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Original article

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Feasibility of comprehensive genotyping specimens from radial endobronchial ultrasonography and electromagnetic navigation bronchoscopy

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Take Home Message: Radial EBUS and EMN bronchoscopies are safe and sensitive procedures for lung cancer diagnosis. Cytology is highly complementary with histology. These small samples are however not suitable for an exhaustive molecular testing in 30% of cases.

Abstract

Introduction: Mini-invasive bronchoscopic techniques (such as radial endobronchial ultrasonography (rEBUS) and electromagnetic navigation (EMN)) have been developed to reach the peripheral lung but result in small samples. The feasibility of an adequate molecular testing from these specimens has been very little studied.

Methods: We retrospectively reviewed EMN and rEBUS procedures performed in patients diagnosed with lung cancer in our institution in 2017 and 2018. We analyzed the sensitivity for rEBUS and EMN and each sampling method, and the feasibility of a comprehensive molecular testing.

Results: 317 rEBUS and 14 EMN were performed. Median sizes of tumors were 16 and 32 mm for EMN and rEBUS, respectively. Overall sensitivity for rEBUS and EMN was 84.3%. Cytology was found to be complementary with biopsies, with 13.3% of cancer diagnosed on cytology while biopsies were negative. Complication rate was 2.4% (pneumothorax 1.5%, mild hemoptysis 0.9%). Genotyping (immunohistochemistry (IHC) for *ROS1* and *ALK* followed by FISH if positive and hybrid capture next-generation sequencing (NGS) covering 48-genes), when ordered (n=188), was feasible in 69.1% (*EGFR* 17.7%, *KRAS* 31.7%, *BRAF* 4.8%, *ALK* 1.2%, *MET* 3.1%, *HER2* 0.8%). PD-L1 expression, when ordered (n=232), could be analyzed in 94% of cases. 56.9% (33/58) of patients for whom genotyping was not feasible underwent a second sampling (12 pretreatment, 21 at progression), allowing for the detection of 6 actionable genotypes (5 *EGFR*, 1 *MET*).

Conclusion: rEBUS and EMN are sensitive and safe procedures that result in limited samples, often not suitable for genotyping, highlighting the importance to integrate liquid biopsy in routine.

Introduction

Mechanisms of oncogenesis in lung cancer have been largely deciphered over the past 20 years. Lung adenocarcinoma can now be considered as a cluster of discrete molecular subtypes, the majority being defined by a single alteration of an oncogenic driver. Multiplex genotyping and high-throughput genomic profiling by next-generation sequencing (NGS) is thus increasingly refining molecular diagnoses [1]. In addition, immune checkpoint inhibitors also requires tissue for the analysis of the tumor microenvironment, in particular PD-L1 (programmed death-ligand 1) expression [2].

There is currently a paradox between the need to obtain significant amount of sample to test an increasing number of biomarkers and the development of bronchoscopic minimally invasive techniques, resulting in small tissue samples with limited amounts of DNA [3]. 20 to 30% of EBUS-TBNA (endobronchial ultrasound transbronchial needle aspiration) nodes samples are rejected for genotyping due to lack of tissue [4,5]. For peripheral lesions, bronchoscopy currently constitutes the preferred approach as it is less invasive than radio-guided biopsies (6). The sensitivity of the main technologies, electromagnetic navigation (EMN) and radial EBUS (rEBUS), is 75%, with very few complications [6,7]. The feasibility of adequate molecular testing of these specimens has however been less studied, in limited series [8] and without next generation sequencing (NGS) [9].

In this study we aimed to study on a large cohort: *i)* the sensitivity of rEBUS and EMN, distinguishing cytology (brushings, washings) and histology (forceps biopsies) yields; *ii)* the feasibility of an exhaustive genotyping (including NGS) on these specimens, as well as of PD-L1 expression analysis; *iii)* the impact of the latter on patients' management (rate and results of second biopsies).

Methods

Patients

We retrospectively studied consecutive patients who underwent as a first diagnostic procedure a bronchoscopy with rEBUS or EMN in 2017 and 2018 in the bronchoscopy unit of Toulouse University Hospital and were subsequently diagnosed with lung cancer, either by the procedure or later on. We reviewed data from all patients undergoing rEBUS or EMN in the Orbis™ Clinical Information System (AGFA Healthcare™), and the Occitanie oncology comprehensive database (http://www.onco-occitanie.fr). Patients gave their consent for this retrospective study and data were de-identified.

Sampling

EMNs (Superdimension system; Covidien, MA, USA) were performed under general anesthesia through a laryngeal mask. This technology combines virtual navigation imaging with sensing of the position of a bronchoscopic catheter, matching virtual and real bronchial trees. Brushings and biopsies were performed through the guiding catheter after reaching the lesion.

Radial EBUS were performed under local or general anesthesia through a flexible bronchoscope. Dedicated brush and forceps were used through the 2.0 mm diameter GuideSheath to sample the lesions after detection of the lesion using the radial ultrasonic miniature probe (UM-S20-17S).

For both techniques, the guiding catheter was rinsed at the end of the procedure with saline for cytology.

Sample handling: diagnosis and molecular testing

Both cytology (brushing and catheter rinse) and histology (biopsies) samples were used for morphological diagnosis. Molecular testing was performed after DNA extraction from sections cut from cell (cytology) or FFPE (tissue) blocks, and included a first screening of *EGFR* (Epidermal

growth factor receptor) common mutations by the Cobas® technique (Kit ROCHE Cobas DNA Sample Preparation Kit), IHC (immunohistochemistry) for ROS1 (Clone D4D6 Ozyme) and ALK (anaplastic lymphoma kinase, Clone 1A4 Diagomics) rearrangements followed by FISH (fluorescence in situ hybridization) if positive (IQFISH break-apart probe, Dako Omnis)), and in a second time, a hybrid capture NGS, covering a 48-genes panel (Roche Sequencing (Kapa/SeqCap), MiSeq DX Illumina). PD-L1 expression was assessed on biopsies and surgically resected specimens using IHC (Clone QR1, Quartett, Diagomics).

Outcomes

The primary outcome was the feasibility of an exhaustive molecular testing. Secondary outcomes were: *i)* the overall sensitivity of the procedures and the sensitivities of cytology and histology specimens, *ii)* the number of patients undergoing second biopsy (before or after first line treatment) and the molecular profile on these second samples, *iii)* The feasibility of PD-L1 expression analysis and the concordance with tissue.

Statistical analysis

Data were summarized by frequency and percentage for categorical variables and by median and range for continuous variables. Comparisons between groups were done using chi-2 test or Fisher test for qualitative values. Comparison between biopsy and surgical specimens was done using Mac Nemar test for paired qualitative data. For all statistical tests, differences were considered significant at the 5% level. All statistical analyses were conducted using STATA 16.1 software.

Results

Population

331 patients underwent rEBUS (n=317) or EMN (n=14) procedures in 2017 and 2018. Median age was 68 years old. 67.1% were male, 13.9% non or light smokers (< 10 pack years). The main characteristics of the population are reported in **Table 1**.

	N (%)
Age (years) (n = 331)	
Median	68.0
(Range)	(42.0 : 87.0)
< 70 years	177 (53.5%)
≥ 70 years	154 (46.5%)
Sex (n = 331)	
Male	222 (67.1%)
Female	109 (32.9%)
Tobacco (n = 331)	
Non-smoker	28 (8.5%)
<10 pack years	18 (5.4%)
10-30 pack years	126 (38.1%)
>30 pack years	159 (48.0%)

Table 1: Characteristics of the population

Median size of the sampled lesion was 32mm [min-max : 9-100] and was ≥ 30mm for 205 patients (63.3%). For EMN patients, median size was 16mm [min-max : 10- 30]. The characteristics of the lesions are detailed in **Table 2.** 39.5%, 24.7% and 35.8% of patients had a metastatic, locally

advanced (IIIA-IIIB) and localized lesion (I-IIB), respectively. Adenocarcinoma was the most frequent histology. Other subtypes are reported in **Figure 1A**.

	n (%)
Lesion size (mm)	
All (median, range)	32 (9.0: 100.0)
rEBUS	32 (9.0: 100.0)
EMN	16 (10: 30.0)
UNK	46 (16.1%)
Lesion size (n = 316)	
<20mm	50 (15.8%)
≥20mm	266 (84.2%)
UNK	15
Lesion size (n = 324)	
<30mm	119 (36.7%)
≥30mm	205 (63.3%)
UNK	7
Stage (n = 324)	
I-IIB	116 (35.8%)
IIIA	43 (13.3%)
IIIB	37 (11.4%)
IV	128 (39.5%)
UNK	7
Histology (n = 331)	
Adenocarcinoma	223 (67.4%)
Squamous	72 (21.8%)
Small cell	9 (2.7%)
Carcinoid	8 (2.4%)
Undifferentiated	6 (1.8%)
Large cell.	4 (1.2%)
Other	4 (1.2%)
UNK	5 (1.5%)

Table 2: Characteristics of the disease

rEBUS: radial endobronchial ultrasonography, EMN: electromagnetic navigation, UNK: unknown

Diagnosis

Overall sensitivity was 84.3% (279/331); 85.4% (271/317) for rEBUS and 57.1% (8/14) for EMN. When performed, sensitivity of histology and cytology samples were 73.8% (234/317) and 77.5% (234/302), respectively (**Table 3**). When both were performed, sensitivity was 86.7% (255/294) and diagnosis was obtained on cytology only in 13.3% (39/294) and on histology only in 9.2% (27/294). When tumor size was available (n=310), sensitivity was 89.4% (93/107) for lesions < 30 mm; 85.5% (171/203) for lesions ≥ 30 mm.

	N (%)
Overall (84.3%)	**279/331**
rEBUS (85,4%)	271/317
EMN (57.1%)	8/14
Cytology (77.5%)	234/302
brushings (72.7%)	208/286
rinse (71%)	193/272
Histology (73.8%)	234/317

Table 3: Diagnostic yield of bronchoscopy for peripheral lesions

rEBUS: radial endobronchial ultrasonography, EMN: electromagnetic navigation

Complications

8 complications (2.4%) were reported, 5 (1.5%) pneumothoraxes including 1 requiring chest tube insertion (0.3%); 3 (0.9%) mild hemoptysis, one of which required an additional day of hospitalization.

Feasibility of genotyping and PD-L1 expression analysis

Tumor genotyping was ordered for 188/331 patients (56.8%, including 49 stage I-II, 30 stage IIIA and 108 stage IIIB-IV, 1 missing) and was not feasible in 30.9% (58/188) of cases due to exhausted tumor tissue (either no block left after the diagnostic steps, or insufficient DNA amount (< 5 ng) after extraction). Feasibility tended to be higher for advanced stages (72.5%) compared to stage I-II (59.2%, p=0.08). Because tissue from biopsies was exhausted, genotyping was performed on cytology samples in 5.3% of cases. The main genotypes of interest identified are summarized in **Figure 1B**: 17.7% *EGFR*, 31.7% *KRAS* (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), 4.3% *STK11* (Serine/Threonine Kinase 11), 4.8% *BRAF*, 3.1% *MET* and 0.8% *HER2* (Human Epidermal Growth Factor Receptor-2) mutations. *ALK* and *ROS1* rearrangements could be tested using IHC for 167/188 (88.8%) of patients (1.2% *ALK*, confirmed by FISH, no *ROS1*).

PD-L1 expression analysis was ordered for 232 patients and was feasible in 94% of cases (218/232). Expression by at least 1% and 50% of tumor cells were detected in 49.5% and 22.7% of patients, respectively. Matched surgically resected pieces and tissue biopsies were available for 15 patients, showing a good concordance for the 50% threshold (3/3 tested positive and 12/12 tested negative in both specimens). Of the 8 patients tested greater than 1% on surgically resected specimens, 3 had tested negative on small biopsies.

Second biopsies (**Figure 2**)

33/58 (56.9%) patients for whom genotyping was not possible underwent a second sampling, straight away (n=12) or at progression after first line treatment (n=21). A screening plasma genotyping, limited to *EGFR* (Cobas), was proposed, completed with tissue when negative. The modalities and results of second biopsy procedures are detailed in the flow chart presented in **Figure 2**. Overall, on plasma or second tissue biopsy genotyping, 5 additional *EGFR* (2 in plasma, 3 on tissue), 1 non-V600E (pD594G) *BRAF*, 1 *MET*, and 5 *KRAS* mutations were detected, before or after first-line treatment (**Figure 2**).

Discussion

In this study, we extensively studied the performance of two bronchoscopy procedures (rEBUS and EMN) for peripheral lesions. We focused not only on the well investigated diagnostic accuracy of these tools, but mostly on their limitations, in particular the pitfall of these scarce specimens in the era of personalized medicine.

First, we report an excellent sensitivity (84.3%) of bronchoscopy for peripheral lesions, and confirm the favorable safety profile [10] (1,5% pneumothorax, only 1 requiring chest tube insertion (0.3%)). These results appear slightly above what is usually reported [6,7,11], likely in part linked to a high median size of the lesions (32 mm), the systematic use of the guidesheath with rEBUS, and the learning curve of a technique widely used in our institution since 2014. The sensitivity is higher in our experience for rEBUS (85.4%) compared with EMN (57.1%), a technology we only use for complex situations (twisted path to reach the lesion, ground glass nodules (less visible in ultrasonography [12]) and smaller nodules (median 16 mm compared to 32 mm for rEBUS in our cohort)).

An important point of our results is the complementarity between histology and cytology. Noteworthy, when both were done, 13.3% and 9.2% of diagnoses were obtained solely with cytology

or histology alone, respectively, with a combined sensitivity of 86.7%. We thus strongly suggest to systematically obtain cytology samples during rEBUS or EMN. The forceps are sometimes difficult to correctly open in distal airways. Brushing when biopsies are poorly productive can, in our experience, help open thin airway walls and increase the yield of rEBUS, in particular for eccentric lesions. This complementarity of cytology with forceps had already been suggested for transbronchial needle aspiration (TBNA) [13], a tool less used. TBNA were performed in 16.5% of cases in the AQuIRE registry, in part because the needle cannot always navigate sharp turns, and was found very useful for eccentric lesions (diagnostic in 9.5% when biopsies are negative) [14].

Another interesting point is the equivalent sensitivity for smaller lesions (89.4% for lesions < 30 mm compared to 85.5% for lesions ≥ 30 mm). This is an attractive result that should reinforce the place of these mini-invasive bronchoscopic diagnostic (and potentially therapeutic [15]) procedures for lung nodules, especially after the recent positive outcomes of the lung cancer screening NELSON trial [16].

But the main aim of our study was to investigate the ability of rEBUS and EMN to provide sufficient material for adequate molecular testing. A comprehensive genotyping is now required before treating patients with advanced non-small cell lung cancer (NSCLC), with an increasing number of available targeted therapies, creating a paradox with the development of mini-invasive bronchoscopic procedures that results in limited material, often exhausted by the routine diagnostic steps. Up to 25% of patients receive treatment without knowledge of their mutational status [17].

In our study, 30.9% of samples obtained by rEBUS or EMN were not suitable for an adequate molecular testing (sequential screening for the most prevalent genotypes followed with NGS) due to exhausted tissue after the diagnostic steps. Few studies have reported higher feasibilities using these specimens. Guisier et al showed on a retrospective analysis of 111 patients that a multiplex analysis (without NGS) could be performed in 79% of rEBUS samples, cytology being more challenging [9].

Others have showed excellent feasibilities for molecular testing but only focusing on one (*EGFR* [11,18]) or few (*EGFR*, *KRAS*, *BRAF* [8]) specific genotypes. To our knowledge, this is the first study investigating the ability of these small specimens to provide a full molecular profile, including NGS, *ALK* and *ROS1* rearrangements and PD-L1 expression.

We tried to investigate the impact of the limited feasibility of genotyping on patients' management (**Figure 2**). 33 of the 58 patients for whom genotyping was not possible underwent a second sampling, (12 before any treatment, 21 at progression), including 31 tissue invasive biopsies, i.e. 16.5% of patients for whom a genotyping was ordered (31/188). 2 *EGFR* mutations were detected in blood, avoiding a tissue biopsy, and 4 additional "actionable" genotypes were found on second tissue biopsies (3 *EGFR*, 1 *MET*).

Obviously, these results only apply to the specific handling of our samples (DNA extraction from formalin-fixed specimens, hybrid capture NGS, MiSeq DX Illumina platform) and other approaches may result in a higher yield. There are several perspectives to compensate for the limited amount of tissue generated by mini-invasive sampling procedures in lung cancer: *i)* liquid biopsy (which was limited in our center in 2017 and 2018 to *EGFR* detection by Cobas in ctDNA) could only avoid 2 tissue biopsies in our study, but may represent a much more appealing approach in the future with the development of multiple circulating tumor DNA NGS platforms that cover all genotypes, including not only mutations but also amplifications or gene fusions with sensitivities ranging from 70% to 80% [19,20]; *ii)* cryobiopsy, with the development of thin cryoprobes suited for peripheral lesions, could provide larger tissue specimens [21,22]; *iii)* The use of non-formalin tissue fixation [23]; *iv)* an alternative handling of cytology specimens, with in particular the use of the free-floating DNA present in their supernatant [24], may increase the overall yield of pauci-cellular biospecimens [25].

Finally, we have studied the feasibility of PD-L1 expression assessment on rEBUS and EMN samples, and confirmed it was highly feasible (94%) [26]. We didn't have enough matched surgically specimens to draw conclusions (n=15) but all > 50% PD-L1 expressions detected on surgically resected specimens were correctly assessed on the small specimens, while 3 patients who tested negative had a > 1% expression on the surgical specimen, which tend to confirm that small samples can underestimate PD-L1 expression [27,28].

In conclusion, this study confirms the good sensitivity of bronchoscopy with rEBUS and EMN for lung cancer diagnosis, even for small lesions (< 30 mm), and its safety, and strongly highlights the complementarity of cytology with histology. It however demonstrates that these small samples are not suitable for an exhaustive molecular testing in 30% of cases, a significant issue given the multiplication of targetable genomic alterations. This pitfall could however be compensated by new techniques (rEBUS-guided cryobiopsy) providing larger samples, the use of cytology supernatant's free-floating DNA and most of all, the implementation of plasma NGS that will in a near future limit the yield of second biopsy for genotyping.

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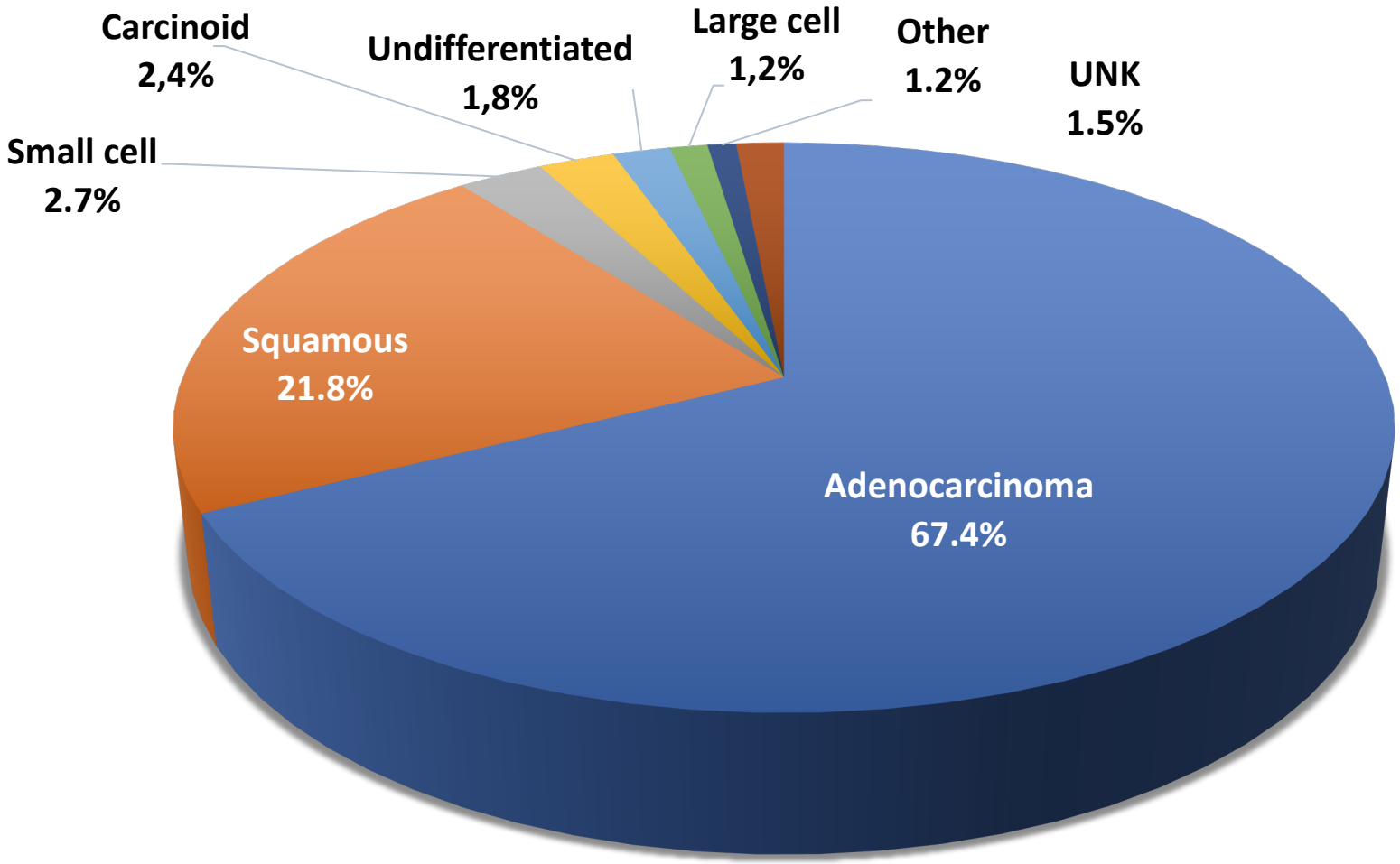
Carcinoid
2,4%
Undifferentiated
1,8%
Large cell
1,2%
Other
1.2%
UNK
1.5%
Small cell
2.7%
Squamous
21.8%
Adenocarcinoma
67.4%

1A

Other
4,80%
ROS1
0%
HER2
0,80%
ALK
1,20%
MET
3,10%
BRAF
4,80%
EGFR
17,70%
KRAS
31,70%

1B

Figure 1: Histological subtypes and mutational status the lung cancer diagnosed by rEBUS and EMN

rEBUS: radial endobronchial ultrasonography; EMN: electromagnetic navigation

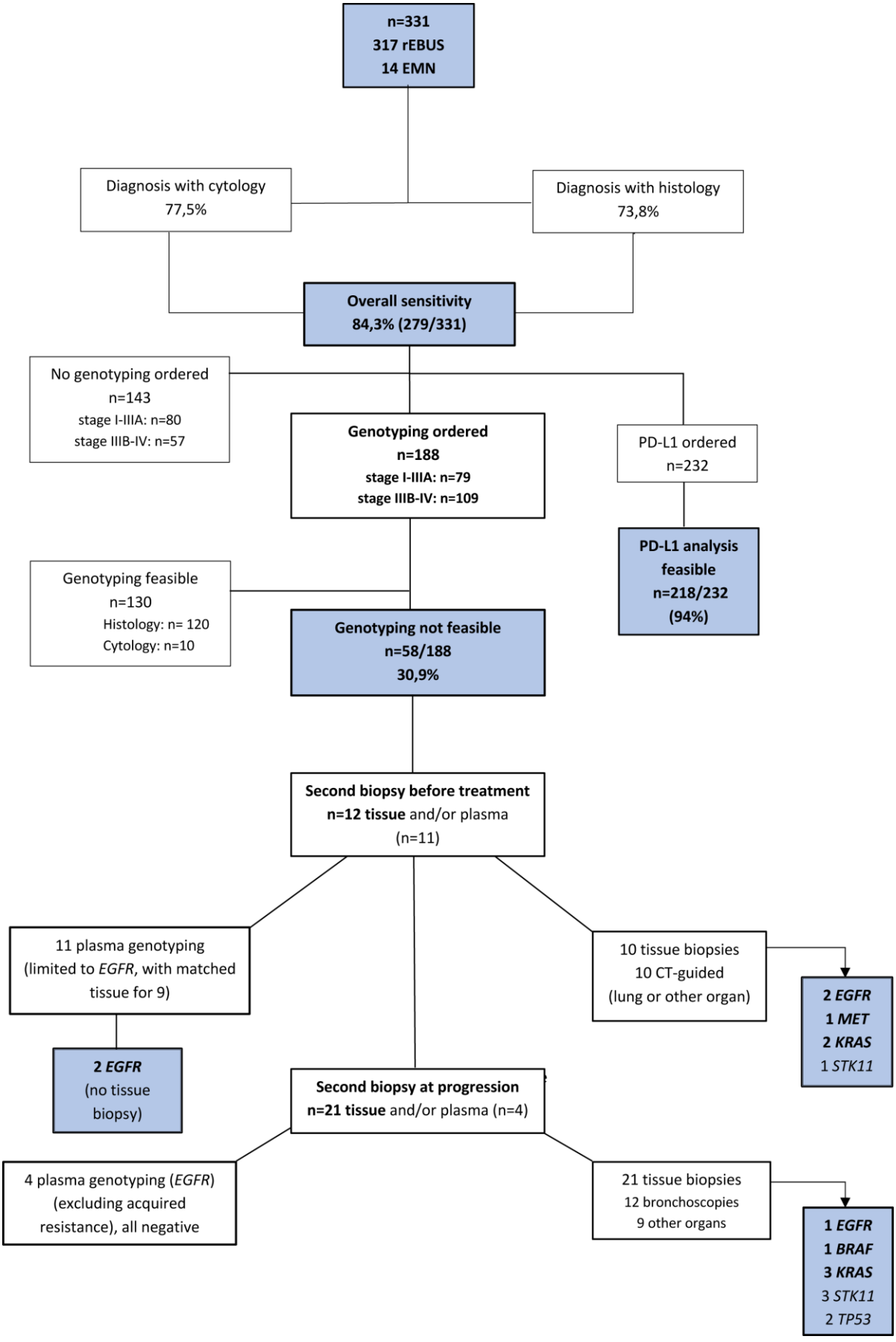
n=331
317 rEBUS
14 EMN
Diagnosis with cytology
77,5%
Diagnosis with histology
73,8%
Overall sensitivity
84,3% (279/331)
No genotyping ordered
n=143
stage I-IIIA: n=80
stage IIIB-IV: n=57
Genotyping ordered
n=188
stage I-IIIA: n=79
stage IIIB-IV: n=109
PD-L1 ordered
n=232
PD-L1 analysis feasible
n=218/232
(94%)
Genotyping feasible
n=130
Histology: n= 120
Cytology: n=10
Genotyping not feasible
n=58/188
30,9%
Second biopsy before treatment
n=12 tissue and/or plasma (n=11)
11 plasma genotyping
(limited to EGFR, with matched tissue for 9)
2 EGFR
(no tissue biopsy)
10 tissue biopsies
10 CT-guided
(lung or other organ)
2 EGFR
1 MET
2 KRAS
1 STK11
Second biopsy at progression
n=21 tissue and/or plasma (n=4)
4 plasma genotyping (EGFR)
(excluding acquired resistance), all negative
21 tissue biopsies
12 bronchoscopies
9 other organs
1 EGFR
1 BRAF
3 KRAS
3 STK11
2 TP53

Figure 2: Flow chart of the study

rEBUS: radial endobronchial ultrasonography; EMN: electromagnetic navigation; CT-guided: guided by computed tomography