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ABSTRACT

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

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

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

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

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

ABSTRACT FRANCAIS

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

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

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

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

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

INTRODUCTION

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

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

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

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

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

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

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

MATERIALS AND METHODS

Clinical samples and ethical considerations

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

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

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

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

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

Cell Lines and culture conditions

The human A549, H322, H441, H1650 lung adenocarcinoma cell lines were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (A549) or RPMI-1640 (H322, H441, H1650) with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (0.1 mg/mL) (Life Technologies) at 37°C in an atmosphere containing 5% CO_2. For experiments, cell lines were grown in a serum-free medium and treated for 1 hour with or without 4 µg/ml calpain 1 (Merck Chemicals) or 10 µg/ml calpastatin (Sigma Aldrich).

Cell isolation

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

Chemokine and cytokine quantification in BALF samples

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

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

Immunohistochemistry

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

ELISA assays

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

Immunofluorescence

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

Statistical analysis

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

RESULTS

Extracellular 1 Calpain

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

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

Source of secreted calpain 1

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

High Calpain 1 concentration is a negative prognostic factor

High Calpain 1 concentration is associated with tumor progression

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

High Calpain 1 concentration is associated with neutrophilic inflammation

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

High Calpain 1 concentration is associated with poor survival

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

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

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

TLR2 is expressed on neutrophils and tumor cells

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

Expression of membranous TLR2 was studied by flow cytometry analysis on epithelial cells from lung cancer lines. Eleven percent to 24% of the A549, 16% to 37% of the H322, 18% to 43% of the H1650 and 48% to 87% of the H441cell lines had a membranous expression of TLR2 (*n* = 3) **(Figure 2b).**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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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

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

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

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

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

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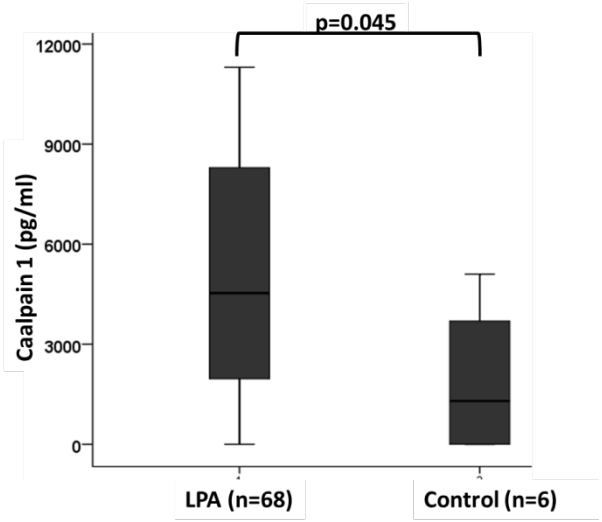
Conflict of interest: None

1. a)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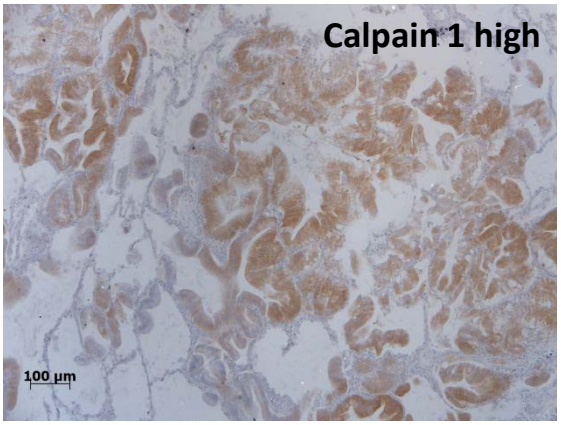
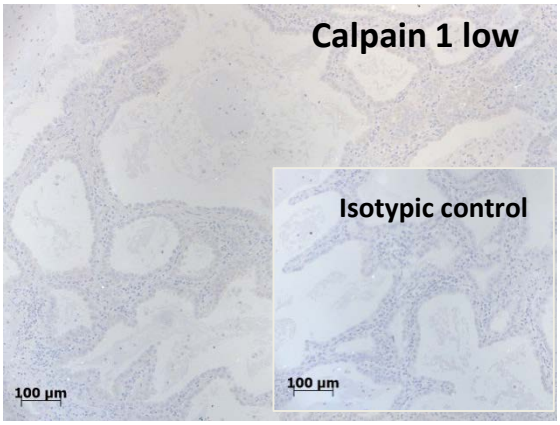
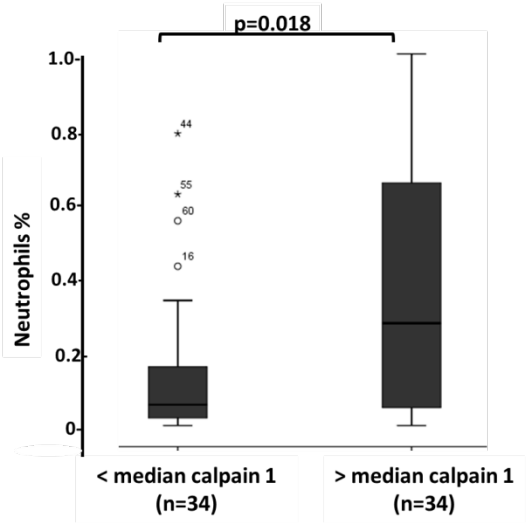
12000
9000
6000
3000
0
Caalpain 1 (pg/ml)
LPA (n=68)
Control (n=6)

b)

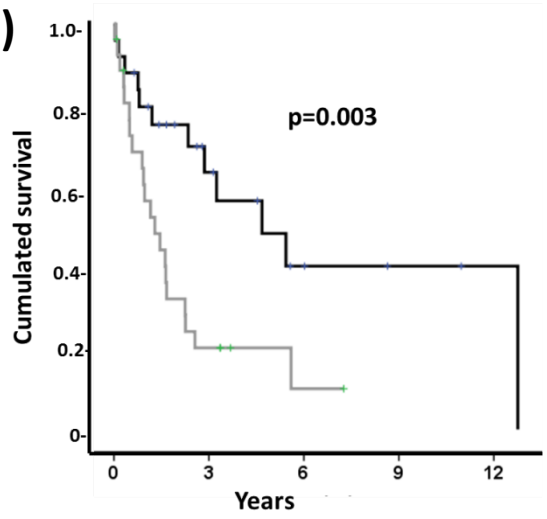

Calpain 1 high
100 µm


Calpain 1 low
Isotypic control
100 µm
100 µm

c)

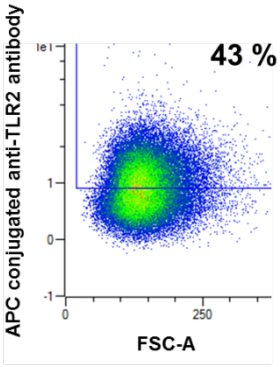

p=0.018
1.0
0.8
0.6
0.4
0.2
0
Neutrophils %
44
55
60
16
< median calpain 1 (n=34)
> median calpain 1 (n=34)

d)

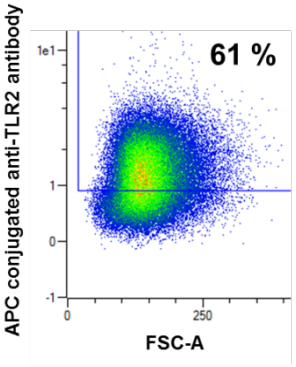

1.0
0.8
0.6
0.4
0.2
0
Cumulated survival
p=0.003
0
3
6
9
12
Years

2. a)

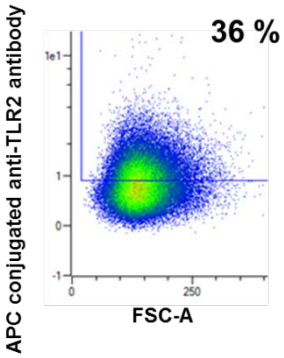
Basal condition



Calpastatin (10 µg/ml)

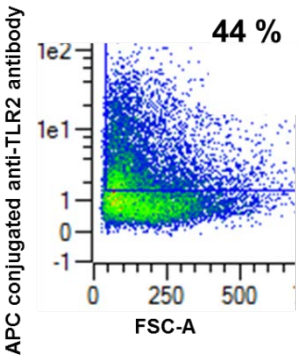


Calpain (4 µg/ml)

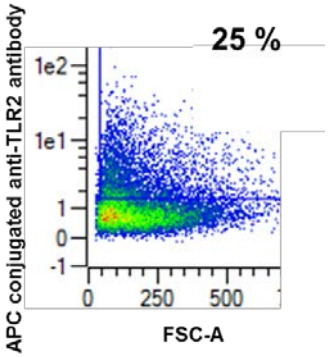


b)

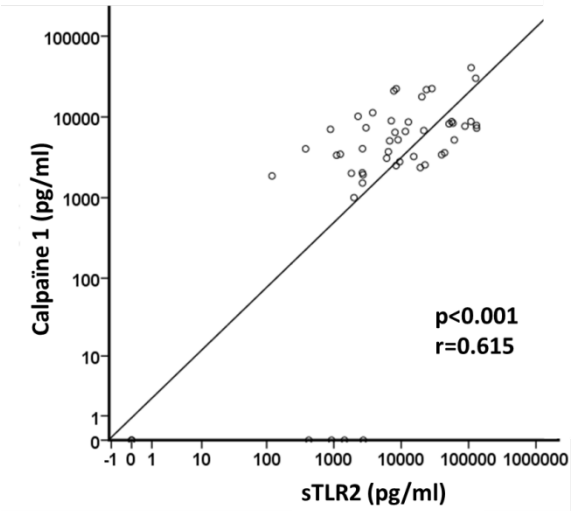
Basal condition



Calpain (4 µg/ml)



c)



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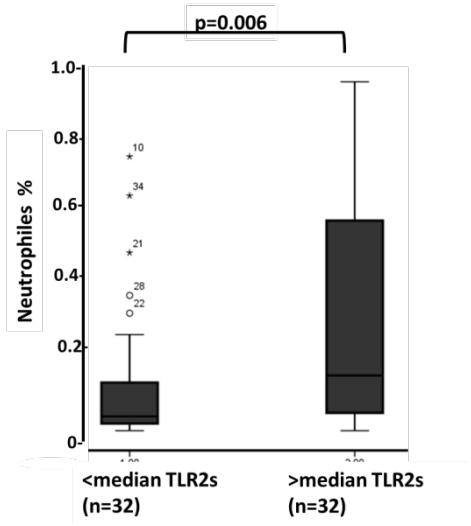
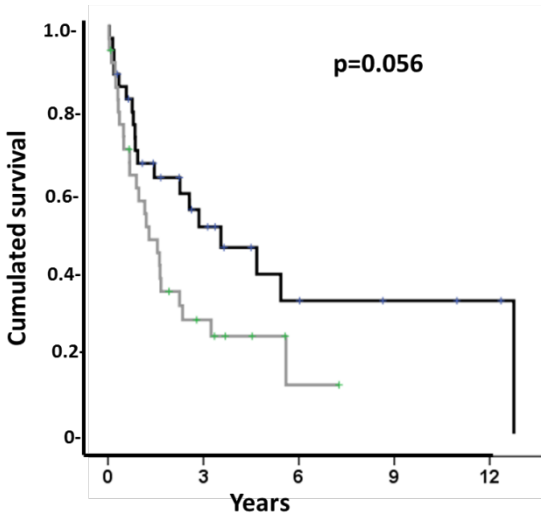
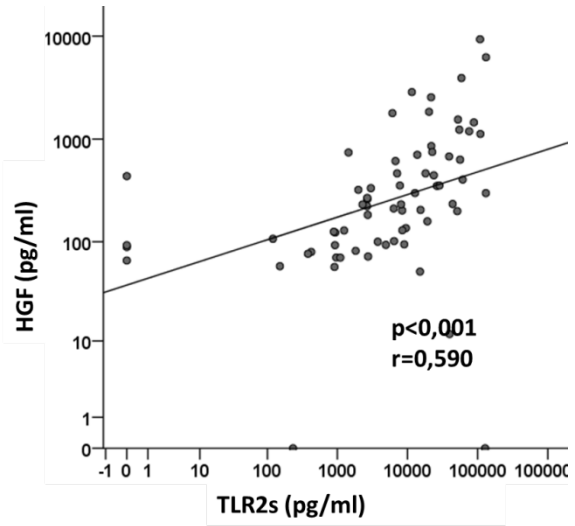
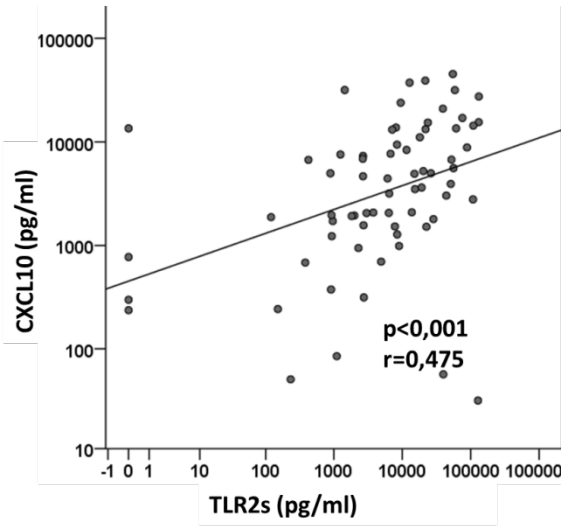

p=0.006
Neutrophiles %
1.0
0.8
0.6
0.4
0.2
0
<median TLR2s
(n=32)
>median TLR2s
(n=32)

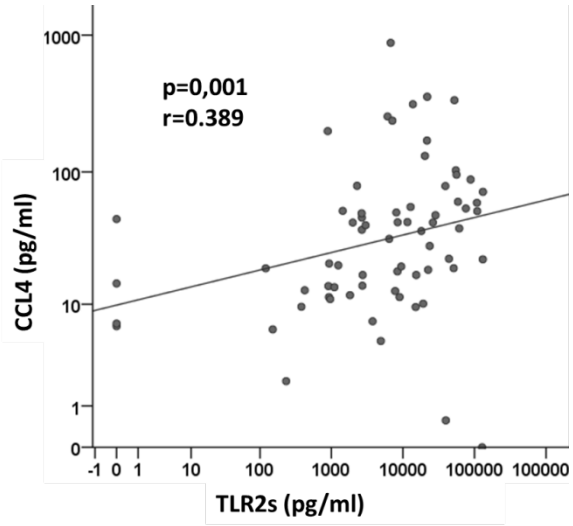
b)

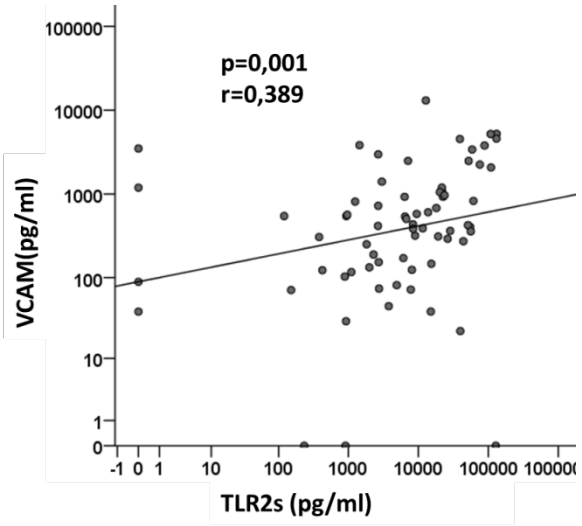

p=0.056
Cumulated survival
1.0
0.8
0.6
0.4
0.2
0
0
3
6
9
12
Years

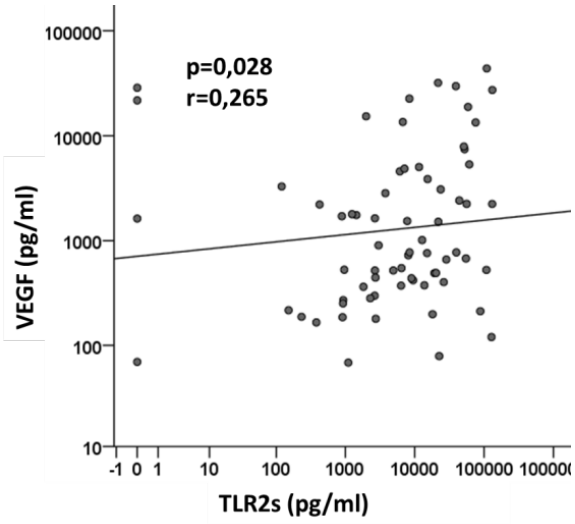
c)


HGF (pg/ml)
p<0,001
r=0,590
TLR2s (pg/ml)


CXCL10 (pg/ml)
p<0,001
r=0,475
TLR2s (pg/ml)


CCL4 (pg/ml)
p=0,001
r=0.389
TLR2s (pg/ml)


VCAM(pg/ml)
p=0,001
r=0,389
TLR2s (pg/ml)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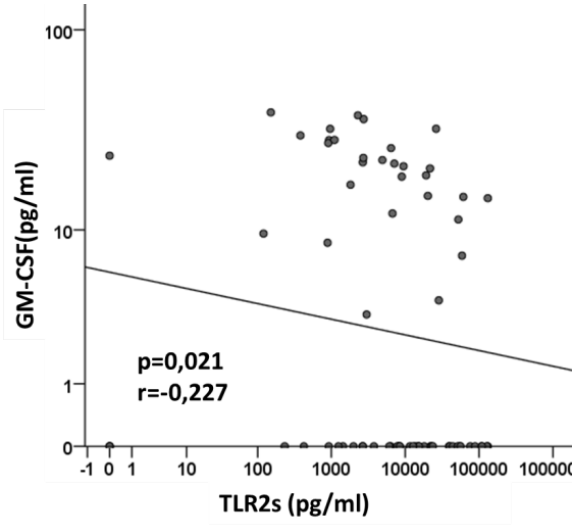
r=0,265
VEGF (pg/ml)
TLR2s (pg/ml)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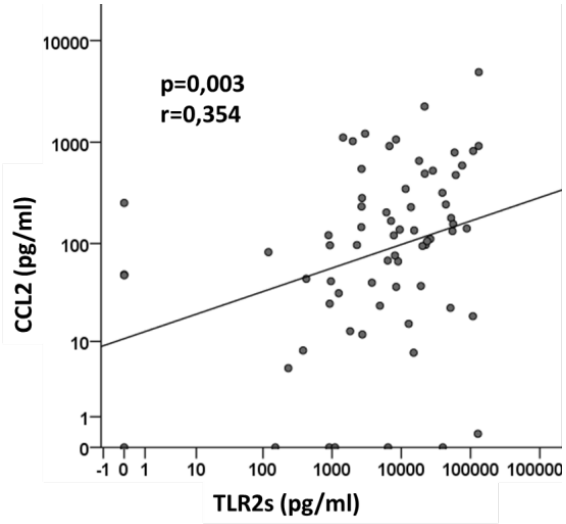
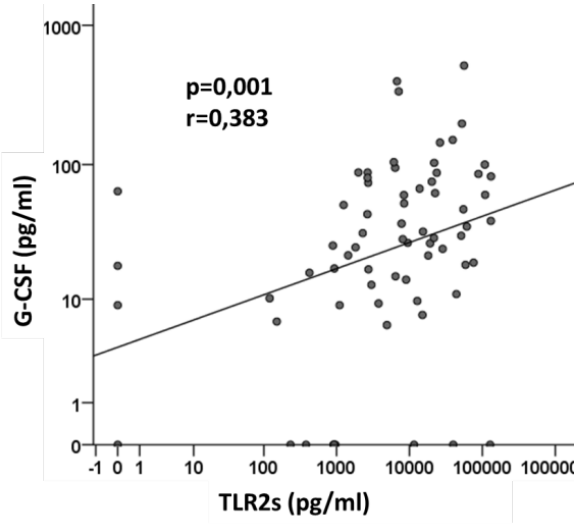
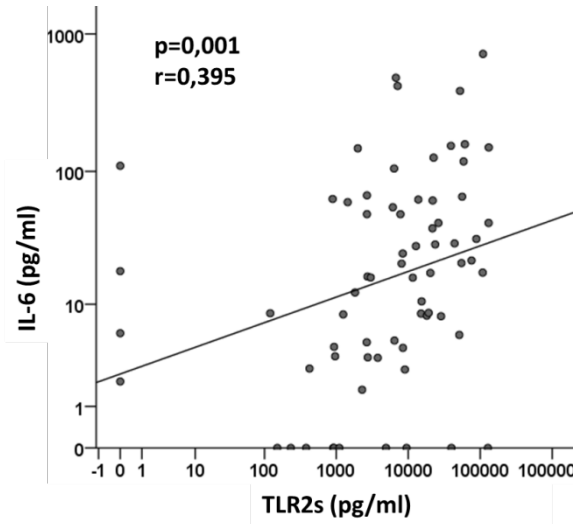
r=-0,227
GM-CSF(pg/ml)
TLR2s (pg/ml)


p=0,003
r=0,354
CCL2 (pg/ml)
TLR2s (pg/ml)


p=0,001
r=0,383
G-CSF (pg/ml)
TLR2s (pg/ml)


p=0,001
r=0,395
IL-6 (pg/ml)
TLR2s (pg/ml)

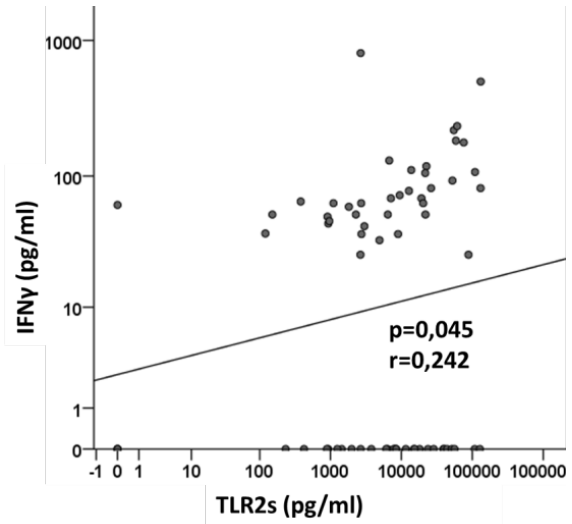

p=0,045
r=0,242
IFNγ (pg/ml)
TLR2s (pg/ml)

Table Is : 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/mm3	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 IIIs. 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
Calpain 1					
>median	34	0.398 (0.212-0.749)	**0.003**	0.712 (0.345-1.472)	0.360
<median	34	1			
Gender					
Men	38	0.555 (0.297-1.036)	**0.061**	2.197 (1.106-4.367)	**0.025**
Women	30				
Age	68	1.019 (0.989-1.048)	0.2		
Tobacco status					
Smoker	51	0.860 (0.440-1.681)	0.6		
Non Smoker	17				
Performance Status					
>0	27	0.393 (0.209-0.738)	**0.004**	0.860 (0.384-1.951)	0.782
0	41				
Stage					
IV	40	0.286 (0.145-0.561)	**<0.001**	0.207 (0.097-0.442)	**<0.001**
I-III	28				
Neutrophils median	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