UNIVERSITY OF GENOA
FACULTY OF MEDICINE AND SURGERY
DEGREE COURSE IN MEDICINE AND SURGERY

Front-line liquid biopsy for early molecular assessment and treatment of hospitalized lung cancer patients

Biopsia liquida di prima linea per la valutazione molecolare e il trattamento precoce dei pazienti ospedalizzati affetti da cancro al polmone

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1. Epidemiology of Lung Cancer

The incidence of lung cancer in Italy in 2020 has been estimated at 41,000 new diagnoses divided into 27,550 men and 13,300 women, making it the second most frequent neoplasm in men (15%) and the third in women (6%).

In 2021, 34,000 deaths from lung cancer were estimated (men = 23,100; women = 10,900). [2]

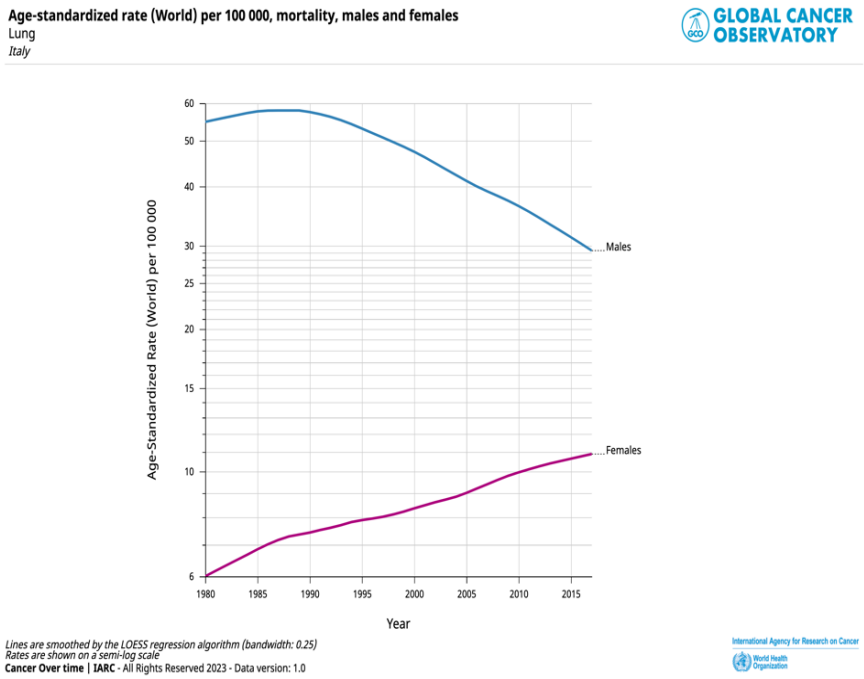
An important fact to note is that in both incidence and mortality in males, there has been a decrease of 11.2% since 2015, while in females it is 5.2% higher in 2020 and this correlates with an increase in smoking habits since the 1980s. [3]

In fact, cigarette smoking is identified as the main risk factor to which 85-90% of cases of new onset can be attributed, closely related to the duration of the habit, the number of cigarettes and the concentration of tar contained. [3]

In second place is radon when combined with cigarette smoke (15% of cases). [4]

Unfortunately, due to the absence of early symptoms and effective screening methods for detecting the disease in its early stages, patients are often identified as having advanced-stage lung cancer, which negatively impacts overall mortality rates.

Only a quarter of patients are diagnosed at an early stage because they are typically asymptomatic, and their neoplasms are incidentally identified during the evaluation of unrelated issues. Currently, more than half of the patients have advanced lung cancer at the time of diagnosis, coming to the attention of the doctor due to symptoms related to the primary tumor, metastases to distant sites, or paraneoplastic syndromes. The most common symptoms are cough, hemoptysis, and dyspnea. [13]



2. Anatomopathological classification

Anatomopathological definition is essential for structuring an appropriate treatment plan.

The fundamental distinction concerns two distinct groups: non-small cell lung cancer (NSCLC), accounting for 85-90% of all lung neoplasms, and small cell lung cancer (SCLC).

According to the WHO classification we can identify four main histotypes:

I. adenocarcinoma: malignant epithelial tumor with glandular differentiation. It often presents peripheral localization in the airways, more typical in women and non-smokers.
Immunohistochemically we often find it positive for thyroid transcription factor TTF1, cytokeratin 7 and napsin.

II. squamous cell carcinoma: malignant epithelial tumor with keratinization and/or presence of intercellular bridges. Mostly presents central localization (pulmonary hilum) in the airways, highly related to smokers.
Immunohistochemically we often find it positive for p63, p40, high molecular weight cytokeratin and desmocollin-3.

III. large cell carcinoma: represents a diagnosis of exclusion from the other variants; lacks distinctive cytological features

IV. small cell carcinoma: a tumor of epithelial origin with relatively small cells and little cytoplasm. Related to smokers with positivity for cytokeratin, chromogranin A, TTF1 and by a high proliferative index usually expressed with Ki67 > 70%.

National and international guidelines recommend that all patients with stage IIIb-IIIc and stage IV lung adenocarcinoma should be screened for EGFR (Epidermal Growth Factor Receptor) mutations, translocations of ALK (Anaplastic Lymphoma Kinase) and ROS-1 (c-ros1), mutations in BRAF (v-Raf murine Sarcoma Viral Oncogene Homolog B) and evaluation of PD-L1 (programmed Death-Ligand 1), the latter for adenocarcinomas and squamous cell carcinomas.
Other molecular alterations with clinical relevance are rearrangements of the RET and NTRK 1-3 genes, amplifications or mutations of exon 14 of MET or exon 2 of the KRAS gene. [5]

3. Diagnosis and staging

The diagnostic procedure in suspected lung cancer includes an accurate reconstruction of the clinical history (including tobacco habit, weight variations and performance status), complete physical examination to which laboratory and radiological examinations are supplemented by performing semi-invasive procedures to obtain tissue samples for typing. [5]

The first-level radiological investigation is by X-ray, which in the case of the presence of masses or nodules must be further investigated by CT scan.

Computed axial tomography (possibly with contrast medium, unless absolutely contraindicated) should be done in the chest and upper abdomen if any other secondary localizations are present.

If the CT scan turns out to be negative, an 18F-FDG PET-CT (positron emission tomography with 18-fluoro-desoxyglucose combined with CT) would still be necessary. [3]

In case of positivity, however, the CT scan allows us to assess the extent of lymph node involvement and presence of any metastases.

It is necessary to perform encephalic CT scans at the beginning of the staging; indeed, this procedure is mandatory for patients with stage II or higher NSCLC.

The anatomic extent of cancer is described by the TNM staging system, which has three components: T the extent of the primary tumor, N regional lymph nodes involvement and M distant metastases. [6]

The TNM classification has several purposes: definition of the appropriate treatment for the patient, prognostic indication, eligibility and stratification of patients within clinical trials. [7].

It is only possible to reach a diagnosis of certainty by means of anatomopathological findings.

The choice of procedure depends on the location of the primary tumor (i.e. whether it is central or peripheral).

If the lesion is central, thus accessible through endoscopy, or localized in the first inner third of the lung, it will characterized by bronchoscopy with endobronchial ultrasound (EBUS).

In peripheral lesions, on the other hand, which are not visible endoscopically, cyto-histological characterization is more complex and is done using other imaging techniques to guide bronchoscopy sampling. [3]

Table A- TNM Classification of Lung Cancer, Eighth Edition

Primary Tumor (T)	
TX	The primary tumor cannot be assessed, or the tumor is defined by the presence of malignant cells in sputum or bronchial washings but is not visualized on imaging or bronchoscopy.
T0	No evidence of tumor.
Tis	Carcinoma in situ. Squamous cell carcinoma in situ (SCIS). Adenocarcinoma in situ (AIS): Adenocarcinoma with a pure lepidic pattern, ≤3 cm in its greatest dimension
T1	Tumor size ≤3 cm, surrounded by lung or visceral pleura, without evidence of proximal bronchial invasion on bronchoscopy (i.e., not in the main bronchus)
T1mi	Minimally invasive adenocarcinoma: Adenocarcinoma (≤3 cm in greatest dimension) with predominantly lepidic pattern and ≤5 mm invasion in greatest dimension
T1a	umor ≤1 cm in greatest dimension; a superficial tumor with any invasion size whose invasive component is limited to the bronchial wall and may extend proximally to the main bronchus is also classified as T1a, but these tumors are rare
T1b	Tumor >1 cm but ≤2 cm in greatest dimension
T1c	Tumor >2 cm but ≤3 cm in greatest dimension
T2	Tumor >3 cm but ≤5 cm or any of the following: • Involves the main bronchus regardless of distance from the carina but without carinal invasion. • Invades the visceral pleura (PL1 or PL2). • Associated with atelectasis or obstructive pneumonitis extending to the

	hilar region involving part or all of the lung -
T2a	Tumor >3 cm but ≤4 cm in greatest dimension
T2b	Tumor >4 cm but ≤5 cm in greatest dimension
T3	Tumor >5 cm but ≤7 cm in greatest dimension or directly invades any of the following: parietal pleura (PL3), chest wall (including superior sulcus tumors), diaphragm, phrenic nerve, parietal pericardium, or separate tumor nodules in the same lobe as the primary tumor
T4	Tumor >7 cm or any size tumor with invasion of any of the following: diaphragm, mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, or carina; or separate tumor nodules in a different ipsilateral lobe. Regional Lymph Nodes (N)
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis.
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes, and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph nodes
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph nodes
Distant Metastasis (M)	
M0	No distant metastasis
M1	Presence of distant metastasis
M1a	Discrete metastasis in a single contralateral lobe; tumor with malignant pleural or pericardial nodules or malignant pleural or pericardial effusion.
M1b	Single extrathoracic metastasis in a single organ or involvement of a single nonregional lymph node
M1c	Multiple extrathoracic metastases in a single organ or multiple organs

By combining the TNM information, one can gather information and understand what stage of the disease the patient is in.

Table B- Stages and Prognostic Groups, Eighth Edition

Stage	**T**	**N**	**M**
0	Tis	N0	M0
IA1	T1mi	N0	M0
	T1a	N0	M0
IA2	T1b	N0	M0
IA3	T1c	N0	M0
IB	T2a	N0	M0

IIA	T2b	N0	M0
IIB	T1a	N1	M0
	T1b	N1	M0
	T1c	N1	M0
	T2a	N1	M0
	T2b	N1	M0
	T3	N0	M0
IIIA	T1a	N2	M0
	T1b	N2	M0
	T1c	N2	M0
	T2a	N2	M0
	T2b	N2	M0
	T3	N1	M0
	T4	N0	M0
	T4	N1	M0
IIIB	T1a	N3	M0
	T1b	N3	M0
	T1c	N3	M0
	T2a	N3	M0
	T2b	N3	M0
	T3	N2	M0
	T4	N2	M0
IIIC	T3	N3	M0
	T4	N3	M0
IVA	Any T	Any N	M1a
	Any T	Any N	M1b
IVB	Any T	Any N	M1c

4. Treatment

- **Treatment of early-stage and locally advanced NSCLC**

  At clinical stages I and II, NSCLC is considered an early-stage neoplasm and can benefit from radical surgical treatment. According to the recent eighth edition of the Tumor Node Metastasis classification (TNM 8), 5-year survival for radically resected stage I ranges from 68% to 92% and for stage II from 53% to 60%.

  The standard surgical procedure for the fit patient is pulmonary lobectomy with radical lymphadenectomy.

At least three mediastinal lymph node stations (always including the subcarinals) should be removed along with hilar and intrapulmonary lymph nodes.

The various lymph nodes removed in this way must be correctly indicated and the various lymph node stations sent separately for histological examination. [8]

When curative surgery is proposed, such a procedure needs to be performed with the aim of achieving surgical radicality, i.e. characterized by:

- Exeresis of the tumor with margin of surrounding healthy tissue, ascertained histologically;
- Absence of neoplastic residues on the section rhyme;
- Excision of loco-regional lymphatic stations. [3]

Evidence shows that, after radical surgery, patients with pathological stage II-III, a performance status of 0-1, no significant comorbidities and good physical recovery after surgery can receive adjuvant chemotherapy with 4 cycles of platinum-based chemotherapy, as this approach is reported to increase long-term survival. [3] By contrast, in patients with stage IA-IB, follow-up is sufficient.

In the case of stage IIIA or IIIB NSCLC, the management is significantly more complex and requires multidisciplinary assessments. In this context, the lymph nodal involvement is critical; in case of N1 disease, the usual management involves surgery and subsequent radiation therapy, while resectable N2 disease is usually managed with neoadjuvant chemotherapy and subsequent surgery. By contrast, non-resectable, bulky lymph nodal involvement usually requires combination regimens including concurrent chemotherapy plus radiation therapy, eventually followed by maintenance with immunotherapy. [3]

- ## Treatment of metastatic NSCLC

Treatment without an oncogenic driver

The treatment strategy for a patient with newly diagnosed, metastatic NSCLC (mNSCLC) without an oncogenic driver includes consideration of histology and PD-L1 expression.

When we find high expression of PD-L1 (equal to or greater than 50%), we will use immunotherapy as a single agent. The registered drugs in Italy for this purpose are pembrolizumab, atezolizumab, and cemiplimab. These drugs are IgG4 monoclonal antibodies targeting PD-1 and can be used for a maximum of 35 cycles.

If we find low expression of PD-L1 (0-49%), there are three combination therapies that can be used:

- Cisplatin + pemetrexed + pembrolizumab (for adenocarcinoma)
- Carboplatin + paclitaxel + pembrolizumab (for squamous cell carcinoma)
- Chemotherapy + ipilimumab + nivolumab (for both histologies; chemotherapy is administered for only 2 cycles). This is a slightly different regimen, an alternative to the previously mentioned two options. [8]

Treatment for oncogene-addicted NSCLC

With the biopsy results it is possible to find, in some cases, molecular alterations that can affect treatment, allowing to use a more tailored approach, known as target therapy.

The most relevant oncogenic alterations with therapeutic impact are:

1) EGFR mutation (10-15% in Caucasian patients and up to 40% in Asian patients)

2) ALK rearrangement (3-7%)

3) ROS1 rearrangement (1-2%)

4) BRAF mutation (2-4%)

5) MET mutation (exon 14)

6) KRAS gene mutation (20-30 %),

7) RET rearrangement (1-2%)

8) NTRK fusion (0.5- 1%)

9) HER2 mutations (1-2%). [3]

EGFR

The first described druggable targets in NSCLC are the sensitizing mutations of *EGFR*, occurring in about 12% of NSCLC. Among them, exon-19 deletions and exon-21 point mutations are the two most common gene alterations and are usually targeted by first- (gefitinib, erlotinib), second- (afatinib, dacomitinib), or third- (osimertinib) generation tyrosine kinase inhibitors (TKIs). Currently, third-generation TKI is the main therapeutic option for these patients.
[9]

KRAS

KRAS mutations are the most common oncogenic drivers in NSCLC (20-25%), and among them, *KRAS-G12C* is the most frequent (50%). [11]

Although phase I-III studies demonstrated the efficacy of KRAS-G12C inhibitors, such as sotorasib, such agents do not achieve deep and prolonged clinical responses like other targeted therapies in NSCLC; notably, these agents have recently received approval for use as second-line treatment for advanced NSCLC harboring *KRAS G12C* mutation. [12]

Resistance to KRAS-G12C TKIs can be mediated by secondary *KRAS* mutations (e.g., Y40A, N116H, or A146V; A59G, Q61L, or Y64A) or activation of different signaling pathways by other protein alterations.

Several ongoing studies are considering the efficacy and tolerability of combination therapies to overcome these mechanisms of resistance, associating KRAS-G12C inhibitors with other small molecules or event with immunotherapy); however, clinical data about therapeutic strategies to overcome acquired resistance in this setting of patients are still unavailable.

ALK

The EML4-ALK rearrangement was the first ALK fusion variant identified in NSCLC patients.

Since then, more than 90 fusion partners of ALK have been identified in NSCLC, accounting for 3-7% of all NSCLC cases. Mechanistically, ALK rearrangements lead to the constitutive activation of the ALK kinase and its associated downstream cellular signaling pathways, including RAS-MAPK, PI3K-AKT, and JAK-STAT.

This constitutive activation disrupts normal cellular proliferation and survival processes, resulting in dysregulated cell growth and survival.

Cancers that harbor ALK rearrangements become reliant on ALK signaling for their survival. This dependency on ALK signaling makes ALK a potential therapeutic target in these cancers.

Inhibiting ALK kinase activity can disrupt the dysregulated signaling pathways and halt the aberrant cellular proliferation and survival characteristic of ALK-rearranged cancers. [14]

There are three generations of drugs, namely: crizotinib (first generation), alectinib and brigatinib (second generation), and lorlatinib (third generation).

ROS1

The ROS proto-oncogene 1 (ROS1), also known as c-Ros sarcoma oncogenic factor-receptor tyrosine kinase (ROS1, receptor tyrosine kinase), was discovered through isolation studies of avian sarcoma virus UR2.

It has been demonstrated that UR2 contains a unique genomic sequence called Ros, which is a viral proto-oncogene with distinct carcinogenic effects.

This gene encodes a protein tyrosine kinase consisting of 2347 amino acid residues and is the largest member of the protein tyrosine receptor family.

The Ros sequence possesses protein kinase activity, encoding a tyrosine residue phosphorylated fusion protein called p68gag-ros that is involved in cell transformation.

Studies have reported a high homology between the human C-ros-1 gene exon and the UR2 sarcoma virus v-Ros sequence, and both are associated with tyrosine-specific kinase activity. [15]

Crizotinib is the most used drug for this type of mutation.

BRAF

BRAF activation encompasses V600 and non-V600 mutations, fusions, rearrangements, in-frame deletions, insertions, and co-mutations.

Furthermore, primary and secondary BRAF activations present distinct biological phenotypes, clinical implications, and subsequent treatments.

Primary BRAF activation plays a crucial role in the proliferation and metastasis of non-small cell lung cancer (NSCLC) as a driver gene, whereas secondary activation is associated with acquired resistance to other targeted therapies, particularly epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs).

Treatment options vary depending on the type of BRAF activation.

Targeted therapy, particularly combination therapy with two drugs, (dabrafenib plus trametinib) is an important approach. Additionally, immune checkpoint inhibitors (ICIs) could be considered as another option, as BRAF activation can serve as a positive biomarker for tumor response to ICI therapy. [16]

MET

Met exon 14 skipping mutation have been identified as 3% of adenocarcinoma and it presents as metastatic disease in the majority of cases.

Metex14 was found to be an independent prognostic factor that predicted worse survival compared with patients without Metex14. [19]

RET

RET gene fusions, which result in the formation of RET chimeric fusion proteins with constitutively active intracellular kinase domains, have been identified in around 1-2% of non-small cell lung cancers (NSCLCs), predominantly those with adenocarcinoma histology. This discovery has significantly impacted the treatment landscape for patients with these rare gene alterations.

One characteristic of RET fusion-positive NSCLCs is the frequent presence of brain metastases at the time of advanced stage diagnosis.

The currently available agents for this type of gene fusion are Pralsetinib and Selpercatinib. [20]

NTRK

Neurotrophic tyrosine receptor kinase (NTRK) gene fusions

are found in approximately 0.3% of all solid tumors but are more prevalent in certain rare tumor types.

Tropomyosin receptor kinase (TRK) inhibitors, such as larotrectinib and entrectinib, have been approved as tumor-agnostic therapies for solid tumors that harbor NTRK fusions. [18]

5. The objective of the study

The main objective of our study was to compare feasibility and performance of liquid biopsy in parallel to conventional tissue biopsy in a population of symptomatic patients who experience the first identification of symptomatic thoracic mass highly suggestive for locally advanced or metastatic lung cancer through the Emergency Department of a single Comprehensive Cancer Center. In particular, we aimed at determining whether this approach could result in decreased time from sample collection to completion of molecular analyses. We also assessed longitudinal monitoring through liquid biopsy, for patients with oncogenic drivers identified at baseline, at time to first radiological evaluation and at time to radiological or clinical progression.

6. Materials and methods

Patient population

For this study, we enrolled patients who were admitted to the Emergency Department of IRCCS Ospedale Policlinico San Martino, Genova (Italy) and hospitalized due to symptoms which were subsequently associated with clinical and radiological findings considered highly suggestive for locally advanced or metastatic lung cancer, such as thoracic mass and/or mediastinal lymph node involvement. Inclusion criteria were: *i)* Age 18 years or older; *ii)* Symptomatic and

hospitalized after access in Emergency Department*; iii)* Clinical and radiological suspect of advanced or locally advanced lung cancer; i*v)* No prior diagnosis of metastatic lung cancer.

Notably, impossibility to undergo conventional tissue biopsy due to not reachable tumor site or medical contraindications to biopsy was not considered ineligibility criteria for our study, as we considered this condition as a possibly relevant field to assess the feasibility and performance of liquid biopsy. Each eligible patient concurrently underwent conventional tissue and blood withdrawal for liquid biopsy. Liquid biopsy was performed upon evaluation by a medical oncologist with specific experience in lung cancer, while conventional biopsy was planned at the earliest convenience and included percutaneous biopsy performed by an interventional radiologist, or bronchoscopy performed by an interventional pulmonologist, both with extensive experience in lung cancer diagnosis and regular participation to multidisciplinary disease management team meetings. When a patient was ineligible for tissue biopsy, for clinical reasons or inaccessibility of the thoracic lesion, only liquid biopsy was performed.

Timing of liquid biopsy

Blood samples for liquid biopsy were collected from each patient at baseline, defined as the time of first clinical/radiological evidence of lung cancer, before starting treatment, regardless of tissue biopsy. Furthermore, if an actionable molecular target was detected at baseline, peripheral blood samples were also collected at two additional timelines: *i)* at time of first radiological response

assessment during first line target therapy; ii) at disease progression during first-line targeted therapy. Molecular longitudinal analyses assessed the presence of either the patient's specific gene alteration and its Variant Allele Frequency (VAF) or/and any new gene mutation emerging as molecular resistance during target therapy, according to clinical-radiological response.

Circulating free Nucleic Acid mutational status

Circulating Nucleic Acids (*i.e.*, DNA and RNA) were isolated from 4 mL of plasma using the MagMAX Cell-Free Total Nucleic Acid Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA), followed by quantification with a Qubit 3TM Fluorometer (ThermoFisher Scientific). Targeted NGS was performed by using the Oncomine™ Lung cfTNA Research Assay able of identifying genomic variants (*i.e.*, single nucleotide substitutions and short indels) in *ALK*, *BRAF*, *EGFR*, *ERBB2*, *KRAS*, *MAP2K1*, *MET*, *NRAS*, *PIK3CA*, *ROS1*, and *TP53*; gene fusions in *ALK*, *RET*, *ROS*; *MET* exon 14 skipping and the copy number variants of *MET*. Manual libraries preparation was performed after the cDNA reverse transcriptase step starting from 20 ng to 50 ng of cfTNA as detailed in manufacturer protocol (Oncomine™ Cell-Free Research Assay MAN0017065). Three to six diluted (60pM) libraries were pooled and used for template preparation on Ion Chef System and sequenced on Ion 520™ and/or 530™ chips (ThermoFisher Scientific). Run sequencing was performed on the Ion Torrent GeneStudio™ S5.

Analysis and annotation of variants were locally carried out with Torrent Variant Caller (TVC, version 5.16). In order to detect a variant with a Variant Allele Frequency (VAF) of

0.1%, a Median Read Coverage (MedReadCov) and Median Molecular Coverage (MedMolCov) starting from 25,000 and 2,500 were needed, respectively. The output BAM and VCF. files were initially analyzed on Ion Torrent Suite *v.*5.16.1 and then annotated Ion Reporter v.5.18 (ThermoFisher Scientific).

Conventional biopsy: diagnosis and genotyping

Upon conventional biopsy, tumor samples were processed and analyzed by a pathologist with experience in lung cancer diagnosis. After histologic diagnosis, samples of non-squamous NSCLC underwent further molecular analyses. Routine assessment for *EGFR*, *KRAS*, *BRAF*, and *HER2* aberrations was tested by real-time polymerase chain reaction (RT-PCR) or matrix-assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF/MS); concurrently, *ALK* rearrangements were evaluated by immunohistochemistry (IHC) assay or fluorescence in situ hybridization (FISH), while *ROS1* rearrangements and *MET* amplification were assessed by FISH. Notably, *NTRK* fusions were searched by screening with IHC and subsequent confirmation by NGS, as described by ESMO guidelines. [27]

Finally, PD-L1 expression was assessed by IHC in both squamous and non-squamous NSCLC. Exon 14 skipping mutations of *MET* was not actively searched in tissue biopsy, as it was not included in the MALDI-TOF/MS panel and no targeted agents for *MET* mutations were available in Italy at the time of patient enrollment.

7. Results

Patient population

From January 2022 to January 2023, we enrolled 47

hospitalized patients from our Emergency Department with various symptoms such as pain, dyspnea, cough, hemoptysis, and with first radiological evidence of primary thoracic mass highly suggestive for lung cancer. Patients had locally advanced (n= 2) or advanced disease (n= 45). We selected patients regardless of performance status, smoking habits and history of previous malignancies. The overall clinical features of the study population are detailed in Table 1.

Overall clinical features of study population		
Patients	N: 47	
Age	Median: 73 years	Range: 45-86 years
Gender	**N**	**%**
Male	20	42.6%
Female	27	57.4%
Smoking status	**N**	**%**
Never	17	36.2%
Former smoker	19	40.4%
current smoker	11	23.4%
ECOG PS at hospitalization	**N**	**%**
1	13	27.7%
2	14	29.8%
3	20	42.5%
Disease Stage (radiological)	**N**	**%**
III	45	95.7%
IV	2	4.3%
Symptoms at hospitalization	**N**	**%**
Dyspnea	13	27.7%
Pain	13	27.7%
Cough/Hemoptysis	5	10.6%
Other	16	34.0%

Table 1. Overall baseline clinical features of study population.

Liquid biopsy findings

Liquid biopsy identified gene alterations in 29 patients out of 47 (61.7%) at baseline (Table 2). To date, only a number of these identified molecular features are acknowledged as targetable mutations responsive to first- or second-line

targeted therapy.

Remarkably, baseline liquid biopsy analysis identified *EGFR* mutations in 10 patients (21.3%) while *KRAS* alterations were found in 13 cases (27.7%), including co-mutations. Indeed, in four patients, liquid biopsy revealed coexistent mutations: a) *KRAS Gly12Cys* and *EGFR exon 21 Leu858Arg*; b) *EGFR exon 18 Glu709Ala* and *EGFR exon 19 Leu747_Thr751del*; c) *EGFR exon 20 Ser768Ile* and *EGFR exon 21 Leu858Arg*; d) *HRAS Gln61His* and *PIK3CA Glu545Lys*.

Molecular characterization at baseline liquid biopsy in the global study population	
Mutation Types	**Number**
KRAS	
p.(Gly12Cys)	4 (a)
p.(Gly12Val)	4
p.(Gly12Asp)	2
p.(Gly12Arg)	1
p.(Gly12Phe)	2
EGFR	
Common mutations involving exon 19	
p.(Leu747_Thr751del)	1 (b;c)
p.(Glu746_Ser752delinsVal)	2
p.(Glu746_Ala750)	1
p.(Leu747_Pro753delinsSer)	1
Common mutations involving exon 21	
p.(Leu858Arg)	5 (a;c)
Uncommon Mutations	
exon 18 p.(Glu709Ala)	1 (b)
exon 20 p.(Ser768Ile)	1 (c)
ALK	
EML4-ALk (EML4 exon 6 fused to ALK exon 20)	1
ERBB2	
Mutations involving Exon 20	
p.(Tyr772_Ala775dup)	1
TP53	
exon 7 p.(Arg248Trp)	1
exon 8 p.(Arg280Lys)	1
PIK3CA	
p.(Glu545Lys)	1 (d)
BRAF	
p.(Asp594Gly)	1
HRAS	
p.(Gln61His)	1 (d)

Table 2. Identified molecular alterations at baseline liquid biopsy in the study population, including both actionable and currently non-actionable oncogenic alterations for NSCLC.
Notably, the number of molecular alterations exceeds the number of patients harboring molecular alterations, due to cases of coexistence of multiple alterations in the sample from the same patient.
The following coexistent mutations were reported:
a) *KRAS Gly12Cys* and *EGFR exon 21 Leu858Arg*;
b) *EGFR exon 18 Glu709Ala* and *EGFR exon 19 Leu747_Thr751del*;
c) *EGFR exon 20 Ser768Ile* and *EGFR exon 21 Leu858Arg*;
d) *HRAS Gln61His* and *PIK3CA Glu545Lys*.

Conventional biopsy findings

In 37 out of 47 patients, with favorable performance status and no impairing comorbidities, we also performed

diagnostic tissue biopsy, leading to histological identification of lung adenocarcinoma (n=24), squamous cell carcinoma (n=2), small cell lung cancer- SCLC (n=1), well differentiated neuroendocrine lung cancer (n=1), sarcomatoid cancer (n=2) and not otherwise specified NSCLC (n=5). Additionally, in one case we identified only poorly differentiated epithelial neoplasm, and one sample was not diagnostic for histological evaluation. We obtained complete molecular profile for 27 out of 37 patients. In two cases, molecular analyses were not requested due to non-diagnostic biopsy or inadequate tissue ; in four cases, molecular analyses were not requested upon completion of histological report as the patients' conditions significantly worsened, resulting in ineligibility to active treatments; in one specific case, molecular analyses on tissue were not requested due to histological diagnosis of small cell lung cancer (SCLC), while in two cases, complete molecular analyses were not requested due to squamous histology, as only PD-L1 expression was deemed necessary for patients' management. Notably, in one case (patient #43), molecular analysis on liquid biopsy revealed actionable EGFR mutations before the availability of conventional biopsy histologic report; hence, we did not proceed with request of molecular analyses on tumor tissue and considered liquid biopsy sufficient for starting treatment.

Performance of liquid and conventional biopsy

Comparison of molecular analysis on plasma and tissue samples revealed an almost complete concurrence (92.6%; 25/27), Apart from two cases. In both patients, molecular analysis of liquid biopsy failed to identify the *EGFR* variants that were instead present in the corresponding tumor tissues, *i.e.*, an exon 19 deletion (case #10) and an exon 21

Leu858Arg (case #39). In this last case, we found the *EGFR* exon 21 mutation using the cells freshly isolated from a pleural liquid sampling rather than the diagnostic tissue-derived from the Endobronchial ultrasound transbronchial needle aspiration (EBUS-TBNA). Finally, no false positive results were found, and mutations identified in liquid biopsy were always confirmed in tissues.

With regards to time to completion, liquid biopsy was completed after a median time of 11 days among all the 47 patients. By contrast, time to completion of conventional biopsy, defined as the number of days required from the biopsy to the histologic diagnosis, was 9 days (n= 37 patients); for the 27 patients who underwent molecular analyses on conventional biopsy sample, the time to completion of molecular analyses from request to molecular report was 10 days.

When we considered the subgroup of patients who underwent complete molecular characterization both in liquid and conventional biopsy (n= 27), the median time to completion of liquid biopsy from sample collection to availability of the report was 11 days, while the median time to completion of molecular characterization from the day of conventional biopsy to the availability of the report was 22 days; the difference between the two methods in terms of days was statistically significant (Mann-Whitney $P< 0.0001$).

Days for completion on liquid biopsy and conventional biopsy for all the enrolled patients are summarized in Figure 1, while the comparison between time to complete molecular characterization in liquid biopsy and in conventional biopsy is reported in Figure 2.

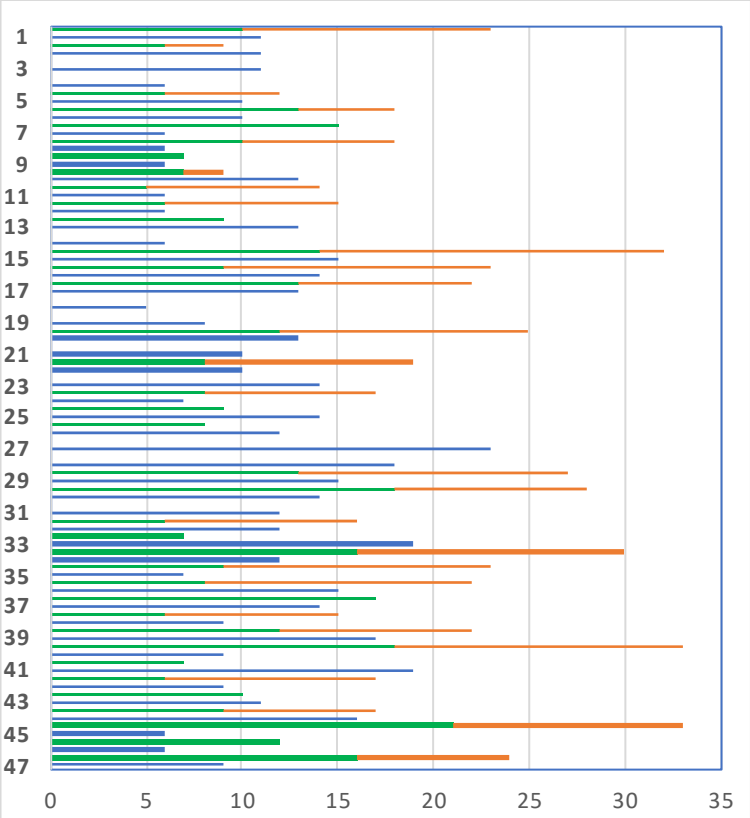


Figure 1. Time from request to availability of the report
for liquid and conventional biopsy
for the whole population of our study (n= 47), in days.
Blue lines represent the time for completion of liquid biopsy,
while green lines represent the time for completion
of conventional. histological diagnosis
and orange lines represent
the time for completion of molecular analyses
on conventional biopsy samples.
Notably, 10 patients did not undergo conventional biopsy and in 10 cases,
molecular analyses were not requested
on conventional biopsy sample.

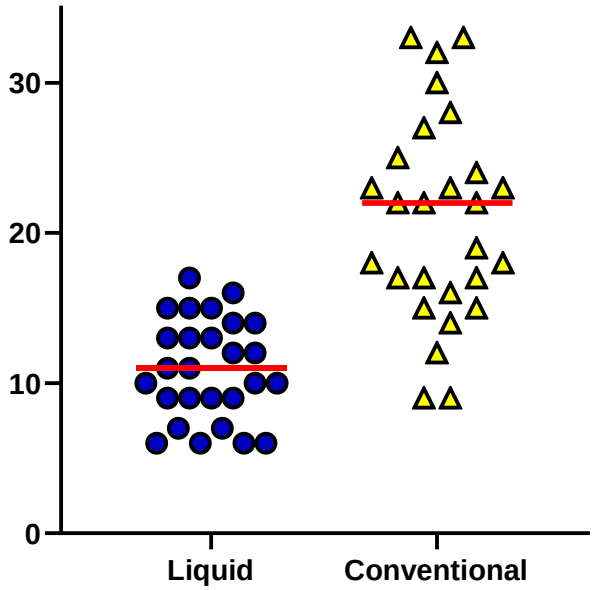


Figure 2: Comparison between time to completion of liquid biopsy vs. conventional molecular characterization among patients for whom molecular analyses were requested on the conventional biopsy sample (n= 27), calculated from sample collection to availability of the molecular report. Liquid biopsy had a shorter completion time in days compared to full molecular characterization on conventional samples (median: 11 vs. 22 days; Mann-Whitney P< 0.0001).

Treatment and longitudinal assessments

Within our patient population, 28 patients received systemic treatments. By contrast, 17 patients did not receive systemic treatment as their clinical conditions made them ineligible for treatment and one patient underwent upfront radical surgery; the treatment of one patient was not reported as he/she was subsequently cured in another hospital. Among the 12 patients harboring *EGFR* mutations, either identified at liquid or conventional biopsy, 10 received first-line treatment with an EGFR inhibitor (osimertinib), while one patient was unable to start treatment due to severe clinical conditions (symptomatic brain metastases which ultimately

led to patient's death) and one patient was treated elsewhere, and we did not obtain additional data. The single patient whose tumor harbored *ALK* rearrangement received first-line treatment with an ALK inhibitor (alectinib).

Notably, four patients for whom actionable oncogenic drivers for first-line treatment were identified only at liquid biopsy, including the aforementioned patient with *ALK* rearrangement, as conventional biopsy was not performed or not diagnostic. Nonetheless, these patients were considered as if such alterations were detected in tumor tissue and managed accordingly. Notably, one patient with *ERBB2* mutation identified at liquid biopsy could not receive targeted therapy since at the time of our analysis specific agents for this genic alteration, such as trastuzumab deruxtecan, were not available in clinical practice.

At the time of the analysis, nine patients with a baseline liquid biopsy positive for a relevant oncogenic driver (irrespective of its actionability in first-line) who received systemic antineoplastic treatment accepted to undergo additional blood collection for the longitudinal analysis. Additionally, we included one patient whose baseline liquid biopsy failed to detect an activating *EGFR* mutation which was identified at conventional biopsy.

Notably, in three cases where baseline liquid biopsy was positive, the repeated liquid biopsy resulted negative for oncogenic drivers, and this occurrence was consistent with radiological benefit from targeted treatment (disease stabilization or partial response). One patient (#14) received single-agent chemotherapy (gemcitabine) in presence of *ERBB2* mutation (as stated earlier); in this case, an emerging *KRAS* mutation was detected during treatment with persistence of *ERBB2* mutation, and the patient developed rapid progression as best response to treatment. In another case (patient #17), a relatively relevant increase in VAF of

EGFR mutation during treatment with osimertinib was not predictive of lack of response, and indeed the patient achieved partial response after an initial stabilization. In this case, subsequent liquid biopsy was able to identify a *PIK3CA* mutation which was not detected at baseline liquid biopsy but was nonetheless present at conventional biopsy; the presence of this mutation did not appear to influence the response to osimertinib. The available longitudinal data are reported in Table 3.

PATIENT ID	BASELINE LIQUID BIOPSY REPORT	FIRST-LINE TREATMENT	LIQUID BIOPSY AT FIRST RADIOLOGICAL EVALUATION	RECIST AT FIRST RADIOLOGICAL EVALUATION	RECIST AT BEST RADIOLOGICAL EVALUATION
1	EGFR c.2573T>G, p.(Leu858Arg) - VAF 1.4%	Osimertinib	Leu858Arg - VAF 0.05%	PR	PR
4	EML4:ALK (exon 6 of EML4 gene fused to exon 20 of ALK gene), 5 molecular families	Alectinib	Wild type	SD	SD
5	EGFR c.2126A>C, p.(Glu709Ala) VAF 10.2%; EGFR c.2240_2254del p.(Leu747_Thr751del) - VAF 11.2%	Osimertinib	Glu709Ala - VAF 1.35%; Del19 - VAF 1.2%	PR	PR
6	KRAS c.35G>T, p.(Gly12Val) - VAF 2.7%	Platinum-based chemotherapy plus pembrolizumab	KRAS Gly12Val - VAF 0.19%	SD	SD
8	KRAS c.35G>A, p. (Gly12Asp) - VAF 7.9%	Pembrolizumab	KRAS Gly12Val - VAF 7.5%	SD	SD
10	Wild type*	Osimertinib	Wild type	SD	PR
14	ERBB2 c.2313_2324dup, p.(Tyr772_Ala775dup) - VAF 2.2%	Single-agent chemotherapy	ERBB2dup - VAF 2.45%; KRAS Gly12Cys - VAF 4.1%	PD	PD
17	EGFR c.2573T>G, p.(Leu858Arg) - VAF 0.2%	Osimertinib	p.(Leu858Arg) - VAF 1.6%; PIK3CA p.(Gly1049Arg) - VAF 6.5%	SD	PR
22	KRAS c.34_35delins, p.(Gly12Phe) - VAF 0.8%	Platinum-based chemotherapy plus pembrolizumab	Wild type	SD	SD
38	EGFR c. 2235_2249del, p.(Glu746_Ala750)del - VAF 18%	Osimertinib	Wild type	SD	PR

Table 3. Longitudinal analysis of liquid biopsy for patients

Legend. RECIST: response evaluation criteria in solid tumors; SD: stable disease; PR: partial response; PD: progressive disease; VAF: variant allele frequency.

*Patient 10 resulted wild type at baseline liquid biopsy, but EGFR mutation was detected at conventional biopsy.

8. Discussion

In our study, a population of 47 consecutive patients, who had been hospitalized through the Emergency Department for symptoms which led to clinical finding of lung cancer, underwent front-line liquid biopsy for detection of oncogenic drivers concurrently with conventional diagnostic work-up. Most patients had locally advanced or metastatic cancer, with the notable exception of one female, never smoker patient, who had stage II lung cancer (which was subsequently diagnosed as a carcinoid). In our study, NGS analysis performed on baseline liquid biopsy was able to detect relevant genic alterations in 28 cases, including 11 patients for whom first-line targeted therapy was potentially available according to current practice guidelines. While this proportion of oncogene-driven tumors might appear to be relatively high as compared to previous reports, the constant improvement of molecular analysis techniques should explain the increased sensitivity of recent studies; additionally, most patients had significantly advanced disease, with higher probability of tumor shredding and release of nucleic acids in the blood stream. [28] [29] [30]

Notably, four out of 11 patients with first-line-actionable oncogenic alterations (activating mutations of *EGFR* of rearrangements of *ALK*) could not undergo conventional biopsy due to physical conditions or had a non-diagnostic conventional biopsy. Furthermore, among the patients who could undergo both biopsies and needed complete molecular analyses, the median time from sample collection to full molecular report was substantially halved (11 days for liquid biopsy and 22 days for conventional biopsy). The net consequence of this approach was that patients with actionable oncogenic drivers for first-line could start earlier a

targeted treatment and also a few patients who could not undergo an actual biopsy were able to receive an active treatment, whereas in normal circumstances such patients would only receive best supportive care.

Additionally, when we could compare liquid and conventional biopsy, the two techniques were generally consistent with each other, with the notable exception of two patients, whose *EGFR* mutation was not detected in blood. In one case, this occurrence may be associated with the absence of extra-thoracic lesions, while in the other case, although bone lesions were present, the burden of extra-thoracic disease was limited, potentially explaining the limited release of neoplastic nucleic acids within the blood stream.

A feature of our study population is represented by the high proportion of patients (n= 17; 36.2%) did not receive treatments, including seven patients who did not undergo conventional biopsy and whose liquid biopsy was not helpful for giving access to targeted therapy and 10 patients who undergo conventional biopsy but still did not receive treatment after molecular assessments. This group is particularly relevant, as the choice to avoid active antineoplastic treatments can generally be either associated with the lack of actionable drivers and low/absent PD-L1 expression for a patient ineligible for chemotherapy, or with progressive worsening of clinical conditions while the physicians wait for molecular analyses. Such a high proportion of candidates to supportive care is not surprising, as we focused our study on patients who had the first evidence of lung cancer upon access to the Emergency Department, hence being characterized by a generally unfavorable prognosis. In such a context, where the eligibility to targeted agents is substantially synonym with the eligibility to antineoplastic treatments for frail patients,

the front-line use of liquid biopsy might improve patient selection, although conventional biopsy is still needed to determine PD-L1 expression and hence eligibility to single-agent immunotherapy.

Globally, our findings support a combined approach for symptomatic, hospitalized patients with first clinical evidence of lung cancer; such approach involves upfront liquid biopsy at the first convenience, in parallel with conventional biopsy (when feasible). If NGS analysis based on liquid biopsy anticipates conventional biopsy and identifies an actionable oncogenic driver with acknowledged first-line therapy, the treatment is expected to get started early, potentially preventing further worsening of clinical conditions and the eventual development of ineligibility to any treatment. If blood-based NGS analysis fails to identify actionable oncogenic drivers and the patient has relevant and progressive symptoms due to lung cancer, a physician might start antineoplastic treatment based on the histologic diagnosis and PD-L1 expression, without having to wait for the full molecular analyses on tumor tissue, as the probability of actionable genic alterations undetected by liquid biopsy is generally low in presence of high extra-thoracic disease burden. One possible limitation to this approach is represented by histologic types such as squamous cell lung cancer and SCLC, for which molecular analyses are not indicated. However, these histo-types are less common than pulmonary adenocarcinoma, thus limiting the potential waste of resources associated with upfront molecular analyses; in addition, some patients with squamous cell lung cancer can still benefit from targeted agents (such as MET inhibitors).

We understand that our study has some relevant limitations. Indeed, it includes a relatively small number of patients treated in a single institution. However, these features

resulted in a consistent approach in terms of patient management and therapeutic choices. Additionally, at the time of data analysis, only few treated patients had undergone disease assessments and longitudinal liquid biopsies, and their results fall beyond the scope of this manuscript. Notably, while the longitudinal assessment was not the focus of this study, we plan to continue patients' enrollment and eventually to develop more robust data on this topic. To date, other publications have been focused on the predictive of prognostic role of longitudinal liquid biopsy assessment, both in terms of qualitative findings and in terms of quantity of circulating-free DNA. [31] [32] [33]

CONCLUSION

In our experience in a population of symptomatic patients with advanced lung cancer support the use of liquid biopsy in conjunction with conventional biopsy, in order to achieve timely histo-molecular characterization and rapid treatment initiation in this unfavored patient population.

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