Journal Pre-proof

Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration for PD-L1 Testing In Non-Small Cell Lung Cancer

Fabio Perrotta, Matthew Nankivell, Bhavani Adizie, Usman Maqsood, Mohamed Elshafi, Syeda Jafri, Andrew D. Lerner, Ian Woolhouse, Mohammed Munavvar, Matthew Evison, Richard Booton, David R. Baldwin, Samuel M. Janes, Keith M. Kerr, Andrea Bianco, Lonny Yarmus, Neal Navani
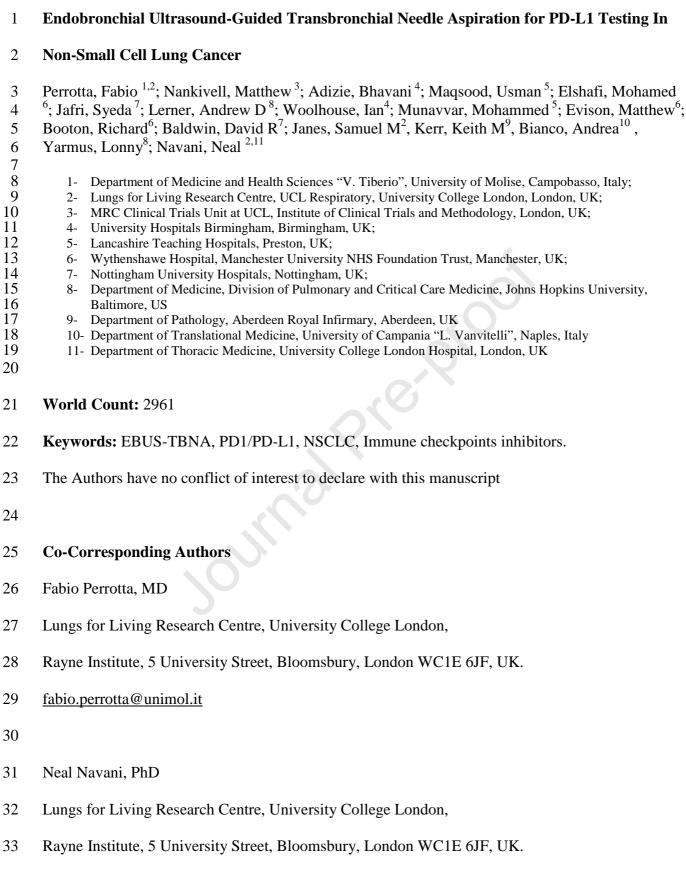
Please cite this article as: Perrotta F, Nankivell M, Adizie B, Maqsood U, Elshafi M, Jafri S, Lerner AD, Woolhouse I, Munavvar M, Evison M, Booton R, Baldwin DR, Janes SM, Kerr KM, Bianco A, Yarmus L, Navani N, Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration for PD-L1 Testing In Non-Small Cell Lung Cancer, *CHEST* (2020), doi: https://doi.org/10.1016/j.chest.2020.04.059.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration for PD-L1 Testing In**

2 **Non-Small Cell Lung Cancer**

3 Perrotta, Fabio [1,2]; Nankivell, Matthew [3]; Adizie, Bhavani [4]; Maqsood, Usman [5]; Elshafi, Mohamed
4 [6]; Jafri, Syeda [7]; Lerner, Andrew D [8]; Woolhouse, Ian[4]; Munavvar, Mohammed [5]; Evison, Matthew[6];
5 Booton, Richard[6]; Baldwin, David R[7]; Janes, Samuel M[2], Kerr, Keith M[9], Bianco, Andrea[10] ,
6 Yarmus, Lonny[8]; Navani, Neal [2,11]

8 1- Department of Medicine and Health Sciences "V. Tiberio", University of Molise, Campobasso, Italy;
9 2- Lungs for Living Research Centre, UCL Respiratory, University College London, London, UK;
10 3- MRC Clinical Trials Unit at UCL, Institute of Clinical Trials and Methodology, London, UK;
11 4- University Hospitals Birmingham, Birmingham, UK;
12 5- Lancashire Teaching Hospitals, Preston, UK;
13 6- Wythenshawe Hospital, Manchester University NHS Foundation Trust, Manchester, UK;
14 7- Nottingham University Hospitals, Nottingham, UK;
15 8- Department of Medicine, Division of Pulmonary and Critical Care Medicine, Johns Hopkins University,
16 Baltimore, US
17 9- Department of Pathology, Aberdeen Royal Infirmary, Aberdeen, UK
18 10- Department of Translational Medicine, University of Campania "L. Vanvitelli", Naples, Italy
19 11- Department of Thoracic Medicine, University College London Hospital, London, UK

21 **World Count:** 2961

23 The Authors have no conflict of interest to declare with this manuscript

25 **Co-Corresponding Authors**

26 Fabio Perrotta, MD

27 Lungs for Living Research Centre, University College London,

28 Rayne Institute, 5 University Street, Bloomsbury, London WC1E 6JF, UK.

29 fabio.perrotta@unimol.it

31 Neal Navani, PhD

32 Lungs for Living Research Centre, University College London,

33 Rayne Institute, 5 University Street, Bloomsbury, London WC1E 6JF, UK.

34 n.navani@ucl.ac.uk

36

37

Email

39   Perrotta Fabio: fabio.perrotta@unimol.it
40   Nankivell Matthew: m.nankivell@ucl.ac.uk
41   Adizie Bhavani: bhavani.adizie@uhb.nhs.uk
42   Maqsood Usman: usman.maqsood@lthtr.nhs.uk
43   Elshafi Mohamed: m.elshafi@nhs.net
44   Jafri Syeda: syeda.jafri@nuh.nhs.uk
45   Lerner Andrew D: alerner@jhu.edu
46   Yarmus Lonny: lyarmus@jhmi.edu
47   Woolhouse Ian: ian.woolhouse@uhb.nhs.uk
48   Munavvar Mohammed: mohammed.munavvar@lthtr.nhs.uk
49   Evison Matthew: m.evison@nhs.net
50   Booton Richard: richard.booton@uhsm.nhs.uk
51   Baldwin David: david.baldwin@nottingham.ac.uk
52   Samuel Janes: s.janes@ucl.ac.uk
53   Keith Kerr: k.kerr@abdn.ac.uk
54   Andrea Bianco: andrea.bianco@unicampania.it
55   Neal Navani: n.navani@ucl.ac.uk

56

57

58

59 **Abbreviation List**
60
61 ALK: anaplastic lymphoma kinase gene
62 CNB: core needle biopsy
63 CT: computed tomography
64 EGFR: epidermal growth factor receptor gene
65 EBUS-TBNA: endobronchial ultrasound-guided transbronchial needle aspiration
66 EUS: endoscopic ultrasonography
67 FNA: fine needle aspiration
68 ICH: immunohistochemistry
69 OR: odds ratio
70 OS: overall survival
71 NOS: not otherwise specified
72 NSCLC: non-small cell lung cancer
73 PD: disease progression
74 PD-1: programmed death-1
75 PD-L1: programmed death-ligand 1
76 PR: partial response
77 TPS: tumor proportion score
78 VATS: video-assisted thoracoscopic surgery
79

Abstract

Rationale: PD-L1 expression on cancer cells is a clinically important biomarker to select NSCLC patients for treatment with PD-1/PD-L1 inhibitors. Clinical trials of immunotherapy in patients with non-small cell lung cancer have required histology for PD-L1 testing, while in clinical practice cytology samples are commonly acquired in patients with advanced disease.

Objectives: This study investigates sampling adequacy of endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) for PD-L1 testing when compared to other methods. Furthermore, the relationship between clinico-pathological characteristics and PD-L1 expression in the study population have been examined.

Methods: Five hundred seventy-seven NSCLC specimens were analysed from consecutive patients with NSCLC across six centres in United Kingdom and one in the United States between January 2015 and December 2016.

Main Results: In the EBUS-TBNA group (189 specimens), the overall percentage of patients with successful PD-L1 testing was 94.7%. There was no significant difference in sampling adequacy with other methods of tissue acquisition. Older subjects had higher failure rates of PD-L1 testing (OR= 1.06, p=0.008). In multivariate analysis, advanced N-stage (p=0.048) and presence of brain metastasis (p<0.001) were associated with high PD-L1 expression.

Conclusion: This large multicenter study shows that EBUS-TBNA provides samples adequate for PD-L1 testing and that advanced N stage and the presence of brain metastasis are associated with high PD-L1 expression.

Introduction

Immune checkpoint inhibitors have demonstrated significant clinical utility in patients with advanced non-small cell lung cancer (NSCLC), and several anti PD-1 and anti PD-L1 monoclonal antibodies have been approved as first or second-line therapies [1–5]. These agents interfere with both costimulatory and co-inhibitory pathways regulating the antigen specific T-cell response [6]. PD-1 is a cell-receptor involved in programmed cell death. The PD-1 receptor binds to the ligands PD-L1 and PD-L2 and results in downregulation of anti-tumor cytolytic T-cell activity, inducing T cell exhaustion and immune tolerance.

The correlation between PD-L1 immunohistochemistry (IHC) expression, measured by the proportion of cancer cells positively staining for PD-L1, and the overall response to anti-PD-1 or anti-PD-L1 agents has been demonstrated in clinical trials. In the landmark KEYNOTE-024 trial[1], Pembrolizumab, an anti-PD-1 agent, resulted in better progression-free survival and overall survival compared to standard chemotherapy in patients with a tumor proportion score of 50% or greater. Therefore, PD-L1 IHC expression is currently used to select patients with advanced lung cancer who may benefit from first line immunotherapy alone. In this study however, core biopsies of tumor were mandated for trial entry[1].

Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is a minimally invasive technique, proven to be effective in obtaining cytology samples suitable for the molecular characterization of NSCLC [7]. However, despite its routine use in clinical practice, patients undergoing tissue acquisition by EBUS-TBNA alone were excluded from immunotherapy trials [8].

We therefore conducted a large, pragmatic, multi-center study to examine whether samples obtained by EBUS-TBNA were suitable for PD-L1 assessment and selection of patients for immune checkpoint inhibition. We compared the diagnostic yield of different methods including cytology samples, small biopsies and lung resections. We also systematically collected patient and procedure characteristics to define factors that predicted a reliable PD-L1 result and PD-L1 expression.

129

Methods

Study design

This study included consecutive patients with known or suspected NSCLC undergoing tissue

acquisition procedures between January 2015 and December 2016 across six centers in the United

Kingdom (University College London Hospital, University Hospital Birmingham, Lancashire

Teaching Hospital, Nottingham University Hospitals, University of South Manchester and

Papworth Hospital, Cambridge) and one center in the United States (Johns Hopkins University).

The specimens were obtained by EBUS-TBNA, percutaneous fine needle aspiration (FNA),

percutaneous core needle biopsy (CNB), medical thoracoscopy, video-assisted thoracoscopic

surgery (VATS) or open thoracotomy. Samples were analyzed and interpreted according to local

protocols and there was no centralized reporting. Genotyping was performed in all non-squamous

NSCLC or in other subtypes according to clinical judgment. Non-squamous NSCLC samples were

prioritized for mutation testing of the epidermal growth factor receptor gene (EGFR),

rearrangement of the anaplastic lymphoma kinase gene (ALK) and ROS-1 re-arrangement where

necessary. Samples were subsequently evaluated for PD-L1 expression.

EBUS-TBNA samples

EBUS-TBNA was performed with a dedicated linear echo-endoscope as previously described [9]. The

procedure at John Hopkins and University College London Hospital were carried out under general

anesthesia in 100% (14/14) and (33/49) 67% of cases respectively. All the other cases were done in

the outpatient setting with patients given moderate sedation with midazolam and fentanyl. In brief,

under direct ultrasound guidance, the lymph node was aspirated using either a 19, 21, 22 or 25-

gauge needle. The site and number of lymph nodes punctured were at the operator's discretion.

Four passes per lymph node were routinely performed in all cases. If these passes did not visually

155 return adequate material, at least 2 more passes from the same lymph node were additionally

156 performed. A suction syringe was applied to the needle during lymph node aspiration. On-site

157 evaluation of samples was not routinely employed. The samples obtained at EBUS-TBNA were

158 expelled from the needle using the stylet and placed into liquid fixative for cell-block processing.

159 The specimen was centrifuged to form a pellet, suspended in agar, fixed in neutral buffered

160 formalin or alcohol-based fixative, and processed as a cell block from which a single hematoxylin

161 and eosin (H&E)-stained section was cut. Further sections were cut and used for IHC staining as

162 required.

163

164 *PD-L1 assessment*

165 All the centers involved in this study used the Dako PD-L1 IHC 22C3 pharmDx

166 immunohistochemical assay (Dako, Glostrup, Denmark). This assay uses a monoclonal antibody

167 (humanized IgG4) that recognize the extracellular domain of PD-L1 to assess PD-L1 expression in

168 formalin-fixed, paraffin embedded (FFPE) tissue. The IHC staining procedure was performed on a

169 Dako Autostainer Link 48 platform with a validated staining protocol. PD-L1 expression was

170 evaluated by tumor proportion score (TPS), which is defined as the percentage of viable tumor cells

171 with at least partial membrane staining relative of all viable tumor cells in the examined section. All

172 other stained cells, such as tumor-associated immune cells, normal/non-neoplastic cells, and

173 necrotic cells, were excluded from evaluation. A minimum of 100 viable tumor cells were required

174 to consider the specimen adequate. The scoring was interpreted as: no PD-L1 expression

175 (TPS<1%); low PD-L1 expression (TPS 1-49%); and high PD-L1 expression (TPS $\geq$ 50%), in line

176 with current clinical practice and immunotherapy licensing. Ethical approval was not required given

177 the observational nature of the study. All data were prospectively recorded in each center, though

178 the study design is retrospective as reported previously[7]. Treatment strategies were fully disclosed

179 to the patients and were discussed in multidisciplinary team meetings.

180

7

181 **ENDPOINTS AND STATISTICAL ANALYSIS**

182 The primary aim of the study was to evaluate the diagnostic performance of PD-L1 testing in

183 specimens obtained by EBUS-TBNA in patients with NSCLC compared to other methods.

184 Secondary endpoints were to define clinico-pathological characteristics associated with a reliable

185 PD-L1 result and also to define clinical features associated with PD-L1 high expression.

186 Associations between baseline characteristics and a successful PD-L1 test were assessed using chi-

187 square tests, chi-square trend tests, and t-tests as appropriate. Baseline variables considered were

188 age, performance status, smoking status, TNM stage, presence of brain metastasis, pathological

189 tumor differentiation, actionable mutations and sampling method. Individual factors associated with

190 PD-L1 level (none, low or high), and with high PD-L1 were assessed using ordinal regression and

191 logistic regression respectively. Predictors of high PD-L1 level were further investigated through a

192 multi-variable model generated using forward selection and backward elimination processes,

193 assessing all variables with a p-value<0.25 on univariate analysis. All statistical calculations were

194 performed using STATA version 16 (StataCorp, College Station, TX).

195

196 **RESULTS**

197 *Study population*

198 Five hundred seventy-seven NSCLC specimens were analyzed from consecutive patients with

199 NSCLC. Three hundred eighteen subjects (55%) were male and the median age of the study

200 population was 68 years (range, 31-96 years). Tissue acquisition techniques included 189 (33%)

201 EBUS or EUS, 72 (12%) endobronchial biopsy, 167 (29%) CT-guided procedures, 124 (21%)

202 surgical excisions or resections, 6 (1%) pleural biopsy and 19 (3%) other site specimens.

203 Demographic and baseline characteristics are summarized in Table 1. Three hundred seventy-eight

204 patients (66%) had a final diagnosis of Adenocarcinoma, 151 (26%) Squamous Cell Carcinoma

205 while 48 (8%) received other diagnoses (Adenosquamous, not otherwise specified (NOS), Large

206 Cell Carcinoma, Other). The presence of EGFR mutations was reported in forty-one patients (7%),

8

207   ALK rearrangement in seven patients (1%) and ROS-1 rearrangement in only one case (<1%). For

208   EBUS-TBNA, 22-gauge needle was used in 78% of cases, 21-gauge needle in 20%, 19 and 25-

209   gauge needle in 1%. Lymph node stations sampled by EBUS-TBNA were reported in

210   Supplementary Table 1. Seven patients (3.7%) who underwent EBUS-TBNA had complications,

211   none of which resulted in early interruption of the procedure. In particular, significant bleeding

212   determined by the operator was documented in six cases (3.2%) while one patient (0.5%)

213   experienced desaturation with early recovery after the procedure. No patients required inpatient

214   admission after the procedure. The complication rates for endobronchial and transbronchial forceps

215   biopsies, CT-guided biopsies and surgery were 4.1%, 9.0% and 11.5%, respectively.

216

217   *PD-L1 assessment*

218   PD-L1 assessment was reported to be feasible in the majority of cases (Table 2). The overall rate of

219   assessment failure was 5% (29 patients). EBUS-TBNA provided adequate sampling for reliable PD-

220   L1 testing in 95% (179/189) of patients. PD-L1 testing was feasible in 155/167 CT guided biopsies

221   (93%), 70/72 endobronchial biopsies (97%), 123/124 surgical specimens (99.2%) and 6/6 pleural

222   biopsies (100%).

223   Failure rate among patients diagnosed with "other" methods was 21% (4/19), resulting in a

224   statistically significant difference when compared to the rest of the study population. Successful

225   PD-L1 assessment rates were similar across the different centers (range, 88.8%-100%). In the

226   EBUS-TBNA group, no differences were observed in yield between 21 and 22-gauge needle

227   (p=0.39). Older age was the only predictor of failure of PD-L1 assessment (OR= 1.06, p=0.008) in

228   univariate analysis. The likelihood of a successful PD-L1 assay did not vary according to study site,

229   gender, ethnicity, smoking status, pack years, performance status, T-stage, N-stage, M-stage,

230   histological subtype, actionable mutations, biopsy type (original vs re-biopsy), presence of brain

231   metastases or receipt of prior radiotherapy. Although the non-squamous samples also underwent

232   analysis for EGFR mutations and ALK and ROS-1 rearrangement, specifically no differences were

9

233 observed in PD-L1 assessment failure rate between adenocarcinoma and squamous cell carcinoma

234 (p=0.825).

235

236

237 *Predictors of PD-L1 expression*

238 PD-L1 tumor proportion staining was negative (<1%) in 234 patients (42.7%), low (1-49%) in 159

239 patients (29.0%), and high (≥ 50%) in 155 patients (28.3%). PD-L1 expression was not influenced

240 by the tissue sampling method (Table 3). However, we found that PD-L1 high expression was

241 associated with the presence of brain metastasis (p= 0.009).   In the model, dividing the study

242 population into high expression (TPS ≥ 50%) versus no or low expression (TPS < 50%) we found

243 that high PD-L1 expression was associated with advanced N-stage (p=0.024), M1 stage (p=0.031),

244 Adenocarcinoma subtype (p=0.023) and presence of brain metastasis (p<0.001). The final

245 multivariate model showed that higher N-stage (p=0.048) and the presence of brain metastasis

246 (p<0.001) were independently associated with high PD-L1 expression.

247

248 *Response to immunotherapy*

249 Fifty-six patients received immune checkpoint inhibitors (44 Pembrolizumab, 10 Nivolumab, 1

250 Atezolizumab, 1 Durvalumab). Table 4 demonstrates the response to immune checkpoint inhibitors

251 according to the line of treatment. 25 (44.6%) patients had disease progression, 20 (35.7%) patients

252 had stable disease, while 11 (19.6%) patients achieved a partial response. All patients with a partial

253 response were observed to have high PD-L1 expression. Disease response was not associated with

254 mode of tissue sampling.

255

256 **DISCUSSION**

257 *PD-L1 expression as predictive biomarker in NSCLC*

258 In the management of advanced NSCLC, molecular subtyping and PD-L1 status assessment have

259 become critical in selecting the most appropriate treatment[10]. Recently, several anti PD-1 and anti

260 PD-L1 agents have been approved by the FDA and EMA for patients with metastatic NSCLC who

261 do not harbor an EGFR mutation or ALK rearrangement both in the first and second line settings.

262 Pembrolizumab has received approval for first-line monotherapy for patients with tumor in which at

263 least 50% of cells express PD-L1 or in second-line treatment for patients with tumor whose at least

264 1% cells express PD-L1 on cell surface[11–14]. However, these trials specifically excluded patients

265 with tissue acquired by EBUS-TBNA despite at least 1/3 of patients having this procedure in

266 clinical practice. In 2016, the Papanicolaou Society of Cytopathology recommended against the use

267 of cytology samples for PD-L1 IHC testing due to insufficient data[15]. Similarly, the Pulmonary

268 Pathology Society[16] highlighted the lack of validation for cytology preparation in PD-L1 testing,

269 though for many patients with advanced NSCLC they are often the only specimens available. PD-

270 L1 analysis is now also required in Europe for patients with stage III disease to receive

271 immunotherapy after concurrent chemoradiotherapy[17]. For these patients, EBUS-TBNA provides an

272 important dual purpose of providing a tissue diagnosis as well as accurately mapping malignant

273 intra-thoracic lymph nodes. In this study, we show that EBUS-TBNA provides samples suitable for

274 PD-L1 testing and that response rates to immunotherapy do not depend upon modality of tissue

275 acquisition.

276 Several limitations of assessing PD-L1 expression are recognized. These include the tumor spatial

277 heterogeneity among different sections of the same sample or at different sites coupled with the

278 dynamic changes in PD-L1 expression over time[18]. However, at this time it represents the only

279 biomarker approved by the regulatory agencies for first line immunotherapy in NSCLC, while

280 others (microsatellite instability, tumor mutation burden, tumor microenvironment, gut

281 microbiome) are currently under investigation or in late stage of development.

282

283 *PD-L1 quantification in EBUS-TBNA*

284 Few studies have investigated the feasibility of PD-L1 assessment by EBUS-FNA[19]. Stoy et al[20]

285 examined the PD-L1 quantification in cytology specimens and they showed successful assessment

286 in 90.9% (20/22) of patients. This study included sixteen EBUS-TBNA, four endobronchial fine

287 needle aspirations and two bronchoscopic-FNA of peripheral nodules; two unsuccessful EBUS tests

288 were because the cell block had <100 cells. They also found a good concordance in two patients

289 who had same site both cytology and histology samples. In another single-center retrospective study

290 collecting 188 patients with lung cancer, Heyman et al. [21] found that cytology specimens were

291 adequate for PD-L1 quantification in 90% of patients, while small biopsy and surgical resection

292 completed assessment rates were 96% and 99%, respectively. Interestingly, only 25 of 214

293 specimens (11.7%) were from EBUS-TBNA, while 36.0% of samples were from surgical resection

294 which is not commonly performed in patients who are currently candidates for immune checkpoint

295 inhibitors. Similar results are described in a larger study which included 252 EBUS-TBNA samples

296 and compared cytology, small biopsies and surgical resections. The authors reported 92% sample

297 adequacy for PD-L1 testing for cytology or small biopsy specimens[22]. In this study the fixation

298 process (formalin only versus methanol/alcohol only versus both) did not influence the PD-L1

299 staining. Very recently, Biswas et al, using the PD-L1 22C3 pharmDx assay, confirmed that EBUS-

300 TBNA was able to allow the PD-L1 quantification in 86% of cases[23]. These studies reflect our

301 findings in 566 patients in whom the rate of failure of PD-L1 testing was 4.8% in the EBUS group.

302 Other studies have investigated the concordance in PD-L1 expression between EBUS-TBNA and

303 other samples[24]. Sakakibara et al.[25] found a good correlation between EBUS samples and surgical

304 samples in both primary (r=0.75; p=0.08, n=6) and metastatic site (r=0.93; p:0.02, n=5); However,

305 the IHC antibody used (EPR1161,Abcam, Cambridge, Massachusetts) was not one of the approved

306 companion assays developed with immune checkpoint inhibitors.

307 An important finding from our study is that older age was associated with a higher chance of a

308 failed PD-L1 assessment. EBUS-TBNA has previously been shown to have an excellent safety

309 profile coupled with an excellent yield for malignancy in older subjects [26,27]. Our data suggest that

310 specimen quality may be inferior in the older patient, perhaps reflecting the challenges of obtaining

311 sufficient diagnostic material in this important group of patients.

312

313 *Clinico-pathological features of PD-L1 expression*

314 In this large multicenter study, we report the novel findings that PD-L1 expression is associated

315 with higher N-stage and the presence of brain metastasis. Previous studies have shown conflicting

316 results. Shimoji et al[28] reported that in 220 patients undergoing surgical resection, PD-L1

317 expression was correlated with younger age, smoking habit and solid pattern in adenocarcinoma

318 subjects, while multivariate analysis however revealed that only the solid adenocarcinoma subtype

319 was an independent predictor of PD-L1 expression. This study however was limited by fact that

320 only patients with early stage disease were included and all samples were from surgical resections.

321 In another article [29] using the E1L3N assay in 297 patients, the authors found that PD-L1 expression

322 on tumor cells was higher in men (p < 0.0001), older (p = 0.0321), smokers (p < 0.0001), high

323 histologic grade (p = 0.0012) and squamous cell histotype (p = 0.0412) patients. More recently, a

324 larger retrospective cohort study of 2402 surgically resected stage I-III NSCLC patients found that

325 PD-L1 positivity was more frequent in never smokers, higher disease stages and larger tumors[30]. In

326 this study, PD-L1 expression in adenocarcinoma patients was associated with better clinical

327 outcomes (OS, time to relapse and relapse-free survival), though these data are heterogeneous

328 among the previous published papers [31–33].

329 Our study confirms findings of a recent metanalysis [34] showing that PD-L1 expression was

330 increased in patients with lymph node metastasis (OR = 1.34, 95% CI: 1.19–1.50, P < 0.001) and

331 TNM stage (OR = 1.45, 95% CI: 1.18–1.78; P < 0.001) but also for the first time that PD-L1 strong

332 positive patients were more likely to have brain metastases. These data are, of great interest as the

333 immune checkpoint inhibitors clinical trials excluded patients with presence of untreated or

334 unstable brain metastasis[1–4,35] and the management of patients with NSCLC and brain metastases is

335 evolving[36].

336

337 *Study limitations*

338 To our knowledge, this is the largest study to assess the feasibility of PD-L1 testing using different

339 modes of tissue acquisition and provides multi-center real world data. However, there are several

340 limitations. First, PD-L1 expression, was evaluated by local pathology units only without any

341 control of inter-observer variability. However, the same approved assay was used in each center. No

342 specific assessment of concordance between EBUS specimens and surgical lymph node sampling

343 was planned in this study. This would have required surgical sampling of intra-thoracic lymph

344 nodes which is currently not standard practice in patients with advanced disease for whom

345 immunotherapy is currently licensed. The study included patients biopsied before the routine

346 approval of checkpoint inhibitors and many of the subjects who received immunotherapy were

347 within clinical trials. Thus, the small number of patients treated with immune checkpoint antibodies

348 did not allow any further consideration of factors that may predict response to immunotherapy.

349

350 **Conclusions**

351 EBUS-TBNA represents an important investigation for tissue acquisition in patients with lung

352 cancer as well as for lymph node staging. In this multicenter study, we have demonstrated that

353 EBUS-TBNA allows adequate sampling for testing PD-L1 in a broad population of NSCLC

354 patients. We have also reported that patients with advanced N-stage and brain metastasis are more

355 likely to express high levels of PD-L1. Finally, these data provide evidence that EBUS-TBNA

356 samples are suitable for complete molecular profiling, including PD-L1 testing, to allow decisions

357 regarding treatments and clinical trial eligibility to be made.

358

359 **Acknowledgement**

366

367 **Authors Contribution**

368 Each author contributed to the study as follows:
369 Conception and design of the work (NN, SMJ, DRB, RB, IW, ADL, KK); Data acquisition (FP,AdB,
370 UM, ME, SJ,LY) ; Statistical Analysis (MN); Drafting the work or revising it critically (FP, MN, ADL,
371 IW, MM, ME, RB, SMJ, KK, AnB, LY,NN). NN is the guarantor of the paper, taking responsibility for
372 the integrity of the work as a whole, from inception to published article.

373

374

375

376
377
378
379
380

381
382 **REFERENCES**

383

384 1. Reck M, Rodríguez-Abreu D, Robinson AG, et al. Pembrolizumab versus Chemotherapy for PD-L1–

385 Positive Non–Small-Cell Lung Cancer. *N Engl J Med*. 2016;375(19):1823-1833.

386 doi:10.1056/NEJMoa1606774

387 2. Borghaei H, Paz-Ares L, Horn L, et al. Nivolumab versus Docetaxel in Advanced Nonsquamous

388 Non-Small-Cell Lung Cancer. *N Engl J Med*. 2015;373(17):1627-1639.

389 doi:10.1056/NEJMoa1507643

390 3. Herbst RS, Baas P, Kim DW, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-

391 positive, advanced non-small-cell lung cancer (KEYNOTE-010): A randomised controlled trial.

392 *Lancet*. 2016;387(10027):1540-1550. doi:10.1016/S0140-6736(15)01281-7

393 4. Fehrenbacher L, Spira A, Ballinger M, et al. Atezolizumab versus docetaxel for patients with

394 previously treated non-small-cell lung cancer (POPLAR): A multicentre, open-label, phase 2

395 randomised controlled trial. *Lancet*. 2016;387(10030):1837-1846. doi:10.1016/S0140-

396 6736(16)00587-0

397 5. Antonia S, Goldberg SB, Balmanoukian A, et al. Safety and antitumour activity of durvalumab plus

398 tremelimumab in non-small cell lung cancer: A multicentre, phase 1b study. *Lancet Oncol*.

399 2016;17(3):299-308. doi:10.1016/S1470-2045(15)00544-6

400 6. Korman AJ, Peggs KS, Allison JP. Checkpoint blockade in cancer immunotherapy. *Adv Immunol*.

401 2006;90:297-339. doi:10.1016/S0065-2776(06)90008-X

402 7. Navani N, Brown JM, Nankivell M, et al. Suitability of endobronchial ultrasound-guided

403 transbronchial needle aspiration specimens for subtyping and genotyping of non-small cell lung

404 cancer: A multicenter study of 774 patients. *Am J Respir Crit Care Med*. 2012;185(12):1316-1322.

405 doi:10.1164/rccm.201202-0294OC

406 8. Beattie J, Yarmus L, Wahidi M, et al. The Immune Landscape of Non-Small-Cell Lung Cancer.

407 Utility of Cytologic and Histologic Samples Obtained through Minimally Invasive Pulmonary

408 Procedures. *Am J Respir Crit Care Med*. 2018;198(1):24-38. doi:10.1164/rccm.201712-2539PP

409 9. Navani N, Nankivell M, Lawrence DR, et al. Lung cancer diagnosis and staging with endobronchial

410 ultrasound-guided transbronchial needle aspiration compared with conventional approaches: an open-

411 label, pragmatic, randomised controlled trial. *Lancet Respir Med*. 2015;3(4):282-289.

412 doi:10.1016/S2213-2600(15)00029-6

413 10. Bianco A, Perrotta F, Barra G, Malapelle U, Rocco D, De Palma R. Prognostic Factors and

414 Biomarkers of Responses to Immune Checkpoint Inhibitors in Lung Cancer. *Int J Mol Sci*.

415 2019;20(19). doi:10.3390/ijms20194931

416 11. Bianco A, Malapelle U, Rocco D, Perrotta F, Mazzarella G. Targeting immune checkpoints in non

417 small cell lung cancer. *Curr Opin Pharmacol*. 2018;40:46-50. doi:10.1016/j.coph.2018.02.006

418 12. Gandhi L, Rodriguez-Abreu D, Gadgeel S, et al. Pembrolizumab plus Chemotherapy in Metastatic

419 Non-Small-Cell Lung Cancer. *N Engl J Med*. 2018;378(22):2078-2092.

420 doi:10.1056/NEJMoa1801005

421 13. Gandhi L, Garassino MC. Pembrolizumab plus Chemotherapy in Lung Cancer. *N Engl J Med*.

422 2018;379(11):e18. doi:10.1056/NEJMc1808567

423 14. Perrotta F, Rocco D, Vitiello F, et al. Immune Checkpoint Blockade for Advanced NSCLC: A New

424 Landscape for Elderly Patients. *Int J Mol Sci*. 2019;20(9). doi:10.3390/ijms20092258

425 15. Layfield LJ, Roy-Chowdhuri S, Baloch Z, et al. Utilization of ancillary studies in the cytologic

426 diagnosis of respiratory lesions: The papanicolaou society of cytopathology consensus

427 recommendations for respiratory cytology. *Diagn Cytopathol*. 2016;44(12):1000-1009.

428 doi:10.1002/dc.23549

429 16. Sholl LM, Aisner DL, Allen TC, et al. Programmed Death Ligand-1 Immunohistochemistry--A New

430 Challenge for Pathologists: A Perspective From Members of the Pulmonary Pathology Society. *Arch

431 Pathol Lab Med*. 2016;140(4):341-344. doi:10.5858/arpa.2015-0506-SA

432 17. Antonia SJ, Villegas A, Daniel D, et al. Durvalumab after Chemoradiotherapy in Stage III Non-

433 Small-Cell Lung Cancer. *N Engl J Med*. 2017;377(20):1919-1929. doi:10.1056/NEJMoa1709937

434 18. Sacher AG, Gandhi L. Biomarkers for the Clinical Use of PD-1/PD-L1 Inhibitors in Non-Small-Cell

435 Lung Cancer: A Review. *JAMA Oncol*. 2016;2(9):1217-1222. doi:10.1001/jamaoncol.2016.0639

436 19. Gosney JR, Boothman A-M, Ratcliffe M, Kerr KM. Cytology for PD-L1 testing: A systematic

437 review. *Lung Cancer*. 2020;141:101-106. doi:10.1016/j.lungcan.2020.01.010

438 20. Stoy SP, Rosen L, Mueller J, Murgu S. Programmed death-ligand 1 testing of lung cancer cytology

439 specimens obtained with bronchoscopy. *Cancer Cytopathol*. 2018;126(2):122-128.

440 doi:10.1002/cncy.21941

441 21. Heymann JJ, Bulman WA, Swinarski D, et al. Programmed death-ligand 1 expression in non-small

442 cell lung carcinoma: Comparison among cytology, small biopsy, and surgical resection specimens.

443 *Cancer Cytopathol*. 2017:1-12. doi:10.1002/cncy.21937

444 22. Wang H, Agulnik J, Kasymjanova G, et al. Cytology cell blocks are suitable for

445 immunohistochemical testing for PD-L1 in lung cancer. *Ann Oncol*. 2018;29(6):1417-1422.

446 doi:10.1093/annonc/mdy126

447 23. Biswas A, Leon ME, Drew P, et al. Clinical performance of endobronchial ultrasound-guided

448 transbronchial needle aspiration for assessing programmed death ligand-1 expression in nonsmall cell

449 lung cancer. *Diagn Cytopathol*. 2018;46(5):378-383. doi:10.1002/dc.23900

450 24. Haragan A, Field JK, Davies MPA, Escriu C, Gruver A, Gosney JR. Lung Cancer Heterogeneity of

451 PD-L1 expression in non-small cell lung cancer : Implications for specimen sampling in predicting

452   treatment response. *Lung Cancer*. 2019;134(April):79-84. doi:10.1016/j.lungcan.2019.06.005

453   25.   Sakakibara R, Inamura K, Tambo Y, et al. EBUS-TBNA as a Promising Method for the Evaluation of

454   Tumor PD-L1 Expression in Lung Cancer. *Clin Lung Cancer*. 2016:3-8.

455   doi:10.1016/j.cllc.2016.12.002

456   26.   Evison M, Crosbie PAJ, Martin J, et al. EBUS-TBNA in elderly patients with lung cancer: Safety and

457   performance outcomes. *J Thorac Oncol*. 2014;9(3):370-376. doi:10.1097/JTO.0000000000000085

458   27.   Okachi S, Imai N, Imaizumi K, et al. Endobronchial ultrasound transbronchial needle aspiration in

459   older people. *Geriatr Gerontol Int*. 2013;13(4):986-992. doi:10.1111/ggi.12043

460   28.   Shimoji M, Shimizu S, Sato K, et al. Lung Cancer Clinical and pathologic features of lung cancer

461   expressing programmed cell death ligand 1 (PD-L1). *Lung Cancer*. 2016;98:69-75.

462   doi:10.1016/j.lungcan.2016.04.021

463   29.   Jiang L, Su X, Zhang T, et al. PD-L1 expression and its relationship with oncogenic drivers in non-

464   small cell lung cancer (NSCLC). *Oncotarget*. 2017;8(16):26845-26857.

465   doi:10.18632/oncotarget.15839

466   30.   Kerr KM, Thunnissen E, Dafni U, et al. A retrospective cohort study of PD-L1 prevalence, molecular

467   associations and clinical outcomes in patients with NSCLC: Results from the European Thoracic

468   Oncology Platform (ETOP) Lungscape Project. *Lung Cancer*. 2019;131:95-103.

469   doi:10.1016/j.lungcan.2019.03.012

470   31.   Mori S, Motoi N, Ninomiya H, et al. High expression of programmed cell death 1 ligand 1 in lung

471   adenocarcinoma is a poor prognostic factor particularly in smokers and wild-type epidermal growth-

472   factor receptor cases. *Pathol Int*. 2017;67(1):37-44. doi:10.1111/pin.12489

473   32.   Takada K, Okamoto T, Shoji F, et al. Clinical Significance of PD-L1 Protein Expression in Surgically

474   Resected Primary Lung Adenocarcinoma. *J Thorac Oncol*. 2016;11(11):1879-1890.

475   doi:10.1016/j.jtho.2016.06.006

476   33.   Cooper WA, Tran T, Vilain RE, et al. PD-L1 expression is a favorable prognostic factor in early stage

477   non-small cell carcinoma. *Lung Cancer*. 2015;89(2):181-188. doi:10.1016/j.lungcan.2015.05.007

478   34.   Zhang M, Li G, Wang Y, et al. Expression in lung cancer and its correlation with driver mutations: A

479   meta-analysis. *Sci Rep*. 2017;7(1):1-10. doi:10.1038/s41598-017-10925-7

480   35.   Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus Docetaxel in Advanced Squamous-Cell

481   Non–Small-Cell Lung Cancer. *N Engl J Med*. 2015;373(2):123-135. doi:10.1056/NEJMoa1504627

482   36.   Goldberg SB, Gettinger SN, Mahajan A, et al. Pembrolizumab for patients with melanoma or non-

483   small-cell lung cancer and untreated brain metastases: early analysis of a non-randomised, open-label,

484   phase 2 trial. *Lancet Oncol*. 2016;17(7):976-983. doi:10.1016/S1470-2045(16)30053-5

485

486

TABLES

Table 1. Baseline characteristics.

		All patients N=577
Age	Median	68
	IQR	61 – 74
	Range	31 – 96
Sex	Male	318 (55%)
	Female	259 (45%)
Ethnicity	Caucasian	429 (88%)
	Other	57 (12%)
	Missing	91
Smoking status	Current	142 (27%)
	Former	312 (59%)
	Never	77 (15%)
	Missing	46
Pack years (those with smoking status data only)	0	77 (15%)
	<20	60 (11%)
	20+	298 (56%)
	Missing (but >0)	96 (18%)
Performance status	0	122 (25%)
	1	262 (53%)
	2	67 (14%)
	3	31 (6%)
	4	9 (2%)
	Missing	86
T-stage	1	85 (16%)
	2	168 (32%)
	3	125 (24%)
	4	151 (29%)
	Missing	48
N-stage	0	121 (23%)
	1	63 (12%)
	2	202 (38%)
	3	142 (27%)
	Missing	49
M-stage	0	218 (42%)
	1	307 (58%)
	Missing	52

		All patients N=577
Histology	Adenocarcinoma	378 (66%)
	Squamous	151 (26%)
	Other	48 (8%)
Sampling method	EBUS/EUS	189 (33%)
	Endobronchial biopsy	72 (12%)
	CT guided biopsy	167 (29%)
	Surgical	124 (21%)
	Pleural	6 (1%)
	Other	19 (3%)
Actionable mutation	ALK	7 (1%)
	EGFR	41 (7%)
	HER-2	2 (<1%)
	ROS1	1 (<1%)
	None	526 (91%)
Brain metastases	No	381 (84%)
	Yes	70 (16%)
	Missing	127
Received radiotherapy	No	310 (58%)
	Yes	221 (42%)
	Missing	46

Table 2. PDL1 assessment success rate.

		N=577
Overall	Overall	548/577 (95%)
Age group*	<60	3/130 (2%)
	60-69	8/187 (4%)
	70-79	13/203 (6%)
	80+	5/57 (9%)

NB. No difference according to sex, ethnicity (caucasian vs others), smoking status, pack years, performance status, T-stage, N-stage, M-stage, Histology, EGFR, biopsy type (original vs re-biopsy), presence of brain metastases, receipt of radiotherapy. Sampling method is non-significant if the "other" group is excluded. Failure rate among the "other" group is 21% (4/19), significantly higher than the other methods. *Odds ratio for age as a continuous variable is 1.06 (p-value=0.008).

Table 2. PDL1 assessment success rate.

		Fail	P-value
Overall	Overall	548/577 (95%)	n\a
Age	<60	3/130 (2%)	0.008*
	60-69	8/187 (4%)	
	70-79	13/203 (6%)	
	80+	5/57 (9%)	
Sex	Male	19/318 (6%)	0.248

		Fail	P-value
	Female	10/259 (4%)	
Ethnicity	Caucasian	0/57 (0%)	0.061
	Other	25/429 (6%)	
Smoking status	Current	3/77 (4%)	0.604[$]
	Former	16/312 (5%)	
	Never	8/142 (6%)	
Pack years (those with smoking status data only)	0	3/77 (4%)	0.511[$]
	<20	6/60 (10%)	
	20+	13/298 (4%)	
Performance status	0	4/122 (3%)	0.086[$]
	1	15/262 (6%)	
	2	5/67 (7%)	
	3	3/31 (10%)	
	4	1/9 (11%)	
T-stage	1	5/85 (6%)	0.949[$]
	2	7/168 (4%)	
	3	8/125 (6%)	
	4	7/151 (5%)	
N-stage	0	6/121 (5%)	0.254[$]
	1	2/63 (3%)	
	2	7/202 (3%)	
	3	11/142 (8%)	
M-stage	0	13/218 (6%)	0.276
	1	12/307 (4%)	
Histology	Adenocarcinoma	23/378 (6%)	0.253
	Squamous	4/151 (3%)	
	Other	2/48 (4%)	
Sampling method	EBUS/EUS	10/189 (5%)	0.098[$]
	Endobronchial biopsy	2/72 (3%)	
	CT guided biopsy	12/167 (7%)	
	Surgical	1/124 (1%)	
	Pleural	0/6 (0%)	
	Other	4/19 (21%)	
Actionable mutation	Any	3/51 (6%)	0.769
	None	26/526 (5%)	

		Fail	P-value
EGFR mutation	No	26/536 (5%)	0.486
	Yes	3/41 (7%)	
Brain metastases	No	22/381 (6%)	0.129
	Yes	1/70 (1%)	
Received radiotherapy	No	20/310 (6%)	0.089
	Yes	7/221 (3%)	

$ For p-value calculation, the "other" group is excluded.
*P-value calculated treating factor as a continuous variable.
$P-value calculated using test for trend.

Table 3. Association with strong PDL1 expression

Factor		None/weak	Strong	P-value
Age	Median	68	67	0.249
	IQR	61-74	59-73	
Sex	Male	215 (72%)	84 (28%)	0.913
	Female	178 (71%)	71 (29%)	
Ethnicity	Caucasian	294 (73%)	110 (27%)	0.140
	Non-Caucasian	36 (63%)	21 (37%)	
Smoking status	Current	93 (69%)	41 (31%)	0.653
	Former	217 (73%)	79 (27%)	
	Never	55 (74%)	19 (26%)	
Pack years	0	55 (74%)	19 (26%)	0.586
	<20	41 (76%)	13 (24%)	
	20+	200 (70%)	85 (30%)	
Performance status	0	87 (74%)	31 (26%)	0.656
	1	176 (71%)	71 (29%)	
	2	42 (68%)	20 (32%)	
	3	19 (68%)	9 (32%)	
	4	4 (50%)	4 (50%)	
T-stage	1	58 (73%)	22 (28%)	0.942
	2	118 (73%)	43 (27%)	
	3	84 (72%)	33 (28%)	
	4	101 (70%)	43 (30%)	
N-stage	0	90 (78%)	25 (22%)	0.024
	1	49 (80%)	12 (20%)	

Factor		None/weak	Strong	P-value
	2	137 (70%)	58 (30%)	
	3	83 (63%)	48 (37%)	
M-stage	0	157 (77%)	48 (23%)	0.031
	1	200 (68%)	95 (32%)	
Histology	Adenocarcinoma	241 (68%)	114 (32%)	0.023
	Squamous	116 (79%)	31 (21%)	
	Other	36 (78%)	10 (22%)	
EGFR mutation	No	366 (72%)	144 (28%)	0.925
	Yes	27 (71%)	11 (29%)	
Any mutation	No	359 (72%)	141 (28%)	0.887
	Yes	34 (71%)	14 (29%)	
Re-biopsy	No	275 (72%)	107 (28%)	0.910
	Yes	75 (71%)	30 (29%)	
Sampling method	EBUS/EUS	120 (67%)	59 (33%)	0.073
	Endobronchial biopsy	49 (70%)	21 (30%)	
	CT guided biopsy	118 (76%)	37 (24%)	
	Surgical	95 (77%)	28 (23%)	
	Pleural	4 (67%)	2 (33%)	
	Other	7 (47%)	8 (53%)	
Brain metastases	No	266 (74%)	93 (26%)	<0.001
	Yes	35 (51%)	34 (49%)	
Received radiotherapy	No	215 (74%)	75 (26%)	0.118
	Yes	145 (68%)	69 (32%)	

Table 4. Response to immunotherapy

Immunotherapy treatment line	Response	All patients (56)
1st line	PR	7 (28%)
	Stable	10 (40%)
	PD	8 (32%)
2nd line	PR	2 (8.3%)
	Stable	7 (29.2%)
	PD	15 (62.5%)
3rd or more	PR	1 (14.3%)
	Stable	1 (14.3%)
	PD	5 (71.4%)