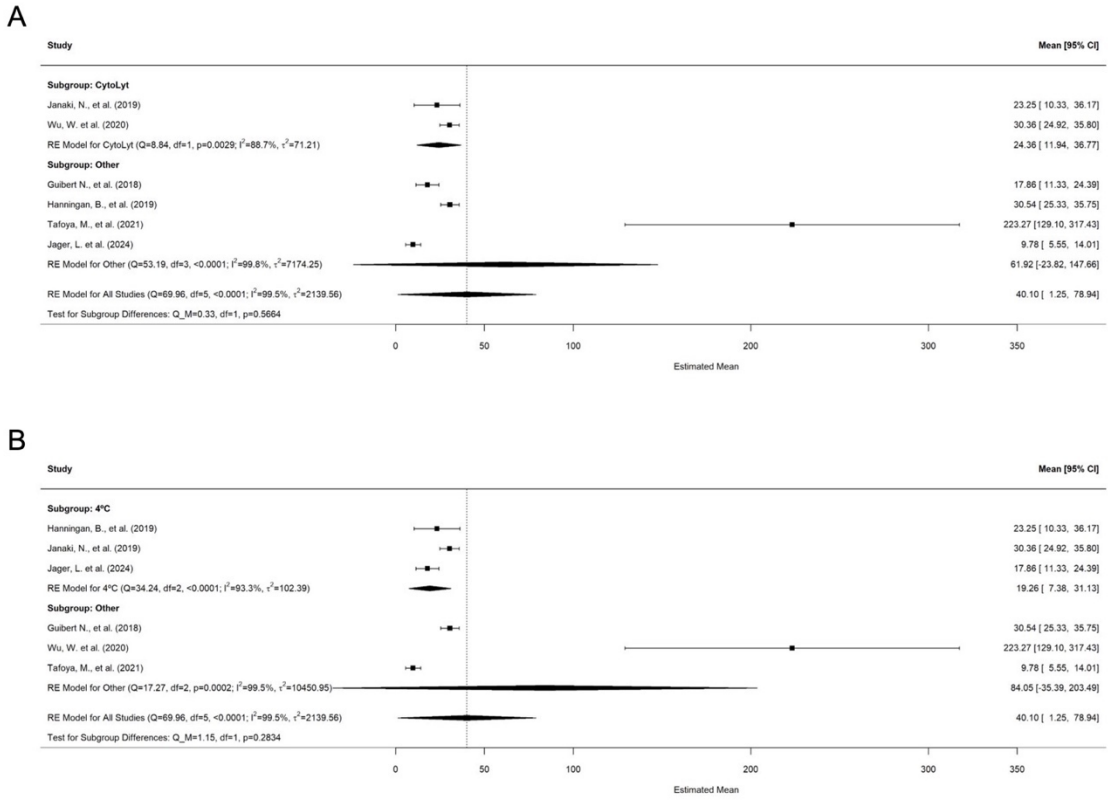

Figure 12 - Forest plots from the meta-analysis, stratified by the Preserving Solution (A) and Storage Temperature (B).
Displaying subgroup estimates and the overall estimate**. A.** When all six studies were combined, the overall random-effects model yielded a mean estimate of 40.10 (95% CI: 1.25–78.94) with very high heterogeneity (I^2 = 99.5%; Q = 69.96; $p < 0.001$; τ^2 = 46.26). For studies using CytoLyt (k = 2), the pooled estimate was 24.36 (95% CI: 11.94–36.77), with significant heterogeneity (I^2 = 88.7%; Q = 8.84; $p < 0.001$; τ^2 = 71.21). In the subgroup with other preservation conditions (k = 4), the pooled estimate was 61.92 (95% CI: −23.82 to 147.66) and heterogeneity remained high (I^2 = 99.8%; Q = 53.19; $p < 0.001$; τ^2 = 7174.25). No statistically significant differences were found between subgroups (QM = 0.33; p = 0.566). **B.** The random-effects model for studies classified in the 4 °C group (k = 3) yielded a pooled estimate of 19.26 (95% CI: 7.38–31.13), with high heterogeneity (I^2 = 93.3%; Q = 34.24; $p < 0.001$; τ^2 = 10.12). In contrast, studies in the other temperature group (k = 3) showed a pooled estimate of 84.05 (95% CI: −35.39 to 203.49; p = 0.1678), with very high heterogeneity (I^2 = 99.5%; Q = 17.27; $p < 0.001$; τ^2 = 102.23). The test for subgroup differences showed QM = 1.15, df = 1, p = 0.2834, indicating no statistically significant difference between groups.

Diagnostic accuracy of SP compared with reference samples was high, with sensitivity 0.95–1.00 and specificity (when estimable) 0.80–1.00. Full agreement was reported in several cohorts (Table 12).

Table 12 - Diagnostic accuracy of supernatant compared to reference standard

Study	TP	FP	FN	TN	Sens.	Specif.	PPV	NPV
Guibert N., *et al.* [114]	12	0	0	0	1.0	NA	1.0	NA
Hanningan, B., *et al.* [118]	73	2	4	8	0.95	0.8	0.97	0.67
Janaki, N., *et al.*[121]	30	0	0	0	1.0	NA	1.0	NA
Wu, W. *et al.* [120]	123	3	3	91	0.98	0.97	0.98	0.97
Tafoya, M., *et al.* [116]	10	0	0	3	1.0	1.0	1.0	1.0
Jager, L. *et al.* [119]	17	0	0	2	1.0	1.0	1.0	1.0

Footnotes: TP (True Positives): Mutations correctly identified in both supernatant (SP) and reference standard (RS); FP (False Positives): Mutations detected in SP but not confirmed in RS; FN (False Negatives): Mutations undetected in SP but present in RS; TN (True Negatives): Samples classified as wild-type in both SP and RS. Sensitivity: TP / (TP + FN), describes the ability of SP to correctly detect mutations present in RS. Specificity: TN / (TN + FP) reflects the ability of SP to correctly classify wild-type cases. PPV (Positive Predictive Value): TP / (TP + FP) reflects the likelihood that a mutation detected in SP is confirmed in RS. NPV (Negative Predictive Value): TN / (TN + FN) expresses the likelihood that a wild-type result in SP corresponds to wild-type in RS. NA (Not Applicable): Specificity and NPV could not be calculated in studies where TN = 0.

Concordance of molecular results between SP and RS was consistently high, with overall Cohen's kappa of 0.947 (95% CI: 0.905–0.989). Mutation-specific concordance was 96.4% for *EGFR*, 97.4% for *KRAS*, and 100% for *ALK*, *ERBB2*, and *MET*. *BRAF* mutations showed slightly lower concordance (81.8%). TAT was reported in five studies, consistently showing 2 to 7 day reductions with SP, most pronounced in the studies by Hannigan et al. and Janaki *et al.* (Table 13).

Table 13 - Summary of turnaround time reduction using supernatant from EBUS-TBNA samples for molecular characterisation of NSCLC

Study	Conventional Tissue Analysis (TAT)	Supernatant Analysis (TAT)	Time Gain (Reduction)	Description
Guibert N., *et al* [114]	Not specified	Not specified	Not quantified	The study mentions that the supernatant provides an immediately available source of fresh DNA, potentially reducing TAT but does not specify exact time reductions.
Hanningan, B., *et al* [118]	5-7 days	2-3 days	2-4 days	NGS analysis of tissue typically takes 5-7 days, while supernatant extraction reduces this to 2-3 days, resulting in a reduction of 2-4 days.
Janaki, N., *et al* [121]	10 ± 5.5 days	3-5 days	5-7.5 days	TAT for tissue samples averages 10 ± 5.5 days, while using supernatant reduces the TAT to 3-5 days, resulting in a reduction of 5-7.5 days.
Gokozan, H., *et al* [117]	12.2 ± 1.8 days	8.5 ± 1.8 days	3.7 ± 1.8 days	Tissue samples take 12.2 ± 1.8 business days, while supernatant reduces the TAT to 8.5± 1.8 days, resulting in a reduction of 3.7 ± 1.8 days.
Jager, L. *et al* [119]	4-10 days	1 day	3-9 days	TAT of NGS results was 1 day for supernatant compared to 4-10 days (mean 6.9 days) for corresponding core biopsies.

Footnotes: Summary of the evidence regarding the reduction in turnaround time (TAT) observed when using supernatant derived from EBUS-TBNA samples. Time gains (reductions) are calculated based on the difference in TAT between conventional tissue analysis and supernatant analysis where specific times are provided.

5. Study V – Feasibility of DNA and RNA Preservation from EBUS-TBNA Supernatant for Molecular Profiling in NSCLC

Results of Study V are presented in two stages: first, we provide the dataset originally analysed and submitted for publication (December 2023 – August 2024). Subsequently, we present the full dataset (December 2023 – December 2024), generated using the same methodology, to complement the published findings with updated results.

In the first phase, a total of 30 patients were screened for study eligibility, with informed consent obtained from all participants. Fifteen patients with NSCLC were included, while the remaining 15 were excluded due to the following reasons: presence of normal lymph node tissue in a staging context (n=9), diagnosis of small-cell lung cancer (n=3), insufficient material for histopathological analysis (n=2), and squamous cell carcinoma not requiring molecular profiling (n=1).

The included cohort demonstrated a male predominance (60%) and a median age of 69 years (range: 42–84). Most patients were current smokers (60%) and the majority had stage IV adenocarcinoma (87%).

The average tumour cell content in cell pellet (CP) samples was 38% (range: 10–70%), rendering all samples adequate for both PD-L1 assessment and NGS. In all 15 cases, both the CP and supernatant fractions were successfully separated. Median DNA and RNA concentrations in CP were 9.58 ng/µL (range: 0.45–50) and 13.6 ng/µL (range: 2.4–56), respectively. By contrast, supernatant phase (SP) samples yielded higher nucleic acid concentrations, with median DNA of 29.9 ng/µL (range: 0.78–51) and median RNA of 52.12 ng/µL (range: 5.95–95). Comparative analysis showed significantly higher DNA and RNA concentrations in SP compared to CP (Wilcoxon test, $p = 0.012$ and $p = 0.005$, respectively). Figure 13 illustrates the distribution of DNA and RNA concentrations in CP and SP.

In CP samples, 16 mutations were identified, of which 7 were clinically actionable, including *EGFR* (n=4), *ERBB2* (n=2), and *KRAS* G12C (n=1). A similar mutation profile was observed in SP, with 19 mutations detected overall, including actionable alterations in *EGFR* (n=3) and *ERBB2* (n=2), along with 2 additional mutations not identified in CP (a *MET* exon 14 skipping event and one additional *KRAS* G12C mutation). Across the 15 cases, concordance between CP and SP was 87%, with identical mutations detected in 13

patients and similar allele frequencies across sample types. Discordant results occurred in two cases: in one, a *KRAS* mutation was detected in SP but not in CP; in another, an *EGFR* exon 20 mutation identified in CP could not be detected in SP due to insufficient material for NGS. Full details of molecular results are provided in Table 14, where the first 15 cases correspond to the dataset submitted for publication, and the additional 5 cases reflect the subsequent phase of patient inclusion.

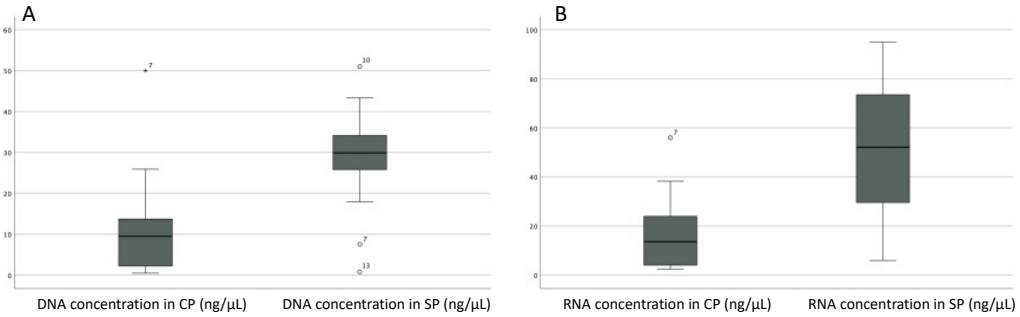

Figure 13 – Boxplot comparing DNA (A) and RNA (B) concentrations (µg/dL) in paired samples from the CP and SP of EBUS-TBNA samples in NSCLC.
Wilcoxon signed rank test results indicate that concentrations of both DNA and RNA were significantly higher in the SP samples compared to CP samples (p = 0.012 for DNA, p = 0.005 for RNA).

The full dataset, collected between December 2023 and December 2024, comprised 38 patients screened for study inclusion after obtaining written informed consent. Among these, 20 patients with histopathologically confirmed NSCLC were included, while 18 were excluded due to predefined reasons, including normal lymph node tissue (n=11), small-cell lung cancer (n=3), insufficient material (n=2), and squamous cell carcinoma not requiring molecular profiling (n=2). The median age of participants was 70 years (range 42–84) and the cohort comprised 12 males (60%) and 8 females (40%). Regarding smoking status, 5 patients (25%) were never-smokers, 6 (30%) were former smokers, and 9 (45%) were current smokers. Performance status (ECOG) was 0 in 13 patients (65%), 1 in 5 patients (25%), and 2 in 2 patients (10%). Most patients presented stage IV disease (n=16; 80%), while 4 patients (20%) had stage III disease. Histological subtypes included adenocarcinoma in 18 patients (90%) and large-cell neuroendocrine carcinoma in 2 patients (10%).

The complete dataset corroborated the preliminary observations regarding nucleic acid recovery, demonstrating higher yields in supernatants compared with paired cell pellets. Median DNA concentration was significantly higher in SP (28.95 ng/µL [range: 0.67–

51.0]) than in CP (9.84 ng/µL [0.45–50.0]; Wilcoxon signed-rank test, p = 0.025). Likewise, median RNA concentration was higher in SP (37.6 ng/µL [4.06–95.0]) compared with CP (15.95 ng/µL [2.4–56.0]; p = 0.007). Overall, these results confirm that SP samples consistently provide superior nucleic acid yields relative to their cellular counterparts. Molecular concordance between paired SP and CP samples was observed in 85% of cases, with only three instances of discordance. In two of these, the discrepancy was attributable to insufficient material in SP, while the remaining case reflected a true molecular mismatch as previously described in the preliminary results dataset, fully detailed in Table 14.

The analysis of turnaround times showed a median interval of 12.5 days (range, 8–19) between the diagnostic procedure and the availability of the histological report, and a median of 8 days (range, 1–15) between the histological report and the release of molecular results. To further explore the potential impact of initiating molecular analysis directly on SP, diagnostic timelines were calculated and compared under two conditions: the observed sequential pathway, in which molecular testing was initiated only after the histological report was available, and a simulated parallel pathway, in which molecular testing would have been started on SP at the time of the diagnostic procedure, while the CP proceeded through the conventional histological workflow. For each patient, the total diagnostic interval in the observed pathway was obtained by summing the time from procedure to histological report and the time from histological report to molecular result, resulting in a median overall diagnostic time of 21.5 days (range 14–31). In the simulated pathway, the total diagnostic time was defined as the longer of the two parallel components (histology vs. molecular), which yielded a median of 13 days (range 8–19). Comparison between the two timelines using the Wilcoxon signed-rank test confirmed a statistically significant reduction with the simulated approach ($Z = -3.927$, $p = 8.6 \times 10^{-5}$). These results are illustrated in Figure 14, which displays boxplots of diagnostic intervals for histology, molecular analysis, the observed total pathway, and the simulated parallel pathway.

Table 14 - Detailed results of the participants and the mutation profile of CP and SP.

Case nº	Age (years)	Gender	CP (% of tumour cells)	CP NGS results (variant and allele frequency*)	SP NGS results (variant and allele frequency*)
1	42	Female	10%	***EGFR*** Exon19 Deletion Glu746_Ala750del (18%)	***EGFR*** Exon19 Deletion Glu746_Ala750del (16.8%) ***MET*** Exon14 Skipping c.2942-1G>A;p (2,7%)
2	49	Male	40%	None detected	***KRAS*** Gly12Cys (33.2%)
3	84	Female	20%	***EGFR*** Exon 20 Insertion Val769_Asp770insPro (14%)	***EGFR*** Exon 20 Insertion Val769_Asp770insPro (10.6%)
4	70	Male	25%	***CTNNB1*** Ser37Cys (16.5%)	***CTNNB1*** Ser37Cys (11%) ***RET*** Val804Met (4.4%)
5	63	Female	70%	***EGFR*** Exon19 Deletion Glu746_Ala750del (43%) ***CDKN2*** Deletion (0.27x)	***EGFR*** Exon19 Deletion Glu746_Ala750del (36.4%) ***CDKN2*** Deletion (0.65x) ***PTEN*** Arg173His (5.8%)
6	83	Female	60%	***ERBB2*** Tyr772_Ala775dup (60.7%) ***ERBB2*** Amplification (6.3x)	***ERBB2*** Tyr772_Ala775dup (67.1%) ***ERBB2*** Amplification (5.34x)
7	80	Female	20%	***EGFR*** Exon 20 Insertion Val769_Asp770insPro (13%)	None detected (insufficient sample)
8	64	Male	50%	***KRAS*** Gly12Cys (28.5%)	***KRAS*** Gly12Cys (43%)
9	73	Male	50%	***TP53*** Glu286Lys (28.6%)	***TP53*** Glu286Lys (24.8%)
10	70	Male	30%	***BRAF*** Asp594_Thr599dup (29.3%)	***BRAF*** Asp594_Thr599dup (37.5%)
11	73	Male	50%	***ERBB2*** Tyr772_Ala775dup (23.3%)	***ERBB2*** Tyr772_Ala775dup (32.7%)

Case nº	Age (years)	Gender	CP (% of tumour cells)	CP NGS results (variant and allele frequency*)	SP NGS results (variant and allele frequency*)
12	64	Male	50%	***TP53*** Val216Leu (66.6%) ***FGFR1*** Amp (5x)	***TP53*** Val216Leu (67.8%) ***FGFR1*** Amp (4.92x)
13	69	Female	10%	None detected	None detected
14	81	Male	50%	***TP53*** Pro152Leu (48%) ***BRAF*** Gly596Arg (21%)	***TP53*** Pro152Leu (56.2%) ***BRAF*** Gly596Arg (29.9%)
15	75	Male	40%	None detected	None detected
16	73	Female	40%	***EGFR*** Exon 21 missense Leu858Arg (13%) ***CTNNB1*** Ser37Phe (4%)	***EGFR*** Exon 21 missense Leu858Arg (9.4%) ***CTNNB1*** Ser37Phe (3.4%)
17	70	Male	10%	***KRAS*** Gly12Cys (40%) ***TP53*** Gly266Va (35%)	***KRAS*** Gly12Cys (35%) ***TP53*** Gly266Va (28%)
18	63	Male	50%	***ALK fusion*** Variant EML4 (4%)	***ALK fusion*** Variant EML4 (3.1%)
19	74	Female	10%	***EGFR*** Exon 21 missense Leu858Arg (5.6%)	***EGFR*** Exon 21 missense Leu858Arg (3.8%) ***TP53*** Val173Met (7%)
20	83	Male	40%	***MET amplification*** Copy numbers: 8.62 ***RAF1*** Ser257Leu (4.4%)	None detected (insufficient sample)

Table 14 (cont.). Footnotes: *Allele frequencies are expressed as a percentage (%) of variant reads detected NGS. Gene designations across the table follow the Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC) standardised nomenclature. “Insufficient sample” indicates that nucleic acid yield in SP was below the minimum threshold for reliable molecular analysis. Cases with multiple alterations list all detected variants in descending order of clinical relevance. CP: cell pellet; SP: supernatant phase.

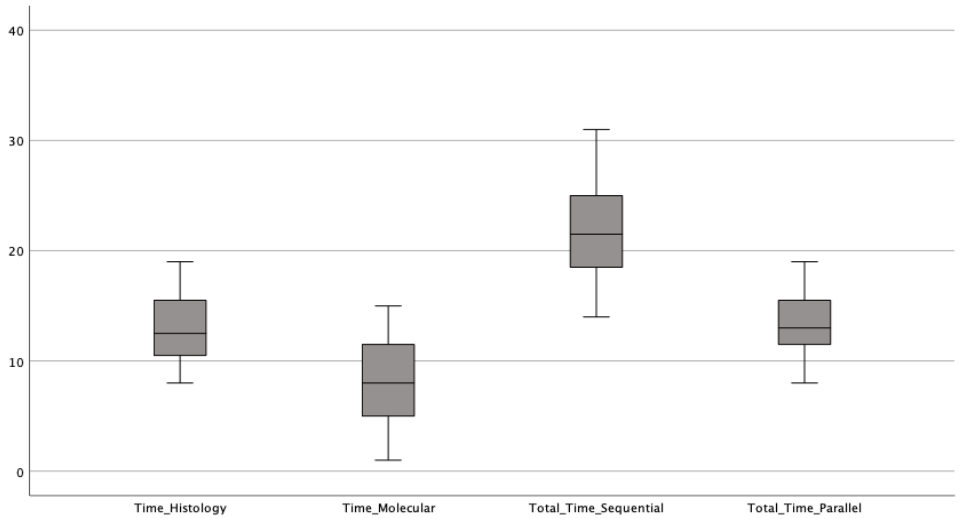

Figure 14 - Boxplots comparing total diagnostic time between the observed sequential pathway (CP) and the simulated parallel pathway (SP).
From left to right, the figure shows the intervals (in days) (i) from procedure to histological report, (ii) from histological report to molecular result, (iii) observed total diagnostic time (sequential approach with CP), and (iv) simulated total diagnostic time (parallel approach with SP). The simulated parallel approach shows a significantly shorter overall diagnostic timeline compared with the observed pathway (Wilcoxon signed-rank test, $p = 8.6 \times 10^{-5}$).

Discussion

The role of EBUS-TBNA has been progressively consolidated in the diagnostic and staging pathways of LC, offering a minimally invasive, accurate, and safe approach for mediastinal and central or peribronchial lesions [59,99]. Its application in NSCLC has gradually expanded to biomarker characterisation, particularly molecular profiling, where it has consistently produced encouraging results [69,100]. However, variability in testing strategies, laboratory workflows, and the limited cellularity of small biopsies may continue to pose challenges to the full exploitation of EBUS-TBNA samples in routine clinical practice.

Within this context, the present thesis provides new evidence on how the diagnostic and molecular potential of EBUS-TBNA can be further optimised. Through a series of complementary studies, it shows that timely access to EBUS-TBNA accelerates biomarker-informed treatment allocation without compromising sample adequacy and may indirectly translate into the survival benefits associated with shorter diagnostic timelines. It also shows that although sequential molecular profiling strategies remain feasible, they risk depleting material for downstream targets. By contrast, broader NGS panels applied to EBUS-TBNA prove consistently feasible and expand the spectrum of actionable findings, enabling broader allocation to targeted therapies and being indirectly associated with survival benefits. Finally, through a systematic literature review and prospective validation using a minimalist approach that preserved clinical workflows, this thesis demonstrates that the routinely discarded supernatant of EBUS-TBNA samples represents a robust and informative source of nucleic acids, further enhancing the value of this procedure in the era of precision oncology.

1. Positioning and Impact of EBUS-TBNA in Real-World Diagnostic Pathways of Advanced NSCLC

The findings from **Study I** of this thesis highlight the central role of streamlined diagnostic workflows in shaping clinical outcomes for patients with advanced NSCLC. Specifically, the time to first diagnostic procedure emerged as an independent predictor of mortality (HR = 1.66, p = 0.016), underscoring the critical importance of minimising delays in the earliest stages of the diagnostic pathway. This observation is particularly

relevant in advanced NSCLC, where delays in diagnosis can adversely affect survival, as therapeutic decisions increasingly rely on timely access to molecular biomarkers. Prolonged intervals between clinical evaluation, diagnostic procedures, and molecular profiling may postpone treatment initiation and ultimately reduce the likelihood of favourable outcomes [49,101].

Within this context, **Study I** clearly demonstrate the clinical relevance of endoscopic approaches such as EBUS-TBNA and EUS-B, which accounted for the majority of diagnostic procedures (51.7%) and were associated with shorter diagnostic intervals compared with transthoracic or surgical biopsies. These advantages likely reflect integrated pulmonology workflows, where patients with suspected LC are initially assessed, thereby reducing logistical barriers associated with interdepartmental referral [76]. Earlier procedural access, as observed with EBUS-TBNA, was associated with expedited initiation of biomarker-informed therapy (p = 0.011). This is particularly relevant in advanced NSCLC, where many patients present with disease-related frailty [102]. In this cohort, 17.6% had ECOG 2, and these patients carried a fourfold higher risk of death (HR = 4.24). For such vulnerable individuals, even modest diagnostic delays may close the window of opportunity to receive systemic therapy, whereas timely diagnosis can represent a critical chance to access treatments capable of altering prognosis [103,104].

Despite these advantages, results from **Study II** reveal that EBUS-TBNA is not consistently prioritised as the preferred source for molecular profiling. In some cases, clinicians favoured alternative samples, including liquid biopsies, even when tissue obtained by EBUS was available. This may partly reflect the study period (2019–2021), when confidence in the molecular adequacy of EBUS-derived samples was still evolving, with gradual recognition of its potential in biomarker assessment [69,105]. Nevertheless, this observation raises an important question about the positioning of EBUS-TBNA within diagnostic pathways. While the technique provides timely access and consistently high adequacy for both histology and biomarker testing, its molecular potential appears to remain under-recognised in daily practice. Such findings call for greater visibility of EBUS-TBNA as a first-line diagnostic tool in thoracic oncology, ensuring its optimal integration into precision oncology workflows [76].

Collectively, these findings emphasise that diagnostic efficiency, supported by minimally invasive procedures such as EBUS-TBNA, not only ensures adequate tissue acquisition but also accelerates treatment initiation, with meaningful implications for survival. At the same time, the lack of prioritisation of EBUS-TBNA derived material for molecular testing exposes a gap between technical feasibility and real-world implementation.

Current guidelines and organisational models of thoracic oncology services should therefore continue to prioritise rapid access to EBUS-TBNA, while strengthening its visibility and continuously improving sample yield to meet the growing demands of modern oncological practice [100,106].

2. Molecular Profiling of EBUS-TBNA Samples: Feasibility of Sequential Testing Strategies and Comparative Performance of Parallel Approaches

The feasibility of EBUS-TBNA as a source for comprehensive molecular characterisation of NSCLC samples is clearly established in **Study I**, where sample adequacy is uniformly high across diagnostic approaches. Histological and immunohistochemical analyses are successful in all cases, PD-L1 assessment in 98.9%, and molecular profiling in 97.6%. No significant differences are observed between procedures for PD-L1 (p = 0.757) or molecular profiling success (p = 0.968), indicating that EBUS-TBNA is not inferior to transthoracic or surgical biopsies, even when these may offer greater tumour representation [69,105].

However, as demonstrated in **Study II**, adequacy can be influenced by the testing strategy applied. Sequential molecular profiling (SMP), although feasible, poses an inherent risk of sample depletion across successive analyses. While *EGFR* testing remains successful in 89.5% and *ALK* in 81.3% of cases, the stepwise consumption of material for histology, PD-L1, and then sequential biomarker assays inevitably reduces availability for downstream markers, echoing concerns described in prior literature [45,69]. This limitation becomes particularly evident in **Study III**, which directly compares SMP with massively parallel next-generation sequencing (MP-NGS). In the sequential approach, adequacy declines progressively, as shown in Table 7 (page 34, Results section). Sequentially tested *EGFR* achieves rates comparable to MP-NGS, but yields decline with each additional marker, reaching the lowest level for *ROS1* (62.2%). This pattern reflects the cumulative consumption of material across the diagnostic cascade. By contrast, MP-NGS consistently achieves complete adequacy across all targets, indicating that the technique itself allows more efficient use of the tissue available from EBUS-TBNA. This efficiency translates into a broader and more informative mutational profile, with NGS detecting nearly twice as many actionable *EGFR* (32.8% vs. 15.5%) and more *ALK* alterations (8.2% vs. 6.7%) compared with SMP. In addition, MP-NGS

identifies a wider mutation spectrum in 88.5% of patients, including concurrent alterations in almost 15%, reflecting the molecular heterogeneity of NSCLC. Importantly, this expanded coverage allows the identification of emerging therapeutic targets such as *RET*, *BRAF*, and *KRAS* G12C, increasingly relevant in the context of precision oncology [39,107,108,109,110].

The clinical impact of this broader discriminative power is evident in **Study III**, where patients tested with MP-NGS are significantly more likely to receive targeted therapies compared with those assessed by SMP (44.3% vs. 22.2%). This aligns with observations from other cohorts and adds to the existing evidence that comprehensive molecular profiling increases therapeutic access and may improve survival [49].

Aside from sample adequacy, which our studies confirm to be consistently high, the temporal dimension of feasibility also emerges as a critical determinant of clinical utility. This is particularly important because, as shown in **Study I**, diagnostic delays in advanced NSCLC are not neutral but translate into worse survival, demonstrating that both adequacy and timeliness must be addressed in equal measure to fully realise the potential of EBUS-TBNA in clinical practice. In this context, across **Studies I** to **III**, the registered median turnaround times consistently exceed the recommended benchmarks [35,72], reflecting a persistent gap between guideline expectations and routine practice [47,50].

Factors such as the cumulative delays inherent to sequential workflows or the external processing required for NGS partly explain these extended timelines. Yet, the challenge is broader and reflects the real-world diagnostic pathway of these patients, in which logistical, organisational, and technical barriers often outweigh the theoretical efficiency of any given methodology. This discrepancy has been consistently described in the literature, where median turnaround times in clinical practice frequently surpass the 10-working-day target established by international recommendations [47,50].

Addressing this limitation will require solutions that transcend individual testing strategies. Moving forward, efforts should focus on the implementation of reflex testing protocols [111], the incorporation of faster in-house sequencing platforms [112], and the optimisation of tissue handling to maximise yield [100,106]. In this regard, the exploration of complementary sources such as the supernatant phase appears particularly promising, not only for enhancing the overall diagnostic performance of EBUS-TBNA but also for accelerating turnaround times.

3. The Emerging Role of EBUS-TBNA Supernatant in Optimising Molecular Yield and Turnaround-Time

The systematic review and meta-analysis presented in **Study IV** provides, for the first time, a comprehensive synthesis of all available evidence on the diagnostic value of supernatant derived from EBUS-TBNA samples. As expected for a relatively recent line of investigation, the number of eligible studies was limited. This highlights that supernatant analysis remains an emerging and underexplored field, building on prior investigations of cytology-derived supernatants across different solid tumours, as recently reviewed by Roy-Chowdhuri [113].

The rationale for exploring the EBUS-TBNA supernatant as a source for molecular profiling rests on both biological and practical grounds. Although processing protocols may vary, EBUS-TBNA samples are typically collected in a liquid medium, which differs across institutions. During subsequent processing, whether through extraction of a tissue core for histology or, more commonly, through centrifugation to generate a cell pellet for cytological analysis, a supernatant phase is invariably produced and conventionally discarded [106]. Since this supernatant remains in direct contact with tumour tissue, it may contain extracellular DNA and RNA in sufficient amount and quality for molecular characterisation [114]. This material is directly derived from the tumour microenvironment, potentially offering a richer tumour signal than blood-based liquid biopsies [115]. In addition, being readily available during routine processing, it offers an opportunity to maximise diagnostic yield without additional procedures [113,115].

Despite the limited number of studies and the considerable heterogeneity in sample processing and preservation protocols, the systematic review and meta-analysis demonstrated consistently high feasibility of using the supernatant phase for molecular profiling. Overall, feasibility rates ranged from 87% to 100%, closely matching those reported for conventional tissue-based molecular assays [114,116,117,118,119,120,121].

A consistent finding in the systematic review is the high DNA yield obtained from supernatant samples. Across studies, the supernatant consistently provides sufficient nucleic acid for downstream molecular profiling. In one study, DNA concentration is even higher in the supernatant than in the paired cell pellet. This observation is also confirmed in our prospective study (**Study V**), where median DNA concentration is higher in the supernatant (28.95 ng/µL [0.67–51.0]) compared with the pellet (9.84 ng/µL [0.45–50.0]; $p = 0.025$), and median RNA concentration shows a similar pattern

(37.6 ng/µL [4.06–95.0] vs. 15.95 ng/µL [2.4–56.0]; $p = 0.007$). These findings suggest that the acellular phase may be enriched in tumour-derived genetic material, possibly due to the accumulation of DNA-rich cellular debris during processing [116].

Beyond nucleic acid recovery, molecular concordance between the supernatant and the cell pellet is consistently high. **Study IV** demonstrates pooled agreement rates exceeding 90% across published series, with a kappa coefficient of 0.947, indicating almost perfect concordance. Discordant cases are uncommon and appear to result from a combination of biological and technical factors, including variability in DNA shedding related to tumour burden [119] and platform-specific differences in sequencing workflows and amplicon design [118]. Temporal and spatial mismatches in sample acquisition contribute to occasional discrepancies, as shown by Hannigan *et al.*, who report lower concordance when EBUS-TBNA supernatants are compared with samples from different sites or time points, reflecting the impact of tumour heterogeneity on molecular profiling. [118]

The consistently strong performance of the supernatant is evident despite substantial heterogeneity across studies, particularly in storage temperatures, preservation media, and centrifugation protocols. This variability limited the ability to conduct quantitative analyses for some parameters. However, where such analyses were feasible, results consistently show that neither storage temperature nor preservative solutions significantly affect DNA yield, reinforcing the reliability of the supernatant across a range of pre-analytical conditions [114,116,118,119,120,121].

Building on this evidence, **Study V** adopts a pragmatic approach by assessing the performance of the supernatant under routine laboratory conditions, using material collected during the standard post-processing of EBUS-TBNA samples and stored at room temperature until analysis. Consistent with the findings of the systematic review, as discussed above, the supernatant demonstrates superior nucleic acid yields, with DNA concentrations significantly higher than those of the paired cell pellets. Importantly, this study also expands the evidence by quantifying RNA, showing markedly higher RNA yields in the supernatant, a parameter that was not systematically assessed in most of the studies included in the review. High molecular concordance is also observed, with matching profiles in 85% of cases. Of the three discordant cases, two are explained by insufficient nucleic acid content in the supernatant related to low sample cellularity, while one represents a true molecular mismatch: the tissue based NGS was negative, whereas the supernatant analysis revealed a *KRAS* G12C mutation. This observation suggests that the supernatant may provide a broader representation of the tumour's molecular landscape, potentially capturing sub clonal variants that are absent or

underrepresented in the cellular fraction. Supporting this hypothesis, three additional cases in Study V showed full concordance for the primary actionable mutation between supernatant and tissue, while also revealing additional mutations in the supernatant that were not detected in the tissue sample. These findings reinforce the concept that the acellular phase may harbour a more comprehensive spectrum of tumour-derived genetic material, offering an opportunity to enhance the detection of clinically relevant alterations [113,115].

One of the most clinically meaningful advantages of integrating the supernatant into molecular workflows is its potential to shorten the turnaround time for molecular results which is also demonstrated in the full dataset analysis of **Study V**. The median interval between the diagnostic procedure and the availability of the histological report is 12.5 days (range, 8–19), with an additional 8 days (range, 1–15) required for the release of molecular results, yielding a median total diagnostic interval of 21.5 days (range, 14–31). To explore the impact of initiating molecular analysis directly from the supernatant, a simulated parallel workflow was modelled in which molecular testing is initiated at the time of the diagnostic procedure, while the paired cellular pellet undergoes conventional histological processing. Under this approach, the median total diagnostic time is reduced to 13 days (range, 8–19), representing a statistically significant reduction compared to the sequential pathway (Wilcoxon signed-rank test, $Z = -3.927$, $p = 8.6 \times 10^{-5}$). These findings highlight the operational efficiency that could be achieved by processing the supernatant in parallel, enabling the earlier availability of molecular data to guide clinical decision-making.

This observation aligns with the findings of the systematic review (**Study IV**), where five studies consistently report meaningful reductions in turnaround time, ranging from 1 to 7.5 days compared to tissue-based workflows [114,117,118,119,121]. These gains are largely attributed to the immediate availability of cfDNA in the supernatant, which enables molecular workflows to start on the day of the procedure, thereby bypassing delays inherent to tissue fixation, embedding, and histopathological review. Importantly, these benefits are achieved without compromising molecular profiling quality, as evidenced by the high concordance observed across multiple studies.

From both clinical and operational perspectives, integrating supernatant analysis into parallel diagnostic workflows represents a pragmatic and efficient approach. Allocating histological evaluation to the cellular pellet while initiating molecular testing directly from the supernatant allows the two processes to proceed simultaneously, thereby shortening diagnostic timelines and facilitating faster access to actionable results. This

streamlined strategy aligns with the growing need for rapid and comprehensive molecular profiling in advanced NSCLC, where timely initiation of targeted therapies is critical, as clearly demonstrated in **Study I**.

Furthermore, supernatant analysis addresses the increasing challenge of *tissue pressure* in molecular diagnostics. The increasing demand for expanded DNA and RNA testing often exceeds the material available in small biopsies or cytological specimens, leading in some cases to incomplete molecular panels or the need for repeat procedures. By providing a consistent and high-yield source of nucleic acids, the supernatant complements the cellular fraction, increasing the likelihood of obtaining complete molecular profiles from a single minimally invasive procedure.

4. Limitations and Future Directions

While this thesis provides a comprehensive and multi-angled evaluation of the role of EBUS-TBNA in the diagnostic and molecular characterisation of advanced NSCLC, several limitations should be acknowledged. Most studies were conducted in a single-centre, retrospective setting, which may limit the generalisability of the findings, particularly in healthcare systems with different referral patterns, procedural expertise, or access to advanced molecular testing platforms. Although the real-world nature of the cohorts enhances external validity, variability in institutional resources, operator experience, and laboratory infrastructure may influence diagnostic yield and turnaround times across different clinical settings.

Sample size was another relevant constraint, particularly in the studies assessing molecular concordance and prospective supernatant performance. Smaller cohorts reduced statistical power and precluded stratified analyses for less frequent genomic alterations. Additionally, the rapidly evolving treatment landscape of advanced NSCLC, with the expanding use of targeted agents and broader molecular panels, may have influenced therapeutic allocation and outcome patterns during the study periods [49,122].

Technical variability across institutions also represents an important challenge. In the systematic review and meta-analysis, heterogeneity in pre-analytical workflows, including centrifugation speeds, preservation solutions, storage conditions, and nucleic acid extraction protocols, hindered direct comparisons and limited the ability to define optimal processing strategies for the supernatant phase. The prospective validation under routine laboratory conditions mitigated some of these uncertainties, but larger,

multicentre prospective studies are needed to confirm performance metrics and refine standard operating procedures. Moreover, while preliminary evidence suggests that the supernatant may capture a broader spectrum of tumour heterogeneity, these observations require validation in larger cohorts with clinically annotated outcomes. Furthermore, cost-effectiveness, workflow adaptation, and resource allocation were not directly assessed in these studies and should be prioritised in future research, particularly in lower-volume or resource-limited centres.

Looking forward, different lines of investigation are warranted. Prospective multicentre studies should validate the integrated diagnostic workflow proposed in this thesis, evaluating its impact on time-to-treatment, treatment allocation, and patient outcomes in real-world clinical settings. Standardisation of supernatant handling, from collection and storage to nucleic acid extraction and sequencing, is essential to ensure reproducibility and scalability. Additionally, studies assessing the economic and operational implications of parallel testing strategies as explored in the prospective study will be key to guiding implementation. Finally, although not directly addressed in this work, the ongoing evolution of advanced bronchoscopic techniques, such as ultrathin bronchoscopy, navigational bronchoscopy, cone beam CT, and robotic assisted bronchoscopy, offers an opportunity to further enhance the diagnostic workflows of NSCLC. These technologies, by improving access to peripheral lung lesions, could complement the integrated model proposed in this thesis, expanding the reach of minimally invasive sampling, and enabling parallel molecular testing strategies for a broader spectrum of NSCLC presentations. Future studies combining these modalities with optimised molecular workflows could help refine and accelerate precision oncology pathways.

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Conclusions

This thesis offers a comprehensive evaluation of the role of EBUS-TBNA in the diagnostic and molecular workflow of advanced NSCLC. Integrating findings from five complementary studies, this thesis demonstrates that EBUS-TBNA consistently provides high diagnostic adequacy for morphological, immunohistochemical, and molecular characterisation, while delivering clear logistical advantages that shorten time-to-diagnosis and enable earlier access to targeted therapies.

The work highlights the limitations of sequential testing strategies, showing how they can lead to material exhaustion and delays, and supports the adoption of massively parallel molecular profiling to maximise actionable mutation detection and optimise treatment allocation. Importantly, it establishes the supernatant phase, typically discarded during routine processing, as a valuable complementary source of DNA and RNA with high concordance to tissue, increased molecular yield, and potential to reduce turnaround time when integrated into parallel workflows (Figure 15).

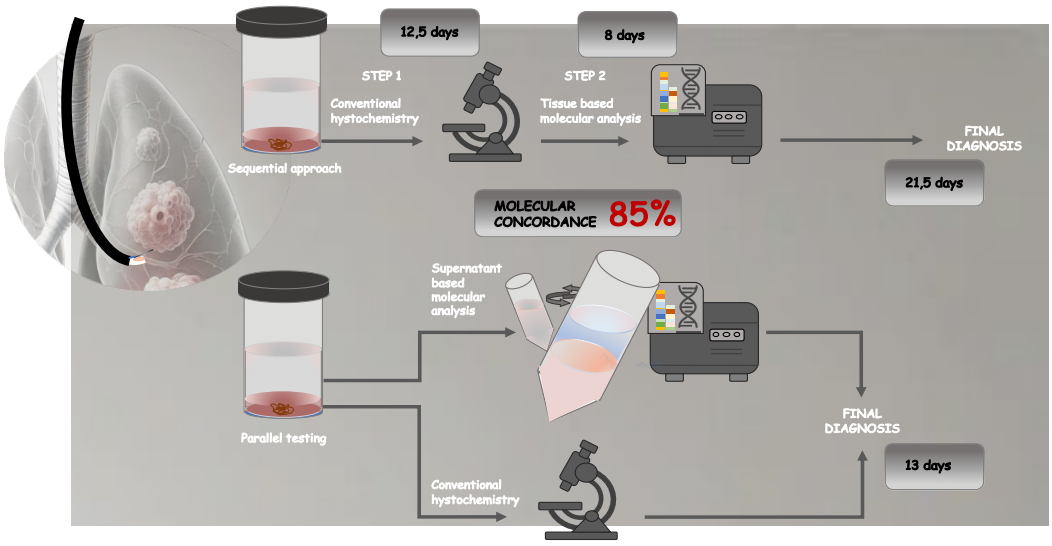

Figure 15 - Proposed integrated diagnostic workflow for advanced NSCLC using EBUS-TBNA supernatant.
This illustration compares the conventional sequential pathway with a parallel workflow in which the EBUS-TBNA cellular pellet is processed for morphology and immunohistochemistry while the supernatant is used for molecular profiling. Parallel processing preserved high molecular concordance and reduced turnaround time.

Framed within an era of escalating biomarker dependence, this thesis presents a practical pathway to alleviate the mounting *tissue pressure* on small biopsies, particularly those obtained by EBUS-TBNA. It advances an integrated diagnostic framework in which the cellular pellet of EBUS-TBNA samples is reserved for histology and immunohistochemistry, while molecular profiling is initiated in parallel from the supernatant phase. This approach maximises tissue efficiency, preserves diagnostic accuracy, and can accelerate the delivery of actionable results, as illustrated in Figure 15.

By converting the routinely discarded supernatant into a reliable, concordant source of tumour DNA and RNA, this provocative strategy holds the potential to broaden the utility of EBUS-TBNA and positions it to meet the rising demands of biomarker-driven thoracic oncology in the coming decades.

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Appendix 1

The five studies forming this thesis are presented sequentially. The candidate is the first author of all. Full texts are provided as annexes, in their published form or, if publication is still pending, in the latest submitted version with the corresponding status indicated at the time of writing.

Study I - ***Real world evidence on diagnostic pathways and biopsy optimisation for PD-L1 and molecular profiling in advanced Non-Small Cell Lung Cancer: A four-year cohort analysis***, Luis Vaz Rodrigues, Joana Oliveira, Joana Duarte, Luis Taborda-Barata, Rosa Cordovilla, Vitor Sousa.

Original article published in the *Jornal Brasileiro de Pneumologia*, (November 2025; https://dx.doi.org/10.36416/1806-3756/e20250188).

J Bras Pneumol. 2025;51(6):e20250188
https://dx.doi.org/10.36416/1806-3756/e20250188
ORIGINAL ARTICLE

Real-world evidence on diagnostic pathways and biopsy optimization for PD-L1 and molecular profiling in advanced non-small cell lung cancer: A four-year cohort analysis

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Submitted: 12 August 2025.
Accepted: 29 August 2025.

Study carried out at the Department of Pulmonology, Francisco Gentil Portuguese Institute of Oncology of Coimbra, Coimbra, Portugal.

ABSTRACT

Objective: Efficient diagnostic pathways in advanced non-small cell lung cancer (NSCLC) are crucial for timely treatment initiation and improved outcomes. This study evaluated the impact of diagnostic delays and the role of minimally invasive techniques in biomarker assessment and survival in a real-world clinical cohort. **Methods:** A retrospective cohort study was conducted involving 205 patients with advanced NSCLC diagnosed between January 2020 and December 2023. Diagnostic procedures included EBUS/EUS-B, transthoracic biopsy, and surgical biopsy. The time-to-diagnostic procedure, time-to-therapy, and survival were analyzed using multivariate models. **Results:** The time interval to the first diagnostic procedure independently predicted mortality (HR=1.66; p=0.016). EBUS and EUS-B achieved significantly shorter diagnostic times (median 8 and 5 days, respectively) compared to transthoracic (20.5 days) and surgical (24.5 days) biopsies. These endoscopic techniques were also associated with shorter time intervals to systemic therapy initiation (p=0.011). Minimally invasive approaches provided sufficient tissue for complete morphological, immunohistochemical, and molecular profiling in most cases, with no significant differences in adequacy among procedures. Patients with actionable mutations had a 44% lower mortality risk (HR=0.56; p=0.013), while high PD-L1 expression was associated with a 56% reduction in mortality risk (HR=0.44; p=0.003). **Conclusions:** Minimally invasive techniques, particularly EBUS and EUS-B, shortened diagnostic delays, ensured adequate biomarker sampling, and enabled earlier initiation of systemic therapy. Since the time-to-diagnosis was independently associated with survival, these approaches may have indirectly contributed to improved outcomes. Our findings highlight the importance of streamlining diagnostic pathways and expanding access to endoscopic methods to optimize care in advanced NSCLC.

Keywords: Non-small cell lung cancer; Diagnostic pathways; EBUS; EUS-B; Molecular profiling; Survival analysis.

INTRODUCTION

Lung cancer (LC) remains the leading cause of cancer-related mortality worldwide,[1] largely due to the high prevalence of late-stage diagnoses and consequent low survival rates.[2] NSCLC, which accounts for more than 85% of LC cases,[3] has become a central focus of clinical research and therapeutic innovation.[4,5] The development of targeted therapies for NSCLC has underscored the importance of precision-based diagnostic strategies, particularly in advanced stages.[5,6] In this context, molecular and immune profiling are essential to identify actionable biomarkers and guide treatment decisions,[7–9] yet their implementation continues to pose significant challenges in real-world clinical settings.

Comprehensive immunohistochemical and molecular profiling in advanced NSCLC often requires a myriad of invasive diagnostic approaches.[8,10,11] The choice of procedure is determined by a dynamic interplay between patient-specific factors—such as lesion location and overall health status—and healthcare resources, including equipment availability, staff expertise, and referral access.[12–14] These factors vary substantially across healthcare settings and are expected to evolve

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Financial support: No funding was received for this study.
Conflicts of interest: None to declare.

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ISSN 1806-3756

with ongoing technological innovation[14] and changing patterns of disease presentation.[8,15]

Although a growing body of research has explored the role of diagnostic pathways and biopsy techniques in the histological and molecular profiling of NSCLC,[9,10–12] evidence from large, long-term cohorts remains limited. This four-year cohort study aimed to address this gap by evaluating multiple diagnostic pathways for immunohistochemistry, programmed death ligand 1 (PD-L1), and actionable molecular targets. By analyzing outcomes related to the utility and timelines of various biopsy modalities, we sought to provide critical insights into how diagnostic strategies influence therapeutic decision-making and patient survival, thereby supporting the evidence-based selection of efficient diagnostic pathways in advanced NSCLC.

METHODS

This retrospective cohort study included patients diagnosed with TNM stage IV NSCLC[16] between January 2020 and December 2023. Eligibility criteria required histopathological confirmation and attempted assessment of immunohistochemical and molecular profiling, as per clinical indication.[8] The study was approved by the Ethics Committee of the Francisco Gentil Portuguese Institute of Oncology of Coimbra (approval No. 23-2022, November 15, 2022), and informed consent was obtained from all participants or their legal representatives.

Baseline demographic and clinical characteristics included age at diagnosis, sex, smoking status, and performance status according to the Eastern Cooperative Oncology Group (ECOG) scale. The initial diagnostic procedure was defined based on the modality that enabled tissue acquisition for histopathological and biomarker evaluation. The procedures included videobronchoscopy, endobronchial ultrasound (EBUS), endoscopic transesophageal ultrasound using the echobronchoscope (EUS-B), computed tomography (CT) or ultrasound-guided transthoracic biopsies (TTB), surgical biopsies, and pleural procedures. The origin of the sample (primary lung tumor, lymph nodes, or metastatic lesion) was also documented.

For EBUS and EUS-B, samples were obtained using dedicated 22G needles, with at least three passes per lymph node station, in accordance with institutional protocol. Cytology smears and cell blocks were prepared, with the latter fixed in 10% neutral buffered formalin for 24–48 hours and embedded in paraffin (FFPE), serving as the primary material for histological, immunohistochemical, and molecular analyses. All other biopsy types were also processed as FFPE following the same protocol. Rapid On-Site Evaluation (ROSE) was not available for any sample type during the study period. All diagnoses were reviewed by a board-certified pathologist.

In addition to histological subtype classification, PD-L1 expression was evaluated by immunohistochemistry using the PD-L1 22C3 pharmDx assay on the Dako Autostainer Link 48 platform (Agilent Technologies, USA). Analyses were performed on FFPE tissue samples, and PD-L1 expression was reported as the percentage of tumor cells exhibiting positive membrane staining, in accordance with the reporting standards in place at the time of data collection.

Molecular profiling was performed using next-generation sequencing (NGS) on FFPE tumor samples with ≥10% tumor content. Nucleic acids were extracted with the MagMAX™ FFPE DNA/RNA Ultra Kit (Thermo Fisher Scientific, USA) and quantified using a Qubit® 3.0 fluorometer. Sequencing was carried out on the Genexus platform (Thermo Fisher Scientific, USA) with the Oncomine™ Precision Assay GX, which enables the detection of mutations, copy number variations, and gene fusions across 50 cancer-related genes.

Key time intervals were systematically measured in days, and included: (1) the time from the initial clinical evaluation to the diagnostic procedure, serving as an indicator of procedural accessibility; (2) the time from the procedure to availability of histopathological and PD-L1 results; and (3) the time from histological diagnosis to the completion of the comprehensive molecular profiling, reflecting laboratory processing intervals.

The time to first-line therapy, treatment modalities, and survival outcomes—defined as the time interval to the last follow-up or death—were also analyzed in relation to diagnostic pathways and molecular findings.

Statistical analyses were performed using SPSS version 23 (IBM Corp., USA), with a significance threshold set at $p<0.05$. The Shapiro-Wilk test assessed the normality of continuous variables, and non-parametric methods were applied given the non-normal distribution. Descriptive statistics are presented as median and range (for age) and as median and interquartile range (IQR) for time intervals. The Kruskal-Wallis test compared time intervals, and the Fisher-Freeman-Halton exact test evaluated diagnostic yield for PD-L1 and molecular profiling. Overall survival was analyzed using Kaplan-Meier curves and the log-rank test. A multivariate Cox regression model identified independent predictors of survival and estimated hazard ratios (HR).

RESULTS

The study cohort consisted of 205 patients, all diagnosed with stage IV NSCLC, of whom 74 (36.1%) were classified as stage IVA and 131 (63.9%) as stage IVB. Most patients were male (122, 59.5%), with females accounting for 83 cases (40.5%). The median age at diagnosis was 68 years (range: 38–89 years). All patients had an ECOG performance status ≤2. Detailed clinical and demographic characteristics of the patients are provided in Table 1.

The most frequently adopted diagnostic procedures were EBUS, performed in 56 patients (27.3%), and EUS-B, in 50 patients (24.4%), totaling 106 cases (51.7%). Among the EBUS samples, 34 (60.7%) were

Table 1. Demographic and clinical characteristics of the included patients.

Variable	Count (n = 205 patients)
Sex, n (%)	
Male	122 (59.5)
Female	83 (40.5)
Age, median (min; max)	68 (38; 89)
Smoking history, n (%)	
Never smoker	67 (32.7)
Former smoker	67 (32.7)
Current smoker	71 (34.6)
Histology, n (%)	
Adenocarcinoma	178 (86.8)
Adenosquamous carcinoma[#]	11 (5.4)
Squamous cell carcinoma	8 (3.9)
Combined adenocarcinoma and neuroendocrine carcinoma	8 (3.9)
Stage, n (%)	
IVA	74 (36.1)
IVB	131 (63.9)
ECOG performance status, n (%)	
0	91 (44.4)
1	78 (38)
2	36 (17.6)
PD-L1, n (%)	
Positive ≥50%	70 (34.15)
Positive <50%	65 (31.7)
Negative	70 (34.15)
Systemic therapies*, n (%)	
Targeted therapy alone	44 (21.5)
Chemotherapy alone	43 (21)
Chemotherapy combined with immunotherapy	24 (11.7)
Immunotherapy alone	18 (8.8)
Chemotherapy followed by targeted therapy	7 (3.4)
Targeted therapy followed by immunotherapy	3 (1.5)

*A detailed description of the systemic therapies is provided in the Supplementary Material. In addition to the listed therapeutic strategies, 17 patients initiated some form of palliative radiotherapy but did not proceed to further systemic treatment due to clinical deterioration, and 49 patients received upfront best supportive care due to clinical decline throughout the course of the diagnostic pathway. #Among the 11 adenosquamous carcinoma cases, 7 were initially diagnosed using minimally invasive procedures (EBUS-TBNA = 4, EUS-B = 2, TTB = 1), with 6 subsequently confirmed via broader surgical or metastatic site biopsies. The remaining 4 cases were diagnosed from surgical specimens. All diagnoses were reviewed by an experienced thoracic pathologist.

obtained from mediastinal or hilar lymph nodes and 22 (39.3%) from primary tumors. For EUS-B, 38 samples (76.0%) were from mediastinal lymph nodes, 8 (16.0%) from primary tumors, and 4 (8.0%) from left adrenal metastases. Less frequently, TTB was conducted in 40 patients (19.5%), including 6 ultrasound-guided and 34 CT-guided procedures. Videobronchoscopy was carried out in 34 patients (16.6%), with 5 procedures assisted by radial EBUS. Surgical biopsies targeted extrathoracic lymph nodes (n=6), brain metastases (n=2), lung tumors (n=2), subcutaneous nodules (n=2), bone (n=2), pleura (n=1), and muscle (n=1), totaling 16 cases (7.8%). Pleural procedures comprised 5 thoracoscopies and 4 thoracenteses (4.4%). Figure 1 illustrates the distribution of diagnostic procedures.

Adenocarcinoma was the most common subtype of NSCLC, identified in 178 patients (86.8%), followed by adenosquamous carcinoma (5.4%), squamous cell carcinoma (3.9%), and combined adenocarcinoma with neuroendocrine carcinoma (3.9%). All procedures yielded sufficient material for histological and immunohistochemical characterization across the entire cohort. PD-L1 expression was assessed in all but two cases with insufficient material (1 EUS-B and 1 TTB). High expression (≥50%) was observed in 70 patients (34.2%), low expression (<50%) in 65 (31.6%), and negative expression in 70 (34.2%). The Fisher-Freeman-Halton exact test revealed no significant differences among procedures regarding PD-L1 testing success (p=0.757). Table 1 summarizes the histopathological and PD-L1 findings.

Molecular profiling was attempted in all patients, but was inconclusive in five cases (2.4%) due to insufficient material (2 EBUS, 1 EUS-B, 1TTB, and 1 videobronchoscopy). No statistically significant differences were found among the diagnostic procedures regarding molecular characterization (Fisher-Freeman-Halton exact test, p=0.968).

EGFR mutations were detected in 54 patients (26.3%), predominantly exon 19 deletions (34 cases). Other variants included exon 21 mutations (9 cases) and exon 20 insertions (7 cases). *KRAS* mutations were identified in 29 patients (14.1%), comprising G12C (10 cases), G12A (8 cases), G12V (5 cases),

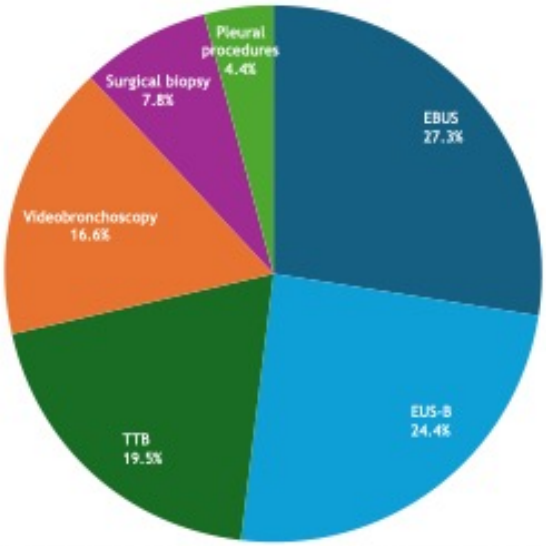

Figure 1. Diagnostic procedures performed. The pie chart illustrates the number of diagnostic procedures conducted, categorized by type. Echoendoscopic procedures (EBUS and EUS-B) were the most frequently performed, followed by TTB and videobronchoscopy. Surgical biopsies and pleural procedures were less frequent.

and rarer variants (G16H, G12P, G12S, and L19P), each observed in a single patient.

Additional driver mutations included *ALK* rearrangements (14 cases, 6.8%), *ERBB2* (8 cases, 3.9%), *TP53* (6 cases, 2.9%), as well as less frequent alterations in *FGFR* (5 cases, 2.4%), *CTNNB1* (5 cases, 2.4%), *ROS1* (2 cases, 1%), *RET* (2 cases, 1%), *BRAF* (2 cases, 1%), *MET* (2 cases, 1%), *CDK4* (2 cases, 1%) and *PIK3CA* (2 cases, 1%). Detailed results are presented in Figure 2.

The overall median time from the initial evaluation to the first diagnostic procedure was 9 days (IQR: 4–18), although it varied significantly among modalities (Kruskal-Wallis test, $p=6.14 \times 10^{-12}$). Surgical biopsies (median: 24.5 days; IQR: 18–37.8) and TTB (median: 20.5 days; IQR: 12–38.3) were associated with the longest delays. In contrast, shorter intervals were observed for EUS-B (median: 5 days; IQR: 1.5–8), videobronchoscopy (median: 6.5 days; IQR: 4.3–9.5), pleural procedures (median: 7 days; IQR: 0–12.5), and EBUS (median: 8 days; IQR: 6–17.5 days).

The median time to obtain histopathological and immunohistochemical results was 11 days (IQR: 8–15.8), with no significant differences among diagnostic approaches (Kruskal-Wallis test, p=0.840).

The overall median time to obtain molecular results was 24 days (IQR: 16–35), with statistically significant differences across modalities (Kruskal-Wallis test, p=0.038). The longest intervals were seen with TTB (median: 31.5 days; IQR: 23–58) and surgical biopsies (median: 28 days; IQR: 14.5–50.5), followed by videobronchoscopy (median: 26.5 days; IQR: 14.3–43). Shorter durations were associated with EUS-B (median: 20 days; IQR: 14–29) and EBUS (median: 24 days; IQR: 15.3–35).

Figure 3 illustrates the differences in median times per diagnostic modality. Detailed time-interval data are provided in Supplementary Tables 1–3.

Most patients received systemic therapies tailored to their molecular and immunohistochemical profiles. Targeted therapy was administered in 21.5% of cases (n=44), while chemotherapy alone was given in 21% (n=43). Combination regimens included targeted therapy followed by immunotherapy (1.5%, n=3), chemotherapy followed by targeted therapy (3.4%, n=7), and chemotherapy combined with immunotherapy (11.7%, n=24). Immunotherapy alone was given to 8.8% of patients (n=18). A detailed overview is provided in Supplementary Table 4.

The time to the initiation of systemic therapy was analyzed by diagnostic procedure and treatment type. Significant differences were observed across the diagnostic modalities (Kruskal-Wallis test, p=0.011), with EUS-B (median: 59 days; IQR: 35.5–75.5), videobronchoscopy (median: 60 days; IQR: 48.5–72.5), and EBUS (median: 61 days; IQR: 49–87) associated with shorter intervals compared to surgical biopsies (median: 81 days; IQR: 50–121) and TTB (median: 82 days; IQR: 55.8–104.8). Targeted therapy showed the longest time to initiation (median: 72 days; IQR: 55–116), whereas chemotherapy combined with immunotherapy had the shortest (median: 61 days; IQR: 48–109). These differences, however, were not statistically significant (Kruskal-Wallis test, p=0.313). Supplementary Table 5 presents a detailed breakdown of these results.

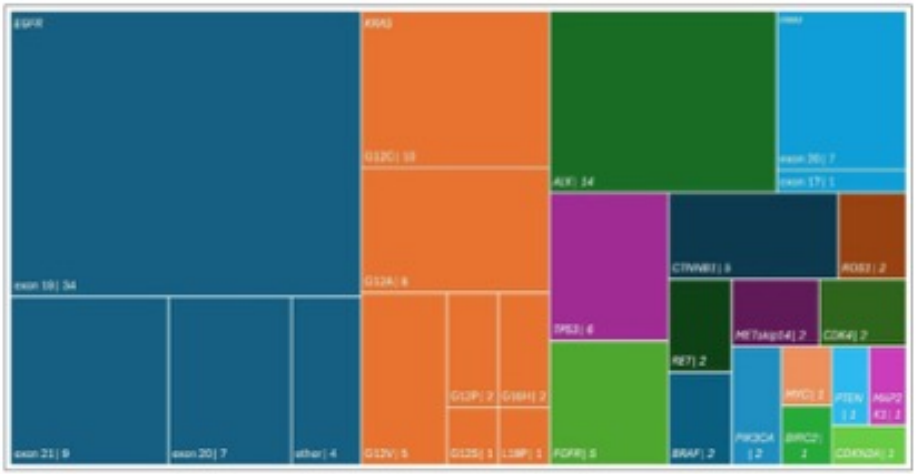

Figure 2. Distribution of mutations in the study population (n=205). Each square represents the absolute number of cases with a specific mutation, scaled proportionally. For *EGFR*, "other" stands for mutations in exons 15 and 17 (two cases) and *EGFR* amplification (two additional cases). Not shown in the figure are two patients that presented with three simultaneous mutations: one with *EGFR* exon 21, *EGFR* exon 5, and *TP53*; and another with *EGFR* exon 19, *CDKN2A*, and *PTEN*. Seven patients presented with two simultaneous mutations: *EGFR* exon 19 and *CTNNB1*; *EGFR* exon 17 and *TP53*; *EGFR* exon 20 and *ERBB2*; *EGFR* exon 20 and *MAP2K1*; *ROS1* and *KRAS* G12A; *KRAS* G12C and *TP53*; and *KRAS* G12C and *FGFR*. Seventy patients had negative molecular findings and were classified as "wild-type" for all tested genes. In five cases, samples were deemed insufficient for molecular analysis (2 EBUS, 1 EUS-B, 1 TTB, 1 videobronchoscopy).

A subset of patients received palliative or supportive interventions. Specifically, 17 (8.3%) initiated palliative radiotherapy but did not proceed to systemic therapy due to rapid clinical deterioration. In addition, 49 patients (23.9%) were allocated to upfront best supportive care, reflecting substantial clinical decline during the diagnostic and staging processes.

Multivariate Cox regression analysis identified several clinical and molecular factors as independent predictors of survival, with the ECOG performance status showing the strongest impact. Patients with ECOG 2 (HR=4.24; 95%CI: 2.38–7.52; $p=1x10^{-7}$) and ECOG 1 (HR=2.29; 95%CI: 1.36–3.87; p=0.002) exhibited worse survival compared to those with ECOG 0. Sub-stage IVB (HR=1.78; 95%CI: 1.12–2.82; p=0.015) and male sex (HR=1.92; 95%CI: 1.36–3.87; p=0.004) were also significantly associated with higher mortality.

Regarding molecular markers, actionable mutations conferred a survival advantage (HR=0.56; 95%CI: 0.35–0.88; p=0.013). Similarly, patients with high PD-L1 expression (≥50%) exhibited better outcomes compared to those with negative PD-L1 expression (HR=0.44; 95%CI: 0.26–0.75; p=0.003).

Diagnostic efficiency, measured by the time to the first diagnostic procedure, was also an independent predictor of mortality, with each additional day of delay modestly increasing the risk (HR=1.15; 95%CI: 1.09–1.28; p=0.045). For practical interpretation, delays were dichotomized into <10 days and ≥10 days, based on the cohort's median time to the first diagnostic procedure (9 days). Delays of 10 days or more were associated with a higher mortality risk (HR=1.66; 95%CI: 1.10–2.50; p=0.016).

Other variables—including age, smoking status, diagnostic modality, time to histological and molecular profiling results, and type of first-line therapy—were not statistically significant. Full results are detailed in Supplementary Table 6. Kaplan-Meier curves were constructed to further evaluate survival according to ECOG performance status, TNM sub-staging, sex, biomarkers, and the time to the diagnostic procedure (Figure 4).

DISCUSSION

Optimizing diagnostic pathways in advanced NSCLC is paramount, as these decisions directly influence clinical timelines, therapeutic allocation, and ultimately, patient survival. This real-world study provides insights into both the challenges and opportunities associated with current diagnostic strategies.

Main findings

The time to the first diagnostic procedure emerged as an independent predictor of mortality (HR = 1.66; p=0.016), highlighting the clinical importance of minimizing delays at this stage. This finding is particularly relevant in advanced NSCLC, where diagnostic delays can adversely affect survival, especially because therapeutic decisions depend on timely access to molecular biomarkers. Prolonged intervals between clinical evaluation, diagnostic procedures, and molecular profiling may postpone

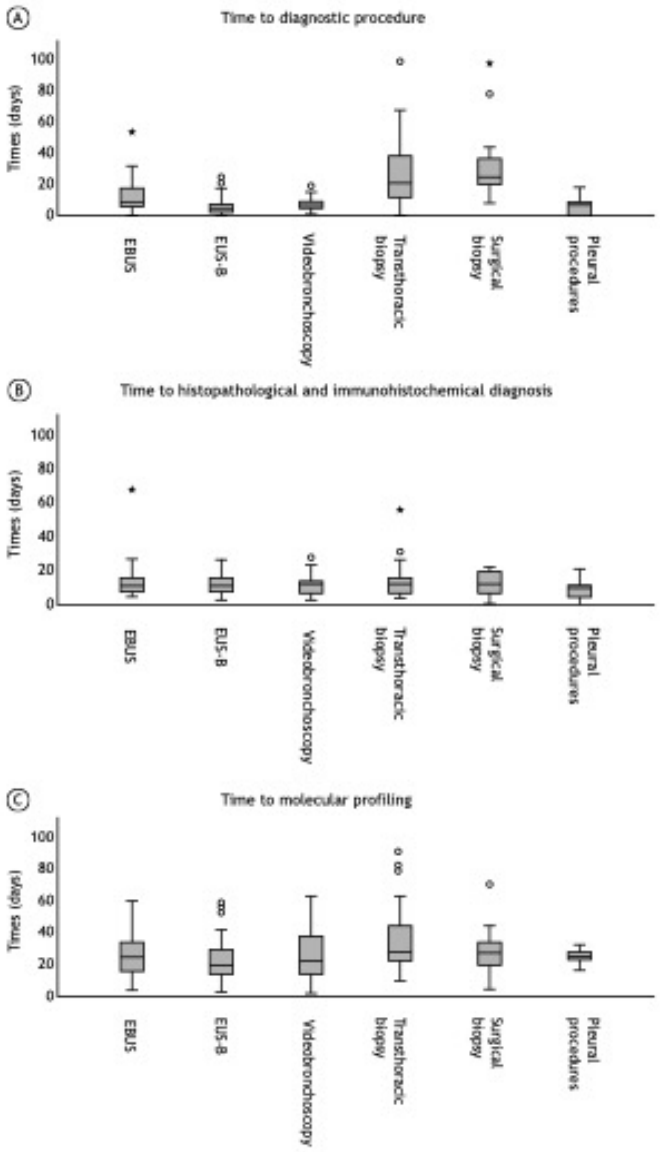

Figure 3. Timeline analysis stratified by diagnostic modality (days). Boxplots illustrating (A) the time to the diagnostic procedure, (B) the time to the histopathological and immunohistochemical diagnosis, and (C) the time to molecular profiling, stratified by modality. Median times indicate significantly faster access for endoscopic techniques (EBUS, EUS-B, and videobronchoscopy) and pleural procedures compared to transthoracic and surgical biopsies. For the time to the diagnostic procedure (A), Kruskal-Wallis testing revealed statistically significant differences across modalities ($p=6.14 \times 10^{-12}$). No significant differences were observed for the histopathological diagnosis (B) ($p=0.840$), while molecular profiling (C) showed significant variability ($p=0.038$), highlighting delays associated with transthoracic and surgical biopsies. Outliers are represented by circles (moderate outliers) and asterisks (extreme outliers), defined as values outside 1.5 and 3 times the interquartile range, respectively.

treatment initiation and reduce the likelihood of favorable outcomes.[17,18] Notably, endoscopic procedures such as EBUS and EUS-B accounted for most diagnostic approaches (51.7%, n=106) and achieved shorter diagnostic times compared to transthoracic or surgical biopsies. This likely reflects

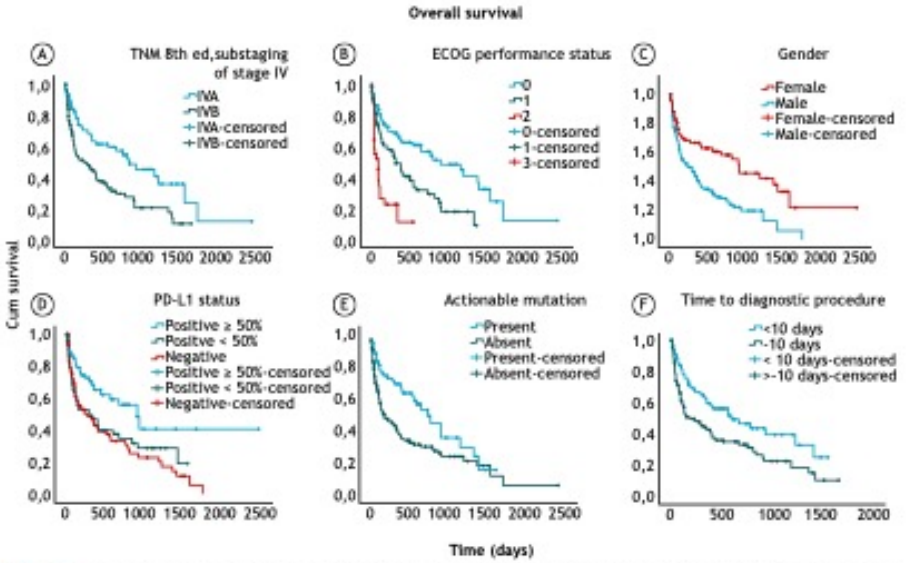

Figure 4. Kaplan-Meier survival curves illustrating overall survival (days), analyzed using the Log-Rank (Mantel-Cox) test. (A) TNM Sub-staging (8th edition): median survival was significantly longer for stage IVA compared to IVB (844 vs. 265 days, p=4.47x10^{-4}); (B) ECOG Performance Status: median survival decreased with worsening ECOG scores: 917 days (ECOG 0), 337 days (ECOG 1), and 97 days (ECOG 2) (p=8.32x10^{-6}); (C) Sex: females had longer median survival than males (917 vs. 295 days, p=2.52x10^{-4}); (D) PD-L1 Expression: patients with high PD-L1 expression (≥50%) had the longest median survival (906 days) compared to low (<50%) or negative PD-L1 expression (299 and 265 days, respectively; p=0.008); (E) Actionable Mutations: patients with actionable mutations had significantly longer median survival than those without (789 vs. 188 days, p=0.003); (F) Time to Diagnostic Procedure: access to the diagnostic procedure in <10 days was associated with longer median survival compared to ≥10 days (597 vs. 188 days, p=0.003).

integrated pulmonology workflows that minimize logistic scheduling delays and reinforces the central role of endoscopic evaluation in efficient NSCLC diagnosis.[18]

Diagnostic workflows and the relevance of comprehensive biomarker testing

Biomarker testing plays a crucial role in guiding treatment decisions in advanced NSCLC.[7–9] In this cohort, actionable mutations (HR=0.56; p=0.013) and high PD-L1 expression (HR=0.44; p=0.003) were associated with improved survival, supporting the clinical benefit of targeted therapies[18] and immune checkpoint inhibitors.[23]

Sample adequacy was high across all diagnostic modalities. Histological and immunohistochemical characterization was achieved in 100% of cases. PD-L1 assessment was possible in 98.9% of samples, while molecular profiling yielded conclusive results in 97.6%. Importantly, no statistically significant differences were observed between procedures for PD-L1 testing (p=0.757) or molecular profiling success (p=0.968), consistent with previous reports.[21,22] However, the time to obtain molecular results was longer for TTB (median: 31.5 days) and surgical biopsies (median: 28 days) than for EBUS (median: 24 days) and EUS-B (median: 20 days), likely reflecting additional logistical delays and the absence of reflex testing protocols that are more efficiently integrated within in-service pulmonology workflows.

Diagnostic efficiency and its impact on treatment allocation and survival

Therapeutic approaches and their implications for survival were also explored in this cohort. Targeted therapy was the most frequently used modality (21.5%), reflecting the increasing reliance on precision medicine in advanced NSCLC. Conversely, 23.9% of patients received only best supportive care due to clinical deterioration, highlighting the challenges of managing a population often characterized by disease-related frailty.[24] The high proportion of patients unable to access systemic therapies due to clinical decline underscores the need for accelerated diagnostic pathways, as delays may preclude timely access to potentially life-prolonging treatments.[25,26]

These findings emphasize the clinical significance of diagnostic efficiency: patients with ECOG 2 had a fourfold increased risk of death compared to those with ECOG 0 (HR=4.24). Notably, endoscopic procedures were associated with shorter times to treatment initiation (p=0.011), further reinforcing their role in expediting therapy and potentially contributing to the survival benefit observed with earlier diagnostic interventions.

Furthermore, these survival disparities extend beyond ECOG performance status to include sex and TNM sub-stage, as demonstrated in our cohort and consistent with established prognostic factors in NSCLC.[16,25] The interplay of these factors highlights the critical importance of early and efficient diagnostic strategies, particularly in vulnerable patient populations.

Study limitations and future directions

Despite its strengths, including a large, homogenous cohort and a real-world setting, the present study has limitations. Its single-center design may restrict the generalizability of findings, particularly in healthcare systems with different referral patterns, diagnostic resources, or access to specialized techniques. Nevertheless, the greater accessibility of interventional pulmonology procedures, such as EBUS and EUS-B, is likely relevant across diverse settings, as most NSCLC patients are initially managed in pulmonology clinics.[27] In contrast, procedures performed by other specialties, such as CT-guided TTB, require interdepartmental coordination, which may introduce delays, as observed in this study.[28]

The study's retrospective design may also have introduced selection bias, potentially explaining the higher-than-expected proportion of adenosquamous carcinoma relative to squamous cell carcinoma, as patients referred for more extensive molecular characterization were likely overrepresented.

Although this study demonstrated that the time to the diagnostic procedure was an independent predictor of mortality, we could not confirm a direct survival advantage for patients undergoing endoscopic procedures as the frontline diagnostic modality, likely due to limited statistical power in stratified analyses. Nonetheless, their contribution to diagnostic timeliness suggests a potential indirect benefit, as part of the multidimensional factors influencing survival.[16,25]

Importantly, procedures such as EBUS and EUS-B, while valuable for mediastinal and central lesions, are less suitable for peripheral lesions, which typically require image-guided approaches such as TTB.[29] Emerging technologies—including ultrathin bronchoscopy, navigation bronchoscopy, cone beam CT, and robotic bronchoscopy—should be explored to expand the diagnostic reach and streamline workflows.[12,30–33] Another limitation is the variability in technological resources and specialized training across institutions. Although EBUS and EUS-B are increasingly accessible, disparities in equipment and expertise may constrain their broader adoption. Standardizing training pathways and ensuring equitable access to advanced diagnostic procedures are essential to overcoming these barriers to care.[34–36] Finally, reliance on minimally invasive procedures may be limited by small tissue samples.[21] In cases requiring multiple analyses, the material may not always sufficient, as observed in this study. One promising strategy to maximize available samples is to repurpose the supernatant phase, which contains free nucleic acids that can be leveraged for molecular profiling. Implementing this approach could enhance diagnostic capacity and reduce delays.[37]

Ultimately, this study highlights that, among the various factors influencing survival in advanced NSCLC, the time to the diagnostic procedure emerges as an independent and modifiable predictor of mortality. Our findings underscore the pivotal role of minimally invasive endoscopic techniques, particularly EBUS and EUS-B, which offer greater accessibility and efficiency in reducing diagnostic delays. The broad integration of these procedures into diagnostic pathways—supported by appropriate training and infrastructure—represents a tangible opportunity to enhance care and improve outcomes for patients with advanced NSCLC.

AUTHOR CONTRIBUTIONS

All authors meet the ICMJE authorship criteria. LVR, RC, LTB, and VS made substantial contributions to the study's conception and design. LVR, JO, and JD were responsible for data collection and acquisition from clinical files. LVR, JO, and LTB contributed significantly to the statistical analysis and interpretation of the data. LVR, RC, LTB, and VS contributed to drafting the manuscript. All authors critically revised the manuscript for intellectual content, approved the final version, and agree to be accountable for all aspects of the work, ensuring its accuracy and integrity.

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Study II - *Feasibility of EBUS-TBNA for the molecular characterisation of non-small cell lung cancer*, Luis Vaz Rodrigues, Marta Viegas, Rosa Cordovilla, Luis Taborda-Barata, Vitor Sousa

Published as a Scientific Letter to the Editor in *Jornal Brasileiro de Pneumologia* (May 2024; doi: https://dx.doi.org/10.36416/1806-3756/e20230193).

J Bras Pneumol. 2024;50(2):e20230193
https://dx.doi.org/10.36416/1806-3756/e20230193

Feasibility of EBUS-TBNA for the molecular characterization of non-small cell lung cancer

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TO THE EDITOR:

The accurate diagnosis of lung cancer (LC) relies on histopathological classification (HC) and molecular characterization (MC) for targeted therapies.[1] Clinicians that deal with LC face the dilemma of how to apply minimally invasive interventions that yield large and well-preserved samples suitable for the demands of the histopathologist and the molecular geneticist.

A milestone in this pathway has been achieved with the introduction of EBUS-TBNA, which is currently the first-choice procedure for mediastinal staging of LC.[2,3] On the other hand, MC of non-small cell LC (NSCLC) is a growing field of research with diverse strategies and heterogenous results.[4-7]

In this study we aimed to evaluate the current clinical practice of a large oncology referral centre concerning the feasibility of EBUS-TBNA-derived samples for MC of NSCLC.

We conducted a retrospective analysis (between January of 2019 and December of 2021) of all patients who underwent EBUS-TBNA for diagnosis and/or staging of NSCLC whose samples proceeded to MC. EBUS-TBNA was performed under general anesthesia with a BF-UC180F endoscope (Olympus, Tokyo, Japan) and 21G needles (ViziShot 2; Olympus). Samples were stored in formaldehyde and were processed as cell blocks for HC. *EGFR* status was determined by real-time polymerase chain reaction. If samples were negative, determination of *ALK* gene rearrangements by fluorescence in-situ hybridization followed.

Procedure and patient-related factors affecting sample adequacy were assessed. Finally, a timeframe was estimated from the initial endoscopic procedure and the final MC.

Descriptive and inferential statistical analysis was performed using the IBM SPSS Statistics software package, version 27 (IBM Corporation, Armonk, NY, USA). A logistic regression model was attempted to ascertain the presence of factors influencing MC results.

A total of 718 patients were subjected to EBUS-TBNA. Of these, 59 (8.2%) proceeded to MC, but only 38 (5.3%) had their MC performed in EBUS-TBNA samples. In the remaining 19 patients, MC was performed in other samples (6 in surgical specimens; 5 in bronchoscopy forceps biopsies; 4 in transthoracic CT-guided biopsies; and 4 in peripheral blood samples).

The patients included (N = 38) were mainly male (n = 25; 65.7%) with a median age of 67 years (range: 40-86 years). Nearly half had a relevant smoking history (12 former smokers and 8 current smokers). Most NSCLC were adenocarcinomas (n = 33; 86.8%), 3 were squamous cell carcinomas (SCC), and 2 were mixed adenocarcinoma and SCC (Table 1). All presented with locally advanced (stage IIIA, in 4; IIIB, in 6; and IIIC, in 3) or metastatic disease (IVA in 12; and IVB in 13). Programmed death-ligand 1 (PD-L1) status was ascertained in all patients and proved to be positive in 44.7% (in 2 patients with SCC and in 15 patients with adenocarcinoma), indeterminate in 5.2% (mixed adenocarcinoma and SCC, in 1; and adenocarcinoma, in 1), and negative in the remainder 50%.

A median of 2 lymph node stations were approached per patient (range: 1-4), with a median number of 3 needle passes (range: 3-8) per lymph node.

Overall, 34 out of the initial 38 cases (89.5%) were satisfactory for *EGFR* mutation testing, whereas 26 out of 32 (81.3%) were suitable for *ALK* rearrangement testing. Clinically relevant *EGFR* mutations were found in 6 patients (15.7%). *ALK* rearrangements were found in 2 cases (Table 1).

Mutated patients were mainly non-smoker males (5 out of 8) and presented with metastatic disease (stage IVB, in 4; and IVA, in 3).

The median time between EBUS-TBNA sampling and final MC was 20 days (range: 7-590 days). An in-depth analysis of this measure showed 11 cases (>30 group) in which this timeframe surpassed 30 days (median: 186 days; range: 48-590 days), whereas that was below 30 days in the ≤30 group (median: 18 days; range: 7-30 days). The patients in the >30 group were mainly in stage III (7 out of 11) whereas those in the ≤30 group were mainly in stage IV (20 out of 27).

MC was pivotal in determining the therapeutic options. All mutated patients were referred for targeted therapy

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ISSN 1806-3756

Table 1. Histopathological classification and mutational profiling of the sample of patients with non-small cell lung cancer (N = 38).

Histopathological classification	Mutational profiling	n (%)
Adenocarcinoma	Non-mutated	26 (68)
	EGFR mutated	5 (13)
	ALK rearrangements	2 (5)
Squamous cell carcinoma	Non-mutated	1 (3)
	EGFR mutated	1 (3)
	ALK rearrangements	1 (3)
Mixed squamous cell carcinoma	Non-mutated	1 (3)
	EGFR mutated	1 (3)

(anti-EGFR, in 6; and anti-ALK, in 2). Immunotherapy was offered as frontline therapy in 15 non-mutated, PD-L1 positive patients. Platinum-based chemotherapy was the option for 15 additional patients. Best supportive care was offered to 2 patients that suffered severe performance status deterioration (ECOG 3 and 4) throughout the course of diagnosis and staging.

Due to the small sample size, a logistic regression model could not be built to ascertain factors associated with the feasibility of EBUS-TBNA for MC. Nevertheless, the 4 cases whose samples were insufficient belonged mainly to the puncture of a single (in 3 cases) or dual (in 1 case) lymph node stations with a median of 4 needle passes per lymph node station (range: 3-8).

This study unveils a clear underutilization of EBUS-TBNA (5.3%) for the MC of NSCLC. Various reasons may account for this. First, NSCLC staging was determinant, since only candidates for systemic therapy (stages III or IV) were referred for MC. Secondly, there was preferential utilization of other biological samples that were perhaps considered more cell enriched, such as surgical or CT-guided biopsies. An intriguing finding of the study was the option for peripheral blood sampling, in 4 cases. The wider accessibility of peripheral blood samples may offer an explanation, but it is still surprising that this material was preferred over EBUS-TBNA samples.

As previously reported,[6,7] EBUS-TBNA was feasible for MC in most cases (89.5% for *EGFR* and 81.3% for *ALK*). Also, in agreement with previous reports,[6,7] *EGFR* analysis outperformed *ALK*. The sequential method applied in this study may offer an explanation. Samples were sequentially used for HC, *EGFR* testing, and only afterwards released for *ALK* testing, which means that only largely cellular samples could suffice all processes.

Two patient groups were identified based on the timeframe of MC. The >30 group mainly included patients with less advanced disease stages who probably undergone multimodal therapeutic strategies in which systemic therapy was likely delayed. In contrast, the ≤30 group included patients with metastatic disease in which systemic therapies came first and hence the prompter need for an up-front MC.

Our study showed a relatively low prevalence of mutations (Table 1). We observed 18% of mutated adenocarcinomas (*EGFR* mutations, in 13%; and *ALK* rearrangements, in 5%), and there was only 1 case of *EGFR*-mutated SCC (3%), as was there 1 case of *EGFR*-mutated mixed SCC and adenocarcinoma (3%), which agrees with publications reflecting Western populations.[8,9]

When assessing factors that could influence the feasibility of molecular profiling EBUS-TBNA samples, we could not safely establish statistically significant relationships. Nevertheless, we observed a trend toward lower yields in patients with a smaller number of lymph nodes approached despite a seemingly higher number of needle passes in these cases. This perhaps reflects the clinical perception of a lower probability of achieving a complete diagnosis in cases when just one lymph node station was approached.

In conclusion, our study highlights the value EBUS-TBNA on obtaining sufficient samples for MC of NSCLC. Nevertheless, questions are raised about sequential approaches and the time required for molecular results, for which additional studies, namely addressing the added value of multiplex simultaneous analysis, are still warranted.[9,10]

Author contributions

LVR, RC, LTB, and VS conceptualized and designed the study. LVR recruited patients and performed the EBUS-TBNA procedures. VS was responsible for histopathological analysis. MV was responsible for molecular analysis. RC and LTB provided input regarding statistical analysis. LVR and LTB were responsible for data collection, major statistical analysis, and main manuscript structure. All authors revised and approved the final manuscript.

CONFLICTS OF INTEREST

None declared.

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Study III - ***Sequential versus massively parallel strategies for molecular characterisation of non-small cell lung cancer samples obtained by endobronchial ultrasound-guided transbronchial needle aspiration***, Luís Vaz Rodrigues, Marta Viegas, Ana Filipa Ladeirinha, Ana Alarcão, Luis Taborda-Barata, Rosa Cordovilla, Vitor Sousa

Published as an Original Paper in *Jornal Brasileiro de Pneumologia* (July 2025; doi: https://dx.doi.org/10.36416/1806-3756/e20250039).

J Bras Pneumol. 2025;51(4):e20250039
https://dx.doi.org/10.36416/1806-3756/e20250039
ORIGINAL ARTICLE

Sequential versus massively parallel strategies for molecular characterization of non-small cell lung cancer samples obtained by endobronchial ultrasound-guided transbronchial needle aspiration

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Submitted: 16 April 2025.
Accepted: 28 April 2025.

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ABSTRACT

Objectives: The advent of massively parallel next-generation sequencing (MP-NGS) offers potential advantages over sequential molecular profiling (SMP) in the management of non-small cell lung cancer (NSCLC). This study compares the two methodologies using samples obtained through endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA), focusing on actionable mutation detection, turnaround time (TAT), and clinical outcomes. **Methods:** A retrospective analysis was conducted on NSCLC patients who underwent EBUS-TBNA and molecular characterization between January 2020 and December 2023. SMP and MP-NGS were compared in terms of actionable mutation detection rates, TAT, and impact on overall survival (OS). **Results:** Among 106 patients, MP-NGS demonstrated a significantly higher detection rate of actionable mutations compared to SMP (40.9% vs. 22.2%, p=0.042). The median TAT was slightly shorter with SMP than with externally outsourced MP-NGS (17 days vs. 23 days, p=0.076). Patients diagnosed via MP-NGS were more frequently allocated to targeted therapies (44.26% vs. 22.2%, p=0.038), which may have positively influenced overall survival (672 days vs. 138 days, p=0.053). **Conclusion:** MP-NGS provided superior diagnostic and clinical advantages over SMP in NSCLC, supporting its adoption as a standard diagnostic approach to enhance personalized therapy and improve patient outcomes.

Keywords: Non-Small Cell Lung Cancer, Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration, Sequential Molecular Profiling, Massively Parallel Next-Generation Sequencing, Actionable Mutations, Personalized Therapy.

INTRODUCTION

Lung cancer (LC) currently ranks first in both incidence and mortality among all types of cancer worldwide,[1] and is closely associated with tobacco epidemics.[2] Non-small cell lung cancer (NSCLC), which accounts for over 85% of all LC cases,[3] remains a diagnostic challenge, as it often presents asymptomatically until advanced stages, when surgery is no longer a viable option.[4] At this point, understanding its subcellular characteristics becomes critical, as this can unveil therapeutic pathways with significantly improved efficacy and safety profiles.[5,6] Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) plays a key role in this context by enabling both the diagnosis and staging of NSCLC[7,8,9] in a minimally invasive manner. The main challenge, however, lies in obtaining adequate samples to meet the requirements of both pathologists and molecular geneticists—fulfilling the threefold goal outlined in clinical guidelines: diagnosis, staging, and molecular characterization in a single procedure.[10]

While EBUS-TBNA is a safe and effective tool for diagnosis and staging,[8,9] its reported yield for molecular profiling is variable, likely due to methodological heterogeneity.[11,12] In a previous study, we found that 89.5% of samples obtained via EBUS-TBNA were satisfactory for *EGFR* testing, but only 81.3% were suitable for *ALK* assessment.[13] In that investigation, the *EGFR* status was determined by real-time polymerase chain reaction (RT-PCR); if the results were negative, *ALK* gene rearrangements were subsequently assessed using fluorescence *in situ*

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ISSN 1806-3756

hybridization (FISH). Despite encouraging results, a key limitation of sequential testing strategies became apparent: depletion of EBUS-TBNA-collected material between tests, particularly affecting downstream markers. With the growing number of clinically relevant molecular markers, a decline in sample utility can be expected when using sequential methods.[14] Therefore, evaluating the potential of massively parallel (MP) molecular analysis—particularly through next-generation sequencing (NGS)—is increasingly relevant.[15] Emerging data support the feasibility of MP-NGS in EBUS-TBNA samples, with reported yields ranging from 86.1% to 98%, depending on the gene panel size.[16,17] However, some variability persists.

Building on these findings, the present study aimed to compare sequential molecular profiling (SMP) with massively parallel next-generation sequencing (MP-NGS) in NSCLC samples obtained via EBUS-TBNA, evaluating feasibility, turnaround time (TAT), treatment strategies, and overall survival (OS). The objective was to clarify the differences between these methods and determine which approach better enhances diagnostic accuracy, reduces TAT, and supports personalized treatment decisions.

METHODS

A cross-sectional cohort study was conducted including patients with stage IV NSCLC, as defined by the 8th edition of the TNM classification,[18] diagnosed between January 2020 and December 2023 at the Francisco Gentil Portuguese Institute of Oncology of Coimbra (IPOC-FG). The cohort was retrospectively established by identifying eligible patients who underwent simultaneous EBUS-TBNA and molecular characterization of NSCLC during this period. Patients were divided into two groups based on the molecular profiling strategy adopted. Between January 2020 and December 2021, SMP was performed in-house, whereas from January 2022 onward, molecular characterization was conducted using outsourced MP-NGS. The two strategies were compared in terms of sample adequacy, mutation detection rates, actionable mutations, and TAT. Additionally, treatment modalities and OS were evaluated.

All patients provided written informed consent, and the study was conducted as part of a PhD project approved by the Ethics Committee of the IPOC-FG (approval No. 23-2022).

EBUS procedures were performed using a BF-UC180F bronchoscope (Olympus, Tokyo, Japan) under general anesthesia, with airway secured via a laryngeal mask. TBNA was carried out using 21G needles (ViziShot 2, Olympus, Tokyo, Japan). In accordance with institutional protocol, at least three needle passages were performed per lesion. Suction use was guided by lymph node vascular patterns[19] and was withheld in cases of grade III/IV vascularity.

Collected specimens were fixed in a 4% aqueous formaldehyde solution, centrifuged at 400×g for 15 min for cell block preparation from the pellet, and subsequently embedded in paraffin for histopathological examination.

SMP followed a stepwise strategy that was performed after immunohistochemistry, including PD-L1 assessment, as previously described by our group.[13] Briefly, the workflow involved RT-PCR for *EGFR* mutation analysis using the Cobas® EGFR Mutation Test v2 (Roche Diagnostics, Mannheim, Germany), a CE-IVD assay designed to detect 42 mutations across exons 18, 19, 20, and 21, including exon 19 deletions, L858R, T790M, G719X, S768I, and exon 20 insertions. Formalin-fixed paraffin-embedded (FFPE) tumor sections (5 µm) were reviewed by a pathologist, and manual microdissection was conducted for samples containing fewer than 10% tumor cells. DNA was extracted using the Cobas® DNA Sample Preparation Kit (Roche Diagnostics, Mannheim, Germany), and amplification/detection was carried out on a Cobas® z480 analyzer (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions.

ALK and *ROS1* rearrangements were evaluated by FISH using 3-µm FFPE tissue sections. Samples with fewer than 100 viable tumor cells were excluded from the analysis. Following standard pretreatment, slides were incubated overnight with SPEC ALK (Z-2124, ZytoVision GmbH, Bremerhaven, Germany) or SPEC ROS1 (Z-2144, ZytoVision GmbH, Bremerhaven, Germany) dual-color break-apart probes. After post-hybridization washing, the slides were analyzed using a Leica DMI6000 B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

For MP-NGS, FFPE tumor blocks with ≥10% tumor content were selected. Genomic DNA/RNA was extracted using the MagMAX™ FFPE DNA/RNA Ultra Kit (Thermo Fisher Scientific, USA), and nucleic acids were quantified with a Qubit® 3.0 fluorometer. Sequencing was performed on the Genexus platform (Thermo Fisher Scientific, USA) using the Oncomine Precision Assay GX, which detects mutations, copy number variations, and fusion variants across 50 cancer-related genes. The results were interpreted using the Oncomine Reporter to identify associated therapies.

To ensure comparability, actionable mutations were defined as *EGFR* mutations, as well as *ALK* and *ROS1* rearrangements, which were consistently tested in both approaches and align with international guidelines for targeted therapies.[8]

Data analysis was performed using IBM SPSS Statistics software (v27.0; IBM Corp., USA). Continuous variables were presented as medians and ranges, while categorical variables were reported as frequencies (n) and percentages (%). The Shapiro-Wilk test was used to assess the normality of continuous variables. Since the variables did not follow a normal distribution, non-parametric methods were employed. Pearson's Chi-Square test was used to compare operational

characteristics between the SMP and MP-NGS groups. TAT, defined as the interval from sample collection to final diagnosis (in days), was analyzed using the Mann-Whitney U test. Kaplan-Meier curves were used to estimate median survival times, and survival distributions were compared using the log-rank test. Multivariate Cox regression analysis was applied to identify independent predictors of survival. Collinearity diagnostics, including the variance inflation factor (VIF), were conducted to confirm the absence of significant multicollinearity. All statistical tests were two-sided, with p-values < 0.05 considered statistically significant.

RESULTS

During the four-year study period, 106 patients with stage IV NSCLC underwent molecular testing on samples obtained via EBUS-TBNA. Of these, 45 were tested using SMP and 61 using MP-NGS.

Patients in both the SMP and MP-NGS groups were predominantly male (62.2% and 60.7%, respectively), with median ages of 67 and 69 years. Adenocarcinoma was the most common histological subtype (SMP: 91.1%; MP-NGS: 88.5%), and the majority of patients were classified as stage IVB (SMP: 68.9%; MP-NGS: 65.57%). No significant epidemiological or clinicopathological differences were observed between groups. Detailed results are presented in Table 1.

Regarding molecular profiling outcomes, adequate samples were obtained in the SMP group for *EGFR* analysis in 93.3% of cases, for *ALK* in 78.4%, and for *ROS1* in 75%, resulting in an overall success rate of 62.2%. Actionable mutations were identified in 22.2% (*EGFR*: 15.6%; *ALK*: 6.7%), while no *ROS1* rearrangements were detected.

In the MP-NGS group, all samples were adequate for molecular analysis. Mutations were detected in 88.5% of cases, with actionable mutations identified in 40.9% (*EGFR*: 32.8%; *ALK*: 8.2%). Similarly, no *ROS1* rearrangements were observed. However, additional relevant mutations were detected, including *HER2* (8.2%), *RET* (1.6%), and *BRAF* (1.6%). *KRAS* mutations were found in 21.3% of cases, with the G12C variant accounting for 8.2%. Details of the mutations are presented in Figure 1.

Table 1. Epidemiological and clinicopathological characteristics of the included patients.

Variable	SMP (n = 45)	MP-NGS (n = 61)	p-value
Sex, n (%)			
Male	28 (62.2)	37 (60.7)	
Female	17 (37.8)	24 (39.3)	0.870*
Age, median (min; max)	67 (38; 84)	69 (42; 86)	0.933#
Smoking history, n (%)			
Never smoker	11 (24.4)	21 (34.4)	
Former smoker	15 (33.3)	19 (31.1)	
Current smoker	19 (42.2)	21 (34.4)	0.519*
ECOG performance status			
0	17 (37.8)	34 (55.7)	
1	18 (40)	19 (31.1)	
2	7 (15.5)	7 (11.5)	
3	3 (6.7)	1 (1.6)	0.223*
Diagnostic procedure			
EBUS alone	28 (62.2)	40 (65.6)	
EBUS and EUS-b	17 (37.8)	21 (34.4)	0.722*
Type of sample			
Lymph node	32 (71.1)	39 (63.9)	
Tumor	11 (24.4)	20 (32.8)	
Left adrenal gland	2 (4.4)	2 (3.3)	0.635*
Histology, n (%)			
Adenocarcinoma	41 (91.1)	54 (88.5)	
Adenosquamous carcinoma† Combined	2 (4.4)	4 (6.6)	
adenocarcinoma and NE carcinoma†	1 (2.2)	3 (4.9)	
Squamous cell carcinoma	1 (2.2)	0	0.556*
Stage, n (%)			
IVA	14 (31.1)	21(34.4)	
IVB	31 (68.9)	40 (65.6)	0.720*

Legend: SMP, Sequential molecular profiling; MP-NGS, Massively parallel–Next generation sequencing; ECOG, Eastern Cooperative Oncology Group; EBUS, Endobronchial Ultrasound; EUS-b, Endoscopic Ultrasound (trans-esophageal) with the echobronchoscope; NE, neuroendocrine; *Pearson's Chi-square test; #Mann-Whitney U test. †In cases classified as adenosquamous carcinoma (n=6) and combined adenocarcinoma with neuroendocrine features (n=4), the diagnosis was suggested based on morphology and immunohistochemistry, performed on FFPE cell blocks obtained by EBUS-TBNA. In five of these cases (3 adenosquamous, 2 combined adenocarcinoma/NE carcinoma), the diagnosis was later confirmed using surgical biopsies from the primary tumor (n=2) or metastatic sites (pleura, n=1; subcutaneous tissue, n=2).

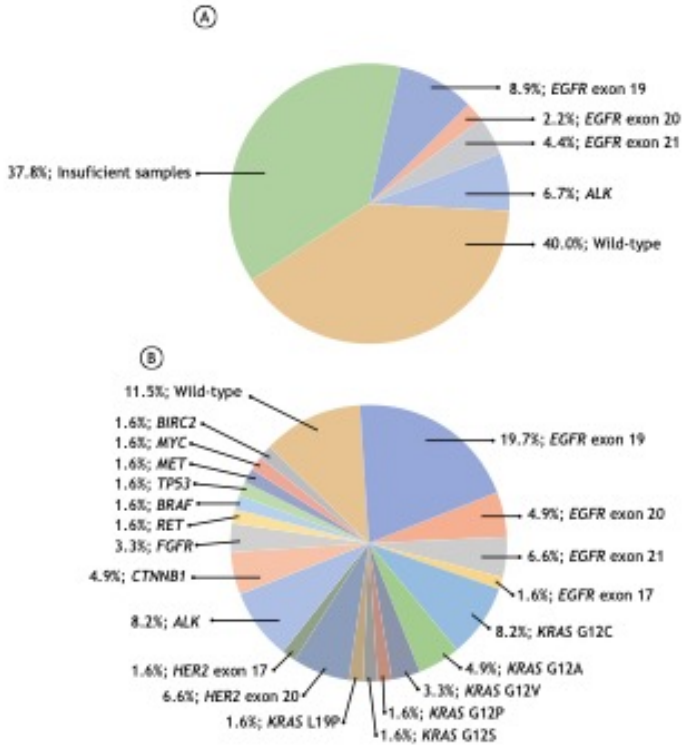

Figure 1. Pie charts illustrating the molecular profiling results in stage IV NSCLC samples using (A) sequential molecular profiling (SMP) and (B) massively parallel–next generation sequencing (MP-NGS). Legend: Aside from the data presented in the charts, 9 patients (14.7%) from the MP-NGS group exhibited complex molecular patterns: 1 harbored three mutations (*EGFR* exon 19, *CDKN2A*, and *PTEN*); 4 combined *EGFR* mutations with a second mutation (2 with *CTNNB1*; 1 with *TP53*; 1 with *PIK3CA*); 4 combined *KRAS* mutations with a second mutation (2 with *FGFR*; 1 with *TP53*; 1 with *BRAF*). These data highlight the superior discriminative power of MP-NGS, the absence of insufficient samples when using this method, and the reduced proportion of cases classified as wild-type.

MP-NGS demonstrated significantly higher success in obtaining sufficient samples for molecular analysis ($p=2 \times 10^{-5}$) and enabled the identification of a significantly greater number of actionable mutations compared to SMP (p=0.042). A comparative summary of the operational characteristics of both methods is shown in Table 2.

The median TAT for positive results was significantly shorter with SMP than with MP-NGS (11 vs. 24 days; p=0.002). Although the overall TAT for SMP was also shorter than that of MP-NGS (17 vs. 23 days), this difference was not statistically significant (p=0.076). Detailed results for these measures are presented in Table 2.

Considering therapeutic options, targeted therapy was administered to 44.3% of patients in the MP-NGS group, compared to 22.2% in the SMP group. Conversely, best supportive care was significantly less frequent in the MP-NGS group (13.1%) than in the SMP group (37.8%).

The differences between the SMP and MP-NGS methods were statistically significant regarding the increased use of targeted therapy (p=0.026) and the reduced utilization of best supportive care (p=0.019). Therapeutic allocation by profiling method (SMP vs. MP-NGS) and the relationship between detected actionable mutations and corresponding targeted therapies are detailed in Figure 2.

The Kaplan-Meier survival analysis revealed significant differences in OS based on the presence of actionable mutations (log-rank p=0.002) and first-line therapy (log-rank p<0.001). Patients with actionable mutations had a median OS of 1128 days, compared to 138 days for those without mutations. First-line targeted therapy was associated with the longest median survival (1128 days), whereas best supportive care was linked to the shortest survival (46 days). Overall, patients in the MP-NGS group exhibited a trend toward improved survival compared to those in the SMP group, with a median OS of 672 days versus 138 days, respectively (log-rank

Table 2. Comparison of molecular profiling techniques: SMP vs. MP-NGS.

Variable	SMP step 1 RT-PCR (*EGFR*)	SMP step 2 FISH (*ALK*)	SMP step 3 FISH (*ROS1*)	SMP Overall results	MP-NGS Overall results	p-value
Patients tested, n (%)	45 (100)	37 (82.2)	24 (53.3)	45 (100)	61 (100)	NA
Adequate samples, n (%)	42 (93.3)	29 (78.4)	18 (75)	28[a] (62.2)	61 (100)	2 x 10^{-5}*
Samples with actionable[b] mutations, n (%)	7 (15.6)	3 (6.7)	0	10 (22.2)	25 (41)	0.042*[$]
Time to positive result[c], median (max; min)	8 (3; 34)	15 (9; 33)	NA	11 (3; 34)	24 (3; 57)	0.002#
Time to final molecular result[c], median (max; min)	15 (9; 33)	17 (3; 58)	23 (3; 58)	17 (3; 58)	23 (3; 57)	0.076#

Legend: SMP, Sequential molecular profiling; RT-PCR, Real-time polymerase chain reaction; *EGRF*, Epidermal growth factor receptor; FISH, Fluorescence *in situ* hybridization; *ALK*, Anaplastic lymphoma kinase; *ROS1*, Proto-oncogene receptor tyrosine kinase; MP-NGS, Massively parallel–Next generation sequencing; NA, not applicable. [a]Overall SMP: combines the positive results of *EGFR* (deemed complete, and that did not require further profiling) and the 18 additional cases where both *ALK* and *ROS1* could be tested, reflecting the sample sufficiency for all tests required to complete the molecular characterization of individual samples. [b]Actionable Mutations: mutations assessed by all three diagnostic methods—*EGFR*, *ALK*, and *ROS1*—were considered actionable. [c]Time to Result: the time, measured in days, from the completion of histopathological evaluation, including PD-L1 staining, to the final result of the molecular study. *Pearson's Chi-square test; *[$] Fisher's Exact test; # Mann-Whitney U test.

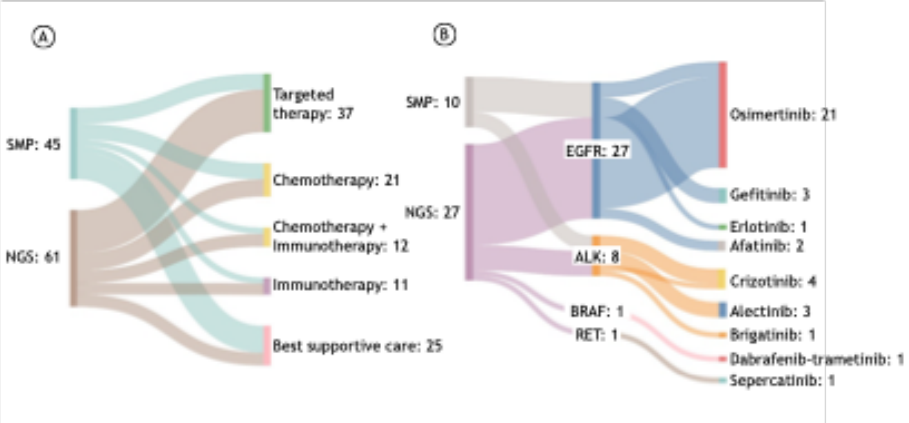

Figure 2. Relationship between molecular profiling strategies and first-line therapeutic choices with detailed targeted therapy selection. Legend: (A) Sankey diagram illustrating the distribution of first-line therapeutic strategies based on the molecular diagnostic method. The figure highlights a significant increase in the use of targeted therapies with MP-NGS compared to SMP (44.3% vs. 22.2%; p=0.026; Pearson's Chi-square test) and a notable reduction in the use of best supportive care strategies with MP-NGS compared to SMP (13.1% vs. 37.8%; p=0.019, Pearson's Chi-square test). (B) Sankey diagram detailing the targetable mutations identified by each method and their corresponding therapies. This panel underscores the superior discriminatory capacity of MP-NGS, which identified more actionable mutations and facilitated greater use of targeted therapies.

p=0.053). According to the Cox proportional hazards model, the presence of actionable mutations remained an independent predictor of improved survival (HR: 0.48; 95% CI: 0.25–0.96; p=0.027), whereas the molecular diagnostic method (MP-NGS vs. SMP; HR: 0.99; p=0.924) and first-line therapy (HR: 1.13 across therapy types; p=0.588) were not statistically significant. All VIF values were below 5, indicating acceptable multicollinearity. Full details are available in Supplementary Tables 1 and 2 and Supplementary Figure 1.

DISCUSSION

The present study offers a detailed comparative analysis of two molecular profiling strategies—SMP and MP-NGS—using minimally invasive EBUS-TBNA-derived samples from patients with stage IV NSCLC. Our findings highlight the superior performance of MP-NGS in identifying actionable mutations, detecting a wider array of genetic alterations, and facilitating access to personalized therapies, which may contribute to improved clinical outcomes, including a potential survival benefit.

The clinical and epidemiological characteristics of our cohort are consistent with those reported in similar patient populations,[20,21] supporting the representativeness of our findings. While the retrospective nature of this study limited control over participant inclusion and group allocation, the comparative analysis of clinical and epidemiological variables revealed no significant differences between the two groups (Table 1), further reinforcing the internal validity of our results.

When comparing the performance of both methodologies, our findings highlight the superiority of MP-NGS over SMP in optimizing the use of EBUS-TBNA-derived samples. MP-NGS achieved a significantly higher sample adequacy rate (100% vs. 62.2%; $p=2 \times 10^{-5}$; Table 2) and identified more actionable mutations in *EGFR* (32.8%) and *ALK* (8.2%) compared to SMP (15.5% and 6.7%, respectively) (Table 2; Figure 1). Moreover, MP-NGS detected a broader spectrum of mutations in 88.5% of patients, with 14.7% harboring more than one, underscoring its enhanced sensitivity and efficiency in identifying emerging actionable targets.[22,23,24]

In order to directly compare the two methods, this study restricted the definition of actionable mutations to *EGRF*, *ALK*, and *ROS1*, in accordance with the minimum requirements outlined in international guidelines.[6] However, the field of targeted therapy for NSCLC continues to evolve, with new actionable mutations being identified regularly.[25] For instance, *RET* rearrangements and *BRAF* mutations—assessed only through MP-NGS in our sample—are already targetable,[26,27,28] as observed in our cohort (Figure 2). Additionally, MP-NGS identified *KRAS* mutations, including the G12C variant in 8.2% of patients, which are increasingly actionable with inhibitors such as sotorasib, showing promising clinical outcomes.[29,30] Furthermore, the simultaneous mutations identified via MP-NGS in several patients (Figure 1) highlight the heterogeneity of NSCLC and open possibilities for sequentially targeting multiple pathways, reinforcing the value of this profiling method.[31]

One notable finding in our study was the progressive decline in sample adequacy throughout the sequential steps of the SMP method, with the lowest adequacy observed for *ROS1* testing (62.2%) (Table 2). This trend aligns with previous reports[11,13] and underscores the critical challenge of sample exhaustion, which is particularly relevant when dealing with limited material such as EBUS-TBNA-derived specimens. Sample depletion often results from the hierarchical testing order, in which IHC, PD-L1 assessment, and *EGFR* analysis are prioritized, frequently leaving insufficient material for FISH-based *ALK* and *ROS1* evaluations.[32] An indirect indicator of this limitation is the discrepancy in *ALK* mutation detection rates between MP-NGS (8.2%) and SMP (6.7%). Similar findings have been reported in other studies, particularly when *ALK* is assessed by IHC, which is prone to false negatives.[25,33] FISH, on the other hand, is generally highly sensitive and specific, provided that samples have adequate tumor content.[25] Although the lower detection rate observed in the SMP group may partly reflect random heterogeneity inherent to the study's retrospective design, we hypothesize that it also stems from the intrinsic limitations of EBUS-TBNA's sampling capacity, compounded by the issue of sample exhaustion discussed above. Notably, MP-NGS effectively overcame these challenges, achieving a sample adequacy rate of 100%.

The median TAT was 17 days for SMP and 23 days for MP-NGS. Although this difference was not statistically significant, the shorter TAT for SMP likely reflects cases in which positive *EGFR* results concluded testing early, eliminating the need for further molecular analyses (Table 2). Additionally, unlike SMP, which was performed in-house, MP-NGS was outsourced, leading to longer processing times due to shipping and external handling—an issue previously documented in the literature.[34] When compared with international guidelines and published benchmarks,[35] these differences become more pronounced. Most studies report median TATs for NGS of around 10 days,[36] which is substantially shorter than the values observed in our cohort. These discrepancies highlight real-world challenges in the timely diagnosis and treatment of NSCLC, especially in institutions where advanced molecular platforms are either not fully integrated or rely on external laboratories. Addressing these limitations will require coordinated strategies to optimize molecular workflows, including wider adoption of in-house MP-NGS platforms and reflex testing protocols to accelerate result turnaround times.[37] In parallel, the development of ultra-rapid multiplex PCR platforms represents a promising complementary approach.[38] These emerging technologies may enable broader genomic profiling—in some cases using existing RT-PCR infrastructure[38]—with the potential to deliver clinically actionable results within a markedly reduced TAT.

The treatment data revealed distinct patterns between the two profiling methods. The MP-NGS group received more targeted therapies (44.26% vs. 22.2%; $p=0.038$), suggesting that MP-NGS may facilitate more personalized treatment strategies by identifying a broader range of actionable mutations (Figure 2), which may have influenced survival outcomes. Indeed, the Kaplan-Meier analysis showed a trend toward improved survival in the MP-NGS group (median OS: 672 vs. 138 days; log-rank $p=0.053$). Although this difference did not remain significant in the multivariable Cox model (HR: 0.99; $p=0.924$), the presence of actionable mutations was independently associated with OS in both models. As previously documented,[39,40] this finding suggests that the survival advantage associated with MP-NGS is primarily mediated by factors such as the identification of actionable mutations and improved access to targeted therapies (Supplementary Tables 1 and 2; Supplementary Figure 1), ultimately reinforcing the clinical value of this method.

This study has some limitations inherent to its retrospective and uncontrolled design. Additionally, the relatively small sample size and the evolving treatment landscape of NSCLC—particularly the growing use of targeted therapies—may have influenced the outcomes.[39,40]

In spite of these constraints, the real-world nature of this study provides valuable insights into the clinical management of advanced NSCLC. Specifically, our findings highlight the superior performance of MP-NGS over SMP in detecting actionable mutations and facilitating access to personalized treatments. Although MP-NGS was associated with a longer TAT due to external processing requirements, its broader mutation coverage and greater sensitivity underscore its clinical utility in the evolving field of personalized NSCLC therapy. Moreover, the observed trend toward improved survival in the MP-NGS group further supports the potential advantages of this method over SMP, particularly in scenarios where only limited samples are available from minimally invasive procedures such as EBUS-TBNA.

Future research should focus on evaluating the cost-effectiveness and accessibility of MP-NGS, particularly in less specialized centers, to guide strategies for its broader and more effective implementation. Additionally, as molecular diagnostics continue to evolve, future studies should explore the comparative performance, feasibility, and clinical impact of emerging genomic technologies alongside MP-NGS.

AUTHOR CONTRIBUTIONS

Study conceptualization, formulation of the research questions, and writing of the manuscript: LVR, RC, LTB, and VS. Execution of EBUS-TBNA procedures and collection of data from clinical files: LVR. Supervision of the sequential molecular profiling protocol, assistance with data collection, and critical review of the database: MV. Development of the MP-NGS protocol, data review, and assurance of database completeness: AA, AFL, and VS. Critical review of the results, statistical analysis, and final content of the manuscript: LVR and LTB. All authors read and approved the final version of the manuscript and agree to be accountable for all aspects of the work, ensuring the accuracy and integrity of the research.

FINANCIAL SUPPORT

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICTS OF INTEREST

The authors declare not to have any conflicts of interest that may be considered to influence directly or indirectly the content of the manuscript.

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Study IV - ***Supernatant from EBUS-TBNA Samples for Molecular Profiling in NSCLC: A Systematic Review and Meta-Analysis***, Luis Vaz Rodrigues, Joana Oliveira, Tiago Maricoto, Luis Taborda-Barata, Rosa Cordovilla, Vitor Sousa.

Systematic Review and Meta-Analysis published in *European Respiratory Review* (December 2025; doi: https://doi.org/10.1183/16000617.0127-2025)

EUROPEAN RESPIRATORY REVIEW
REVIEW
L. VAZ RODRIGUES ET AL.

Supernatant from endobronchial ultrasound-guided transbronchial needle aspiration samples for molecular profiling in NSCLC: a systematic review and meta-analysis

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Supernatant derived from EBUS-TBNA samples is a feasible, underused source for molecular profiling in NSCLC, with high concordance to tissue and potential to reduce re-biopsies and accelerate treatment decisions https://bit.ly/4n6FLPx

Cite this article as: Vaz Rodrigues L, Oliveira J, Maricoto T, *et al*. Supernatant from endobronchial ultrasound-guided transbronchial needle aspiration samples for molecular profiling in NSCLC: a systematic review and meta-analysis. *Eur Respir Rev* 2025; 34: 250127 [DOI: 10.1183/16000617.0127-2025].

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Received: 31 May 2025
Accepted: 30 Aug 2025

Abstract

Background Molecular profiling has become essential in the management of nonsmall cell lung cancer (NSCLC). While endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is a cornerstone in diagnosis, tissue scarcity may hinder comprehensive testing. Recent studies suggest that supernatant from EBUS-TBNA could serve as an alternative source for molecular analysis.

Methods A systematic review and meta-analysis were conducted according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PROSPERO identifier CRD42024600046). Studies from 2000 to 2024 evaluating the feasibility, molecular concordance and turnaround time of EBUS-TBNA supernatant for molecular profiling in NSCLC were included. Outcomes included DNA yield, detection of actionable mutations and agreement with tissue-based results.

Results Seven studies (n=506 patients) were included. Feasibility of molecular analysis using supernatant was high (87–100%). DNA yields varied across studies. In pooled analysis, storage temperature and preservation solution had no significant effect, while individual studies reported lower yields with high-speed centrifugation. Concordance with tissue samples ranged from 83% to 100%, with Cohen's κ 0.947 (95% CI 0.905–0.989), indicating an almost perfect agreement. Supernatant samples demonstrated faster turnaround times (reduction of 1–7.5 days).

Conclusions EBUS-TBNA supernatant is a feasible and accurate source for molecular testing in NSCLC, with high concordance and shorter turnaround times. Standardisation of protocols is required before broader implementation in clinical practice.

Introduction

Lung cancer remains the leading cause of cancer-related mortality worldwide [1], with nonsmall cell lung cancer (NSCLC) representing the most prevalent subtype [2, 3]. The diagnosis of lung cancer remains a challenge due to the asymptomatic nature of early-stage disease and the nonspecific symptoms that emerge later, often leading to delayed diagnoses [4, 5]. As a result, most patients are diagnosed at advanced stages

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when surgical resection is no longer an option [5, 6]. Advances in NSCLC management have highlighted the critical role of molecular diagnostics in enabling personalised therapies. ~50% of NSCLC cases harbour targetable genetic alterations that influence the response to treatment [3, 7, 8]. These findings have propelled the adoption of precision medicine strategies, improving survival, reducing treatment toxicity, and enhancing patients' quality of life [9–11]. However, implementing molecular diagnostics in routine clinical practice requires high-quality biological samples [12].

Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) has emerged as a cornerstone technique for the diagnosis and staging of NSCLC, offering high sensitivity and specificity [13, 14]. Its minimally invasive nature makes it an optimal choice for sample acquisition in patients with advanced disease [12, 15]. Traditionally, tissue and cytology samples obtained *via* EBUS-TBNA have been used for histopathological and molecular testing [15]. Nevertheless, the growing demand for increasingly detailed molecular profiling to guide therapeutic decisions places additional pressure on these samples, which are often limited in quantity and quality [12]. Furthermore, the standard diagnostic workflow prioritises histology, with molecular testing conducted sequentially, resulting in prolonged time to treatment initiation [16, 17].

These challenges have sparked interest in using the supernatant phase from EBUS-TBNA samples as an alternative source for molecular testing. In fact, circulating tumour DNA (ctDNA) is already established as an alternative source for molecular testing and has demonstrated utility in various biological fluids [18–20]. However, ctDNA often presents limitations in DNA quantity, compromising its reliability for molecular analysis [19].

To address this challenge, researchers have proposed utilising discarded biological material, such as supernatant from EBUS-TBNA, as a novel source of ctDNA for molecular profiling. Early studies suggest that supernatant-based molecular diagnostics may offer comparable results to traditional tissue samples while preserving the cellular components and potentially improving diagnostic efficiency and patient outcomes [21–28].

This systematic review aims to evaluate the feasibility, diagnostic yield and molecular performance of EBUS-TBNA supernatant as a source for molecular testing in NSCLC. By comparing its results with conventional tissue samples, we seek to determine its potential role in the evolving landscape of minimally invasive diagnostics and precision medicine in NSCLC.

Material and methods

Study design

This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [29] to ensure methodological rigour and transparency. The protocol was prospectively registered in the International Prospective Register of Systematic Reviews (PROSPERO) (identifier CRD42024600046). The review was structured according to the PICO framework, as follows.

- Population (P): patients with suspected or confirmed NSCLC undergoing EBUS-TBNA.
- Intervention (I): molecular profiling performed on supernatant from EBUS-TBNA.
- Comparison (C): molecular results from supernatant samples *versus* a reference standard of conventional tissue or cytology-based specimens.
- Outcomes (O): measures of feasibility, diagnostic yield, molecular concordance between sample types, turnaround time.

Search strategy

A comprehensive search of peer-reviewed literature was performed across major biomedical databases, including PubMed, Embase, Cochrane Library, Connected Papers, and ClinicalKey, covering studies published from January 2000 to the present. This timeframe was chosen to reflect the period since the introduction of EBUS-TBNA in 2002, to ensure the inclusion of all studies that investigated the use of supernatant obtained from EBUS-TBNA samples for molecular profiling in NSCLC. No language restrictions were applied; non-English articles were translated as needed.

Additional searches were conducted in trial registries such as www.ClinicalTrials.gov, ISRCTN, and the Australian New Zealand Clinical Trials Registry (anzctr.org.au) to identify unpublished or ongoing studies. Grey literature, including conference abstracts, theses, and dissertations, was explored using platforms such as OpenGrey (www.opengrey.eu) and OAIster (www.oclc.org/en/oaister.html). Reference lists of included studies were screened manually to identify additional relevant publications.

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The search strategy included combinations of terms such as ("nonsmall cell lung cancer" OR "lung cancer") AND ("EBUS-TBNA" OR "endobronchial ultrasound") AND ("molecular profiling" OR "molecular testing" OR "NGS") AND ("supernatant"). Full details of the search terms and Boolean operators are provided in the supplementary material (appendix S1).

Eligibility

Inclusion criteria

Studies were included according to the following criteria: 1) research studies published from 2000 onward; 2) studies involving NSCLC patients undergoing EBUS-TBNA; 3) comparison of molecular findings from EBUS-TBNA supernatant *versus* reference standard; 4) studies reporting diagnostic outcomes, including sensitivity, specificity, predictive values or molecular yield (*e.g.* detection of *EGFR*, *ALK* or *KRAS* mutations); 5) observational studies (prospective and retrospective cohorts, case–control studies), diagnostic accuracy studies and randomised controlled trials; and 6) full-text articles and abstracts containing sufficient data for analysis.

Exclusion criteria

Studies excluded were 1) studies from before the year 2000; 2) studies involving samples obtained exclusively by methods other than EBUS-TBNA; 3) studies focusing on cancers other than NSCLC; 4) narrative reviews, editorials or commentaries unless those presenting novel data.

Outcomes

Primary outcome

The primary outcome was the feasibility of molecular profiling from EBUS-TBNA supernatant, defined as the ability to obtain DNA of sufficient quantity and quality for molecular analysis, compared to reference standard. Diagnostic performance was also assessed (sensitivity, specificity, predictive values), using reference standard specimens for comparison.

Secondary outcomes

Pre-analytical factors: impact of preservation medium, storage temperature and centrifugation on feasibility and molecular yield.

Molecular concordance: agreement between molecular results obtained from supernatant and reference standard, quantified as percentage concordance and using Cohen's κ coefficient when applicable.

Detection of actionable mutations: proportion of supernatant samples with clinically relevant genetic alterations, as defined by current guidelines [3, 30].

Turnaround time (TAT): time, in days, required to obtain molecular results from supernatant compared to reference standard.

Screening and selection process

References were imported into Rayyan [31], a web-based review tool, to remove duplicates. Screening was conducted in two stages. First, two independent reviewers (L. Vaz Rodrigues and J. Oliveira) assessed all titles and abstracts for relevance. Second, potentially eligible studies were retrieved in full and independently evaluated against the inclusion criteria by the same reviewers.

Discrepancies in study selection were resolved through discussion and consensus. If disagreements persisted, a third reviewer (T. Maricoto) was consulted. Selection flow and exclusion reasons are detailed in the PRISMA 2022 diagram (figure 1).

Data extraction

Two independent reviewers (L. Vaz Rodrigues and J. Oliveira) extracted and synthesised data using a standardised form, piloted on a subset of studies to ensure consistency. The extracted data included the following.

1) Study characteristics: authors, year of publication, study design and location.
2) Patient characteristics: sample size, age, sex, NSCLC subtype, stage, smoking history and comorbidities (if reported).
3) Diagnostic methods: EBUS-TBNA technique (needle gauge, number of passes), collection methods for supernatant and reference standard samples.

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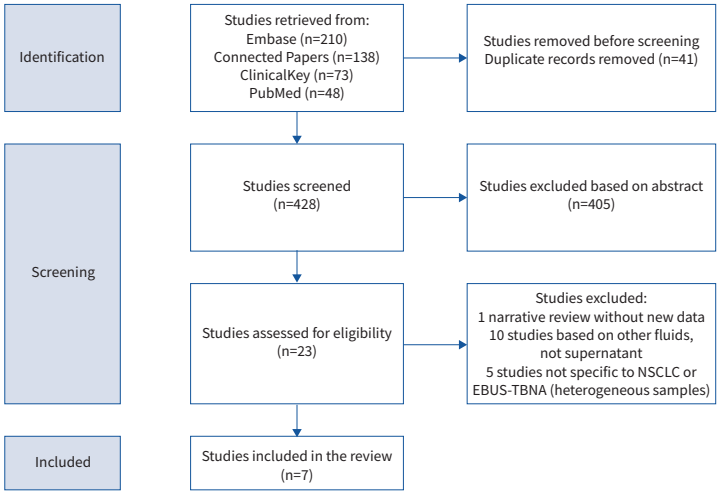

FIGURE 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow diagram of the systematic review process. The diagram illustrates the selection process of studies included in the systematic review. A total of 469 records were retrieved from four databases (PubMed, Embase, Connected Papers and ClinicalKey). After removing 41 duplicate records, 428 studies were screened based on title and abstract, leading to the exclusion of 405 studies that did not meet the inclusion criteria. Following full-text assessment of 23 studies, an additional 16 were excluded due to irrelevance to the research question, use of nonsupernatant samples, or heterogeneous study populations. Ultimately, seven studies were included in the final review. NSCLC: nonsmall cell lung cancer; EBUS-TBNA: endobronchial ultrasound-guided transbronchial needle aspiration.

4) DNA and RNA concentration (if reported) in supernatant and reference standard, preserving solution and pre-processing details (storage time, storage temperature, centrifugation speed and duration). Molecular techniques applied and detected mutations were also recorded.
5) Outcomes: feasibility; concordance between supernatant and reference standard results; detection of actionable mutations and TAT.

Disagreements were resolved by consensus or, if needed, by a third reviewer (T. Maricoto). Data were compiled into a central database for descriptive and inferential analysis.

Risk-of-bias assessment

The methodological quality of the included studies was assessed using the Cochrane Risk of Bias Tool (RoB 2.0) [32] for RCTs and the Newcastle–Ottawa Scale (NOS) for observational studies [33]. Studies were rated as low, moderate or high risk based on selection, performance, detection, attrition and reporting domains. Assessments were conducted independently by L. Vaz Rodrigues and J. Oliveira, with disagreements resolved by consensus or, if necessary, consultation with a third reviewer (T. Maricoto).

Data synthesis and analysis

A qualitative synthesis was performed to summarise the included studies. Study characteristics, methods and outcomes were summarised in a descriptive table (table 1). Narrative synthesis was used to compare results and explore factors influencing molecular profiling using EBUS-TBNA supernatant.

Most studies reported outcomes as median (range). To allow data aggregation, means and standard deviations were estimated from medians using the method by Hozo *et al*. [34]. Using the estimated means, standard deviations and sample sizes, a restricted random-effects meta-analysis was performed with the metafor package in R software (version 4.4.1) [35].

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TABLE 1 Characteristics of studies evaluating the feasibility of molecular testing using endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) supernatant, including study population and diagnostic performance

First author (year) [reference] country	Population	Type of paper and research design	Age years	Gender	Diagnostic methods (type and count)	Histological classification (type and count)	Needle gauge (EBUS)	ROSE	Molecular method; reference standard	Feasibility of supernatant %	Agreement (supernatant *versus* reference standard) %
Guibert (2018) [23] USA	17	Short communication; prospective	NS	NS	EBUS (16) CT-TTB (1)	ADC (12); SQCC (1); SCC (1); nonmalignant (3)	NS	NS	NGS and ddPCR; smears and cell-block	100	100
Hanningan (2019) [24] USA	150	Original article; prospective	68 (31–92)	Male 64 (43) Female 86 (57)	EBUS and CT-TTB (NS)	ADC (97); SQCC (25); NSCC NOS (6); poorly differentiated carcinoma (8); sarcomatoid carcinoma (1); carcinoid (3); SCC (10)	20–25 G	Yes	NGS and ddPCR; core-biopsy and cfDNA	90	97[#] 89[¶] 83[+] 100[§]
Janaki (2019) [25] USA	30	Original article; prospective	71 (48–82)	Male 18 (60) Female 12 (40)	EBUS	ADC (24); SQCC (2); Mx melanoma (2); Mx breast (2)	20–25 G	Yes	NGS; cell-block or previous surgical biopsy	100	100
Gokozan (2020) [27] USA	62	Original article; retrospective	68 (39–91)	Male 24 (40) Female 37 (60)	EBUS	ADC (37); NSCC NOS (19); SQCC (5); NEC (1)	20–25 G	Yes	NGS; cell-block	100	NS
Wu (2020) [26] China	214	Original article; prospective	63 (38–90)	Male 120 (56) Female 94 (44)	EBUS and CT-TTB (NS)	ADC (184); SQCC (9); NSCC NOS (21)	20–25 G	Yes	NGS and ARMS-PCR; cell-block	100	97
Tapoya (2021) [21] USA	14	Original article; prospective	NS	NS	EBUS (16); bronchial washings (3); bronchial brushing (3); BAL (1)	NSCLC NOS (23)	NS	Yes	NGS; cell-block or FFPE biopsy	87	100
Jager (2024) [28] USA	19	Original article; prospective	65 (50–95)	Male 9 (47) Female 10 (53)	EBUS	ADC (11); SQCC (3); Mx breast (2); Mx prostate (1); Mx renal (1); poorly differentiated NSCC (1)	19–22 G	Yes	NGS; cell-block	100	100

Data are presented as n, median (range) or n (%). ROSE: rapid on-site evaluation; NS: not specified; CT-TTB: computed tomography-guided transthoracic biopsy; ADC: adenocarcinoma; SQCC: squamous cell carcinoma; SCC: small cell carcinoma; NGS: next-generation sequencing; ddPCR: droplet digital PCR; NSCC: nonsmall cell carcinoma; NOS: not otherwise specified; cfDNA: circulating free DNA; Mx: metastatic; NEC: neuroendocrine carcinoma; ARMS-PCR: amplification refractory mutation system PCR; BAL: bronchoalveolar lavage; NSCLC: nonsmall cell lung cancer; FFPE: formalin-fixed paraffin-embedded. Categorisation of agreement rates based on the relationship between the supernatant and reference standard: [#]: same site/same day (n=35); [¶]: different site/same day (n=9); [+]: different site/different day (n=23); [§]: cfDNA/different day (n=7).

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Subgroup analyses by "preservative solution" and "storage temperature" were conducted to evaluate differences.

Between-study heterogeneity was assessed using the Q statistic, I^2 index, and the τ^2 estimate. Meta-regression was used to test subgroup differences with preservative solution and temperature as moderators.

Agreement between supernatant and reference standard was assessed by direct comparison, defined as the percentage of identical molecular alterations in both samples. Cohen's κ was computed for subgroups with sample size ⩾30 samples and κ-values were interpreted using standard thresholds: values >0.80 indicated almost perfect agreement, 0.61–0.80 substantial agreement, and 0.41–0.60 moderate agreement.

Additionally, a detailed analysis of diagnostic accuracy was conducted by extracting the number of true positives, false positives, false negatives and true negatives from each study when available. All pooled estimates are reported with 95% confidence intervals.

Results

Overview of eligible studies

A total of 469 articles were initially identified through database searches based on the pre-defined eligibility criteria. After removing 41 duplicates, 428 articles remained for screening. Titles and abstracts were reviewed by two investigators (L. Vaz Rodrigues and J. Oliveira). Studies were excluded if they were unrelated to NSCLC molecular profiling with EBUS-TBNA supernatant, had ineligible design, or fell outside the defined timeframe. After this initial screening, 23 articles were selected for full-text review.

Following detailed assessment, 16 studies were excluded. One was a narrative review with no original data. 10 analysed needle lavage samples, but not supernatant. Five included heterogeneous sample populations without clearly distinguishing NSCLC or EBUS-TBNA-derived samples.

Seven studies met eligibility criteria and were included. The PRISMA flow diagram (figure 1) summarises the selection process. Table 1 provides an overview of the methodological features of the included studies. All seven studies were published between 2018 and 2024 and were mostly prospective observational studies, conducted in the USA (six studies) and China (one study). The reference standard varied across studies, with most relying on cell-block preparations, while others utilised core biopsies, smears or previous surgical biopsy specimens for comparison.

Risk-of-bias assessment

As all included studies were observational, risk of bias was assessed using the NOS. Overall, six studies were rated as low risk, while one had a moderate risk, as comparator samples were frequently derived from different anatomical sites or collected at different times potentially limiting the reliability of molecular concordance. Assessment was conducted independently by two reviewers (L. Vaz Rodrigues and J. Oliveira), with full agreement. A detailed breakdown is provided in supplementary table S1.

Data synthesis

Study population and diagnostic methodologies

Across the included studies, there was a cumulative total of 506 patients (range 17–214 per study) with a median age ranging from 63 to 72 years. The proportion of male patients varied from 37% to 60%, while female representation ranged from 40% to 63%. Smoking status was not detailed in any of the included studies. Only Wu *et al*. [26] reported tumour staging using the 8th edition of the TNM (tumour, node, metastasis) classification [36]. In that cohort (n=214), most patients were stage IV (n=160), followed by stage III (n=28), II (n=10) and I (n=16). EBUS-TBNA was the primary diagnostic method applied in all studies, often combined with computed tomography-guided transthoracic biopsy or other bronchoscopy techniques. Histological classification varied, with adenocarcinoma being the most common diagnosis, followed by squamous cell carcinoma and NSCLC not otherwise specified. Needle gauges (G) for EBUS-TBNA ranged from 19 G to 25 G, and rapid on-site evaluation was reported in four studies. Only Hannigan *et al*. [24] reported the number of needle passes, averaging two to three per case. Next-generation sequencing (NGS) was used in all studies: alone in four, combined with droplet digital (dd)PCR in two, and with amplification refractory mutation system (ARMS)-PCR in one. The key methodological details of the included studies are summarised in table 1. Based on these data, the following sections provide a detailed analysis of the diagnostic performance, molecular testing feasibility and the impact of methodological variables on the results.

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Feasibility of supernatant based molecular testing

All seven studies confirmed that EBUS-TBNA supernatant is a viable source for molecular testing, with feasibility rates ranging from 87% to 100%. This finding was consistent throughout the different studies, despite variability in sample processing protocols, including storage conditions (−80°C to 5°C), centrifugation speeds (reported as 400–10 000×*g* or 600–16 000 rpm), and preservation media (CytoLyt, RPMI, physiological saline or ThinPrep). While no clear trend was observed between storage temperature and feasibility, studies employing multistage centrifugation protocols consistently reported 100% feasibility, whereas those using single-step centrifugation had the lowest feasibility rates (87–90%).

Feasibility was also evaluated in relation to molecular testing methods. Most studies employed NGS as the primary analysis technique, while some incorporated ddPCR [23, 24] or ARMS-PCR [26]. Among studies using NGS exclusively, feasibility ranged from 87% to 100% [25, 27, 28]. In studies combining NGS with ddPCR or ARMS-PCR, feasibility was between 90% and 100%. Given the narrow range of feasibility across all molecular methods, no meaningful differences were observed.

DNA yield from supernatant samples

Six of the seven included studies reported quantitative DNA yields from supernatant, showing substantial variability. Median values ranged from 0.77 ng·µL^{-1} [26] to 85.5 ng·µL^{-1} [21]. Gokozan *et al.* [27] did not report DNA yield.

Notably, high-speed centrifugation protocols, such as those used by Wu *et al.* [26] (10 000 rpm) and Jager *et al.* [28] (16 000 rpm), were associated with lower DNA yields (0.77–4.7 ng·µL^{-1}), compared to moderate-speed processing (*e.g.* 600×*g* Hannigan *et al.* [24]), which yielded 6.8 ng·µL^{-1}.

Differences in preservative solutions and storage conditions were evident. While most studies utilised CytoLyt or physiological saline, Hannigan *et al.* [24] employed RPMI medium, and Tafoya *et al.* [21] used ThinPrep. Storage temperatures varied from −80°C [23] to 5°C [21], while storage durations ranged from <1 day [28] to a median of 14 days [25]. Table 2 summarises the protocols for sample handling and DNA yield from EBUS-TBNA supernatant, illustrating considerable heterogeneity in centrifugation, storage and preservation media across studies.

Pooled DNA yields were calculated across six studies and stratified by preservation medium and storage temperature.

For preservation medium, studies using CytoLyt (k=2) showed a pooled mean DNA yield of 24.36 ng·µL^{-1} (95% CI 11.94–36.77), with high heterogeneity (I^2=88.7%; Q=8.84; p<0.001; τ^2=71.21). Studies using other media (k=4) had a pooled mean of 61.92 ng·µL^{-1} (95% CI −23.82–147.66; I^2=99.8%; Q=53.19; p<0.001; τ^2=7174.25). Combining all six studies, the overall pooled mean was 40.10 ng·µL^{-1} (95% CI 1.25–78.94; I^2=99.5%; Q=69.96; p<0.001; τ^2=2139.56). Despite high intragroup heterogeneity, subgroup differences were not statistically significant (Q=0.33; p=0.5664). Figure 2a shows the forest plot stratified by preservation medium.

A separate analysis by storage temperature is shown in figure 2b. Studies using 4°C (k=3) had a pooled mean yield of 19.26 ng·µL^{-1} (95% CI 7.38–31.13; I^2=93.3%; Q=34.24; p<0.0001; τ^2=102.39). Studies using other temperatures (k=3) showed a pooled mean of 84.05 ng·µL^{-1} (95% CI −35.39–203.49; I^2=99.5%; Q=17.27; p=0.0002; τ^2=10 450.95). The overall model again indicated significant heterogeneity (I^2=99.5%; Q=69.96; p<0.0001; τ^2=2139.56), with a combined mean of 40.10 ng·µL^{-1} (95% CI 1.25–78.94). As with preservation medium, no significant difference was found between temperature subgroups (Q=1.15; p=0.2834).

Diagnostic accuracy of supernatant

All included studies utilised either cytology cell-blocks or histological tissue biopsies as the reference standard for molecular analysis. Sensitivity of supernatant molecular analysis compared to reference standard was consistently high, ranging from 0.95 to 1.00, with full concordance in several studies (supplementary table S2). Guibert *et al.* [23] and Janaki *et al.* [25] reported 100% agreement between supernatant and reference standard for all mutations analysed. Wu *et al.* [26] reported a sensitivity of 0.98, with six discordant cases (three false positives and three false negatives). Specificity, when measurable, ranged from 0.80 to 1.00. However, specificity could not be determined in Guibert *et al.* [23] and Janaki *et al.* [25] due to the absence of true negative cases. Due to heterogeneity in study design, variations in sample size, and the absence of true negative cases in some studies, pooled sensitivity and specificity

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TABLE 2 Sample handling and DNA yield from endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) supernatant: centrifugation protocols, storage conditions and preservant solutions across studies

First author (year) [reference]	Sample handling					Supernatant DNA yield ng·µL^{-1}
	Preservant solution	Storage temperature	Storage time days	1st centrifugation	2nd centrifugation	
Guibert (2018) [23]	CytoLyt or Physiological saline	−80°C	NS	400×*g* duration NS	1500×*g* duration NS	4.8 (0.3–79)
Hanningan (2019) [24]	Needle rinse in RPMI	4°C	NS	600×*g* for 10 min	NS	6.8 (0.2–107)[#]
Janaki (2019) [25]	CytoLyt	4°C	14 (7–29)	1200×*g* for 5 min	10 000×*g* for 5 min	6.6 (0.4–56.4)[¶]
Wu (2020) [26]	CytoLyt	−20°C	NS	3000 rpm for 5 min	10 000 rpm for 10 min	0.77 (<0.05–120)
Tafoya (2021) [21]	ThinPrep	5°C	6 (3–41)	NS	NS	85.5 (<1–699)
Jager (2024) [28]	Physiological saline	4°C	<1[+]	2000 rpm for 10 min	16 000 rpm for 10 min	4.7 (0.1–28.6)[§]

Data are presented as median (range), unless otherwise stated. NS: not specified. [#]: DNA concentration (ng·µL^{-1}) was calculated by dividing the total extracted DNA (ng) by the reported elution volume (60 µL), as stated in the original study; [¶]: DNA concentration (ng·µL^{-1}) was calculated using the elution volume (50 µL) specified in the Maxwell RSC Nucleic Acid Purification Kit, as referenced in the original study; [+]: molecular testing of the supernatant was initiated within 24 h of sample collection; [§]: DNA concentration (ng·µL^{-1}) was calculated by dividing the total extracted DNA (ng) by the elution volume (15 µL), based on the MagMAX Cell-Free Total Nucleic Acid Isolation Kit protocol .

estimates were not calculated to prevent potential bias. Instead, individual study results were reported in detail to ensure accuracy and preserve the integrity of the findings (supplementary table S2).

Comparison of molecular profiling results in supernatant and reference standard

The agreement between supernatant and reference standard samples was consistently high, with concordance rates ranging from 83% to 100%, depending on study design and sample comparison criteria (table 1).

Most studies reported full agreement in mutation profiling between supernatant and reference standard. However, Hannigan *et al.* [24] noted variability based on biopsy conditions: concordance was 97% when samples were collected from the same site and day, decreasing to 89% when obtained from a different site on the same day, and to 83% when from a different site and day.

Across all studies, Cohen's κ was 0.947 (95% CI 0.905–0.989), indicating almost perfect agreement between supernatant and reference standard molecular results.

Regarding the detection of clinically actionable mutations, a total of six studies provided disaggregated mutation-level data enabling direct comparison between supernatant and reference standard results (supplementary table S3). One of these studies [26] focused exclusively on *EGFR* mutations in a cohort of 214 patients and did not report data on other genes.

EGFR mutations were reported in 169 individuals, corresponding to 33.4% of the total cohort. In one study, involving 214 patients, *EGFR* mutations were identified in 120 (56.1%) cases, although specific mutation subtypes were not detailed [26]. In the remaining studies, where individual *EGFR* variants were specified, the most frequently reported were L858R (18 cases, 3.6%), followed by T790M (eight cases, 1.6%) and exon 19 deletions (five cases, 1.0%). The concordance between supernatant and reference standard for *EGFR* mutations was 96.4% (163 out of 169 cases), with six discordant results (1.2% of the total cohort). Cohen's κ for *EGFR* was 0.974 (95% CI 0.948–0.999), indicating almost perfect agreement.

KRAS mutations were identified in 39 patients across the included studies, corresponding to 13.4% of the 292 patients tested for this gene. The most frequently reported mutations were G12C (14 cases, 4.8%), G12D (eight cases, 2.7%), and G12A (five cases, 1.7%). Concordant detection occurred in 38 (97.4%) out of 39 cases, with a Cohen's κ of 0.971 (95% CI 0.913–1.000), indicating almost perfect agreement.

ALK rearrangements were reported in four (1.4%) patients across the included studies, all of which were consistently detected in both supernatant and reference standard samples, corresponding to 100%

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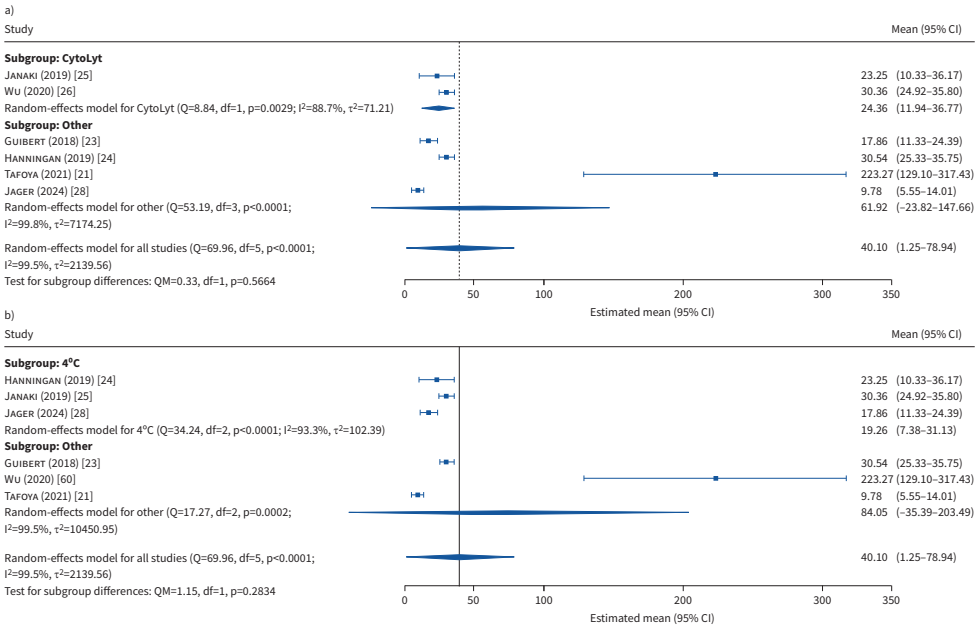

FIGURE 2 Forest plots from the meta-analysis, stratified by a) preservant solution and b) storage temperature variables, displaying subgroup estimates and the overall estimate. a) When all six studies were combined, the overall random-effects model yielded a mean estimate of 40.10 (95% CI 1.25–78.94) with very high heterogeneity (I^2=99.5%; Q=69.96; p<0.001; τ^2=46.26). For studies using CytoLyt (k=2), the pooled estimate was 24.36 (95% CI 11.94–36.77), with significant heterogeneity (I^2=88.7%; Q=8.84; p<0.001; τ^2=71.21). In the subgroup with other preservation conditions (k=4), the pooled estimate was 61.92 (95% CI −23.82–147.66) and heterogeneity remained high (I^2=99.8%; Q=53.19; p<0.001; τ^2=7174.25). No statistically significant differences were found between subgroups (QM=0.33; p=0.566). b) The random-effects model for studies classified in the 4°C group (k=3) yielded a pooled estimate of 19.26 (95% CI 7.38–31.13), with high heterogeneity (I^2=93.3%; Q=34.24; p<0.001; τ^2=10.12). In contrast, studies in the other temperature group (k=3) showed a pooled estimate of 84.05 (95% CI −35.39–203.49; p=0.1678), with very high heterogeneity (I^2=99.5%; Q=17.27; p<0.001; τ^2=102.23). The test for subgroup differences showed QM=1.15, df=1, p=0.2834, indicating no statistically significant difference between groups.

concordance. *ERBB2* alterations, including exon 20 insertions and gene amplifications, were identified in seven (2.4%) patients, also with complete agreement between supernatant and reference standard. *MET* exon 14 skipping was reported in a single case (0.3%) in the study by Janaki *et al.* [25], with concordant detection in both sample types. *BRAF* mutations were detected in 11 (3.8%) patients and included variants such as V600E, V600R, D594N, N581S, K601N and G466E. Of these, nine were identified in both supernatant and reference standard, while two were detected only in the reference standard, resulting in a concordance of 81.8%. Other molecular alterations with potential clinical or prognostic relevance (such as those involving *TP53*, *STK11*, *PIK3CA*, *IDH1*, *PTEN*, *FGFR* family genes, *CDKN2A* and *SMAD4*) were also frequently reported across studies, with high rates of agreement between supernatant and reference standard (supplementary table S3). Due to the low sample size of the subgroups, κ-statistics were not performed for *ALK*, *ERBB2*, *MET*, *BRAF* and other less frequent molecular alterations.

Turnaround time

The use of supernatant derived from EBUS-TBNA samples for molecular characterisation of NSCLC demonstrated a consistent reduction in TAT across several studies. Guibert *et al.* [23] highlighted that supernatant offers an immediate source of fresh DNA, potentially decreasing TAT compared to traditional cell-block material. Hannigan *et al.* [24] reported a significant reduction, with tissue-based NGS requiring 5–7 days *versus* 2–3 days for supernatant samples. Similarly, Janaki *et al.* [25] observed a decrease from

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10±5.5 days to 3–5 days with the use of supernatant. Gokozan *et al.* [27] also reported a reduction in TAT from 12.2±5.3 days (reference standard) to 8.5±1.8 days (supernatant). Additionally, Jager *et al.* [28] noted a qualitative reduction in TAT by 1 day when using supernatant, compared to the 4–10 days required for the reference standard. These findings are summarised in table 3. Due to the limited number of studies reporting TAT with consistent metrics and the heterogeneity in how TAT was measured, no statistical pooling or meta-analysis was conducted.

Discussion

This systematic review and meta-analysis demonstrates that supernatant obtained from EBUS-TBNA samples is a feasible and reliable source for molecular profiling in NSCLC. Across the seven included studies, feasibility rates ranged from 87% to 100%. Despite heterogeneity in sample handling protocols, DNA yields were consistently sufficient for downstream molecular techniques. Moreover, the diagnostic performance of supernatant samples was comparable to that of conventional tissue or cytology-based specimens. Molecular concordance between supernatant and reference standard ranged from 83% to 100%, with a pooled Cohen's κ of 0.947, reflecting almost perfect agreement. Notably, actionable mutations, including *EGFR*, *KRAS*, *ALK*, *ERBB2*, *MET* and *BRAF*, were detected in supernatant with high accuracy when compared to reference standard, further supporting its utility in therapeutic decision-making. Additionally, most studies reported a reduction in TAT for molecular results when using supernatant, with differences ranging from 2 to 7 days. These findings reinforce the potential of supernatant as a robust and time-efficient alternative or complementary sample in molecular diagnostic workflows.

Minimally invasive diagnostic approaches play a critical role in the molecular characterisation of NSCLC, with EBUS-TBNA now established as a cornerstone technique [37–39]. Beyond its pivotal role in mediastinal staging, EBUS-TBNA has proven highly effective for obtaining diagnostic material in NSCLC, allowing simultaneous histopathological confirmation and molecular profiling from a single procedure [39–41]. However, limited sample volume and cellularity may constrain biomarker testing, particularly in advanced disease where comprehensive profiling is essential for therapeutic decisions [41, 42].

To address the issue of sample exhaustion, liquid biopsy has emerged as a complementary strategy [18]. While plasma-derived ctDNA is widely used in advanced NSCLC, its sensitivity varies significantly by mutation type [20, 43]. Alternative biological sources beyond plasma have therefore been investigated to enhance the diagnostic sensitivity of liquid biopsy approaches, including pleural fluid in patients with malignant pleural effusions [44] and bronchial lavage fluid obtained during bronchoscopy [45]. In the context of liquid biopsy strategies, supernatant derived from different cytological sample preparations is emerging as a valuable, reliable and most often underutilised source for molecular testing, as was recently emphasised in a narrative review by Roy-Chowdhuri [46].

TABLE 3 Detailed summary of turnaround time (TAT) reduction using supernatant from endobronchial ultrasound-guided transbronchial needle aspiration samples for molecular characterisation of nonsmall cell lung cancer

First author (year) [reference]	Conventional tissue analysis (TAT) days	Supernatant analysis (TAT) days	Time gain (reduction) days	Description
Guibert (2018) [23]	Not specified	Not specified	Not quantified	The study mentions that the supernatant provides an immediately available source of fresh DNA, potentially reducing TAT, but does not specify exact time reductions
Hanningan (2019) [24]	5–7	2–3	2–4	NGS analysis of tissue typically takes 5–7 days, while supernatant extraction reduces this to 2–3 days, resulting in a reduction of 2–4 days
Janaki (2019) [25]	10±5.5	3–5	5–7.5	TAT for tissue samples averages 10±5.5 days, while using supernatant reduces the TAT to 3–5 days, resulting in a reduction of 5–7.5 days
Gokozan (2020) [27]	12.2±5.3	8.5±1.8	3.7±0.8	Tissue samples take 12.2±5.3 business days, while supernatant reduces the TAT to 8.5±1.8 days, resulting in a reduction of 3.7±0.8 days
Jager (2024) [28]	4–10	1	3–9	TAT of NGS results was 1 day for supernatant compared to 4–10 days (mean 6.9 days) for corresponding core biopsies

Time gains (reductions) are calculated based on the difference in TAT between conventional tissue analysis and supernatant analysis where specific times are provided. NGS: next-generation sequencing.

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Among these alternatives, supernatant derived from EBUS-TBNA samples offers several advantages. Unlike peripheral blood, which reflects DNA shed systemically, EBUS-TBNA supernatant is collected directly from the tumour microenvironment during sample processing. This proximity may result in a higher concentration and greater representativeness of tumour-specific genetic material. Our systematic review supports this hypothesis, with feasibility rates between 87% and 100% across all included studies. Interestingly, one study documented higher DNA concentrations in supernatant than in the corresponding cellular pellet, suggesting that the acellular phase may retain significant amounts of tumour-derived nucleic acids, possibly due to the accumulation of DNA-rich cellular debris [21]. However, the DNA yield from supernatant samples showed notable variability, which appears to be influenced by pre-analytical factors such as sample processing protocols, storage conditions, centrifugation speeds and preservation media.

The studies included in this review revealed substantial heterogeneity in the pre-analytical processing of EBUS-TBNA supernatant samples, particularly regarding storage temperature, preservative media and centrifugation protocols (table 2). This variability significantly affected the comparability of studies and the strength of pooled results. Despite the methodological heterogeneity, subgroup analyses for storage temperature and preservative solutions were feasible and showed no statistically significant differences in DNA yield. However, the associated high heterogeneity limits the generalisability of these findings (figure 2). The impact of methodological heterogeneity was even more pronounced for other variables, particularly centrifugation protocols. Speeds, g-forces and processing times were inconsistently reported across studies. Due to this lack of standardisation, no formal meta-analysis could be performed for this parameter. Nevertheless, individual studies suggested that high-speed centrifugation may negatively impact DNA yield. Wu *et al.* [26], using a high-speed centrifugation protocol (10 000 rpm), reported the lowest DNA concentration (0.77 $ng \cdot \mu L^{-1}$), whereas Hannigan *et al.* [24], employing a more moderate protocol (1500 rpm), achieved higher yields (6.8 $ng \cdot \mu L^{-1}$). One possible explanation is that excessive centrifugal force may induce DNA fragmentation, reducing yield, an effect described in other contexts, including spermatozoa [47] and *Escherichia coli* DNA studies [48]. While this hypothesis is biologically plausible, the findings must be interpreted with caution. The wide methodological variability between studies precludes firm conclusions regarding optimal pre-analytical protocols and limits the broader applicability of individual observations.

Additionally, this heterogeneity prevented a formal meta-analysis of diagnostic accuracy metrics, such as sensitivity and specificity. Differences in the definition of reference standard, variability in the molecular targets assessed, and frequent absence of true negative cases made pooled estimates methodologically unsound. Available individual data are presented in supplementary table S2, but should be interpreted cautiously and within the methodological context of each study. Overall, these inconsistencies underscore the need for prospective studies adopting standardised workflows to enable reproducibility and improve data comparability.

Despite the methodological heterogeneity, this review found consistently high concordance between the molecular profiling results obtained from EBUS-TBNA supernatant and reference standard. Specifically, actionable mutations such as *EGFR* and *KRAS* were reliably detected in supernatant, with agreement rates ranging from 83% to 100% and pooled Cohen's κ-values nearing 1.0, indicating almost perfect agreement. Discordant cases were rare and may be attributed to several biological and technical factors, such as low tumour cellularity in individual samples [28], variability in DNA shedding related to tumour stage, burden and vascularisation [49], or differences in sequencing platforms and amplicon design [24]. Temporal and spatial mismatches in sample acquisition also affected agreement. This was illustrated in the study by Hannigan *et al.* [24], where lower concordance was observed when reference standard samples were obtained from different sites and at different times. Such discrepancies probably reflect not only technical factors, but also biological variability, as spatial and temporal heterogeneity in solid tumours can lead to distinct molecular profiles and introduce bias when comparing samples [50, 51]. In addition, the limited number of studies restricted subgroup analyses for less frequent mutations such as *ALK*, *ERBB2*, *BRAF* and *MET*. Although numerical concordance was high, their low cumulative frequency prevented the calculation of Cohen's κ, underscoring the need for larger datasets and standardised reporting to support robust comparisons for these targets.

The molecular alterations analysed in this review are among the most clinically relevant in NSCLC and are prioritised in current treatment algorithms by both the National Comprehensive Cancer Network [3, 17] and the European Society for Medical Oncology [30, 52]. Importantly, the molecular landscape of NSCLC continues to evolve, driven by the discovery of new predictive biomarkers, resistance mechanisms and therapeutic targets [53]. As precision oncology advances, the scope and complexity of molecular testing is

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expected to expand further. In this dynamic context, sample exhaustion has emerged as a growing barrier to timely and complete molecular profiling [15, 42]. This underscores the importance of maximising the utility of all available biological specimens, particularly those obtained through minimally invasive techniques such as EBUS-TBNA. In this context, the supernatant, routinely discarded during conventional processing, emerges as a valuable and underutilised resource. As highlighted in our review, supernatant consistently provided sufficient nucleic acids and delivered high diagnostic performance. These findings align with previous studies and reinforce its potential to reduce the need for additional procedures [54].

One of the most promising and clinically meaningful advantages of using supernatant from EBUS-TBNA samples is the potential to reduce TAT for molecular results. Although heterogeneity among the included studies precluded data pooling, five studies [23–25, 27, 28] reported meaningful reductions in TAT, ranging from 1 to 7.5 days when compared to reference standard. This gain is largely attributed to the immediate availability of ctDNA in supernatant and the ability to initiate molecular workflows on the same day of sample collection, bypassing delays associated with tissue processing. Reducing TAT is not a trivial matter, as delays in initiating systemic therapy can negatively impact prognosis, particularly in patients with rapidly progressing disease [55]. Interestingly, although clinical guidelines recommend a TAT of <10 working days [16], real-world studies often report longer intervals, reflecting logistical and institutional barriers [56–58]. These delays represent a well-recognised obstacle to the implementation of precision oncology [58, 59] and have been associated with reduced survival in large cohort studies [60]. These discrepancies between molecular testing guideline recommendations and real-world clinical practice highlights the need for diagnostic strategies that are not only biologically effective but also operationally efficient, such as reflex test protocols, or the early incorporation of liquid biopsy approaches [58, 59]. Alternatively, integrating supernatant from EBUS-TBNA samples into molecular workflows may offer a pragmatic solution capable of reducing TAT while simultaneously minimising the need for additional sample acquisition or repeat biopsies. These advantages may be particularly valuable in resource-limited settings, where access to invasive procedures, histological processing, or comprehensive molecular platforms is often constrained [37, 61]. In such contexts, the use of supernatant may help reduce the need for re-biopsies and accelerate molecular diagnostics using existing resources, although this was outside the scope of the present review and remains to be demonstrated in dedicated studies.

This study has some limitations that should be acknowledged. First, only seven studies met our inclusion criteria, which were specifically restricted to NSCLC and supernatant derived from EBUS-TBNA samples. While this narrow scope allowed for focused analysis, it also limited the breadth of available evidence and reduced the statistical power of subgroup analyses. Additionally, heterogeneity in the methodologies used across studies (centrifugation speed, storage time, preservation solutions and DNA extraction protocols) hindered direct comparisons and constrained our ability to identify the most effective practices for optimising DNA yield. Consequently, subgroup analyses were feasible only for a limited number of parameters (specifically, storage temperature and preservation medium) whereas the greater variability observed in others, such as centrifugation protocols, precluded formal meta-analysis. As a result, no definitive conclusions can be drawn about optimal pre-analytical workflows, and the generalisability of DNA yield findings remains limited.

The limited number of included studies also restricted the ability to perform mutation-specific concordance analyses, particularly for alterations with lower frequency. While *EGFR* and *KRAS* mutations were sufficiently represented to support more robust statistical evaluation, other clinically relevant targets, such as *ALK*, *BRAF*, *ERBB2* and *MET*, were reported too infrequently to allow pooled analysis.

Despite these constraints, to our knowledge, our systematic review and meta-analysis represents the first comprehensive synthesis focused on the use of EBUS-TBNA supernatant for molecular profiling in NSCLC. The findings are encouraging, demonstrating high feasibility, strong agreement with conventional samples, and potential operational advantages, including reduced TAT. These results support the integration of supernatant into diagnostic workflows as a complementary or rescue material, especially valuable when tissue is limited or exhausted.

Future studies should aim to standardise supernatant processing protocols and validate their use in larger, prospective cohorts. In addition to evaluating technical performance, future research should also explore clinical implementation pathways and assess real-world impact on turnaround times, treatment decision-making, and patient related outcomes. Establishing best practices will be essential to support broader adoption and fully harness the diagnostic potential of this minimally invasive, high-yield biospecimen in precision oncology.

https://doi.org/10.1183/16000617.0127-2025

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Points for clinical practice

- The supernatant phase from EBUS-TBNA samples is a reliable and underutilised source for molecular profiling in NSCLC, with successful DNA extraction reported in 87% to 100% of cases and near-perfect concordance with tissue-based reference standards (Cohen's κ up to 0.974).
- Supernatant consistently provides sufficient DNA for next-generation sequencing and other molecular platforms, even when pre-analytical conditions vary.
- Supernatant use may reduce the need for re-biopsies, optimise available material, and accelerate turnaround time for molecular results, with reported reductions of up to 7.5 days.
- This practice may further enhance the strategic role of EBUS-TBNA as a cornerstone in minimally invasive precision oncology, aligning diagnostic efficiency with the growing complexity of biomarker testing.

Questions for future research

- What are the optimal pre-analytical protocols (*e.g.* centrifugation speed, storage temperature, preservation medium) to maximise DNA yield from EBUS-TBNA supernatant?
- Could the integration of supernatant analysis consistently reduce time-to-treatment and improve survival in prospective clinical pathways?
- What are the cost-effectiveness and scalability implications of adopting supernatant as a standard component in NSCLC molecular diagnostics?

Provenance: Submitted article, peer reviewed.

The systematic review protocol was registered with PROSPERO (https://www.crd.york.ac.uk/prospero/) with identifier: CRD42024600046.

Author contributions: Study conception and design: L. Vaz Rodrigues, V. Sousa, R. Cordovilla, L. Taborda-Barata. Data collection and analysis: L. Vaz Rodrigues, J. Oliveira, T. Maricoto. Statistical analysis: L. Vaz Rodrigues, J. Oliveira, L. Taborda-Barata. Drafting of the manuscript: L. Vaz Rodrigues, J. Oliveira. Critical revision of the manuscript for important intellectual content: All authors. All authors have read and approved the final version of the manuscript.

Conflict of interest: None of the authors have any conflicts of interest to disclose related to the publication of this manuscript. No financial, personal, or professional relationships exist that could inappropriately influence or bias the content, interpretation, or conclusions of this work.

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Study V – *Feasibility of DNA and RNA preservation from EBUS-TBNA supernatant for molecular profiling in Non-Small Cell Lung Cancer*, Luís Vaz Rodrigues, Joana Oliveira, Ana Filipa Ladeirinha, Ana Alarcão, Luis Taborda-Barata, Rosa Cordovilla, Vitor Sousa

Original Paper published in the format of Rapid-Communication, in *Pulmonology* (November 2025; https://doi.org/10.1080/25310429.2025.2588834).

PULMONOLOGY JOURNAL

Pulmonology

ISSN: 2531-0429 (Print) 2531-0437 (Online) Journal homepage: www.tandfonline.com/journals/tpul20

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To cite this article: Luís Vaz Rodrigues, Joana Oliveira, Ana Filipa Ladeirinha, Ana Alarcão, Luis Taborda-Barata, Rosa Cordovilla & Vitor Sousa (2025) Feasibility of DNA and RNA preservation from EBUS-TBNA supernatant for molecular profiling in non-small cell lung cancer, Pulmonology, 31:1, 2588834, DOI: 10.1080/25310429.2025.2588834

To link to this article: https://doi.org/10.1080/25310429.2025.2588834

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PULMONOLOGY
2025, VOL. 31, NO. 1, 2588834
https://doi.org/10.1080/25310429.2025.2588834

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Feasibility of DNA and RNA preservation from EBUS-TBNA supernatant for molecular profiling in non-small cell lung cancer

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ABSTRACT

Background: Endobronchial ultrasound-transbronchial needle aspiration (EBUS-TBNA) is used to diagnose and stage Non-Small Cell Lung Cancer (NSCLC), where the supernatant is discarded.

Research question: Can DNA/RNA extracted directly from formaldehyde-preserved EBUS-TBNA supernatant, provide sufficient and reliable molecular profiling in NSCLC?

Study design and methods: This prospective study included patients with advanced NSCLC (stage III-IV) undergoing EBUS-TBNA to compare DNA/RNA quantification, NGS feasibility and molecular findings between the supernatant phase (Sp) and the cell pellet (Cp).

Results: The median DNA and RNA concentrations were significantly higher in Sp than Cp, with Sp showing 29.9 ng/µL DNA and 52.12 ng/µL RNA versus 9.58 ng/µL and 13.6 ng/µL in Cp (Wilcoxon signed rank test, $p = 0.012$ and $p = 0.005$). MP in Cp identified 16 mutations (7 actionable), while Sp detected 19 mutations, including two additional actionable mutations. Concordance between Cp and Sp was 87%, with identical mutations in 13 cases and discrepancies in two cases.

Conclusions: Sp obtained from EBUS-TBNA is a rich source of tumour DNA and RNA, with high feasibility for NGS. There is strong concordance of NGS results between Cp and Sp, supporting Sp as a complementary/alternative source for molecular profiling in NSCLC, potentially reducing the need for additional biopsies.

ARTICLE HISTORY
Received 25 November 2024
Accepted 6 November 2025

KEYWORDS
Non-small cell lung cancer; endobronchial ultrasound-guided transbronchial needle aspiration; supernatant phase; molecular profiling; DNA/RNA preservation

Introduction

Endobronchial ultrasound-transbronchial needle aspiration (EBUS-TBNA) is widely used for diagnosing and staging non-small cell lung cancer (NSCLC).[1] Traditionally, only the cellular pellet (Cp) from EBUS-TBNA is used for molecular analysis, while the supernatant phase (Sp) is discarded. However, tumour DNA can shed into the surrounding environment as cell-free circulating tumour DNA (ctDNA),[2] enabling molecular profiling (MP) of solid tumours using alternative samples like peripheral blood or urine.[3]

This suggests ctDNA may also be present in the supernatant of EBUS-TBNA samples, which is currently discarded. Previous studies tested this hypothesis using Sp from lung carcinoma samples, showing promising results compared to tissue-based MP.[4–6] In these studies, Sp preparation required adjustments to the processing protocol, implying changes to standard practices.

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/25310429.2025.2588834.

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Our study aimed to assess the feasibility of obtaining adequate DNA and RNA for MP from Sp preserved directly in buffered formaldehyde, without altering the standard sample collection and pre-processing workflow.

Methods

In this ongoing prospective study, we are enrolling patients over 18 years old with histopathologically confirmed advanced-stage (III-IV) NSCLC referred for EBUS-TBNA and requiring MP as per clinical indication.[7] Exclusion criteria align with general contraindications for EBUS-TBNA.[8] Procedures are performed using a BF-UC180F bronchoscope (Olympus, Tokyo, Japan) under general anaesthesia, with a laryngeal mask securing the airway. TBNA is conducted with 21 G ViziShot 2 needles (Olympus, Tokyo, Japan), and aspirated material is preserved directly in buffered formalin. As rapid on-site cytology evaluation (ROSE) is not available at our institution, a minimum of four needle passes are performed per target lesion, following international guidelines.[9] Samples are centrifuged to separate the Cp, used for paraffin-embedded cell blocks. The Sp, typically discarded, is further centrifuged, washed with ethanol and processed for DNA and RNA extraction. Molecular profiling of both Cp and Sp is conducted via next-generation sequencing (NGS) using the Genexus platform and the Oncomine Precision Assay GX (ThermoFisher Scientific, US).

Comparative analysis of the DNA and RNA concentrations between the Cp and Sp were assessed using the non-parametric Wilcoxon signed-rank test due to the small sample size. Additionally, absolute and percentage agreement between molecular findings in the Cp and Sp were calculated to evaluate concordance. Statistical significance was set at $p < 0.05$.

All participants provide written informed consent, and the study is approved by the Ethics Committee of the Francisco Gentil Portuguese Oncology Institute of Coimbra (approval number 23–2022). Full details of the methodology are provided in the supplementary material.

Results

Between December 2023 and August 2024, we screened a total of 30 patients for study inclusion, obtaining informed consent for each. Among these, 15 NSCLC patients were included, while 15 were excluded due to the following reasons: presence of normal lymph node tissue in a staging context ($n = 9$), diagnosis of small cell lung cancer ($n = 3$), insufficient material for histopathologic analysis ($n = 2$) and squamous cell carcinoma not requiring MP ($n = 1$).

Patient characteristics

Included participants had a male predominance (60%) and a median age of 69 years (range: 42–84). Most patients were current smokers (60%) and had stage IV adenocarcinoma (87%). Full details of the participants included are presented in Table 1. EBUS-TBNA procedures were performed with a median of 4 needle passes per case (range: 4–6), with no differences between diagnostic and staging procedures.

DNA and RNA quantification

The average tumour cell content in Cp samples was 38% (range: 10%–70%), making all samples suitable for both PD-L1 and NGS analysis. In all 15 included cases, it was possible to separate both the Cp and Sp. In Cp, the median DNA and RNA concentrations were 9.58ng/µL (0.45–50) and 13.6ng/µL (2.4–56), respectively. By contrast, Sp showed higher mean concentrations, with median DNA at 29.9 ng/µL (0.78–51) and RNA at 52.12 ng/µL (5.95–95). Comparative analysis demonstrated statistically higher DNA and RNA concentrations in Sp than in Cp (Wilcoxon test, $p = 0.012$ and $p = 0.005$, respectively) (Figure 1).

Molecular profiling outcomes

In Cp samples, we identified 16 mutations, 7 of which were actionable, including *EGFR* ($n = 4$), *ERBB2* ($n = 2$) and *KRAS* G12C ($n = 1$). In Sp samples, a similar mutation profile was observed, with 19 mutations detected

Table 1. Detailed results of the participants included and the mutation profile of both cell pellet (Cp) and supernatant phase (Sp).

Case n°	Age (years)	Gender	Cp (% of tumour cells)	Cp NGS results (variant and allele frequency)	Sp NGS results (variant and allele frequency)
1	42	Female	10%	***EGFR*** Exon19 Deletion Glu746_Ala750del (18%)	***EGFR*** Exon19 Deletion Glu746_Ala750del (16.8%) ***MET*** Exon14 Skipping c.2942-1G>A;p (2,7%)
2	49	Male	40%	None detected	***KRAS*** Gly12Cys (33.2%)
3	84	Female	20%	***EGFR*** Exon 20 Insertion Val769_Asp770insPro (14%)	***EGFR*** Exon 20 Insertion Val769_Asp770insPro (10.6%)
4	70	Male	25%	***CTNNB1*** Ser37Cys (16.5%)	***CTNNB1*** Ser37Cys (11%) ***RET*** Val804Met (4.4%)
5	63	Female	70%	***EGFR*** Exon19 Deletion Glu746_Ala750del (43%) ***CDKN2*** Deletion (0.27x)	***EGFR*** Exon19 Deletion Glu746_Ala750del (36.4%) ***CDKN2*** Deletion (0.65x) ***PTEN*** Arg173His (5.8%)
6	83	Female	60%	***ERBB2*** Tyr772_Ala775dup (60.7%) ***ERBB2*** Amplification (6.3x)	***ERBB2*** Tyr772_Ala775dup (67.1%) ***ERBB2*** Amplification (5.34x)
7	80	Female	20%	***EGFR*** Exon 20 Insertion Val769_Asp770insPro (13%)	None detected (insuficient sample)
8	64	Male	50%	***KRAS*** Gly12Cys (28.5%)	***KRAS*** Gly12Cys (43%)
9	73	Male	50%	***TP53*** Glu286Lys (28.6%)	***TP53*** Glu286Lys (24.8%)
10	70	Male	30%	***BRAF*** Asp594_Thr599dup (29.3%)	***BRAF*** Asp594_Thr599dup (37.5%)
11	73	Male	50%	***ERBB2*** Tyr772_Ala775dup (23.3%)	***ERBB2*** Tyr772_Ala775dup (32.7%)
12	64	Male	50%	***TP53*** Val216Leu (66.6%) ***FGFR1*** Amp (5x)	***TP53*** Val216Leu (67.8%) ***FGFR1*** Amp (4.92x)
13	69	Female	10%	None detected	None detected
14	81	Male	50%	***TP53*** Pro152Leu (48%) ***BRAF*** Gly596Arg (21%)	***TP53*** Pro152Leu (56.2%) ***BRAF*** Gly596Arg (29.9%)
15	75	Male	40%	None detected	None detected

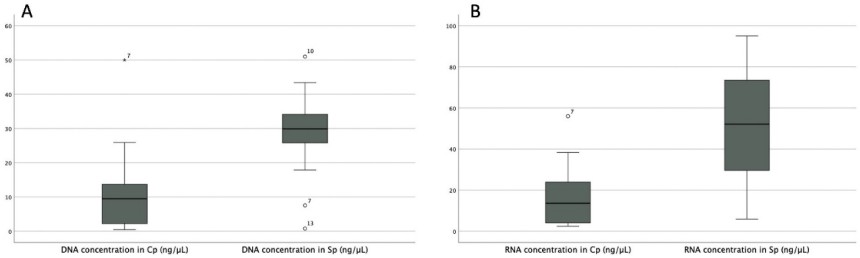

Figure 1. Boxplot comparing DNA (a) and RNA (b) concentrations (µg/dL) in paired samples from the cell pellet (Cp) and the supernatant phase (Sp) of EBUS-TBNA samples in NSCLC. Wilcoxon signed rank test results indicate that concentrations of both DNA and RNA were significantly higher in the Sp samples compared to Cp samples ($p = 0.012$ for DNA, $p = 0.005$ for RNA).

overall, including actionable mutations in *EGFR* ($n = 3$) and *ERBB2* ($n = 2$), along with 2 additional mutations not identified in Cp (*MET* Exon 14 Skipping in one patient and one additional *KRAS* G12C mutation that was not identified in the corresponding Cp). Across the 15 cases, concordance between Cp and Sp samples was 87%, with identical mutations observed in 13 cases, with similar allele frequencies. Discrepancies occurred in two cases: in one case, a *KRAS* mutation was detected in Sp but not in Cp, while in another, an *EGFR* exon 20 mutation found in Cp was undetected in Sp due to insufficient sample material for NGS. The full details of the NGS results in both Cp and Sp are presented in Table 1.

Discussion

Our preliminary findings suggest that Sp derived directly from EBUS-TBNA is a feasible and reliable source for DNA and RNA extraction, suitable for NGS-based MP in NSCLC. Notably, Sp yielded higher DNA and RNA concentrations, indicating that the liquid phase from EBUS-TBNA samples may complement or serve as an alternative to Cp, especially in cases with limited tissue yield. Furthermore, our findings indicate that Sp does not merely replicate Cp results but provides additional molecular insights. In three cases, Sp identified mutations not detected in Cp, while in two cases, mutations were exclusively found in either Sp or Cp. These results align with current literature on liquid biopsy and supernatant-based studies, which highlight extracellular nucleic acids as accurate molecular sources and reinforce ctDNA's role in capturing tumour heterogeneity.[2–6] The high concordance of NGS results between Cp and Sp, alongside additional actionable mutations identified in Sp, underscores Sp's potential as an adjunct to Cp in clinical practice, which could simplify diagnostic pathways and reduce turnaround times by offering a secondary source for MP without necessitating repeat biopsies. This could be particularly advantageous in advanced NSCLC, where rapid and comprehensive molecular characterisation is essential for therapeutic decision-making.[10,11]

Notably, our analysis utilised material preserved directly in buffered formaldehyde, with no apparent interference in viable DNA/RNA extraction for NGS. This approach contrasts with previous studies that required modifications in sample processing,[4–6] yet yielded similar molecular profiling results, further supporting the feasibility of implementing Sp analysis in routine practice without altering existing workflows. This could streamline diagnostics by avoiding additional processing steps required in other studies.

While promising, the study's small sample size limits its conclusions. To address this, we are actively expanding the ongoing prospective cohort to further explore Sp's ability to capture the full range of actionable alterations in NSCLC. Future research should also address how storage duration and technical factors impact DNA/RNA integrity to enhance Sp's clinical utility.

Acknowledgments

We extend our sincere gratitude to the bronchoscopy nurses and auxiliary staff, whose expertise and dedication were essential to the success of the EBUS-TBNA procedures. We also thank the technical teams in the pathology and genetics laboratories for their meticulous work in processing the samples, ensuring the quality and reliability of our data. Additionally, we would like to acknowledge Dr Luis Cirnes from the Institute of Molecular Pathology and Immunology at the University of Porto (IPATIMUP) for sharing DNA and RNA quantification results from the cell pellet analysis in two cases, which provided valuable complementary data. Lastly, we are profoundly grateful to all the patients who generously consented to participate in this study, making this research possible.

Author contributions

LVR, **RC**, **LTB**, and **VS** conceptualised the study, formulated the research questions, and contributed to writing the manuscript. **LVR** performed all EBUS-TBNA procedures and was responsible for collecting data from clinical files. **AA**, **AFL**, and **VS** were responsible for the molecular profiling protocol, cell pellet and supernatant phase preparation, DNA and RNA extraction and quantification and NGS methodology applied. **JO**, **LTB** and **RC** assisted with data collection, statistical analysis and critically reviewed the database. All authors provided critical review of the results, statistical analysis, and final content of the manuscript. All authors read and approved the final version of the manuscript and agree to be accountable for all aspects of the work, ensuring the accuracy and integrity of the research.

Disclosure statement

The authors declare not to have any conflicts of interest that may be considered to influence directly or indirectly the content of the manuscript.

Funding

This study was supported by a grant from ThermoFisher Portugal, which provided funding for the NGS analysis of the supernatant samples from the study participants.

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Artificial intelligence involvement

The authors declare that the material present on this paper has not been produced with the help of any artificial intelligence software or tool.

Data availability statement

The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request.

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Appendix 2

The following is the list of publications co-authored by the candidate that indirectly contributed to the development of this thesis and are referenced throughout the manuscript where relevant.

1. ***Pre- and post-COVID practice of interventional pulmonology in adults in Portugal***. Fernando Guedes, António Jorge Ferreira, Jorge Dionísio, **Luis Vaz Rodrigues**, Antonio Bugalho

Published as Original Paper in *Pulmonology*. 2024 Nov-Dec;30(6):537-545. Epub 2022 Mar 23.PMID: 35339419
DOI: 10.1016/j.pulmoe.2022.02.009

This publication provides an overview of interventional pulmonology practices in Portugal before and after the COVID-19 pandemic, with a particular focus on procedural availability and changes in clinical activity. It highlights the role of EBUS-TBNA, while unveiling persistent regional disparities in access to the procedure. The candidate contributed to all stages of the project, including the preparation and dissemination of questionnaires, processing and interpretation of the collected data, and the drafting and critical revision of the manuscript.

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2. ***Linear endosonography in lung cancer: a comprehensive review.*** Antonio Bugalho, Fernando Guedes, Francisco Freitas, **Luis Vaz Rodrigues**, Paul Frost Clementsen, Ralf Eberhardt, José Cepeda Ribeiro

Published as a Narrative Review in *Portuguese Journal of Cardiac, Thoracic and Vascular Surgery*. 2022 Apr 11;29(1):35-43. PMID: 35471220 Review.
DOI: 10.48729/pjctvs.239

This article reviews the expanding role of EBUS-TBNA in the diagnosis and staging of lung cancer. It emphasises its ability to provide high-quality samples for morphological assessment and its growing application in biomarker testing. The candidate contributed to the drafting and critical revision of the manuscript.

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3. ***EBUS-Guided Transbronchial Mediastinal Cryobiopsy: A Novel Technique for Diagnosing Mediastinal Lesions-Single-Center Experience.*** Márcia Araújo, **Luís Rodrigues**, Paulo Matos, Michele De Santis

Published as a Scientific Letter to the Editor, in *Archivos de Bronconeumologia*, 2024 Sep;60(9):593-594. Epub 2024 May 28. PMID: 38853118
DOI: 10.1016/j.arbres.2024.05.023

This study analyses a cohort of patients who underwent EBUS-guided mediastinal cryobiopsy, highlighting its added value in diagnostic yield. By demonstrating how obtaining larger tissue samples can enhance diagnostic performance, this work complements the focus of the thesis, which explores alternative strategies, such as optimising molecular workflows and leveraging the supernatant phase, to maximise the efficiency and utility of EBUS-TBNA samples. The candidate contributed to all phases of the project, including study design, performance of procedures, data collection, statistical analysis, and support in the drafting and critical revision of the manuscript.

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4. ***Bronchoalveolar Lavage Proteomics in Patients with Suspected Lung Cancer.*** Ana Sofia Carvalho, Célia Marina Cuco, Carla Lavareda, Francisco Miguel, Mafalda Ventura, Sónia Almeida, Paula Pinto, Tiago Tavares de Abreu, **Luís Vaz Rodrigues**, Susana Seixas, Cristina Bárbara, Mikel Azkargorta, Felix Elortza, Júlio Semedo, John K Field, Leonor Mota, Rune Matthiesen

Published as Original Paper at *Scientific Reports*, 2017 Feb 7:7:42190. PMID: 28169345
DOI: 10.1038/srep42190

This work explores the potential of non-conventional liquid biopsy sources, specifically bronchoalveolar lavage, for biomarker discovery in patients with suspected NSCLC, aligning with the thesis by reinforcing the value of tumour-proximal samples and innovative liquid biopsy approaches for molecular and biomarker analysis. The candidate contributed to various stages of the project, including participant selection, data analysis, and critical review of the manuscript.

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5. ***Is the Proteome of Bronchoalveolar Lavage Extracellular Vesicles a Marker of Advanced Lung Cancer?*** Ana Sofia Carvalho, Maria Carolina Strano Moraes, Chan Hyun Na, Ivo Fierro-Monti, Andreia Henriques, Sara Zahedi, Cristian Bodo, Erin M Tranfield, Ana Laura Sousa, Ana Farinho, **Luís Vaz Rodrigues**, Paula Pinto, Cristina Bárbara, Leonor Mota, Tiago Tavares de Abreu, Júlio Semedo, Susana Seixas, Prashant Kumar, Bruno Costa-Silva, Akhilesh Pandey, Rune Matthiesen

Published as Original Paper in *Cancers*, 2020 Nov 20;12(11):3450. PMID: 33233545; PMCID: PMC7699733
DOI: 10.3390/cancers12113450

This work investigates the proteomic profile of extracellular vesicles from bronchoalveolar lavage in lung cancer, revealing associations with advanced disease stage and poor survival. It aligns with the thesis by highlighting the potential of tumour-proximal, non-conventional samples for biomarker discovery and prognostic assessment, complementing the exploration of EBUS-TBNA supernatant for molecular profiling in NSCLC. The candidate participated in multiple phases of the work, notably in the selection of participants, support in data interpretation, and the critical revision of the manuscript, contributing to the integration of clinical and molecular perspectives.
