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SYSTEMIC AND TUMOR TH1 AND TH2 INFLAMMATORY PROFILE AND MACROPHAGES IN LUNG CANCER: INFLUENCE OF UNDERLYING CHRONIC RESPIRATORY DISEASE
--Manuscript Draft--

Abstract:	Chronic respiratory conditions, especially chronic obstructive pulmonary disease (COPD), and inflammatory events underlie lung cancer (LC). Objectives: We hypothesized that profiles of Th1 and Th2 cytokines and M1 and M2 macrophages are differentially expressed in lung tumors and blood of patients with non-small cell LC (NSCLC) with and without COPD and that M1/M2 specifically may influence their survival. Methods: In blood, inflammatory cytokines (ELISA) were quantified in 80 LC patients (LC-COPD, 60 and LC-only, 20) and lung specimens (tumor and non-tumor) from those undergoing thoracotomy (LC-COPD and LC-only, 20/group). Results: In LC-COPD compared to LC patients, systemic levels of TNF-alpha, IL-2, TGF-beta, and IL-10 were increased, whereas VEGF and IL-4 levels decreased. In lung tumors, TNF-alpha, TGF-beta, and IL-10 levels were greater than in non-tumor parenchyma in LC-COPD, while IL-2 and VEGF levels were higher in tumors of both LC-only and LC-COPD. Compared to non-tumor lung, M1 macrophage counts were reduced, while M2 were increased in tumors of both patient groups, and M1/M2 was greater in LC-COPD than LC-only. M1 and M2 counts did not influence patients' survival. Conclusions: The

relative predominance of Th1 cytokines and M1 macrophages in the blood and tumors of patients with underlying COPD imply that a stronger proinflammatory pattern exists in these patients. Inflammation should not be targeted systematically in all patients with LC. Screening for the presence of underlying respiratory diseases and identification of the specific inflammatory pattern should be carried out in patients with LC, at least in early stages of their disease.

SYSTEMIC AND TUMOR TH1 AND TH2 INFLAMMATORY PROFILE AND MACROPHAGES IN LUNG CANCER: INFLUENCE OF UNDERLYING CHRONIC RESPIRATORY DISEASE

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Short title: COPD influences inflammatory pattern in lung cancer

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25 **ABSTRACT**

26 Chronic respiratory conditions, especially chronic obstructive pulmonary disease

27 (COPD), and inflammatory events underlie lung cancer (LC). **Objectives:** We

28 hypothesized that profiles of Th1 and Th2 cytokines and M1 and M2 macrophages are

29 differentially expressed in lung tumors and blood of patients with non-small cell LC

30 (NSCLC) with and without COPD and that M1/M2 specifically may influence their

31 survival. **Methods:** In blood, inflammatory cytokines (ELISA) were quantified in 80

32 LC patients (LC-COPD, 60 and LC-only, 20) and lung specimens (tumor and non-

33 tumor) from those undergoing thoracotomy (LC-COPD and LC-only, 20/group).

34 **Results**: In LC-COPD compared to LC patients, systemic levels of TNF-alpha, IL-2,

35 TGF-beta, and IL-10 were increased, whereas VEGF and IL-4 levels decreased. In lung

36 tumors, TNF-alpha, TGF-beta, and IL-10 levels were greater than in non-tumor

37 parenchyma in LC-COPD, while IL-2 and VEGF levels were higher in tumors of both

38 LC-only and LC-COPD. Compared to non-tumor lung, M1 macrophage counts were

39 reduced, while M2 were increased in tumors of both patient groups, and M1/M2 was

40 greater in LC-COPD than LC-only. M1 and M2 counts did not influence patients'

41 survival. **Conclusions:** The relative predominance of Th1 cytokines and M1

42 macrophages in the blood and tumors of patients with underlying COPD imply that a

43 stronger proinflammatory pattern exists in these patients. Inflammation should not be

44 targeted systematically in all patients with LC. Screening for the presence of underlying

45 respiratory diseases and identification of the specific inflammatory pattern should be

46 carried out in patients with LC, at least in early stages of their disease.

47 **Word count:** 250

48 **KEY WORDS:** lung cancer; chronic respiratory conditions; Th1 and Th2 cytokines;

49 M1 and M2 macrophages; immune system; systemic and lung compartments

50

INTRODUCTION

In cancer-related mortality, lung cancer (LC) continues to be the most common cause of death worldwide[1-5], accounting for almost one third of deaths in certain regions[6]. Underlying respiratory conditions such as chronic obstructive pulmonary disease (COPD), which is also a highly prevalent disorder in industrialized countries, has been consistently associated with LC occurrence[7-11]. Airway obstruction and emphysema are, indeed, important risk factors for LC[7-11]. Identification of the biological mechanisms that render patients with chronic lung diseases more susceptible to LC development remains to be fully elucidated.

In this regard, despite the complex interactions observed among inflammation, immunity and lung tumor development, chronic inflammation has already been identified as a potential trigger in the process of tumorigenesis. As such, chronic inflammatory insults in the airways and lungs of respiratory patients may favor the risk of LC as also shown to occur in other cancer types such as pancreas, esophagus, and stomach[12-14]. In this regard, induction of several interleukins (IL) and cyclooxygenase-2 activity was suggested to contribute to the neoplastic transformation in patients with COPD[15-17]. Importantly, those inflammatory molecules may interfere with regulatory cell mechanisms such as repair, angiogenesis, and apoptosis, which favor the neoproliferative process[15-17]. Furthermore, cytokines and growth factors such as tumor necrosis factor (TNF)-alpha, IL-2, IL-4, IL-10, vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-beta, and epidermal growth factor receptor (EGFR) were also shown to promote tumor growth and metastasis in patients with underlying respiratory conditions[18-22].

Tumor immune surveillance also seems to play an important role in LC development including patients with chronic respiratory diseases[23]. Interestingly, Th1

76 lymphocytes, which release TNF-alpha, IL-2, and interferon-gamma, exert antitumor

77 effects, while Th2 cells, which mainly produce IL-4, were shown to favor tumor growth

78 by inhibiting the host immune system[24]. Tumorigenesis and relapse may rely on

79 alterations in Th1 and Th2 cytokines in patients[20;25;26]. In line with this, in patients with

80 LC, systemic Th2 cytokine levels were increased, whereas those of Th1 were

81 decreased[20]. Importantly, a change in Th1 and Th2 cytokines was seen in the same

82 patients after surgical treatment of the lung tumor, suggesting that these cytokines may

83 play a significant role in tumor progression[20].

84   In tumor microenvironment, type 1 (M1) and type 2 (M2) polarized macrophage

85 subtypes play a role in tumorigenesis through the regulation of several functions such as

86 cell adhesion, apoptosis, and senescence[25-27]. Furthermore, M1 macrophages were

87 shown to act in the initial process of tumorigenesis, exerting anti-tumorigenic effects,

88 while M2 macrophages were the predominant cells of established tumors[28]. Besides,

89 decreased M1/M2 macrophages may also influence tumor survival[29]. Whether the

90 pattern of chronic inflammatory events including the subtypes of macrophages may

91 differ in the tumors of LC patients with underlying respiratory diseases such as COPD

92 remains an open question. Answers to these questions may offer insight into that may

93 help design immunotherapeutic strategies for the better management of LC.

94   On the basis of this, we hypothesized that Th1 and Th2 cytokine profiles and M1

95 and M2 macrophages are differentially expressed in the lung tumor and non-tumor

96 parenchyma, and blood of patients with LC with and without COPD and that

97 specifically M1/M2 ratio may influence their survival. Hence, the study objectives were

98 defined as follows. In plasma, tumor lesions and non-tumor lung of non-small cell LC

99 (NSCLC) patients with and without underlying COPD, to determine: 1) protein levels

100   of Th1 and Th2 cytokines, 2) M1 and M2 macrophage counts in the lung, and 3)

101   survival of the patients according to numbers of M1 and M2 macrophages.

102

METHODS

103

104   (See the online supplement for detailed information on all methodologies including

105   statistical analysis).

Study design and patient recruitment

106

107   This is a prospective, cross-sectional study, in which patients were recruited

108   consecutively from the Lung Cancer Clinic of the Respiratory Medicine Department at

109   *Hospital del Mar* (Barcelona, Spain). For the purpose of the investigation, 80 Caucasian

110   patients with LC were recruited consecutively before having received any treatment for

111   their lung neoplasm from the weekly LC board meeting. Blood samples were obtained

112   at the time of diagnostic confirmation of LC in all 80 patients. These patients were

113   further subdivided *post-hoc* into two groups according to the presence of underlying

114   COPD, which was diagnosed on the basis of current guidelines[30-33]: 1) 60 patients with

115   LC who also had COPD (LC-COPD group, 1 female) and 2) 20 patients with LC

116   without COPD (LC-only group, 7 females). In LC-COPD patients, 57 males and 1

117   female and in LC-only group 13 males and 7 females simultaneously participated in a

118   previous study aimed to assess redox balance in lung tumors[34]. Moreover, from the

119   same study cohort, in the group of patients who underwent thoracotomy for the surgical

120   resection of their lung neoplasms (clinical indication according to guidelines for

121   diagnosis and management of lung cancer[30-33], specimens from the tumor and non-

122   tumor lung parenchyma were also obtained in all cases (n=40) and were further

123   subdivided *post-hoc* as follows: 1) 20 patients with LC and COPD (LC-COPD group,

124   all males) and 2) 20 patients with LC without COPD (LC-only group, 7 females).

125 Therefore, in these two groups of patients (LC-only and LC-COPD), blood and lung

126 specimens were available for the study. Twenty males (LC-COPD) and both 8 males

127 and 4 females (LC-only) also participated in the previous study[34].

128 Histological diagnosis and staging (tumor, node, metastasis, TNM) of LC were

129 confirmed in all patients[30-33]. Exclusion criteria were as follows: SCLC patients, chronic

130 cardiovascular, chronic metabolic and clot system disorders, signs of severe bronchial

131 inflammation and/or infection (bronchoscopy), current or recent invasive mechanical

132 ventilation, and chronic oxygen therapy. Approval was obtained from the institutional

133 Ethics Committee on Human Investigation (*Hospital del Mar–IMIM*, Barcelona) in

134 accordance with the World Medical Association guidelines (Helsinki Declaration of

135 2008) for research on human beings. Informed written consent was obtained from all

136 patients.

137 **Clinical assessment**

138 Lung function parameters were assessed in all patients following standard procedures.

139 Body composition evaluation included the assessment of body mass index (BMI) and

140 fat-free max index (FFMI) by bioelectrical impedance. Nutritional parameters were also

141 evaluated through conventional blood tests.

142 **Sample collection**

143 Blood sample specimens were obtained in all the recruited patients (n=80) from the arm

144 vein after an overnight fasting period. Moreover, in all patients undergoing thoracotomy

145 (n=40), lung specimens were obtained from both tumor and non-tumor surrounding

146 parenchyma during the surgery, in which standard technical procedures were followed

147 by the specialized thoracic surgeons. The expert pathologist selected a fragment of lung

148 tumor and non-tumor specimens of approximately 10×10 mm^2 size from the fresh

149 samples after a careful collection of the specimens required for diagnosis purposes.

Sample preservation

151 Lung specimens were immediately frozen in liquid nitrogen and stored in the −80ºC

152 freezer (under permanent alarm control) for further analysis or immersed in an alcohol-

153 formol bath to be embedded in paraffin. Blood samples were centrifuged and frozen at

154 −80 °C until further analyses. Frozen tissues were used for enzyme-linked

155 immunosorbent assay (ELISA) techniques, while paraffin-embedded lung sections,

156 which were gently provided by *Parc de Salut MAR* Biobank (MARBiobanc, Barcelona),

157 were used for the assessment of macrophage counts (immunohistochemical analyses).

Molecular biology analyses

159 *Quantification of cytokines and growth factors in plasma using ELISA*. Protein levels of

160 TNF-alpha, VEGF, IL-2, IL-10, interferon-gamma, TGF-beta and IL-4 were quantified

161 in blood (plasma) from all patients (ELISA kits, Gen-probe Diaclone SAS, Besançon,

162 France) following previous studies [15;35;36].

163 *Quantification of cytokines and growth factors in lung tissue (tumor and non-tumor)*

164 *using ELISA*. Protein levels of TNF-alpha, VEGF, IL-2, IL-10, interferon-gamma,

165 epidermal growth factor (EGFR), TGF-beta and IL-4 were quantified in lung specimens

166 from all patients (ELISA kits, Raybiotech Inc, Norcross GA, and Cloud-Clone Corp,

167 Houston, USA), following previous studies [15;35;36].

168 *Counts and types of macrophages in lung specimens (tumor and non-tumor)*. M1 and

169 M2 macrophages were identified on three-micrometer lung paraffin-embedded sections

170 using double-staining immunohistochemical procedures (Envision DuoFLEX

171 Doublestain System, Dako North America Inc., Carpinteria, CA, USA) following the

172 manufacturer's instructions and previous studies[37-39].

Statistical analyses

174 All statistical analyses were performed using the software SPSS 15.0 (SPSS Inc,

175 Chicago, IL, USA). Data are expressed as mean (standard deviation). The normality of

176 the study variables was explored using Shapiro-Wilk test. In order to test the potential

177 effects of cigarette smoking (CS) on the study results, LC-COPD were further

178 subdivided into moderate (n=25) and heavy (n=35) smokers, in which 60 packs-year

179 was the cut-off value (median). On the basis of a standard power statistics established at

180 a minimum of 80% and assuming an alpha error of 0.05, the statistical power was

181 sufficiently high to detect minimum differences between the two study groups in the

182 target variables (plasma cytokines and macrophage subtypes). The sample size was

183 calculated on the basis of these parameters, which required a minimum of 40-50 patients

184 for the plasma cytokines analyses and 15 patients for the analyses conducted in the lung

185 specimens in order to detect potential differences in the study variables between the two

186 groups.

187 Differences between groups in the study variables were assessed using one-way

188 analysis of variance (ANOVA) and Tukey's *post-hoc* analysis was used to adjust for

189 multiple comparisons. Differences between study groups for qualitative variables were

190 explored using the Chi-square test. Statistical significance was established at $P \leq 0.05$.

191 Variables from the two compartments (blood and lungs) were evaluated independently.

192

193 **RESULTS**

194 **Clinical characteristics**

195 Clinical and functional characteristics of all LC-COPD and LC-only patients recruited

196 in the study are shown in Tables 1 and 2 (all patients as a group and only patients

197 undergoing thoracotomy, respectively). The number of LC-COPD patients was higher

198 than LC-only and were mostly males in both groups (Tables 1 and 2). No significant

199 differences were found in age or BMI between LC-COPD and LC-only patients (Tables

200   1 and 2). Smoking history differed between LC-COPD and LC-only patients (Table 1).

201   The functional parameters diffusion lung capacity for carbon monoxide (DL_{CO}), forced

202   expiratory volume in one second (FEV_1), FEV_1/forced vital capacity (FVC) were

203   significantly decreased in LC-COPD compared to LC patients (Tables 1 and 2). No

204   significant differences were found in these parameters between heavy and moderate

205   smokers within the LC-COPD group (Tables 1 and 2). Moreover, no statistically

206   significant differences were found in either TNM or histological subtypes between LC-

207   COPD and LC-only groups (Tables 1 and 2). In LC-COPD compared to LC patients,

208   levels of albumin and fibrinogen were decreased, whereas those of C-reactive protein

209   (CRP) and globular sedimentation (GSV) were increased (Tables 1 and 2). Furthermore,

210   body weight loss was greater in LC-COPD, especially in heavy smokers, compared to

211   LC-only patients (Table 1).

212   **Systemic levels of cytokines**

213   In LC-COPD, levels of TNF-alpha and IL-2 levels were greater compared to LC-only

214   patients (Figures 1A-1B). Systemic levels of interferon-gamma did not differ among the

215   study groups (Figure 1C). In the *post-hoc* analyses, levels of IL-2 were higher in heavy

216   smokers compared to moderate smokers, whereas no differences were observed in either

217   TNF-alpha or interferon-gamma levels between these groups (Figures 1A-1C). In LC-

218   COPD patients, levels of VEGF and IL-4 were significantly lower, while those of TGF-

219   beta and IL-10 were higher compared to LC-only patients (Figures 1D-1G). Levels of

220   the cytokines VEGF, IL-4, TGF-beta and IL-10 did not differ between heavy and

221   moderate smokers in LC-COPD patients (Figures 1D-1G). No significant associations

222   were found between systemic levels of the study cytokines and the patients' survival

223   (data not shown).

224   **Levels of cytokines and growth factors in the lung specimens**

225 *Tumor versus non-tumor parenchyma in LC-COPD and LC patients.* In LC-COPD

226 patients and especially in moderate smokers, TNF-alpha levels were significantly

227 increased in tumor lesions compared to non-tumor specimens, while no differences were

228 seen in LC-only patients (Figure 2A). IL-2 levels were significantly greater in tumor

229 lesions of both LC and LC-COPD patients compared to non-tumor specimens (Figure

230 2B). No differences were found in interferon-gamma levels between tumor and non-

231 tumor lungs in any of the study groups (Figure 2C). VEGF levels were significantly

232 higher in tumor lesions compared to the non-tumor lungs in all LC-COPD and LC-only

233 patients (Figure 2D). In the latter patients, levels of TGF-beta were increased in tumor

234 lesions compared to non-tumor specimens (Figure 2E). No differences were found in

235 IL-4 levels between tumor and non-tumor lungs in any of the study patient groups

236 (Figure 2F). In all LC-COPD patients, IL-10 levels were greater in the tumors than in

237 the non-tumor lungs (Figure 2G). In both groups of patients, epidermal growth factor

238 receptor (EGFR) levels were significantly increased in tumor specimens compared to

239 non-tumor lesions (Figure 2H), and no differences were seen between moderated and

240 heavy smokers. Finally, No significant correlations were found in any of the study

241 cytokines between blood and lung compartments.

242 *Differences between LC-COPD and LC in either tumor lesions or non-tumor specimens.*

243 No significant differences were found in TNF-alpha, IL-2 and interferon-gamma levels

244 between LC-COPD (both heavy and moderate smokers) and LC-only patients in either

245 tumor or non-tumor lungs (Figures 2A-2C). In LC-COPD compared to LC-only

246 patients, levels of VEGF, TGF-beta, and IL-10 were greater in the tumor lesions

247 (Figures 2D, 2E, and 2G). Levels of IL-4 did not significantly differ between LC-COPD

248 and LC-only patients in either tumor or non-tumor lungs (Figure 2F). No significant

249 differences were found in VEGF, TGF-beta, IL-4, IL-10 or EGFR levels between

250 moderate and heavy smokers within the LC-COPD group of patients (Figures 2D-2H).

251 No significant associations were found between levels in the lungs of the study

252 cytokines or growth factors and the patients' survival (data not shown).

253 *M1 and M2 macrophage subtypes in the lung.* In both LC-COPD and LC-only groups,

254 M2 macrophages were increased, while M1 and M1/M2 ratio were decreased in lung

255 tumors compared to non-tumor specimens (Figures 3A-3B, Table 3). In tumor

256 specimens, M1/M2 was significantly greater in LC-COPD than in LC-only patients

257 (Table 3). No significant associations were found between M1, M2 or M1/M2 and

258 patients' survival in any study group (Figure E1A-E1B).

259

260 **DISCUSSION**

261 In the current study, the main findings were that patients with LC-COPD exhibited a

262 moderate airway obstruction and functional emphysema; adenocarcinoma was the

263 predominant histological type, and those who smoked more showed a significantly

264 greater loss of body weight. Moreover, in LC-COPD compared to LC-only patients,

265 systemic levels of Th1 cytokines TNF-alpha, IL-2 and those of Th2 cytokines TGF-beta

266 and IL-10 were increased, whereas those of VEGF and IL-4 were decreased with no

267 significant differences in interferon-gamma levels. In LC-COPD patients, levels of

268 TNF-alpha, TGF-beta, and IL-10 were greater in tumors than in non-tumor lungs, while

269 a significant rise in IL-2 and VEGF levels was seen in the tumors of both groups.

270 Moreover, VEGF, TGF-beta, and IL-10 levels were increased in the tumors of LC-

271 COPD than in those of LC-only patients. In the tumors of both groups, M1

272 macrophages and M1/M2 were reduced, while M1/M2 was significantly greater in LC-

273 COPD patients. Smoking history did not influence the differences in the study

274 parameters between groups. Importantly, no correlations between lung and blood

275 compartments were observed for any of the study variables in the two groups of

276 patients. In view of these findings, the study hypothesis was confirmed to a great extent.

277   CD4+ T lymphocytes are divided into T helper (Th)1 or Th2 cells on the basis of

278 the secreted cytokines. Th1 lymphocytes release TNF-alpha, IL-2, and interferon-

279 gamma which exert antitumor effects in humans, while Th2 cells produce IL-4 and IL-

280 10 that favor tumor growth by inhibiting the host immune system[24;25]. In patients with

281 several types of cancer, alterations of the Th1/Th2 immunological balance have been

282 detected in tumorigenesis and cancer relapse[20;25;26]. In the present investigation,

283 systemic levels of TNF-alpha, IL-2, TGF-beta, and IL-10 were significantly increased

284 in patients with underlying COPD compared to those without any respiratory condition.

285 These findings suggest that Th1 systemic immunological response and regulatory T-

286 lymphocyte cytokines (TGF-beta and IL-10) were significantly enhanced in patients

287 bearing the two conditions: COPD and LC. It is likely that in COPD, systemic

288 inflammation as a result of chronic CS exposure drives a general Th1 response in the

289 patients, which was not observed in LC patients without COPD.

290   Importantly, in the lung tumors of LC patients with underlying COPD, levels of

291 the cytokines TNF-alpha, TGF-beta, and IL-10 were significantly greater than those

292 encountered in the surrounding non-tumor lung. Moreover, levels of VEGF, TGF-beta,

293 and IL-10 were also significantly higher in the tumors of LC-COPD than in LC-only

294 patients. Interestingly, levels of IL-4 did not differ in lung specimens among the study

295 groups. Collectively, these findings suggest that in COPD, chronic inflammation in the

296 lungs is characterized by the release of Th1 and regulatory T-lymphocytes, which may

297 protect patients against tumor development and progression. Indeed, similar findings

298 were reported in the bronchial epithelium of patients with LC and COPD[15].

299 Nonetheless, a previous study[20] showed that in patients with NSCLC, systemic levels of

300 Th2 cytokines were increased, while those of Th1 cytokines were reduced compared to

301 healthy subjects. In the same investigation[20], NSCLC patients were shown to lower

302 systemic Th2 cytokine levels in response to lung tumor resection, and the relapse rates

303 were significantly greater in those patients with persistent abnormal levels of IL-4.

304 Importantly, in that study[20], patients were not analyzed according to the presence of

305 COPD or smoking history. In fact, this analysis is a major novel contribution of the

306 current investigation.

307 EGFR is the cell-surface receptor of the EGF family protein ligands in several cell

308 types that contribute to the correct development of glands in tissues. Mutations of

309 EGFR that induce its overexpression are involved in tumorigenesis such as LC. Despite

310 that the efficacy of anti-EGFR therapies (namely EGFR tyrosine kinase inhibitors and

311 anti-EGFR monoclonal antibodies) remain controversial in certain NSCLC types,

312 especially squamous LC, beneficial effects have been observed in recent studies, in

313 which adenocarcinoma was the predominant histological subtype[40;41]. Moreover,

314 durable clinical responses beyond five years have also been proposed in response to

315 pathway-targeted immunotherapy as a result of treatment of NSCLC patients with a

316 novel EGF-directed agent[42]. As in previous studies[40;41;43], in the present investigation, in

317 both groups of patients, EGFR levels were also significantly greater in the tumor lesions

318 than in the surrounding non-tumor parenchyma. Identification of potential mutations of

319 EGFR gen, however, was beyond the scope of the current study and will remain the

320 focus of research in future investigations.

321 Macrophages may exert proinflammatory or anti-inflammatory functions

322 depending on the secreted cytokines. In tumors, the inflammatory infiltrates are mostly

323 represented by this type of cells. In general, M1 macrophages favor inflammation,

324 whereas M2 macrophages promote anti-inflammatory actions and tissue repair. While

325 M1 cells fight against tumor development, M2 macrophages exert the opposite effects,

326 by promoting cancer growth, survival, progression, and dissemination[25]. In keeping

327 with, in the tumor lesions of both study groups of patients, the number of M1

328 macrophages was reduced, while that of M2 was increased. Hence, the ratio of M1 to

329 M2 cells was also significantly lower in the tumors than in the surrounding non-tumor

330 lung in all groups of patients. Indeed, interaction of factors such as the stage of the

331 tumor and the local microenvironment has been shown to determine macrophage

332 phenotype and tumor progression[25]. Importantly, in the study, tumors of LC patients

333 with underlying COPD exhibited a significantly greater M1/M2 ratio than those of

334 patients without COPD. These results suggest that the relative predominance of M1

335 phenotype in tumors of LC-COPD patients may imply a better prognosis in these

336 patients compared to those with no COPD. Nonetheless, the number of macrophages or

337 M1/M2 ratio was not associated with the patients' survival in any of the study groups

338 (Figure E1A-E1B) as was shown to occur in other types of tumors[29]. It is likely that the

339 statistically significant difference of M1/M2 ratio in tumors between the two study

340 groups may not be of sufficient biological relevance to modify the prognosis of patients

341 with LC.

342 Importantly, in the last few years, a better and progressive understanding of the

343 complex interactions between the immune system, inflammation and carcinogenesis

344 including LC treatment has led to the development of novel immunotherapeutic

345 agents[44;45]. Nonetheless, the efficacy of these immunomodulatory drugs is hampered by

346 the acquired resistance following certain periods of time[44;45]. Hence, further insight into

347 the potential contribution of the immune system to lung carcinogenesis is required in

348 order to design immunotherapeutic strategies that will ensure a longer lasting control of

349 the disease. Cytokines, monoclonal antibodies, tumor and dendritic cell vaccines, and

350 checkpoint inhibitors are examples of the passive and active immunotherapy agents that

351 are currently used in clinical settings[44-46]. Future research in this field will shed light

352 into further mechanisms that will selectively target tumor cells in patients with LC.

Study limitations

354 A first limitation in the study refers to the relatively lower number of lung specimens

355 analyzed in the study compared to the number of blood samples. Nevertheless, for

356 ethical reasons, tumor and non-tumor lung specimens could only be obtained from

357 patients undergoing thoracotomy for the treatment of their lung neoplasm from the

358 established cohort of patients that participated in this investigation, from whom blood

359 samples had been collected. Furthermore, the study variables could have also been

360 analyzed using other approaches in which the potential predictive value of the analyzed

361 markers may have been estimated. However, the investigation was aimed to identify

362 whether underlying COPD influences the expression of Th1 and Th2 cytokines in

363 patients with LC. Another approach would be to analyze the expression of the target

364 variables on the basis of histological subtypes. In this regard, no significant differences

365 were detected in any of the clinical or biological variables between the two groups.

366 Another limitation is the relatively low numbers of females in both groups of

367 patients. This is because the prevalence of LC in female patients, especially in those

368 patients with underlying COPD is still very low in our geographical region[7;9;10]. Despite

369 these limitations, the main findings confirmed the study hypothesis to a great extent.

Conclusions

371 A differential expression profile of inflammatory markers and cells has been identified

372 in lung tumors and blood compartment in LC patients bearing a chronic respiratory

373 condition, irrespective of smoking history. The relative predominance of Th1 cytokines

374 and M1 macrophages in the blood and tumors of patients with underlying COPD imply

375   that a stronger proinflammatory pattern exists in these patients. These findings have

376   potential clinical implications as inflammation should not be targeted systematically in

377   all patients with LC. Screening for the presence of underlying respiratory diseases and

378   identification of the specific inflammatory pattern should be carried out in patients with

379   LC, at least in early stages of their disease.

380

381

382 **ACKNOWLEDGMENTS**

401

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542   **FIGURE LEGENDS**

543   **Figure 1**

544   **A)** Mean values and standard deviation of blood TNF-alpha levels are shown in

545   the graph. As described in methods, comparisons were made between LC

546   and LC-COPD, and among the latter patients, analyses were also made

547   between heavy and moderate smokers. TNF-alpha levels were significantly

548   higher in LC-COPD than in LC patients. Levels of TNF-alpha did not

549   significantly differ between heavy and moderate smokers.

550   **B)** Mean values and standard deviation of blood IL-2 levels are shown in the

551   graph. As described in methods, comparisons were made between LC and

552   LC-COPD, and among the latter patients, analyses were also made between

553   heavy and moderate smokers. IL-2 levels were significantly increased in LC-

554   COPD compared to LC patients. IL-2 levels were also higher in heavy

555   smokers than in moderate smokers in LC-COPD patients.

556   **C)** Mean values and standard deviation of blood interferon-gamma levels are

557   shown in the graph. As described in methods, comparisons were made

558   between LC and LC-COPD, and among the latter patients, analyses were

559   also made between heavy and moderate smokers. Interferon-gamma levels

560   did not significantly differ among the study groups.

561   **D)** Mean values and standard deviation of blood VEGF levels are shown in the

562   graph. As described in methods, comparisons were made between LC and

563   LC-COPD, and among the latter patients, analyses were also made between

564   heavy and moderate smokers. VEGF levels were significantly decreased in

565   LC-COPD than in LC patients. Levels of VEGF did not significantly differ

566   between heavy and moderate smokers.

E) Mean values and standard deviation of blood IL-4 levels are shown in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. IL-4 levels were significantly reduced in LC-COPD than in LC patients. Levels of IL-4 did not significantly differ between heavy and moderate smokers.

F) Mean values and standard deviation of blood TGF-beta levels are shown in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. Levels of TGF-beta were significantly greater in LC-COPD than in LC patients. Levels of TGF-beta did not significantly differ between heavy and moderate smokers.

G) Mean values and standard deviation of blood IL-10 levels are shown in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. IL-10 levels were significantly increased in LC-COPD than in LC patients. Levels of IL-10 did not significantly differ between heavy and moderate smokers.

Figure 2

A) Mean values and standard deviation of lung TNF-alpha levels are depicted in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. Moreover, TNF-alpha levels detected in non-tumor (NT) and tumor (T) lungs are also represented in graphs for all the groups for the purpose of the

592   comparisons. Levels of TNF-alpha were significantly higher in the T

593   lesions compared to NT lungs in LC-COPD, especially in moderate

594   smokers. TNF-alpha levels did not significantly differ between T and NT

595   in LC patients or between LC-COPD and LC patients. No significantly

596   differences were found in TNF-alpha levels between heavy and moderate

597   smokers in either T or NT lungs.

598   **B)** Mean values and standard deviation of lung IL-2 levels are depicted in

599   the graph. As described in methods, comparisons were made between LC

600   and LC-COPD, and among the latter patients, analyses were also made

601   between heavy and moderate smokers. Moreover, IL-2 levels detected in

602   NT and T lungs are also represented in graphs for all the groups for the

603   purpose of the comparisons. Levels of IL-2 were significantly greater in

604   T lesions compared to NT lung specimens in both LC and LC-COPD

605   patients. IL-2 levels did not significantly differ between LC-COPD and

606   LC patients or between moderate and heavy smokers.

607   **C)** Mean values and standard deviation of lung interferon-gamma levels are

608   depicted in the graph. As described in methods, comparisons were made

609   between LC and LC-COPD, and among the latter patients, analyses were

610   also made between heavy and moderate smokers. Moreover, interferon-

611   gamma levels detected in NT and T lungs are also represented in graphs

612   for all the groups for the purpose of the comparisons. Interferon-gamma

613   levels did not significantly differ in any of the study groups of patients.

614   **D)** Mean values and standard deviation of lung VEGF levels are depicted in

615   the graph. As described in methods, comparisons were made between LC

616   and LC-COPD, and among the latter patients, analyses were also made

617   between heavy and moderate smokers. Moreover, VEGF levels detected

618   in NT and T lungs are also represented in graphs for all the groups for the

619   purpose of the comparisons. Levels of VEGF were significantly

620   increased in T lesions compared to NT lung specimens in both LC and

621   LC-COPD patients, especially in heavy and moderate smokers. Lung

622   VEGF levels were also higher in the T lesions of LC-COPD than in LC

623   patients. No significant differences were found between heavy and

624   moderate smokers.

625   **E)** Mean values and standard deviation of lung TGF-beta levels are depicted

626   in the graph. As described in methods, comparisons were made between

627   LC and LC-COPD, and among the latter patients, analyses were also

628   made between heavy and moderate smokers. Moreover, TGF-beta levels

629   detected in NT and T lungs are also represented in graphs for all the

630   groups for the purpose of the comparisons. Levels of TGF-beta were

631   significantly greater in T lesions than in NT lung specimens of LC-

632   COPD patients. TGF-beta levels were also increased in T lesions of LC-

633   COPD compared to LC patients. No significantly differences were found

634   between T and NT in LC patients or between heavy and moderate

635   smokers.

636   **F)** Mean values and standard deviation of lung IL-4 levels are depicted in

637   the graph. As described in methods, comparisons were made between LC

638   and LC-COPD, and among the latter patients, analyses were also made

639   between heavy and moderate smokers. Moreover, IL-4 levels detected in

640   NT and T lungs are also represented in graphs for all the groups for the

641   purpose of the comparisons. Levels of IL-4 did not significantly differ

642   among the study groups of patients.

643   **G)** Mean values and standard deviation of lung IL-10 levels are depicted in

644   the graph. As described in methods, comparisons were made between LC

645   and LC-COPD, and among the latter patients, analyses were also made

646   between heavy and moderate smokers. Moreover, IL-10 levels detected

647   in NT and T lungs are also represented in graphs for all the groups for the

648   purpose of the comparisons. Levels of IL-10 were significantly greater in

649   T lesions compared to NT lung specimens in LC-COPD patients,

650   especially in moderate and heavy smokers. IL-10 levels were also

651   increased in T parenchyma of LC-COPD compared to LC patients. No

652   significant differences were found between T and NT in LC patients or

653   between heavy and moderate smokers.

654   **H)** Mean values and standard deviation of lung EGFR levels are depicted in

655   the graph. As described in methods, comparisons were made between LC

656   and LC-COPD, and among the latter patients, analyses were also made

657   between heavy and moderate smokers. Moreover, EGFR levels detected

658   in NT and T lungs are also represented in graphs for all the groups for the

659   purpose of the comparisons. Levels of EGFR were significantly higher in

660   T lesions compared to NT lung specimens in both LC and LC-COPD

661   patients. No significant differences were observed in EGFR levels

662   between LC-COPD and LC patients or between heavy and moderate

663   smokers.

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665

666 **Figure 3:**

667   A) Representative examples of double immunohistochemical staining for M1

668   macrophages (CD68-HLA positively stained macrophages) in non-tumor (left-

669   hand side panel) and tumor (right hand-side panel) lung specimens. All types of

670   macrophages ($CD68^+$) are stained only in brown color (black arrow) whereas

671   M1 macrophages ($CD68^+$-HLA^+) are specifically stained with brown and red

672   color (red arrow).

673   **B)** Representative examples of double immunohistochemical staining for M2

674   macrophages (CD68-CD206 positively stained macrophages, black arrows) in

675   non-tumor (left-hand side panel) and tumor (right hand-side panel) lung

676   specimens. All types of macrophages ($CD68^+$) are stained only in brown color

677   (black arrow) whereas M2 macrophages ($CD68^+$-$CD206^+$) are specifically

678   stained with brown and red color (red arrow).

679

Table 1. Clinical and functional characteristics of all the study patients.

Anthropometric variables	Lung Cancer N=20	Lung Cancer-COPD N=60	Lung Cancer-COPD moderate smokers N=25	Lung Cancer-COPD heavy smokers N=35
Age, years	64 (12)	68 (11)	68 (9)	67 (11)
Male, N, / Female, N	13 / 7	59 / 1	24 / 1	35/0
BMI, Kg/m^2	26 (5)	24 (3)	25 (3)	25 (4)
Smoking history				
Current: N, %	12, 60	38, 63 **	10, 40	28, 80 §§
Ex-smoker: N, %	5, 25	22, 37 **	15, 60	7, 20 §§
Never Smoker: N,%	3, 15	0, 0 **	0, 0	0, 0
Packs-year	53 (25)	65 (23) *	44 (9)	78 (19) §§§
Lung function testing				
FEV$_1$, % pred	92 (8)	57 (12) ***	60 (13)	57 (12)
FEV$_1$/FVC, % pred	77 (6)	60 (8) ***	61 (5)	60 (9)
DLCO, % pred	88 (12)	69 (18) ***	70 (19)	68 (18)
KCO, % pred	88 (11)	80 (21)	81 (21)	80 (21)
TNM staging				
Stage IA: N, %	5, 25	9, 15	5, 20	4, 12
Stage IB: N, %	0, 0	10, 16	5, 20	5, 14
Stage IIA: N, %	1, 5	5, 8	2, 8	3, 9
Stage IIB: N, %	4, 20	4, 7	2, 8	2, 6
Stage IIIA: N, %	3, 15	9, 15	3, 12	6, 17
Stage IIIB: N, %	4, 20	7, 12	1, 4	6, 17
Stage IV: N, %	3, 15	16, 27	7, 28	9, 25
Histological diagnosis				
Squamous cell carcinoma: N, %	6, 30	27, 45	12, 48	15, 43
Adenocarcinoma: N, %	11, 55	24, 40	10, 40	14, 40
Others: N, %	3, 15	9, 15	3, 12	6, 17
Blood parameters				
Total leukocytes /microL	8.3 10^3 (1.9 10^3)	8.4 10^3 (2.2 10^3)	8.2 10^3 (2.3 103)	8.7 10^3 (2.1 103)

Total Neutrophils /microL	$5.7 \cdot 10^3$ ($2.1 \cdot 10^3$)	$5.9 \cdot 10^3$ ($1.8 \cdot 10^3$)	$5.8 \cdot 10^3$ ($1.8 \cdot 10^3$)	5.9 ($1.8 \cdot 10^3$)
Total lymphocytes/microL	$1.7 \cdot 103$ (650)	$1.7 \cdot 103$ (676)	$1.4 \cdot 10^3$ (635)	$1.8 \cdot 10^3$ (681)
Albumin (g/dL)	4.4 (0.4)	4.1 (0.5) *	4.2 (0.50)	4.1 (0.5)
Total proteins (g/dL)	7.4 (0.5)	7.3 (0.4)	7.2 (0.6)	7.5 (0.6)
Fibrinogen (mg/dL)	485 (99)	453 (152)	430 (151.7)	473 (153)
CRP (mg/dL)	1.1 (1.1)	4.9 (6.8) *	5.1 (7)	4.8 (6.8)
GSV (mm/h)	20 (7.4)	28.8 (27.9)	26.2 (28.5)	30.4 (28)
Ceruloplasmin (g/dL)	26.2 (4.8)	30.7 (7.7)	35 (11)	29.1 (6.1)
Body weight loss, kg				
0, N, %	18, 90	36, 60 ***	18, 72	18, 52 §§§
1-4, N, %	2, 10	8, 14 ***	3, 12	5, 14§§§
5-8, N, %	0, 0	14, 23 ***	4, 16	10, 28 §§§
9-12, N, %	0, 0	2, 3 ***	0, 0	2, 6 §§§

Continuous variables are presented as mean (standard desviation), while categorical variables are presented as the number of patients in each group and percentage of the total population.

Definition of abbreviations: N, number; kg, kilograms; m, meters; BMI, body mass index; FEV$_1$, forced expiratory volume in the first second; pred, predicted; FVC, forced vital capacity; DLco, carbon monoxide transfer; K$_{CO}$, Krogh transfer factor; TNM, tumor, nodes, metastasis; CRP, C-reactive protein; GSV, globular sedimentation velocity; L, liter.

Statistical analyses and significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between LC-COPD patients as a group and LC-only patients; § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$ between LC-COPD heavy smokers and LC-COPD moderate smokers in the post-hoc analyses. Comparisons of the clinical and physiological variables between LC-COPD as a group and LC-only patients, as well as between the two groups of LC-COPD patients (moderate and heavy smokers) were assessed using the Student's T-test. Differences between the study groups for the qualitative variables were assessed using the Chi-square test.

Table 2. Clinical and functional characteristics of the study patients undergoing thoracotomy.

Anthropometric variables	Lung Cancer N=20	Lung Cancer-COPD N=20	Lung Cancer-COPD moderate smokers N=9	Lung Cancer-COPD heavy smokers N=11
Age, years	64 (12)	65 (9)	63 (10)	66 (9)
Male, N/ Female, N	13 / 7	20 / 0	9 / 0	11 / 0
BMI, Kg/m^2	26 (5)	26 (5)	25 (4)	27 (3)
Smoking history				
Current: N, %	12, 60	14, 70	6, 67	8, 73
Ex-smoker: N, %	5, 25	6, 30	3, 33	3, 27
Never Smoker: N,%	3, 15	0, 0 *	0, 0	0, 0
Packs-year	53 (25)	57 (20)	39 (6)	71 (14) § § §
Lung function testing				
FEV_1 , % pred	92 (8)	61 (14) ***	64 (14)	59 (14)
FEV_1/FVC, % pred	77 (6)	60 (8) ***	59 (8)	62 (8)
DLCO, % pred	88 (12)	72 (21) ***	69 (22)	74 (21)
KCO, % pred	88 (11)	73 (17)***	70 (16)	75 (19)
TNM staging				
Stage IA: N, %	5, 25	5, 25	3, 34	2, 18
Stage IB: N, %	0, 0	4, 20	2, 22	2, 18
Stage IIA: N, %	1, 5	6, 30	2, 22	4, 37
Stage IIB: N, %	4, 20	2, 10	2, 22	0, 0
Stage IIIA: N, %	3, 15	3, 15	0, 0	3, 27
Stage IIIB: N, %	4, 20	0, 0	0, 0	0, 0
Stage IV: N, %	3, 15	0, 0	0, 0	0, 0
Histological diagnosis				
Squamous cell carcinoma: N, %	6, 30	5, 25	2, 22	3, 27
Adenocarcinoma: N, %	11, 55	14, 70	7, 78	7, 63
Others: N, %	3, 15	1, 5	0, 0	1, 10
Blood parameters				
Total leukocytes /microL	8.3 10^3 (1.9 10^3)	9.2 10^3 (2.1 10^3)	9.4 10^3 (2.3 103)	9.1 10^3 (2.1 103)

Total Neutrophils /microL	$5.7 \cdot 10^3$ ($2.1 \cdot 10^3$)	$6.3 \cdot 10^3$ ($1.7 \cdot 10^3$)	$6.2 \cdot 10^3$ ($1.5 \cdot 10^3$)	$6.3 \cdot 10^3$ ($1.9 \cdot 10^3$)
Total lymphocytes/microL	$1.7 \cdot 10^3$ (650)	$2.1 \cdot 10^3$ (617)	2.0 (801)	$2.0 \cdot 10^3$ (457)
Albumin (g/dL)	4.4 (0.4)	4.0 (0.5) **	4.0 (0.6)	4.1 (0.5)
Total proteins (g/dL)	7.4 (0.5)	7.1 (0.6)	6.9 (0.7)	7.3 (0.4)
Fibrinogen (mg/dL)	485 (99)	409 (77) **	377 (60)	436 (84)
CRP (mg/dL)	1.1 (1.1)	1.7 (1.2)	1 (0.4)	2 (2.6)
GSV (mm/h)	20 (7.4)	30 (16.8) *	28.7 (23)	30 (14.6)
Ceruloplasmin (g/dL)	26.2 (4.8)	26.5 (4.8)	26 (4.18)	26.6(5.3)
Body weight loss, kg				
0, N, %	18, 90	17, 85	7, 78	10, 90
1-4, N, %	2, 10	1, 5	1, 11	0, 0
5-8, N, %	0, 0	2, 10	1, 11	1, 10
9-12, N, %	0, 0	0, 0	0, 0	0, 0

Continuous variables are presented as mean (standard desviation), while categorical variables are presented as the number of patients in each group and percentage of the total population.

Definition of abbreviations: N, number; kg, kilograms; m, meters; BMI, body mass index; FEV_1, forced expiratory volume in the first second; pred, predicted; FVC, forced vital capacity; DLco, carbon monoxide transfer; K_{CO}, Krogh transfer factor; TNM, tumor, nodes, metastasis; CRP, C-reactive protein; GSV, globular sedimentation velocity; L, liter.

Statistical analyses and significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ between LC-COPD patients as a group and LC-only patients; § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$ between LC-COPD heavy smokers and LC-COPD moderate smokers in the post-hoc analyses. Comparisons of the clinical and physiological variables between LC-COPD as a group and LC-only patients, as well as between the two groups of LC-COPD patients (moderate and heavy smokers) were assessed using the Student's T-test. Differences between the study groups for the qualitative variables were assessed using the Chi-square test.

**Table 3. Counts of positive cells for specific markers of M1 and M2 macrophages.**

|	Lung Cancer		Lung Cancer-COPD	
	Non-tumor	Tumor	Non-tumor	Tumor
M1/micrometer^2	0.83 (0.22)	0.23 (0.18) ¶¶¶	0.78 (0.22)	0.25 (0.17) ¶¶¶
M2/micrometer^2	0.21 (0.19)	0.59 (0.33) ¶¶¶	0.24 (0.17)	0.41 (0.31) ¶¶
Ratio M1 / M2	6.10 (3.6)	0.30 (0.27) ¶¶¶	6.20 (5.7)	0.70 (0.5) ¶¶¶, *

Data are presented as mean (standard deviation).

Statistical analyses and significance: ¶¶ p<0.01, ¶¶¶ p<0.001 between tumor and non-tumor lung specimens in either LC or LC-COPD groups of patients; * p<0.05 between LC-COPD tumor and LC-only tumor.

Definition of abbreviations: M1, macrophages type 1 (HLA$^+$ cells); M2, macrophages type 2 (CD206$^+$cells).
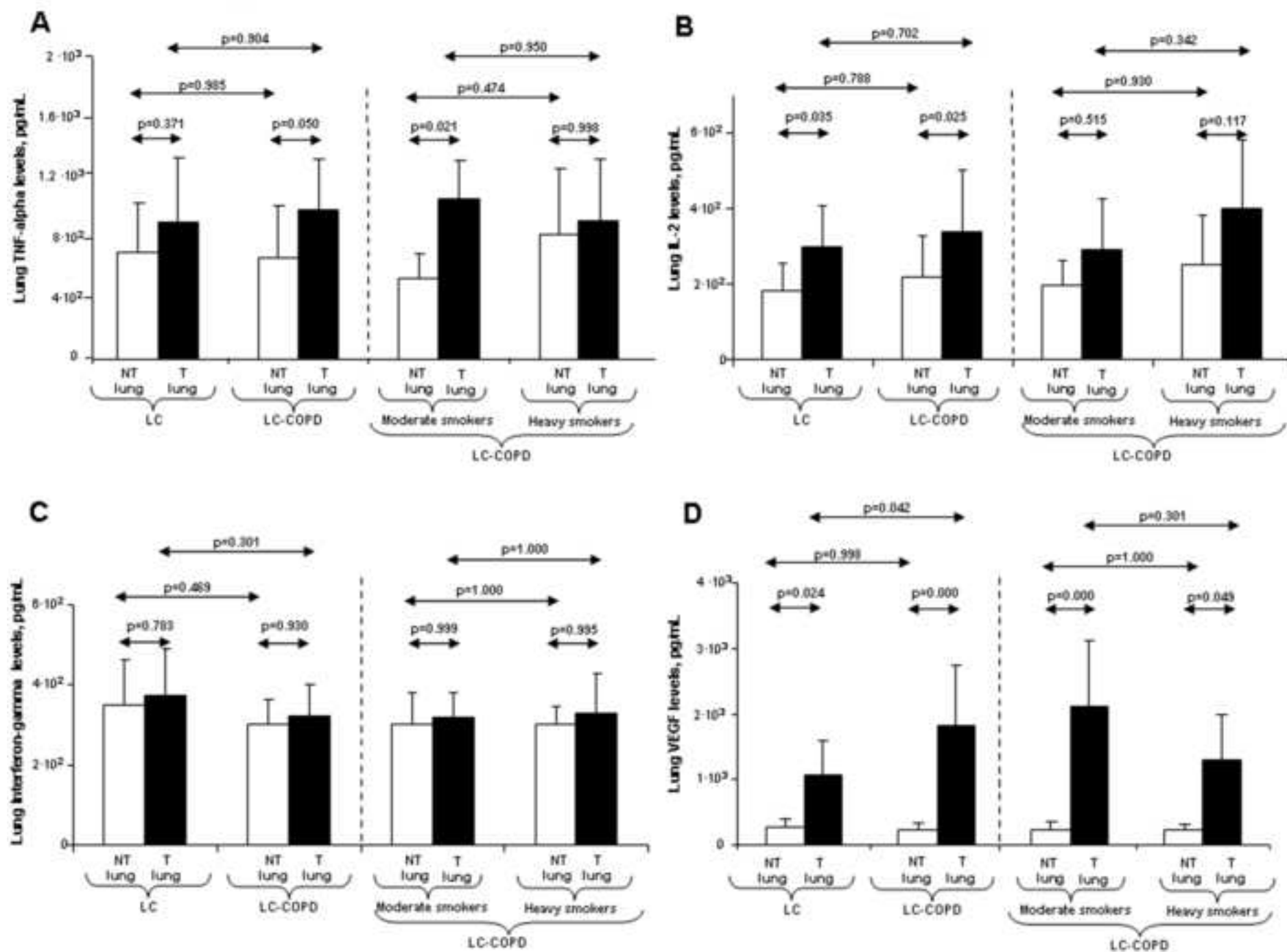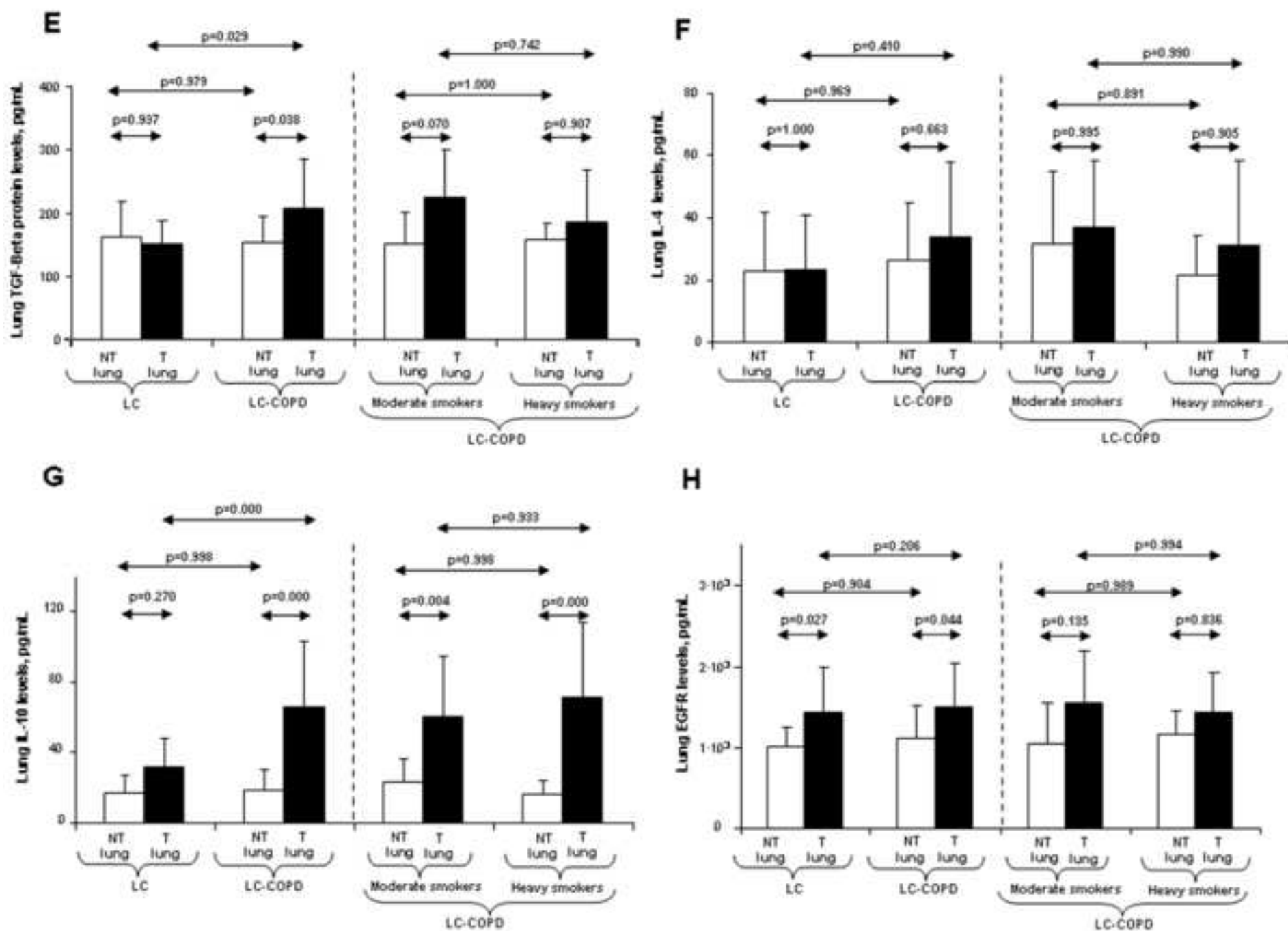
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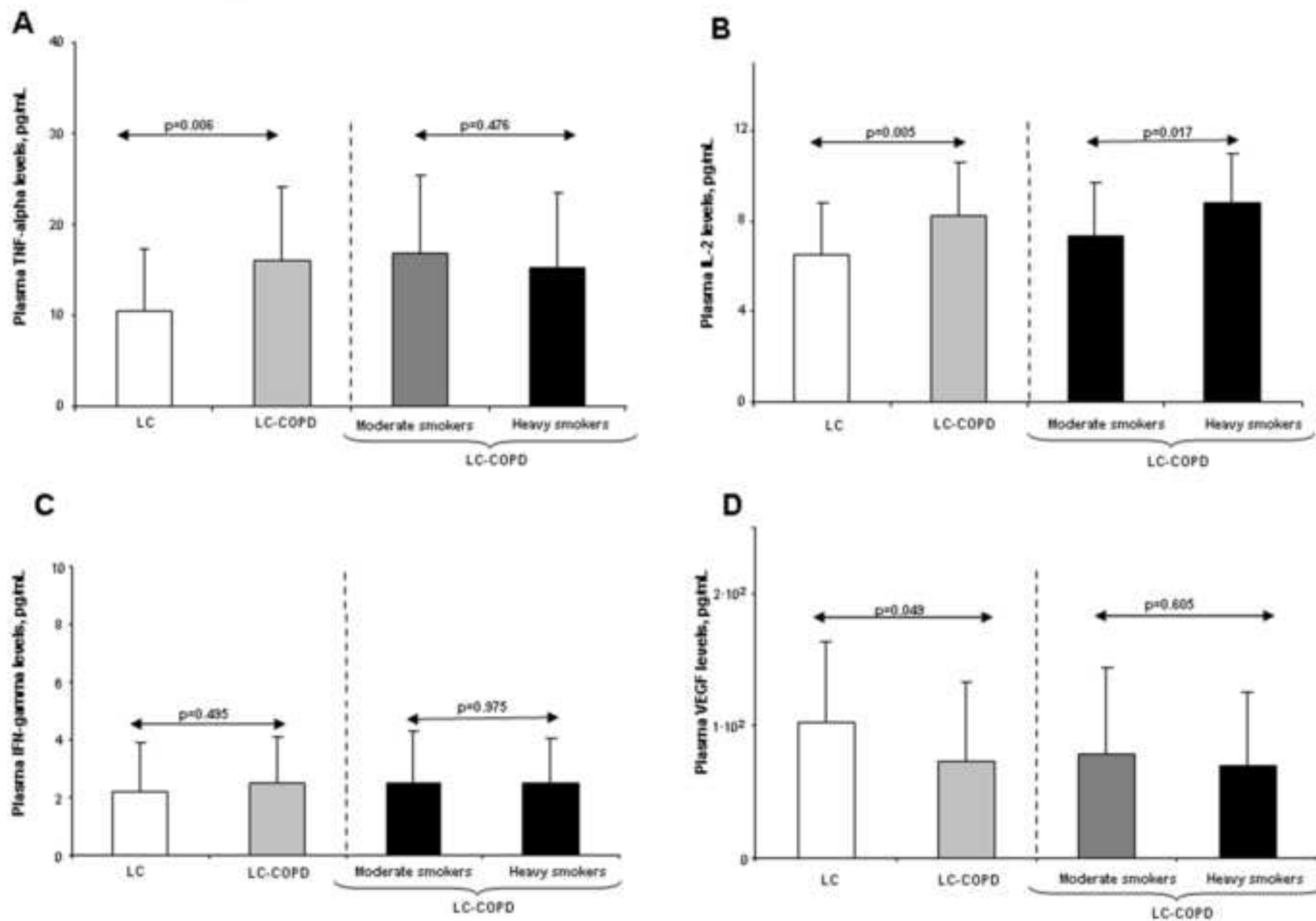
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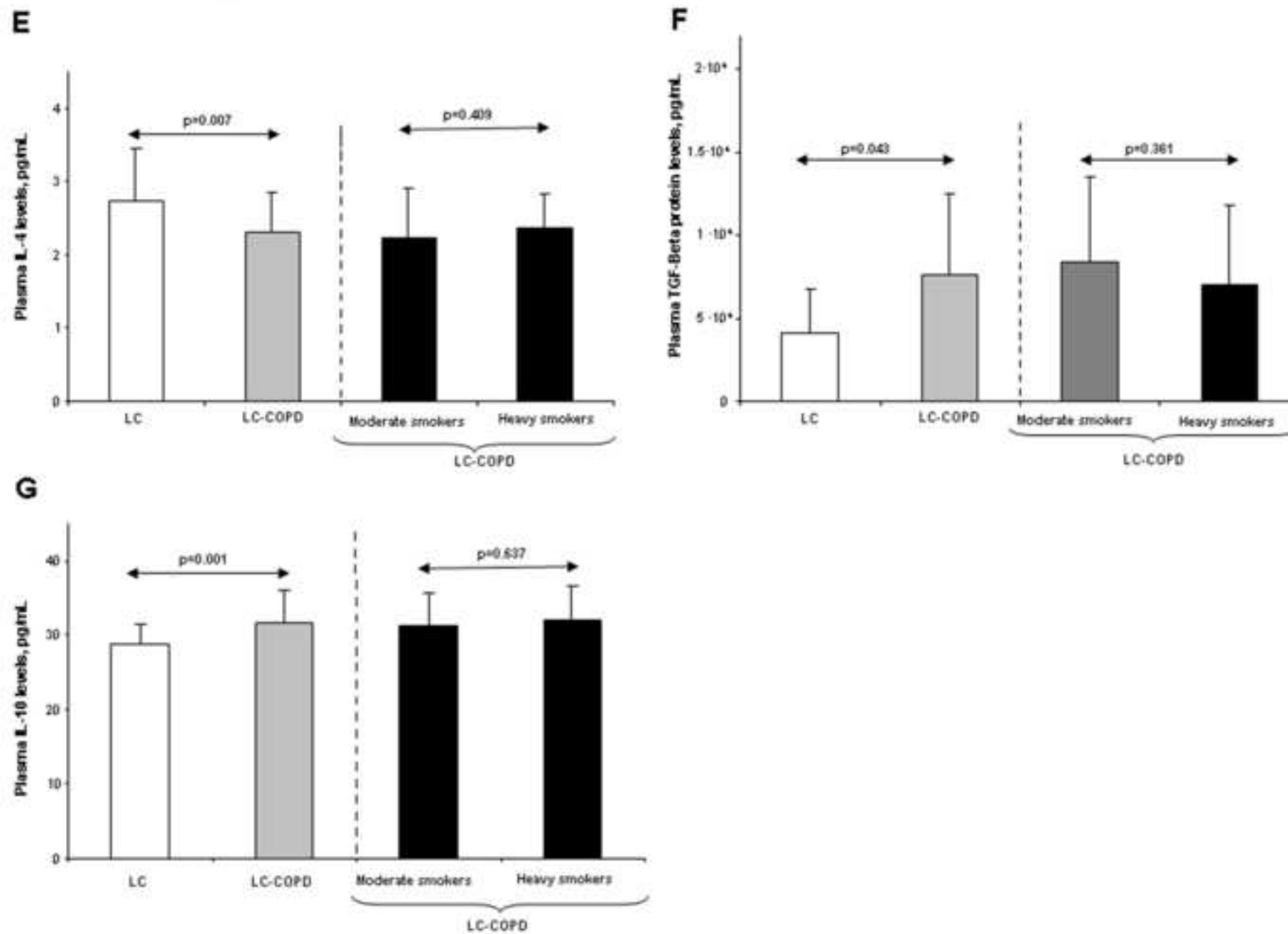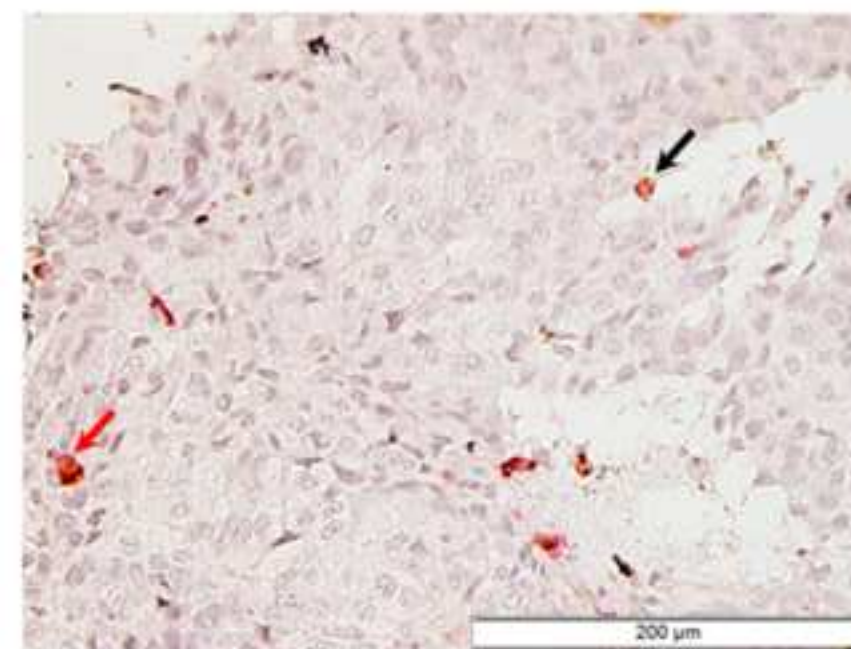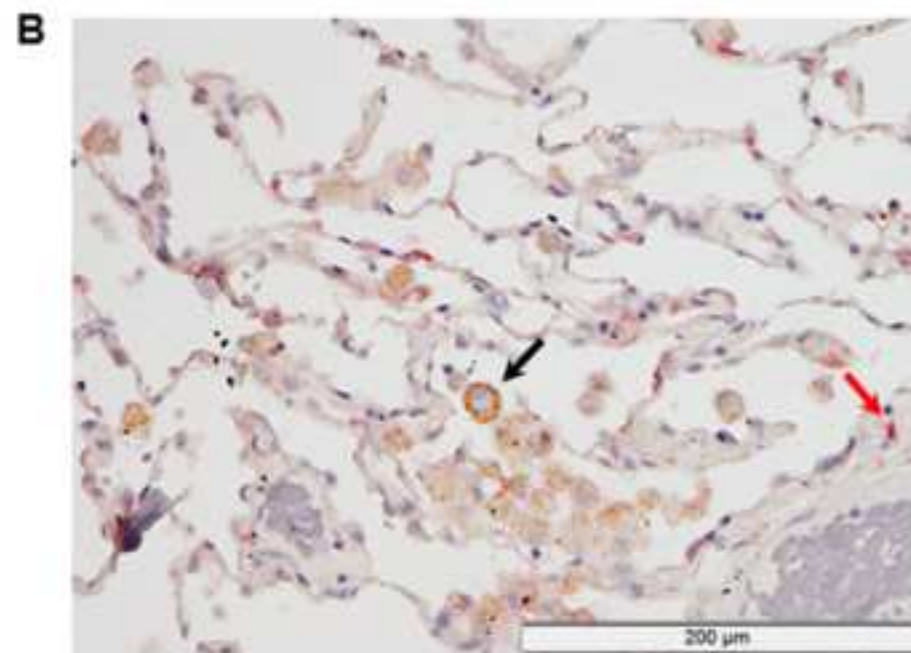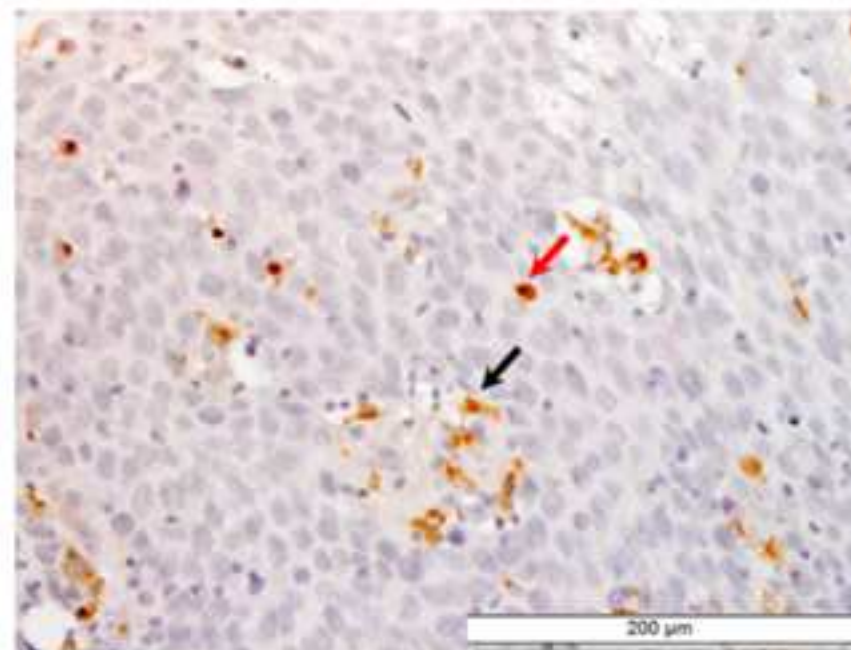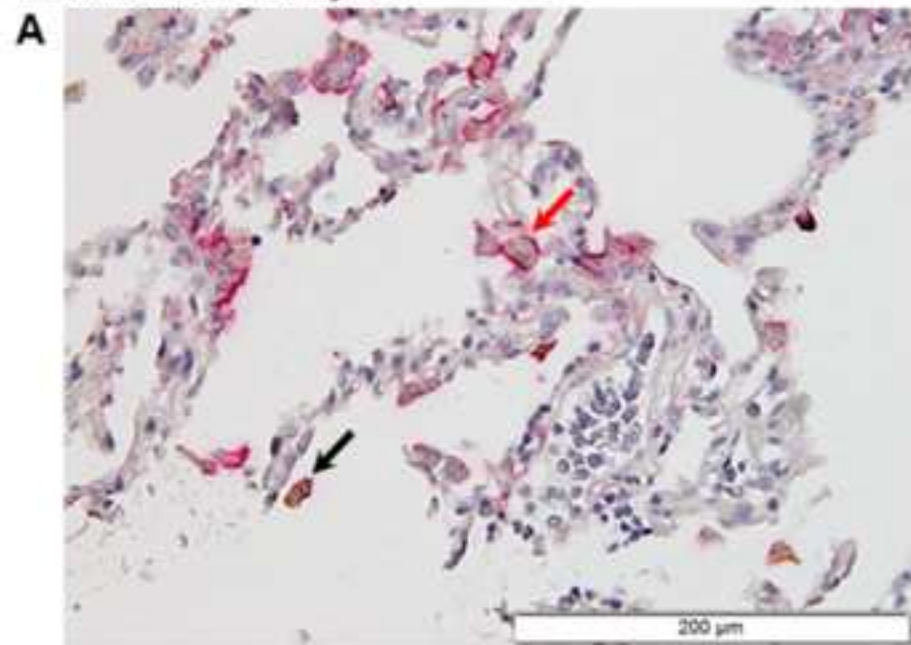
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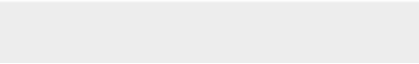
M. Mateu-Jimenez *et al.* Figure 2

M. Mateu-Jimenez *et al.* Figure 3

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1   **SYSTEMIC AND TUMOR TH1 AND TH2 INFLAMMATORY PROFILE AND**

2   **MACROPHAGES IN LUNG CANCER: INFLUENCE OF UNDERLYING**

3   **CHRONIC RESPIRATORY DISEASE**

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20   **Short title:** COPD influences inflammatory pattern in lung cancer

21   **Word count:** 3,725 words

22

23

24

25   **ABSTRACT**

26   Chronic respiratory conditions, especially chronic obstructive pulmonary disease

27   (COPD), and inflammatory events underlie lung cancer (LC). **Objectives:** We

28   hypothesized that profiles of Th1 and Th2 cytokines and M1 and M2 macrophages are

29   differentially expressed in lung tumors and blood of patients with non-small cell LC

30   (NSCLC) with and without COPD and that M1/M2 specifically may influence their

31   survival. **Methods:** In blood, inflammatory cytokines (ELISA) were quantified in 80

32   LC patients (LC-COPD, 60 and LC-only, 20) and lung specimens (tumor and non-

33   tumor) from those undergoing thoracotomy (LC-COPD and LC-only, 20/group).

34   **Results**: In LC-COPD compared to LC patients, systemic levels of TNF-alpha, IL-2,

35   TGF-beta, and IL-10 were increased, whereas VEGF and IL-4 levels decreased. In lung

36   tumors, TNF-alpha, TGF-beta, and IL-10 levels were greater than in non-tumor

37   parenchyma in LC-COPD, while IL-2 and VEGF levels were higher in tumors of both

38   LC-only and LC-COPD. Compared to non-tumor lung, M1 macrophage counts were

39   reduced, while M2 were increased in tumors of both patient groups, and M1/M2 was

40   greater in LC-COPD than LC-only. M1 and M2 counts did not influence patients'

41   survival. **Conclusions:** The relative predominance of Th1 cytokines and M1

42   macrophages in the blood and tumors of patients with underlying COPD imply that a

43   stronger proinflammatory pattern exists in these patients. Inflammation should not be

44   targeted systematically in all patients with LC. Screening for the presence of underlying

45   respiratory diseases and identification of the specific inflammatory pattern should be

46   carried out in patients with LC, at least in early stages of their disease.

47   Word count: 250

48   **KEY WORDS:** lung cancer; chronic respiratory conditions; Th1 and Th2 cytokines;

49   M1 and M2 macrophages; immune system; systemic and lung compartments

50

INTRODUCTION

In cancer-related mortality, lung cancer (LC) continues to be the most common cause of death worldwide[1-5], accounting for almost one third of deaths in certain regions[6]. Underlying respiratory conditions such as chronic obstructive pulmonary disease (COPD), which is also a highly prevalent disorder in industrialized countries, has been consistently associated with LC occurrence[7-11]. Airway obstruction and emphysema are, indeed, important risk factors for LC[7-11]. Identification of the biological mechanisms that render patients with chronic lung diseases more susceptible to LC development remains to be fully elucidated.

In this regard, despite the complex interactions observed among inflammation, immunity and lung tumor development, chronic inflammation has already been identified as a potential trigger in the process of tumorigenesis. As such, chronic inflammatory insults in the airways and lungs of respiratory patients may favor the risk of LC as also shown to occur in other cancer types such as pancreas, esophagus, and stomach[12-14]. In this regard, induction of several interleukins (IL) and cyclooxygenase-2 activity was suggested to contribute to the neoplastic transformation in patients with COPD[15-17]. Importantly, those inflammatory molecules may interfere with regulatory cell mechanisms such as repair, angiogenesis, and apoptosis, which favor the neoproliferative process[15-17]. Furthermore, cytokines and growth factors such as tumor necrosis factor (TNF)-alpha, IL-2, IL-4, IL-10, vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-beta, and epidermal growth factor receptor (EGFR) were also shown to promote tumor growth and metastasis in patients with underlying respiratory conditions[18-22].

Tumor immune surveillance also seems to play an important role in LC development including patients with chronic respiratory diseases[23]. Interestingly, Th1

76 lymphocytes, which release TNF-alpha, IL-2, and interferon-gamma, exert antitumor

77 effects, while Th2 cells, which mainly produce IL-4, were shown to favor tumor growth

78 by inhibiting the host immune system[24]. Tumorigenesis and relapse may rely on

79 alterations in Th1 and Th2 cytokines in patients[20;25;26]. In line with this, in patients with

80 LC, systemic Th2 cytokine levels were increased, whereas those of Th1 were

81 decreased[20]. Importantly, a change in Th1 and Th2 cytokines was seen in the same

82 patients after surgical treatment of the lung tumor, suggesting that these cytokines may

83 play a significant role in tumor progression[20].

84 In tumor microenvironment, type 1 (M1) and type 2 (M2) polarized macrophage

85 subtypes play a role in tumorigenesis through the regulation of several functions such as

86 cell adhesion, apoptosis, and senescence[25-27]. Furthermore, M1 macrophages were

87 shown to act in the initial process of tumorigenesis, exerting anti-tumorigenic effects,

88 while M2 macrophages were the predominant cells of established tumors[28]. Besides,

89 decreased M1/M2 macrophages may also influence tumor survival[29]. Whether the

90 pattern of chronic inflammatory events including the subtypes of macrophages may

91 differ in the tumors of LC patients with underlying respiratory diseases such as COPD

92 remains an open question. Answers to these questions may offer insight into that may

93 help design immunotherapeutic strategies for the better management of LC.

94 On the basis of this, we hypothesized that Th1 and Th2 cytokine profiles and M1

95 and M2 macrophages are differentially expressed in the lung tumor and non-tumor

96 parenchyma, and blood of patients with LC with and without COPD and that

97 specifically M1/M2 ratio may influence their survival. Hence, the study objectives were

98 defined as follows. In plasma, tumor lesions and non-tumor lung of non-small cell LC

99 (NSCLC) patients with and without underlying COPD, to determine: 1) protein levels

100   of Th1 and Th2 cytokines, 2) M1 and M2 macrophage counts in the lung, and 3)

101   survival of the patients according to numbers of M1 and M2 macrophages.

102

103   **METHODS**

104   (See the online supplement for detailed information on all methodologies including

105   statistical analysis).

106   **Study design and patient recruitment**

107   This is a prospective, cross-sectional study, in which patients were recruited

108   consecutively from the Lung Cancer Clinic of the Respiratory Medicine Department at

109   *Hospital del Mar* (Barcelona, Spain). For the purpose of the investigation, 80 Caucasian

110   patients with LC were recruited consecutively before having received any treatment for

111   their lung neoplasm from the weekly LC board meeting. Blood samples were obtained

112   at the time of diagnostic confirmation of LC in all 80 patients. These patients were

113   further subdivided *post-hoc* into two groups according to the presence of underlying

114   COPD, which was diagnosed on the basis of current guidelines[30-33]: 1) 60 patients with

115   LC who also had COPD (LC-COPD group, 1 female) and 2) 20 patients with LC

116   without COPD (LC-only group, 7 females). In LC-COPD patients, 57 males and 1

117   female and in LC-only group 13 males and 7 females simultaneously participated in a

118   previous study aimed to assess redox balance in lung tumors[34]. Moreover, from the

119   same study cohort, in the group of patients who underwent thoracotomy for the surgical

120   resection of their lung neoplasms (clinical indication according to guidelines for

121   diagnosis and management of lung cancer[30-33], specimens from the tumor and non-

122   tumor lung parenchyma were also obtained in all cases (n=40) and were further

123   subdivided *post-hoc* as follows: 1) 20 patients with LC and COPD (LC-COPD group,

124   all males) and 2) 20 patients with LC without COPD (LC-only group, 7 females).

125   Therefore, in these two groups of patients (LC-only and LC-COPD), blood and lung

126   specimens were available for the study. Twenty males (LC-COPD) and both 8 males

127   and 4 females (LC-only) also participated in the previous study[34].

128   Histological diagnosis and staging (tumor, node, metastasis, TNM) of LC were

129   confirmed in all patients[30-33]. Exclusion criteria were as follows: SCLC patients, chronic

130   cardiovascular, chronic metabolic and clot system disorders, signs of severe bronchial

131   inflammation and/or infection (bronchoscopy), current or recent invasive mechanical

132   ventilation, and chronic oxygen therapy. Approval was obtained from the institutional

133   Ethics Committee on Human Investigation (*Hospital del Mar–IMIM*, Barcelona) in

134   accordance with the World Medical Association guidelines (Helsinki Declaration of

135   2008) for research on human beings. Informed written consent was obtained from all

136   patients.

137   **Clinical assessment**

138   Lung function parameters were assessed in all patients following standard procedures.

139   Body composition evaluation included the assessment of body mass index (BMI) and

140   fat-free max index (FFMI) by bioelectrical impedance. Nutritional parameters were also

141   evaluated through conventional blood tests.

142   **Sample collection**

143   Blood sample specimens were obtained in all the recruited patients (n=80) from the arm

144   vein after an overnight fasting period. Moreover, in all patients undergoing thoracotomy

145   (n=40), lung specimens were obtained from both tumor and non-tumor surrounding

146   parenchyma during the surgery, in which standard technical procedures were followed

147   by the specialized thoracic surgeons. The expert pathologist selected a fragment of lung

148   tumor and non-tumor specimens of approximately 10x10 mm^2 size from the fresh

149   samples after a careful collection of the specimens required for diagnosis purposes.

150 **Sample preservation**

151 Lung specimens were immediately frozen in liquid nitrogen and stored in the −80ºC

152 freezer (under permanent alarm control) for further analysis or immersed in an alcohol-

153 formol bath to be embedded in paraffin. Blood samples were centrifuged and frozen at

154 −80 °C until further analyses. Frozen tissues were used for enzyme-linked

155 immunosorbent assay (ELISA) techniques, while paraffin-embedded lung sections,

156 which were gently provided by *Parc de Salut MAR* Biobank (MARBiobanc, Barcelona),

157 were used for the assessment of macrophage counts (immunohistochemical analyses).

158 **Molecular biology analyses**

159 *Quantification of cytokines and growth factors in plasma using ELISA.* Protein levels of

160 TNF-alpha, VEGF, IL-2, IL-10, interferon-gamma, TGF-beta and IL-4 were quantified

161 in blood (plasma) from all patients (ELISA kits, Gen-probe Diaclone SAS, Besançon,

162 France) following previous studies [15;35;36].

163 *Quantification of cytokines and growth factors in lung tissue (tumor and non-tumor)*

164 *using ELISA.* Protein levels of TNF-alpha, VEGF, IL-2, IL-10, interferon-gamma,

165 epidermal growth factor (EGFR), TGF-beta and IL-4 were quantified in lung specimens

166 from all patients (ELISA kits, Raybiotech Inc, Norcross GA, and Cloud-Clone Corp,

167 Houston, USA), following previous studies [15;35;36].

168 *Counts and types of macrophages in lung specimens (tumor and non-tumor).* M1 and

169 M2 macrophages were identified on three-micrometer lung paraffin-embedded sections

170 using double-staining immunohistochemical procedures (Envision DuoFLEX

171 Doublestain System, Dako North America Inc., Carpinteria, CA, USA) following the

172 manufacturer's instructions and previous studies[37-39].

173 **Statistical analyses**

174 All statistical analyses were performed using the software SPSS 15.0 (SPSS Inc,

175   Chicago, IL, USA). Data are expressed as mean (standard deviation). The normality of

176   the study variables was explored using Shapiro-Wilk test. In order to test the potential

177   effects of cigarette smoking (CS) on the study results, LC-COPD were further

178   subdivided into moderate (n=25) and heavy (n=35) smokers, in which 60 packs-year

179   was the cut-off value (median). On the basis of a standard power statistics established at

180   a minimum of 80% and assuming an alpha error of 0.05, the statistical power was

181   sufficiently high to detect minimum differences between the two study groups in the

182   target variables (plasma cytokines and macrophage subtypes). The sample size was

183   calculated on the basis of these parameters, which required a minimum of 40-50 patients

184   for the plasma cytokines analyses and 15 patients for the analyses conducted in the lung

185   specimens in order to detect potential differences in the study variables between the two

186   groups.

187   Differences between groups in the study variables were assessed using one-way

188   analysis of variance (ANOVA) and Tukey's *post-hoc* analysis was used to adjust for

189   multiple comparisons. Differences between study groups for qualitative variables were

190   explored using the Chi-square test. Statistical significance was established at $P \leq 0.05$.

191   Variables from the two compartments (blood and lungs) were evaluated independently.

192

193   **RESULTS**

194   **Clinical characteristics**

195   Clinical and functional characteristics of all LC-COPD and LC-only patients recruited

196   in the study are shown in Tables 1 and 2 (all patients as a group and only patients

197   undergoing thoracotomy, respectively). The number of LC-COPD patients was higher

198   than LC-only and were mostly males in both groups (Tables 1 and 2). No significant

199   differences were found in age or BMI between LC-COPD and LC-only patients (Tables

200   1 and 2). Smoking history differed between LC-COPD and LC-only patients (Table 1).

201   The functional parameters diffusion lung capacity for carbon monoxide (DL_{CO}), forced

202   expiratory volume in one second (FEV_1), FEV_1/forced vital capacity (FVC) were

203   significantly decreased in LC-COPD compared to LC patients (Tables 1 and 2). No

204   significant differences were found in these parameters between heavy and moderate

205   smokers within the LC-COPD group (Tables 1 and 2). Moreover, no statistically

206   significant differences were found in either TNM or histological subtypes between LC-

207   COPD and LC-only groups (Tables 1 and 2). In LC-COPD compared to LC patients,

208   levels of albumin and fibrinogen were decreased, whereas those of C-reactive protein

209   (CRP) and globular sedimentation (GSV) were increased (Tables 1 and 2). Furthermore,

210   body weight loss was greater in LC-COPD, especially in heavy smokers, compared to

211   LC-only patients (Table 1).

212   **Systemic levels of cytokines**

213   In LC-COPD, levels of TNF-alpha and IL-2 levels were greater compared to LC-only

214   patients (Figures 1A-1B). Systemic levels of interferon-gamma did not differ among the

215   study groups (Figure 1C). In the *post-hoc* analyses, levels of IL-2 were higher in heavy

216   smokers compared to moderate smokers, whereas no differences were observed in either

217   TNF-alpha or interferon-gamma levels between these groups (Figures 1A-1C). In LC-

218   COPD patients, levels of VEGF and IL-4 were significantly lower, while those of TGF-

219   beta and IL-10 were higher compared to LC-only patients (Figures 1D-1G). Levels of

220   the cytokines VEGF, IL-4, TGF-beta and IL-10 did not differ between heavy and

221   moderate smokers in LC-COPD patients (Figures 1D-1G). No significant associations

222   were found between systemic levels of the study cytokines and the patients' survival

223   (data not shown).

224   **Levels of cytokines and growth factors in the lung specimens**

225   *Tumor versus non-tumor parenchyma in LC-COPD and LC patients.* In LC-COPD

226   patients and especially in moderate smokers, TNF-alpha levels were significantly

227   increased in tumor lesions compared to non-tumor specimens, while no differences were

228   seen in LC-only patients (Figure 2A). IL-2 levels were significantly greater in tumor

229   lesions of both LC and LC-COPD patients compared to non-tumor specimens (Figure

230   2B). No differences were found in interferon-gamma levels between tumor and non-

231   tumor lungs in any of the study groups (Figure 2C). VEGF levels were significantly

232   higher in tumor lesions compared to the non-tumor lungs in all LC-COPD and LC-only

233   patients (Figure 2D). In the latter patients, levels of TGF-beta were increased in tumor

234   lesions compared to non-tumor specimens (Figure 2E). No differences were found in

235   IL-4 levels between tumor and non-tumor lungs in any of the study patient groups

236   (Figure 2F). In all LC-COPD patients, IL-10 levels were greater in the tumors than in

237   the non-tumor lungs (Figure 2G). In both groups of patients, epidermal growth factor

238   receptor (EGFR) levels were significantly increased in tumor specimens compared to

239   non-tumor lesions (Figure 2H), and no differences were seen between moderated and

240   heavy smokers. Finally, No significant correlations were found in any of the study

241   cytokines between blood and lung compartments.

242   *Differences between LC-COPD and LC in either tumor lesions or non-tumor specimens.*

243   No significant differences were found in TNF-alpha, IL-2 and interferon-gamma levels

244   between LC-COPD (both heavy and moderate smokers) and LC-only patients in either

245   tumor or non-tumor lungs (Figures 2A-2C). In LC-COPD compared to LC-only

246   patients, levels of VEGF, TGF-beta, and IL-10 were greater in the tumor lesions

247   (Figures 2D, 2E, and 2G). Levels of IL-4 did not significantly differ between LC-COPD

248   and LC-only patients in either tumor or non-tumor lungs (Figure 2F). No significant

249   differences were found in VEGF, TGF-beta, IL-4, IL-10 or EGFR levels between

moderate and heavy smokers within the LC-COPD group of patients (Figures 2D-2H). No significant associations were found between levels in the lungs of the study cytokines or growth factors and the patients' survival (data not shown).

M1 and M2 macrophage subtypes in the lung. In both LC-COPD and LC-only groups, M2 macrophages were increased, while M1 and M1/M2 ratio were decreased in lung tumors compared to non-tumor specimens (Figures 3A-3B, Table 3). In tumor specimens, M1/M2 was significantly greater in LC-COPD than in LC-only patients (Table 3). No significant associations were found between M1, M2 or M1/M2 and patients' survival in any study group (Figure E1A-E1B).

DISCUSSION

In the current study, the main findings were that patients with LC-COPD exhibited a moderate airway obstruction and functional emphysema; adenocarcinoma was the predominant histological type, and those who smoked more showed a significantly greater loss of body weight. Moreover, in LC-COPD compared to LC-only patients, systemic levels of Th1 cytokines TNF-alpha, IL-2 and those of Th2 cytokines TGF-beta and IL-10 were increased, whereas those of VEGF and IL-4 were decreased with no significant differences in interferon-gamma levels. In LC-COPD patients, levels of TNF-alpha, TGF-beta, and IL-10 were greater in tumors than in non-tumor lungs, while a significant rise in IL-2 and VEGF levels was seen in the tumors of both groups. Moreover, VEGF, TGF-beta, and IL-10 levels were increased in the tumors of LC-COPD than in those of LC-only patients. In the tumors of both groups, M1 macrophages and M1/M2 were reduced, while M1/M2 was significantly greater in LC-COPD patients. Smoking history did not influence the differences in the study parameters between groups. Importantly, no correlations between lung and blood

275 compartments were observed for any of the study variables in the two groups of
276 patients. In view of these findings, the study hypothesis was confirmed to a great extent.
277 CD4+ T lymphocytes are divided into T helper (Th)1 or Th2 cells on the basis of
278 the secreted cytokines. Th1 lymphocytes release TNF-alpha, IL-2, and interferon-
279 gamma which exert antitumor effects in humans, while Th2 cells produce IL-4 and IL-
280 10 that favor tumor growth by inhibiting the host immune system[24;25]. In patients with
281 several types of cancer, alterations of the Th1/Th2 immunological balance have been
282 detected in tumorigenesis and cancer relapse[20;25;26]. In the present investigation,
283 systemic levels of TNF-alpha, IL-2, TGF-beta, and IL-10 were significantly increased
284 in patients with underlying COPD compared to those without any respiratory condition.
285 These findings suggest that Th1 systemic immunological response and regulatory T-
286 lymphocyte cytokines (TGF-beta and IL-10) were significantly enhanced in patients
287 bearing the two conditions: COPD and LC. It is likely that in COPD, systemic
288 inflammation as a result of chronic CS exposure drives a general Th1 response in the
289 patients, which was not observed in LC patients without COPD.

290 Importantly, in the lung tumors of LC patients with underlying COPD, levels of
291 the cytokines TNF-alpha, TGF-beta, and IL-10 were significantly greater than those
292 encountered in the surrounding non-tumor lung. Moreover, levels of VEGF, TGF-beta,
293 and IL-10 were also significantly higher in the tumors of LC-COPD than in LC-only
294 patients. Interestingly, levels of IL-4 did not differ in lung specimens among the study
295 groups. Collectively, these findings suggest that in COPD, chronic inflammation in the
296 lungs is characterized by the release of Th1 and regulatory T-lymphocytes, which may
297 protect patients against tumor development and progression. Indeed, similar findings
298 were reported in the bronchial epithelium of patients with LC and COPD[15].
299 Nonetheless, a previous study[20] showed that in patients with NSCLC, systemic levels of

300 Th2 cytokines were increased, while those of Th1 cytokines were reduced compared to

301 healthy subjects. In the same investigation[20], NSCLC patients were shown to lower

302 systemic Th2 cytokine levels in response to lung tumor resection, and the relapse rates

303 were significantly greater in those patients with persistent abnormal levels of IL-4.

304 Importantly, in that study[20], patients were not analyzed according to the presence of

305 COPD or smoking history. In fact, this analysis is a major novel contribution of the

306 current investigation.

307 EGFR is the cell-surface receptor of the EGF family protein ligands in several cell

308 types that contribute to the correct development of glands in tissues. Mutations of

309 EGFR that induce its overexpression are involved in tumorigenesis such as LC. Despite

310 that the efficacy of anti-EGFR therapies (namely EGFR tyrosine kinase inhibitors and

311 anti-EGFR monoclonal antibodies) remain controversial in certain NSCLC types,

312 especially squamous LC, beneficial effects have been observed in recent studies, in

313 which adenocarcinoma was the predominant histological subtype[40;41]. Moreover,

314 durable clinical responses beyond five years have also been proposed in response to

315 pathway-targeted immunotherapy as a result of treatment of NSCLC patients with a

316 novel EGF-directed agent[42]. As in previous studies[40;41;43], in the present investigation, in

317 both groups of patients, EGFR levels were also significantly greater in the tumor lesions

318 than in the surrounding non-tumor parenchyma. Identification of potential mutations of

319 EGFR gen, however, was beyond the scope of the current study and will remain the

320 focus of research in future investigations.

321 Macrophages may exert proinflammatory or anti-inflammatory functions

322 depending on the secreted cytokines. In tumors, the inflammatory infiltrates are mostly

323 represented by this type of cells. In general, M1 macrophages favor inflammation,

324 whereas M2 macrophages promote anti-inflammatory actions and tissue repair. While

325 M1 cells fight against tumor development, M2 macrophages exert the opposite effects,

326 by promoting cancer growth, survival, progression, and dissemination[25]. In keeping

327 with, in the tumor lesions of both study groups of patients, the number of M1

328 macrophages was reduced, while that of M2 was increased. Hence, the ratio of M1 to

329 M2 cells was also significantly lower in the tumors than in the surrounding non-tumor

330 lung in all groups of patients. Indeed, interaction of factors such as the stage of the

331 tumor and the local microenvironment has been shown to determine macrophage

332 phenotype and tumor progression[25]. Importantly, in the study, tumors of LC patients

333 with underlying COPD exhibited a significantly greater M1/M2 ratio than those of

334 patients without COPD. These results suggest that the relative predominance of M1

335 phenotype in tumors of LC-COPD patients may imply a better prognosis in these

336 patients compared to those with no COPD. Nonetheless, the number of macrophages or

337 M1/M2 ratio was not associated with the patients' survival in any of the study groups

338 (Figure E1A-E1B) as was shown to occur in other types of tumors[29]. It is likely that the

339 statistically significant difference of M1/M2 ratio in tumors between the two study

340 groups may not be of sufficient biological relevance to modify the prognosis of patients

341 with LC.

342 Importantly, in the last few years, a better and progressive understanding of the

343 complex interactions between the immune system, inflammation and carcinogenesis

344 including LC treatment has led to the development of novel immunotherapeutic

345 agents[44;45]. Nonetheless, the efficacy of these immunomodulatory drugs is hampered by

346 the acquired resistance following certain periods of time[44;45]. Hence, further insight into

347 the potential contribution of the immune system to lung carcinogenesis is required in

348 order to design immunotherapeutic strategies that will ensure a longer lasting control of

349 the disease. Cytokines, monoclonal antibodies, tumor and dendritic cell vaccines, and

350 checkpoint inhibitors are examples of the passive and active immunotherapy agents that

351 are currently used in clinical settings[44-46]. Future research in this field will shed light

352 into further mechanisms that will selectively target tumor cells in patients with LC.

Study limitations

354 A first limitation in the study refers to the relatively lower number of lung specimens

355 analyzed in the study compared to the number of blood samples. Nevertheless, for

356 ethical reasons, tumor and non-tumor lung specimens could only be obtained from

357 patients undergoing thoracotomy for the treatment of their lung neoplasm from the

358 established cohort of patients that participated in this investigation, from whom blood

359 samples had been collected. Furthermore, the study variables could have also been

360 analyzed using other approaches in which the potential predictive value of the analyzed

361 markers may have been estimated. However, the investigation was aimed to identify

362 whether underlying COPD influences the expression of Th1 and Th2 cytokines in

363 patients with LC. Another approach would be to analyze the expression of the target

364 variables on the basis of histological subtypes. In this regard, no significant differences

365 were detected in any of the clinical or biological variables between the two groups.

366 Another limitation is the relatively low numbers of females in both groups of

367 patients. This is because the prevalence of LC in female patients, especially in those

368 patients with underlying COPD is still very low in our geographical region[7;9;10]. Despite

369 these limitations, the main findings confirmed the study hypothesis to a great extent.

Conclusions

371 A differential expression profile of inflammatory markers and cells has been identified

372 in lung tumors and blood compartment in LC patients bearing a chronic respiratory

373 condition, irrespective of smoking history. The relative predominance of Th1 cytokines

374 and M1 macrophages in the blood and tumors of patients with underlying COPD imply

375 that a stronger proinflammatory pattern exists in these patients. These findings have

376 potential clinical implications as inflammation should not be targeted systematically in

377 all patients with LC. Screening for the presence of underlying respiratory diseases and

378 identification of the specific inflammatory pattern should be carried out in patients with

379 LC, at least in early stages of their disease.

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FIGURE LEGENDS

Figure 1

A) Mean values and standard deviation of blood TNF-alpha levels are shown in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. TNF-alpha levels were significantly higher in LC-COPD than in LC patients. Levels of TNF-alpha did not significantly differ between heavy and moderate smokers.

B) Mean values and standard deviation of blood IL-2 levels are shown in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. IL-2 levels were significantly increased in LC-COPD compared to LC patients. IL-2 levels were also higher in heavy smokers than in moderate smokers in LC-COPD patients.

C) Mean values and standard deviation of blood interferon-gamma levels are shown in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. Interferon-gamma levels did not significantly differ among the study groups.

D) Mean values and standard deviation of blood VEGF levels are shown in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. VEGF levels were significantly decreased in LC-COPD than in LC patients. Levels of VEGF did not significantly differ between heavy and moderate smokers.

E) Mean values and standard deviation of blood IL-4 levels are shown in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. IL-4 levels were significantly reduced in LC-COPD than in LC patients. Levels of IL-4 did not significantly differ between heavy and moderate smokers.

F) Mean values and standard deviation of blood TGF-beta levels are shown in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. Levels of TGF-beta were significantly greater in LC-COPD than in LC patients. Levels of TGF-beta did not significantly differ between heavy and moderate smokers.

G) Mean values and standard deviation of blood IL-10 levels are shown in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. IL-10 levels were significantly increased in LC-COPD than in LC patients. Levels of IL-10 did not significantly differ between heavy and moderate smokers.

Figure 2

A) Mean values and standard deviation of lung TNF-alpha levels are depicted in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. Moreover, TNF-alpha levels detected in non-tumor (NT) and tumor (T) lungs are also represented in graphs for all the groups for the purpose of the

comparisons. Levels of TNF-alpha were significantly higher in the T lesions compared to NT lungs in LC-COPD, especially in moderate smokers. TNF-alpha levels did not significantly differ between T and NT in LC patients or between LC-COPD and LC patients. No significantly differences were found in TNF-alpha levels between heavy and moderate smokers in either T or NT lungs.

B) Mean values and standard deviation of lung IL-2 levels are depicted in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. Moreover, IL-2 levels detected in NT and T lungs are also represented in graphs for all the groups for the purpose of the comparisons. Levels of IL-2 were significantly greater in T lesions compared to NT lung specimens in both LC and LC-COPD patients. IL-2 levels did not significantly differ between LC-COPD and LC patients or between moderate and heavy smokers.

C) Mean values and standard deviation of lung interferon-gamma levels are depicted in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. Moreover, interferon-gamma levels detected in NT and T lungs are also represented in graphs for all the groups for the purpose of the comparisons. Interferon-gamma levels did not significantly differ in any of the study groups of patients.

D) Mean values and standard deviation of lung VEGF levels are depicted in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made

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between heavy and moderate smokers. Moreover, VEGF levels detected in NT and T lungs are also represented in graphs for all the groups for the purpose of the comparisons. Levels of VEGF were significantly increased in T lesions compared to NT lung specimens in both LC and LC-COPD patients, especially in heavy and moderate smokers. Lung VEGF levels were also higher in the T lesions of LC-COPD than in LC patients. No significant differences were found between heavy and moderate smokers.

E) Mean values and standard deviation of lung TGF-beta levels are depicted in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. Moreover, TGF-beta levels detected in NT and T lungs are also represented in graphs for all the groups for the purpose of the comparisons. Levels of TGF-beta were significantly greater in T lesions than in NT lung specimens of LC-COPD patients. TGF-beta levels were also increased in T lesions of LC-COPD compared to LC patients. No significantly differences were found between T and NT in LC patients or between heavy and moderate smokers.

F) Mean values and standard deviation of lung IL-4 levels are depicted in the graph. As described in methods, comparisons were made between LC and LC-COPD, and among the latter patients, analyses were also made between heavy and moderate smokers. Moreover, IL-4 levels detected in NT and T lungs are also represented in graphs for all the groups for the

641   purpose of the comparisons. Levels of IL-4 did not significantly differ

642   among the study groups of patients.

643 **G)** Mean values and standard deviation of lung IL-10 levels are depicted in

644   the graph. As described in methods, comparisons were made between LC

645   and LC-COPD, and among the latter patients, analyses were also made

646   between heavy and moderate smokers. Moreover, IL-10 levels detected

647   in NT and T lungs are also represented in graphs for all the groups for the

648   purpose of the comparisons. Levels of IL-10 were significantly greater in

649   T lesions compared to NT lung specimens in LC-COPD patients,

650   especially in moderate and heavy smokers. IL-10 levels were also

651   increased in T parenchyma of LC-COPD compared to LC patients. No

652   significant differences were found between T and NT in LC patients or

653   between heavy and moderate smokers.

654 **H)** Mean values and standard deviation of lung EGFR levels are depicted in

655   the graph. As described in methods, comparisons were made between LC

656   and LC-COPD, and among the latter patients, analyses were also made

657   between heavy and moderate smokers. Moreover, EGFR levels detected

658   in NT and T lungs are also represented in graphs for all the groups for the

659   purpose of the comparisons. Levels of EGFR were significantly higher in

660   T lesions compared to NT lung specimens in both LC and LC-COPD

661   patients. No significant differences were observed in EGFR levels

662   between LC-COPD and LC patients or between heavy and moderate

663   smokers.

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Figure 3:

A) Representative examples of double immunohistochemical staining for M1 macrophages (CD68-HLA positively stained macrophages) in non-tumor (left-hand side panel) and tumor (right hand-side panel) lung specimens. All types of macrophages (CD68$^+$) are stained only in brown color (black arrow) whereas M1 macrophages (CD68$^+$-HLA$^+$) are specifically stained with brown and red color (red arrow).

B) Representative examples of double immunohistochemical staining for M2 macrophages (CD68-CD206 positively stained macrophages, black arrows) in non-tumor (left-hand side panel) and tumor (right hand-side panel) lung specimens. All types of macrophages (CD68$^+$) are stained only in brown color (black arrow) whereas M2 macrophages (CD68$^+$-CD206$^+$) are specifically stained with brown and red color (red arrow).

Table 1. Clinical and functional characteristics of all the study patients.

Anthropometric variables	Lung Cancer N=20	Lung Cancer-COPD N=60	Lung Cancer-COPD moderate smokers N=25	Lung Cancer-COPD heavy smokers N=35
Age, years	64 (12)	68 (11)	68 (9)	67 (11)
Male, N, / Female, N	13 / 7	59 / 1	24 / 1	35/0
BMI, Kg/m^2	26 (5)	24 (3)	25 (3)	25 (4)
Smoking history				
Current: N, %	12, 60	38, 63 **	10, 40	28, 80 §§
Ex-smoker: N, %	5, 25	22, 37 **	15, 60	7, 20 §§
Never Smoker: N,%	3, 15	0, 0 **	0, 0	0, 0
Packs-year	53 (25)	65 (23) *	44 (9)	78 (19) §§§
Lung function testing				
FEV_1 , % pred	92 (8)	57 (12) ***	60 (13)	57 (12)
FEV_1/FVC, % pred	77 (6)	60 (8) ***	61 (5)	60 (9)
DLCO, % pred	88 (12)	69 (18) ***	70 (19)	68 (18)
KCO, % pred	88 (11)	80 (21)	81 (21)	80 (21)
TNM staging				
Stage IA: N, %	5, 25	9, 15	5, 20	4, 12
Stage IB: N, %	0, 0	10, 16	5, 20	5, 14
Stage IIA: N, %	1, 5	5, 8	2, 8	3, 9
Stage IIB: N, %	4, 20	4, 7	2, 8	2, 6
Stage IIIA: N, %	3, 15	9, 15	3, 12	6, 17
Stage IIIB: N, %	4, 20	7, 12	1, 4	6, 17
Stage IV: N, %	3, 15	16, 27	7, 28	9, 25
Histological diagnosis				
Squamous cell carcinoma: N, %	6, 30	27, 45	12, 48	15, 43
Adenocarcinoma: N, %	11, 55	24, 40	10, 40	14, 40
Others: N, %	3, 15	9, 15	3, 12	6, 17
Blood parameters				
Total leukocytes /microL	$8.3\ 10^3$ ($1.9\ 10^3$)	$8.4\ 10^3$ ($2.2\ 10^3$)	$8.2\ 10^3$ (2.3 103)	$8.7\ 10^3$ (2.1 103)

Total Neutrophils /microL	$5.7 \cdot 10^3$ ($2.1 \cdot 10^3$)	$5.9 \cdot 10^3$ ($1.8 \cdot 10^3$)	$5.8 \cdot 10^3$ ($1.8 \cdot 10^3$)	5.9 ($1.8 \cdot 10^3$)
Total lymphocytes/microL	$1.7 \cdot 103$ (650)	$1.7 \cdot 103$ (676)	$1.4 \cdot 10^3$ (635)	$1.8 \cdot 10^3$ (681)
Albumin (g/dL)	4.4 (0.4)	4.1 (0.5) *	4.2 (0.50)	4.1 (0.5)
Total proteins (g/dL)	7.4 (0.5)	7.3 (0.4)	7.2 (0.6)	7.5 (0.6)
Fibrinogen (mg/dL)	485 (99)	453 (152)	430 (151.7)	473 (153)
CRP (mg/dL)	1.1 (1.1)	4.9 (6.8) *	5.1 (7)	4.8 (6.8)
GSV (mm/h)	20 (7.4)	28.8 (27.9)	26.2 (28.5)	30.4 (28)
Ceruloplasmin (g/dL)	26.2 (4.8)	30.7 (7.7)	35 (11)	29.1 (6.1)
Body weight loss, kg				
0, N, %	18, 90	36, 60 ***	18, 72	18, 52 §§§
1-4, N, %	2, 10	8, 14 ***	3, 12	5, 14§§§
5-8, N, %	0, 0	14, 23 ***	4, 16	10, 28 §§§
9-12, N, %	0, 0	2, 3 ***	0, 0	2, 6 §§§

Continuous variables are presented as mean (standard desviation), while categorical variables are presented as the number of patients in each group and percentage of the total population.

Definition of abbreviations: N, number; kg, kilograms; m, meters; BMI, body mass index; FEV_1, forced expiratory volume in the first second; pred, predicted; FVC, forced vital capacity; DLco, carbon monoxide transfer; K_{CO}, Krogh transfer factor; TNM, tumor, nodes, metastasis; CRP, C-reactive protein; GSV, globular sedimentation velocity; L, liter.

Statistical analyses and significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ between LC-COPD patients as a group and LC-only patients; § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$ between LC-COPD heavy smokers and LC-COPD moderate smokers in the post-hoc analyses. Comparisons of the clinical and physiological variables between LC-COPD as a group and LC-only patients, as well as between the two groups of LC-COPD patients (moderate and heavy smokers) were assessed using the Student's T-test. Differences between the study groups for the qualitative variables were assessed using the Chi-square test.

Table 2. Clinical and functional characteristics of the study patients undergoing thoracotomy.

Anthropometric variables	Lung Cancer N=20	Lung Cancer-COPD N=20	Lung Cancer-COPD moderate smokers N=9	Lung Cancer-COPD heavy smokers N=11
Age, years	64 (12)	65 (9)	63 (10)	66 (9)
Male, N/ Female, N	13 / 7	20 / 0	9 / 0	11 / 0
BMI, Kg/m^2	26 (5)	26 (5)	25 (4)	27 (3)
Smoking history				
Current: N, %	12, 60	14, 70	6, 67	8, 73
Ex-smoker: N, %	5, 25	6, 30	3, 33	3, 27
Never Smoker: N,%	3, 15	0, 0 *	0, 0	0, 0
Packs-year	53 (25)	57 (20)	39 (6)	71 (14) § § §
Lung function testing				
FEV$_1$, % pred	92 (8)	61 (14) ***	64 (14)	59 (14)
FEV$_1$/FVC, % pred	77 (6)	60 (8) ***	59 (8)	62 (8)
DLCO, % pred	88 (12)	72 (21) ***	69 (22)	74 (21)
KCO, % pred	88 (11)	73 (17)***	70 (16)	75 (19)
TNM staging				
Stage IA: N, %	5, 25	5, 25	3, 34	2, 18
Stage IB: N, %	0, 0	4, 20	2, 22	2, 18
Stage IIA: N, %	1, 5	6, 30	2, 22	4, 37
Stage IIB: N, %	4, 20	2, 10	2, 22	0, 0
Stage IIIA: N, %	3, 15	3, 15	0, 0	3, 27
Stage IIIB: N, %	4, 20	0, 0	0, 0	0, 0
Stage IV: N, %	3, 15	0, 0	0, 0	0, 0
Histological diagnosis				
Squamous cell carcinoma: N, %	6, 30	5, 25	2, 22	3, 27
Adenocarcinoma: N, %	11, 55	14, 70	7, 78	7, 63
Others: N, %	3, 15	1, 5	0, 0	1, 10
Blood parameters				
Total leukocytes /microL	8.3 10^3 (1.9 10^3)	9.2 10^3 (2.1 10^3)	9.4 10^3 (2.3 103)	9.1 10^3 (2.1 103)

Total Neutrophils /microL	$5.7 \, 10^3 \, (2.1 \, 10^3)$	$6.3 \, 10^3 \, (1.7 \, 10^3)$	$6.2 \, 10^3 \, (1.5 \, 103)$	$6.3 \, 10^3 \, (1.9 \, 103)$
Total lymphocytes/microL	1.7 103 (650)	$2.1 \cdot 10^3 \, (617)$	2.0 (801)	$2.0 \, 10^3 \, (457)$
Albumin (g/dL)	4.4 (0.4)	4.0 (0.5) **	4.0 (0.6)	4.1 (0.5)
Total proteins (g/dL)	7.4 (0.5)	7.1 (0.6)	6.9 (0.7)	7.3 (0.4)
Fibrinogen (mg/dL)	485 (99)	409 (77) **	377 (60)	436 (84)
CRP (mg/dL)	1.1 (1.1)	1.7 (1.2)	1 (0.4)	2 (2.6)
GSV (mm/h)	20 (7.4)	30 (16.8) *	28.7 (23)	30 (14.6)
Ceruloplasmin (g/dL)	26.2 (4.8)	26.5 (4.8)	26 (4.18)	26.6(5.3)
Body weight loss, kg				
0, N, %	18, 90	17, 85	7, 78	10, 90
1-4, N, %	2, 10	1, 5	1, 11	0, 0
5-8, N, %	0, 0	2, 10	1, 11	1, 10
9-12, N, %	0, 0	0, 0	0, 0	0, 0

Continuous variables are presented as mean (standard desviation), while categorical variables are presented as the number of patients in each group and percentage of the total population.

Definition of abbreviations: N, number; kg, kilograms; m, meters; BMI, body mass index; FEV_1, forced expiratory volume in the first second; pred, predicted; FVC, forced vital capacity; DLco, carbon monoxide transfer; K_{CO}, Krogh transfer factor; TNM, tumor, nodes, metastasis; CRP, C-reactive protein; GSV, globular sedimentation velocity; L, liter.

Statistical analyses and significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ between LC-COPD patients as a group and LC-only patients; § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$ between LC-COPD heavy smokers and LC-COPD moderate smokers in the post-hoc analyses. Comparisons of the clinical and physiological variables between LC-COPD as a group and LC-only patients, as well as between the two groups of LC-COPD patients (moderate and heavy smokers) were assessed using the Student's T-test. Differences between the study groups for the qualitative variables were assessed using the Chi-square test.

Table 3. Counts of positive cells for specific markers of M1 and M2 macrophages.

	Lung Cancer		Lung Cancer-COPD	
	Non-tumor	Tumor	Non-tumor	Tumor
M1/micrometer^2	0.83 (0.22)	0.23 (0.18) ¶¶¶	0.78 (0.22)	0.25 (0.17) ¶¶¶
M2/micrometer^2	0.21 (0.19)	0.59 (0.33) ¶¶¶	0.24 (0.17)	0.41 (0.31) ¶¶
Ratio M1 / M2	6.10 (3.6)	0.30 (0.27) ¶¶¶	6.20 (5.7)	0.70 (0.5) ¶¶¶, *

Data are presented as mean (standard deviation).

Statistical analyses and significance: ¶¶ $p < 0.01$, ¶¶¶ $p < 0.001$ between tumor and non-tumor lung specimens in either LC or LC-COPD groups of patients; * $p < 0.05$ between LC-COPD tumor and LC-only tumor.

Definition of abbreviations: M1, macrophages type 1 (HLA$^+$ cells); M2, macrophages type 2 (CD206$^+$cells).