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1 **Calpain 1 in bronchoalveolar lavage fluid is associated with poor prognosis in lepidic**
2 **predominant pulmonary adenocarcinoma**

3

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22

23 **ABSTRACT**

24 Calpain 1 is a pro inflammatory calcium-activated cysteine protease, which can be partly
25 externalized. Extracellular calpains limit inflammatory processes and promote tissue repair,
26 through cell proliferation and migration. Toll like receptor (TLR) 2 has been identified as a
27 target of extracellular calpains in lymphocytes. The aim was to investigate the externalization
28 of calpain 1 and the release of soluble TLR2 during tumor progression of pulmonary lepidic
29 predominant adenocarcinoma (LPA).

30 Extracellular calpain 1, soluble fragment of TLR2 and cytokines were analyzed by ELISA in
31 bronchoalveolar lavage fluid (BALF) supernatants from patients with LPA (n=68). Source of
32 calpain was analyzed by immunohistochemistry and soluble TLR2 by flow cytometry on
33 polymorphonuclear neutrophils (PMN) and human lung cancer cell lines.

34 Extracellular calpain 1, secreted by tumor cells, was associated to tumor progression,
35 neutrophilic inflammation, with a poor prognostic factor on survival (p=0.003). TLR2 was
36 expressed on PMN and tumor cells and decreased after calpain exposure. Soluble fragment of
37 TLR2 in BALF supernatants was correlated to the extracellular calpain 1 concentration
38 (r=0.624; p<0.001), and its high level was associated with tumor progression and a pro-
39 inflammatory environment.

40 Extracellular calpain 1 secreted by tumor cells, could participate in inflammatory
41 microenvironment and tumor progression through TLR2 in LPA.

42

43 **Key words:** calpain – toll like receptor 2 – lung cancer – polymorphonuclear neutrophils -
44 adenocarcinoma

45

46 **ABSTRACT FRANCAIS**

47 La calpaïne 1 est une protéase à cystéine activée par le calcium, qui peut être partiellement
48 externalisée. Les calpaines extracellulaires favorisent la résolution de l'inflammation et la
49 réparation des tissus, à travers la prolifération et la migration cellulaire. Le récepteur Toll like
50 (TLR) 2 a été identifié comme une cible des calpaines extracellulaires dans les lymphocytes.
51 L'objectif est d'étudier le rôle de la calpaïne extracellulaire 1 dans la progression tumorale de
52 l'adénocarcinome pulmonaire lepidique (ADL).

53 La calpaïne extracellulaire, le fragment soluble de TLR2, et les cytokines étaient analysés par
54 ELISA dans les surnageants de lavage bronchoalvéolaire (LBA) de patients atteints d'ADL (n
55 = 68). La source de calpaïne était analysée par immunohistochimie. TLR2, cible de la
56 calpaïne extracellulaire était étudiée par cytométrie de flux sur les polynucléaires neutrophiles
57 (PNN) et des lignées humaines de cancer bronchiques.

58 Calpaïne 1 extracellulaire, sécrétée par les cellules tumorales, était associée à la progression
59 tumorale, l'inflammation à neutrophiles, avec un facteur de mauvais pronostic de survie (p =
60 0,003). TLR2 était exprimé sur les cellules tumorales ou les PNN avec une diminution
61 d'expression après traitement par calpaïne. Le fragment soluble de TLR2 était corrélée à la
62 concentration extracellulaire de calpaïne 1 dans les surnageants de LBA (r = 0,624; p <0,001).
63 Le fragment soluble de TLR2 élevé était associé à la progression tumorale et un
64 environnement pro-inflammatoire

65 La calpain extracellulaire sécrétée par la cellule tumorale, favorise un microenvironnement
66 inflammatoire et la progression tumorale médiée par TLR2 dans ADL.

67 **Mots clé:** calpaïne– TLR 2– cancer bronchique – polynucléaire neutrophile - adénocarcinoma

68

69 INTRODUCTION

70 Calpains are ubiquitous cytosolic calcium-activated cysteine proteases (1). Two main
71 isoforms are ubiquitously expressed: calpain 1 which requires micromolar and Calpain 2
72 millimolar Ca^{2+} concentrations for activity. Their activity is tightly controlled by calpastatin, a
73 specific endogenous calpain inhibitor (1).

74 Calpains have several biological effects that could play an important role in cancer
75 biology. They promote i) cell mobility by modifying the distribution of cytoskeletal anchors
76 to the cell membrane (2)(3), ii) activation of inflammatory cells by the NF- κ B transcription
77 factor signaling pathway (4)(5), iii) tumor vascularization by VEGF response (6), iv) cell
78 proliferation, although this role remains controversial (7)(8).

79 However, the prognostic impact of calpains and their effect in cancer remain
80 controversial. Increased calpain expression is associated with poor prognosis in lung, stomach
81 and breast cancer, while it is associated with a good prognosis in ovarian and pancreatic
82 cancer (9)(10)(11)(12)(13). In a mouse model of melanoma, calpains did not present a
83 significant tumor effect, as calpains inhibited cell proliferation, but promoted cell migration
84 and metastasis (14).

85 Although calpains are considered as intracellular enzymes, few studies show that they
86 are partly externalized. Calpains are secreted by lymphocytes, macrophages, and endothelial
87 cells among other cells (15)(16). When externalized, they seem to promote inflammation
88 resolution and tissue repair. For instance, externalized calpains activate anti-inflammatory
89 cytokines (TGF- β) (17) and inhibit pro inflammatory proteins such as chemerins or IL-17
90 (18)(19). In addition, extracellular calpains participate in epithelium and endothelium
91 regeneration after ischemic or inflammatory damage (16)(20)(21). Recently, our team
92 demonstrated that extracellular calpains also cleave Toll like receptor 2 (TLR2) on human and

93 murine lymphocytes, thereby limiting IL-17 expression (19). Finally, no data on extracellular
94 calpain are available in oncology.

95 Lung adenocarcinomas are the most frequent histological type of non-small cell lung
96 cancer (NSCLC) (22). Pure lepidic pulmonary adenocarcinomas are characterized by a
97 proliferation of terminal unit cells with no evidence of stromal, pleural, or vascular invasion
98 (22)(23). As a result, diagnosis could only be established after comprehensive pathological
99 analysis of a surgical specimen. Invasive lung adenocarcinomas consist of a mixture of
100 different histological patterns referred to as lepidic, acinar, solid, papillary or micropapillary
101 (22). In lepidic predominant adenocarcinoma (LPA), tumor progression by aerogenous
102 spreading explains its propensity for multicentric and bilateral lung involvement with
103 respiratory signs at diagnosis, pulmonary relapse, and death as a result of respiratory failure.
104 (23)(24). These features also explain the so-called “pneumonic” presentation of the disease on
105 chest X-ray as well as the lack of solid lesions. LPA are characterized by an intense
106 inflammatory reaction involving complex interactions between tumor and inflammatory cells
107 (25)(26)(27)(28)(29).

108 As inflammation and proliferation are major features in lung carcinogenesis, the aim
109 of this study was to determine whether calpain 1 exteriorization is associated with tumor
110 progression of LPA. We analyzed bronchoalveolar lavage fluid (BALF) supernatants from
111 patients with LPA and identified extracellular calpain 1 and its target, TLR2, as negative
112 prognostic factors.

113

114 MATERIALS AND METHODS

115 Clinical samples and ethical considerations

116 The database from the Chest Department at Tenon Hospital (Assistance Publique-Hôpitaux de
117 Paris) was retrospectively searched for all patients with LPA with pneumonic presentation,
118 diagnosed between January 1992 and July 2010. To be included, patients had to have a
119 histologically proven lung adenocarcinoma with a pneumonic presentation on chest X-ray as
120 well as consolidation seen on a computed tomography [CT] scan. The chest X-ray and CT
121 scan performed at diagnosis were reviewed by two investigators (MD, MW) before including
122 the data.

123 Ninety-two patients with LPA were diagnosed and followed-up in the Tenon Hospital
124 Chest Department (Assistance Publique-Hôpitaux de Paris, Paris, France). Clinical findings
125 are summarized in supplementary data (Table S1). For all patients, diagnosis was assessed by
126 a lung cancer pathologist (MA), based on the 2011 IASLC/ATS/ERS classification of lung
127 adenocarcinoma (22). The disease was classified according to the seventh International TNM
128 Classification System for Lung Cancer (30). Follow-up data were recorded until death. A
129 surgical exeresis was performed in 54 patients. For surgical samples, predominant and minor
130 histological patterns were specified, i.e., lepidic predominant adenocarcinoma, or, papillary,
131 acinar, micropapillary. For small samples, any identifiable pattern present was described..

132 BALF was used as a diagnostic procedure and performed as previously described (26). After
133 diagnostic procedure, the remaining BALF was spun, and the supernatant aseptically
134 separated and stored at -80°C. A frozen BALF supernatant sample was available in 68
135 patients. BALF supernatants from controls were obtained from six subjects undergoing
136 diagnostic procedures. They were four men and three women aged 61 ± 7 years. Three were

137 smokers. None had a history of neoplastic disease and all had normal results of BALF
138 analysis.

139 All patients signed a research approval informed consent permitting analyses of their
140 biological samples. All informed consents were collected and stored in the Department of
141 Pathology, Tumorotheque des Hôpitaux Universitaires de l'Est Parisien (AP-HP). This study
142 was approved by the Ethics of Human Research Committee of our institution.

143 **Cell Lines and culture conditions**

144 The human A549, H322, H441, H1650 lung adenocarcinoma cell lines were purchased from
145 the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified
146 Eagle Medium (DMEM) (A549) or RPMI-1640 (H322, H441, H1650) with 10% fetal bovine
147 serum (FBS), penicillin (100 U/mL), and streptomycin (0.1 mg/mL) (Life Technologies) at
148 37°C in an atmosphere containing 5% CO₂. For experiments, cell lines were grown in a
149 serum-free medium and treated for 1 hour with or without 4 µg/ml calpain 1 (Merck
150 Chemicals) or 10 µg/ml calpastatin (Sigma Aldrich).

151 **Cell isolation**

152 Peripheral blood polymorphonuclear neutrophils (PMN) were isolated from peripheral blood
153 of healthy volunteers by means of density gradient centrifugation (PMN cell separation
154 medium, Eurobio). PMNs were separated from erythrocytes by hypotonic shock and washed
155 thrice in sterile saline.

156 **Chemokine and cytokine quantification in BALF samples**

157 Chemokine and cytokine concentrations in BALF supernatants were quantified using Bio-
158 Plex multiplex bead-based assays with Bio-Plex Pro™ Human Cytokine 27-Plex

159 Immunoassay and three individual assays for human VCAM-1, GRO α and HGF (Bio-Rad
160 Laboratories) as previously reported (31).

161 **Immunohistochemistry**

162 Formalin-fixed, paraffin-embedded 3 μ m tissue sections from surgical specimens were used
163 for calpain immunohistochemical (IHC) studies. After rehydration, deparaffinized sections
164 were pretreated by epitope retrieval solution, endogenous peroxidase activity was quenched
165 and a non-specific binding sites blocking was performed. Sections were incubated with
166 primary antibody anti-calpain 1 monoclonal antibody (clone P-6) (Santa Cruz, Clinisciences;
167 1:400) 90 min at room temperature. Sections were incubated with Dako Envision+ System-
168 HRP labelled polymer anti-mouse and revealed by diaminobenzidine. Appropriate isotype
169 mouse IgG1 (Dako) was used as negative control. Two investigators (NR and MA) blinded to
170 clinico-pathological variables evaluated immunostaining independently. The H-scores (0–
171 300) were ascribed as previously reported (32).

172 **ELISA assays**

173 Calpain 1 (Cloud-Clone Corp, Euromedex) and soluble TLR2 (R&D Systems, Bio-Techne)
174 expressions were determined in BALF by ELISA detection kits according to the
175 manufacturer's instructions.

176 **Immunofluorescence**

177 Cells at a concentration of $10^6/100$ μ l were incubated 1h at 4°C with APC-conjugated anti-
178 human TLR2 (Miltenyi Biotec) or control antibody expression. Data were collected on a
179 MACSQuant cytofluorometer (Miltenyi Biotec) and analyzed with FlowJo software
180 (TreeStar).

181 **Statistical analysis**

182 For quantitative variables, results were expressed as median (Q25-75). Clinical data were
183 compared according to the median concentration of calpain or TLR2s in BALF supernatant:
184 high group in patients with concentration above the median and low group in patients having
185 a concentration below the median. Comparisons were made using the Mann-Whitney non-
186 parametric tests. For qualitative variables, the χ^2 test was used for comparisons and
187 Spearman's coefficient (ρ) for correlation studies. The survival time was defined as delay
188 from diagnosis to death or to the cutoff date, defined as August 2011. Survival rates were
189 calculated with the Kaplan-Meier method, and survival curves were compared using the log-
190 rank test. Variables with p -value below 0.1 in univariate analysis were tested in the
191 multivariate Cox model using a backward stepwise variable selection. A p value below 0.05
192 was considered significant. Data were processed using SPSS 20.0 software (IBM
193 Corporation).

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196

197 **RESULTS**

198 **Extracellular 1 Calpain**

199 **Calpain 1 concentration is higher in BALF supernatant of LPA than controls**

200 Median extracellular calpain 1 concentration, measured by ELISA, was significantly higher in
201 BALF supernatants of LPA than those of controls ($p = 0.045$) (**Figure 1a**). In BALF
202 supernatants of LPA ($n = 68$), median calpain 1 concentration was 4029 pg / ml [Q25-75:
203 1947-8327] compared to controls ($n = 6$) 1299 pg / ml [Q25-75: 0-4044].

204 **Source of secreted calpain 1**

205 To investigate the source of calpain 1 secretion into the BALF, an immunohistochemistry
206 analysis of the expression of calpain 1 was performed on surgical specimens of LPA, three
207 with high and 3 with low calpain 1 concentrations. An heterogeneous cytoplasmic expression
208 with a membrane reinforcement of calpain 1 on tumor cells was noted with an H-score ranged
209 from 0 to 270 (**Figure 1b**). No nuclear staining was detected. No expression was detected on
210 inflammatory infiltrate, macrophages, lymphocytes or PMN. Endothelial and bronchial cells
211 also expressed calpain 1 in their cytoplasm. Calpain 1 would be secreted mainly by tumor
212 cells.

213 **High Calpain 1 concentration is a negative prognostic factor**

214 *High Calpain 1 concentration is associated with tumor progression*

215 A high calpain 1 concentration was significantly associated with metastatic stage (**Table 1**).
216 In the calpain 1 high group, 76.5% (26/34) patients had a metastatic stage compared to 23.5%
217 (8/34) in the low group ($p = 0.003$). There were no significant differences according to sex,
218 smoking status, age or Performans status.

219 *High Calpain 1 concentration is associated with neutrophilic inflammation*

220 In the calpain 1 high group, 32 ± 6.0 % of PMNs were detected versus 14 ± 3.4 % in 1
221 calpain low group ($p = 0.018$) (**Figure 1c**). PMN are a negative prognostic factor in LPA

222 ***High Calpain 1 concentration is associated with poor survival***

223 Survival was significantly shorter in the calpain 1 high group (1.3 years; CI: 0.5 to 3.2 years)
224 than the calpain 1 low group (3.2 years; CI: 0.8 12.7 years) ($p = 0.003$) (**Figure 1 d**).
225 Multivariate analysis included all variables with $p < 0.1$ in univariate analysis: metastatic stage
226 ($p < 0.001$), Performans Status ($p = 0.003$), PMN ($p = 0.003$), sex ($p = 0.07$) and calpain 1
227 high group ($p = 0.002$). In multivariate analysis, only the metastatic stage ($p < 0.001$) and male
228 gender ($p = 0.025$) were associated with a significant decrease in survival (Table S2).

229

230 **Extracellular calpain 1 mechanisms of action: toll like receptor 2 as target**
231 **of extracellular calpain 1**

232 As there was a positive association between PMN and high calpain 1 concentration, we
233 hypothesized that calpains cleave TLR2 on PMN and / or tumor cells. Thus, the expression of
234 membranous TLR2 was investigated in these two cell types.

235 **TLR2 is expressed on neutrophils and tumor cells**

236 Expression of membranous TLR2 was studied by flow cytometry analysis on isolated PMN
237 from healthy volunteers. Forty three percent to 74% of PMN had a membranous expression of
238 TLR2 ($n = 3$) (**Figure 2 a**).

239 Expression of membranous TLR2 was studied by flow cytometry analysis on epithelial cells
240 from lung cancer lines. Eleven percent to 24% of the A549, 16% to 37% of the H322, 18% to
241 43% of the H1650 and 48% to 87% of the H441 cell lines had a membranous expression of
242 TLR2 ($n = 3$) (**Figure 2b**).

243 **TLR2 is cleaved by extracellular calpain 1 on neutrophils and tumor cells**

244 As TLR2 is expressed on PMN and tumor cells, we questioned whether extracellular calpain 1
245 cleaves TLR2 on these cell types.

246 Analysis by flow cytometry of PMN showed a decrease of membranous TLR2 expression
247 after 1 hour exposure to calpain 1 (4 μ g/ml) as compared to BSA (control). This decrease
248 reached 30% (IC25-75: 23 to 37%) ($n = 3$) compared to the basal expression. Conversely,
249 TLR2 expression increased by 42% compared to basal expression after treatment by calpain
250 inhibitor, calpastatin (10 μ g / ml) (**Figure 2a**).

251 Analysis by flow cytometry of epithelial cell lines from lung cancer showed a decrease of
252 membranous TLR2 expression after 1 hour exposure to calpain 1 (4 μ g/ml) of 14% (IC25-75:
253 9 to 18%) ($n = 3$) for the A549, of 13% (IC25-75: 3 to 23%) ($n = 3$) for the H441, of 20%
254 (IC25-75: 23 to 37%) ($n = 3$) for the H1650 and of 43% (IC25-75: 35 to 50%) ($n = 3$) for the
255 H322 cell lines compared to the basal expression (**Figure 2b**).

256 **Soluble fragment of TLR2 is correlated to the extracellular calpain 1 concentration in** 257 **the BALF supernatants**

258 Because TLR2 expressed on PMN and tumor cells is cleaved by extracellular calpain 1, we
259 determined the concentration of the soluble fragment of TLR2 (TLR2s) by ELISA in BALF
260 supernatants of LPA patients. The concentration of TLR2s was strongly correlated to the
261 concentration of extracellular calpain 1 in BALF supernatant ($r = 0.624$, $p < 0.001$) (**Figure**
262 **2c**).

263

264 **High soluble fragment of TLR2 is associated with a negative prognosis in** 265 **patients with LPA**

266 We showed that high extracellular calpain 1 concentration was associated with a poor
267 prognosis. Because the membranous TLR2 is the target of extracellular calpain 1, we
268 wondered whether soluble fragment of TLR2 was also associated with prognosis.

269 **High soluble fragment of TLR2 in BALF supernatants is associated with tumor** 270 **progression**

271 A high concentration of TLR2s was significantly associated with metastatic stage. In the
272 TLR2s high group, there were more patients with crackles compared to the TLR2s low group
273 (67%, 20/34 vs 27%, 8/34; $p = 0.002$), more patients with bronchorrhea (41%, 14/34 vs 6%,
274 2/34; $p = 0.001$) and bilateral pulmonary involvement (70%, 24/34 vs 30%, 12/34; $p = 0.004$).
275 Fewer patients had a surgical treatment in the TLR2s high group compared to the TLR2s low
276 group (26% vs 65% 9/34, 22/34, $p = 0.002$). There were no significant differences by sex,
277 smoking status, age or Performans Status.

278 **High soluble fragment of TLR2 in BALF supernatants is associated with neutrophilic** 279 **inflammation and positive tumor cytology**

280 In the TLR2s high group, 32.5±% PMN were detected compared to 13.8% in the TLR2 low
281 group ($p = 0.006$) (**Figure 3a**). In the TLR2s high group, 62% (18/34) positive tumor
282 cytology were detected in BALF compared to 30% (10/34) in the TLR2 low group ($p =$
283 0.012).

284 **High soluble fragment of TLR2 tends to be a poor prognostic factor**

285 Survival tended to be shorter in the high TLR2s group (1.2 years; Q25-75: 0.5 to 3.2 years)
286 than the low TLR2s group (3.5 years; Q25-75: 0.8 to 12,7ans) ($p = 0.056$) (**Figure 3b**). In
287 multivariate analyses, all variables with $p < 0.1$ in univariate analysis were included. Only
288 metastatic stage ($p < 0.001$) and male gender (0.001) were associated with a significant
289 decrease in survival (Data not shown).

290 **High soluble fragment of TLR2 is associated with a pro-inflammatory environment**

291 Among analyzed cytokines and growth factors, concentrations of HGF, CXCL10, IL-6,
292 VCAM, CCL4, G-CSF, CCL2, GM-CSF, IFN- γ and VEGF were significantly correlated to
293 the TLR2s concentration (**Figure 3c**). All of these cytokines, except for GM-CSF, had a high
294 concentration in the case of high soluble TLR2 concentration. The cytokines CXCL 8, CCL5
295 and CXCL1 were not correlated to TLR2s (data not shown).

296

297

298

299 **DISCUSSION**

300 Our findings indicate a negative prognosis of extracellular calpain 1 in LPA. Calpain 1 is
301 secreted by cancer cells and cleaves membranous TLR2 on PMN or cancer cells. Soluble
302 fragment of TLR2 is associated with a pro inflammatory tumor environment.

303 No data are available on extracellular calpains in cancer. Unlike intracellular calpain,
304 extracellular calpains are associated with inflammation resolution and tissue repair
305 (19)(20)(21). However in patients with LPA, high extracellular calpain 1 is significantly
306 associated with metastasis, alveolar inflammation and an unfavorable prognosis.

307 Analysis of surgical specimens identified tumor cells as source of secreted calpain 1.
308 This externalization could be linked to cell death, apoptosis or necrosis. It could involve either
309 the formation of microparticles (33) or the passage through the cell membrane via channels,
310 such as ABCA1 transporter (19). ATP-binding cassette (ABC) transporters are a family of
311 transmembrane proteins that transport a wide range of substrates, including lipids and the
312 surfactant through the biological membranes (34). As ABCA1 is expressed in the lung and
313 has showed its role in the secretion of calpain in lymphocytes, ABCA1 might be the calpain
314 transporter in LPA. Further studies including immunohistochemistry analyses of ABCA1 will
315 be performed.

316 As extracellular calpain 1 concentration in BALF supernatant was a poor prognostic
317 factor, we investigated the mechanism of action of extracellular calpain 1. Recently, our team
318 identified TLR2 as a target for extracellular calpain in human lymphocytes (19). Once
319 externalized, calpains cleave the extracellular domain of TLR2 and release a soluble form of
320 this receptor. In LPA, prognosis is determined by both PMN and tumor cells (26)(27)(29). We
321 hypothesized that calpains cleave TLR2 on PMN and/or tumor cells. Several arguments
322 support this hypothesis. Flow cytometry analysis demonstrated that PMN and tumor cells
323 expressed the membranous TLR2. Treatment of PMN and tumor cells by calpain1 resulted in

324 a decrease of membranous TLR2 expression. Analysis of the soluble fraction of TLR2 by
325 ELISA was highly correlated to the concentration of extracellular calpain 1 in the supernatant
326 of BALF in LPA.

327 As for the extracellular calpain 1, a high concentration of TLR2s is significantly
328 associated to neutrophilic inflammation and metastatic tumor stage. Neutrophilic
329 inflammation is associated to tumor progression in LPA (23)(26)(29). Various mechanisms of
330 action of neutrophils have been reported: they release mutagenic free radicals,
331 proinflammatory growth factors such as HGF (hepatocyte growth factor) which is the ligand
332 of the Met receptor that promotes cell proliferation and migration (26). The role of TLR2
333 cleavage by calpains on tumor progression remains to be demonstrated using *in vitro*
334 functional tests.

335 Recent data suggest in a mouse model that TLR2 plays a key role in tumor progression
336 of lung cancer by macrophages activation via TLR2, secretion of TNF- α and tumor growth
337 promotion (35). In LPA, TLR2s is significantly correlated with pro inflammatory
338 environment including HGF, CCL 4, CXCL10, IL-6, G-CSF, GM-CSF, CCL2, IFN gamma,
339 VEGF and VCAM-1. All of these cytokines, except for GM-CSF, are positively correlated
340 with TLR2s.

341 Our findings suggest that Calpain 1 is secreted by cancer cells use the innate immune
342 system to generate an inflammatory microenvironment supporting tumor growth.

343

344

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458

459

460 **Table I: Clinical characteristics of patients with LPA according to median**
 461 **extracellular calpain 1 and soluble fragment of TLR2 concentrations in BALF**
 462 **supernatant**

	Calpain 1			sTLR2		
	< (n=34)	> (n=34)	p	< (n=34)	> (n=34)	p
Age (years. mean +/- SEM)	66.7±11	65.2 ±12	NS	66.7±14	65.1±10	NS
Gender						
Female	16 (47%)	14 (59%)	NS	22 (65%)	16 (47%)	NS
Male	18 (53%)	20 (41%)		12 (35%)	18 (53%)	
Smoking status						
Never smoked	10 (29%)	7 (21%)	NS	7 (21%)	10 (29%)	NS
Former or current	24 (71%)	27 (79%)		27 (79%)	24 (71%)	
Bronchorrhea	6 (19%)	9 (30%)	NS	2 (6%)	6 (41%)	0.001
Crackles	7 (23%)	22 (73%)	<0.001	8 (27%)	20 (67%)	0.002
Performans Status						
0	22 (69%)	17 (50%)	NS	20 (61%)	20 (61%)	NS
≥1	10 (30%)	17 (50%)		13(39%)	13 (39%)	
Stage						
I-III	20(59%)	8 (23.5%)	0.003	22(65%)	7 (21%)	<0.001
IV	14 (41%)	26 (76.5%)		12 (35%)	27 (79%)	
Surgery	20 (59%)	11 (32%)	0.025	22 (65%)	9 (26.5%)	0.002
LPA						
Mucinous variant	16 (48%)	20 (71%)	NS	15 (50%)	21 (68%)	NS
Positive cytology	12 (39%)	17 (55%)	NS	10 (30%)	18 (62%)	0.012
BAL (mean+/-SEM)						
Cell count/mm3	505 000 ±165 180	587 931 ±96 745	0.01	502 666±151 813	566 666 ±128 251	NS
Macrophages (%)	72±3.5	48±5.2	<0.001	73±4	48±5.0	<0.001
Neutrophils (%)	14±3.4	32±6.0	0.018	13.8±3.7	32.5±5.8	0.006
Lymphocytes (%)	12±1.7	17±3.4	NS	12±1.8	16.9±3.3	NS

463 LPA= lepidic predominant adenocarcinoma, BLAF= bronchoalveolar lavage fluid,

464

465

466

467 **Figure 1.** Extracellular calpain 1. **A.** The concentration was assessed by ELISA in 68 BALF
468 supernatants from LPA and 6 controls (Mann Whitney Test). Each sample was assessed in
469 duplicate. Line, median; Column, Q25-Q75; Bars, min, max. **B.** Source of calpains: High/low
470 cytoplasmic Calpain 1 staining (brown) in LPA and isotopic control. **C.** Relative value of
471 neutrophil (%) in BALF supernatant in patients with LPA according to <or> median
472 extracellular calpain 1 concentration (Mann Whitney test). Line, median; Column, Q25-Q75;
473 Bars, min, max. **D.** Survival curve (Kaplain Meier) of patients with < median (---) vs >
474 median (---) calpain 1 concentration in BALF supernatant (log rank test)

475

476 **Figure 2.** Membranous TLR2 target of extracellular calpain 1. **A.** TLR2 expression on PMN
477 by flow cytometric analysis using APC conjugated anti-TLR2 antibody with a basic
478 condition, treatment for 1h by calpain (4 ug/ml) or calpain inhibitor calpastatin (10 ug/ml) **B.**
479 TLR2 expression by flow cytometry analysis using APC conjugated TLR2 antibody on
480 epithelial tumor cell lines with a basic condition and treatment for 1h by calpain (4 ug/ml).
481 This figure shows the results of three different experiments. **C.** Correlation between the
482 concentration of extracellular calpain 1 (pg/ml) and soluble fragment of TLR2s (pg/ml) in
483 BALF supernatants of LPA (Rho Spearman test)

484

485 **Figure 3.** Soluble fragment of TLR2 associated to tumor progression. **A.** Relative value of
486 neutrophil (%) in BALF supernatant in patients with LPA according to <or> median TLR2s
487 concentration (Mann Whitney test). Line, median; Column, Q25-Q75; Bars, min, max. **B.**
488 Survival curve (Kaplain Meier) of patients with < median (---) vs > median (---) TLR2s
489 concentration in BALF supernatant (log rank test) **C.** Correlation between TLR2s and HGF,

490 CXCL10, IL-6, VCAM, CCL4, G-CSF, CCL2, GM-CSF, G-CSF, VEGF, IFN- γ (Rho
491 Spearman test).

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496 tumeurs spontanées chez l'animal pour la recherche translationnelle en cancérologie »

497 **Conflict of interest:** None

498

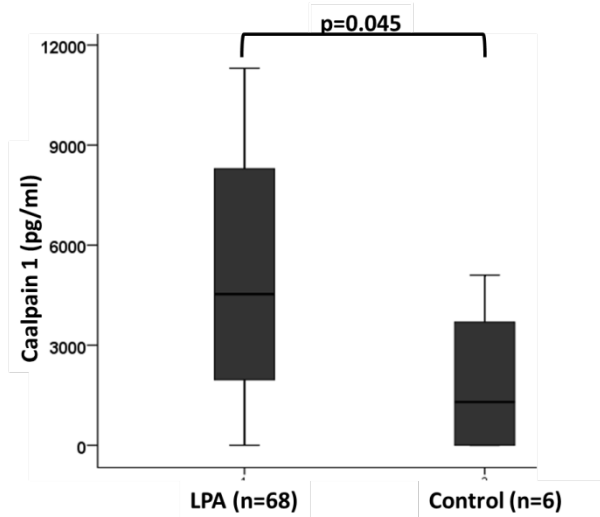
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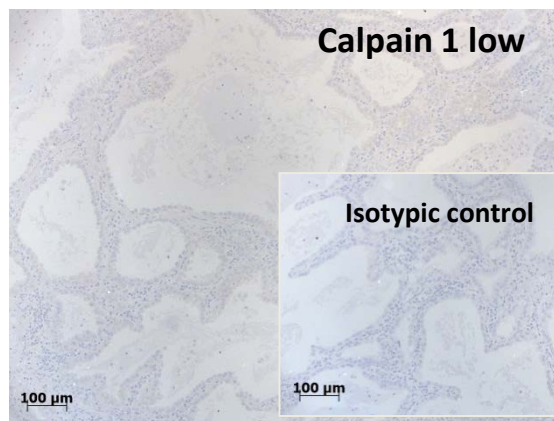
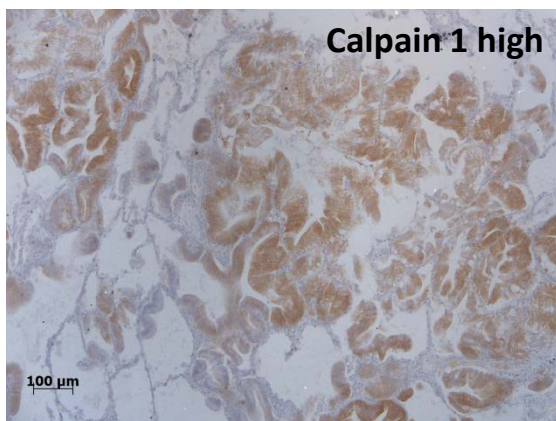
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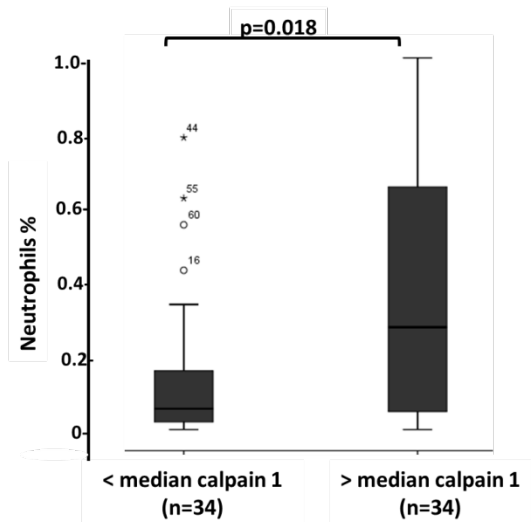
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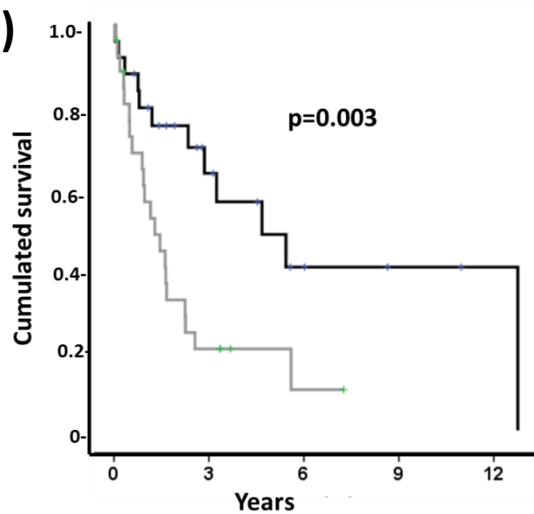
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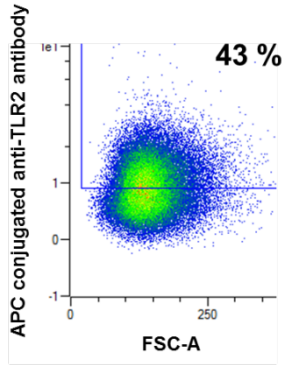
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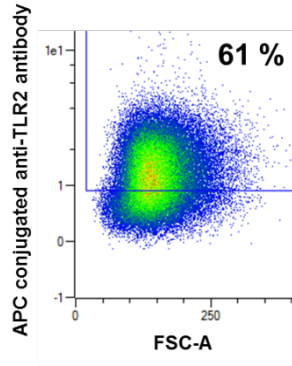
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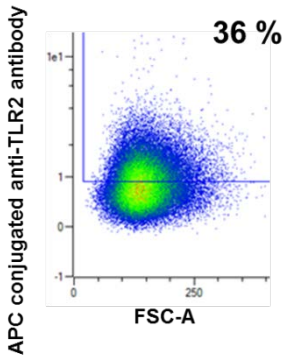
2. a) Basal condition



Calpastatin (10 µg/ml)

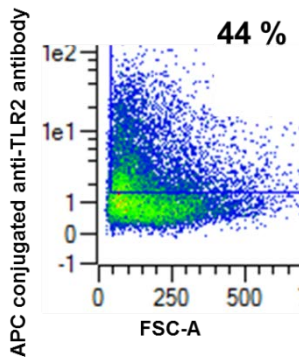


Calpain (4 µg/ml)

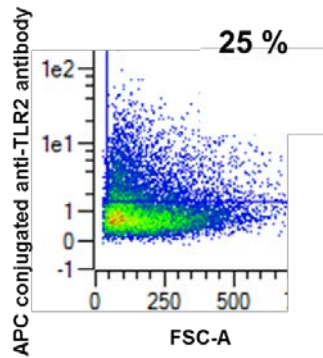


b)

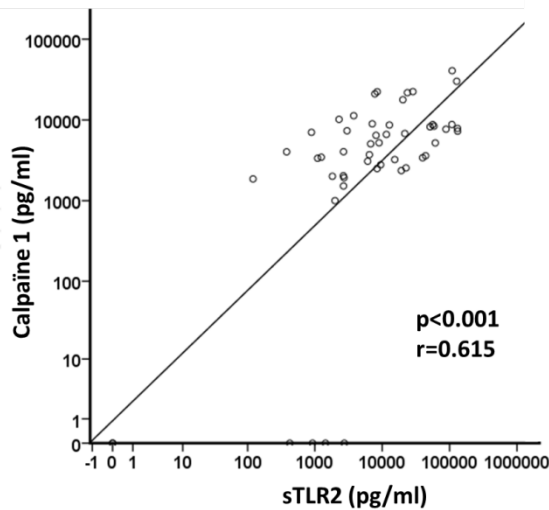
Basal condition



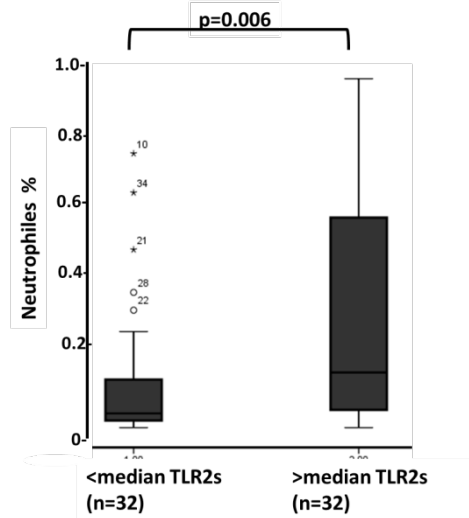
Calpain (4 µg/ml)



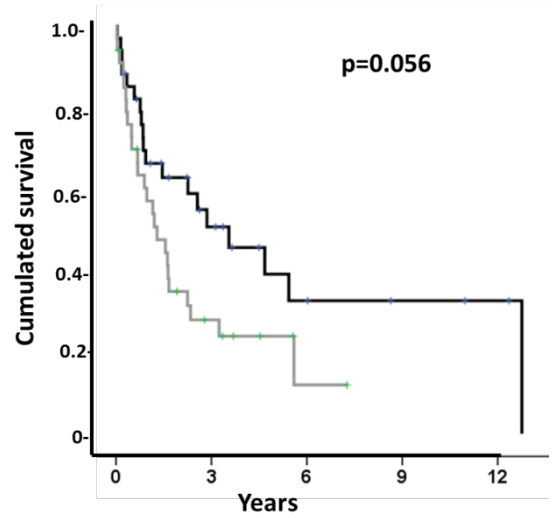
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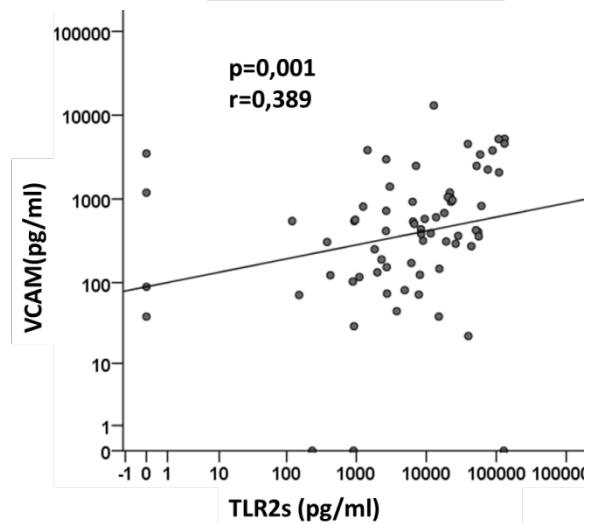
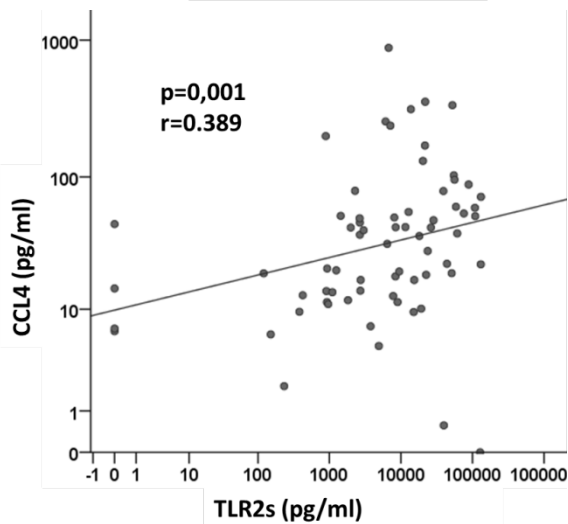
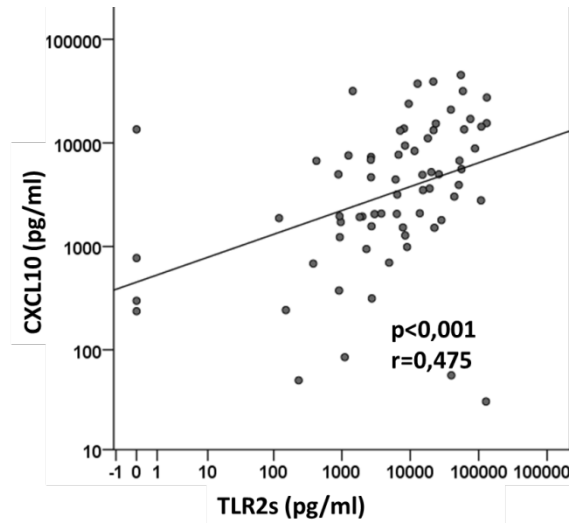
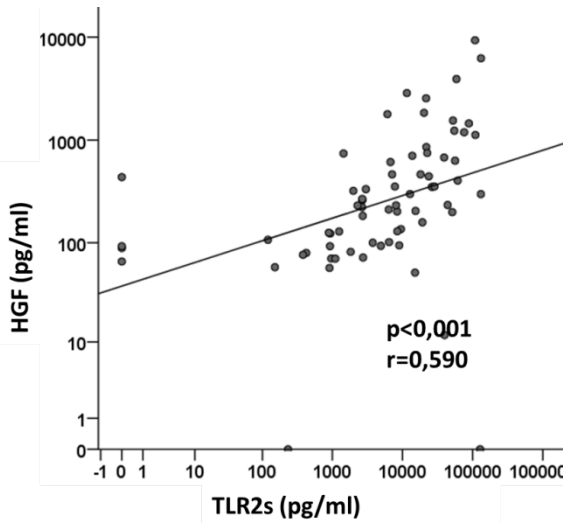
3.a)



b)



c)



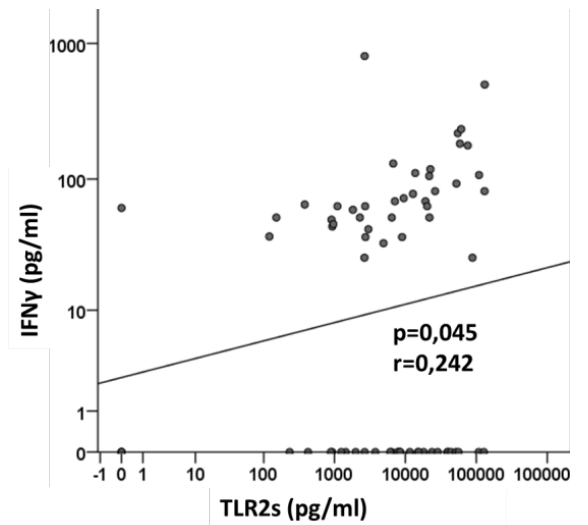
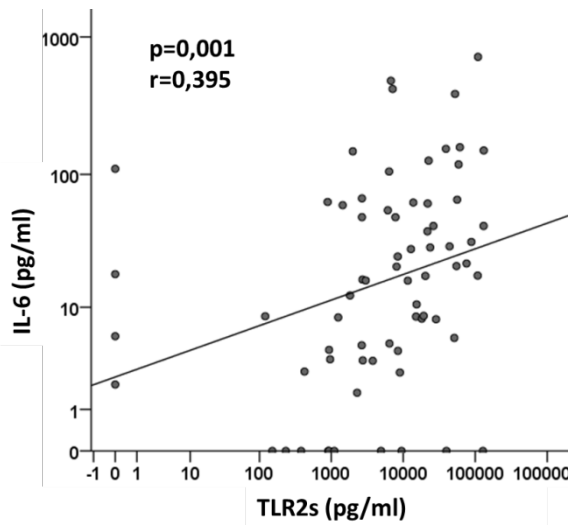
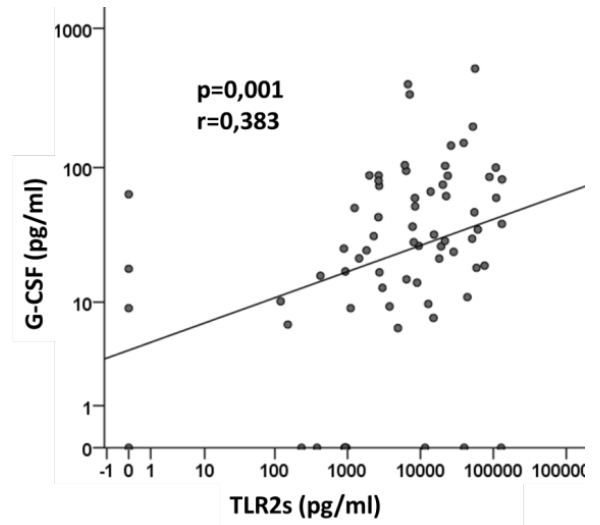
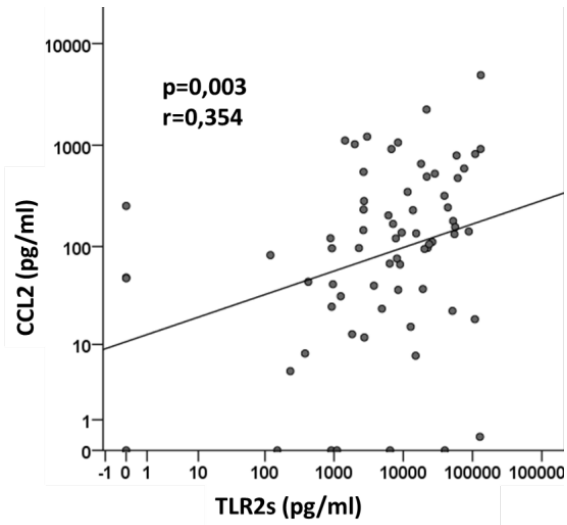
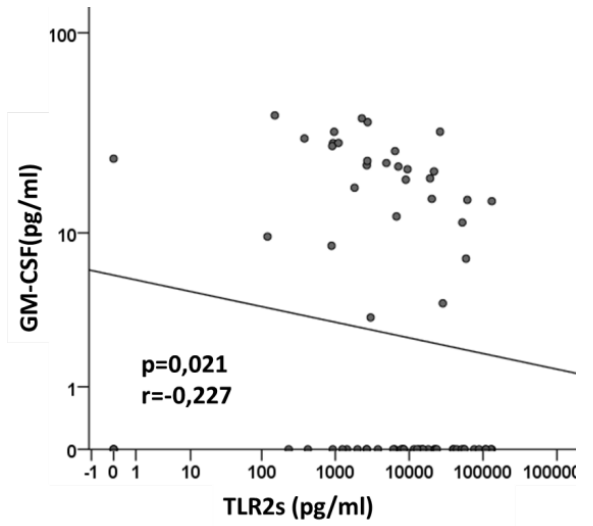
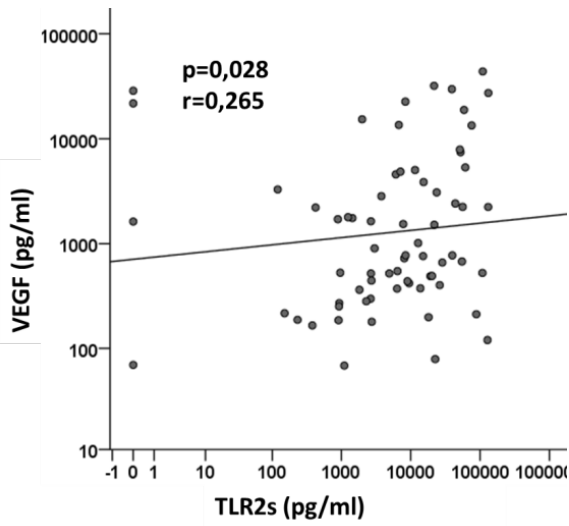


Table 1s : Clinical characteristics of the cohort

	Patients (n=68)
Age (years, av±SEM)	65±1
Gender	
Female	30 (44%)
Male	38 (56%)
Smoking status	
Never smoked	17 (25%)
Former or current	51 (75%)
Bronchorrhea	15 (25%)
Rale	29 (48%)
Performans Status	
0	39 (59%)
>0	27 (41%)
Bilateral lesions	31 (46%)
Stage	
I-III	28 (41%)
IV	40 (59%)
Surgery	31 (46%)
LPA	
Mucinous subtype	36 (59%)
Positive cytology	29 (47%)
BAL	
Cell count/mm ³	593 709 ± 107 809
Macrophages (%)	60±3.5
Neutrophils (%)	24±3.5
Lymphocytes (%)	14±1.8
Mutations (n=37)	
EGFR	3 (8%)
RAS	4 (11%)
ALK	0

Table III. Uni and multivariate analyses of factors associated to survival

Variable	Nbr patients	Hazard ratio (95%CI) Univariate	p-value	hazard ratio (95%CI) Multivariate	p-value
<i>Calpain 1</i>					
>median	34	0.398 (0.212-0.749)	0.003	0.712 (0.345-1.472)	0.360
<median	34	1			
<i>Gender</i>					
Men	38	0.555 (0.297-1.036)	0.061	2.197 (1.106-4.367)	0.025
Women	30				
<i>Age</i>	68	1.019 (0.989-1.048)	0.2		
<i>Tobacco status</i>					
Smoker	51	0.860 (0.440-1.681)	0.6		
Non Smoker	17				
<i>Performance Status</i>					
>0	27	0.393 (0.209-0.738)	0.004	0.860 (0.384-1.951)	0.782
0	41				
<i>Stage</i>					
IV	40	0.286 (0.145-0.561)	<0.001	0.207 (0.097-0.442)	<0.001
I-III	28				
<i>Neutrophils median</i>	63	5.328 (1.758-156.144)	0.003	2.133 (0.653-6.974)	0.210

Variables with p <0.1 in univariate analysis were included in the multivariate analysis by descending likelihood method. CI = 95% confidence interval

