Archivos de Bronconeumologia

MARKERS OF STROMA IN LUNG CANCER: INFLUENCE OF COPD
MARCADORES DEL ESTROMA EN EL CÁNCER DE PULMÓN: INFLUENCIA DE LA EPOC

--Borrador del manuscrito--

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Palabras clave:	Lung cancer; copd; stroma; cancer-associated fibroblasts; extracellular matrix; endothelial cell marker CD31
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Resumen:	Background: Stroma, mainly composed by fibroblasts, extracellular matrix (ECM) and vessels, may play a role in tumorigenesis and cancer progression. Chronic Obstructive Pulmonary Disease (COPD) is an independent risk factor for LC. We hypothesized that markers of fibroblasts, ECM and endothelial cells may differ in tumors of LC patients with/without COPD. Methods: Markers of cultured cancer-associated fibroblasts and normal fibroblasts [CAFs and NFs, respectively, vimentin and alpha-smooth muscle actin (SMA) markers, immunofluorescence in cultured lung fibroblasts], ECM, and endothelial cells (type I collagen and CD31 markers, respectively, immunohistochemistry) were identified in lung tumor and non-tumor specimens (thoracotomy for lung tumor resection) from 15 LC-COPD patients and 15 LC-only patients. Results: Numbers of CAFs significantly increased, while those of NFs significantly decreased in tumor samples compared to non-tumor specimens of both LC and LC-COPD patients. Endothelial cells (CD31) significantly decreased in tumor samples compared to non-tumor specimens only in LC patients. No significant differences were seen in levels of type I collagen in any samples or study groups. Conclusions: Vascular endothelial marker CD31 expression was reduced in tumors of non-COPD patients, while type I collagen levels did not differ between groups. A rise in CAFs levels was detected in lung tumors of patients irrespective of airway obstruction. Low levels of CD31 may have implications in the overall survival of LC patients, especially in those without underlying airway obstruction. Identification of CD31 role as a prognostic and therapeutic biomarker in lung tumors of patients with underlying respiratory diseases warrants attention.
Respuesta a los revisores:	LETTER TO THE EDITOR CC: igo01m@gmail.com Apreciados autores: C1

Todos los revisores consideran que la versión revisada de su manuscrito"MARKERS OF STROMA IN LUNG CANCER: INFLUENCE OF COPD MARCADORES DEL ESTROMA EN EL CÁNCER DE PULMÓN: INFLUENCIA DE LA EPOC"(Ref. ARBR-D-20-00463R1 ha sido muy mejorada, sin embargo aún existen algunos problemas que deberán subsanarse en una nueva revisión previamente a la obtención de la aceptación definitiva.

A su vez deberán preparar un documento aparte donde se dé respuesta a todos y a cada uno de los comentarios de los revisores, incluyendo los propios comentarios planteados por todos los revisores. Los cambios introducidos en la versión revisada deberán estar debidamente marcados para ser visualizados fácilmente por los revisores.

Esperamos recibir su versión revisada en los próximos 15 días. En el caso de que no sea así y no se nos notifique por adelantado entenderemos que desestima el envío de la versión revisada de su manuscrito.

R1
Estimado Editor:
Agradecemos al Editor que nos haya concedido la oportunidad de revisar y modificar nuestro manuscrito siguiendo las indicaciones precisas de los revisores. En la versión revisada se ha modificado el texto ampliamente, así como se ha añadido información solicitada por el revisor.
Se ha modificado el manuscrito según los comentarios del revisor # 3. Concretamente se ha modificado en algunos párrafos, no en todos, la palabra COPD y se ha utilizado la expresión “airway obstruction” y “obstrucción al flujo aéreo” en el resumen en español. No hemos modificado la expresión en todo el texto, puesto que los pacientes de nuestro estudio sí fueron diagnosticados por los autores reclutadores (neumólogos) de EPOC. Para ello se siguieron los criterios diagnósticos descritos en las normativas vigentes (citas # 27 & 28, en la Metodología del estudio). Por dichas razones solamente se ha procedido a modificar el texto en aquellos párrafos y/o secciones donde el uso de COPD o “airway obstruction” era indistinto. Se ha mantenido la expresión COPD en la definición de los grupos indicados en las Tablas y en las Figuras. Finalmente, cabe señalar que el recuento de palabras se ha visto incrementado de 3.235 a 3.251 (versión revisada). En la versión revisada, el texto modificado aparece en letra roja y marcado en color verde, para distinguirlo fácilmente del resto de texto, que ya había sido modificado en la versión revisada previa.
El manuscrito revisado se envía de nuevo a la revista para su consideración por el Editor dentro del marco temporal establecido en su carta.

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MARKERS OF STROMA IN LUNG CANCER: INFLUENCE OF COPD

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Word count: 3,251

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Competing interests declared by all the authors: None.

Authors' contributions: Study conception and design: EB, VC; Patient assessment and recruitment: JT, VC, DRC, MMJ, ARF, RA, LP; Molecular biology analyses: JT, DRC, KA; Statistical analyses and data interpretation: XD, JT, DRC, EB; Manuscript drafting and intellectual input: EB, JT; Manuscript writing final version: EB.

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ABSTRACT

Background: Stroma, mainly composed by fibroblasts, extracellular matrix (ECM) and vessels, may play a role in tumorigenesis and cancer progression. Chronic Obstructive Pulmonary Disease (COPD) is an independent risk factor for LC. We hypothesized that markers of fibroblasts, ECM and endothelial cells may differ in tumors of LC patients with/without COPD. **Methods:** Markers of cultured cancer-associated fibroblasts and normal fibroblasts [CAFs and NFs, respectively, vimentin and alpha-smooth muscle actin (SMA) markers, immunofluorescence in cultured lung fibroblasts], ECM, and endothelial cells (type I collagen and CD31 markers, respectively, immunohistochemistry) were identified in lung tumor and non-tumor specimens (thoracotomy for lung tumor resection) from 15 LC-COPD patients and 15 LC-only patients. **Results:** Numbers of CAFs significantly increased, while those of NFs significantly decreased in tumor samples compared to non-tumor specimens of both LC and LC-COPD patients. Endothelial cells (CD31) significantly decreased in tumor samples compared to non-tumor specimens only in LC patients. No significant differences were seen in levels of type I collagen in any samples or study groups. **Conclusions:** Vascular endothelial marker CD31 expression was reduced in tumors of non-COPD patients, while type I collagen levels did not differ between groups. A rise in CAFs levels was detected in lung tumors of patients irrespective of airway obstruction. Low levels of CD31 may have implications in the overall survival of LC patients, especially in those without underlying airway obstruction. Identification of CD31 role as a prognostic and therapeutic biomarker in lung tumors of patients with underlying respiratory diseases warrants attention. **Word count:** 248

KEY WORDS: lung cancer; COPD; stroma; cancer-associated fibroblasts; extracellular matrix; endothelial cell marker CD31

INTRODUCTION

Despite recent advances, non-small cell lung cancer (NSCLC) still leads to a great mortality in most of the continents[1,2], reaching up to one third of deaths in certain countries[1,3]. Clinical factors such as chronic obstructive pulmonary disease (COPD) or airway obstruction underlie the pathophysiology of LC in many patients[1,4–6]. Several relevant investigations have clearly demonstrated that airway obstruction and emphysema render the patients more susceptible to the development of LC[7–9]. Despite this consolidated knowledge, full elucidation of the underlying biological features is still underway.

In the airways, lungs, and blood compartment of patients with LC and underlying COPD, mechanisms such as redox imbalance, inflammatory events, epigenetics, and immune alterations were shown to be disrupted compared to LC patients with no COPD[10]. As a result of the interaction of those biological events with key cellular processes, namely angiogenesis, cell death and repair, and the cell survival machinery, COPD patients are more prone to lung tumorigenesis[11].

Stroma is defined as the part of a tissue or organ that confers mainly structure with no specific function, and is mainly composed by blood vessels, nerves, and connective tissue. In LC, several components such as extracellular matrix (ECM), endothelial cells, and cancer-associated fibroblasts (CAFs) play a significant role in tumorigenesis and cancer progression[12]. CAFs are a major component of the stroma in tumors. Growth factors, hormones, and cytokines mediate the tumor cell proliferation favored by CAFs. The most specific and widely used marker of CAFs is alpha-smooth muscle actin (SMA), which is indeed a specific marker of myofibroblasts[12]. The differentiation process of epithelial cells into mesenchymal cells is known as

epithelial-mesenchymal transition (EMT), characterized by the appearance of mesenchymal properties[13,14]. Interestingly, CAFs may also regulate EMT [13].

Extracellular macromolecules such as collagen, enzymes, and glycoproteins conform a specific network of the ECM, which is also involved in tumor development and progression[15]. In cancer stroma, collagen was demonstrated to be the most abundant protein[16]. Importantly, type I collagen promotes growth of cancer cells, invasion, and distant metastasis, thus favoring tumor progression[17], as well as resistance to therapy[18]. Whether a distinct expression of extracellular matrix markers or CAFs may take place in the stroma of lung tumor samples of patients with COPD remains to be answered.

The formation of new vessels in tumors can be identified using specific markers such as platelet endothelial cell adhesion molecule also known as cluster of differentiation (CD) 31. CD31 is involved in several physiological processes, namely maintenance of vascular endothelial and inflammatory cell functions and is also expressed in tumor cells[19]. In fact, the immunohistochemical measurement of CD31 expression can be reliably used as a marker of neoangiogenesis in tumors[20]. In mice with experimental airway inflammation mimicking COPD, an immunosuppressive microenvironment of the lung tumors was characterized by increased angiogenesis[21]. Whether differences in CD31 expression may exist in tumors of patients with COPD compared to non-COPD remain to be identified.

On this basis, we hypothesized that in LC patients with airway obstruction, cancer stroma as analyzed using specific markers may differ from tumors of patients with no underlying COPD. Accordingly, our objectives were to determine in lung tumors and non-tumor specimens (control samples) of LC patients with and without COPD the following parameters: 1) CAFs and non-tumor fibroblasts (cultured fibroblasts), 2) type

I collagen as a marker of extracellular matrix, and 3) CD31 expression levels as a marker of endothelial cells and blood vessels.

METHODS

Study design and ethics

This is a cross-sectional, prospective study designed following the World Medical Association guidelines (Seventh revision of the Declaration of Helsinki, Fortaleza, Brazil, 2013)[22] for research on human beings and was approved by the institutional Ethics Committee on Human Investigation (protocol # 2008/3390/I, Hospital del Mar–IMIM, Barcelona, Spain). All patients invited to participate in the study signed the informed written consent. The current investigation followed the international STROBE guidelines[23].

Patients were prospectively recruited from the Lung Cancer Clinic of the Respiratory Medicine Department at *Hospital del Mar* (Barcelona, Spain). All the patients were part of the *Lung Cancer Mar Cohort*. For this observational study, 30 patients with LC were recruited in 2019. Candidates for tumor resection underwent pulmonary surgery prior to administration of any sort of adjuvant therapy. LC diagnosis and staging were established by histological confirmation and classified according to currently available guidelines for the diagnosis and management of LC[24,25]. TNM (tumor, node, and metastasis) staging was defined as stated in the 8th edition Lung Cancer Stage Classification[26]. COPD diagnosis was established as a post-bronchodilator forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) ≤ 0.7 which is not fully reversible by spirometry according to currently available guidelines for diagnosis and management of COPD[27,28]. Exclusion criteria were: small cell lung cancer (SCLC), chronic cardiovascular disease, restrictive lung disease, metabolic, immune

disease, or clot system disorders, signs of severe inflammation and/or bronchial infection (bronchoscopy), current or recent invasive mechanical ventilation, or long-term oxygen therapy.

Specimens from the tumor and non-tumor lungs were collected from all the study subjects. Patients were further subdivided *post-hoc* into two groups on the basis of underlying COPD: 1) 15 patients with LC and COPD (LC-COPD group) and 2) 15 patients with LC without COPD (LC group).

Clinical assessment

In all patients, lung function parameters were assessed following standard procedures. Diagnosis and severity of patients with COPD were determined according to currently available guidelines[6]. Nutritional evaluation included the assessment of body mass index (BMI) and nutritional blood parameters from all patients.

Sample collection and preservation

Lung samples were obtained from tumors and the surrounding non-tumor parenchyma following standard technical procedures during thoracotomy for the standard care in the treatment of lung tumors. In all patients, the expert pulmonary pathologist selected tumor and non-tumor lung specimens of approximately 10x10 mm^2 area from the fresh samples as previously validated[4,29]. Non-tumor specimens were collected as far as possible from the lung to the tumor resection margins (average >7 cm). Fragments of both tumor and non-tumor specimens were fixed in formalin and embedded in paraffin blocks until further use. Another fragment was harvested in Dulbecco's Modified Eagle Medium (DMEM) with 1% of penicillin, streptomycin, and fungiozone for the cell culture process.

Cell culture

Fresh human tumor and non-tumor lung samples were placed in Dulbecco's Modified Eagle Medium (DMEM) with 1% of penicillin, streptomycin, and fungiozone immediately after obtaining lung specimens and transported on ice to the molecular laboratory. Tumor and non-tumor specimens were minced finely and digested in 1% collagenase type I (Sigma-Aldrich, St. Louis, MO) at 37°C for two hours with occasional agitation. Then the digested tissue was centrifuged at 1,200 rpm for two minutes. Cell suspensions were cultured on culture plates in proliferation medium consisting of the mixture of DMEM-medium, 10% fetal bovine serum, and 1% penicillin-streptomycin-fungizone solution at 37 °C in a 5% CO_2 atmosphere. The culture medium was changed after 48 hours to remove unattached cells and debris in suspension. Cells were subcultured with 0.025% trypsin (Life Technologies, California, USA) and 0.01% EDTA when they reached 50-80% confluence for ten minutes. All the study experiments were performed on the cultured cells between passages 1 and 2 of the primary cultures to perform immunofluorescence as described below.

Immunoflorescence staining of CAFs and NFs

CAFs and NFs were identified by analyzing the fibroblast‐specific protein vimentin and alpha-SMA (CAFs). Briefly, cells were fixed with acetone and methanol (1:1) on the slides at -20°C for ten minutes, and were then washed with PBS three times. Subsequently, slides were incubated with blocking solution (50mM Tris with PH=7.5, 150 Mm NaCl, 0.01% Triton, 1% bovine serum albumin and 1% skimmed milk powder) for one hour at room temperature in a humidifed chamber. Subsequently, primary antibodies incubation with anti-alpha SMA antibody (anti-alpha-SMA antibody, Santa Cruz) and anti-vimentin antibody (anti-vimentin antibody, Santa Cruz) was performed overnight at 4°C in the chamber. After washing with PBS three times,

slides were incubated with corresponding secondary antibodies diluted in PBS for one hour: anti-mouse IgG FITC (Invitrogen, Thermo Fisher Scientific) and anti-rabbit IgG A647 (Invitrogen, Thermo Fisher Scientific) at room temperature. Finally, the sections were mounted using the fluorescent mounting medium 4',6-diamidino-2-phenylindole (DAPI) G-Fluoromount medium (Southern Biotech, Birmingham, AL, USA), which specifically stained DNA (allowing identification of all nuclei) in the cell sections. A fluorescence microscope (x 40 objectives, Nikon Eclipse Ni, Nikon, Tokyo, Japan) coupled with a digitizing camera was used to identify and count the number of fibroblasts (30 fields) in each study sample. Results were expressed as the percentage of either both alpha-SMA and vimentin positively stained fibroblasts for identification of CAFs or vimentin-only positively stained for detection of NFs to the total number of counted fibroblasts in the 30 fields. Results are reported separately for both CAFs and NFs in each type of lung specimen and patient group.

Markers of ECM and endothelial cells using immunohistochemistry

Type I collagen and endothelial cells were identified on three-micrometer lung tumor and non-tumor cross-sections using immunohistochemical procedures as previously described[10,29]. Following deparaffinization, lung cross-sections were immersed in preheated antigen retrieval solution of ethylenediaminetetraacetic acid (EDTA, pH 9), incubated at 95ºC for 40 minutes to be then cooled down to room temperature. Slides were washed over the following steps with phosphate buffer saline (PBS). Endogenous peroxidase activity was blocked with 6% hydrogen peroxide for 15 minutes. Primary antibody incubation with anti-collagen I antibody (anti-collagen I antibody, Abcam, Cambridge, UK) and anti-CD31antibody (anti-CD31 antibody, Abcam, Cambridge, UK) was performed for one hour. Slides were incubated with biotinylated universal secondary antibody for 30 minutes followed by a 30-minute

incubation with HRP-streptavidin and diaminobenzidine for five minutes (kit LSAB+HRP Dako Cytomation Inc., Carpinteria, CA, USA) as a substrate. Hematoxylin counterstaining was performed for two minutes and slides were dehydrated and mounted for conventional microscopy. Images of the stained lung sections (tumor and non-tumor) were captured with a light microscope (Olympus, Series BX50F3, Olympus Optical Co., Hamburg, Germany) coupled with an image-digitizing camera (Pixera Studio, version 1.0.4, Pixera Corporation, Los Gatos, CA, USA).

Expression of the markers collagen and CD31 was estimated as the percentage of type I collagen and CD31 using the semiquantitative immunohistochemical scoring system (Hscore) according to methodologies previously published[30]. Type I collagen and CD31 staining in the tumor and non-tumor specimens was established according to the following categories: Hscore 0 (indicated the absence of staining) and Hscore 1 (indicated the presence of staining). Data are shown as the percentage of both positively and negatively stained structures for all the histological sections in both tumor and non-tumor lung specimens.

Statistical analyses

The normality of the study variables was tested using the Shapiro-Wilk test. The marker CD31 was used to estimate sample size. For the one-way analysis of variance (ANOVA) of one factor, considering the between-group variance to be 10486.7 and the within-group error equal to 3160.9, a minimum of 12 patients (24 patients in total) per type of sample (tumor and non-tumor) sufficed to reach an 80% power given an alpha error of 0.05. The software Stata/MP release 15 (StataCorp LLC, College Station, Texas, USA) was used for sample size calculation. Clinical variables are shown in a Table. Qualitative variables are represented as frequencies (number and percentage), while quantitative variables are shown as mean and standard deviations. Differences in

clinical variables between LC and LC-COPD groups of patients were assessed using Student's T-test. Differences among the different biological variables were estimated using ANOVA and Tukey's post-hoc to adjust for multiple comparisons for the two sample types (tumor and non-tumor) and the two patient groups. A subanalysis in which only never-smokers and non-smokers were analyzed was conducted. Moreover, one-way covariance (ANCOVA) was also used to adjust for cigarette smoking history in the analyses of all the biological. Statistical significance was established at $P \leq 0.05$. All statistical analyses were conducted using the software Statistical Package for the Social Science (SPSS, version 23, SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical characteristics

Clinical and functional characteristics of LC and LC-COPD patients are shown in Table 1. Age, sex, or BMI did not significantly differ between the two groups of patients. Ex-smokers and the number of pack-years were significantly greater in LC-COPD patients compared to LC patients, while the number of never smokers was significantly greater in the latter group (Table 1). The lung functional parameters FEV_1, FEV_1/FVC, DL_{CO} and K_{CO} were significantly lower in LC-COPD than in LC patients (Table 1). Most of the patients were in GOLD stages I and II (93%, Table 1). TNM staging or histological subtypes did not significantly differ between the two groups. In LC-COPD compared to LC patients, the levels of total leukocytes and neutrophils significantly increased, while levels of albumin significantly decreased. Total proteins, fibrinogen, C-reactive protein, globular sedimentation velocity, and body weight loss did not differ between LC-COPD and LC patients.

Levels of CAFs increased in tumor specimens

Compared to non-tumor lungs, levels of alpha-SMA significantly increased in tumor specimens both in LC and LC-COPD patients, while levels of vimentin significantly decreased in tumor samples in both groups of patients (Figure 1 and Figure 2).

Levels of the fibroblast markers alpha-SMA (marker of CAFs) and vimentin (marker of NFs) did not significantly differ in either tumor or non-tumor specimens between LC-COPD and LC patients (Figure 1 and Figure 2).

The subanalysis of the patients according to either GOLD stages or cigarette smoking history revealed identical results to those shown when the entire population was analyzed as a whole (data not shown).

Markers of collagen and endothelial cells in lung specimens

Levels of the ECM marker type I collagen and those of the endothelial marker CD31 did not significantly differ in either tumor or non-tumor lungs between the two patient groups (Figures 3 and 4, respectively).

Levels of type I collagen did not differ between tumor and non-tumor samples in any study groups of patients (Figure 3). Importantly, in LC patients, levels of Hscore 1 (presence of staining) of CD31 significantly declined in tumor specimens compared to non-tumor samples, whereas those of Hscore 0 (absence of staning) increased (Figure 4). In LC-COPD, no significant differences were seen in CD31 marker leves between tumor and non-tumor samples (Figure 4).

The subanalysis of the patients according to either GOLD stages or cigarette smoking history revealed identical results to those shown when the entire population was analyzed as a whole (data not shown).

DISCUSSION

In the current investigation, the main findings were that levels of the endothelial marker CD31 significantly decreased in tumors of LC patients, but not in tumors of patients with airway obstruction. In both groups of patients, a rise in the expression of CAFs was seen in lung tumors. Levels of type I collagen in tumor and non-tumor lungs did not differ between patient groups. The most relevant findings collected in the study are discussed below.

CAFs play a crucial role in cancer cell invasion through several mechanisms[31]. Vimentin, which is expressed in normal mesenchymal cells, maintains cellular integrity and provides resistance against stress. Its function has also been proposed in different cancer cell types including LC[32]. In the present investigation, the expression of CAFs was significantly greater in the tumor specimens in both groups of LC patients with and without COPD. No significant differences in the levels of cultured CAFs in tumor lungs were seen between the study groups of patients. These findings suggest that CAFs are similarly expressed in lung tumors regardless of underlying airway obstruction. They also imply that fibroblasts are not likely to be involved in an accelerated process of cancer invasion and progression in patients with airway obstruction. Conversely, the percentage of fibroblasts-expressing vimentin-only was significantly reduced in the tumors of both groups of patients. These results also reinforce the concept that CAFs are likely to be a predominant feature of the stroma in lung tumor progression in the patients regardless of the presence of airway obstruction.

Whether a similar profile of CAFs expression can be detected in lung tumors of patients with other underlying respiratory diseases remains to be elucidated. In idiopathic pulmonary fibrosis, myofibroblasts are persistently activated, which secrete collagen type I, and express alpha-SMA fibers, thus they may favor lung

tumorigenesis[35]. Conversely, in patients with non-cystic fibrosis bronchiectasis, a lower or no risk of LC was demonstrated[33,34].

Activated myofibroblasts synthesize extracellular components that contribute to the remodeling of the ECM taking place during carcinogenesis. As such CAFs secrete type I collagen, which plays an important role in tumor development, growth, and epithelial-mesenchymal transition[36]. Moreover, overall survival correlated with low levels of expression of type I collagen and cancer cell differentiation[36]. In the present study, expression levels of collagen did not significantly differ between tumor and non-tumor samples or between the study groups. These findings suggest that collagen was not a major driver in lung tumor development in these patients, probably because well-differentiated tumor types were analyzed in the study.

CD31 is a glycoprotein expressed in endothelial cells, leukocytes, T cells, and platelets[20]. CD31 is also expressed in lung tumors[37]. In the current investigation, a significant decline in CD31 expression levels (Hscore 1) was detected in the tumor specimens of patients with LC, while in patients with underlying airway obstruction no significant differences were seen between lung tumor and non-tumor samples. These findings imply that the vascular endothelial component of stroma was probably involved in the prognosis of LC in patients with and without COPD. In fact, 47% of LC-COPD and 80% of LC patients are still alive in this series (10-year follow-up, data not shown). In keeping with, CD31 has proven to be a useful marker to evaluate angiogenesis in lung tumors[38] as well as to monitor the response to specific anti-angiogenic molecules such as vascular endothelial growth factor (VEGF) in clinical settings[38,39]. In this regard, several investigations have demonstrated that VEGF inhibitors, through reduced angiogenesis (CD31 marker among others), are currently prescribed as single agents in the third-line treatment of patients with NSCLC[38,39].

Study limitations

A limitation in the study was the relatively reduced number of analyzed patients. Nonetheless, calculations of sample size estimated 12 patients in each group (24 in total), thus the number of patients included was sufficient to detect statistically significant differences in the study. The degree of airway obstruction might have influenced the study results. However, as most of the patients were in GOLD stages I and particularly II, COPD severity did not exert any significant impact on the results. Almost half of the patients were non-smokers, thus cigarette smoking might have influenced the study results. Nevertheless, a subanalysis in which non-smokers and ex-smokers were included revealed identical results to those obtained with the entire population.

If non-tumor samples had been obtained from a closer distance from the tumors, the profile of biological events might have differed as shown previously for other components of the extracellular matrix (integrins) that probably play a significant role in recurrence[40]. Nonetheless, this was not explored in the present study, and warrants further attention.

Conclusions

Within the stroma, the expression of the vascular endothelial marker CD31 was reduced in tumors of patients without airway obstruction, while expression levels of the ECM component type I collagen did not differ between patient groups. A rise in the levels of CAFs was detected in the lung tumors of patients irrespective of underlying airway obstruction.

Low levels of CD31 may have implications in the overall survival of LC patients, especially in those without underlying airway obstruction. Investigations aiming to decipher the specific role of CD31 as a predictor of survival and as a biomarker to

monitor anti-angiogenic agents in lung tumors of patients with underlying respiratory diseases are warranted.

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FIGURE LEGENDS

Figure 1A and 1B: Representative examples of immunofluorescence staining of the markers DAPI (upper panel), vimentin (upper middle panel), alpha-SMA (lower middle panel), and CAFs (positively stained for both vimentin and alpha-SMA, bottom panel) in cultured fibroblasts obtained from non-tumor and tumor specimens of LC and LC-COPD patients. Definition of abbreviations: DAPI, 4', 6-diamidino-2-phenylindole; alpha-SMA, alpha-smooth muscle actin; CAFs, cancer-associated fibroblasts; LC, lung cancer; COPD, chronic obstructive pulmonary disease.

Figure 2A and 2B: Mean values and standard deviations (SD) of levels of the markers vimentin and vimentin and alpha-SMA as measured by percentage of the total fibroblasts. Definition of abbreviations: alpha-SMA, alpha-smooth muscle actin; LC, lung cancer; COPD, chronic obstructive pulmonary disease. Statistical analyses: **, $p \leq 0.01$ between tumor (T) and non-tumor (NT) lungs in both LC and LC-COPD patients.

Figure 3: A) Representative examples of immunohistochemical staining for type I collagen in tumor and non-tumor specimens (collagen I-positively stained) in LC and LC-COPD patients, respectively. Black arrows point towards areas stained in blue with hematoxylin (negatively-stained for collagen), while red arrows point towards positively-stained areas (brown color). **B)** Mean and standard deviations (SD) of levels of type I collagen in tumor and non-tumor of both groups as measured using histoscores (see Methods). Definition of abbreviations: Hscore, histochemical score; LC, lung cancer; COPD, chronic obstructive pulmonary disease. Statistical analyses:

n.s., no significance between tumor (T) and non-tumor (NT) lungs in either LC or LC-COPD patients.

Figure 4: A) Representative examples of immunohistochemical staining for CD31 in tumor and non-tumor specimens (CD31-positively stained) in LC and LC-COPD patients, respectively. Black arrows point towards areas stained in blue with hematoxylin (negatively-stained for CD31), while red arrows point towards positively-stained areas (brown color). **B)** Mean and standard deviations (SD) of levels of CD31 in tumor and non-tumor of both groups as measured using specific histoscores (see Methods). Definition of abbreviations: Hscore, histochemical score; LC, lung cancer; COPD, chronic obstructive pulmonary disease. Statistical analyses: **, $p \leq 0.01$ between tumor (T) and non-tumor (NT) lungs in LC patients; *n.s.*, no significance between tumor (T) and non-tumor (NT) lungs in LC-COPD patients.

ABSTRACT

Background: Stroma, mainly composed by fibroblasts, extracellular matrix (ECM) and vessels, may play a role in tumorigenesis and cancer progression. Chronic Obstructive Pulmonary Disease (COPD) is an independent risk factor for LC. We hypothesized that markers of fibroblasts, ECM and endothelial cells may differ in tumors of LC patients with/without COPD. **Methods:** Markers of cultured cancer-associated fibroblasts and normal fibroblasts [CAFs and NFs, respectively, vimentin and alpha-smooth muscle actin (SMA) markers, immunofluorescence in cultured lung fibroblasts], ECM, and endothelial cells (type I collagen and CD31 markers, respectively, immunohistochemistry) were identified in lung tumor and non-tumor specimens (thoracotomy for lung tumor resection) from 15 LC-COPD patients and 15 LC-only patients. **Results:** Numbers of CAFs significantly increased, while those of NFs significantly decreased in tumor samples compared to non-tumor specimens of both LC and LC-COPD patients. Endothelial cells (CD31) significantly decreased in tumor samples compared to non-tumor specimens only in LC patients. No significant differences were seen in levels of type I collagen in any samples or study groups. **Conclusions:** Vascular endothelial marker CD31 expression was reduced in tumors of non-COPD patients, while type I collagen levels did not differ between groups. A rise in CAFs levels was detected in lung tumors of patients irrespective of airway obstruction. Low levels of CD31 may have implications in the overall survival of LC patients, especially in those without underlying airway obstruction. Identification of CD31 role as a prognostic and therapeutic biomarker in lung tumors of patients with underlying respiratory diseases warrants attention. **Word count:** 248

KEY WORDS: lung cancer; COPD; stroma; cancer-associated fibroblasts; extracellular matrix; endothelial cell marker CD31

INTRODUCTION

Despite recent advances, non-small cell lung cancer (NSCLC) still leads to a great mortality in most of the continents[1,2], reaching up to one third of deaths in certain countries[1,3]. Clinical factors such as chronic obstructive pulmonary disease (COPD) or airway obstruction underlie the pathophysiology of LC in many patients[1,4–6]. Several relevant investigations have clearly demonstrated that airway obstruction and emphysema render the patients more susceptible to the development of LC[7–9]. Despite this consolidated knowledge, full elucidation of the underlying biological features is still underway.

In the airways, lungs, and blood compartment of patients with LC and underlying COPD, mechanisms such as redox imbalance, inflammatory events, epigenetics, and immune alterations were shown to be disrupted compared to LC patients with no COPD[10]. As a result of the interaction of those biological events with key cellular processes, namely angiogenesis, cell death and repair, and the cell survival machinery, COPD patients are more prone to lung tumorigenesis[11].

Stroma is defined as the part of a tissue or organ that confers mainly structure with no specific function, and is mainly composed by blood vessels, nerves, and connective tissue. In LC, several components such as extracellular matrix (ECM), endothelial cells, and cancer-associated fibroblasts (CAFs) play a significant role in tumorigenesis and cancer progression[12]. CAFs are a major component of the stroma in tumors. Growth factors, hormones, and cytokines mediate the tumor cell proliferation favored by CAFs. The most specific and widely used marker of CAFs is alpha-smooth muscle actin (SMA), which is indeed a specific marker of myofibroblasts[12]. The differentiation process of epithelial cells into mesenchymal cells is known as epithelial-mesenchymal transition (EMT), characterized by the appearance of mesenchymal properties[13,14]. Interestingly, CAFs may also regulate EMT [13].

Extracellular macromolecules such as collagen, enzymes, and glycoproteins conform a specific network of the ECM, which is also involved in tumor development and progression[15]. In cancer stroma, collagen was demonstrated to be the most abundant protein[16]. Importantly, type I collagen promotes growth of cancer cells, invasion, and distant metastasis, thus favoring tumor progression[17], as well as resistance to therapy[18]. Whether a distinct expression of extracellular matrix markers or CAFs may take place in the stroma of lung tumor samples of patients with COPD remains to be answered.

The formation of new vessels in tumors can be identified using specific markers such as platelet endothelial cell adhesion molecule also known as cluster of differentiation (CD) 31. CD31 is involved in several physiological processes, namely maintenance of vascular endothelial and inflammatory cell functions and is also expressed in tumor cells[19]. In fact, the immunohistochemical measurement of CD31 expression can be reliably used as a marker of neoangiogenesis in tumors[20]. In mice with experimental airway inflammation mimicking COPD, an immunosuppressive microenvironment of the lung tumors was characterized by increased angiogenesis[21]. Whether differences in CD31 expression may exist in tumors of patients with COPD compared to non-COPD remain to be identified.

On this basis, we hypothesized that in LC patients with airway obstruction, cancer stroma as analyzed using specific markers may differ from tumors of patients with no underlying COPD. Accordingly, our objectives were to determine in lung tumors and non-tumor specimens (control samples) of LC patients with and without COPD the following parameters: 1) CAFs and non-tumor fibroblasts (cultured fibroblasts), 2) type I collagen as a marker of extracellular matrix, and 3) CD31 expression levels as a marker of endothelial cells and blood vessels.

METHODS

Study design and ethics

This is a cross-sectional, prospective study designed following the World Medical Association guidelines (Seventh revision of the Declaration of Helsinki, Fortaleza, Brazil, 2013)[22] for research on human beings and was approved by the institutional Ethics Committee on Human Investigation (protocol # 2008/3390/I, Hospital del Mar–IMIM, Barcelona, Spain). All patients invited to participate in the study signed the informed written consent. The current investigation followed the international STROBE guidelines[23].

Patients were prospectively recruited from the Lung Cancer Clinic of the Respiratory Medicine Department at *Hospital del Mar* (Barcelona, Spain). All the patients were part of the *Lung Cancer Mar Cohort*. For this observational study, 30 patients with LC were recruited in 2019. Candidates for tumor resection underwent pulmonary surgery prior to administration of any sort of adjuvant therapy. LC diagnosis and staging were established by histological confirmation and classified according to currently available guidelines for the diagnosis and management of LC[24,25]. TNM (tumor, node, and metastasis) staging was defined as stated in the 8th edition Lung Cancer Stage Classification[26]. COPD diagnosis was established as a post-bronchodilator forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) $\leq$ 0.7 which is not fully reversible by spirometry according to currently available guidelines for diagnosis and management of COPD[27,28]. Exclusion criteria were: small cell lung cancer (SCLC), chronic cardiovascular disease, restrictive lung disease, metabolic, immune disease, or clot system disorders, signs of severe inflammation and/or bronchial infection (bronchoscopy), current or recent invasive mechanical ventilation, or long-term oxygen therapy.

Specimens from the tumor and non-tumor lungs were collected from all the study subjects. Patients were further subdivided *post-hoc* into two groups on the basis of underlying COPD: 1) 15 patients with LC and COPD (LC-COPD group) and 2) 15 patients with LC without COPD (LC group).

Clinical assessment

In all patients, lung function parameters were assessed following standard procedures. Diagnosis and severity of patients with COPD were determined according to currently available guidelines[6]. Nutritional evaluation included the assessment of body mass index (BMI) and nutritional blood parameters from all patients.

Sample collection and preservation

Lung samples were obtained from tumors and the surrounding non-tumor parenchyma following standard technical procedures during thoracotomy for the standard care in the treatment of lung tumors. In all patients, the expert pulmonary pathologist selected tumor and non-tumor lung specimens of approximately 10x10 mm^2 area from the fresh samples as previously validated[4,29]. Non-tumor specimens were collected as far as possible from the lung to the tumor resection margins (average >7 cm). Fragments of both tumor and non-tumor specimens were fixed in formalin and embedded in paraffin blocks until further use. Another fragment was harvested in Dulbecco's Modified Eagle Medium (DMEM) with 1% of penicillin, streptomycin, and fungiozone for the cell culture process.

Cell culture

Fresh human tumor and non-tumor lung samples were placed in Dulbecco's Modified Eagle Medium (DMEM) with 1% of penicillin, streptomycin, and fungiozone

immediately after obtaining lung specimens and transported on ice to the molecular laboratory. Tumor and non-tumor specimens were minced finely and digested in 1% collagenase type I (Sigma-Aldrich, St. Louis, MO) at 37°C for two hours with occasional agitation. Then the digested tissue was centrifuged at 1,200 rpm for two minutes. Cell suspensions were cultured on culture plates in proliferation medium consisting of the mixture of DMEM-medium, 10% fetal bovine serum, and 1% penicillin-streptomycin-fungizone solution at 37 °C in a 5% CO_2 atmosphere. The culture medium was changed after 48 hours to remove unattached cells and debris in suspension. Cells were subcultured with 0.025% trypsin (Life Technologies, California, USA) and 0.01% EDTA when they reached 50-80% confluence for ten minutes. All the study experiments were performed on the cultured cells between passages 1 and 2 of the primary cultures to perform immunofluorescence as described below.

Immunoflorescence staining of CAFs and NFs

CAFs and NFs were identified by analyzing the fibroblast‐specific protein vimentin and alpha-SMA (CAFs). Briefly, cells were fixed with acetone and methanol (1:1) on the slides at -20°C for ten minutes, and were then washed with PBS three times. Subsequently, slides were incubated with blocking solution (50mM Tris with PH=7.5, 150 Mm NaCl, 0.01% Triton, 1% bovine serum albumin and 1% skimmed milk powder) for one hour at room temperature in a humidifed chamber. Subsequently, primary antibodies incubation with anti-alpha SMA antibody (anti-alpha-SMA antibody, Santa Cruz) and anti-vimentin antibody (anti-vimentin antibody, Santa Cruz) was performed overnight at 4°C in the chamber. After washing with PBS three times, slides were incubated with corresponding secondary antibodies diluted in PBS for one hour: anti-mouse IgG FITC (Invitrogen, Thermo Fisher Scientific) and anti-rabbit IgG A647 (Invitrogen, Thermo Fisher Scientific) at room temperature. Finally, the sections were

mounted using the fluorescent mounting medium 4',6-diamidino-2-phenylindole (DAPI) G-Fluoromount medium (Southern Biotech, Birmingham, AL, USA), which specifically stained DNA (allowing identification of all nuclei) in the cell sections. A fluorescence microscope (x 40 objectives, Nikon Eclipse Ni, Nikon, Tokyo, Japan) coupled with a digitizing camera was used to identify and count the number of fibroblasts (30 fields) in each study sample. Results were expressed as the percentage of either both alpha-SMA and vimentin positively stained fibroblasts for identification of CAFs or vimentin-only positively stained for detection of NFs to the total number of counted fibroblasts in the 30 fields. Results are reported separately for both CAFs and NFs in each type of lung specimen and patient group.

Markers of ECM and endothelial cells using immunohistochemistry

Type I collagen and endothelial cells were identified on three-micrometer lung tumor and non-tumor cross-sections using immunohistochemical procedures as previously described[10,29]. Following deparaffinization, lung cross-sections were immersed in preheated antigen retrieval solution of ethylenediaminetetraacetic acid (EDTA, pH 9), incubated at 95ºC for 40 minutes to be then cooled down to room temperature. Slides were washed over the following steps with phosphate buffer saline (PBS). Endogenous peroxidase activity was blocked with 6% hydrogen peroxide for 15 minutes. Primary antibody incubation with anti-collagen I antibody (anti-collagen I antibody, Abcam, Cambridge, UK) and anti-CD31antibody (anti-CD31 antibody, Abcam, Cambridge, UK) was performed for one hour. Slides were incubated with biotinylated universal secondary antibody for 30 minutes followed by a 30-minute incubation with HRP-streptavidin and diaminobenzidine for five minutes (kit LSAB+HRP Dako Cytomation Inc., Carpinteria, CA, USA) as a substrate. Hematoxylin counterstaining was performed for two minutes and slides were dehydrated and mounted for conventional microscopy. Images of the

stained lung sections (tumor and non-tumor) were captured with a light microscope (Olympus, Series BX50F3, Olympus Optical Co., Hamburg, Germany) coupled with an image-digitizing camera (Pixera Studio, version 1.0.4, Pixera Corporation, Los Gatos, CA, USA).

Expression of the markers collagen and CD31 was estimated as the percentage of type I collagen and CD31 using the semiquantitative immunohistochemical scoring system (Hscore) according to methodologies previously published[30]. Type I collagen and CD31 staining in the tumor and non-tumor specimens was established according to the following categories: Hscore 0 (indicated the absence of staining) and Hscore 1 (indicated the presence of staining). Data are shown as the percentage of both positively and negatively stained structures for all the histological sections in both tumor and non-tumor lung specimens.

Statistical analyses

The normality of the study variables was tested using the Shapiro-Wilk test. The marker CD31 was used to estimate sample size. For the one-way analysis of variance (ANOVA) of one factor, considering the between-group variance to be 10486.7 and the within-group error equal to 3160.9, a minimum of 12 patients (24 patients in total) per type of sample (tumor and non-tumor) sufficed to reach an 80% power given an alpha error of 0.05. The software Stata/MP release 15 (StataCorp LLC, College Station, Texas, USA) was used for sample size calculation. Clinical variables are shown in a Table. Qualitative variables are represented as frequencies (number and percentage), while quantitative variables are shown as mean and standard deviations. Differences in clinical variables between LC and LC-COPD groups of patients were assessed using Student's T-test. Differences among the different biological variables were estimated using ANOVA and Tukey's post-hoc to adjust for multiple comparisons for the two sample types (tumor

and non-tumor) and the two patient groups. A subanalysis in which only never-smokers and non-smokers were analyzed was conducted. Moreover, one-way covariance (ANCOVA) was also used to adjust for cigarette smoking history in the analyses of all the biological. Statistical significance was established at $P \leq 0.05$. All statistical analyses were conducted using the software Statistical Package for the Social Science (SPSS, version 23, SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical characteristics

Clinical and functional characteristics of LC and LC-COPD patients are shown in Table 1. Age, sex, or BMI did not significantly differ between the two groups of patients. Ex-smokers and the number of pack-years were significantly greater in LC-COPD patients compared to LC patients, while the number of never smokers was significantly greater in the latter group (Table 1). The lung functional parameters FEV_1, FEV_1/FVC, DL_{CO} and K_{CO} were significantly lower in LC-COPD than in LC patients (Table 1). Most of the patients were in GOLD stages I and II (93%, Table 1). TNM staging or histological subtypes did not significantly differ between the two groups. In LC-COPD compared to LC patients, the levels of total leukocytes and neutrophils significantly increased, while levels of albumin significantly decreased. Total proteins, fibrinogen, C-reactive protein, globular sedimentation velocity, and body weight loss did not differ between LC-COPD and LC patients.

Levels of CAFs increased in tumor specimens

Compared to non-tumor lungs, levels of alpha-SMA significantly increased in tumor specimens both in LC and LC-COPD patients, while levels of vimentin

significantly decreased in tumor samples in both groups of patients (Figure 1 and Figure 2).

Levels of the fibroblast markers alpha-SMA (marker of CAFs) and vimentin (marker of NFs) did not significantly differ in either tumor or non-tumor specimens between LC-COPD and LC patients (Figure 1 and Figure 2).

The subanalysis of the patients according to either GOLD stages or cigarette smoking history revealed identical results to those shown when the entire population was analyzed as a whole (data not shown).

Markers of collagen and endothelial cells in lung specimens

Levels of the ECM marker type I collagen and those of the endothelial marker CD31 did not significantly differ in either tumor or non-tumor lungs between the two patient groups (Figures 3 and 4, respectively).

Levels of type I collagen did not differ between tumor and non-tumor samples in any study groups of patients (Figure 3). Importantly, in LC patients, levels of Hscore 1 (presence of staining) of CD31 significantly declined in tumor specimens compared to non-tumor samples, whereas those of Hscore 0 (absence of staning) increased (Figure 4). In LC-COPD, no significant differences were seen in CD31 marker leves between tumor and non-tumor samples (Figure 4).

The subanalysis of the patients according to either GOLD stages or cigarette smoking history revealed identical results to those shown when the entire population was analyzed as a whole (data not shown).

DISCUSSION

In the current investigation, the main findings were that levels of the endothelial marker CD31 significantly decreased in tumors of LC patients, but not in tumors of

patients with airway obstruction. In both groups of patients, a rise in the expression of CAFs was seen in lung tumors. Levels of type I collagen in tumor and non-tumor lungs did not differ between patient groups. The most relevant findings collected in the study are discussed below.

CAFs play a crucial role in cancer cell invasion through several mechanisms[31]. Vimentin, which is expressed in normal mesenchymal cells, maintains cellular integrity and provides resistance against stress. Its function has also been proposed in different cancer cell types including LC[32]. In the present investigation, the expression of CAFs was significantly greater in the tumor specimens in both groups of LC patients with and without COPD. No significant differences in the levels of cultured CAFs in tumor lungs were seen between the study groups of patients. These findings suggest that CAFs are similarly expressed in lung tumors regardless of underlying airway obstruction. They also imply that fibroblasts are not likely to be involved in an accelerated process of cancer invasion and progression in patients with airway obstruction. Conversely, the percentage of fibroblasts-expressing vimentin-only was significantly reduced in the tumors of both groups of patients. These results also reinforce the concept that CAFs are likely to be a predominant feature of the stroma in lung tumor progression in the patients regardless of the presence of airway obstruction.

Whether a similar profile of CAFs expression can be detected in lung tumors of patients with other underlying respiratory diseases remains to be elucidated. In idiopathic pulmonary fibrosis, myofibroblasts are persistently activated, which secrete collagen type I, and express alpha-SMA fibers, thus they may favor lung tumorigenesis[35]. Conversely, in patients with non-cystic fibrosis bronchiectasis, a lower or no risk of LC was demonstrated[33,34].

Activated myofibroblasts synthesize extracellular components that contribute to the remodeling of the ECM taking place during carcinogenesis. As such CAFs secrete type I collagen, which plays an important role in tumor development, growth, and epithelial-mesenchymal transition[36]. Moreover, overall survival correlated with low levels of expression of type I collagen and cancer cell differentiation[36]. In the present study, expression levels of collagen did not significantly differ between tumor and non-tumor samples or between the study groups. These findings suggest that collagen was not a major driver in lung tumor development in these patients, probably because well-differentiated tumor types were analyzed in the study.

CD31 is a glycoprotein expressed in endothelial cells, leukocytes, T cells, and platelets[20]. CD31 is also expressed in lung tumors[37]. In the current investigation, a significant decline in CD31 expression levels (Hscore 1) was detected in the tumor specimens of patients with LC, while in patients with underlying airway obstruction no significant differences were seen between lung tumor and non-tumor samples. These findings imply that the vascular endothelial component of stroma was probably involved in the prognosis of LC in patients with and without COPD. In fact, 47% of LC-COPD and 80% of LC patients are still alive in this series (10-year follow-up, data not shown). In keeping with, CD31 has proven to be a useful marker to evaluate angiogenesis in lung tumors[38] as well as to monitor the response to specific anti-angiogenic molecules such as vascular endothelial growth factor (VEGF) in clinical settings[38,39]. In this regard, several investigations have demonstrated that VEGF inhibitors, through reduced angiogenesis (CD31 marker among others), are currently prescribed as single agents in the third-line treatment of patients with NSCLC[38,39].

Study limitations

A limitation in the study was the relatively reduced number of analyzed patients. Nonetheless, calculations of sample size estimated 12 patients in each group (24 in total), thus the number of patients included was sufficient to detect statistically significant differences in the study. The degree of airway obstruction might have influenced the study results. However, as most of the patients were in GOLD stages I and particularly II, COPD severity did not exert any significant impact on the results. Almost half of the patients were non-smokers, thus cigarette smoking might have influenced the study results. Nevertheless, a subanalysis in which non-smokers and ex-smokers were included revealed identical results to those obtained with the entire population.

If non-tumor samples had been obtained from a closer distance from the tumors, the profile of biological events might have differed as shown previously for other components of the extracellular matrix (integrins) that probably play a significant role in recurrence[40]. Nonetheless, this was not explored in the present study, and warrants further attention.

Conclusions

Within the stroma, the expression of the vascular endothelial marker CD31 was reduced in tumors of patients without airway obstruction, while expression levels of the ECM component type I collagen did not differ between patient groups. A rise in the levels of CAFs was detected in the lung tumors of patients irrespective of underlying airway obstruction.

Low levels of CD31 may have implications in the overall survival of LC patients, especially in those without underlying airway obstruction. Investigations aiming to decipher the specific role of CD31 as a predictor of survival and as a biomarker to monitor anti-angiogenic agents in lung tumors of patients with underlying respiratory diseases are warranted.

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FIGURE LEGENDS

Figure 1A and 1B: Representative examples of immunofluorescence staining of the markers DAPI (upper panel), vimentin (upper middle panel), alpha-SMA (lower middle panel), and CAFs (positively stained for both vimentin and alpha-SMA, bottom panel) in cultured fibroblasts obtained from non-tumor and tumor specimens of LC and LC-COPD patients. Definition of abbreviations: DAPI, 4', 6-diamidino-2-phenylindole; alpha-SMA, alpha-smooth muscle actin; CAFs, cancer-associated fibroblasts; LC, lung cancer; COPD, chronic obstructive pulmonary disease.

Figure 2A and 2B: Mean values and standard deviations (SD) of levels of the markers vimentin and vimentin and alpha-SMA as measured by percentage of the total fibroblasts. Definition of abbreviations: alpha-SMA, alpha-smooth muscle actin; LC, lung cancer; COPD, chronic obstructive pulmonary disease. Statistical analyses: **, $p \leq 0.01$ between tumor (T) and non-tumor (NT) lungs in both LC and LC-COPD patients.

Figure 3: A) Representative examples of immunohistochemical staining for type I collagen in tumor and non-tumor specimens (collagen I-positively stained) in LC and LC-COPD patients, respectively. Black arrows point towards areas stained in blue with hematoxylin (negatively-stained for collagen), while red arrows point towards positively-stained areas (brown color). **B)** Mean and standard deviations (SD) of levels of type I collagen in tumor and non-tumor of both groups as measured using histoscores (see Methods). Definition of abbreviations: Hscore, histochemical score; LC, lung cancer; COPD, chronic obstructive pulmonary disease. Statistical analyses: *n.s.*, no significance between tumor (T) and non-tumor (NT) lungs in either LC or LC-COPD patients.

Figure 4: A) Representative examples of immunohistochemical staining for CD31 in tumor and non-tumor specimens (CD31-positively stained) in LC and LC-COPD patients, respectively. Black arrows point towards areas stained in blue with hematoxylin (negatively-stained for CD31), while red arrows point towards positively-stained areas (brown color). **B)** Mean and standard deviations (SD) of levels of CD31 in tumor and non-tumor of both groups as measured using specific histoscores (see Methods). Definition of abbreviations: Hscore, histochemical score; LC, lung cancer; COPD, chronic obstructive pulmonary disease. Statistical analyses: **, $p \leq 0.01$ between tumor (T) and non-tumor (NT) lungs in LC patients; *n.s.*, no significance between tumor (T) and non-tumor (NT) lungs in LC-COPD patients.

Tabla (Table)

Table 1. Clinical and functional characteristics of the study patients

Anthropometric variables	**LC (N=15)**	**LC-COPD (N=15)**
Age, years	67 (10)	67 (8)
Male, N / Female, N	8 / 7	12 / 3
BMI, kg/m^2	27 (5)	26 (4)
Smoking history		
Current: N, %	8, 53	8, 53
Ex-smoker: N, %	0, 0	7, 47***
Never smoker: N, %	7, 47	0, 0***
Pack-years	24 (18)	56 (25)**
Lung function parameters		
FEV_1 , %	89 (11)	67 (14)***
FEV_1/FVC, %	76 (5)	59 (9)***
DL_{CO}, %	84 (11)	60 (15)***
K_{CO}, %	85 (11)	59 (15)***
GOLD stage		
GOLD Stage I : N, %	NA	2, 13
GOLD Stage II : N, %	NA	12, 80
GOLD Stage III : N, %	NA	1, 7
GOLD Stage IV : N, %	NA	0, 0
TNM staging		
Stage 0+ I: N, %	8, 53	8, 53
Stage II+III: N, %	7, 47	7, 47
Stage IV: N, %	0, 0	0, 0
Histological diagnosis		
Squamous cell carcinoma: N, %	3, 20.0	4, 26.6
Adenocarcinoma: N, %	10, 66.7	10, 66.7
Others: N, %	2, 13.3	1, 6.7
Blood parameters		
Total leucocytes/µL	6.46 (1.29)×10^3	8.88 (1.84) ×10^3***
Total neutrophils/µL	4.01 (1.22)×10^3	5.88 (1.74) ×10^3*
Total lymphocytes/µL	1.89 (0.55)×10^3	2.06 (0.81) ×10^3
Albumin (g/dL)	4.4 (0.22)	4.0 (0.60)*
Total proteins (g/dL)	6.9 (0.50)	6.4 (0.74)
Fibrinogen (mg/dL)	441 (160)	416 (58)
CRP (mg/dL)	3.03 (5.85)	6.63 (8.61)
GSV (mm/h)	11 (9)	23 (20)
Body weight loss, kg		
0, N, %	14, 93.3	14, 93.3
1-5, N, %	0, 0	0, 0
6-10, N, %	1, 6.7	1, 6.7

Continuous variables are presented as mean (standard deviation) while categorical variables are presented as the number of patients in each group and the percentage in the study group total population. *Definition of abbreviations*: N, number; kg, kilograms; m, metres; BMI, body mass index; FEV_1, forced expiratory volume in one second; FVC,

forced vital capacity; DL_{CO}, carbon monoxide transfer; K_{CO}, Krogh transfer factor; GOLD: Global initiative for Chronic Obstructive Lung Disease; NA, not applicable; TNM, tumor, nodes, metastasis; CRP, C-reactive protein; GSV, globular sedimentation velocity; L, liter. Statistical analyses and significance: * $p<0.05$, *** $p<0.001$ between LC-COPD patients and LC patients.

Figura (Figure)

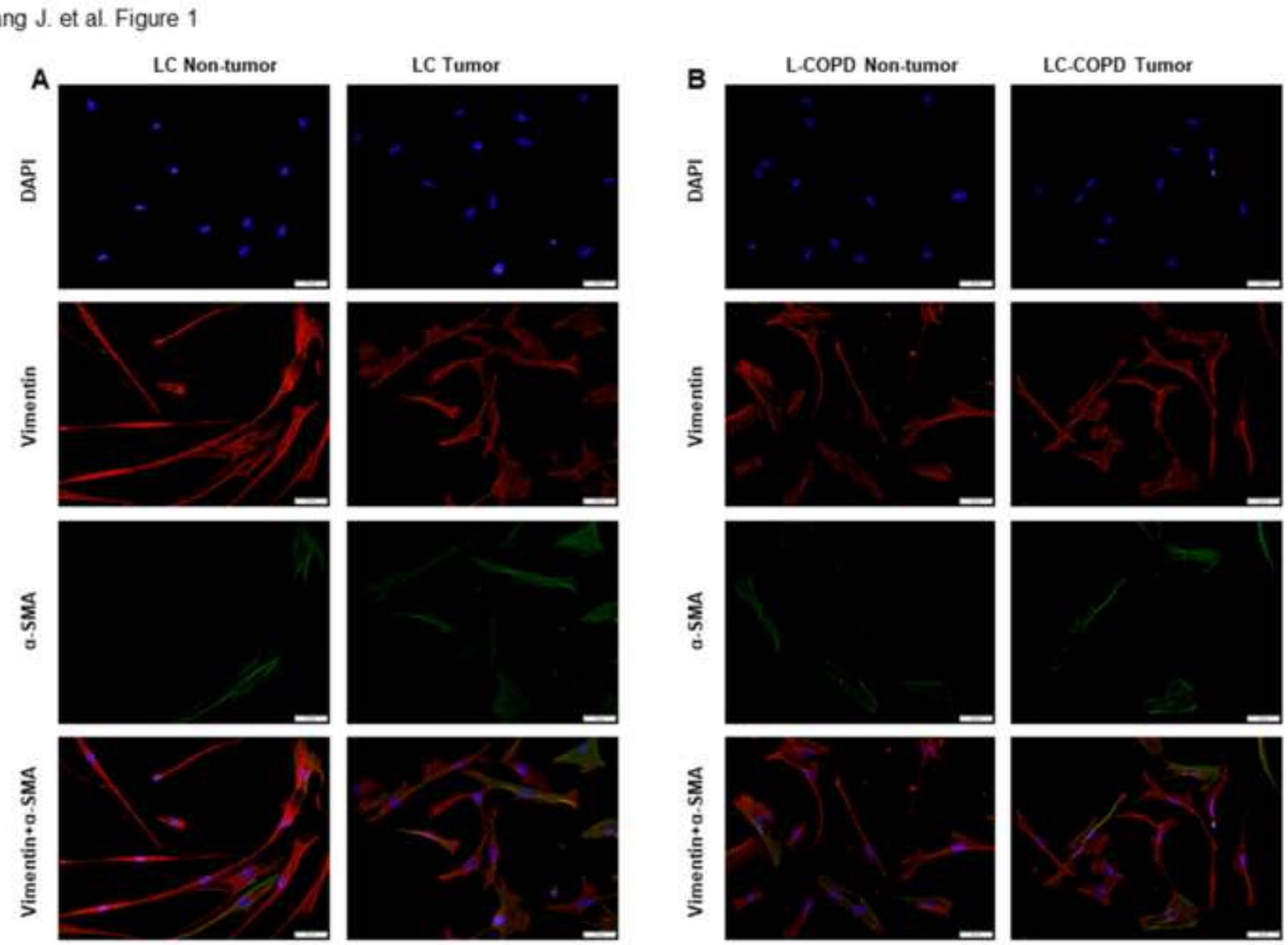


Figura (Figure)

Tang J. et al. Figure 2

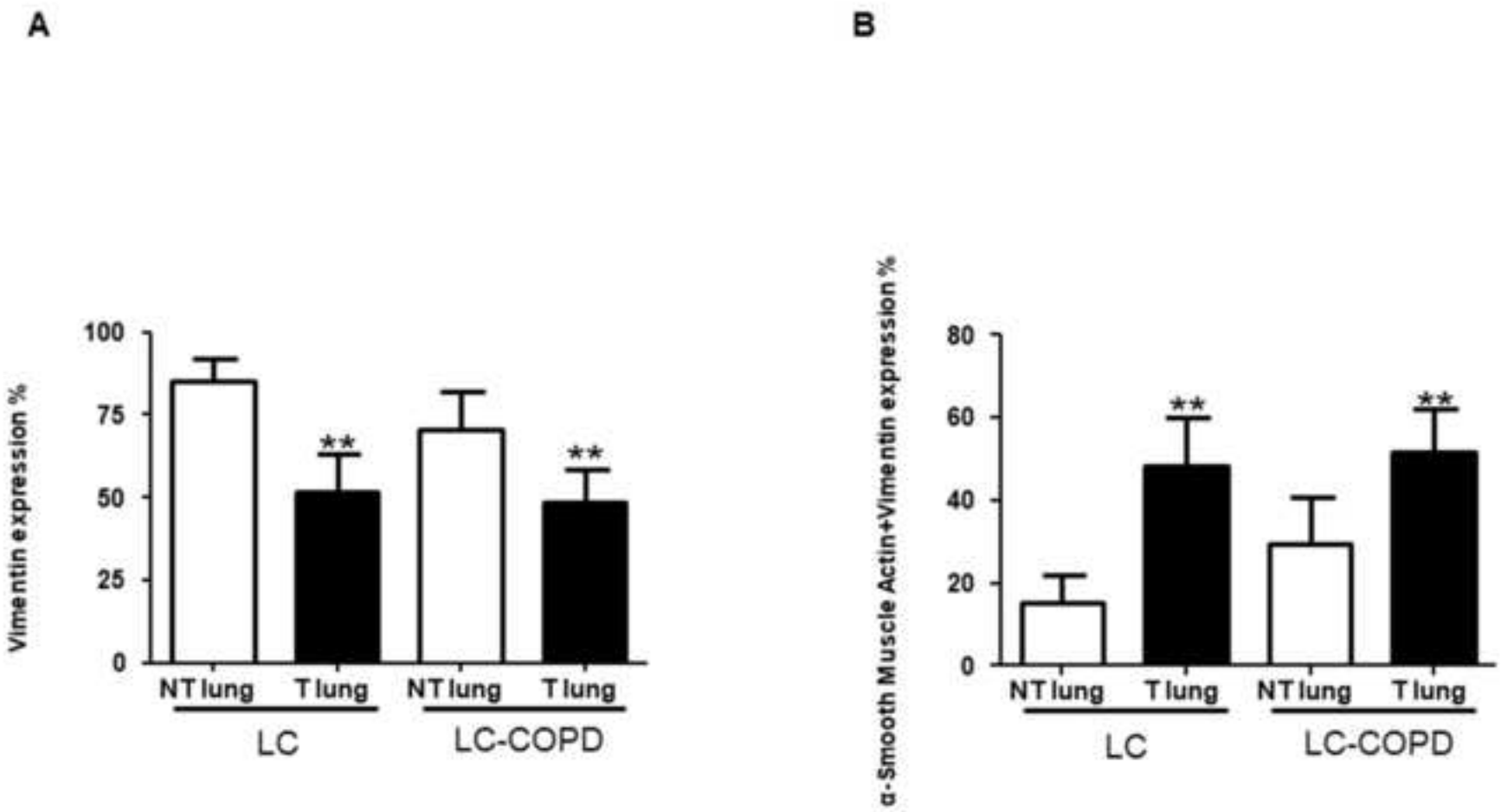


Figura (Figure)

Tang J. et al. Figure 3

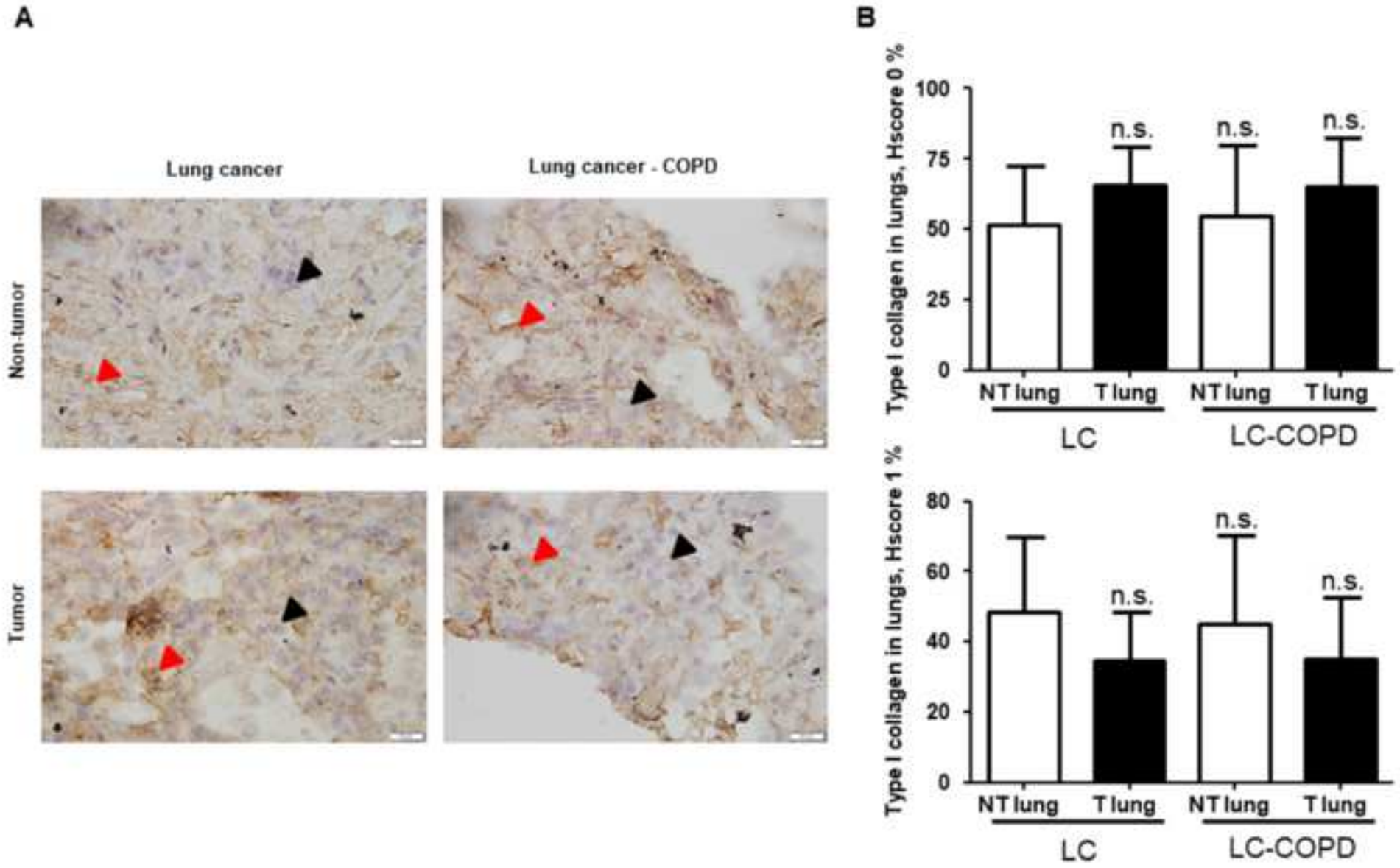
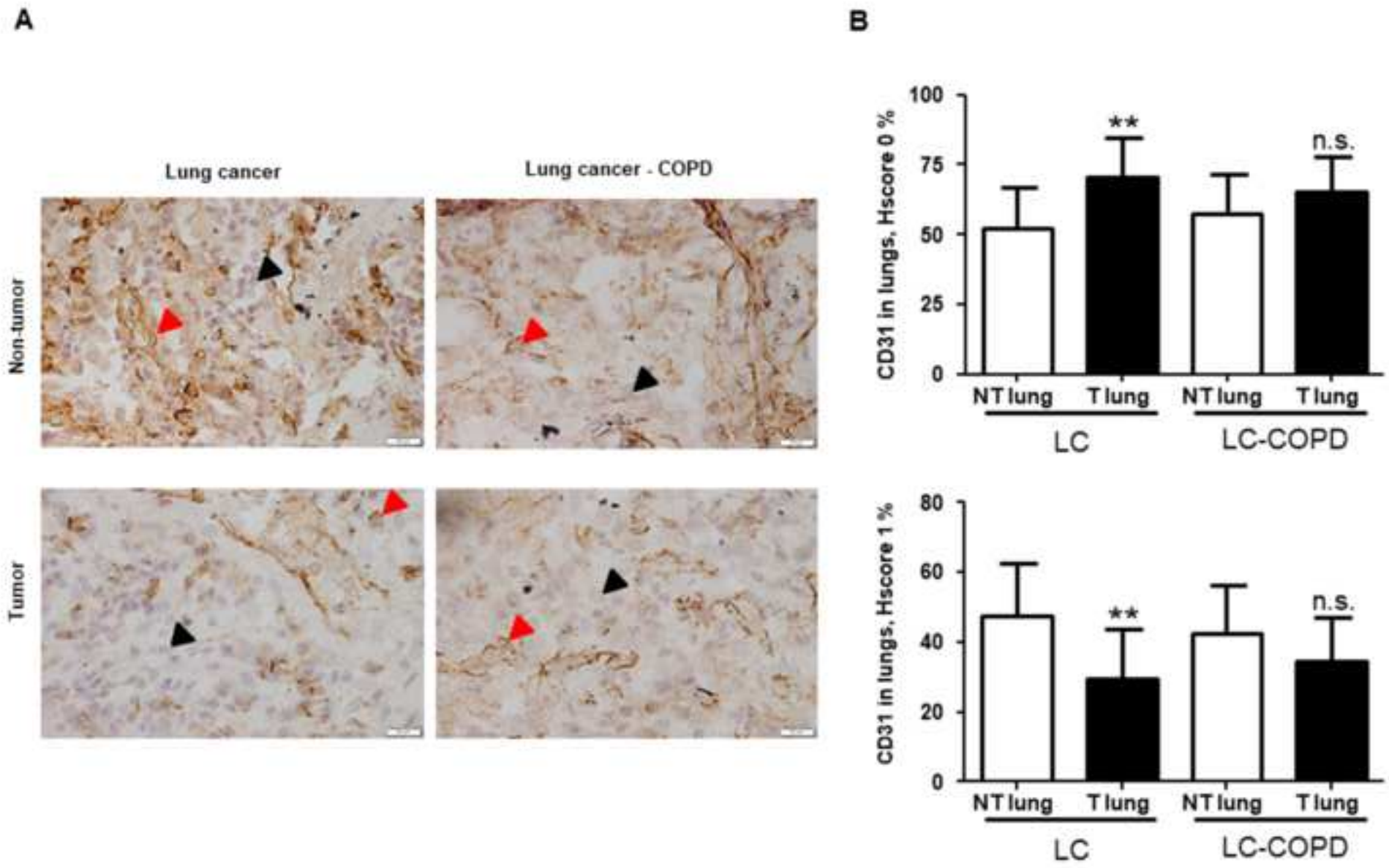


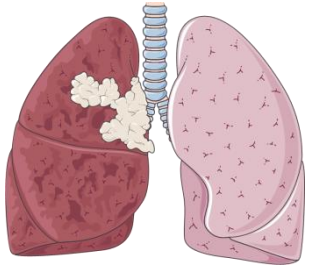
Figura (Figure)

Tang J. et al. Figure 4



Graphical/visual abstract

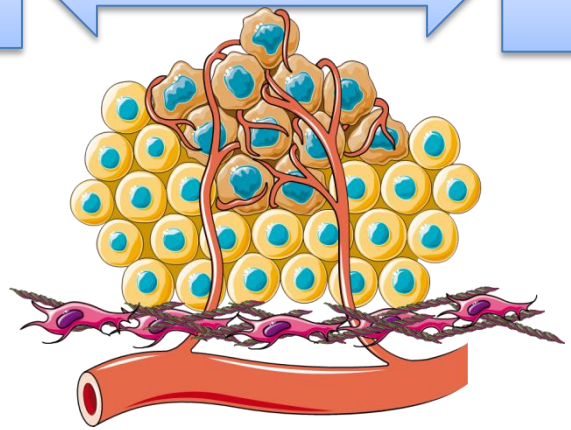
GRAPHICAL ABSTRACT



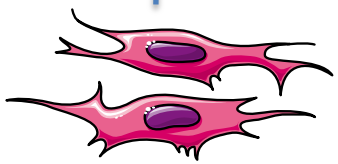
LC patients (n=15)

VERSUS

LC-COPD patients (n=15)



Tumor Stroma



CAFs

Type I Collagen

Endothelial cells

COPD did not influence the components of stroma within the tumor microenvironment in LC patients

Ética de la publicación

1. ¿Su trabajo ha comportado experimentación en animales?:
No

2. ¿En su trabajo intervienen pacientes o sujetos humanos?:
Sí

- Si la respuesta es afirmativa, por favor, mencione el comité ético que aprobó la investigación.:
institutional Ethics Committee on Human Investigation (protocol # 2008/3390/I, Hospital del Mar-IMIM, Barcelona)
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Sí