Universitat Autònoma de Barcelona

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PhD programme in Medicine

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UAB Doctoral Thesis/2021, Barcelona

For my parents and my brother

Agradecimientos

Los cuatro años de la tesis doctoral no son tan largos cuando ya estoy en el final del maratón. Hay momentos difíciles que sin vuestras ayudas y acompañamiento todo esto no habría sido posible. Gracias por haberme ayudado tanto en la vida profesional como en la vida personal. Gracias por haber aparecido en mi vida.

Antes de todo, querría agradecer a mi directora Dra. Esther Barreiro por su tutoría y persistente ayuda.

A la Dra. **Esther Barreiro**, por aceptarme a formar parte de su grupo de investigación y enseñarme cómo hacer la investigación. Muchas gracias por las oportunidades que me ha dado, por su enseñanza, su confianza, su dirección y sus esfuerzos en todos los artículos publicados y la revisión de la presente tesis doctoral. También doy las gracias por cuidarme mucho y sus consejos de cómo vivir en Barcelona.

Al Dr. **Juan Pedro-Botet Montoya** por haber aceptado de ser mi tutor. Muchas gracias por su confianza y su ayuda durante toda mi tesis.

Al Dr. **Víctor Curull** por su conceptualización del proyecto y el reclutamiento de todos los pacientes.

Al Dr. **José Yélamos** por su ayuda constante y valiosas recomendaciones en parte de mis experimentos.

Agradezco mucho a mis compañeros del laboratorio, **Daniel, Xuejie, Liyun, María Guitart, Laura, Anna, María Pérez, Antonio, Adrián, Carmen, Klara, Kati y Mariela** y por enseñarme las técnicas del laboratorio, sus ayudas tanto en el laboratorio como en la vida. Y gracias a mi compañera del piso **Xiaotong Xi** por su acompañamiento y apoyo. Gracias a todos vosotros, mis amigos.

Agradezco mucho al Dr. **Xavier Duran**, estadístico de nuestro centro, gracias por su ayuda y valiosos consejos para con todos los análisis estadísticos, que no han sido pocos.

También doy las gracias a todos los miembros del Hospital del Mar, entre ellos el Dr. **Alberto Rodríguez**, el Dr. **Rafael Aguiló** y la Dra. **Lara Pijuán** por sus aportaciones y ayuda con las muestras de los pacientes y la guía con las tinciones histológicas.

También doy las gracias a todos los **pacientes** que han participado en los diversos proyectos y estudios que conforman mi tesis doctoral.

Quisiera añadir también que los trabajos que conforman mi tesis doctoral han sido presentados a diversos congresos nacionales e internacionales (10 en total) tales como *European Respiratory Society* (ERS) International Congress, *American Thoracic Society* (ATS) International Congress, Jornadas Docentes del Centro de Investigación en Red de Enfermedades Respiratorias (CIBERES), Barcelona-Boston Lung Conference, Congreso Nacional de la Sociedad Española de Neumología y Cirugía Torácica (SEPAR) y Congreso *de la Societat Catalana de Pneumologia* (SOCAP). Algunos de los trabajos como: *Immunotherapy with monoclonal antibodies in lung cancer of mice: oxidative stress and other biological events* (XII Jornada de Formación CIBERES 2019), *Perfil Inmunológico y Supervicencia en Pacientes con Cáncer de Pulmón: Papel de la EPOC* (53º Congreso Nacional Virtual SEPAR, 2020) han sido premiados cada uno de ellos mediante una ayuda para asistir a un congreso nacional o internacional de la especialidad correspondiente (ámbito de las enfermedades respiratorias).

Al final, también quiero expresar mi agradecimiento a mis padres y hermano, aunque estamos lejos y con 7 horas de diferencia, sé que estáis siempre a mi lado.

¡A todos vosotros, muchas gracias!

ABBREVIATIONS

WHO: World Health Organization

NSCLC: non-small cell lung cancer

SCLC: small cell lung cancer

SCC: squamous cell lung carcinoma

CS: cigarette smoking

COPD: chronic obstructive pulmonary disease

GOLD: global initiative for chronic obstructive lung disease

GBD: global burden of disease

mMRC: modified British Medical Research

CAT: COPD assessment test

SEPAR: Spanish Society of Pulmonary and Thoracic Surgery

GesEPOC: guía española de la enfermedad pulmonar obstructiva crónica

LCS: lung cancer screening

LDCT: low-dose computed tomography

USPSTF: U.S. Preventive Services Task Force

MDA: malondialdehyde

SOD: superoxide dismutase

NF- κB: nuclear factor kB

miR: microRNA

TME: tumor microenvironment

ADP: adenosine di-phosphate

ECM: extracellular matrix

NK: natural killer

PD-L1: programmed death-ligand 1

Th: T helper

M: macrophage

IFN-γ: interferon-gamma

TNF: tumor necrosis factor

TGF: tumor growth factor

GM-CSF: granulocyte-macrophage colony-stimulating factor

MHC: major histocompatibility complex

BALF: bronchoalveolar lavage fluid

TILs: tumor-infiltrating lymphocytes

T reg: regulatory T cells

CTLA: cytotoxic T lymphocyte antigen

Tim: T-cell immunoglobulin domain and mucin domain

TLS: tertiary lymphoid structures

GC: germinal center

CXCL: chemokine (C-X-C motif) ligand

Bregs: regulatory B cells

MDSC: myeloid-derived suppressor cells

TAM: tumor-associated macrophage

SLO: secondary lymphoid structures

HEVs: high endothelial venules

IL: interleukin

nTregs: natural regulatory T cells

iTregs: induced regulatory T cells

APC: antigen presenting cells

CAF: cancer-associated fibroblast

SMA: smooth muscle actin

FAP: fibroblast activation protein

EMT: epithelial-mesenchymal transition

MMP: matrix metalloproteinases

LOX: lysyl oxidase

VEGF: vascular endothelial growth factor

BM: basement membrane

IM: interstitial matrix

EC: endothelial cell

Ang: angiopoietins

PDGF: platelet-derived growth factor

FGF: fibroblast growth factor

PECAM: Platelet Endothelial Cell Adhesion Molecule-1

OS: overall survival

SSB: single-strand DNA

BER: base excision repair

SSR: strand break repair

NER: nucleotide excision repair

HR: homologous recombination

NHEJ: non-homologous end joining

MMR: mismatch repair

PARP: poly ADP-ribose polymerase

VATS: video-associated thoracoscopic surgery

EGFR: epidermal growth factor receptor

KRAS: Kirsten rat sarcoma viral oncogene

ALK: anaplastic lymphoma kinase

BAX: BCL2 Associated X

BAK: BCL2-killer

DC: dendritic cell

LAG3: lymphocyte activation gene

ICI: immune checkpint inhibitor

BRAC: breast cancer gene

FDA: Food and Drug Administration

SLFN: Schlafen Family Member

PSF: progression-free survival

TMB: tumor mutational burden

ROS: reactive oxygen specie

TABLE OF CONTENT

ABSTRACT

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Background: Lung cancer (LC) is a leading cause of death worldwide. Chronic obstructive pulmonary disease (COPD) is a highly prevalent lung disease. COPD has been well established as an independent risk factor for lung tumorigenesis in patients. However, the biological mechanisms that explain the possible associations between lung cancer and COPD remain to be fully elucidated.

Hypothesis: The tumor microenvironment components (immune profile, stroma, cytokines, and PARP activation) may differ in tumors of lung cancer patients with and without COPD. Immunotherapy may also reduce tumor burden through several biological events.

Objectives: 1) Studies in patients: to elucidate the role of the biological events: tumor microenvironment, immune cell composition, stroma characteristics, and PARP overactivation in the process of tumorigenesis in tumors of patients with and without underlying COPD; 2) Mouse study: to evaluate the effects of immunotherapy on tumor burden through the analyses of several biological mechanisms such as oxidative stress, apoptosis, and autophagy.

Methods: Two models were used: 1) Studies in patients: 90 LC patients with underlying COPD and 43 LC-only patients were recruited from 2008 to 2019 from the *Lung Cancer Mar Cohort*, Barcelona. Lung tumor and the surrounding non-tumor lung specimens were obtained from all study patients through thoracotomy or video-assisted thoracoscopic surgery (VATS) prior to chemotherapy and/or radiotherapy; 2) Mouse study: Two groups of wild-type BALB/C mice with experimental lung cancer (subcutaneous inoculation of LP07 adenocarcinoma cells in the left flank of mice) were established: treated and non-treated mice, n=9/group. In the treatment group, lung cancer mice were treated with a cocktail of monoclonal antibodies (intraperitoneal injection, anti-PD-L1, anti-CTLA-4, anti-CD19, and anti-CD137). Lung tumors were obtained from all mice. Biological analysis: laboratory techniques such as western-blot, immunohistochemistry,

ELISA, cell culture, and immunofluorescence were used to assess the target biological markers in each study.

Results: 1) Studies in patients: lung tumors of patients with underlying COPD showed lower levels of tertiary lymphoid structures (TLSs) compared to lung cancer only patients. Moreover, lower levels of TLS and B cells in lung tumors were associated with poorer 10-year overall survival rates of patients, especially in those with underlying COPD. In tumor stroma, the presence of COPD did not elicit any significant difference in levels of extracellular matrix, cancer-associated fibroblasts or endothelial cells. In addition, DNA damage and PARP activation levels were higher only in lung tumors of patients with underlying COPD, while PARP-1 and PARP-2 enzyme expression levels were lower in lung tumors compared to non-tumor specimens irrespective of the presence of COPD. 2) Mouse study: treatment with immunotherapy reduced tumor burden through increased levels of oxidative stress, apoptosis, autophagy, and signaling pathways such as NF-kB and sirtuin-1 in tumors of the treated mice compared to tumors of non-treated animals.

Conclusions: Tumor immune microenvironment, stroma components, and PARP are differentially expressed in lung tumors of lung cancer patients with underlying COPD. The reduction in TLS and GC formation, the rise in DNA damage, and PARP overactivation probably contribute to the greater susceptibility of COPD patients to develop lung tumors. In mice treated with the combination of monoclonal antibodies, increased levels of oxidative stress along with activated apoptosis and autophagy may be part of the mechanisms whereby immunotherapy may reduce tumor burden. In conclusion, the presence of COPD should be considered when designing therapeutic strategies of lung cancer including immunotherapy as well as PARP activity inhibition.

RESUMEN

RESUMEN

Introducción: El cáncer de pulmón (CP) constituye una de las principales causas de muerte en el mundo. La enfermedad pulmonar obstructiva crónica (EPOC) es de alta prevalencia. La EPOC es un factor de riesgo independiente para el desarrollo de CP en los pacientes. A pesar de los avances recientes, aun quedan mecanismos por dilucidar que ayuden a comprender mejor las relaciones entre EPOC y CP.

Hipótesis: Los componentes del microambiente tumoral (el perfil inmunitario, el estroma, las citocinas y la activación de PARP) pueden diferir en los tumores de pacientes con CP con y sin EPOC. La inmunoterapia también puede reducir la carga tumoral a través de varios mecanismos biológicos.

Objetivos: 1) Estudios en pacientes: estudiar el papel de los siguientes mecanismos biológicos: el microambiente tumoral, las células inmunes, las características del estroma y la sobreactivación de PARP en el proceso de tumorigénesis en pacientes con CP con y sin EPOC. 2) Estudio en ratones: evaluar los efectos de la inmunoterapia en el tamño tumoral mediante el análisis de varios mecanismos biológicos como el estrés oxidativo, la apoptosis y la autofagia.

Métodos: Se utilizaron dos modelos: 1) Estudios en pacientes: se reclutaron 90 pacientes con EPOC-CP y 43 pacientes solo con CP procedentes de *la Cohorte Cáncer de Pulmón Mar,* Barcelona, desde el año 2008 hasta el 2019. Las muestras pulmonares tumorales y no tumorales se obtuvieron en todos los pacientes mediante toracotomía o cirugía toracoscópica asistida por video (VATS), siempre previo a la quimioterapia y/o radioterapia. 2) Estudio en ratones: se establecieron dos grupos de ratones BALB /c con CP inducido mediante la inoculación subcutánea de células de adenocarcinoma pulmonar LP07: ratones tratados y no tratados, n= 9/grupo. A los ratones tratados con CP se les administró mediante inyección intraperitoneal un cóctel de anticuerpos monoclonales (anti-PD-L1, anti-CTLA-4, anti-CD19 y anti-CD137) y una solución

tampón (PBS) a los ratones control (con tumor sin tratamiento). Se obtuvieron los tumores en todos los ratones al final del estudio (30 días). Análisis biológico: se utilizaron técnicas de laboratorio como el Western-blot, la inmunohistoquímica, el ELISA, los cultivos celulares, y la inmunofluorescencia para evaluar los marcadores biológicos objeto de estudio en cada modelo y tipos de muestras.

Resultados: 1) Estudios en pacientes: los tumores pulmonares de pacientes con EPOC mostraron niveles más bajos de estructuras linfoides terciarias (ETLs) y centros germinales (CG) respecto de los pacientes sin EPOC. Además, los niveles más bajos de ELTs y células B en los tumores pulmonares se asociaron con una peor supervivencia a 10 años, especialmente en aquéllos con EPOC. En el estroma tumoral, la presencia de EPOC no se asoció a diferencias en los componentes del estroma tales como la matriz extracelular, los fibroblastos asociados al cáncer o las células endoteliales. Además, los niveles de daño del ADN y la consiguiente activación de PARP estaban más elevados solamente en los tumores pulmonares de los pacientes con EPOC, mientras que la expresión de las enzimas PARP-1 y PARP-2 estaban disminuidas en los tumores pulmonares respecto de las muestras no tumorales, independientemente de la presencia de EPOC. 2) Estudio en ratones: la inmunoterapia redujo la carga tumoral a través del aumento de los niveles del estrés oxidativo, apoptosis, autofagia y de vías de señalización como NF-kB y sirtuin-1 en tumores de los ratones tratados respecto de los tumores procedentes de ratones con CP sin inmunoterapia.

Conclusiones: El microambiente inmunológico tumoral, los componentes del estroma y la actividad de PARP se expresan de forma claramente diferenciada en los tumores pulmonares de pacientes con CP con EPOC respecto de los pacientes sin EPOC. La reducción en la formación de ELTs y CGs, el aumento en el daño del ADN y la sobreactivación de PARP probablemente contribuyan a la mayor susceptibilidad para desarrollar CP observada en pacientes con EPOC. En ratones tratados con la combinación de anticuerpos monoclonales (inmunoterapia), el aumento de los niveles de estrés oxidativo junto con la activación de apoptosis y autofagia pueden ser parte de los mecanismos mediante los cuales la inmunoterapia reduce la carga tumoral. En resumen, la

presencia de EPOC debe tenerse en cuenta en el diseño de terapias para el CP, incluidas la inmunoterapia y la inhibición de la activación de PARP.

1. INTRODUCTION

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1.1 Lung cancer

Lung cancer is the uncontrolled growth of malignant cells in the lungs and airways. Lung cancer is one of the leading causes of cancer incidence and mortality worldwide. According to the World Health Organization (WHO) GLOBOCAN, lung cancer is the third most common cancer type after breast and prostate cancer of both sexes and the first in cancer death in 2018 (1). In China, lung cancer remains the most incident and deadly cancer type (1) (Figure 1).

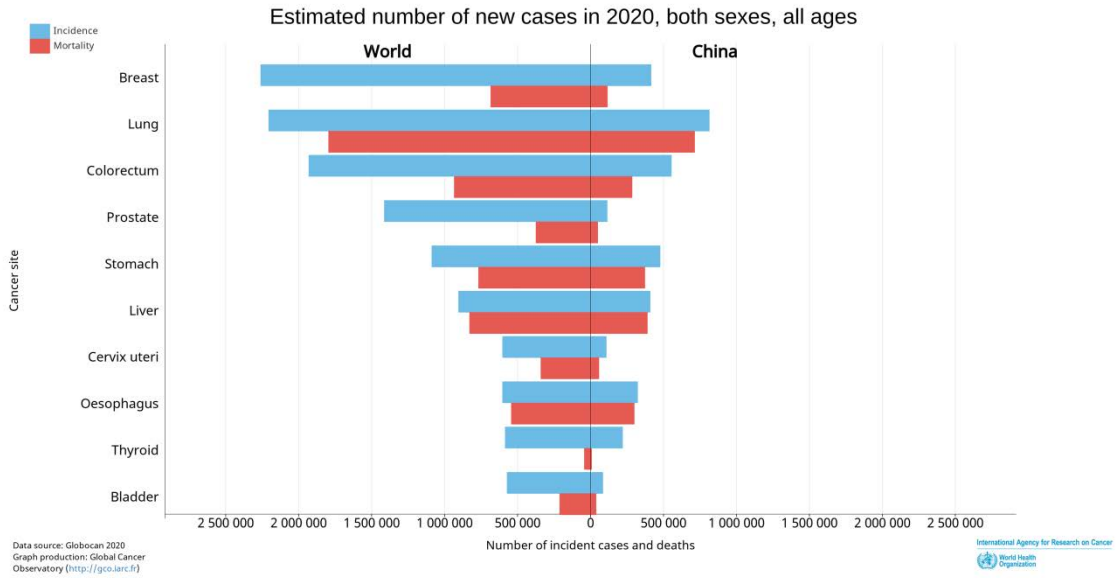


Figure 1. Bar chat of first ten cancer types for incidence and mortality all over the world and in China in 2020. Data source: GLOBOCAN 2020.

With the increasing awareness of the importance of quitting smoking, the application of smokefree policies, and the improvement of medical diagnosis and treatment, lung cancer mortality has declined by 4% per year from 2008 to 2017. However, patients' 5-year survival rates remain very poor with only 19%, which is one of the worst survival outcomes of all cancer types (2,3). This may be due to the delays in lung cancer diagnosis in asymptomatic or non-specific cases.

The principal symptoms of lung cancer are progressing cough, chest pain, hemoptysis, asthenia, weight and appetite loss, and paraneoplastic syndromes (4). However, most lung cancer patients do not present any symptoms until the

disease has spread. Studies showed that over two-thirds of the lung cancers had been diagnosed at advanced stages (stage III-IV) when the curative treatment is no longer suitable for them (5,6).

1.1.1 Lung cancer histological classification

Two main lung cancer histological subtypes are established: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is the most common subtype which composes about 85% of all lung cancer cases while only 15% of lung cancer cases are SCLC (7,8). NSCLC is also the studied cancer type of the current thesis.

NSCLC is furtherly classified into three subtypes: 1) adenocarcinoma, the most common type of lung cancer, arises from epithelial of the small airway and type II alveolar cells in the peripheral lung, accounts for about 40% of lung cancers; 2) squamous cell lung carcinoma (SCC), arises from neuroendocrine cells in the center of the lungs, compromises 25-30% of all lung cancer cases and is closely correlated with a smoking history; and 3) large-cell lung carcinoma, the undifferentiated malignant tumor arising from transformed epithelial cells in the central lungs, constitutes 5-10% of all lung cancer cases (9). Due to the new, light-yield cigarettes, lung cancer has shifted from trachea and bronchus to peripheral lung, and histologically from SCC to adenocarcinoma (10) (Figure 2).

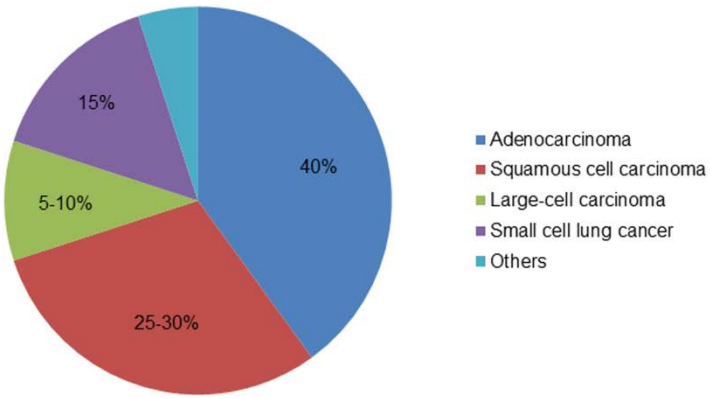


Figure 2. Pie chart of lung cancer histological subtypes.

1.1.2 Risk factors for lung cancer

Lung cancer is the consequence of the interaction between exposure to etiologic agents and individual intrinsic susceptibility. Among the etiologic agents, cigarette smoking (CS) is the most important and evident factor for all major histological types of lung cancer (11). Almost 80% of the lung cancer cases in men and 50% in women are related to CS. For smokers, the risk to develop lung cancer is 20-50 fold higher compared to never-smokers (12).

Cigarette smoke contains more than 7,000 chemicals and about 70 of them are carcinogenic (13). When these carcinogenic chemicals enter the respiratory tract, they can injury the ciliated cells of the respiratory epithelium directly or indirectly by the induced oxidative stress and DNA damages and then lead to carcinogenesis (14). In addition to voluntary tobacco smoking, passive exposure to cigarette smoke is another smoking-related cause of lung cancer. Certainly, smoking cessation can successfully reduce the lung cancer risk, but studies showed the excess risk continues even in long-term smoking quitters during the whole life (15).

Apart from CS, other etiologic agents such as occupational exposures, outdoor and indoor air pollution, physical activity, diet, and genetic factors also demonstrated to contribute to lung cancer development (16).

Importantly, the previous existing lung diseases also have been demonstrated to increase lung cancer risk (17). In a large cohort of 716,872 subjects, patients with tuberculosis showed 11 fold higher of lung cancer incidence than those without this entity, and the risk is even higher in those with combined tuberculosis and chronic obstructive pulmonary disease (COPD) (18). Bronchiectasis is also shown to associate with lung cancer. A large cohort of 57,576 patients with bronchiectasis proffered a 2.36 fold increased risk of lung cancer compared to the control population without this pathology (19). The presence of pneumonia is also linked with higher lung cancer risk (20,21). The association between asthma and lung cancer is debatable as some studies showed higher risks (22,23) of lung cancer in asthmatic patients while others demonstrated inverse or weak relations (17,24). The role of COPD as a risk factor

for lung cancer has been well established. This association will be introduced in detail in the two following sections.

1.2 Chronic obstructive pulmonary disease (COPD)

COPD is defined as “a common, preventable and treatable disease characterized by persistent respiratory symptoms and airflow limitation due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases” by the Global Initiative for Chronic Obstructive Lung Disease (GOLD), 2019 (25).

COPD is the fourth leading cause of death worldwide and will rank third in 2030 according to WHO estimation (25,26). Global Burden of Disease (GBD) had reported 251 million new cases of COPD worldwide in 2016 (27) and also claimed that COPD had already been the third leading cause of death globally in 2017, which had occurred thirteen years earlier than predicted WHO (28). In China, a large cohort estimated that 8.6% of adults have COPD, which means almost 100 million Chinese are suffering from this disease (29). In Spain EPISCAN II recently reported a prevalence of 11.8% of COPD burden in a large cohort of 12,825 subjects (30).

The principal symptoms of COPD include shortness of breath especially during physical activities, chronic cough with/without mucus, recurrent respiratory infections, and other symptoms such as wheezing, chest tightness, and lack of energy (25,31). The diagnosis of COPD is confirmed by the presence of irreversible airflow limitation with a post-bronchodilator $FEV_1/FVC<0.70$ through a spirometry test (25,32).

Similar to lung cancer, CS is also the principal risk factor for COPD development in addition to ageing, genetic susceptibility (alpha-1-antitrypsin deficiency), and other irritants such as air pollution, second / third-hand smoke, fumes and heating fuels (33–35).

GOLD guideline is the most widely used to classify COPD. COPD patients are classified as A, B, C, D depending on the combination of three factors: the severity of airflow limitation based on post-bronchodilator FEV_1, dyspnea severity (using modified British Medical Research Council (mMRC), symptoms using the

COPD assessment test (CAT™) and exacerbation history in the previous year (Figure 3) (25).

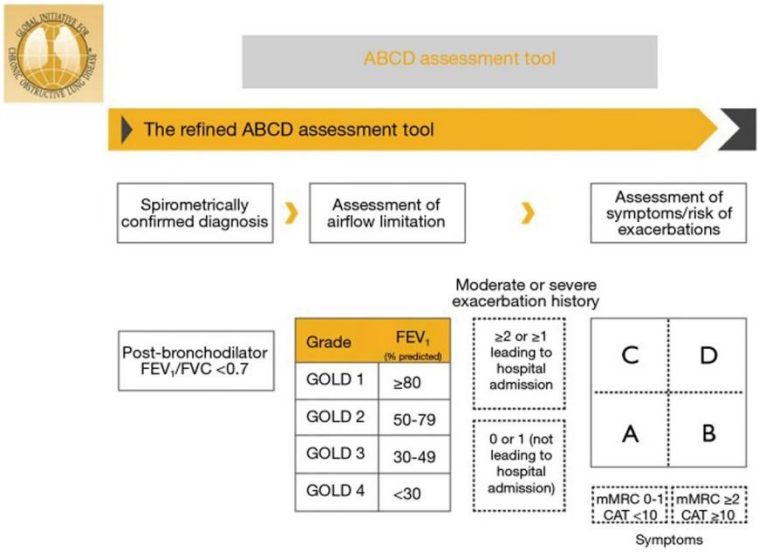


Figure 3. The refined COPD GOLD ABCD assessment tool (adapted from Global Initiative for Chronic Obstructive Lung Disease 2019 guideline (25).

In 2012, the Spanish Society of Pulmonary and Thoracic Surgery (SEPAR) published the first clinical practice COPD guideline (GesEPOC). This guideline provides the COPD phenotype based on the patients' clinical manifestations and exacerbation frequency to guide the personalized treatment (36,37). In the recent GesEPOC guideline of 2017, COPD is classified in four clinical phenotypes: non-exacerbator, mixed COPD-asthma, exacerbator with emphysema, and exacerbator with chronic bronchitis as shown in the adapted Figure 4 (38).

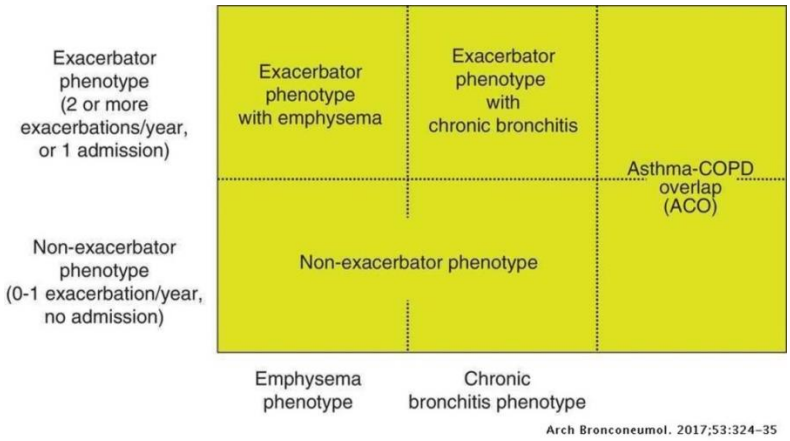


Figure 4. COPD phenotypes according to GesEPOC guideline of 2017 adapted from (38).

1.3 Associations between lung cancer and COPD

Lung cancer and COPD share the common crucial risk factor-cigarette smoking. Among the extensive smoking-associated diseases, COPD is the most common one, and lung cancer is the most deadly one. COPD prevalence in lung cancer is between 8% to 51% (39,40). Importantly, 65-80% of COPD may be under-diagnosed worldwide (41,42). Meanwhile, the over-diagnosis of COPD also challenges public health, between 30% to 60% of previously diagnosed COPD patients actually have normal spirometric lung function parameters (41,42).

For decades, the high prevalence of COPD in lung cancer patients was attributed to the common etiological factor-smoking. However, in 1986 Skillrud et al. firstly reported that COPD per se was an independent risk factor to develop lung cancer (43). Since then, numerous epidemiological studies have extensively strengthened this evidence (44–46). In a recent study with 334,548 individuals without baseline lung cancer, after a median followed-up of 7 years, COPD patients presented a high risk of lung cancer irrespectively of their smoking status (47). In non-smokers, COPD also promoted a 2.67 fold higher risk of lung cancer development compared to non-smokers without COPD (47). In addition, both emphysema and chronic airway obstruction conditions were demonstrated to be associated with higher susceptibility to lung cancer regardless of CS, especially the phenotype emphysema (48,49).

1.3.1 Lung cancer screening in COPD

For lung cancer screening (LCS), low-dose computed tomography (LDCT) is recommended in current and ex-smokers (who have quit smoking within the past 15 years) having a 30 pack-years smoking history annually by the U.S. Preventive Services Task Force (USPSTF) (50). This screening strategy properly reduced lung cancer mortality compared to the radiography screening group (51). However, the decreasing trend of lung cancer patients meeting the USPSTF criteria in a large cohort from 1984 to 2011 indicating the need for a more sensitive screen approach (52). For saving more lives, in 2020 the American College of Radiology recommends extending the LCS criteria to 20 pack-years and 20 years of quit-smoking history (53). Furthermore, since the high lung cancer risk in COPD patients, several screening studies were conducted in COPD patients. Lowry et al.

demonstrated better life expectancy screening lung cancer in COPD patients who smoked ≥1 pack per year than the current LCS recommendation (53). Torres et al. validated a COPD lung cancer score (COPD-LUCSS) as a good predictor of lung cancer risk in COPD patients (54). Interestingly, as the population eligible for LCS also are those at risk of COPD, Ruparel et al. found that adding spirometry in LCS could reduce the high COPD under-diagnosis rates (55).

1.3.2 Common mechanisms in lung cancer and COPD

Despite the strong clinical and epidemiological evidence of the associations of lung cancer and COPD, the biological mechanisms that link these two entities remain unclear. Although cigarette smoke can directly or indirectly trigger both pathologies, only 15-25% of smokers are estimated to develop COPD, and 10-15% of smokers develop lung cancer in their lifetime. Importantly, 15-20% of lung cancer in men and 50% of lung cancer in women are never-smokers (56). These results indicate other intrinsic mechanisms may be the cause of the association between lung cancer and COPD.

Indeed, lung cancer and COPD present opposite natures. Lung cancer is a condition characterized by uncontrolled continuous cell proliferation, avoidance of apoptosis, and promotion of new blood vessels (57). Conversely, COPD, especially the subtype emphysema consists of matrix structure destruction, alveolar septal cell apoptosis, and loss of blood supply (58). Notably, these two pathologies share some key altered mechanisms such as oxidative stress, inflammation, immune profile, genetic and epigenetic regulation, epithelial-mesenchymal transition, DNA damage, and repair. These shared mechanisms may be candidates to explain the links between these two diseases.

Oxidative and noxious stress is a key pathogenetic mechanism in COPD (59). These stress processes occur when the excessive free radicals in cigarette smoke exceed the cell antioxidant capacity to clean them (60). Oxidative and noxious stress can promote DNA damages in cells which may result in carcinogenesis if unrepaired or improperly repaired (60). These stress conditions can also degrade tumor suppressor proteins which subsequently increase cell division, decrease cell apoptosis and DNA repair and contribute to tumor development and progression (61). Interestingly, Mateu-Jiménez et al. found that protein oxidation forms include

malondialdehyde (MDA) protein adduct, species superoxide anion, protein carbonylation, and nitration levels increased while antioxidant enzyme superoxide dismutase (SOD) 2 and glutathione levels decreased in NSCLC patients with underlying COPD compared to those without this condition (62). These results may indicate that the oxidative and noxious stress may be enrolled in COPD susceptibility to lung cancer development.

Chronic inflammation is an important feature in COPD. The critical role of chronic inflammation in cancer development is well established in different cancer types (63,64). In general, neutrophil released reactive oxygen species might damage DNA and trigger tumorigenesis. In addition, the key inflammation-induced carcinogenesis mediator NF- κB is overactivated in COPD (62). NF- κB signaling pathway was demonstrated to control epithelial-mesenchymal transition, upregulate the matrix metalloproteinases and vascular endothelial growth factor, and suppress tumor suppressor p53 which may trigger tumorigenesis and tumor progression (65). Other inflammation and proliferation common pathways such as PI3K (66) and Wnt (67) pathways which are important drivers of cell proliferation and apoptosis suppression were also increased in COPD patients. All these pieces of evidence may add tools to explain the chronic lung inflammation in COPD patients to trigger carcinogenesis.

Additionally, epigenetic events may also play a role in COPD and lung cancer links. Cigarette smoke, oxidative stress, and inflammation are proven to promote genome instability and also epigenetic changes (68). Epigenetic changes are those heritable gene expression modifications which no alter DNA sequence. The most important epigenetic changes are DNA hypermethylation, histone modifications, and microRNAs alteration and subsequently, alter their downstream mechanisms (68). Mateu-Jiménez et al. found that DNA methylation and expression levels of miR-21, miR-210, miR-let7c increased along with downregulated downstream markers *PTEN, MARCKs, TPM-1, PDCD4, SPRY-2, ETS-1, ZEB-2, FGFRL-1, EFNA-3, and k-RAS* expression levels in lung tumors of COPD patients compared to those without COPD (68). Downregulation of these genes may upregulate their target cell processes and cause cell proliferation,

tumor development and metastasis indicating the epigenetic links between COPD and lung cancer (Figure 5).

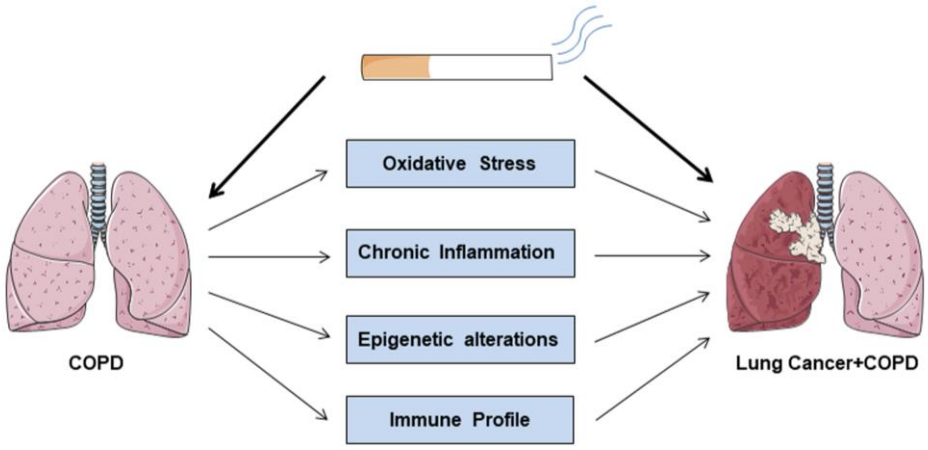


Figure 5. Biological mechanisms of previous studies to link COPD and lung cancer.

Apart from the above explained biological mechanisms linking lung cancer and COPD, the tumor microenvironment (TME), in particular, the immune TME and DNA repair enzyme-poly (ADP) ribose polymerase family also play crucial roles in both pathologies (69,70). These mechanisms are studied in lung cancer patients with and without underlying COPD in the present thesis. The scientific evidences of these mechanisms are explained in the following sections.

1.4 Lung cancer and COPD pathogenesis-TME

In recent years, the complexity of tumors has been recognized to exceed that of normal tissues, and tumor is not considered as a homogeneous collection of tumor cells that grow uncontrollably (71). In fact, when cancer cells are established in the injury field, they interact constantly with the surrounding non-malignant components and create the tumor microenvironment (TME) (Figure 6) (71).

As healthy organs, solid tumors also contain two regions: a parenchyma region and a stroma region. But the basal lamina between these two regions is incomplete in tumors unlike in the healthy organs, they are poorly defined and closely interacted (71). This crosstalk between tumor cells and their microenvironment is dynamic and constant: cancer cells can change their microenvironment by secreting extracellular signals. Vice versa, the TME can also

affect tumor growth and progression. In all cancer stages, TME changes according to the requirement of the cancer cells and always favors cancer progression (72). The components of TME are recognized as cancer hallmarks of tumor proliferation, angiogenesis, invasion, metastasis, and also therapeutic resistance. In lung cancer, studies also demonstrated the predictive and therapeutic role of the TME (73).

The non-malignant of the TME include immune cells, blood vessels, extracellular matrix (ECM), fibroblasts, pericytes, adipocytes, and the secreted mediators (Figure 6) (74). In the current thesis, the TME, especially, the immune TME, the stroma components, several cytokines, DNA damage and repair mechanisms are studied in patients to elucidate the links between COPD and lung cancer. A short introduction of these elements will show below.

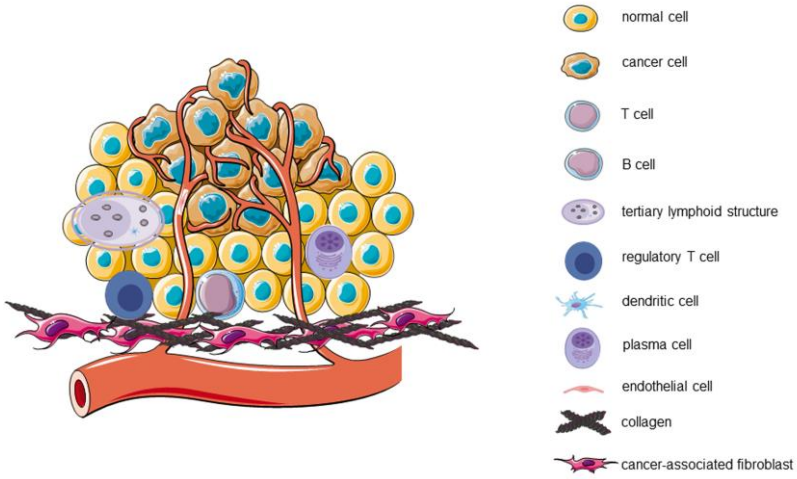


Figure 6. Tumor microenvironment.

1.4.1 Immune TME

The immune system is a crucial defense tool of the host against foreign invaders (75). The immune system comprises two major components: the innate immunity and the adaptive immunity. Innate immunity, the non-specific and antigen-independent immune system, is the first line of defense of the host (75). It contains natural killer cells (NK), macrophages, neutrophils, dendritic cells, mast cells, basophils, and eosinophils, and responds immediately after pathogen invasion. The second defense line, the adaptive immunity, is specific and antigen-

dependent. It is comprised principally of T and B lymphocytes which induce highly specific, long-lasting immune response (75). The tumor-infiltrating immune cells are composed principally of T and B lymphocytes along with macrophages, a few dendritic cells, and natural killer cells within the lung microenvironment (76).

However, the immune system may also promote tumor development through a continuous dynamic process between cancer cells and the immune cells, namely immune-editing (77). In both lung cancer and COPD conditions, patients have similar dysfunctional immune cell profiles including the dysregulation of T cells, neutrophils, and macrophages, also present T-cell exhaustion with increased programmed death-ligand 1 $(PD\text{-}L1)^{+}$ cells levels, increased regulatory T cells, and myeloid-derived suppressor cells (78). Nonetheless, the characteristics of the immune cells differ in COPD patients from those in lung cancer conditions. In lung cancer, as the most solid tumor types, immune cells are polarized towards a type 2 T helper (Th2) phenotype, and with the corresponding activated type 2 macrophage (M2) phenotype (70). But in COPD, the predominant immune cell phenotype is Th1, and the released cytokine interferon-gamma induces a mixed phenotype of macrophages which show cytotoxic profile (70).

Interestingly, Mateu-Jiménez et al. showed that in blood and lung tumor samples of early stages of lung cancer-COPD patients, Th1 cytokines (TNF-alfa, IL-2) levels and the ratio of M1/M2 macrophages increased, while Th2 cytokines (TGF-beta, IL-10) decreased compared to lung cancer only patients (79). These data suggest that COPD altered the tumor immune profile from the Th2 phenotype (pro-tumor effect) to the Th1 phenotype (anti-tumor effect) in the early stages of lung cancer (79). The specific role of the immune system in LC and the influence of COPD should be further investigated. The major components of the immune cells in lung cancer and COPD conditions are briefly described below.

1.4.1.1 Innate immune cells: Natural Killer

Natural killer (NK) belongs to the innate immune system along with other components such as physical anatomical barriers, dendritic cells, and leucocytes (80). They are the third-largest population of lymphocytes after T and B cells, account for 5-15% of peripheral blood mononuclear cells in humans (81). Mostly,

NKs present in the blood, lymph nodes, spleen, tonsils, thymus, and bone marrow. In human lungs, NKs represents about 10% of the lymphocytes in healthy conditions (82). During infected and tumor conditions, they can migrate to the infected tissue regulating by different classes of chemoattractants (83).

NKs are not only cell killers but also immune regulators. One of the main functions of NKs is natural cytotoxicity (84). They can exert anti-viral and anti-tumor responses through two mechanisms: 1) release cytotoxic molecules granzymes and perforin leading to cell lysis or apoptosis without pre-stimulation; 2) NKs activation which is induced by the antibody-opsonized infected cells can release the cytolytic granules and cause cell apoptosis (85).

Another important role of the NKs is the regulation of the immune system by their released cytokines. Principally, NKs secrete interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), IL-10, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF). These cytokines can stimulate dendritic cells and influence immune cell polarization. Additionally, IFN-γ presents anti-tumor properties by the induction of major histocompatibility complex (MHC) I expression and activation of cytotoxic T lymphocytes (86).

In cancer conditions, NKs produce cytolytic molecules and antitumor cytokines such as perforin, granzymes, and IFN-γ which are crucial to inhibit tumor progression (87). Studies have demonstrated that NKs infiltration degree and the NKs-derived IFN-γ levels are positively associated with patients' survival rates in different cancer types including lung cancer (87,88). However, cancer cells can release immune-suppressive cytokines such as IL-10 and TGF- β to repress NKs function (89). In lung cancer patients, higher levels of IL-10 and TGF- β were observed in lung tumors and associated with poor prognosis (89). Furthermore, a recent study reported that lung cancer patients treated with the combined immunotherapy of NKs and anti-PD-1 showed longer overall survival than those with only anti-PD-1antibody (90).

In chronic respiratory conditions, NKs levels significantly increased in bronchoalveolar lavage fluid (BALF) and peripheral blood with increased cytotoxicity and granzyme B levels of COPD patients and smokers compared to

healthy subjects (91,92). This increased level of NKs may contribute to COPD susceptibility, while the role of NKs in lung cancer patients with underlying COPD is still unclear. Additionally, NK cell-based therapy may have a promising therapeutic potential in lung cancer and COPD patients.

1.4.1.2 Adaptive immune cells

Among all the tumor-infiltrating immune cells, T and B lymphocytes represent two-thirds of them (93). They can elicit dynamic responses according to their cell subtypes during tumor progression. Evidence strengthens the important impact of tumor-infiltrating lymphocytes (TILs) on the clinical outcomes of different cancer conditions including lung cancer (94–98).

1.4.1.2.1 Tumor-infiltrating T lymphocytes (TILs-T)

As the major force of adaptive immunity, the multifaceted functions of tumor-infiltrating T lymphocytes (TILs-T) are well studied. T lymphocytes contain two principal subtypes: $CD4^+$ helper T cell and $CD8^+$ cytotoxic T cell (99). The $CD4^+$ helper T cells are composed of Th1, Th2, Th17, and T regulatory (T reg) subsets (100). For most solid tumor types, $CD8^+$ cytotoxic T cells and $CD4^+$ Th1 cells are involved in type I immune response and show an anti-tumor effect (101). While $CD4^+$ Th2 cells, Th17, and regulatory T cells (Treg) present pro-tumor function and are associated with detrimental prognosis in patients (102).

In addition, T cell functions are compromised in chronic inflammation and tumor circumstances as a result of persistent expressions of immune-checkpoints such as (PD-L1), cytotoxic T lymphocyte antigen 4 (CTLA-4), T-cell immunoglobulin domain, and mucin domain-3 (Tim-3) among others (103). Studies showed that T cell-targeted immunotherapies with immune checkpoints inhibitors and chimeric antigen receptor-T cell therapy improved clinical outputs in lung cancer patients (104–106). Nonetheless, just a section of cancer patients can benefit from TILs-T cells based immunotherapy, the resistance and the significant toxicity indicating the need for other therapeutic methods (107).

1.4.1.1.2 Tumor-infiltrating B lymphocytes (TILs-B)

In contrast to TILs-T, B lymphocytes in the TME are much less investigated. Recently, TILs-B are getting more and more attention in cancer immunology (108).

Studies on the impact of TILs-B on clinical outcomes of several cancer types suggest that TILs-B as a new hallmark in cancer development and progression (109).

Like TILs-T, TILs-B cells are also a heterogeneous population with diverse phenotypes contributing to their pro-tumor and anti-tumor effects. There are two principal TILs-B subtypes in TME: $CD20^+$ B cells which include germinal center B, naïve B, switched-memory B, non-switched memory B, and $CD20^-$ $CD138^+$ plasma cells (110).

In tumors, TILs-B cells are mainly localized in the B cell-rich zone within tertiary lymphoid structures (TLSs) which latterly divide into germinal center (GC) area and mantle zone (111). TILs-B maintain TLSs structure and maturation in TME through the secretion of cytokines such as B lymphocyte chemoattractant CXCL13 and lymphotoxin (112). In lung cancer, both TILs-B cells and TLSs levels are associated with better long-term survival rates (113,114).

The principal function of B cell is the involvement in the humoral immune response by producing plasma cells and antibodies which can detect and react against tumor-associated antigens (110). The presence of tumor-infiltrating plasma cells and immunoglobulins is associated with a favorable prognosis in lung cancer patients suggesting the anti-tumor role of TILs-B cells (115). Also, TILs-B cells were demonstrated to prompt T cell activation and expansion probably by antigen presentation in NSCLC (116).

Apart from the protective role, TILs-B cells also show tumor-promoting function mainly through the regulatory B cells (Bregs) phenotype (117). Bregs can cause immunosuppression through IL-10 and TGF-ß (46). They may also increase the expression of the immune regulatory ligand as PD-L1 and CTLA-4 to suppress T and NKs functions (118,119). Moreover, Bregs can attenuate the anti-tumor immune effects inducing Treg subtype conversion, and promote the interaction with myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAMs), subsequently lead to carcinogenesis (120–123). In lung cancer, recent studies reported that TILs-B cells are present in all cancer stages, and differ among stages and histological subtypes (124,125).

1.4.1.2.3 Tertiary lymphoid structures

Another component getting more and more attention is the tertiary lymphoid structures (TLSs). They are aggregates of immune cells formed in non-lymphoid sites under long-lasting pathological conditions such as autoimmune diseases, infection, chronic inflammation, or cancer (113). Similar to the secondary lymphoid organs (SLO) such as lymph nodes and spleen, TLSs also contain a T cell-rich zone composed by a cluster of T cells and mature dendritic cells, and a B cell-rich area characterized by naïve B cells follicles with GC surrounded with plasma cells (Figure 7) (126). Both zones present high endothelial venules (HEVs), the particular blood vessels which secrete cytokines such as CCL19, CCK21, CXCL12 and, CXCL13 to recruit lymphocytes to the infected sites (126). Differently to SLOs, TLSs lack a capsule and afferent lymphatic vessels. Moreover, they are transitory and resolved after antigen elimination(127).

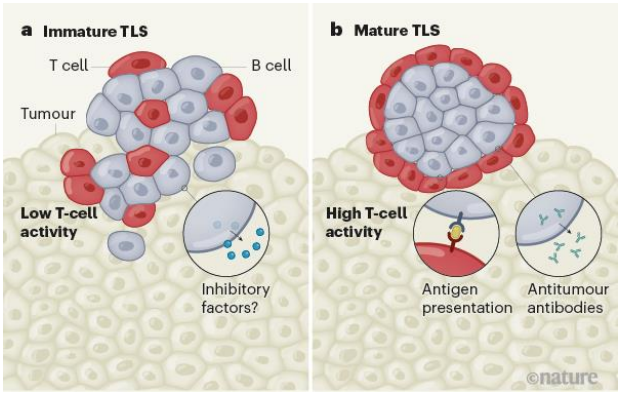


Figure 7. Immature and matures TLSs (adapted from (128)).

TLSs are classified as mature and immature subtypes according to the formation of a clear called GC as in the SLOs (Figure 3) (128,129). GC is the transient center region in the B cell-rich zone where factories the majority of activated B lymphocytes in the lymphoid structures after antigen exposure (130). They are crucial for the innate humoral immune response due to the dynamical development of mature B cells, B cells expressing antibodies, antibody-secreting plasma cells, and durable memory B cells during antigen invasion(131).

Increasing evidence indicates the favorable prognostic value of TLSs in various cancer types including LC, colorectal cancer, pancreatic cancer, oral squamous cell carcinoma, breast cancer, ovarian cancer, hepatocellular cancer,

melanoma and stomach cancer (132,133). Also, the presence of TLSs and B cells in tumors promoted immunotherapy response in melanoma, sarcoma, and renal cell carcinoma patients (128,133–135).

In chronic respiratory conditions, TLSs formed in the lungs may directly be due to the microbial colonizers within tobacco, or the smoke-induced inflammatory cytokines IL-17 or other lymphoid tissue inducer cells (136). In COPD, TLSs may play both protective and pathological roles (137). Studies showed that induced TLSs enhanced anti-viral responses and reduced lung damage (138). Meanwhile, it is also illustrated that TLSs may contribute to perpetuating inflammation influencing lung innate immunity and signal COPD progression(139). However, the mechanism and prognostic values of TLSs in COPD remain to be elucidated. The specific role of TLSs in lung cancer patients with underlying COPD is also poorly known.

1.4.1.2.4 Regulatory T cells

To regulate the immune system and maintain self-antigens tolerance, our body disposes of the suppressor cells to counteract other immune cells, the Regulatory T cells (Tregs). As indicated by the name, Tregs are a subgroup of T cells in charge of regulating and suppressing T cells, B cells, and dendritic cells (140). They are crucial to control immune homeostasis and prevent autoimmune diseases (141).

Tregs are identified as $CD4^+$, $CD25^+$, and the transcriptional factor Forkhead box P3 (Foxp3) $^+$ expressed cells. Among them, CD4 and CD25 are the cell surface markers, Foxp3 is the intracellular biomarker and more specific for Tregs. Two main types of Tregs are identified: 1) natural Tregs (nTregs) or thymus-derived Tregs, account for almost 10% of peripheral CD4 cells, and 2) induced Tregs (iTregs) or peripherally-induced T-regs, are differentiated from antigens stimulated naïve CD4 T cells (142).

Tregs secret inhibitory cytokines IL-10, TGF-β, and IL-35 to inhibit effector T cell signaling and regulate IFN-γ function (143). Tregs also release cytolytic molecules granzymes to kill T cells and NKs which leads to immunosuppression (144). Moreover, studies showed that Tregs can reduce the activity of antigen-

presenting cells (APC) competing for binding to the ligands CD 80 and CD 86 on APCs with T cells and lead to immune tolerance (145). These mechanisms are essential in avoiding excessive immune responses in healthy individuals. However, in pathological conditions, the balance between immune activation and suppression is broken. In autoimmune conditions such as asthma and allergy, Tregs are fewer and functionally defective in allergic and asthmatic individuals (146,147).

In the context of chronic inflammation and tumor conditions, Tregs mainly migrate to the inflamed tissues to suppress the immune cell responses (148). In chronic respiratory conditions, Tregs levels significantly increased in the blood of COPD patients compared to healthy control individuals (149). Moreover, the authors found that the Tregs in COPD patients had a significantly greater expression of CTLA-4 and exhibited an augmented suppressive effect on effector T cells (149). These findings indicate the highly expressed defective Tregs may explain the perpetuate inflammation in COPD conditions (149).

In terms of tumor conditions, Treg cells are one of the major obstacles to the application of immunotherapy. Tregs can prevent anti-tumor immunity development by impeding cancer immune surveillance and contribute to carcinogenesis and tumor progression (150). In TME, Tregs, as well as the immune checkpoints such as PD-1 and CTLA-4 are overexpressed in several cancer types including lung cancer (151–154). The increased levels of Tregs in tumors were also shown to associate with higher histological staging and poorer prognosis in lung cancer patients (155). Up to now, the efficacy of Treg cell-targeted therapeutic strategies such as Fc-Optimized anti-CD25 and anti-Foxp3 monoclonal antibodies has been evaluated in preclinical models (156,157). However, similar to other immune checkpoint inhibitors, only a few patients show clinical efficiency to them. Future studies should dedicate to elucidate which Tregs biomarkers are responsible for the immune evasion in tumors in order to improve the therapy effectiveness, especially in those with underlying chronic respiratory conditions.

1.4.2 Tumor stroma

Another important part of the TME is the non-malignant components around the tumor cells, namely tumor stroma. Tumor stroma contains: 1) non-malignant cells, such as cancer-associated fibroblasts (CAFs), endothelial cells and pericytes of vessels, mesenchymal cells, innate and adaptive immune cells and, 2) the extracellular matrix (ECM) which consists of proteins such as collagens, proteoglycans, and glycoproteins. The main components of the tumor stroma are described below.

1.4.2.1 Cancer-associated fibroblasts (CAFs)

Fibroblasts are the principal cellular component of stroma (158,159). In normal tissues, fibroblasts locate within the interstitial membrane of ECM and maintain inactive states (160). During wound healing and fibrosis, fibroblasts become activated namely “myofibroblasts” expressing specific markers: α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), and unspecific markers: vimentin, fibronectin, desmin, etc (161). These activated fibroblasts cause organ fibrosis and produce growth factors, cytokines, chemokines, and immune modulators (162). When the wound healing process completes, myofibroblasts die via apoptosis. However, in cancer conditions, as “a wound that never heals”, these activated fibroblasts also called cancer-associated fibroblasts (CAFs) are perpetually activated and never go to apoptosis. Studies showed that the increased level of CAFs promoted tumor progression, invasion, and metastasis in several cancer types, including lung cancer (163–166).

CAFs also have been shown to induce epithelial-mesenchymal transition (EMT). They promote tumor progression through cytokines such as TGF-β, interleukin (IL)-6, IL-10, and miR-33b in breast cancer, bladder cancer, and lung cancer respectively (167–169). Furthermore, CAFs are the major resource of the fibrillar ECM proteins type I, III, and V collagens and fibronectin (170). Meanwhile, CAFs also secrete ECM-degrading proteases such as matrix metalloproteinases (MMPs) and LOX-proteins which maintain the homeostasis of ECM by degrading the matrix and play roles in tumor progression and invasion (170,171). The secreted MMPs by CAFs induce angiogenesis through the release of the key angiogenic factor vascular endothelial growth factor (VEGF) within the degraded

matrix (161). The increased accumulation of collagen in stroma can cause desmoplasia and stiffness of tumor lead to reduction of drug delivery and cause drug resistance (172,173).

1.4.2.2 Extracellular matrix (ECM)

The ECM is the non-cellular three-dimensional networks within tissues providing physical support and regulate a lot of cellular processes (174). The ECM includes two types of matrices: the basement membrane (BM) and the interstitial matrix (IM) (172,174). The BM is a thin extracellular matrix separating epithelial and endothelial cells. Its principal functions are structural scaffolds and cell behavior regulation. While IM plays a major paper in cell adhesion, cell-cell communication, angiogenesis, and cell proliferation (175). The ECM principally contains two types of macromolecules: 1) fibrous proteins such as collagens, elastins, fibronectins, and laminins, and 2) proteoglycans which present hydrated gel forms, are the principal components of extracellular interstitial space in tissue. In ECM, the major component is collagen, a superfamily with 28 members that occupies about 30% of total protein mass in mammals, and type I collagen is the most abundant subtype of the collagen family (176). EMC is a highly dynamic structure with constant remodeling (175). Unsuitable synthesis or degradation of ECM molecules may interrupt tissue morphogenesis, differentiation, and homeostasis (177).

During carcinogenesis, the dynamics of ECM are altered. Cancer cells and the transformed fibroblasts CAFs secrete higher levels of metalloproteinases (MMPs) which degrade the BM. They also produce the malformation of ECM with loosely woven and non-planar even bent collagen (178). All these aspects make the BM weaker and allow the escape of cancer cells from the primary site and cause metastasis (179). Furthermore, CAFs are the major source to synthesize ECM proteins with tumors, they are shown to increase the production of network-forming collagens as type I collagen, the major components of IM collagens (71). In turn, the increased IM collagens induce tumor cell invasion and cause tumor progression.

1.4.2.3 Endothelial cells

Tumor angiogenesis, the formation of new blood vessels within tumors is a hallmark of cancer (180). The neovasculature provides oxygen and nutrients to

tumors which are indispensable for tumor growth and progression (181). Unlike normal blood vessels, tumor vasculature is morphologically and functionally different: the vessels are immature, tortuous, hyperpermeable and lack vascular mural cells (smooth muscle cells and pericytes), the endothelial wall is irregular, the basement membrane is flawed or discontinuous, the venules are not clearly identified with blood flow patchy and even bidirectional (182,183). These abnormal tumor vasculatures lead to inadequate responses to inflammatory stimuli and limit immune cell distribution and tumor cell intravasation during tumor metastasis (184).

Endothelium, the inner lining of arteries, veins, capillaries, and lymphatic vessels, is a single layer consists of endothelial cells (ECs) (185). In healthy adults, ECs remain quiescent for prolonged periods and proliferate only once every 150 days (186). However, in hypoxic conditions as cancer and wounds, angiogenesis is turned on by the pro-angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietins (Ang), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) (187).

Tumor ECs present a proangiogenic phenotype as cancer cells increase the level of matrix metalloproteinases (MMPs) which degrade the ECM and permit ECs migration. Furthermore, angiogenic growth factors such as VEGF are associated with tumor progression (188).

The membrane protein of ECs CD31, also known as Platelet Endothelial Cell Adhesion Molecule-1 (PECAM) has been evaluated to reliably measure the intratumoral microvessels density in several cancer types including LC (189). But the role of CD31 in cancers is still controversial. Some studies showed that CD 31 could promote tumor metastasis and are associated with poorer prognosis in several cancer types but not in lung cancer (188). Studies also demonstrated no significant influence of CD31 on cancer patients' survival (190,191). However, studies also reported that a high level of CD31 had associated with better overall survival (OS) in lung cancer and pancreatic cancer might be due to the better delivery of the specific immune cells and drugs to the tumor niche (191–193). Larger cohorts are warranted to identify the specific role of intratumoral microvessel density marker CD31 in lung cancer and also the influence of COPD on patients' prognosis.

1.4.3 DNA damage and DNA repair

1.4.3.1 DNA damage

As above mentioned, DNA damage and repair influence tumor initiation and progression. DNA damage, the chemical alteration of the basic structure of DNA, impedes the proper replication mechanism of the DNA (Figure 8) (76). In daily life, cigarette smoke, biomass fume, air pollution, radiation among other factors can directly or indirectly cause an imbalance between oxidants and antioxidants namely oxidative stress, and injure epithelial cells of the respiratory tract (76). Subsequently, the induced DNA damage and inflammation in respiratory tract cells are key to tumor initiation and progression and also contribute to chronic respiratory disorders (194).

DNA damage occurs daily in human cells. Approximately 70,000 new DNA lesions naturally appeared per day in each cell (194). Almost 75% of these DNA lesions are on one of the two strands of the DNA double helix, namely single-strand DNA breaks (SSBs) (195). They are mainly caused by the oxidative damage releasing compounds during endogenous metabolic or hydrolytic cellular processes (195). The rest of damages are double-strand DNA breaks principally induced by exogenous environmental factors such as ionizing radiation, ultraviolet radiation, chemical agents, aromatic amines, and toxins (196). DNA injury can cause genome instability and lead to mutations.

Fortunately, our body possesses DNA repair tools to reverse this damage to keep the genome integrity. In human mainly exist six DNA repair pathways: base excision repair (BER), single strand break repair (SSR), nucleotide excision repair (NER), homologous recombination (HR), non-homologous end joining (NHEJ), and mismatch repair (MMR) (197). Among them, Poly (ADP-ribose) polymerase (PARP) 1 and PARP-2 are two nucleic enzymes that play crucial roles in DNA damages detection and repair (198). They are the main members of the super PARP family of 18 enzymes. Once DNA damage occurs, PARPs rapidly sense them and then catalyze the synthesis and transfer of ADP-ribose from the ADP donor NAD^+ onto the amino acid residues such glutamate, aspartate or lysine of acceptor proteins, called poly(ADP-ribosyl)ation (Figure 8) (199).

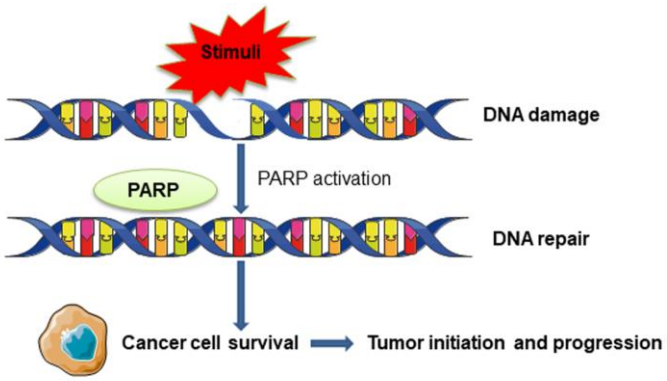


Figure 8. DNA damage and PARP activation .

1.4.3.2 PARP-1

In the super PARP family, PARP-1, also called poly (ADP-ribose) synthase or poly (ADP-ribose) transferase (ARTD1), is responsible for 85% to 90% of the activity of the total PARP family. PARP-1 is a 113 kDa protein, contains three well defined structural domains: DNA binding domain comprises two zinc fingers to define a DNA-break-sensing motif, an automodification domain and a catalytic domain (200). When SSB occurred in DNA structures, PARP-1 detects it rapidly, binds to the damaged DNA, then starts the synthesis of poly ADP-ribose chain and then transfer them onto the target protein (201). PARP-1 also involves in double-strand DNA break repair via homologous recombination (HR) and non-homologous end joining (NHEJ) (202).

1.4.3.3 PARP-2

PARP-2, a 62 kDa nucleic enzyme, contains an N-terminal region, a central WGR domain, and a C-terminal catalytic domain. Unlike PARP-1, the N-terminal region of PARP-2 does not comprise zinc fingers. Along with PARP-1 and PARP-3, PARP-2 is also triggered by DNA SSBs via PARylation (203). PARP-2 functions are overlapping with PARP-1, but PARP-2 binds less efficiently to DNA SSBs, instead, identifies gaps and flap structures (204).

However, this DNA repair mechanism is not always efficient. When the DNA damage level is mild, the activation of PARP can reverse these damages and maintain cell survival. But when these damages are severe and persistent, these accumulated injuries are no longer totally repaired. Moreover the persistent injured condition also leads to PARP overactivation. In this circumstance, the substrate

NAD^+ is depleted and the accumulation of the PAP polymers leads to parthanato, a caspase-independent and apoptosis-inducing factor dependent cell death (205). Parthanato is identified in both chronic respiratory conditions and cancers and may play important roles in disease initiation and progression. Also, the unrepaired DNA damages can produce changes in the DNA sequence, resulting in epigenetic alterations and mutations and leads to carcinogenesis (205).

1.5 Lung cancer treatment

Surgery is the elective procedure of the first line for patients with early-stage (stage I-II) lung cancer (206). In these stages, patients are usually asymptomatic and lung cancer is diagnosed by screening or an accident finding for other reasons (207). The complete lung tumor resection with curative purpose through a traditional thoracotomy or video-assisted thoracoscopic surgery (VATS) is indicated for them (206). Nowadays VATS is more frequent as better long-term outcomes have been reported with it (208).

As abovementioned, over two-thirds of lung cancer patients had been diagnosed at advanced stages (stage III-IV) when surgery is no longer suitable for them (5,6). In these stages, cancer cells spread to lymph node and/or to distant sites, tumors cannot be eliminated totally by surgery, so chemotherapy with platinum doublets or combined chemotherapy and radiotherapy is recommended according to the healthy status of these patients, and the prognosis is not good in them (209,210).

Due to the development of technology as the emergence of next-generation sequencing and other omics-based platforms allow us to understand better the diseases at the molecular level. Precision medicine also appears with new therapeutic options for specific patients. In lung cancer field, epidermal growth factor receptor (EGFR) gene mutations, Kirsten rat sarcoma viral oncogene (KRAS) mutations, anaplastic lymphoma kinase (ALK), and ROS1 rearrangements are the most investigated specific genomic aberrations with molecular targeted therapies (211). In stage IV lung cancer patients with these mutations, targeted therapy has been demonstrated to efficiently improve their survival rates. Until now, treatment targeted EGFR and ALK mutations have been approved in advanced NSCLC patients with the corresponding genomic mutations (212–214).

Additionally, the angiogenic inhibitor bevacizumab which targets the vascular endothelial growth factors (VEGF) has been approved in NSCLC patients (215). However, the role of the targeted agents has not been evaluated properly in the early stages of lung cancer and only a small part of cancer patients have benefited from the molecular targeted therapies (216,217).

1.5.1 Immunotherapy

With a better understanding of the tumor immunological microenvironment, in the last decade, immunotherapy based on “immune normalization” with the mechanism of action to restore the suppressed immune system has demonstrated effectiveness on various cancer therapy with fewer immune-related adverse events (218,219). This new strategy has opened a new way towards the world of conquering cancer.

Immunotherapy mostly bases on the inhibition of the immune checkpoints, the molecules on the surface of the immune cells to regulate the immune response. Cancer cells can take advantage of these immune checkpoints to make them “self-cells” which can’t be recognized by the host immune system and avoid being attacked (220). The most studied and with clinical application immune checkpoints are PD-L1, PD-1 and CTLA-4. The inhibition of these immune checkpoints can restore the suppressed immune system to recognize cancer cells and kill them.

Once the suppressed immune cells are restored, they can induce target cancer cells to undergo the downstream programmed cell death or apoptosis, the type I cell death. Apoptosis can be avtivated through two principal pathways: the extrinsic and the intrinsic pathways (221). The extrinsic pathway can be triggered by oxidative stress and other stressors. While the intrinsic signaling pathway is regulated by proapoptotic markers BAX and/or BAK, and antiapoptotic proteins BCL-2 and caspase which are overexpressed in more than half of all cancer types (157).

Additionally, autophagy, the type II cell death associates with both innate and adaptive immunity. It is a double-edged sword in cancers. On one hand, autophagy has been demonstrated to contribute to antigen presentation via the MHC class II complex by dendritic cells and also antigen processing for MHC class I presentation, and subsequently priming T lymphocytes (222). Moreover,

autophagy can also drive the development of other immune components such as B cells, DCs, and the differentiation of plasma cells with the enhancement of antigen presentation (222). But on the other hand, autophagy also can promote tumor progression. In the advanced stages of cancer, the upregulated autophagy can absorb energy and nutrition from degraded cellular components and promote cancer cell proliferation and cause tumor progression and metastasis (223).

So far four PD-1 inhibitors pembrolizumab, nivolumab, atezolizumab, and durvalumab have been approved in metastatic or extent stage in both NSCLC and SCLC patients by U.S.FDA. Moreover, the combination of a PD-1 inhibitor nivolumab, a CTLA-4 inhibitor ipilimumab, and platinum-doublet chemotherapy was also approved as first-line treatment for patients with metastatic or recurrent NSCLC without mutations in EGFR or ALK mutations by U.S.FDA recently (224). Meanwhile, other immune checkpoints such as T-cell immunoglobulin domain and mucin domain 3 (TIM-3), lymphocyte activation gene-3 (LAG3) are also gathered attention as the appearance of drug resistance to the current immune checkpoints inhibitors (225).

Apart from the ICIs aimed to normalize the suppressed immune response, there is also immunotherapy based on immune enhancement strategies. For instance, the anti-CD20 and anti-CD19 antibodies are used for B cell lymphoblastic leukemia and lymphoma (226). The monoclonal antibody anti-CD137 boosting NK and T cells also show anti-cancer immunity in mouse and human models in several cancer types including lung cancer (227). However, similarly to targeted therapies in cancer, only a small part of cancer patients have benefited from immunotherapy. We are still facing the opportunity and challenge to find the appropriate patients who will be beneficial from immunotherapy (228). In this context, the impact of COPD on the effectiveness of immunotherapy in lung cancer patients has not been established yet.

1.5.2 PARP inhibition

As aforementioned, almost all cancers are the consequence of somatic and epigenetic mutations which are principally caused by DNA damages. In cancer cells, frequently one of the DNA repair pathways is dysfunctional, this malfunction may be compensated by another DNA repair pathway. This may be one reason for

the resistance to DNA-damaging based radiotherapy and chemotherapy (229). When other DNA repair pathways are blocked, cancer cells die. This process is the so-called synthetic lethality. When the mutation occurs in either gene alone, the cell survives, but when mutations occur simultaneously on both genes, cell dies (230).

The first successful application of synthetic lethality is the treatment with PARP inhibitors in cancers with BRCA gene mutations (231). In BRCA mutated cancer cells, the HR DNA repair pathway is deficient, with the inhibition of the second DNA repair pathway by PARP inhibitors, inducing double-strand breaks which lead to cancer cell death via apoptosis (231,232). Studies showed that PARP inhibitors significantly improved progression-free survival of patients with ovarian cancer and breast cancer (233,234). Up to now, four PARP inhibitors (olaparib, niraparib, rucaparib, and talazoparib) have been approved for advanced ovarian cancer and breast cancer patients with BRCA mutations by the U.S.FDA.

Until now, PARP inhibitors have not been approved in lung cancer treatment. In a phase II trial in SCLC patients, the combination of PARP inhibitors with chemotherapy showed improved overall response rates. Interestingly, in patients with SLFN11-positive SCLC, this combination significantly improves patients' PSF and OS (235). Nevertheless, in NSCLC no significant beneficial effects of PARP inhibitor olaparib in combination with gefitinib were shown in a phase II trial of 182 patients. Notably, none of these studies have distinguished patients with the presence of underlying COPD. These results may indicate a more personalized therapeutic indication of PARP inhibitors in lung cancer patients and the necessity to identify the appropriate NSCLC candidates who may benefit from PARP inhibitors (236).

In chronic respiratory conditions, the chronic inflammation with excessive oxidative reactive species induces DNA damages which result in PARP overactivation (237). Moreover, PARP-1 was shown to regulate NF-κB cytokines, inducible nitric-oxide synthase and promote inflammation (238,239). And PARP-1 inhibitor olaparib has been shown to ameliorate lung emphysema and inflammation induced by elastase in mice model (240). The effects of PARP

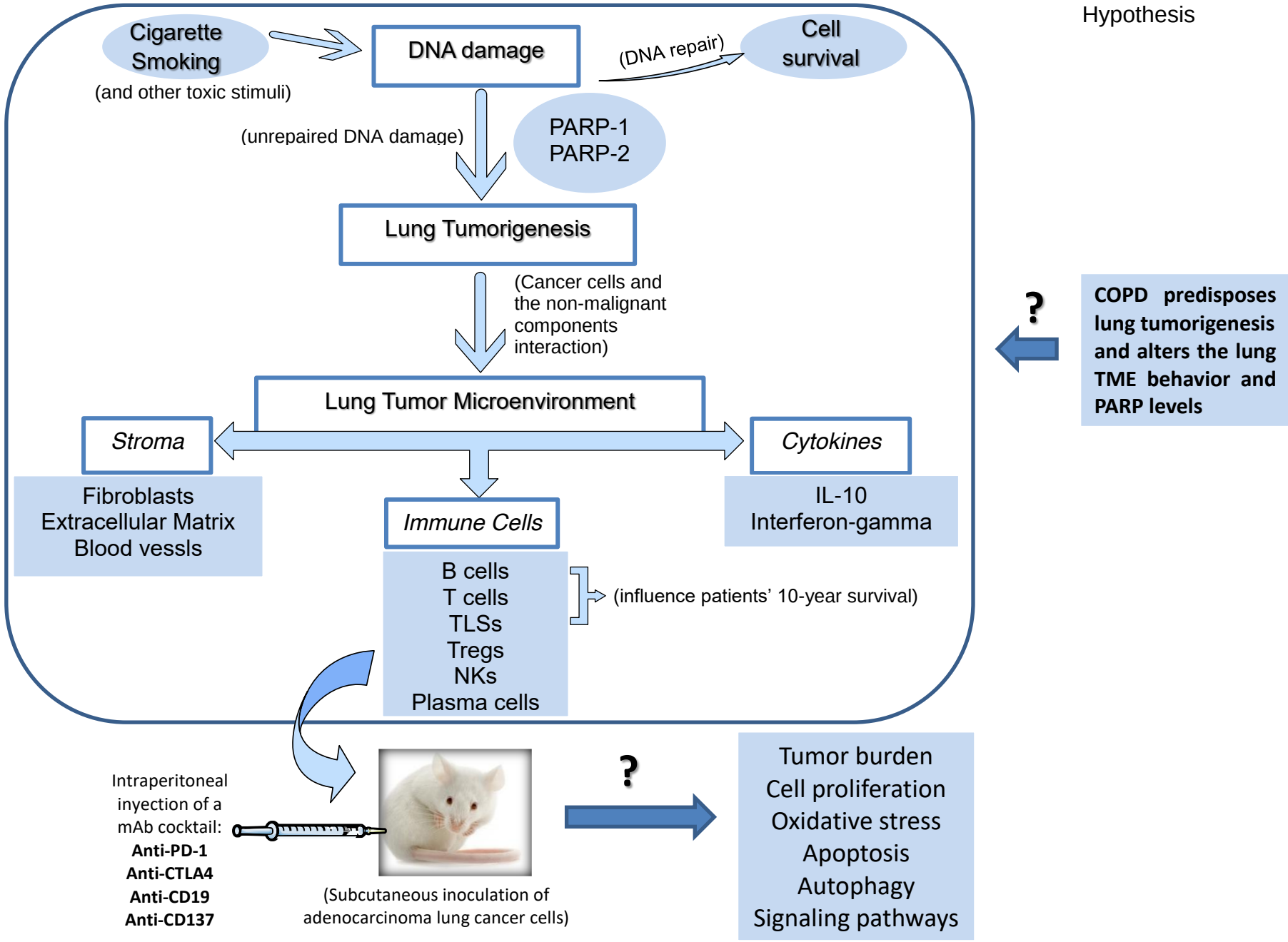
enzymes and PARP inhibitors in lung cancer patients with underlying COPD remain to be elucidated.

2. HYPOTHESIS

2. HYPOTHESIS

In the current thesis, structural and biological features/events that have been shown to participate in the process of tumorigenesis have been studied in tumors of patients with and without COPD: tumor micronenvironment, particular immune cell composition and stroma structures, and PARP overactivation. In mice with experimental lung adenocarcinoma, the treatment with immune therapy may affect tumor progression through several biological mechanisms such as oxidative stress, apoptosis, and autophagy. This was also explored in the present thesis.

Hypothesis


Cigarette Smoking
(and other toxic stimuli)
DNA damage
(DNA repair)
Cell survival
(unrepaired DNA damage)
PARP-1
PARP-2
Lung Tumorigenesis
(Cancer cells and the non-malignant components interaction)
Lung Tumor Microenvironment
Stroma
Fibroblasts
Extracellular Matrix
Blood vessls
Immune Cells
B cells
T cells
TLSs
Tregs
NKs
Plasma cells
(influence patients' 10-year survival)
Cytokines
IL-10
Interferon-gamma
?
COPD predisposes lung tumorigenesis and alters the lung TME behavior and PARP levels
Intraperitoneal inyection of a mAb cocktail:
Anti-PD-1
Anti-CTLA4
Anti-CD19
Anti-CD137
(Subcutaneous inoculation of adenocarcinoma lung cancer cells)
?
Tumor burden
Cell proliferation
Oxidative stress
Apoptosis
Autophagy
Signaling pathways

3. OBJECTIVES

3. OBJECTIVES

3.1 Main objective

To explore the biological mechanisms related to the tumor microenvironment (immune profile, stroma, and PARP activation). Moreover, the mechanisms whereby immunotherapy contributed to reduced tumor burden were also studied in mice.

3.2 Secondary objectives

1. To analyze innate and adaptive immune components and cytokine levels and the associations between the studied immune components and patients' 10-year OS.

To achieve this objective, the corresponding studies were carried out:

Study #1. B Cells and tertiary lymphoid structures influence survival in lung cancer patients with resectable tumors.

Study #2. Immune cell subtypes and cytokines in lung tumor microenvironment: influence of COPD.

2. To assess cell proliferation rates, immune tumor microenvironment, oxidative stress, antioxidant enzymes, apoptosis, autophagy and signaling in a mice model with experimental tumors.

To achieve this objective, the corresponding study was carried out:

Study #3. Immunotherapy with monoclonal antibodies in lung cancer of mice: oxidative stress and other biological events.

3. To assess the stroma component: cancer-associated fibroblast, extracellular matrix, and endothelial cells in patients.

To achieve these objectives, the corresponding study was carried out:

Study #4. Markers of stroma in lung cancer: influence of COPD

4. To evaluate DNA damage, PARP expression and activity in patients.

To achieve these objectives, the corresponding study was carried out:

Study #5. Increased PARP activity and DNA damage in NSCLC patients: the influence of COPD.

4. COMPENDIUM OF PUBLICATIONS

4.1 First Study

Title:

B cells and Tertiary Lymphoid Structures influence Survival in Lung cancer Patients with Resectable Tumors

Authors:

Jun Tang, Daniel Ramis-Cabrer, Víctor Curull, Xuejie Wang, Mercé Mateu-Jiménez, Lara Pijuan, Xavier Duran, Liyun Qin, Alberto Rodríguez-Fuster, Rafael Aguiló and **Esther Barreiro**.

Journal:

Cancers. 2020 Sep 16; 12 (9):2644.

DOI: 10.3390/cancers12092644

PMID: 32947928

Impact Factor:

6.126 (2019), Quartile: Q1

cancers

MDPI

Article

B Cells and Tertiary Lymphoid Structures Influence Survival in Lung Cancer Patients with Resectable Tumors

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Received: 5 August 2020; Accepted: 11 September 2020; Published: 16 September 2020

Simple Summary: Nowadays, humans still die of lung cancer (LC), a disease mainly related to cigarette smoking (CS). Smokers also develop chronic bronchitis, namely chronic obstructive pulmonary disease (COPD). Environmental factors and a natural predisposition from the patients' sides may render them more prone to develop tumors derived from CS. Thus, a great number of patients may suffer from chronic bronchitis and LC simultaneously. Chronic respiratory diseases are also important risks factors for LC. The immune system, among other biological mechanisms, protect our cells from infections and cancer development. Several immune structures and cells may be altered in the tumors of patients with COPD as opposed to lung tumors of patients with no underlying respiratory disease. A total of 133 patients with LC participated in the study: 93 with underlying COPD. Several structures (tertiary lymphoid structures, TLS) and T and B lymphocytes were analyzed in the lung tumor and non-tumor areas (specimens obtained during surgical extirpation of the tumors). We found that in LC patients with COPD, compared to those without it, fewer numbers of TLSs and B cells were detected, and those patients died significantly earlier. These results have implications in the diagnosis and treatment options of lung tumors in patients with underlying respiratory diseases.

Abstract: Immune profile of B and T cells and tertiary lymphoid structures (TLSs) may differ in tumors of lung cancer (LC) patients with/without chronic obstructive pulmonary disease (COPD), and may also influence patient survival. We sought to analyze: (1) TLSs, germinal centers (GCs), B and T cells, and (2) associations of the immune biomarkers with the patients' 10-year overall survival (OS). TLSs (numbers and area), B [cluster of differentiation (CD) 20], and T (CD3), and GCs cells were identified in both tumor and non-tumor specimens (thoracotomy) from 90 LC-COPD patients and 43 LC-only patients. Ten-year OS was analyzed in the patients. Immune profile in tumors of LC-COPD versus LC: TLS numbers and areas significantly decreased in tumors of LC-COPD compared to LC patients. No significant differences were observed in tumors between LC-COPD and LC patients for B

or T cells. Immune profile in tumors versus non-tumor specimens: TLS areas and B cells significantly increased, T cells significantly decreased in tumors of both LC and LC-COPD patients. Survival: in LC-COPD patients: greater area of TLSs and proportion of B cells were associated with longer survival rates. The immune tumor microenvironment differs in patients with underlying COPD and these different phenotypes may eventually impact the response to immunotherapy in patients with LC.

Keywords: lung cancer; chronic respiratory diseases; tertiary lymphoid structures; B cells; overall survival

1. Introduction

Lung cancer (LC) is still the most common cause of death worldwide [1–5], accounting for almost one-third of deaths in certain geographical areas [6]. Chronic respiratory diseases, such as chronic obstructive pulmonary disease (COPD), which is also a highly prevalent condition in certain regions, has been consistently associated with LC incidence [7,8]. Airway obstruction and emphysema are, indeed, important risk factors for LC [7,8]. Assessment of the biological mechanisms that render patients with chronic lung diseases more susceptible to LC development remains to be fully elucidated.

In the process of tumorigenesis, inflammatory events interact with several cellular mechanisms such as angiogenesis, apoptosis, cell repair, and distant metastasis, which are promoted by cytokines and growth factors [9,10]. Tumor microenvironment is also crucial in the development of LC, its progression, and response to therapy in clinical settings. Immune surveillance is relevant to the microenvironment of the tumor lesions as it may interfere with disease progression. Antitumor effects are exerted by T helper (Th) 1lymphocytes, whereas Th2 cells may inhibit the host immune system, thus, favoring tumor development and growth [11]. LC relapse and response to immunotherapy also rely on the balance between Th1 and Th2 immune phenotype [9,12–14]. Moreover, Th1 and Th2 immune response may vary in patients with underlying respiratory diseases [15,16]. In accordance, a previous study clearly demonstrated that Th1 cytokines were predominant in the tumors of patients with LC and underlying COPD, suggesting that these patients exhibited a greater inflammatory profile that might be beneficial in response to certain therapies [10]. The specific pattern of immune cells present in lung tumor specimens of patients with LC and COPD remains unanswered.

Tertiary lymphoid structures (TLSs), which share identical characteristics to lymph nodes, are encountered in inflamed and infected tissues and in tumors. They are characterized by the presence of a T cell area, germinal centers, and proliferating B cells among other structures [17–19]. In COPD patients, a greater number of TLSs were demonstrated in lung tissues [20]. Whether TLSs may be involved in LC development in patients with COPD is still debatable. Our hypothesis was that tumor microenvironment, as assessed by the profile of TLSs and the number of B and T cells, may differ in tumors of patients with underlying COPD compared to those without this disease, and these differences may also influence patient survival. Hence, our objectives were that in lung tumors and non-tumor specimens of LC patients with and without COPD: (1) TLSs, germinal centers (GCs), and B and T cells were explored, and (2) associations of these immune biomarkers with the patients' 10-year overall survival (OS) were assessed. All of the patients were clinically followed up, to a maximum period of 10 years, for the analyses of the survival.

2. Results

2.1. Clinical Characteristics of the Study Patients

Table 1 describes all clinical and functional features of both LC and LC-COPD patients. The number of LC-COPD patients was greater than that of LC only patients (two-fold), with predominance

of male patients. No significant differences were seen in age or body mass index (BMI) between LC-COPD and LC patients. Expectedly, the percentage of ex-smokers and the number of packs–year were significantly higher in LC-COPD patients than LC patients, while the number of never-smokers was significantly greater in the latter group (Table 1). As expected, lung functional parameters were significantly lower in LC-COPD patients than in LC patients (Table 1). The majority (91%) of LC-COPD patients were in Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) I and II stages. In addition, no differences were observed in tumor, node, and metastasis (TNM) staging or histological subtypes between both groups of patients. Compared to LC only patients, total leucocyte, neutrophil, and lymphocyte, levels were significantly higher in LC-COPD patients. No significant differences were found in levels of albumin, total proteins, fibrinogen, C-reactive protein (CRP), globular sedimentation (GSV), and body weight loss between the two study groups of patients.

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

Anthropometric Variables	**Lung Cancer (*n* = 43)**	**Lung Cancer-COPD (*n* = 90)**
Age, years	65 (12)	67 (8)
Male, N/Female, *N*	17/26	78/12 ***
BMI, kg/m^2	27 (4)	26 (4)
Smoking history		
Current: *N*, %	13, 30	43, 48
Ex-smoker: *N*, %	8, 19	44, 49 **
Never smoker: *N*, %	22, 51	3, 3 ***
Pack-years	17 (22)	56 (25) ***
Lung function parameters		
FEV_1	90 (12)	67 (15) ***
FEV_1/FVC, %	75 (6)	61 (9) ***
DLco, %	85 (14)	67 (18) ***
Kco, %	85 (12)	69 (17) ***
GOLD Stage		
GOLD Stage I: *N*, %	NA	19, 21
GOLD Stage II: *N*, %	NA	63, 70
GOLD Stage III: *N*, %	NA	8, 9
TNM staging		
Stage 0–II: *N*, %	37, 86	73, 81.1
Stage III: *N*, %	6, 14	13, 14.5
Stage IV: *N*, %	0, 0	4, 4.4
Histological diagnosis		
Squamous cell carcinoma: *N*, %	5, 12	16, 17.8
Adenocarcinoma: *N*, %	32, 74	68, 75.6
Others: *N*, %	6, 14	6, 6.7

Table 1. *Cont.*

Anthropometric Variables	Lung Cancer (n = 43)	Lung Cancer-COPD (n = 90)
Blood parameters		
Total leucocytes/μL	7.39 (2.42) × 10^3	9.17 (2.93) × 10^3 ***
Total neutrophils/μL	4.82 (2.49) × 10^3	6.01 (2.61) × 10^3 **
Total lymphocytes/μL	1.76 (0.78) × 10^3	2.32 (1.61) × 10^3 *
Albumin (g/dL)	4.3 (0.4)	4.1 (0.6)
Total proteins (g/dL)	7.0 (0.6)	6.8 (0.8)
Fibrinogen (mg/dL)	420 (130)	454 (151)
CRP (mg/dL)	6.5 (8.3)	7.5 (13.1)
GSV (mm/h)	27 (14)	27 (16)
Body weight loss, kg		
0, *N*, %	40, 93	82, 91
1–5, *N*, %	1, 2	3, 3
6–10, *N*, %	2, 5	5, 6

Continuous variables are shown as mean and standard deviation, while categorical variables are described as the number of patients in each group and the percentage in the study group with respect to the total population. Definition of abbreviations: N, number; kg, kilograms; m, meters; 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 Pulmonary Disease; NA, not applicable; TNM, tumor, nodes, metastasis; CRP, C-reactive protein; GSV, globular sedimentation velocity; L, liter; COPD, chronic obstructive pulmonary disease. Statistical analyses and significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between LC-COPD patients and LC patients.

2.2. Number and Area of TLSs and Number of GCs in Lung Samples

2.2.1. Differences between LC-COPD and LC in Either Tumor or Non-Tumor Lung Samples

Both numbers of TLSs corrected by area (TLSs/mm^2) and total area of TLSs (mm^2) significantly decreased in the tumors of LC-COPD patients compared to LC group (Figure 1A–C). The number of GCs also significantly declined in LC-COPD patients compared to LC patients (Table 2 and Figure 1D).

2.2.2. Differences between Tumor and Non-Tumor Lung Samples in LC-COPD and LC Patients

Compared to non-tumor specimens, both numbers and areas of TLSs were significantly higher in tumor lungs than in non-tumor lungs in both study groups (Figure 1A–C). The GCs number also significantly increased in lung tumors compared to non-tumor specimens in both study groups (Table 2 and Figure 1D).

Table 2. Number of germinal centers within tertiary lymphoid structures.

Germinal Centers	Lung Cancer (n = 18)		Lung Cancer-COPD (n = 43)	
	NT Lung	T Lung	NT Lung	T Lung
0, *n* (%)	17 (94)	10 (56) *	43 (100)	36 (84) **,§
>1, *n* (%)	1 (6)	8 (44) *	0 (0)	7 (16) **,§

Values are represented as number and percentage of the total samples in both tumor (T) and non-tumor (NT) samples in both LC and LC-COPD groups of patients. Statistical analyses and significance: * $p < 0.05$, ** $p < 0.01$ between tumor and non-tumor lung specimens in either LC or LC-COPD groups of patients, § $p < 0.05$ in tumor lung specimens between LC and LC-COPD patients. The digit 0 means absence of germinal centers (GCs) in the samples.

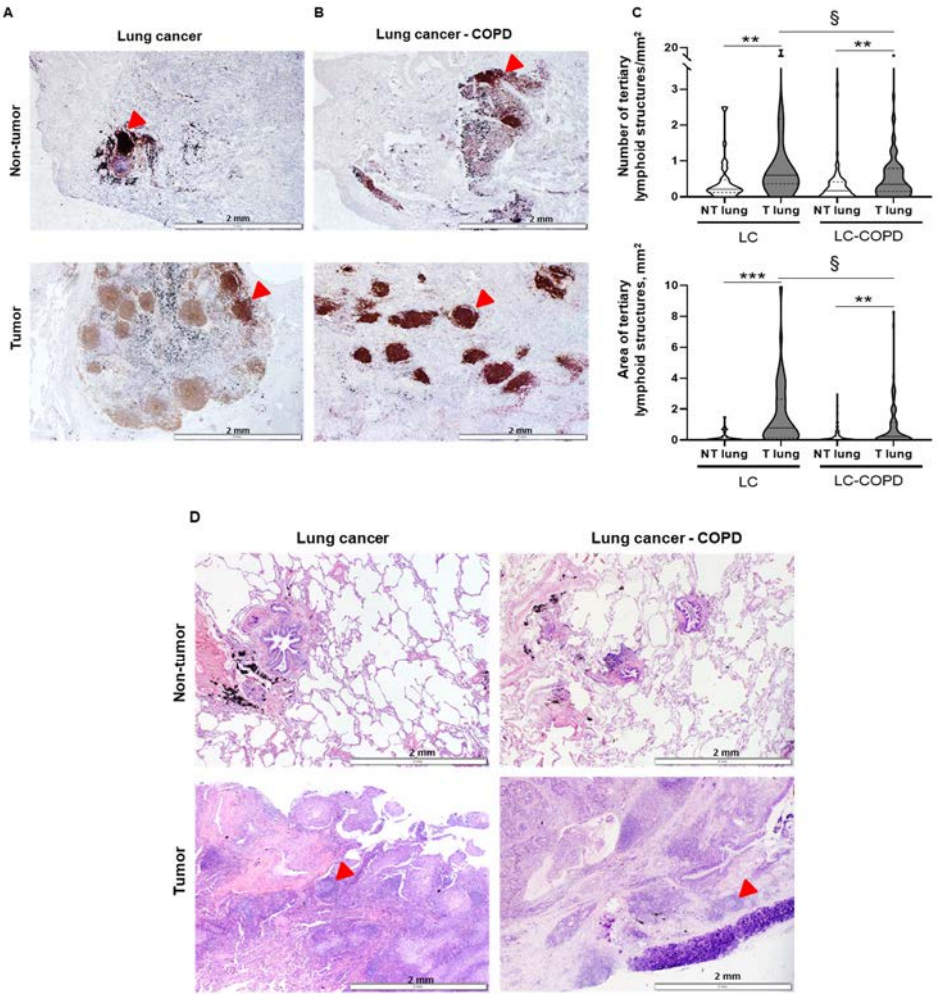

Figure 1. TLSs and germinal centers in tumor and non-tumor lungs of patients. (**A**,**B**) Representative examples of double immunohistochemical staining for TLSs indicated by red arrows in lung cancer (LC) and LC-COPD patients, respectively. (**C**) Violin plot with median (continuous line) and interquartile range (discontinuous line) of the number of TLSs corrected by area (number/mm², top panel) and total area of TLSs (mm², bottom panel), respectively. Comparisons were made between the non-tumor (NT) and tumor (T) samples, and the LC and LC-COPD groups of patients. Statistical significance: **, $p \leq 0.01$; ***, $p \leq 0.001$ between tumor and non-tumor lungs in either LC or LC-COPD patients, §, $p \leq 0.05$ in tumor samples between LC and LC-COPD patient groups. (**D**) Representative examples of hematoxylin and eosin staining for the germinal centers contained within the TLSs in LC and LC-COPD patients. Red arrows point towards germinal centers. Definition of abbreviations: TLSs, tertiary lymphoid structures.

2.3. T and B Cell Levels in Lung Samples

2.3.1. Differences between LC-COPD and LC in either Tumor or Non-Tumor Lung Samples

Total numbers of T cells/μm² and B cells/μm² did not differ in either tumor or non-tumor specimens between LC-COPD and LC patients (Figure 2A–C).

2.3.2. Differences between Tumor and Non-Tumor Lung Samples in LC-COPD and LC Patients

The number of T cells/μm² was significantly lower in tumor samples compared to non-tumor lungs of both groups of patients (Figures 2A–C, top panel). Total numbers of B cells/μm² were

significantly greater in the tumors compared to non-tumor lungs in both LC and LC-COPD groups of patients (Figures 2A–C, bottom panel).

Among LC-COPD patients, statistically significant associations were seen between the percentage of T cells in lung tumor specimens and forced expiratory volume in one second/forced vital capacity (FEV_1/FVC) ($R = 0.228$ and $p = 0.032$) and the percentage of B cells and FEV_1/FVC ($R = -0.370$ and $p < 0.001$, Figure 2D).

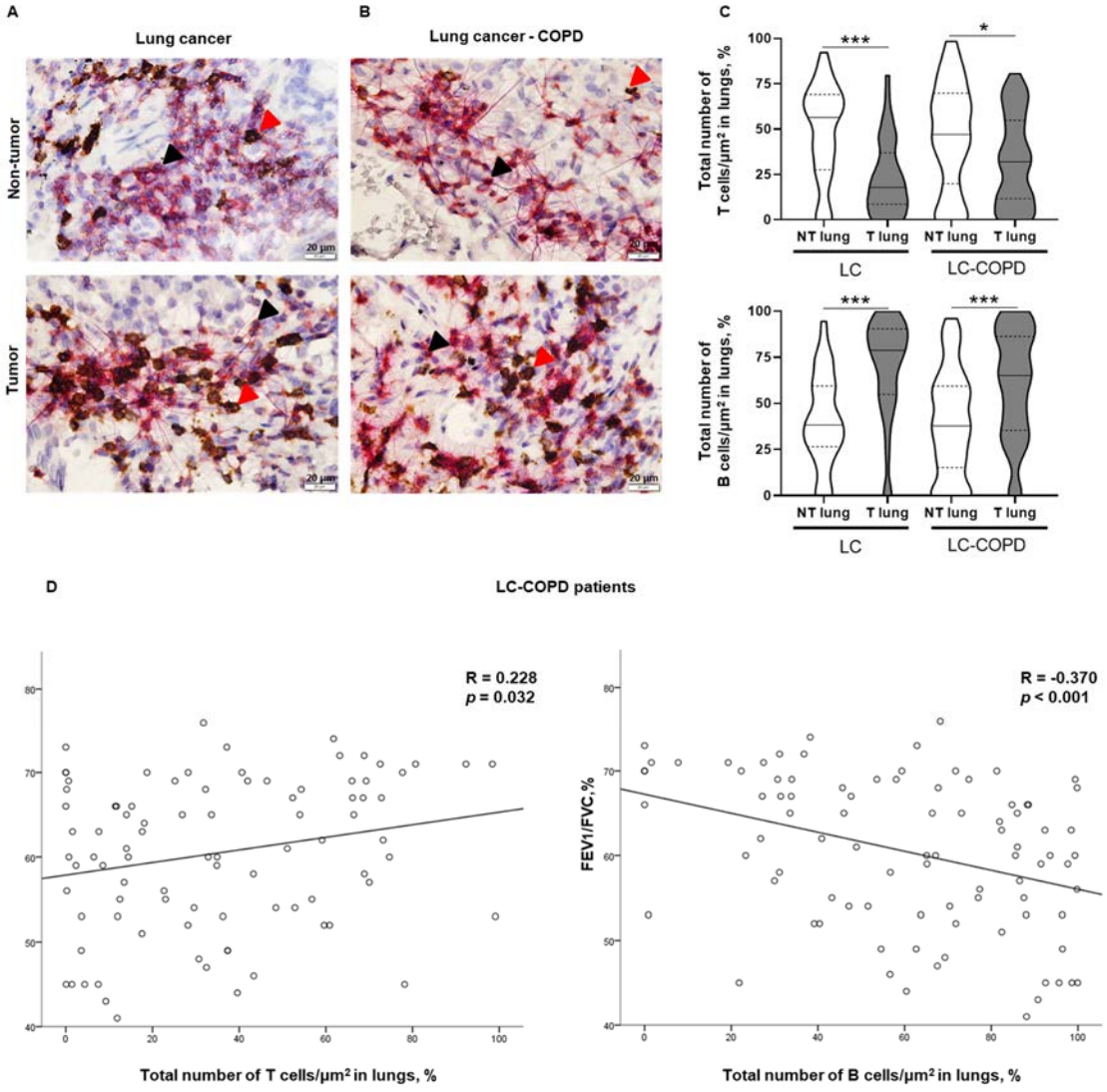

Figure 2. T and B cell counts in tumor and non-tumor lungs of patients and their correlations with lung function parameters. (**A**,**B**) Representative double immunohistochemical staining sections of T and B cells in non-tumor and tumor lung specimens of LC and LC-COPD patients, respectively. T cells (CD3+) indicated by black arrows were stained in red color, and B cells (CD20+) indicated by red arrows were stained in brown color, respectively. (**C**) Violin plot with median (continuous line) and interquartile range (discontinuous line) of the number of T cells (top panel) and B cells (bottom panel) as indicated by the percentage of T and B cells in the total measured area respectively. Black-stained regions within the lungs correspond to anthracosis. (**D**) Statistically significant correlations between FEV_1/FVC and T cell numbers (positive), and B cell numbers (inverse) in LC-COPD patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; CD, cluster of differentiation; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity. Statistical significance: ***, $p \leq 0.001$; *, $p < 0.05$ between tumor and non-tumor samples in both study groups.

2.3.2. Differences between Tumor and Non-Tumor Lung Samples in LC-COPD and LC Patients

2.4. Associations of T cells with OS in LC and LC-COPD Patients

The number of T cells/μm^2 was significantly greater in tumor samples compared to non-tumor lungs of both groups of patients (Figure 2A–C, top panel). Total numbers of B cells/μm^2 were significantly greater in the tumors compared to non-tumor lungs in both LC and LC-COPD groups of patients (Figure 2A–C, bottom panel).

Among LC-COPD patients, statistically significant associations were seen between the percentage of T cells in lung tumor specimens and forced expiratory volume in one second/forced vital capacity

(FEV_1/FVC) (R = 0.228 and p = 0.032) and the percentage of B cells and FEV_1/FVC (R = −0.370 and $p <$ 0.001, Figure 2D).

2.4. Associations of TLSs with OS in LC and LC-COPD Patients

Cancers **2020**, *12*, 2644 7 of 17

When all patients were analyzed together, a lower number of TLSs (cut-off: 1.944/mm^2) was associated with a poorer 10-year survival (Figure 3A). When patients were subdivided according to the presence of COPD, no significant differences were observed between a low number of TLSs and survival (Figure 3B). As to the area of TLSs in the tumors (cut-off value: 1.112 mm^2), a significantly worse survival was observed in the patients with lower levels of TLS area (Figure 3C). When patients were subdivided according to underlying COPD, smaller TLS areas in the tumors were also significantly associated with significantly poorer survival than those with greater areas of TLSs (Figure 3D). Moreover, when patients were stratified according to COPD severity (GOLD I and II stages), smaller areas of TLSs were also associated with a significantly poorer survival (Figure 3E). Interestingly, the presence of underlying COPD in this cohort was also significantly associated with a lower 10-year patients' survival as shown in Figure 3F.

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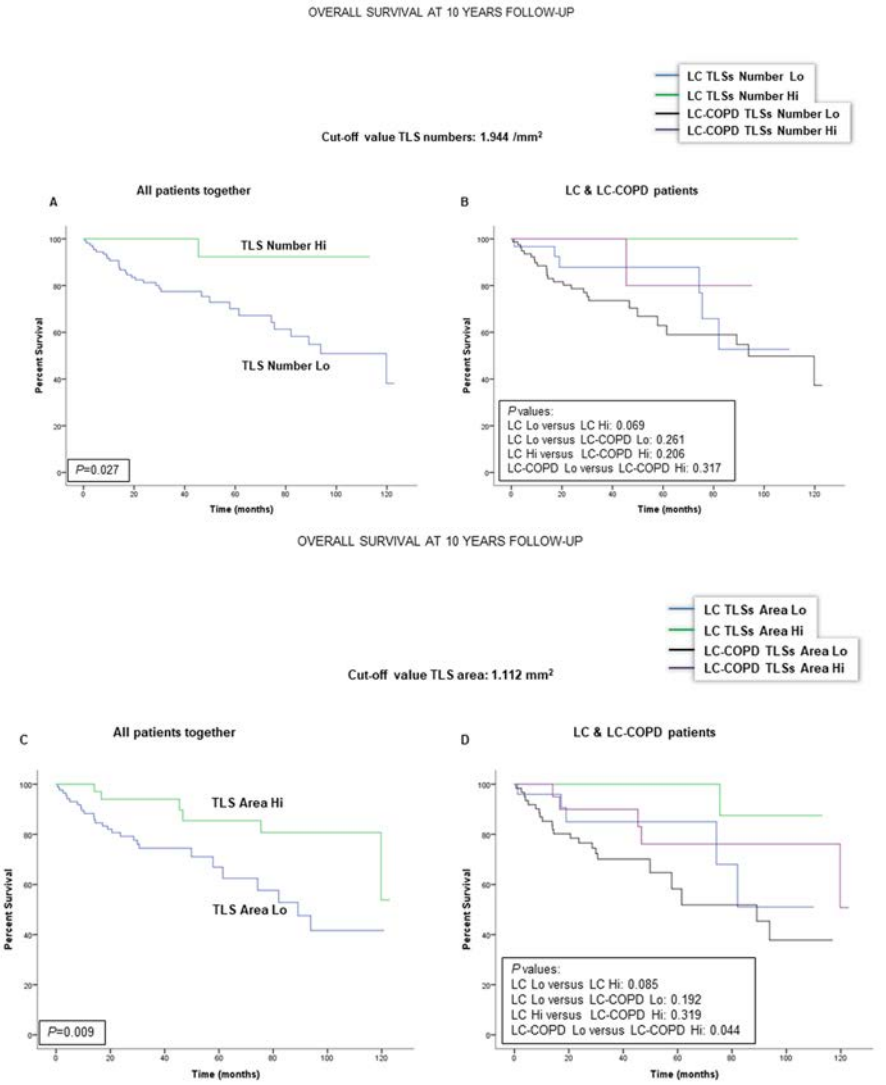

Figure 3. *Cont*.

Cancers **2020**, *12*, x 8 of 17

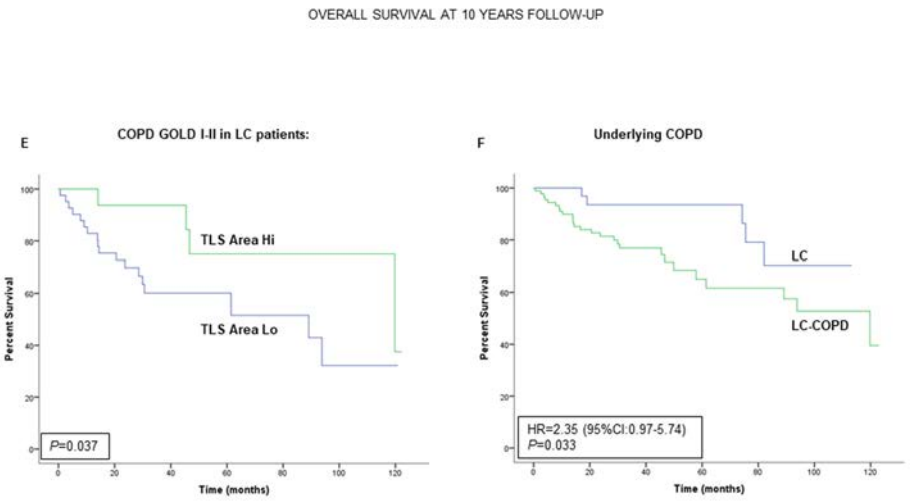

Figure 3. Kaplan-Meier survival curves of the two groups of patients according to TLS levels in tumors. (**A**) Kaplan-Meier survival curves for overall survival (OS) in all patients based on the cut-off value of the number of TLSs (above and below the cut-off value: 1.944/mm^2). Patients with lower numbers had a significantly worse survival. (**B**) Kaplan-Meier survival curves for OS in LC patients with and without COPD based on the cut-off value of the number of TLSs (above and below the cut-off value: 1.944/mm^2). No significant differences were detected. (**C**) Kaplan-Meier survival curves for OS in all patients based on the cut-off value of the total area of TLSs (above and below the cut-off value: 1.112 mm^2). Patients with smaller areas of TLSs had a significantly worse survival. (**D**) Kaplan-Meier survival curves for OS in LC patients with and without COPD based on the total area of TLSs (above and below the cut-off value: 1.112 mm^2). Smaller areas of TLSs were significantly associated with poorer survival among LC-COPD patients. (**E**) Kaplan-Meier survival curves for OS in LC-COPD patients with GOLD I-II stages based on the total area of the TLSs (above and below the cut-off value: 1.112 mm^2). Patients with smaller areas of TLSs had a significantly worse survival. (**F**) Kaplan-Meier survival curves for OS in LC patients according to the presence of underlying COPD. COPD per se was associated with a worse survival among the study patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; Hi, high level (above cut-off value); Lo, low level (below cut-off value); TLS, tertiary lymphoid structures; GOLD, global initiative for chronic obstructive lung disease.

2.5. Associations of B and T Cells with Survival in LC and LC-COPD Patients

Patient 10-year survival was not significantly modified by the levels of T cells (cut-off: 38.08 cells × μm^{-2}, %) in the tumors in either LC or LC-COPD patients (Figure 4A,B). When all patients were analyzed together, lower numbers of B cells in the tumors (cut-off: 90.80 cells × μm^{-2}, %) were significantly associated with a significantly worse survival (Figure 4C). Interestingly, in patients with LC-COPD, but not in those with LC, lower levels of B cells in the tumors were significantly associated with poorer survival than in patients with higher levels of B cells (Figure 4D).

2.5. Associations of B and T Cells with Survival in LC and LC-COPD Patients

Patient 10-year survival was not significantly modified by the levels of T cells (cut-off: 38.08 cells × μm^{-2}, %) in the tumors in either LC or LC-COPD patients (Figure 4A,B). When all patients were analyzed together, lower numbers of B cells in the tumors (cut-off: 90.80 cells × μm^{-2}, %) were significantly associated with a significantly worse survival (Figure 4C). Interestingly, in patients with LC-COPD, but not in those with LC, lower levels of B cells in the tumors were significantly associated with poorer survival than in patients with higher levels of B cells (Figure 4D).

Cancers **2020**, *12*, x 9 of 17

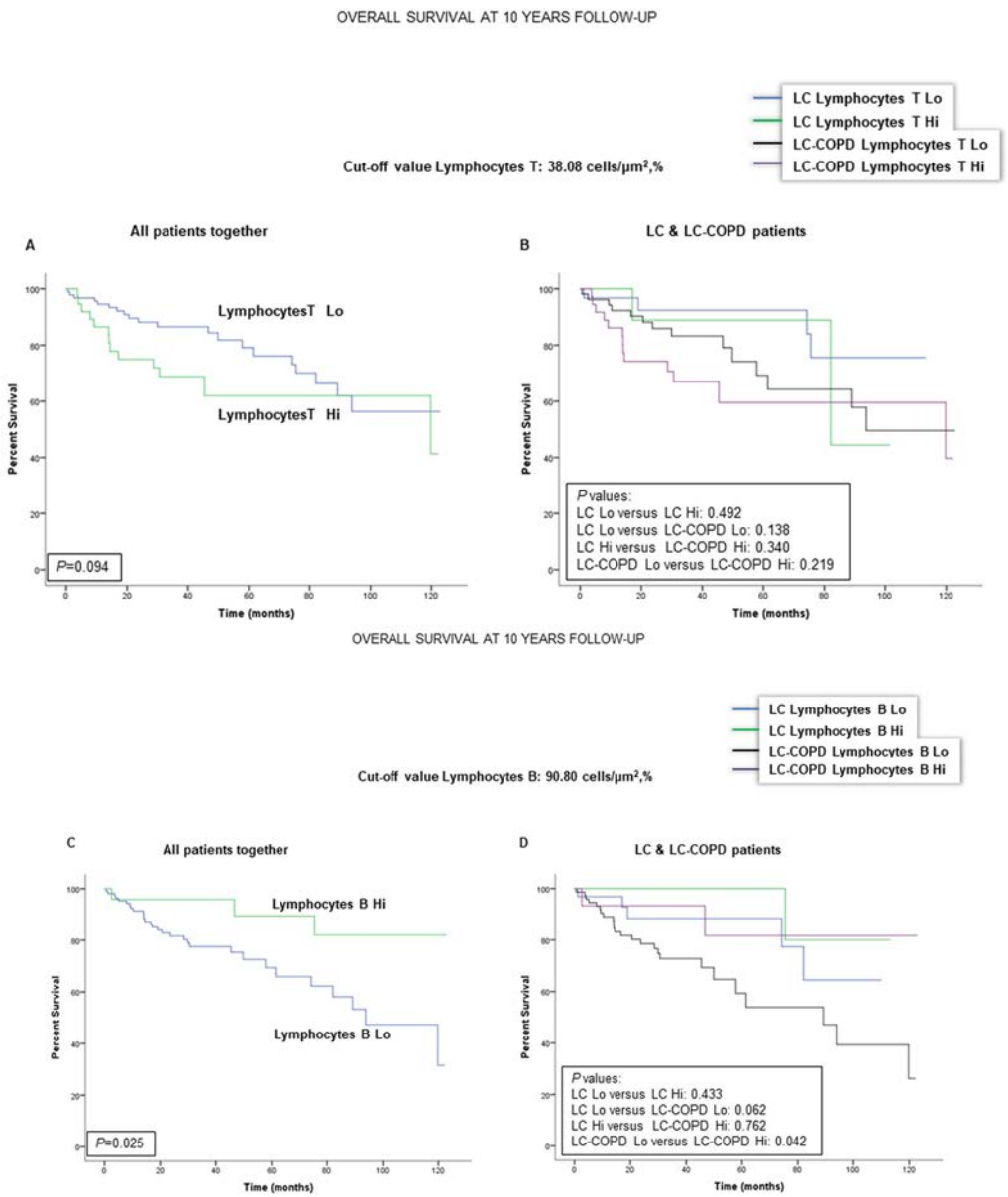

Figure 4. Kaplan–Meier survival curves of the two groups of patients according to T and B cell counts in tumors. (**A**) Kaplan–Meier survival curves for OS in all patients based on the percentage of T cell lymphocytes (above and below the cut-off value: 38.08 cells/µm², %). (**B**) Kaplan–Meier survival curves for OS in LC patients with and without COPD based on the percentage of T lymphocytes (above and below the cut-off value: 38.08 cells/µm², %). Significant differences were observed in any of the patient groups according to the cut-off value of the percentage of T cells within the TLSs. (**C**) Kaplan–Meier survival curves for OS in all patients based on the percentage of B cell lymphocytes (above and below the cut-off value: 90.80 cells/µm², %). Patients with lower proportions of B cells had a significantly worse survival. (**D**) Kaplan–Meier survival curves for OS in LC patients with and without COPD based on the percentage of B lymphocytes (above and below the cut-off value: 90.80 cells/µm², %). Smaller proportions of B cells were significantly associated with poorer survival among LC-COPD patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; Hi, high level (above cut-off value); Lo, low level (below cut-off value).

3. Discussion

In the current study, the main findings were that, in tumor specimens of patients with LC and underlying COPD, the numbers of TLSs and GCs were reduced. Smaller areas of TLSs and lower numbers of B cells were associated with a poorer 10-year survival of the patients, and this was also

3. Discussion

In the current study, the main findings were that, in tumor specimens of patients with LC and underlying COPD, the numbers of TLSs and GCs were reduced. Smaller areas of TLSs and lower numbers of B cells were associated with a poorer 10-year survival of the patients, and this was also related to the severity of the COPD as measured by GOLD stage. Moreover, the presence of a chronic respiratory disease, such as COPD, per se, was also associated with a worse survival among all the patients with LC. The main results encountered in the study are discussed below.

TLSs are organized similarly to lymph nodes or spleen and their function in tissues is probably linked to underlying inflammation. In fact, TLSs are present in organs of chronic inflammatory diseases and are characterized by lymphoid genesis. TLSs are composed by large B cell follicles surrounded by T cells, which may contain dendritic cells [21]. Greater numbers of TLSs were detected in the small airways [22,23] and lungs [15,20,24] of patients with COPD and in the lungs of mice exposed to chronic cigarette smoke [24]. Furthermore, B-cell infiltration in TLSs was also shown to perpetuate inflammatory events in lung specimens that may lead to COPD progression in the patients [16].

The occurrence of TLSs has also been demonstrated in tumor samples of patients with NSCLC mainly characterized by the presence of follicular B cells, mature dendritic cells, and T cells [17,25]. In those studies, the density of mature dendritic cells was shown to correlate with better clinical outcomes in patients with early stages of NSCLC [17,25]. The same authors [19] also demonstrated that B cell density within the TLSs may also be a surrogate for the patients' long-term survival in early stages of NSCLC, implying a role for B-cell mediated immunity in these patients.

In the current investigation, the numbers and area of TLSs were significantly reduced in lung tumor specimens of patients with COPD compared to those without this condition. Moreover, the numbers of GCs, sites of B cell proliferation and differentiation, were also significantly lower in the tumor samples of the COPD patients than in those without this disease. These results are in line with the decline in the number and area of the TLSs, implying that patients with underlying COPD may be less immunocompetent against tumorigenesis. Interestingly, the proportions of B cells were increased in the lung tumors of both groups of patients, with no significant differences between them, while T-cell counts within the TLSs declined in the tumor specimens, with no effect of COPD on those numbers. In fact, B cells were shown to have prognostic value regardless of the numbers of CD8+ T cells in tumors [26]. T cells may become exhausted in tumors including patients with COPD [27], which shows the existing correlations between immune checkpoints and TLSs [26]. These results are similar to those previously reported [27] in a retrospective study of patients with LC, in which a significant proportion of the patients were also COPD. Nonetheless, these results are somehow counter to previous reports in which the number of several types of T cells were increased in tumors of patients with COPD [28]. The methodologies (immunohistochemistry versus flow cytometry) employed in each study, the approaches (prospective versus retrospective cohorts of patients) used in each case, and the degree of the airway obstruction may account for discrepancies among investigations [27,28]. Importantly, the proportions of B cells within the TLSs were greater in the tumor samples than in the tumor-free parenchyma. These findings are in agreement with those previously shown in tumor tissues (sarcoma and melanoma), in which a great amount of B cells was also identified in patients [26,29,30].

In the study, all of the patients from the Lung Cancer Mar Cohort were prospectively followed up to ten years. When all LC patients were analyzed together, a reduced number of TLSs in the lung tumor specimens was associated with a poorer survival in the patients compared to those with greater numbers of TLSs. Furthermore, a smaller area of TLSs in the lung tumors was also associated with a worse prognosis, especially in the patients with underlying COPD. Likewise, a low proportion of B cells in the lung tumors also correlated with a poorer survival among patients with COPD. These are relevant findings that are line with recent reports [26,29,30], in which B cell-enriched TLSs were the best prognostic factor among study patients regardless of the proportions of T cells.

Importantly, in those seminal investigations [26,29,30], the presence of TLSs and B cells was associated with greater survival rates, as well with improved response to immunotherapy in patients

with either melanoma [29,30] or sarcoma [26]. In accordance, findings encountered in the present study also demonstrated that greater areas of TLSs and of B cell proportions led to better survival rates in patients with LC that were followed up for a long period of time (10 years). Interestingly, these associations were especially blatant in patients with underlying COPD. In fact, reduced area of TLSs was also significantly associated with a poorer survival when COPD patients were analyzed independently according to the severity of their disease as identified by GOLD stages I and II (91% of all the patients). In addition, patients with underlying COPD were also those who died significantly earlier than patients with no COPD (Figure 3F, hazard ratio: 2.35). Indeed, these are confirmatory findings of what had already been published in previous investigations [31–33], showing that mortality rates were significantly higher in LC patients with underlying COPD [34,35].

Study Limitations

A potential study limitation is related to the relatively reduced number of patients for the amount of variables and subgroups that were analyzed. Nonetheless, the study hypothesis was confirmed. Additionally, the number of patients was correct, according to the estimations made using statistical analyses as described in Methods. The specific role of cigarette smoking was not assessed in the study, despite that its burden was significantly higher in the LC-COPD patients. However, in a multivariate analysis, in which the variable packs–year was also included, no significant differences were observed between the two groups of patients. These results are in line with those showed by Mark et al. [28], who reported no significant effects of cigarette smoking on Th1 cell profile in lung tumors.

4. Materials and Methods

4.1. 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) [36] for research on human beings and approved by the institutional Ethics Committee on Human Investigation (protocol # 2008/3390/I, 4 February, 2008, Hospital del Mar–Instituto Hospital del Mar de Investigaciones Médicas, Barcelona, Spain). All patients invited to participate in the study signed the written informed consent.

Patients were prospectively recruited from the Lung Cancer Clinic of the Lung Cancer Unit at Hospital del Mar (Barcelona, Spain). All of the patients were part of the Lung Cancer Mar Cohort that started in 2008. The last patients were enrolled in March 2018. For this observational study, 133 patients with LC were recruited. Candidates for tumor resection underwent pulmonary surgery prior to administration of any sort of adjuvant 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) 90 patients with LC and COPD (LC-COPD group), and 2) 43 patients with LC without COPD (LC group).

LC diagnosis and staging were established by histological confirmation and classified according to currently available guidelines for the diagnosis and management of LC [37,38]. Tumor, node, and metastasis (TNM) staging was defined as stated in the 8th edition of the Lung Cancer Stage Classification [39]. In all cases, pre-operative staging was performed using chest and upper abdomen Computed Tomography (CT) scan and Fluoro-deoxy-glucose positron emission tomography/computed tomography (PET) body-scan. When suspected mediastinal lymph-node involvement, a fiber optic bronchoscopy with endo-bronchial ultra-sound (EBUS), and trans-tracheal biopsy of the suspected nodes were performed. In case of negative results, a surgical exploration of the mediastinum: cervical video-assisted mediastinal lymphadenectomy (VAMLA) and/or anterior mediastinotomy were performed, the latter depending on the location of the suspected nodes. Notwithstanding, in all surgical cases, intra-operative systematic hilar and mediastinal lymphadenectomy (at least, ipsilateral paratracheal, subcarinal, and ipsilateral pulmonary ligament) was performed as previously recommended [40,41]. Standard clinical guidelines were used to establish the selection of patients and contraindications for thoracic surgery as previously described [42]. Decisions on the best therapeutic

approach were always made during the weekly meetings of the Multidisciplinary Lung Cancer Committee. Lung tumor resections were applied using classical thoracotomy for all the patients in this study. In the present study, exclusion criteria were: small cell lung cancer (SCLC), severe malnutrition status, chronic cardiovascular disease, metabolic or clot system disorders, signs of severe inflammation and/or bronchial infection (bronchoscopy), current or recent invasive mechanical ventilation, or long-term oxygen therapy. The presence/absence of these diseases was confirmed using standard clinical tests: exercise capacity electrocardiogram, clinical examination, blood tests, bronchoscopy, and echocardiography.

4.2. Clinical Assessment

In all patients, lung function parameters were assessed following standard procedures. Diagnosis and severity of patients with COPD were determined according to current guidelines [5,43]. Nutritional evaluation included the assessment of body mass index (BMI) and nutritional blood parameters from all patients.

4.3. 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 10×10 mm^2 area from the fresh samples. Non-tumor specimens were collected as far distal to the tumor margins as possible (average >7 cm). Fragments of both tumor and non-tumor specimens were fixed in formalin and embedded in paraffin blocks until further use.

4.4. Identification of B Cells, T Cells, and TLSs in the Lung Specimens

B cells, T cells, and TLSs were identified on three-micrometer lung tumor and non-tumor cross-sections using double-staining immunohistochemical procedures (EnVision DuoFLEX Doublestain System, Dako North America Inc., Carpinteria, CA, USA) following the manufacturer's instructions and previous study [10,44,45]. B and T cells were identified by staining of the lung samples with specific antibodies for B cells (anti-CD20 antibody, clone L26, Dako) and T cells (anti-CD3 antibody, Dako). Following deparaffinization, lung sample cross-sections were immersed in preheated antigen-retrieval solution (Dako high pH solution) at 95 °C for 20 min to be then allowed to cool down to room temperature. Slides were washed several times with wash buffer (Dako wash buffer solution). Endogenous peroxidase activity was blocked for 15 min with Dako endogenous enzyme blocking agent. Samples were incubated with anti-human CD3 rabbit polyclonal primary antibody for 40 min. The second incubation was performed for one hour with anti-human CD20 mouse monoclonal antibody. Dextran polymer (EnVision DuoFLEX, Dako) was used as the secondary antibody. Samples were subsequently incubated for 20 min with horseradish peroxidase for mouse monoclonal (CD20) and alkaline phosphatase for rabbit polyclonal (CD3) antibodies. Slides were gently washed and incubated for 10 min with diaminobenzidine (EnVision DuoFLEX, 3,3'-Diaminobenzidine) as a chromogen for the mouse monoclonal antibody (brown reaction product; anti-CD20 antibody) and with liquid permanent red (EnVision DuoFLEX LPR) as a chromogen for the rabbit polyclonal antibody (red reaction product; anti-CD3 antibody).

All procedures were conducted at room temperature. Hematoxylin counterstaining was performed for two minutes, and slides were mounted for conventional microscopy. Images were taken under 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). The number of cells and total area (μm^2) were measured in each of the lung specimens (both tumor and non-tumor samples) using the Image J software (National Institute of Health, Maryland, MD, USA).

In each lung section, the total amount of B cells (CD20-positively-stained) and T cells (CD3-positively-stained) were quantified blindly by two independent observers who were previously

trained for that purpose. Data are presented as the percentage of either B or T cells separately in the measured area in both tumor and non-tumor lung specimens (% B cells/μm^2 and % T cells/μm^2, respectively).

Numbers of TLSs were also manually counted by two independent trained observers after identification of the cell types (B and T cells) that composed these structures using Image J software (National Institute of Health). In addition, total area (mm^2) of each TLSs was also measured in both tumor and non-tumor specimens using Image J software. Data are presented as the number of TLSs in the measured area in both tumor and non-tumor samples (number of TLSs/mm^2) and as the mean area of all the identified and counted TLSs (mm^2).

4.5. Identification of GCs in TLSs of Lung Specimens

In a subgroup of patients (n = 61), the presence of GCs within the TLSs was also specifically evaluated in each lung tumor and non-tumor specimens on three-micrometer sections using hematoxylin and eosin staining by two independent observers [10,46,47]. 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). GCs were selected by the presence of two separate topographic zones: 1) one dark-stained area, which was characterized by a dense population of lymphocytes, and 2) a light-stained area, which was characterized by a low-density lymphocyte site. Data are expressed as the number of GCs in all study groups of patients.

4.6. Statistical Analyses

The normality of the study variables was examined using the Shapiro-Wilk test. For an initial descriptive analysis of clinical parameters, qualitative variables were described as frequencies (number and percentage) and quantitative variables as mean and standard deviation. Differences between LC and LC-COPD were assessed using Student's t-test or Mann-Whitney U tests for parametric and non-parametric variables, respectively. Chi-square test was used to assess differences between the two groups for the categorical variables.

Differences among the different biological variables were explored using the Kruskal-Wallis equality-of-populations rank test, followed by Dunn's Pairwise Comparison test (Sidák adjustment) for the two sample types and patient groups.

OS was defined as the time from the date of diagnosis of LC to the date of death from this disease or the last follow-up, which was completed in December 2018. The median follow-up duration was 37.9 months (P25 = 20.0 months, P75 = 65.4 months). Patients were followed up to a maximum period of 10 years. Patients who did not died of lung cancer were excluded in the investigation.

Threshold analysis was carried out for each continuous biological variable to determine the best cut-off point as predictor of OS, which was the endpoint in the study. The cut-off point was defined using the web-based software Cutoff Finder [48], which has also been previously used in other studies [49,50]. For each biological variable, we identified the threshold level at which a log-rank test allowed segregation of patients into groups with better and worse survival.

Moreover, taking each variable categorized into two groups, estimated power for two-sample comparisons of survivor functions Log-rank test was applied using the Freedman method. Accepting an alpha risk of 0.05 in a two-sided test with 87 and 38 patients in each group (post hoc subdivision), the statistical power was 100% (both number and area of TLSs), T cells (86%), and 100% (B cells). Kaplan-Meier survival curves were performed for each dichotomized variable (below versus above cutoff values, described as Lo and Hi) and log-rank test p-value was estimated. Pearson's correlation analyses were performed to explore potential correlations between clinical and biological variables. Statistical significance was established at $p \leq 0.05$. All statistical analyses were carried out using the software Stata/MP 15 (StataCorp LLC, Texas, TX, USA).

5. Conclusions

A decline in the surface and numbers of TLSs was observed in lung tumors of patients with underlying COPD, which was significantly associated with a poorer survival in these patients. An increase in B cell proportions was seen within the TLSs in tumors of LC patients with and without chronic respiratory disease, and in the latter group, lower levels of B cells correlated with lower survival. The immune tumor microenvironment differs in patients with underlying COPD and these different phenotypes may eventually impact the response to immunotherapy in patients with LC. Thus, the presence of underlying respiratory conditions should be targeted when designing immune therapeutic strategies in LC.

Author Contributions: Study conception and design: E.B., V.C., L.P.; patient assessment and recruitment and sample collection: J.T., V.C., D.R.-C., X.W., M.M.-J., A.R.-F., R.A., L.P.; pathological diagnosis and tumor identification: L.P.; histological analyses: J.T., D.R.-C., X.W., M.M.-J., L.Q., L.P.; statistical analyses and data interpretation: X.D., J.T., D.R.-C., E.B.; manuscript drafting and intellectual input: E.B., J.T., D.R.-C., V.C., A.R.-F.; manuscript writing final version: E.B. All authors have read and agreed to the published version of the manuscript.

Funding: This study has been supported by FIS 18/00075 and CIBERES (FEDER, ISC-III), SEPAR 2018 and SEPAR 2020, unrestricted research grant from Menarini SA 2018, PT13/0010/0005 (FEDER), CIBERES (Instituto de Salud Carlos III, Spain), and the "Xarxa de Bancs de tumors sponsored by Pla Director d'Oncologia de Catalunya (XBTC)", Catalan Government.

Acknowledgments: The authors are thankful to Cristina Lopez-Rodriguez from the Immunology Unit at Universitat Pompeu Fabra (Barcelona) for her advice with the immunological approaches and experiments, to Mireia Admetlló for her help with the patient clinical assessment, and to Judit Prat for her aid with the counting measurements.

Conflicts of Interest: The authors declare no conflict of interest.

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4.2 Second Study

Title:

Immune Cell Subtypes and Cytokines in Lung Tumor Microenvironment: Influence of COPD

Authors:

Jun Tang, Daniel Ramis-Cabrer, Víctor Curull, Xuejie Wang, Liyun Qin, Mercé Mateu-Jiménez, Xavier Duran, Lara Pijuan, Alberto Rodríguez-Fuster, Rafael Aguiló and **Esther Barreiro**.

Journal:

Cancers. 2020 May 13;12(5):1217.
DOI: 10.3390/cancers12051217
PMID: 32414037

Impact Factor:

6.126 (2019), Quartile: Q1

cancers

MDPI

Article

Immune Cell Subtypes and Cytokines in Lung Tumor Microenvironment: Influence of COPD

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Received: 12 March 2020; Accepted: 9 May 2020; Published: 13 May 2020

Abstract: Background: The immune microenvironment plays a role in tumorigenesis. Chronic Obstructive Pulmonary Disease (COPD) is an independent risk factor for lung cancer (LC). We hypothesized that immune profile characterized by T regulatory (Treg), natural killer (NK), and plasma cells, as well as interleukin (IL)-10 and interferon-gamma, may differ within tumors of LC patients with/without COPD. Methods: Treg (anti-CD3 and anti-forkhead boxP3 antibodies), NK (anti-NCR1 antibody), IgG (anti-CD138-IgG antibody), IgA (anti-CD138-IgA antibody) using immunohistochemistry, and both IL-10 and interferon-gamma (ELISA) were quantified in tumor and non-tumor specimens (thoracotomy for lung tumor resection) from 33 LC–COPD patients and 20 LC-only patients. Results: Immune profile in tumor versus non-tumor specimens: Treg cell counts significantly increased in tumors of both LC and LC–COPD patients, while in tumors of the latter group, IgG-secreting plasma cells significantly decreased and IL-10 increased. No significant differences were seen in levels of NK cells, IgA-secreting cells, IgA/IgG, or interferon-gamma. Immune profile in tumors of LC–COPD versus LC: No significant differences were observed in tumors between LC–COPD and LC patients for any study marker. Conclusions: Immune cell subtypes and cytokines are differentially expressed in lung tumors, and the presence of COPD elicited a decline in IgG-secreting plasma cell levels but not in other cell types.

Keywords: lung cancer; COPD; T regulatory cells; natural killer cells: immunoglobulin-secreting plasma cells; immune tumor microenvironment; IL-10 and interferon-gamma

1. Introduction

Lung cancer (LC) continues to be a major cause of mortality worldwide [1–5]. In certain geographical areas, LC may account for up to one-third of deaths [1–6]. The presence of airway

obstruction is a major risk factor for LC development [1–12]. Specifically, Chronic Obstructive Pulmonary Disease (COPD) and emphysema [13–15] have been demonstrated to favor lung tumorigenesis in the patients [16,17]. The underlying biological mechanisms that render patients with COPD more susceptible to the development of emphysema remain to be fully elucidated.

Several biological events such as increased oxidative stress, inflammation, epigenetics, and tumor microenvironment have been proposed as mechanisms that underlie the process of tumorigenesis in patients with chronic airway obstruction and emphysema [7,18]. Those events interact with key cellular mechanisms, such as angiogenesis, cell repair, and cell death and growth, which may interfere with cell survival, thus promoting tumorigenesis and LC development [7,19].

It has been well established that the tumor microenvironment and immune surveillance play a significant role in cancer initiation and progression [20,21]. Regulatory T cells (Treg) are key in immune tolerance and homeostasis [22,23]. Treg cells infiltrate tumors and suppress antitumor immunity within the tumor microenvironment, thus promoting tumor progression and growth [22,23]. Importantly, it has also been shown that tumor-infiltrating Treg cells express a differential phenotype from that expressed in circulating cells [24,25], which implies that local environmental factors may influence the immunosuppressive function of Treg cells. Whether chronic airway obstruction, such as in COPD, may alter Treg expression remains to be investigated.

Natural killer (NK) cells, which are present in peripheral blood, lymph nodes, spleen, and bone marrow, play important roles in innate and adaptive immune system responses [26,27]. NK cells activate monocytes and cytotoxic T cells and modulate T helper cell polarization, while they may also stimulate or inhibit B cells to produce immunoglobulins [28]. NK cells also release cytokines such as interferon-gamma that inhibit the proliferation of lung tumors [29]. Moreover, tumor cells may also produce immunosuppressive cytokines, namely interleukin (IL)-10 and transforming growth factor (TGF) beta that inhibit the function of NK cells [30–34]. Whether the presence of COPD may modify NK cell counts in tumors remains to be explored. Tumor-infiltrating B cells and antibodies produced within the tumors may also play a role in cancer progression. Furthermore, high levels of IgG and low levels of IgA within lung tumors were associated with better overall survival for certain adenocarcinoma subtypes [35]. Whether the presence of airway obstruction may influence the expression of plasma cells remains unanswered.

On this basis, we hypothesized that, in LC patients with COPD, the immune profile characterized by the expression of Treg cells, NK cells, plasmatic cells, and levels of the cytokines' interferon-gamma and IL-10 within the tumors may differ from LC patients with no underlying COPD. Accordingly, our objectives were to determine in lung tumors and non-tumor specimens of LC patients, with and without COPD, the following parameters: (1) counts of Treg and NK cells, (2) numbers of both IgG- and IGA-secreting plasma cells, and (3) levels of the cytokines IL-10 and interferon-gamma.

2. Methods

2.1. Study Design and Ethics

This is a cross-sectional prospective study designed by following the World Medical Association guidelines (Seventh revision of the Declaration of Helsinki, Fortaleza, Brazil, 2013) [36] for research on human beings and 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.

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, 53 patients with LC were recruited during the years 2017–2019. 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 [37,38]. TNM

(tumor, node, and metastasis) staging was defined as stated in the eighth edition of the Lung Cancer Stage Classification [39]. COPD diagnosis was established as a post-bronchodilator forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) ≤ 0.7, which is not fully reversible by spirometry, according to currently available guidelines for diagnosis and management of COPD [5,40]. Exclusion criteria were as follows: 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) 33 patients with LC and COPD (LC–COPD group) and (2) 20 patients with LC without COPD (LC group).

2.2. Clinical Assessment

In all patients, lung function parameters were assessed by following standard procedures. Diagnosis and severity of patients with COPD were determined according to currently available guidelines [5,40]. Nutritional evaluation included the assessment of body mass index (BMI) and nutritional blood parameters from all patients.

2.3. 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 10×10 mm^2 area from the fresh samples, as previously validated [7–9]. Non-tumor specimens were collected as far as possible from the distal to the tumor 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 snap-frozen immediately in liquid nitrogen and stored at −80 °C for the quantification of the cytokine levels.

2.4. Identification of Treg Cells and Plasma Cells in the Lung Specimens

Treg cells and IgG and IgA immunoglobulins secreting plasma cells were identified on three-micrometer lung tumor and non-tumor cross-sections, using double-staining immunohistochemical procedures (EnVision DuoFLEX Doublestain System, Dako North America Inc., Carpinteria, CA, USA) following the manufacturer's instructions and previous studies [7]. Treg cells were identified through the expression of CD3 and the intracellular transcription factor-forkhead box P3 (FOXP3), using specific antibodies (anti-CD3 and anti-FOXP3 clone 236A/E7, respectively, Dako North America and Abcam, Cambridge, UK, respectively). Plasma cells were identified by using the CD138 marker and the corresponding immunoglobulins A and G (anti-CD138 clone MI15, anti-IgA, and anti-IgG, respectively, Dako North America). Following deparaffinization, lung sample cross-sections were immersed in preheated antigen-retrieval solution (Dako high pH solution) at 95 °C for 20 min, to be then allowed to cool down to room temperature. Slides were washed several times with wash buffer (Dako wash buffer solution). Endogenous peroxidase activity was blocked for minutes with Dako endogenous enzyme blocking agent. Samples were incubated with the corresponding primary antibodies: anti-human CD3 rabbit polyclonal antibody or anti-human CD138 mouse monoclonal antibody for 40 min. The second incubation was performed for 1 h with the corresponding antibody in each case (anti-human FOXP3 mouse monoclonal antibody, anti-human IgA, or IgG rabbit polyclonal antibody). Chain-polymer conjugate technology utilizing enzyme-labeled inert backbone molecule of dextran was used in order to amplify the signal (EnVision DuoFLEX, Dako) [41]. Samples were then incubated with horseradish peroxidase (HRP) for mouse monoclonal antibodies and alkaline phosphatase (AP) for rabbit polyclonal antibodies for 20 minutes. Slides were gently washed and incubated for 10 min with diaminobenzidine (EnVision DuoFLEX DAB+, Carpinteria, CA, USA),

as a chromogen for mouse monoclonal antibodies (brown reaction product; anti-FOXP3 or anti-CD138 antibodies) and liquid permanent red (EnVision DuoFLEX LPR) as a chromogen for rabbit polyclonal antibodies (red reaction product; anti-CD3, anti-IgA or anti-IgG antibodies).

All procedures were conducted at room temperature. Hematoxylin counterstaining was performed for two minutes, and slides were mounted for conventional microscopy. Images were taken under 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). The number of cells and total area (μm^2) were measured in each of the lung specimens (both tumor and non-tumor samples), using Image J software (National Institute of Health, Maryland, MD, USA).

In each lung section, the total amount of Treg cells (both CD3- and FOXP3-positively-stained), plasma cells secreting IgA (CD138- and IgA-positively-stained), and plasma cells secreting IgG (CD138- and IgG-positively stained) were quantified blindly by two independent observers who were previously trained for that purpose (correlation between them $R^2 > 0.90$). In order to ensure the quality and reliability of the results, the discrepant results were measured again by the two independent observers, as many times as a correlation > 0.90 was achieved for each sample and analyzed marker. All the results are presented as follows: (1) as the percentage of Treg cells in the measured area in μm^2 in both tumor and non-tumor lung specimens (% Treg, total number of cells/$\mu m^2 \times 100$), and (2) as the percentage of either IgA or IgG positive plasma cells in the measured area in μm^2 in both tumor and non-tumor lung specimens (% IgA, total number of plasma cells/$\mu m^2 \times 100$ and % IgG, total number of plasma cells/$\mu m^2 \times 100$ respectively). The ratio of IgA to IgG was also calculated by dividing the % of IgA-secreting plasma cells for the given area by the % of IgG-secreting plasma cells within the same area (no units).

2.5. Identification of NK Cells in Lung Specimens

NK cells were identified in the tumor and non-tumor lung specimens on three-micrometer sections, using conventional immunohistochemical procedures as previously described [7]. Following deparaffinization, lung cross-sections were immersed in preheated antigen retrieval solution of ethylenediaminetetraacetic acid (EDTA, pH 8, Sigma-Aldrich, St. Louis, MO, USA), incubated at 95 °C for 20 min, and then cooled down to room temperature. Slides were washed over the following steps with phosphate buffer saline (PBS, Sigma-Aldrich). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min. In order to properly identify NK cells in the lung samples (tumor and non-tumor specimens), NKp46 receptor (encoded by the *ncr*1 gene) was measured by using a specific primary antibody, as also previously reported [42,43]. Thus, primary antibody incubation with anti-natural Cytotoxicity Triggering Receptor 1 (anti-NCR1 protein antibody, Abcam, Cambridge, UK) was performed for one hour. Slides were then incubated with biotinylated universal secondary antibody for 30 min, followed by another 30 min 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, 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). In addition, NCR1-positively-stained cells were counted in the tumor and non-tumor lung specimens of all the patients. The area of the lungs in which NK cells were identified (μm^2) was also measured in both tumor and non-tumor specimens, using Image J software (National Institutes of Health, USA). Data are shown as the percentage of NK cells in the measured area in both tumor and non-tumor lung specimens (% NK cells/$\mu m^2 \times 100$).

2.6. Quantification of Cytokines in Lung Tissue

Protein levels of IL-10 and interferon-gamma were quantified in tumor and non-tumor lung specimens from all the subjects, using specific Enzyme-Linked Immunosorbent Assay (ELISA) kits (Raybiotech Inc, Norcross GA), following the manufacturer's instructions and previous studies [7]. Frozen samples from all the patients were homogenized in lysis buffer. Samples were centrifuged at 1000× *g* for 30 min, the pellet was discarded, and the supernatant was designated as the crude cytoplasmic homogenate. The entire procedures were always conducted at 5 °C (on ice). In the assigned ELISA plates, 100 µL of lung homogenates were added and incubated with the corresponding diluted biotinylated antibody in duplicates. After several washes with washing solution, samples were incubated with HRP, to be subsequently incubated with tetramethylbenzidine (TMB, Raybiotech Inc, Norcross, GA, USA) substrate solution at room temperature, in darkness. Finally, the enzyme reaction (HRP) was suspended by the addition of stop solution reagent to all the samples. A standard curve was always created with each assay run. Absorbance was read in a microplate reader at 450 nm, using 655 nm as a reference filter. Intra- and inter-assay coefficients of variation in lung homogenates ranged from 0.45% to 3.52% and from 0.89% to 3.69% for both IL-10 and in interferon-gamma ELISA experiments, respectively.

2.7. Statistical Analyses

All the statistical analyses were performed by using STATA (software for Statistics and Data Science) software (StataCorp LLC, College Station, TX, USA). The normality of the study variables was tested by using the Shapiro–Wilk test. Clinical variables are expressed in a Table 1. 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 by using the Student's *t*-test. Histological results obtained in the lung preparations are expressed as scatter plots of individual values in which median and interquartile ranges are also shown. Differences between patient groups (LC and LC–COPD) and types of samples (tumor and non-tumor) were assessed by using the Kruskal–Wallis equality-of-populations rank test, followed by Dunn's Pairwise Comparison test (Sidák adjustment). Statistical significance was established at $p \leq 0.05$.

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

Anthropometric Variables	Lung Cancer (n = 20)	Lung Cancer-COPD (n = 33)
Age, years	65 (14)	67 (8)
Male, n/Female, N	10/10	29/4 **
BMI, kg/m^2	28 (4)	25 (4) *
Smoking History		
Current: n, %	8, 40	23, 70 *
Ex-smoker: n, %	3, 15	9, 27
Never smoker: n, %	9, 45	1, 3 ***
Pack-years	18 (22)	56 (27) ***
Lung Function Parameters		
FEV_1, %	88 (9)	68 (15) ***
FEV_1/FVC, %	77 (5)	63 (8) ***
DL_{CO}, %	87 (15)	72 (20) **
KCO, %	89 (13)	73 (18) **
GOLD Stage		
GOLD Stage I: n, %	NA	10, 30
GOLD Stage II: n, %	NA	20, 60
GOLD Stage III: n, %	NA	3, 10
TNM Staging		
Stage 0+ II: n, %	17, 85	28, 84.8
Stage III: n, %	3, 15	3, 9.1
Stage IV: n, %	0, 0	2, 6.1
Histological Diagnosis		
Squamous cell carcinoma: n, %	4, 20	7, 21
Adenocarcinoma: n, %	15, 75	25, 76
Others: n, %	1, 5	1, 3
Blood Parameters		
Total leucocytes/μL	6.39 (1.77) × 10^3	9.52 (2.70) × 10^3 ***
Total neutrophils/μL	3.72 (1.37) × 10^3	6.64 (2.42) × 10^3 ***
Total lymphocytes/μL	1.97 (0.71) × 10^3	2.02 (0.76) × 10^3
Albumin (g/dL)	4.4 (0.2)	4.0 (0.6) **
Total proteins (g/dL)	7.0 (0.4)	6.8 (1.0)
Fibrinogen (mg/dL)	443 (126)	427 (83)
CRP (mg/dL)	3.5 (5.6)	10.5 (19.5)
GSV (mm/h)	23 (10)	26 (16)
Body Weight Loss, kg		
0, n, %	20, 100	30, 91
1–5, n, %	0, 0	1, 3
6–10, n, %	0, 0	2, 6

Continuous variables are presented as mean and standard deviation, while categorical variables are presented as the number of patients in each group and the percentage in the study group with respect to the total population. Definition of abbreviations: N, number; kg, kilograms; m, meters; 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: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between lung cancer–Chronic Obstructive Pulmonary Disease (LC–COPD) patients and LC patients.

3. Results

3.1. Clinical Characteristics

Clinical and functional characteristics of LC and LC–COPD patients that were recruited in the current investigation are shown in Table 1. As expected, the number of LC–COPD patients was higher than those in the group of LC. Age did not significantly differ between the two groups of patients, while BMI was significantly lower in LC–COPD patients compared to LC patients. The number of male patients in the LC–COPD group was greater than in LC patients. As expected, current smokers and the

number of pack/year was 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} in LC–COPD patients were significantly lower than in LC patients (Table 1). Most of the patients with COPD were in GOLD I and II stages (90%). TMN staging or histological subtypes did not significantly differ between the two groups. The number of patients with adjuvant treatment following thoracotomy did not differ between the two study groups. In LC–COPD compared to LC patients, the levels of total leucocytes and neutrophils were significantly increased while levels of albumin significantly decreased. Total proteins, fibrinogen, C-reactive protein (CRP), globular sedimentation velocity (GSV), and body weight loss did not differ between LC–COPD and LC patients.

3.2. Treg and NK Cells in Lung Specimens

3.2.1. Differences between LC–COPD and LC in either Tumor Lesions or Non-Tumor Specimens

Proportions of Treg cells/μm² significantly increased in the tumors compared to non-tumor specimens in both LC and LC–COPD patient groups (Figure 1A,B). However, no significant differences were found in the proportions of NK cells/μm² between the tumor and non-tumor lung specimens in either LC or LC–COPD patients (Figure 2A,B). A subanalysis conducted only in patients with lung adenocarcinoma revealed identical results for this set of experiments

No significant differences were found in the total proportions of Treg cells/um² or NK cells/um² between LC–COPD and LC patients in either tumor or non-tumor specimens (Figures 1 and 2).
A subanalysis conducted only in patients with lung adenocarcinoma revealed identical results for this set of experiments

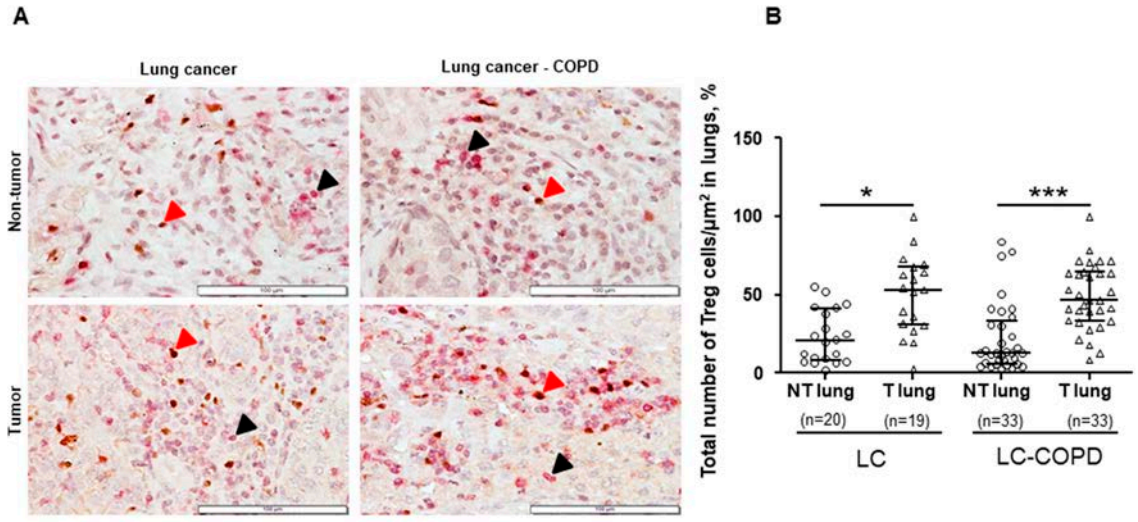
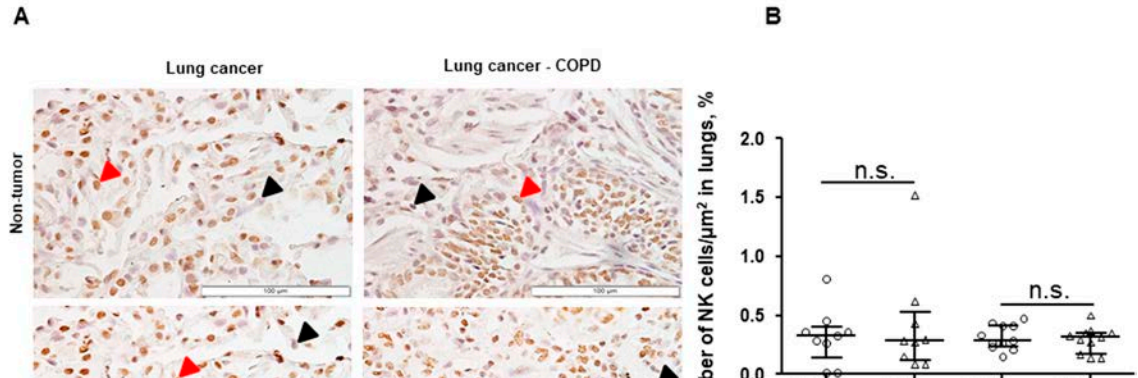


Figure 1. (**A**) Representative examples of double immunohistochemical staining for Treg cells (CD3+FOXP3+ positively stained T cells) in LC and LC–COPD patients, respectively. All types of T cells (CD3+) are stained in only in red (black arrow), while Treg cells (CD3+FOXP3+) are specifically stained with both brown and red (black arrow). (**B**) Median and interquartile ranges between 75th and 25th percentiles of proportions of Treg cells in the total measured area. Comparisons were made between the non-tumor (NT) and tumor (T) samples and the LC and LC–COPD groups. For technical reasons, the number of patients in each group presented in the LC and LC–COPD groups may differ. Statistical significance of * $p \leq 0.05$, ** $p \leq 0.001$ between pairs of samples (tumor and non-tumor) in either LC or LC–COPD patients. Statistical significance of $p < 0.5$, *** $p \leq 0.001$ between COPD and non-obstructive pulmonary disease; LC–COPD, patients with lung cancer and chronic obstructive pulmonary disease; Treg, regulatory T cells; CD, cluster of differentiation; FOXP3, forkhead box P3.

Figure 1. (**A**) Representative examples of double immunohistochemical staining for Treg cells (CD3+FOXP3+ positively stained T cells) in LC and LC–COPD patients, respectively. All types of T cells (CD3+) are stained in only in red (black arrow), while Treg cells (CD3+FOXP3+) are specifically stained with both brown and red (black arrow). (**B**) Median and interquartile ranges between 75th and 25th percentiles of proportions of Treg cells in the total measured area. Comparisons were made between the non-tumor (NT) and tumor (T) samples and the LC and LC–COPD groups. For technical reasons, the number of patients in each group presented in the LC and LC–COPD groups may differ. Statistical significance of * $p \leq 0.05$, ** $p \leq 0.001$ between pairs of samples (tumor and non-tumor) in either LC or LC–COPD patients. Statistical significance of $p < 0.5$, *** $p \leq 0.001$ between COPD and non-obstructive pulmonary disease; LC–COPD, patients with lung cancer and chronic obstructive pulmonary disease; Treg, regulatory T cells; CD, cluster of differentiation; FOXP3, forkhead box P3.



the non-tumor (NT) and tumor (T) samples and the LC and LC–COPD groups. For technical reasons, the number of patients in each group or type of samples (tumor and non-tumor) may differ. Statistical significance: *, $p \leq 0.5$; ***, $p \leq 0.001$ between tumor and non-tumor lungs in either LC or LC–COPD patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; Treg, regulatory T cells; CD, cluster of differentiation; FOXP3, forkhead box P3.

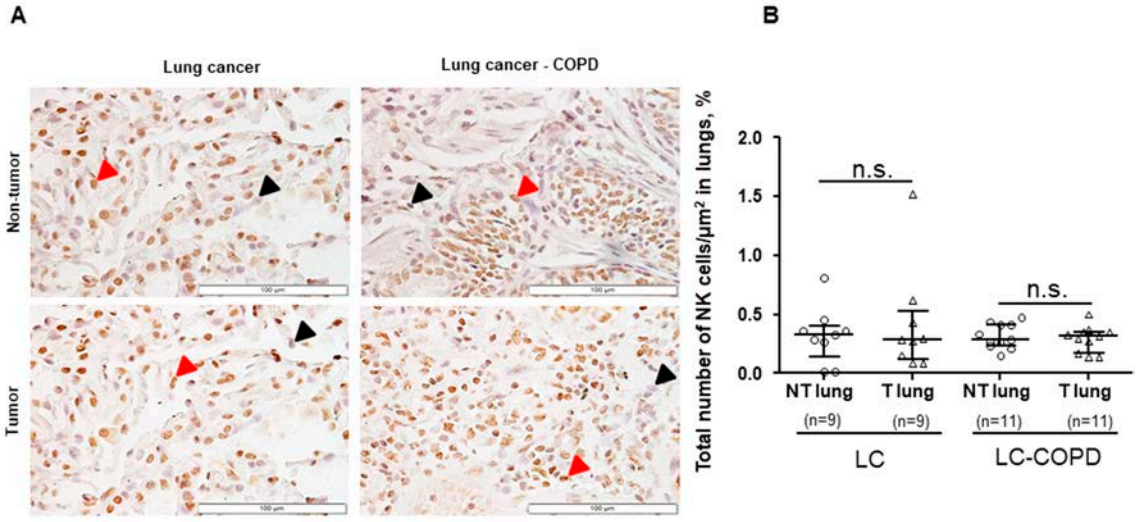


Figure 2. (**A**) Representative examples of double immunohistochemical staining for NK cells (NCR1+) in LC and LC–COPD patients, respectively. Black arrows point toward NK cells negatively stained in blue with hematoxylin, and red arrows point toward NK cells (NCR1+) stained in brown. (**B**) Median and interquartile ranges between 75th and 25th percentiles of proportions of NK cells in the total measured area. Comparisons were made between the non-tumor (NT) and tumor (T) samples and the LC and LC–COPD groups. For technical reasons, the number of patients in each group or type of samples (tumor and non-tumor) may differ. Statistical significance: n.s. No significance between tumor and non-tumor lungs in either LC or LC–COPD patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; CD, cluster of differentiation, NK, natural killer; NCR1, natural cytotoxicity triggering receptor 1.

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3.2.2. Differences between Tumor and Non-Tumor Parenchyma in LC–COPD and LC Patients

Proportions of Treg cells/µm^2 significantly increased in the tumors compared to non-tumor specimens in both LC and LC–COPD patient groups (Figure 1A,B). However, no significant differences were found in the proportions of NK cells/µm^2 between the tumor and non-tumor lung specimens in either LC or LC–COPD patients (Figure 2A,B). A subanalysis conducted only in patients with lung adenocarcinoma revealed identical results for this set of experiments

3.3. IgG and IgA Secreting Plasma Cells in Lung Specimens

3.3.1. Differences between LC–COPD and LC in either Tumor Lesions or Non-Tumor Specimens

No significant differences were found in the total proportions of IgG-secreting plasma cells/µm^2 or IgA-secreting plasma cells/µm^2 between LC–COPD and LC patients, in either tumor or non-tumor specimens (Figures 3 and 4). A subanalysis conducted only in patients with lung adenocarcinoma revealed identical results for this set of experiments

3.3.2. Differences between Tumor and Non-Tumor Parenchyma in LC–COPD and LC Patients

Proportions of IgG-secreting plasma cells/µm^2 significantly decreased in the tumors compared to non-tumor specimens, only in LC–COPD patients (Figure 3A,B). No significant differences were found in the proportions of IgA-secreting plasma cells/µm^2 between tumor and non-tumor lung specimens in either LC or LC–COPD patients (Figure 4A,B). No significant differences were found in the IgA/IgG ratio between the tumor and non-tumor lung specimens in either LC or LC–COPD patients (Figure 4C). A subanalysis conducted only in patients with lung adenocarcinoma revealed identical results for this set of experiments

Proportions of IgG-secreting plasma cells/μm² significantly decreased in the tumors compared to non-tumor specimens, only in LC–COPD patients (Figure 3A,B). No significant differences were found in the proportions of IgA-secreting plasma cells/μm² between tumor and non-tumor lung specimens in either LC or LC–COPD patients (Figure 4A,B). No significant differences were found in the IgA/IgG ratio between the tumor and non-tumor lung specimens in either LC or LC–COPD patients (Figure 4C). A subanalysis conducted only in patients with lung adenocarcinoma revealed identical results for this set of experiments

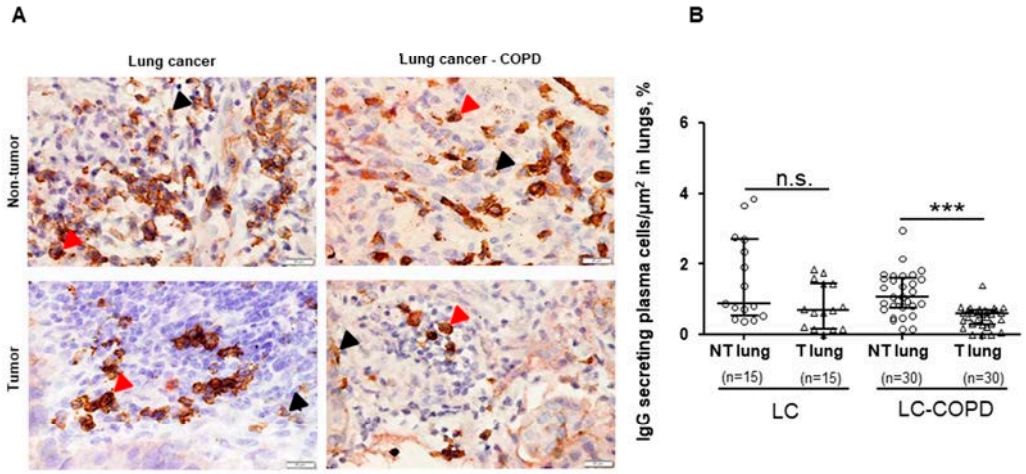

Figure 3. (**A**) Representative examples of double immunohistochemical staining for IgG-secreting plasma cells (CD138-IgG positively stained plasma cells) in LC and LC–COPD patients, respectively. All types of plasma cells (CD138+) are stained only in brown (black arrow), while IgG-secreting plasma cells (CD138+IgG+) are specifically stained with both brown and red. (**B**) Median and interquartile ranges between 75th and 25th percentiles of number of IgG-secreting plasma cells in the total measured area. Black-stained regions within the lungs correspond to anthracosis. Comparisons were made between the non-tumor (NT) and tumor (T) samples and the LC and LC–COPD groups. For technical reasons, the number of patients in each group or type of samples (tumor and non-tumor) may differ. Statistical significance: ***, $p < 0.001$ between tumor and non-tumor lungs in LC–COPD patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; CD, cluster of differentiation; Ig, immunoglobulin.

Figure 3. (A) Representative examples of double immunohistochemical staining for IgG-secreting plasma cells (CD138-IgG positively stained plasma cells) in LC and LC–COPD patients, respectively. All types of plasma cells (CD138+) are stained only in brown (black arrow), while IgG-secreting plasma cells (CD138+IgG+) are specifically stained with both brown and red. (B) Median and interquartile ranges between 75th and 25th percentiles of number of IgG-secreting plasma cells in the total measured area. Black-stained regions within the lungs correspond to anthracosis. Comparisons were made between the non-tumor (NT) and tumor (T) samples and the LC and LC–COPD groups. For technical reasons, the number of patients in each group or type of samples (tumor and non-tumor) may differ. Statistical significance: ***, p < 0.001 between tumor and non-tumor lungs in LC–COPD patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; CD, cluster of differentiation; Ig, immunoglobulin.

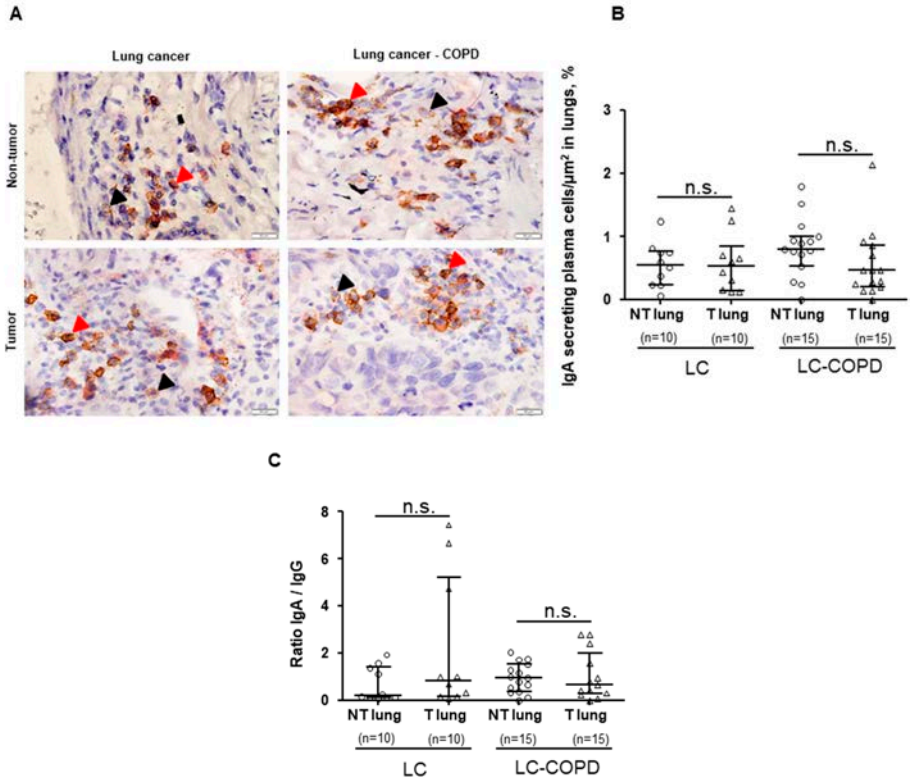

Figure 4. (**A**) Representative examples of double immunohistochemical staining for IgA-secreting plasma cells (CD138-IgA positively stained plasma cells) in LC and LC–COPD patients, respectively. All types of plasma cells (CD138+) are stained only in brown (black arrow), while IgA-secreting plasma cells (CD138+IgA+) are specifically stained with both brown and red. (**B**) Median and interquartile ranges between 75th and 25th percentiles of number of IgG-secreting plasma cells in the total measured area. Black-stained regions within the lungs correspond to anthracosis. (**C**) Median and interquartile ranges between 75th and 25th percentiles of IgA/IgG ratio in LC and LC–COPD patients, respectively. Comparisons were made between the non-tumor (NT) and tumor (T) samples and the LC and LC–COPD groups. For technical reasons, the number of patients in each group or type of samples (tumor and non-tumor) may differ. Statistical significance: n.s. No significance between tumor and non-tumor lungs in either LC or LC–COPD patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; CD, cluster of

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3.4. Cytokines Levels in Lung Specimens

3.4.1. Differences between LC–COPD and LC in either Tumor Lesions or Non-Tumor Specimens

Protein levels of IL-10 and interferon-gamma cytokines did not significantly differ between LC–COPD and LC in either tumor or non-tumor lung specimens (Figure 5A,B).

subanalysis conducted only in patients with lung adenocarcinoma revealed identical results for this set of experiments

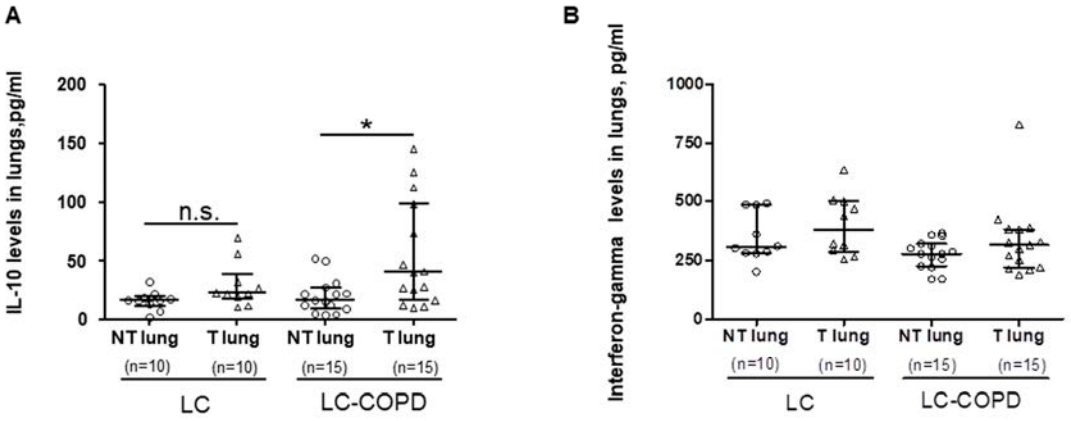

Figure 5. (**A**) Mean values and SD of number IL-10 levels by ELISA in LC and LC–COPD patients, respectively. (**B**) Median and interquartile ranges between 75th and 25th percentiles of number of interferon-gamma levels by ELISA in LC and LC–COPD patients, respectively. Comparisons were made between the non-tumor (NT) and tumor (T) samples and the LC and LC–COPD groups. For technical reasons, the number of patients in each group or type of samples (tumor and non-tumor) may differ. Statistical significance: *, $p \leq 0.5$ between tumor (T) and non-tumor (NT) lungs in LC–COPD patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; IL, interleukin; IFN, interferon. ELISA, enzyme linked immunosorbent assay.

3.4.2. Differences between Tumor and Non-Tumor Parenchyma in LC–COPD and LC Patients.

4. Discussion

Levels of IL-10 significantly increased in the tumors compared to non-tumor specimens only in LC–COPD patients, whereas no significant differences were found in interferon-gamma levels between tumor and non-tumor lung specimens in either LC or LC–COPD patients (Figure 5A,B).

In the current investigation, the number of Treg cells was greater in lung tumors of both groups of patients compared to non-tumor lung specimens. The presence of underlying COPD did not significantly modify Treg counts in the tumors. Treg cells modulate the immune system and maintain immune tolerance and homeostasis, thus preventing the development of autoimmune diseases. In general, the immunosuppressive function of Treg cells is based on the inhibition of proliferation of effector T cells [44]. In the study, Treg cells were most likely responsible for the creation of an immunosuppressive environment within the tumors; the rise in Treg cell counts was detected in a similar fashion in the tumors of both groups of patients.

Interestingly, the cytokine TGF-beta, which was shown to be significantly produced by cancer cells [7], also induces the proliferation and differentiation of Treg cells [45]. IL-10 can also be synthesized by Treg cells, which may favor the production of this cytokine in tumors, even by other cell types [34]. In the present study, a significant rise in IL-10 protein levels was detected in the tumors of the patients with underlying COPD, but not in those without this condition. These findings suggest that COPD patients are probably more prone to favor the expansion and proliferation of Treg cells within lung tumors. Future investigations should aim to explore the precise role of IL-10 and its potential relationships in lung tumorigenesis in patients with chronic airway obstruction, as in COPD. This would enable us to tease out whether the rise in IL-10 plays a significant role or may just be an epiphenomenon.

Levels of interferon-gamma did not differ between tumor and non-tumor specimens in any of the

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Levels of interferon-gamma did not differ between tumor and non-tumor specimens in any of the study groups. However, it has been suggested that interferon-gamma may be a potential useful biomarker for the monitoring of the response to immunotherapy [46]. Differences in clinical staging may account for the discrepancies in levels of interferon-gamma detected in the tumors of the patients in the current study and those in which high levels of this cytokine were seen in tumors of patients with advanced LC staging [46].

NK cells represent 10% of peripheral lymphocytes in patients. They are abundantly expressed in several immune structures, such as bone marrow, spleen, and lymph nodes, and the release of chemoattractants favor their migration to inflammation sites [26,27]. Importantly, NK cells stimulate maturation of dendritic cells and are also relevant for the activation of monocytes and cytotoxic T cells [28]. In the present study, the number of NK cells in tumor specimens did not differ between the two study groups of patients. Moreover, no differences were detected between lung tumor samples and non-tumor lung specimens in any of the study groups. These findings are somehow counter to previous results [47] in which NK cell infiltration degree correlated with overall survival in patients with LC. Furthermore, the tumor microenvironment was also shown to impair NK cell function, characterized by a significant reduction in their tumoricidal capacity [48].

High proportions of IgG and low proportions of IgA were associated with improved overall survival in patients with lung adenocarcinoma with specific mutations [35]. In other cancer types, high IgG proportions within the tumor lesions correlated with better survival rates among the patients [49]. A recent investigation has also demonstrated that the baseline level of anti-BP180 IgG in patients with LC was associated with a better response to immunotherapy and overall survival [50]. Furthermore, the ratio of IgA/IgG was shown to be useful as a biomarker for the early diagnosis of LC [51]. In other studies, however, IgA levels within tumors were not associated with survival in patients with hepatocellular carcinoma [52] or bladder cancer [53]. In the current study, levels of IgG-secreting plasma cells were significantly reduced within the tumor specimens only in LC patients with underlying COPD, but not in LC-only patients. Interestingly, levels of IgA did not differ between tumor and non-tumor specimens in any study group of patients. Altogether, these findings imply that the protective role of IgG was probably blunted in the tumors of the patients in the current investigation. Future studies should focus on whether IgG therapy may be effective for the treatment of lung tumors, specifically in patients with COPD.

Finally, we would like to comment on the fact that other complementary approaches, such as flow cytometry on fresh samples, might also be used in future investigations, with the aim to identify other immune cell types within the lung tumors in COPD patients. Nonetheless, the use of relatively large fresh samples, which are required for flow cytometry, may not always be possible in these types of studies conducted on patients.

5. Conclusions

The proportions of Treg cells increased in tumors of LC patients with and without COPD, while levels of IgG-secreting plasma cells decreased only in the tumors of LC–COPD patients. Protein levels of IL-10 significantly increased in tumors of LC–COPD but not in those without this condition. Levels

of tumor NK cells, IgA-secreting plasma cells, or interferon-gamma did not differ between the two study groups. Immune cell subtypes and cytokines are differentially expressed in lung tumors, and the presence of underlying COPD elicited a significant decline in IgG-secreting plasma cell levels but not in the other cell types.

Author Contributions: Study conception and design, E.B., V.C., and L.P.; patient assessment and recruitment and sample collection, J.T., V.C., D.R.-C., X.W., M.M.-J., A.R.-F., R.A.E., and L.P.; pathological diagnosis and tumor identification, L.P.; histological analyses, J.T., D.R.-C., X.W., M.M.-J., and L.Q.; statistical analyses and data interpretation, X.D., J.T., D.R.-C., and E.B.; manuscript drafting and intellectual input, E.B., J.T., and V.C.; manuscript-writing of final version, E.B. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by FIS 18/00075 and CIBERES (FEDER, ISC-III), SEPAR 2018, unrestricted research grant from Menarini SA 2018, CIBERES (*Instituto de Salud Carlos III*, Spain).

Acknowledgments: The authors are thankful to Professor Cristina Lopez-Rodriguez from the Immunology Unit at *Universitat Pompeu Fabra* (Barcelona) for her advice with the immunological approaches and experiments and to Ms Mireia Admetlló for her help with the patient clinical assessment.

Conflicts of Interest: The authors declare no conflict of interest.

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4.3 Third Study

Title:

Immunotherapy with Monoclonal Antibodies in Lung Cancer of Mice: Oxidative Stress and Other Biological Events

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Journal:

Cancers. 2019 Sep 4;11(9):1301.
DOI: 10.3390/cancers11091301
PMID: 31487876

Impact Factor:

6.126 (2019), Quartile: Q1

cancers

MDPI

Article

Immunotherapy with Monoclonal Antibodies in Lung Cancer of Mice: Oxidative Stress and Other Biological Events

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Received: 7 June 2019; Accepted: 30 August 2019; Published: 4 September 2019

Abstract: *Background:* Lung cancer (LC) is a major leading cause of death worldwide. Immunomodulators that target several immune mechanisms have proven to reduce tumor burden in experimental models through induction of the immune microenvironment. We hypothesized that other biological mechanisms may also favor tumor burden reduction in lung cancer-bearing mice treated with immunomodulators. *Methods:* Tumor weight, area, T cells and tumor growth (immunohistochemistry), oxidative stress, apoptosis, autophagy, and signaling (NF-κB and sirtuin-1) markers were analyzed (immunoblotting) in subcutaneous tumor of BALB/c mice injected with LP07 adenocarcinoma cells treated with monoclonal antibodies (CD-137, CTLA-4, PD-1, and CD-19, N = 9/group) and non-treated control animals. *Results:* Compared to non-treated cancer mice, in tumors of monoclonal-treated animals, tumor area and weight and ki-67 were significantly reduced, while T cell counts, oxidative stress, apoptosis, autophagy, activated p65, and sirtuin-1 markers were increased. *Conclusions:* Immunomodulators elicited a reduction in tumor burden (reduced tumor size and weight) through decreased tumor proliferation and increased oxidative stress, apoptosis, autophagy, and signaling markers, which may have interfered with the immune profile of the tumor microenvironment. Future research should be devoted to the elucidation of the specific contribution of each biological mechanism to the reduced tumor burden.

Keywords: experimental lung cancer; immunomodulators; oxidative stress; autophagy; tumor growth; sirtuin-1

1. Introduction

Lung cancer is the most prevalent cancer worldwide that affects both sexes and has a very high mortality [1]. Despite the development of new therapeutic strategies, patients with lung cancer have an overall survival rate lower than 15% in five years [1–3]. Respiratory conditions such as chronic obstructive pulmonary disease (COPD) and lung fibrosis predispose patients to a greater risk to develop lung cancer, especially non-small cell lung cancer (NSCLC) type [1,2,4–6].

The underlying biology of lung cancer is complex, as several mechanisms may interplay at different stages. For instance, inflammation, which is key in host protection, may promote lung cancer initiation

and malignancy in chronic inflammatory processes such as in patients with COPD [2,5,7–11]. Moreover, oxidative stress was also shown to participate in tumor initiation, promotion, and progression of carcinogenesis in patients with lung cancer, particularly in those with COPD [2,9,12,13]. Besides, inflammatory events and oxidative stress may drive the release of a cascade of cytokines and growth factors, which may favor lung tumorigenesis [2,9,12,13] through interference with biological processes such as apoptosis and autophagy [11,13–15]. In the last few years, the implications of biological mechanisms such as increased oxidative stress, inflammatory events, particularly a Th1-predominant response, and epigenetic events were demonstrated to be differentially expressed in the lung tumors of patients with COPD compared to patients without this respiratory condition [8,11,13,16]. These results are important, since they may help establish a differential profile of patients that may be more or less susceptible to certain therapies.

The immune system defends the host against diseases, including neoplastic transformation. However, cancer cells may evade the host immune system through a process defined as cancer immunoediting [17]. Cancer immune scape results from the action of immunosuppressive pathways that involve membrane receptors that are located in immune cell types along different steps of the cancer-immunity cycle [17–21]. Immune checkpoints enable immune tolerance to prevent autoimmunity events in the host [17–22]. Several immune checkpoints have been identified so far. As such, programmed cell death protein 1 (PD1) is a membrane receptor that promotes immune tolerance through T cell inactivation [18,19,22]. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a disruptor of antigen presentation upon T cell activation [17]. Cluster of differentiation 137 (CD137) is located in several immune cells such as T regulatory cells (Treg) that are responsible for repressing T cell activity [21]. Additionally, B cells present cluster of differentiation 19 (CD19), which can trigger pro- and anti-tumorigenic responses (Table 1) [20].

Table 1. Monoclonal antibodies for the treatment of lung tumors in mice.

Monoclonal Antibodies	Targets	Function
Anti-PD-1	PD-1	PD-1 receptor is expressed in activated T cells and induces immune tolerance by repressing T cell effector function [23,24].
Anti-CTLA-4	CTLA-4	CTLA-4 receptor is expressed in T cells and induces immune tolerance by repressing antigen presentation [23,25].
Anti-CD19	CD-19	CD-19 activates B cells [23,26].
Anti-CD137	CD-137	CD-137 receptor activates $CD8^+$ T and NK cells [21,23].

Definition of abbreviations: PD-1, programmed cell death-1; CTLA-4, cytotoxic T-lymphocyte associated protein-4; CD-137, TNF receptor superfamily member 9; CD-19; B-lymphocyte antigen; NK, natural killer.Specific immune checkpoint inhibitors have been designed, namely monoclonal antibodies that specifically act against these membrane receptors in order to boost the immune microenvironment. The blockade of these inhibitory pathways has been shown to restore the anti-tumor activity of the immune system [17,22,27–32]. The therapeutic efficacy of the combination of different immunomodulatory monoclonal antibodies has been recently demonstrated in animal models of lung cancer, in which the tumor immune microenvironment was specifically explored [18,19]. Furthermore, in previous studies from our group [8,11–13,16,33], the contribution of inflammation and signaling pathways [e.g., nuclear factor (NF)-kB and Sirtuin-1], oxidative stress, autophagy, and apoptosis in response to several pharmacological strategies was shown in mice bearing lung tumors. Oxidative stress was also shown to mediate the response to immunotherapy in colorectal cancer in mice [34] and the chemoresistance in ovarian cancer of patients [35]. Whether similar biological mechanisms can be observed in the tumors of mice treated with a combination of several immunomodulators remains to be identified. Thus, we reasoned that immunomodulators may also exert beneficial effects on tumor burden through biological events other than the immune microenvironment.

On this basis, we hypothesized that treatment of a combination of specific immunomodulatory monoclonal antibodies that included anti-PD1, anti-CTLA-4, anti-CD137, and anti-CD19 may have an effect on tumor progression through several biological mechanisms such as oxidative stress, autophagy, and apoptosis through specific signaling pathways in wild-type lung adenocarcinoma cells of mice [33]. Accordingly, in the current investigation, the main objectives were two-fold: (1) to assess the immune tumor microenvironment (T cells) and (2) to quantify levels of oxidative stress, antioxidant enzymes, apoptosis, autophagy, signaling, and cell proliferation rates in the subcutaneous lung adenocarcinoma tumors of BALB/c mice treated with a combination of immunomodulators (anti-PD1, anti-CTLA-4, anti-CD137, and anti-CD19 monoclonal antibodies). A group of tumor-bearing mice that did not receive treatment with the cocktail of monoclonal antibodies was the control group in the study. This experimental model of NSCLC has been previously well-validated in our group [12,33,36–38].

2. Methods

2.1. Animal Experiments

2.1.1. Experimental Design

The study protocol is illustrated in Figure 1. An animal model with lung cancer was developed through the inoculation of cancer cells from LP07 stable adenocarcinoma cell line derived from P07 lung tumor that spontaneously appeared in BALB/c mice [39–41]. Eighteen female BALB/c mice (8 weeks old, 20 g weight) acquired from Harlan Interfauna Ibérica SL (Barcelona, Spain) received a subcutaneous inoculation of LP07 cells (4×10^5) resuspended in 0.2 mL of minimal essential medium (MEM) in the left flank (Figure 1). After tumor cell inoculation on day 0 of all the mice, they were randomly divided into two independent groups (N = 9/group) to be thereafter followed for 30 days: (1) experimental control group in which mice received an intraperitoneal administration of 0.2 mL phosphate-buffered saline (PBS) every 72 h (non-treated controls group) and (2) mice treated with a combination of monoclonal antibodies (treated lung cancer group) that included anti-PD1 (RMP1-14; Cat. #BE0146, BioXCell, West Lebanon, NH, USA), anti-CTLA-4 (9D9; Cat. #BE0164, BioXCell), anti-CD137 (LOB12.3; Cat. #BE0169, BioXCell), and anti-CD19 (1D3; Cat. #BE0150, BioXCell) antibodies [18,19,21,23–26] (Table 1). A dose of 5×10^{-3} mg/kg/72 h in 0.2 mL PBS was administered to the treated group of lung cancer mice from day 15 (tumors visible) up until day 30 (Figure 1). The intraperitoneal route was chosen in order to mimic administration of this type of therapies in clinical settings [19]. For ethical reasons we were not allowed to extend the study protocol longer than 30 days. Also for ethical reasons, only non-treated tumor-bearing mice administered with the vehicle PBS were used as the control group in the study. Food and water were supplied ad libitum and mice were kept under pathogen-free conditions with a 12:12 h light:dark cycle in the animal facilities placed in the Barcelona Biomedical Research Park (PRBB) premises.

2.1.2. In Vivo Measurements Conducted on the Animals

Food intake and body weight were measured daily in all the study animals. Tumor area was also measured daily using a specific caliper in all the animals.

2.1.3. Sacrifice and Sample Collection

The two experimental groups of mice were sacrificed after 30 days of inoculation of LP07 cells. In each mouse, an intraperitoneal injection of 0.1 mL sodium pentobarbital (60 mg/kg) was inoculated prior to sacrifice. In order to verify total anesthesia depth, the pedal and blink reflexes were assessed in all animals. As the histological features of the subcutaneous tumor and those of the lung metastases are identical in this LP07 mouse model of lung cancer, for practical reasons, the subcutaneous tumor was used for the laboratory experiments. As such, the subcutaneous tumor was extracted from all the mice. A fragment of the tumor specimens was immediately frozen in liquid nitrogen and stored at

−80 °C, while the other fragment was immersed in an alcohol-formol to be thereafter embedded in paraffin until further use.

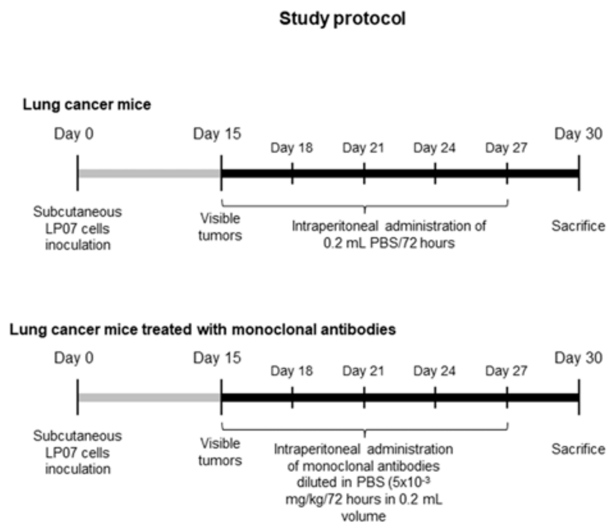

Figure 1. Graphical time-line representation of the non-treated control lung cancer mice and the lung cancer group of animals treated with the combination of monoclonal antibodies. This was a controlled study designed according to the ethical regulations on animal experimentation of the Spanish Legislation (Real Decreto 53/2013, BOE 34/11370–11421), the European Community Directive 2010/63/EU, and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986) at PRBB. The Animal Research Committee approved all animal experiments (Animal Welfare Department in Catalonia, Spain, # EBP-15-1704).

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2.2. Molecular Biology Analyses

2.2.1. Histological Analyses of Tumor Samples

Immunohistochemical techniques were applied on tumor sections in order to explore expression of the proliferation marker Ki-67 and T cells, following previous methodologies [8,12,13,16,33,36–38,42,43]. Briefly, for all the target antigens, tumor cross-sections were deparaffinized and then antigen retrieval was carried out by heating slides in a water bath in Tris/Ethylenediaminetetraacetic acid (EDTA) buffer, pH 9, for 30 min (Ki-67 and CD3) or in a pressure-cooker (CD4, CD8) in 0.1 M citrate buffer, pH 6, for 15 min. Subsequently, samples were blocked with 3% H_2O_2 for 15 min, and immediately afterwards they were blocked with 1% BSA and goat serum for two hours. Slides were then incubated at room temperature for 30 min (CD3) or at 4 °C overnight (Ki-67, CD4, CD8) with the following specific primary antibodies: anti-Ki67 (Merck-Millipore, Darmstadt, Germany), anti-CD3 (DAKO, Glostrup, Denmark), anti-CD4 (Abcam, Cambridge, UK), and anti-CD8 (Abcam). Slides were washed and incubated for 30 min with biotinylated universal secondary antibody, followed by another 30 min incubation with horseradish-conjugated streptavidin and diaminobenzidine for five minutes (kit LSAB+HRP Dako Cytomation Inc., Carpinteria, CA, USA) as a substrate.

Tumor sections were counterstained with hematoxylin for two minutes and were then dehydrated and mounted for conventional microscopy. Images of the stained tumors were taken under a light microscope (Olympus BX 61, Olympus Corporation, Tokyo, Japan) coupled with a camera (Olympus DP 71, Olympus Corporation). The number of Ki-67, CD3, and CD8 cells was expressed as the percentage of the cells that were positively stained for the corresponding markers of the total cells in the tumor preparations. The number of CD4 cells was represented as the number of cells that were positively stained for CD4 in the measured area (expressed in micrometers).

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Terminal deoxynucleotidyl transferase–mediated uridine 5′-triphosphate (UTP) nick- end labelling (TUNEL) assay. In tumor paraffin-embedded sections, apoptotic nuclei were identified using the TUNEL assay

(In Situ Cell Death Detection Kit, POD, Roche Applied Science, Mannheim, Germany) for all study groups following the manufacturer's instructions and previous studies [38,43]. Briefly, this assay is based on the principle that during the apoptosis of nuclei, genomic DNA may yield double-stranded, low molecular weight fragments as well as single-strand breaks (nicks) in high molecular weight DNA. These DNA strand breaks can be identified by labelling 3′-hydroxyl (3′OH) groups with modified nucleotides in an enzymatic reaction. In this assay, deoxynucleotidyl transferase (TdT), which catalyzes the polymerization of nucleotides to free 3′-OH DNA ends, is used to label DNA strand breaks. Briefly, diaphragm and gastrocnemius muscle sections were fixed and permeabilized. Subsequently, they were incubated with the TUNEL reaction mixture that contains terminal TdT and fluorescein-dUTP. During the incubation period, terminal TdT catalyzed the addition of fluorescein-dUTP at free 3′-OH groups in single- and double-stranded DNA. After washing, the label incorporated at the damaged sites of the DNA was marked by anti-fluorescein antibody conjugated with the reporter enzyme peroxidase. After several washes that removed unbound enzyme conjugate, the peroxidase retained in the immune complex was visualized by a substrate reaction. Apoptotic nuclei were brown, while negative nuclei were blue (hematoxylin counterstaining). In each tumor cross-section, the TUNEL-positive nuclei and the total number of nuclei were counted blindly by two independent observers, who were previously trained for that purpose. Results were expressed as the ratio of total TUNEL positively-stained nuclei to the total number of counted nuclei, as also previously reported [38,43]. A minimum amount of 300 nuclei were counted in each tumor preparation. Final results corresponded to the mean value of the counts provided by the two independent observers (concordance rate 95%). Negative control experiments, in which the TUNEL reaction mixture was omitted, were also conducted. Moreover, rat testicles were used as a positive control in these experiments.

2.2.2. Immunoblotting

Protein levels of the target markers were determined using 1D electrophoresis and immunoblotting according to previously published methodologies [8,12,13,16,33,36–38,42]. Frozen tumor samples extracted from mice were homogenized in lysis buffer. The following specific primary antibodies were used to identify the different target markers: protein tyrosine nitration (anti-3-nitrotyrosine antibody, Invitrogen, Eugene, OR, USA), malondialdehyde protein adducts (anti-MDA protein adduct antibody, Academic Bio-Medical Company, Inc., Houston, TX, USA), catalase (anti-catalase antibody, Calbiochem, Darmstadt, Germany), Mn-superoxide dismutase (SOD2, anti-SOD2 antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA), CuZn-superoxide dismutase (SOD1, anti-SOD1 antibody, Santa Cruz), b-cell lymphoma 2 (BCL-2, anti-BCL-2, antibody Santa Cruz), BCL-2 associated X protein (BAX, anti-BAX antibody, Santa Cruz), nucleoporin p62 (anti-p62 antibody, Sigma-Aldrich, St. Louis, MO, USA), beclin-1 (anti-beclin-1 antibody, Santa Cruz), microtubule-associated protein 1 light-chain 3 (LC3B, anti-LC3B antibody, Cell Signaling, MA, USA), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 subunit and phosphorylated p65 subunit (anti NF-κB p65 and p-NF-κB p65 antibodies, Santa Cruz), sirtuin-1 (anti-sirtuin-1 antibody, EMD Millipore, Billerica, MA, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, anti-GAPDH antibody, Santa Cruz) as the protein loading control to confirm identical protein loading among different lanes. Horseradish peroxidase (HRP)-conjugated secondary antibodies and a chemiluminescence kit (Thermo Scientific, Rockford, IL, USA) were used to detect the antigens from all samples. For the sake of comparisons between the two groups, all study samples (×18) were run together in the same mini-cell electrophoresis and transfer boxes, respectively, and the corresponding membranes were detected using chemiluminescence in the same platform under identical exposure times.

Polyvinylidene difluoride (PVDF) membranes were scanned with the Molecular Imager Chemidoc XRS System (Bio-Rad Laboratories, Hercules, CA, USA) using the software Quantity One version 4.6.5 (Bio-Rad Laboratories), and optical densities of target proteins were quantified using the software Image Lab version 2.0.1 (Bio-Rad Laboratories). Final optical densities (arbitrary units) acquired in each group of mice corresponded to the average value of all the samples (lanes). Values of optical

densities (arbitrary units) of LC3B-II/LC3B-I were also calculated as the ratio of LC3B-II (14 kDa) to LC3B-I (16 kDa) protein content. Moreover, all values of the different antigens were calculated as the ratio of the optical densities of the given variable to those of the loading control GAPDH in each study sample, as shown in each figure.

Standard stripping methodologies were employed to detect p62 protein levels in the same PVDF membranes of beclin-1. The loading control GAPDH was also detected using stripping methodologies in the study markers: protein tyrosine nitration, MDA-protein adducts, SOD1, SOD2, catalase, BAX, BCL-2, beclin-1, p62, LC3B, NF-κB p65, pNF-κB p65, and Sirtuin-1. Briefly, primary and secondary antibodies were stripped off proteins using a stripping solution (25 nM glycine, pH 2.0, and 1% Sodium Dodecyl Sulphate (SDS)) for 30 min. Membranes were subsequently washed two consecutive times (10 min each) with phosphate buffered saline and tween (PBST) at room temperature. Immediately afterwards, membranes were blocked with 1% Bovine Serum Albumin (BSA) and incubated with specific primary and secondary antibodies following the abovementioned procedures.

2.3. Statistical Analysis

Using specific software (StudySize 2.0, CreoStat HB, Frolunda, Sweden) and assuming an alpha error of 0.05 and a minimum of 80% of standard power statistics, the sample size (N = 9/group) was sufficiently great to identify a difference of 700 and 0.6 points in both tumor area and weight variables between groups, respectively. The Shapiro–Wilk test was used to check the normality of the study variables. Therefore, data are expressed as mean and standard deviation in both tables and figures. The Statistical Package for the Social Sciences (SPSS, version 22, SPSS Inc., Chicago, IL, USA) was used to compare the study variables between the two study groups using the unpaired Student's t-test, and statistical significance was established at $p \leq 0.05$.

3. Results

3.1. Monoclonal Antibodies Improved Tumor Burden and Body Weight in Mice

As illustrated in Table 2 and Figure 2A, by the end day (30) of the study protocol, in the lung cancer mice compared to non-treated control mice, treatment with the cocktail of monoclonal antibodies had significantly improved the following variables: final body weight, body weight gain with and without tumor, tumor weight (34% reduction), and tumor area (64% reduction). Importantly, levels of Ki-67-positive nuclei were significantly lower (27% reduction), while TUNEL-positively stained nuclei were significantly higher (127% increase) in the tumors of mice treated with the monoclonal antibodies compared to those detected in the non-treated control animals (Figure 2B,C).

Table 2. Physiological and tumor characteristics in the study groups of mice.

Variables	Lung Cancer Mice	Lung Cancer + Monoclonal Antibodies Mice
Initial body weight (g)	20.41 (1.22)	20.34 (0.79)
Final body weight (g)	19.35 (2.25)	21.39 (1.57), *
Body weight gain (%)	−4.27 (10.47)	+5.16 (6.33), *
Body weight gain without tumor (%)	−15.06 (11.28)	−2.66 (8.35), *
Tumor weight (g)	2.38 (0.75)	1.57 (0.89), *

Variables are presented as mean (standard deviation). Statistical significance: * $p \leq 0.05$ between the two study groups of mice.

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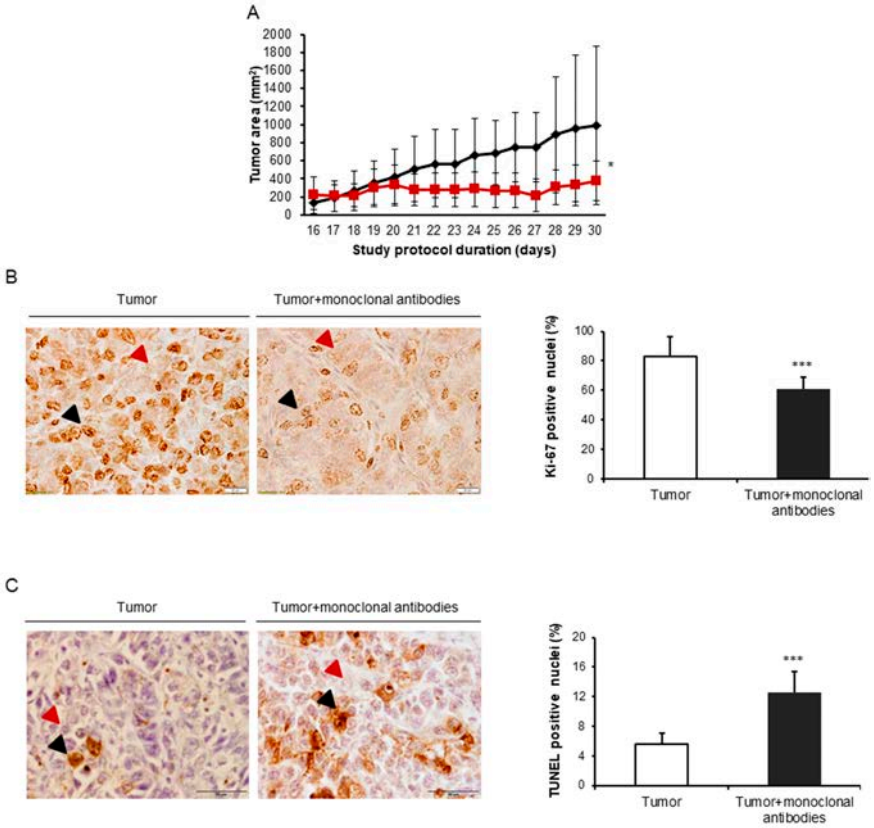

Figure 2. (**A**) Mean values and standard deviation of subcutaneous tumor area (mm^2) of non-treated control lung cancer group of mice (black color) and lung cancer mice treated with monoclonal antibodies group (red color) during the study protocol. (**B**) Representative histological sections (40×) and mean values and standard deviation of Ki-67 in the subcutaneous tumors of the non-treated control mice and the treated lung cancer animals. Black arrowheads point towards Ki-67-positively stained nuclei (brown color), while red arrowheads point towards Ki-67 negatively stained nuclei (purple color). (**C**) Representative histological sections (40×) and mean values and standard deviation of TUNEL in the subcutaneous tumors of the non-treated control mice and the treated lung cancer animals. Black arrowheads point towards TUNEL-positively stained nuclei (brown color), while red arrowheads point towards TUNEL-negatively stained nuclei (purple color). Statistical significance: *: $p \leq 0.05$ and ***: $p \leq 0.001$ between lung cancer mice compared to the lung cancer-monoclonal antibodies group.

3.2. Immune Microenvironment in Response to the Immunomodulators

In tumors of mice treated with the monoclonal antibodies, the number of T cells (CD3, CD8, and CD4) was significantly greater than that observed in the non-treated animals (Table 3 and Figure 3).

Table 3. Immune microenvironment in the study groups of mice.

T Cells	Lung Cancer Mice	Lung Cancer + Monoclonal Antibodies Mice
CD3 + cells (%)	8.34 (0.91)	11.30 (0.78), ***
CD4 + (cells/μm^2)	1.99×10^{-6} (0.44×10^{-6})	3.37×10^{-6} (1.49×10^{-6}), *
CD8 + cells (%)	5.16 (1.35)	7.86 (1.04), ***

Values are expressed as mean (standard deviation). Statistical significance: * $p \leq 0.05$ and *** $p \leq 0.001$ between the two experimental groups of mice.

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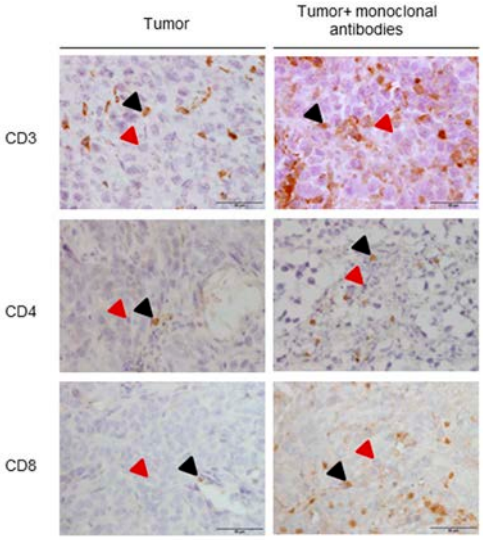

Figure 3. Representative examples of immunohistochemistry staining for CD3, CD4, and CD8, in tumor samples of the different study groups of mice. All types of T cells (CD3, CD8, and CD4) are stained with brown color (black arrows), while negative nuclei are stained in purple color (red arrows).

3.3. Tumor Oxidative Stress in Response to the Immunomodulators

Compared to non-treated mice, protein tyrosine nitration and oxidation (MDA-protein adducts) and cytosolic SOD1 levels were significantly greater in the tumors of the mice treated with the monoclonal antibodies, while no significant differences were detected in mitochondrial SOD2 or catalase protein levels between the two study groups (Figures 4A,B and 5A–C).

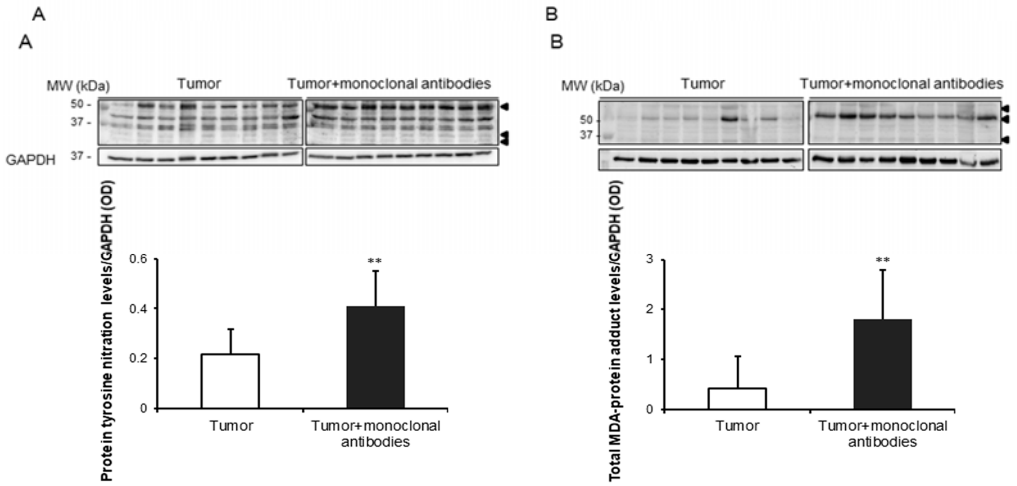

Figure 4. (**A**) Representative immunoblots and mean values and standard deviation of total protein tyrosine nitration levels/GAPDH in subcutaneous tumors of lung cancer mice as measured by optical densities. (**B**) Representative immunoblots and mean values and standard deviation of total MDA protein adduct levels/GAPDH in subcutaneous tumors of lung cancer mice as measured by optical densities. Representative GAPDH is shown as the loading control. Statistical significance is represented as follows: **: $p \leq 0.01$ between non-treated controls ($N = 9$) in white bars and treated lung cancer ($N = 9$) mice in black bars. Definition of abbreviations: MDA, malondialdehyde; GAPDH, glyceraldehyde-3-phospate dehydrogenase; OD, optical densities.

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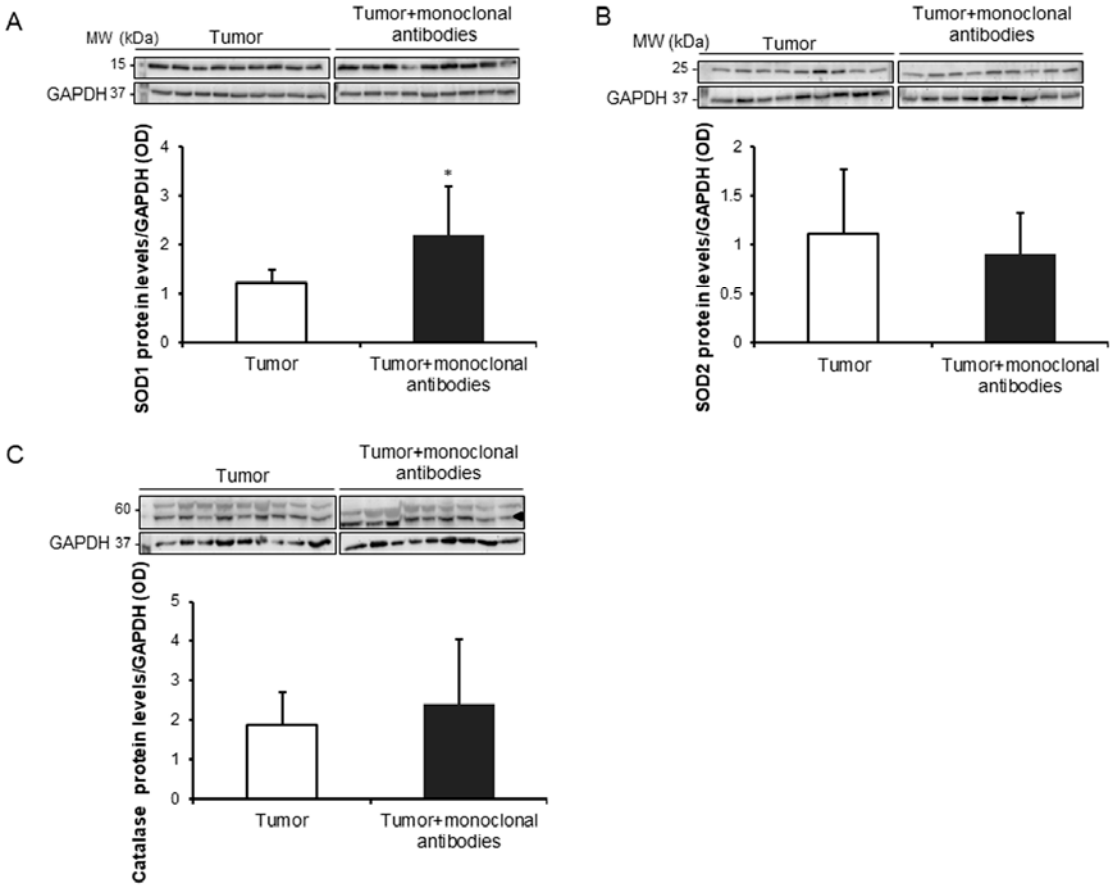

Figure 5. (**A**) Representative immunoblots and mean values and standard deviation of SOD1 protein levels/GAPDH in subcutaneous tumors of lung cancer mice as measured by optical densities. (**B**) Representative immunoblots and mean values and standard deviation of SOD2 protein levels/GAPDH in subcutaneous tumors of lung cancer (LC) mice as measured by optical densities. (**C**) Mean values and standard deviation of catalase protein levels/GAPDH in subcutaneous tumors of lung cancer mice as measured by optical densities. Representative GAPDH is shown as the loading control. Statistical significance is represented as follows: * $p \leq 0.05$ between non-treated controls ($N = 9$) in white bars and treated lung cancer ($N = 9$) mice in black bars. Definition of abbreviations: SOD1, Cu,Zn-superoxide dismutase; SOD2, Mn-superoxide dismutase; GAPDH, glyceraldehyde-3-phospate dehydrogenase; OD, optical densities.

3.4. Tumor Apoptosis and Autophagy Markers in Response to Immunomodulators

Treatment of the mice with the cocktail of monoclonal antibodies induced a significant rise in BAX protein levels in the tumors compared to non-treated animals, while no significant differences were found in tumor BCL-2 protein levels between the two study groups (Figure 6A,B). Treatment with monoclonal antibodies did not induce any significant difference in protein expression levels of either beclin-1 or p62, whereas the ratio of LC3-II to LC3-I was significantly increased in the tumors of the treated mice (Figure 7A–C).

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Cancers **2019**, *11*, 1301 10 of 16
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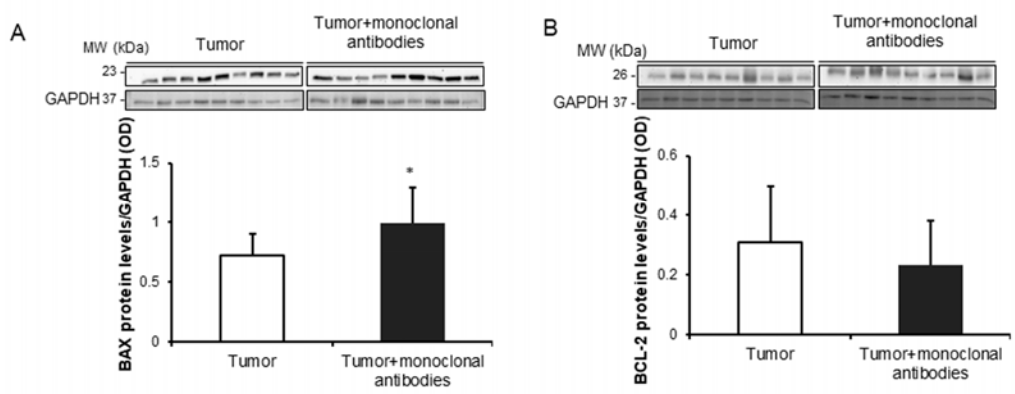

Figure 6. (**A**) Representative immunoblots and mean values and standard deviation of BAX protein levels/GAPDH in subcutaneous tumors of lung cancer mice as measured by optical densities. (**B**) Representative immunoblots and mean values and standard deviation of BCL-2 protein levels/GAPDH in subcutaneous tumors of LC mice as measured by optical densities. Representative GAPDH is shown as the loading control. Statistical significance is represented as follows: $^{*}p \leq 0.05$ between non-treated controls ($N = 9$) in white bars and treated lung cancer ($N = 9$) mice in black bars. Definition of abbreviations: BAX, BCL-2 associated X protein; BCL-2, b-cell lymphoma 2; GAPDH, glyceraldehyde-3-phospate dehydrogenase; OD, optical densities.

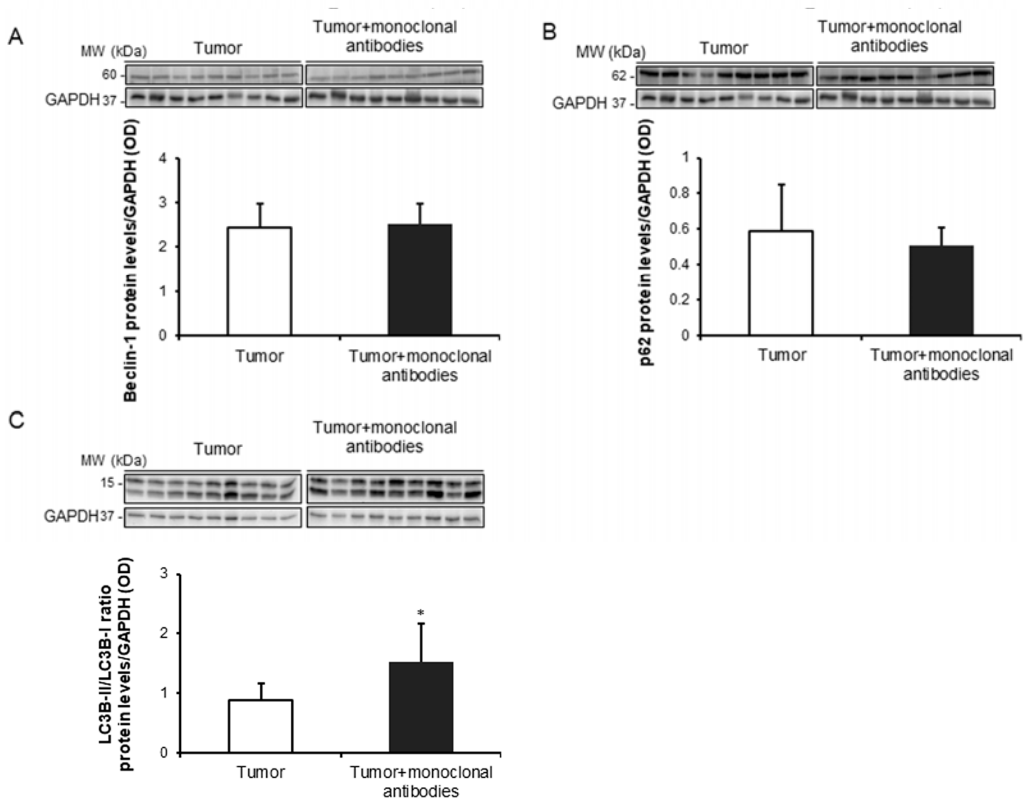

Figure 7. (**A**) Representative immunoblots and mean values and standard deviation of Beclin-1 protein levels/GAPDH in subcutaneous tumors of LC mice as measured by optical densities. (**B**) Representative immunoblots and mean values and standard deviation of p62 protein levels/GAPDH in subcutaneous tumors of LC mice as measured by optical densities. (**C**) Representative immunoblots and mean values and standard deviation of autophagy LC3-II protein levels/GAPDH in subcutaneous tumors of LC mice as measured by optical densities. Representative GAPDH is shown as the loading control. Statistical significance is represented as follows: $^{*}p \leq 0.05$ between non-treated controls ($N = 9$) in white bars and treated lung cancer ($N = 9$) mice in black bars. Definition of abbreviations: p62, nucleoporin p62; LC3, microtube-associated protein 1 light chain 3; GAPDH, glyceraldehyde-3-phospate dehydrogenase; OD, optical densities.

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3.5. Effects of the Immunomodulators on Signaling Markers in Tumors

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Protein levels of the ratio of p-p65 subunit to total p65 of the NF-κB signaling pathway were significantly greater in the tumors of the mice treated with the monoclonal antibodies than in those of non-treated rodents (Figure 8A). Protein levels of the deacetylase sirtuin-1 were significantly increased in the tumors of the treated mice compared to non-treated animals (Figure 8B).

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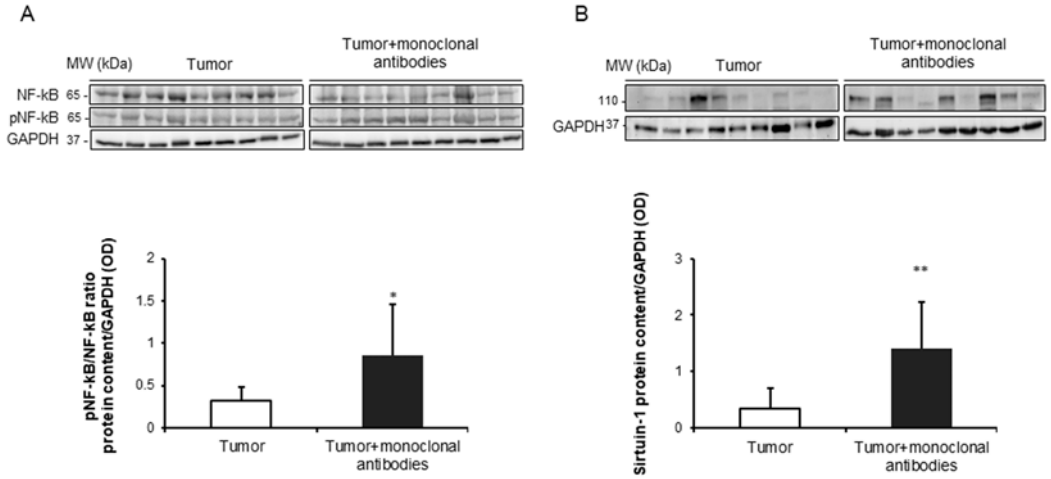

Figure 8. (**A**) Representative immunoblots and mean values and standard deviation of ratio of pNF-κB/NF-κB protein levels/GAPDH in subcutaneous tumors of lung cancer mice as measured by optical densities. (**B**) Representative immunoblots and mean values and standard deviation of sirtuin-1 protein levels/GAPDH in subcutaneous tumors of lung cancer mice as measured by optical densities. Representative GAPDH is shown as the loading control. Statistical significance is represented as follows: and *: $p \leq 0.05$ and **: $p \leq 0.01$ between non-treated controls ($N = 9$) in white bars and treated lung cancer ($N = 9$) mice in black bars. Definition of abbreviations: NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; GAPDH, glyceraldehyde-3-phospate dehydrogenase; OD, optical densities.

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4. Discussion

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In the tumors of the mice treated with the cocktail of monoclonal antibodies, the area was significantly reduced at the end of the study period. These are relevant findings that confirm the efficacy of the treatment with the immunomodulators of the tumor cells in this experimental model of lung cancer in mice. These results are in line with those previously reported, in which a complete regression of the tumors (melanoma and lung cancer) was attained following intratumoral treatment of the mice with the same cocktail of monoclonal antibodies [18,19]. In the present study, the numbers of T cell subtypes also significantly increased in the tumors of the mice that received treatment with the checkpoint inhibitors.

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As opposed to previous investigations [18,19], the immunomodulators were injected intraperitoneally with the aim to mimic the treatments applied in patients, in whom drugs are usually administered systemically. In this investigation, a complete regression of the tumors was not achieved, probably as a result of the systemic administration of the drugs compared to previous reports [18,19], in which the monoclonal antibodies were administered locally. Nonetheless, in the tumor-bearing mice that received the medical treatment with the immunomodulators a substantial reduction in tumor burden as measured by both tumor weight (34%) and area (64%) was observed.

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This leads to the conclusion that the treatment, the doses, and the route were effectively administered in the current study.

Interestingly, the number of tumor proliferating cells was also significantly lower in the tumors of the mice treated with the monoclonal antibodies than in the non-treated tumor-bearing rodents. These results suggest that cell cycle arrest probably due to alterations in cyclin expression levels may account for the reduced levels of Ki-67-positively stained nuclei encountered in the adenocarcinoma cells of the treated tumor-bearing mice. Moreover, these findings are also in agreement with previous investigations, in which expression levels of Ki-67 were significantly reduced (34%) in the tumors of mice treated with several selective inhibitors of cell survival pathways [12], in those from transgenic mice deficient for either poly(Adenosine Diphosphate Ribose (ADP)-ribose)) polymerases (PARP)-1 or -2 enzymes [33], and in those of rodents treated with pharmacological inhibitors of PARP activity [44]. Taken together, these results are also very consistent with the findings reported herein: reduced tumor area and weight in the tumor-bearing mice treated with the immunomodulators at the end of the study period.

Oxidative stress was assessed using several indirect markers in the tumor cells of both groups of mice. Importantly, levels of protein tyrosine nitration and total MDA-protein adducts were significantly greater in the tumor cells of the mice that received treatment with the monoclonal antibodies. These results are in agreement with those previously observed in another investigation, in which protein oxidation levels were also increased in the tumors of Parp-$1^{-/-}$ and Parp-$2^{-/-}$ mice [33]. In the present investigation, levels of the antioxidant enzyme SOD1, but not those of SOD2 or catalase, were significantly greater in the tumors of the lung cancer-bearing mice treated with the monoclonal antibodies. These results are in line with those encountered in the tumors of mice treated with the proteasome inhibitor bortezomib [12]. Furthermore, accumulation of reactive oxygen species (ROS) and glutathione depletion were also shown in tumor cells of mice with colorectal cancer [34], and an oxidative stress-associated mechanism of T cell activation was observed in the stroma of ovarian and colon tumor samples in patients as well [35,45,46].

The rise in the expression of cytosolic SOD1 levels may have been a response to counterbalance the deleterious effects of increased oxidative stress in the tumor cells as previously suggested [12]. Altogether, a rise in several oxidative stress markers was observed in the tumors of the mice treated with the cocktail of monoclonal antibodies. These findings may imply that in response to treatment with the immunomodulators, oxidative stress may drive cell cycle arrest and tumor cell death independently of the immune response [47].

Oxidative stress may also trigger several important cellular pathways such as cell death, apoptosis, and autophagy through signaling pathways such as the redox sensitive NF-κB pathway. In this regard, the ratio levels of active p65 (phosphorylated) to total p65 were greater in the tumors of animals treated with the monoclonal antibodies than in the non-treated mice. Importantly, levels of TUNEL-positive nuclei were also significantly increased in tumors of the mice treated with the immunomodulators. Additionally, Bax protein levels also increased in the tumors of mice treated with the monoclonal antibodies, while no differences in Bcl2 levels were seen between the study groups. These results are consistent with those previously reported, in which different therapeutic strategies also elicited a rise in proapoptotic markers [12,33]. The increase in apoptotic markers of cancer cells was also demonstrated in previous investigations in which the animals were treated with selective inhibitors of PARP activity [44,48–50].

A rise in the autophagy marker LC3B was observed in the tumor cells of the mice treated with the immunomodulators compared to non-treated control rodents. These results imply that autophagy may also mediate the reduced tumor burden observed in the mice that received treatment with the cocktail of monoclonal antibodies. In fact, similar results were previously demonstrated in the tumors of mice that were genetically deficient for either PARP-1 or PARP-2 proteins, especially the latter [33].

The deacetylase sirtuin-1 may play a role in autophagy as a result of its upstream regulation of LC3B [51]. In the current study, a significant rise in protein levels of sirtuin-1 was detected in the

tumor cells of the mice treated with the monoclonal antibodies compared to non-treated animals. On the other hand, sirtuin-1 may also play a role in the regulation of tumor microenvironment of the immune cells [52]. These results imply that sirtuin-1 probably interfered with immune cells [52], leading to changes in the tumor microenvironment (from Th2 type to Th1 immunity) as previously demonstrated [18,19]. This may further contribute to the reduced tumor burden observed in the mice treated with the monoclonal antibodies. Despite the relevance of this question, it will have to be fully elucidated in future investigations as it was clearly beyond the objectives of the current investigation.

Study Limitations

Limitations inherent to the use of an animal experimental model may have occurred in the current investigation as compared to clinical studies. This may partly preclude the generalization of the present study results to clinical settings. Moreover, other limitations may be related to the type of tumor cells and the animal background as well as the type of laboratory techniques employed to identify the different immune cells compared to previous investigations [18,19]. Procedures beyond the histology, such as flow cytometry, may be useful to selectively identify the type and number of the cells contained in the tumors. Nonetheless, these experiments would have required a completely different experimental approach at the time of conducting the animal experiments and when collecting the tumor specimens. On the other hand, the histological approach enabled us to identify topographically the presence of the T cells within the cancer specimens, thus confirming that they were, indeed, part of the tumor microenvironment.

Another possible limitation in the study would be related to the lack of additional control groups of mice, such as animals administered with isotype-matched antibodies to confirm the selectivity and specificity of the immunotherapy. Nevertheless, as the efficacy and selectivity of the antibodies used in the present investigation had already been demonstrated in previous studies [18,19] and for ethical reasons, no additional control groups were included. Despite all these limitations, the experiments reported herein shed light onto novel mechanisms whereby immunotherapy may exert beneficial effects in lung adenocarcinoma tumors.

5. Conclusions

We have demonstrated that immunomodulators with different mechanisms of action elicited a reduction in the tumor burden as measured by tumor size and weight through several biological mechanisms, namely decreased tumor proliferation rates and increased T cell counts, oxidative stress, apoptosis, autophagy, and signaling pathways, which may have interfered with the immune profile of the tumor microenvironment. Future research should be devoted to the elucidation of the specific contribution of each mechanism (reduced tumor proliferation, increased tumor degradation, and stimulation of the immune tumor microenvironment) to the reduced tumor burden seen in this animal model of lung cancer. These findings may have potential therapeutic implications in patients under treatment with immunomodulators for their lung neoplasms.

Author Contributions: Study conception and design: E.B., J.T., D.R.-C.; Animal experiments and sample collection: D.R.-C., J.T.; Molecular biology and histological analyses: J.T., D.R.-C., X.W.; Statistical analyses and data interpretation: J.T., D.R.-C., E.B.; manuscript drafting and intellectual input: E.B., J.T., D.R.-C.; manuscript writing final version: E.B.

Funding: This study has been supported by CIBERES; FIS 14/00713 (FEDER); FIS 18/00075 (FEDER); SEPAR 2016; SEPAR 2018; FUCAP 2016; Unrestricted grant from Menarini SA 2018 (Spain), and GlaxoSmithKline SA (Spain) 2018.

Acknowledgments: The authors are very grateful to Xavier Duran for his contribution to the statistical analyses of the study results and to Anna Salazar-Degracia for her help with part of the animal experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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4.4 Fourth Study

Title:

Markers of Stroma in Lung Cancer: Influence of COPD

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Journal:

Archivos de Bronconeumología. 2019 Sep 4;11(9):1301.
DOI: 2020 Sep 24;S0300-2896(20)30311-2.
PMID: 33077297

Impact Factor:

4.957 (2019), Quartile: Q1

Arch Bronconeumol. 2020;xxx(xx):xxx–xxx

Sociedad Española de Neumología y Cirugía Torácica SEPAR

ARCHIVOS DE Bronconeumología

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Original

Markers of Stroma in Lung Cancer: Influence of COPD

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ARTICLE INFO

Article history:
Received 18 May 2020
Accepted 4 September 2020
Available online xxx

Keywords:
Lung cancer
COPD
Stroma
Cancer-associated fibroblasts
Extracellular matrix
Endothelial cell marker CD31

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.

Marcadores estromales en el cáncer de pulmón: la influencia de la enfermedad pulmonar obstructiva crónica

Palabras clave:
Cáncer de pulmón
EPOC
Estroma
Fibroblastos asociados al cáncer
Matriz extracelular
Marcador de células endoteliales CD31

RESUMEN

Antecedentes: El estroma, compuesto principalmente por fibroblastos, matriz extracelular (MEC) y vasos, puede desempeñar un papel en la génesis tumoral y la progresión del cáncer. La enfermedad pulmonar obstructiva crónica (EPOC) es un factor de riesgo independiente para el carcinoma de pulmón (CP). Nuestra hipótesis fue que los marcadores de fibroblastos, MEC y células endoteliales pueden variar en los tumores de los pacientes con CP con o sin EPOC.

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https://doi.org/10.1016/j.arbres.2020.09.005
0300-2896/

Métodos: Se identificaron los marcadores de fibroblastos asociados al cáncer y los fibroblastos normales cultivados (FAC y FN, respectivamente; marcadores: vimentina y α-actina del músculo liso [SMA por sus siglas en inglés]; inmunofluorescencia en fibroblastos de pulmón cultivados) y marcadores de la MEC y las células endoteliales (marcadores: colágeno tipo I y CD31, respectivamente; inmunohistoquímica) en muestras de pulmón tumoral y no tumoral (toracotomía para resección de tumores pulmonares) de 15 pacientes con EPOC-CP y 15 pacientes con solo CP.
Resultados: El número de FAC aumentó de forma significativa, mientras que el de FN disminuyó significativamente en las muestras tumorales en comparación con las muestras no tumorales de pacientes con CP y EPOC-CP. Las células endoteliales (CD31) disminuyeron también de forma significativa en las muestras tumorales en comparación con las muestras no tumorales solo en los pacientes con CP. No se observaron diferencias significativas en los niveles de colágeno tipo I en ninguna muestra o grupo de estudio.
Conclusiones: La expresión del marcador vascular endotelial CD31 se redujo en los tumores de los pacientes sin EPOC, mientras que los niveles de colágeno tipo I no difirieron entre los grupos. Se detectó un aumento en los niveles de FAC en los tumores de pulmón de los pacientes, con independencia de la presencia de obstrucción de las vías respiratorias. Los niveles bajos de CD31 pueden tener implicaciones en la supervivencia general de los pacientes con CP, en especial, en aquellos sin obstrucción subyacente de las vías respiratorias. Convendría estudiar e identificar el papel del CD31 como biomarcador terapéutico y de pronóstico en los tumores de pulmón de pacientes con enfermedades respiratorias subyacentes.

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,

Please cite this article in press as: Tang J, et al. Markers of Stroma in Lung Cancer: Influence of COPD. Arch Bronconeumol. 2020. https://doi.org/10.1016/j.arbres.2020.09.005

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 (FEV1)/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% CO2 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 (50 mM 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 95 °C 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

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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 ex-smokers and non-smokers 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.

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)***
DLCO, %	84 (11)	60 (15)***
KCO, %	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.

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

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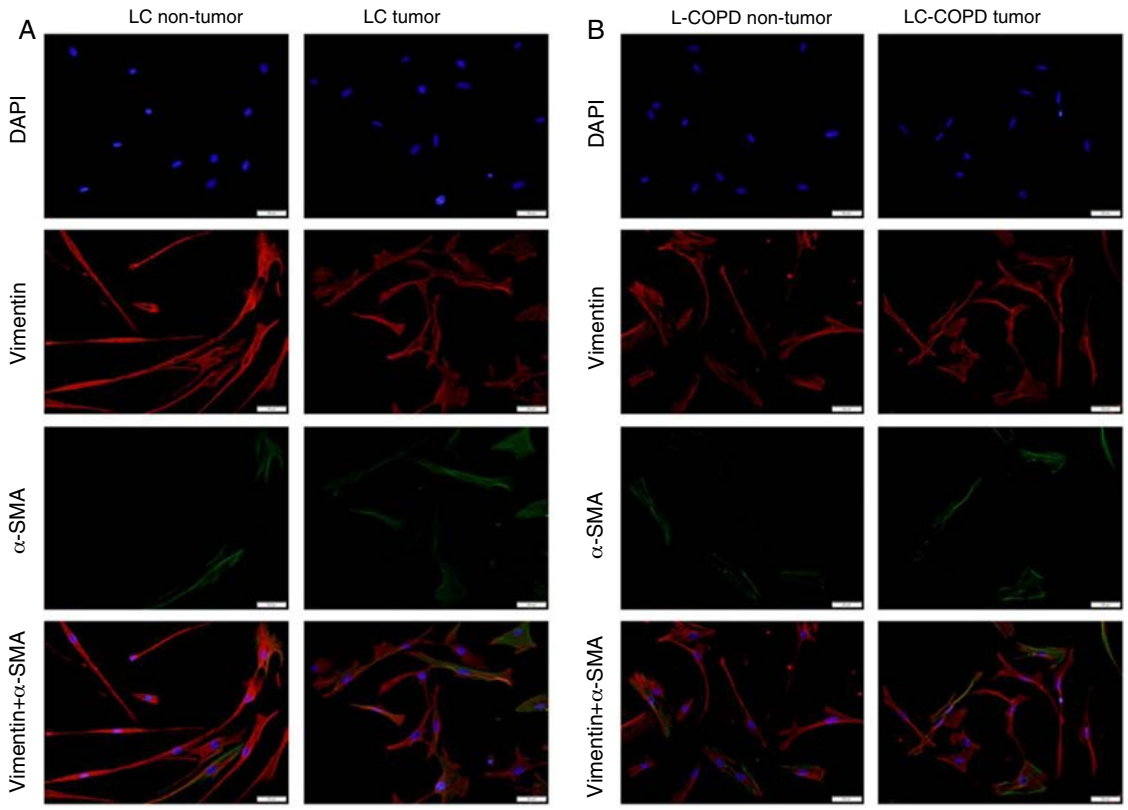

Figure 1. A and B: Representative examples of immunofluorescence staining of the markers DAPI (upper panel), vimentin (upper middle panel), alpha-SMA (lower middle panel), and CAFs (positively stained for both vimentin and alpha-SMA, bottom panel) in cultured fibroblasts obtained from non-tumor and tumor 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.

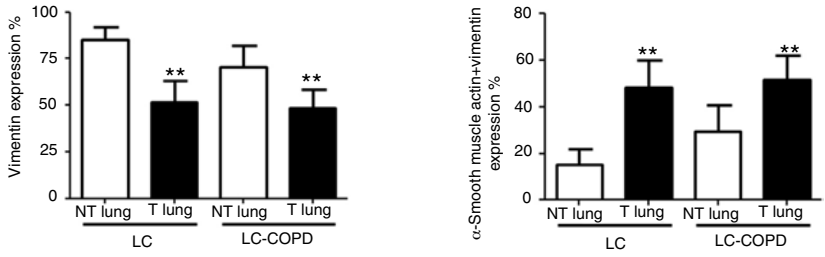

Figure 2. A and B: Mean values and standard deviations (SD) of levels of the markers vimentin and vimentin and alpha-SMA as measured by percentage of the total fibroblasts. Definition of abbreviations: alpha-SMA, alpha-smooth muscle actin; LC, lung cancer; COPD, chronic obstructive pulmonary disease.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

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

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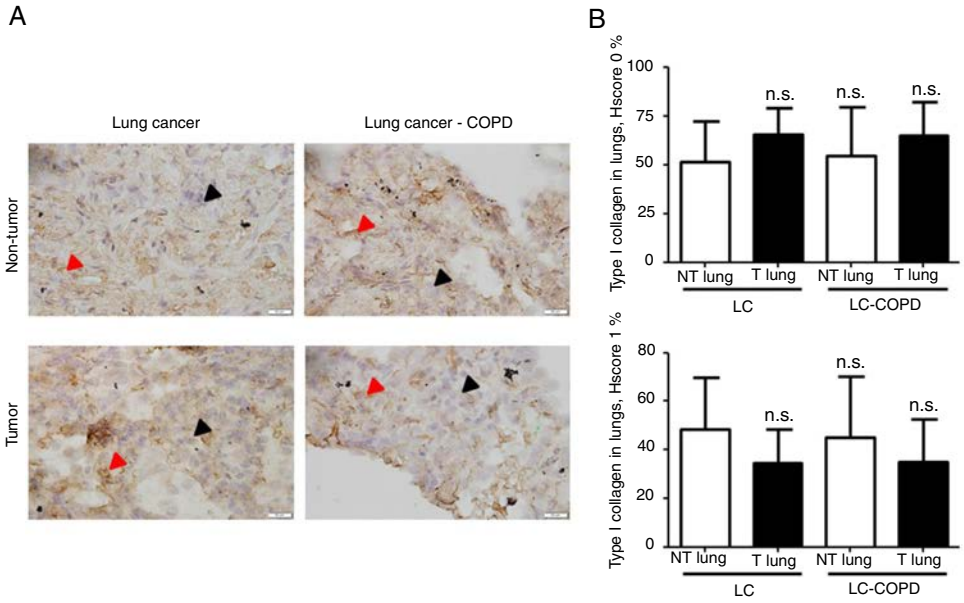

Figure 3. A) Representative examples of immunohistochemical staining for type I collagen in tumor and non-tumor specimens (collagen I-positively stained) in LC and LC-COPD patients, respectively. Black arrows point towards areas stained in blue with hematoxylin (negatively-stained for collagen), while 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.

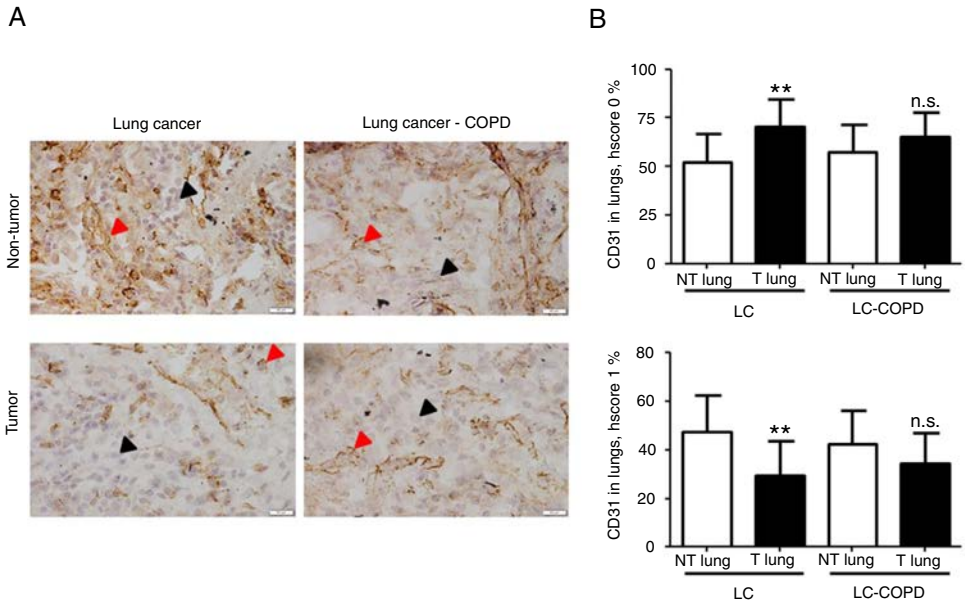

Figure 4. A) Representative examples of immunohistochemical staining for CD31 in tumor and non-tumor specimens (CD31-positively stained) in LC and LC-COPD patients, respectively. Black arrows point towards areas stained in blue with hematoxylin (negatively-stained for CD31), while red arrows point towards 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.

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

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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.

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.

Sources of funding

This study has been supported by FIS 18/00075 (FEDER, ISC-III) & CIBERES (ISC-III), SEPAR 2018 and SEPAR 2020, and an unrestricted research grant from Menarini SA 2018 (Spain).

Competing interests declared by all the authors

None.

Acknowledgments

The authors are thankful to Ms. Mireia Admetlló and Esmeralda Hernández for their help with the patient clinical assessment.

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4.5 Fifth Study

Title:

Increased PARP Activity and DNA Damage in NSCLC Patients: The Influence of COPD

Authors:

Jun Tang, Víctor Curull, Xuejie Wang, Coral Ampurdanés, Xavier Duran, Lara Pijuan, Alberto Rodríguez-Fuster, Rafael Aguiló, José Yélamos and **Esther Barreiro**.

Journal:

Cancers. 2020 Nov 11;12(11):3333.
DOI: 10.3390/cancers11091301
PMID: 33187221

Impact Factor:

6.126 (2019), Quartile: Q1

cancers

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Article

Increased PARP Activity and DNA Damage in NSCLC Patients: The Influence of COPD

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Received: 22 October 2020; Accepted: 9 November 2020; Published: 11 November 2020

Simple Summary: Many people still die of lung cancer (LC), a disease that is mainly related to cigarette smoking. Smokers may also develop chronic obstructive pulmonary disease (COPD). COPD is a risk factor per se for LC. Cigarette smoking and other chemicals injure DNA on a daily basis. A repair mechanism based on PARP-1 and PARP-2 activity can restore damaged DNA to keep cells alive. However, cancer cells also take advantage of this mechanism to survive. Fifteen LC-COPD and 15 LC patients were enrolled in this study to elucidate whether COPD influences DNA damage-dependent PARP activity in lung tumors. DNA damage, PARP activity, PARP-1 and PARP-2 expression were analyzed in tumor and non-tumor lungs obtained during surgical resection of the lung tumors. DNA damage and PARP activity were increased only in tumors in LC-COPD patients. However, PARP-1 and PARP-2 expression decreased in tumors of both patient groups. LC patients with COPD may benefit from PARP inhibitor therapies.

Abstract: (1) *Background*: Lung cancer (LC) is a major leading cause of death worldwide. Poly (ADP-ribose) polymerase (PARP)-1 and PARP-2 are key players in cancer. We aimed to assess PARP-1 and PARP-2 expression and activity and DNA damage in tumors and non-tumor lungs from patients with/without chronic obstructive pulmonary disease (COPD). (2) *Methods*: Lung tumor and non-tumor specimens were obtained through video-assisted thoracoscopic surgery (VATS) in LC patients with/without underlying COPD (two groups of patients, n = 15/group). PARP-1 and PARP-2 expression (ELISA), PARP activity (PARP colorimetric assay kit) and DNA damage (immunohistochemistry) levels were identified in all samples. (3) *Results*: Both PARP-1 and PARP-2 expression levels were significantly lower in lung tumors (irrespective of COPD)compared to non-tumor specimens, while DNA damage and PARP activity levels significantly increased in lung tumors compared to non-tumor specimens only in LC-COPD patients. PARP-2 expression was positively correlated with smoking burden in LC-COPD patients. (4) *Conclusions*: In lung tumors of COPD patients, an overactivation of PARP enzyme was observed. A decline in PARP-1 and PARP-2

protein expression was seen in lung tumors irrespective of COPD. Other phenotypic features (airway obstruction) beyond cancer may account for the increase in PARP activity seen in the tumors of patients with underlying COPD.

Keywords: lung cancer; DNA damage; PARP-1 and PARP-2 expression; PARP activity; COPD

1. Introduction

Lung cancer (LC) is a leading cause of death worldwide [1–4]. Surgical resection of lung tumors continues to be the main elective treatment in LC patients [5–7]. However, this approach cannot be applied to patients with advanced stages. Other options such as chemotherapy and immune therapy with or without other biological or pharmacological agents are indicated in these cases [3].

Several mechanisms and risk factors are involved in the pathophysiology of non-small cell LC (NSCLC) [6]. Underlying respiratory conditions favor the incidence of lung tumors in patients, especially in those with emphysema [8,9]. Mechanisms such as inflammation, oxidative stress, epigenetics alterations, and the tumor microenvironment underlie the pathophysiology of tumor development in patients with chronic respiratory disorders including stromal structures and immune cell profiles, and have an impact on the patients' survival [10–12]. Indeed, chronic obstructive pulmonary disease (COPD) is a major risk factor for LC development [9,13].

Interestingly, poly (ADP-ribose) polymerase-1 (PARP-1) has also been shown to play a significant role in elastase-induced lung inflammation and emphysema in a mouse model of COPD [14]. PARP-1 and PARP-2 catalytically cleave NAD^+ and transfer ADP-ribose moieties onto specific amino residues of acceptor proteins in response to DNA damage. This process, termed poly ADP-ribosylation (PARylation), forms poly (ADP-ribose) polymers (PAR), which vary in size and branching. PAR elicit functional and structural changes in the target proteins [15–17]. PARP activity is upregulated in response to DNA damage to maintain DNA stability, integrity, and repair [18]. Accordingly, PARP inhibitors have emerged as promising therapeutic tools in cancer. They may act as potentiators of chemotherapeutic agents, immune therapy, and radiotherapy [19]. They can also be administered alone in tumors characterized by breast cancer (BRCA) gene mutations [20]. Accordingly, PARP inhibitors are currently in use for the treatment of several cancer types such as breast and ovarian cancer [21]. In small cell lung cancer (SCLC), the combination of PARP inhibitors and platinum based-chemotherapy showed superior efficacy compared to chemotherapy alone in a preclinical model [22]. Whether PARP-1 and PARP-2 expression and activity are also involved in LC development in patients with underlying COPD remains an open question.

On this basis, we hypothesized that PARP-1 and PARP-2 expression and activity may be increased in lung tumors of patients with COPD. Thus, we explored: (1) DNA damage, (2) PARP-1 and PARP-2 protein expression and PARP activity, and (3) correlations between clinical and biological variables in lung tumors of patients with and without COPD. A group of LC cancer patients with no COPD was included as a control group for the purpose of comparison.

2. Results

2.1. Clinical and Functional Characteristics of Study Patients

The clinical and functional characteristics of all the study patients are shown in Table 1. Anthropometric variables such as age, gender and BMI did not show any significant difference between LC and LC-COPD patients. The number of male patients in the LC-COPD group was significantly larger than that in the LC group (Table 1). The cigarette smoking burden variable, pack-years was significantly higher in LC-COPD patients than in LC patients, while the number of non-smokers was significantly larger in the LC group (Table 1). The lung functional parameters FEV_1,

FEV_1/FVC, DL_{CO} and K_{CO} were significantly reduced in LC-COPD patients compared to LC patients (Table 1). The majority of the COPD patients were in GOLD stages I and II (86.6%). TNM staging or histological subtypes did not significantly differ between the study groups. The levels of blood parameters such as total leucocytes, neutrophils, lymphocytes, albumin, total proteins and body weight loss did not significantly differ between the study patients.

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

Anthropometric Variables	LC (*N* = 15)	LC-COPD (*N* = 15)
Age, years	64 (11)	67 (9)
Male, *N*/Female, *N*	6/9	10/5
BMI, kg/m^2	27 (4)	28 (7)
Smoking history		
Current: *N*, %	5, 33.3	6, 40
Ex-smoker: *N*, %	5, 33.3	9, 60
Never smoker: *N*, %	5, 33.3	0 *
Pack-years	23 (23)	61 (23) ***
Lung function parameters		
FEV_1	88 (23)	70 (20) *
FEV_1/FVC, %	76 (5)	58 (11) ***
DLCO, %	85 (17)	57 (13) ***
KCO, %	83 (13)	55 (10) ***
GOLD stage		
GOLD stage I: *N*, %	NA	5, 33.3
GOLD stage II: *N*, %	NA	8, 53.3
GOLD stage III: *N*, %	NA	2, 13.3
GOLD stage IV: *N*, %	NA	0, 0
TNM staging		
Stage I: *N*, %	5, 33.3	7, 46.7
Stage II: *N*, %	4, 26.7	6, 40
Stage III: *N*, %	6, 40	2, 13.3
Histological diagnosis		
Squamous cell carcinoma: *N*, %	0, 0	0, 0
Adenocarcinoma: *N*, %	15, 100	15, 100
Others: *N*, %	0, 0	0, 0
Blood parameters		
Total leucocytes/µL	9.3 (3.5) × 10^3	10.1 (4.4) × 10^3
Total neutrophils/µL	6.8 (3.9) × 10^3	7.5 (4.7) × 10^3
Total lymphocytes/µL	1.8 (0.8) × 10^3	1.8 (0.9) × 10^3
Albumin (g/dL)	4.3 (0.4)	4.4 (0.5)
Total proteins (g/dL)	6.9 (0.4)	7.1 (0.6)
Body weight loss, kg		
0, *N*, %	13, 86.6	12, 80
1–5, *N*, %	1,6.7	1, 6.7
6–10, *N*, %	1,6,7	2, 13.3

Continuous variables are presented as mean and standard deviation while categorical variables are presented as the number of patients in each group and the percentage in the study group with respect to the total population. Definition of abbreviations: N, number; kg, kilograms; m, meters; 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; L, liter. Statistical analyses and significance: * $p < 0.05$, *** $p < 0.001$ between LC-COPD patients and LC patients.

2.2. DNA Damage Increased in Lung Tumors of COPD Patients

In LC-COPD patients, the percentage of γ-H2AX positive cells, a marker of DNA damage, was significantly higher in the tumors compared to non-tumor lung samples (Figure 1). On the contrary, the levels of DNA damage did not significantly differ between tumor and non-tumor specimens in LC patients (Figure 1).

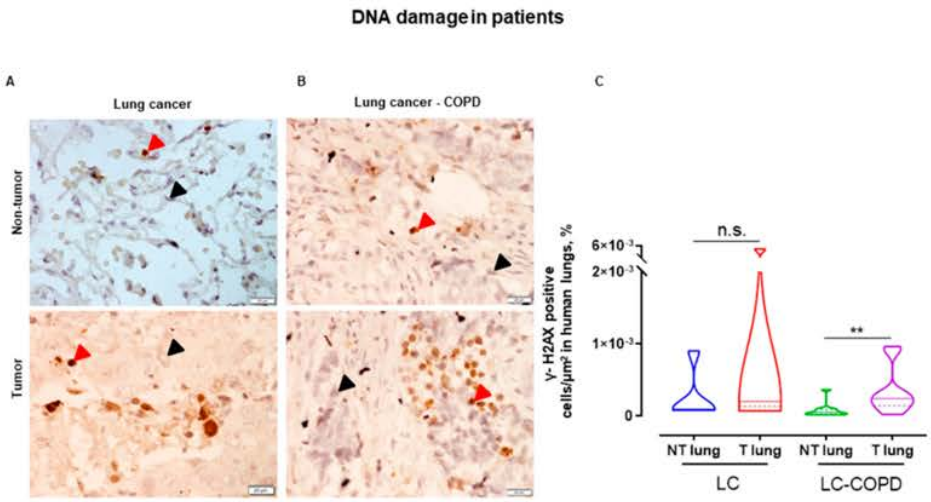

Figure 1. (**A**,**B**) Representative immunohistochemical staining sections of γ-H2AX in non-tumor and tumor lung specimens of LC and LC-COPD patients. Red arrows point towards γ -H2AX cells, which were positively stained in brown, and black arrows point towards γ -H2AX cells that remained stained in blue (hematoxylin counterstaining). (**C**) Violin plots with median (continuous line) and interquartile ranges (discontinuous lines) of the number of γ -H2AX positively stained cells in the total measured area. Statistical significance: n.s., no significance, ** $p < 0.01$ between non-tumor and tumor samples in LC-COPD patients. Definition of abbreviations: PARP, poly (ADP-ribose) polymerase; LC, lung cancer; COPD, chronic obstructive pulmonary disease.

2.3. PARP Activity Increased in Lung Tumors of COPD Patients

Consistent with the DNA damage results, a significant rise in PARP activity was also detected in lung tumor specimens of LC-COPD patients compared to control non-tumor samples (Figure 2). However, in LC patients, no significant differences in PARP activity were seen between tumor and non-tumor specimens (Figure 2).

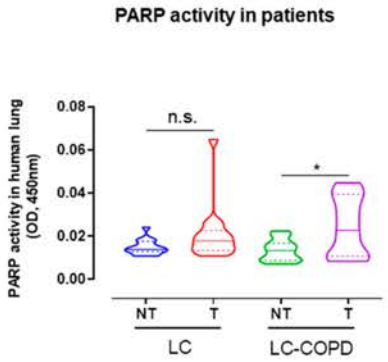

Figure 2. Violin plots with median (continuous lines) and interquartile ranges (discontinuous lines) of PARP activity levels in non-tumor and tumor lung specimens of LC and LC-COPD patients. Statistical significance: n.s., no significance, * $p < 0.05$ between non-tumor and tumor specimens of LC-COPD patients. Definition of abbreviations: PARP, poly (ADP-ribose) polymerase; OD, optical densities; LC, lung cancer; COPD, chronic obstructive pulmonary disease.

2.4. PARP Expression Decreased in Lung Tumors of All the Study Patients

A significant decline was observed in PARP-1 and PARP-2 protein expression levels in tumors of both LC and LC-COPD patients compared to the respective non-tumor samples in both groups (Figure 3).

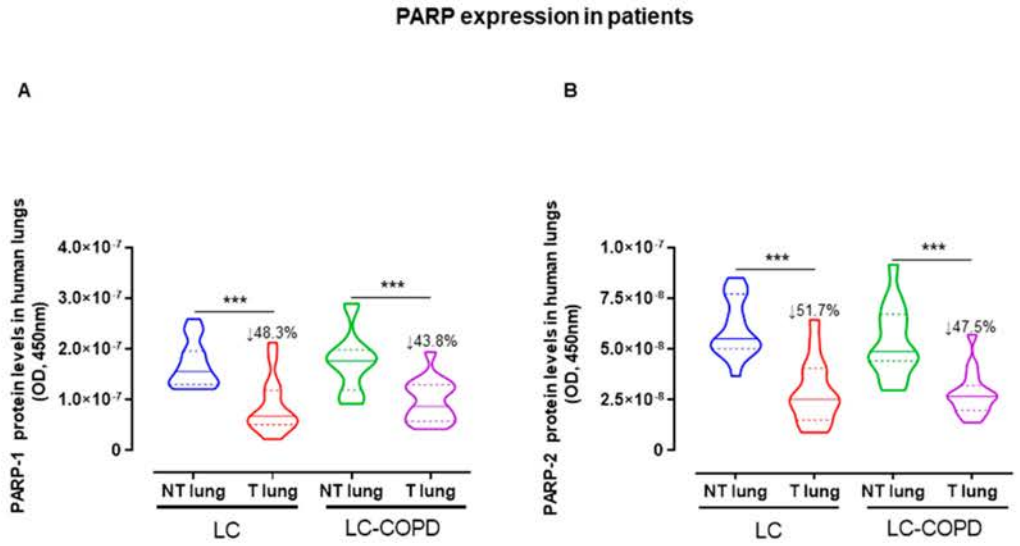

Figure 3. (**A**,**B**) Violin plots with median (continuous line) and interquartile ranges (discontinuous lines) of PARP-1 and PARP-2 protein levels assessed by ELISA in LC and LC-COPD patients, respectively. Statistical significance: *** $p \leq 0.001$ between tumor (T) and non-tumor (NT) specimens in LC and LC-COPD groups. Definition of abbreviations: PARP, poly (ADP-ribose) polymerase; OD, optical densities; LC, lung cancer; COPD, chronic obstructive pulmonary disease.

2.5. Influence of Staging in PARP Activity and Expression in LC and LC-COPD Patients

Interestingly, the variable staging of lung tumors did not significantly influence the PARP activity levels detected in the lung tumors when all the patients were analyzed as a whole (Figure 4).

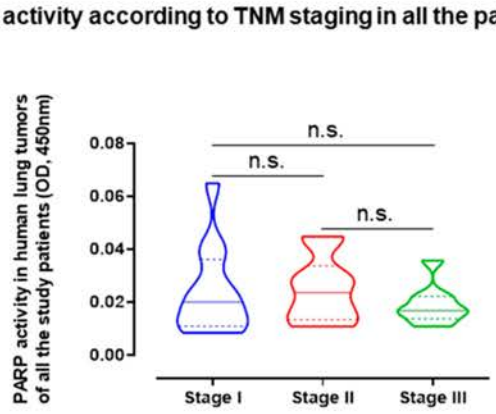

Figure 4. Violin plots with median (continuous lines) and interquartile ranges (discontinuous lines) of PARP activity levels in lung tumors according to cancer stages of all the study patients. Statistical significance: n.s., no significance among cancer stages of patients. Definition of abbreviations: PARP, poly-ADP ribose polymerase; OD, optical densities.

The influence of LC staging in the PARP protein expression levels found in tumor specimens of all of the patients taken as a whole, revealed that patients in stage I exhibited the greatest amount

of PARP-1 expression compared to the other LC stages (Figure 5A). Importantly, these results were also found in the LC patients with underlying COPD (Figure 5B), while PARP-1 expression was not modified according to staging in the group of LC patients (Figure 5C).

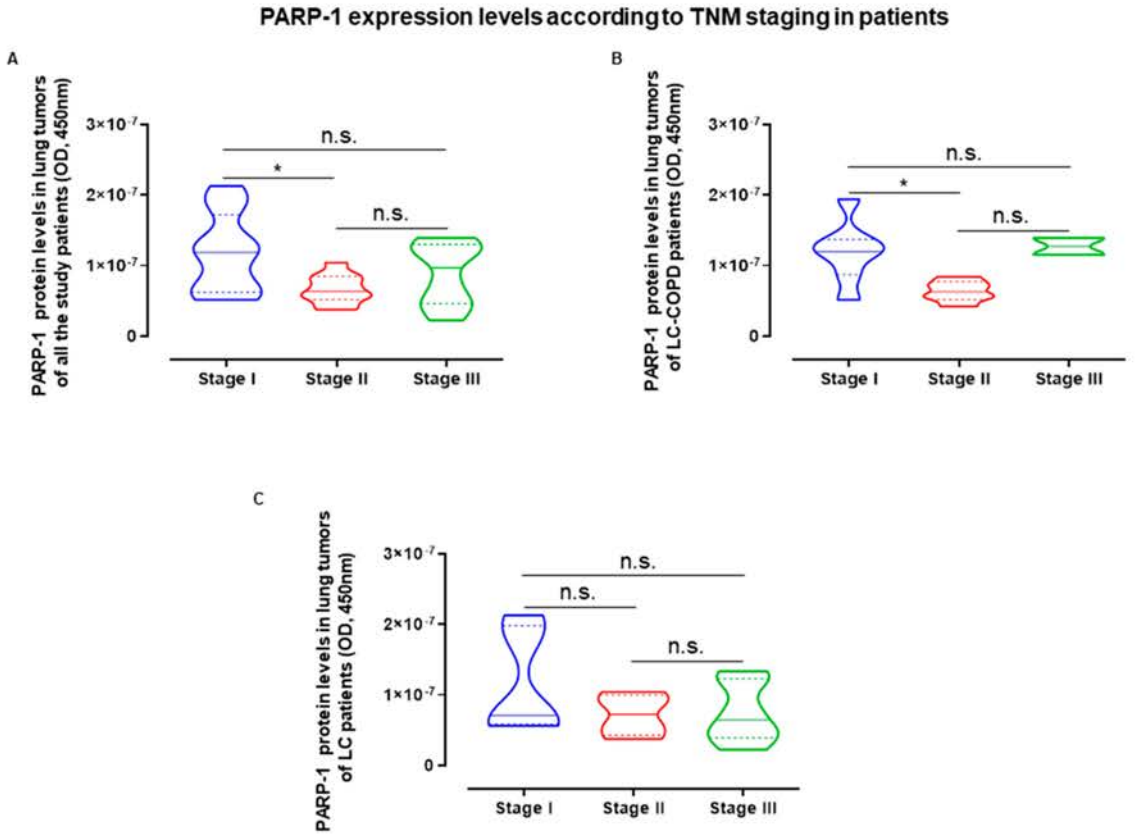

Figure 5. (**A**–**C**) Violin plots with median (continuous lines) and interquartile ranges (discontinuous lines) of PARP-1 protein levels in lung tumors according to cancer stages in all the study patients, LC-COPD and LC patients respectively. Statistical significance: * $p < 0.05$, n.s., no significance among cancer stages of patients. Definition of abbreviations: PARP, poly-ADP ribose polymerase; OD, optical densities.

The LC staging variable did not influence lung tumor PARP-2 expression when all the patients were analyzed as a whole (Figure 6A). Additionally, no significant associations were found between LC staging and PARP-2 protein content in the lung tumors in either LC-COPD (Figure 6B) or LC patients (Figure 6C).

2.6. PARP-2 Expression in Lung Tumors of COPD Patients Correlates with Cigarette Smoking

A significant positive correlation was found between the burden of cigarette smoking as indicated by the variable, pack-years and levels of PARP-2 expression in lung tumors of LC patients with underlying COPD (Figure 7).

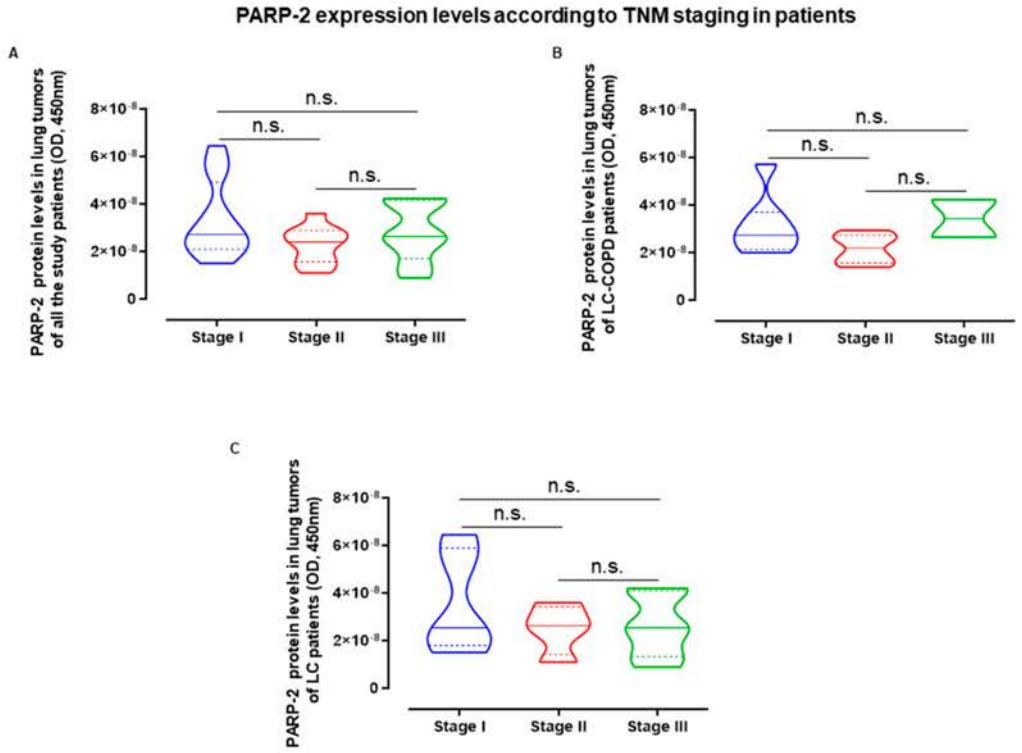

Figure 6. (**A**–**C**) Violin plots with median (continuous lines) and interquartile ranges (discontinuous lines) of PARP-2 expression levels in lung tumors according to cancer stages of all the study patients, LC-COPD and LC patients respectively. Statistical significance: n.s., no significance among cancer stages of patients. Definition of abbreviations: PARP, poly-ADP ribose polymerase; OD, optical densities.

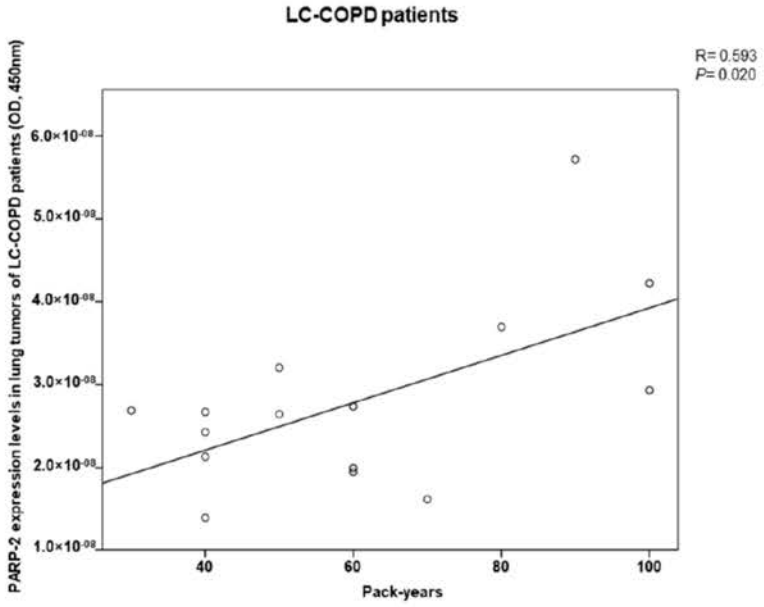

Figure 7. A significant positive correlation was detected between PARP-2 expression levels in lung tumors and pack-years in LC-COPD patients. Definition of abbreviations: poly (ADP-ribose) polymerase; LC, lung cancer; COPD, chronic obstructive respiratory disease.

3. Discussion

In tumors of patients with COPD compared to those of patients with no COPD, a rise in DNA damage and PARP activity was observed, while PARP-1 and PARP-2 protein expression levels decreased.

The tumors of LC-COPD patients with stage I exhibited greater expression levels of PARP-1 enzyme whereas in patients with LC-only, no differences were seen in either PARP-1 or PARP-2 expression levels according to different stages. Moreover, PARP-2 expression in lung tumor specimens significantly correlated with cigarette smoking burden among LC-COPD patients.

PARP-1 is the most relevant enzyme of the PARP family as its activity accounts for 85–90% of poly (ADP-ribosyl)ation in cells [23,24]. PARP activity plays a significant role in DNA repair through the induction of post-translational modifications of the target proteins by the transfer of ADP ribose moieties using NAD^+ as a substrate [23]. Other effects of the overaction of PARP include the occurrence of necrosis in tissues in response to persistent DNA damage. As such necrosis may take place as a result of depletion of the substrate NAD^+ [25].

In the current investigation, levels of DNA damage, as measured by γ-H2AX significantly increased in the tumors of LC-COPD, suggesting that severe injury of the DNA took place in the tumor cells of these patients. Likewise, the overactivation of PARP activity took place only in the tumors of the same patients. Moreover, a significant positive correlation between pack-years and PARP-2 expression was only observed in the LC-COPD patients. These are relevant findings that suggest that chronic cigarette smoking induces DNA damage, which may be counterbalanced by PARP activity. In fact, DNA damage and PARP-1 overactivation induced the parthanatos pathway of cell death as a result of the exposure of human bronchial epithelial cells to cigarette smoke [26]. Moreover, in patients with stable COPD, systemic PARP-1 activation was also observed in the lymphocytes along with increased inflammation and oxidative stress [27].

Importantly, the expression of PARP-1 and PARP-2 was significantly lower (the decrease ranged from 44% to 52%) but did not disappear in the tumors of both groups of patients, irrespective of COPD. Interestingly, despite the reduced expression of PARP-1 and PARP-2, overall PARP activity was maintained and even significantly increased in the tumors of the patients with the underlying respiratory condition. These findings are in agreement with previous results, in which the protein content of PARP isoforms did not influence PARP activity in several cancer cell lines [28]. Biological mechanisms such as endogenous activation or repression and/or post-translational modifications may account for the lack of correlations between PARP enzyme activity and protein expression levels.

Furthermore, the findings of the present investigation suggest that stimuli beyond the cancer phenotype were most likely part of the pathophysiology of the overaction of PARP activity. In keeping with this, elastase-induced emphysema was shown to increase PARP activity in mouse lungs in an experimental model of COPD [14]. Taken together, these results point to a potential role of PARP inhibitors in the treatment of LC patients, particularly in those with underlying respiratory conditions as tumors of those without COPD did not experience an increase in PARP activity. This scenario might account for the lack of significant beneficial effects of the PARP inhibitor, olaparib in combination with gefitinib in a phase 2 trial in NSCLC patients [29].

In summary, we studied two different groups of patients with LC. The differential phenotypic features of the patients associated with the underlying respiratory disease evidenced that these aspects should be taken into account when designing the best therapeutic strategies for the management of LC patients, especially as the burden of DNA damage and the activity of PARP enzymes were only significantly greater in the tumor specimens of patients with underlying COPD.

Study Limitations

A potential limitation of this study was the relatively low number of patients analyzed in the investigation. Nonetheless, it should be mentioned that very selective inclusion and exclusion criteria were established to recruit the patients. Moreover, patients had to undergo video-assisted thoracoscopic surgery (VATS) for the resection of their lung neoplasm, which is not the case for all LC patients seen in specialized clinics. Additionally, sample size was calculated ad hoc by the statistician in the group. As such, a minimum of 13 patients/group was estimated to be necessary to fulfill the study objectives. Furthermore, as 15 patients/group were finally recruited, the power of the study was 83.90%, and PARP

activity was the target variable. All the results obtained in the present investigation, including the association analyses are based on the analysis of the 15 patients recruited in each group.

4. Materials and Methods

4.1. Study Design and Ethics

This is a cross-sectional, prospective study designed according to the World Medical Association guidelines (Seventh revision of the Declaration of Helsinki, Fortaleza, Brazil, 2013) for human subjects involved in medical investigations. The study was approved by the institutional Ethics Committee on Human Investigation (protocol # 2008/3390/I, at Hospital del Mar–IMIM, Barcelona, Spain). All the participants of the study signed the informed written consent.

Patients were prospectively recruited from the Lung Cancer Clinic at Hospital del Mar (Barcelona, Spain). All the participants were part of the Lung Cancer Mar Cohort. For the purpose of the current study, 30 patients with LC were recruited in 2019. Candidate patients for tumor resection underwent VATS prior to the administration of any kind of adjuvant therapy. Tumor and non-tumor lung specimens were collected from all the study participants.

LC diagnosis and staging were established by histological confirmation and classified in accordance with currently available guidelines for the diagnosis and management of LC [30,31]. TNM (tumor, node, and metastasis) staging was defined as stated in the 8th edition of the Lung Cancer Stage Classification [32]. 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 [33,34]. 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. The presence/absence of these diseases was confirmed using standard clinical tests: clinical examination, blood tests, bronchoscopy, electrocardiogram, echocardiography, and exercise capacity evaluation. Patients were further subdivided post hoc into two groups based on the presence of COPD: (1) 15 LC patients with COPD (LC-COPD group) and (2) 15 LC patients without COPD (LC group).

4.2. Clinical Assessment

Lung function parameters were evaluated according to standard procedures in all the study patients. In patients with underlying COPD, the diagnosis and severity were determined in accordance with currently available guidelines [35,36]. A nutritional evaluation was done for all patients including body mass index (BMI) and blood nutritional parameters.

4.3. Collection and Preservation of Samples

Lung specimens were obtained from tumors and the surrounding non-tumor parenchyma following standard technical procedures during VATS for the standard care in the treatment of lung tumors. In all of the study patients, the expert pulmonary pathologist selected an approximately 10×10 mm^2 area of tumor and non-tumor specimen from the fresh lung samples. Non-tumor specimens were obtained as far distal to the tumor margins as possible (average >7 cm). Fragments of both tumor and non-tumor samples were fixed in formalin and embedded in paraffin blocks until further use. Another fragment was frozen immediately in liquid nitrogen and preserved at −80 °C for the measurement of protein levels.

4.4. Molecular Biology Analysis

4.4.1. DNA Damage in Lungs of the Study Patients Using Immunohistochemistry

DNA damage was assessed in tumor and non-tumor lung specimens by the presence of γ-H2AX, a hallmark of DNA damage [37] using conventional immunohistochemistry as previously described [10,38]. Briefly, paraffin-embedded specimens were cut into three-micrometer sections on a microtome. Following deparaffinization, lung sections were submerged in pre-heated antigen-retrieval solution of in Citrate Buffer (PH = 6) in a pressure cooker for 15 min and then slides were gradually cooled to room temperature. After rinsing with distilled water three times, slides were treated with 3% hydrogen peroxide for ten minutes to block endogenous peroxidase activity. Then, slides were incubated with blocking buffer (PBS 1% bovine serum albumin) for one hour at room temperature and with anti-γ-H2AX primary antibody (anti-γ-H2AX, Millipore) at 4 °C overnight. The next day, after being washed three times with PBS, the slides were incubated with biotinylated universal secondary antibody for one hour and the detection process was assessed using HRP-streptavidin for five minutes. After two minutes of hematoxylin counterstaining, slides were dehydrated and mounted with dibutylphthalate polystyrene xylene (DPX) mounting medium for conventional microscopy examination. Images of the stained tumor and non-tumor lung sections of patients were obtained under 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). In addition, anti-γ-H2AX positively stained cells in all of the samples were counted independently by two previously trained investigators. The area of the lung sections was measured using Image J software (National Institutes of Health, Bethesda, MD, USA) in all stained slides. Data are presented as the percentage of anti-γ-H2AX cells in the measured area in all the non-tumor and tumor lung specimens of patients.

4.4.2. PARP Activity in Lungs of the Study Patients

PARP enzyme activities in human lung samples were estimated using the higher throughput (HT) 96 test size Universal Colorimetric PARP Assay Kit with Histone-Coated Strip Wells (Trevigen, Gaitherburg, MD, USA) according to the manufacturer's instructions and previous studies [39]. The amount of total protein levels in lung homogenates were quantified using NanoDrop ND1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) with duplicates of each sample. Briefly, all histone-coated strip wells were rehydrated with PARP buffer for 30 min. A standard curve was always run with each assay along with the samples. The identical amounts of proteins (80 μg) from lung homogenates were added and incubated with 25 μL PARP cocktail (mixture of PARP cocktail, activated DNA and PARP buffer) for one hour at room temperature. Then, wells were washed twice with 1 × PBS and 0.1% Triton X-100 and twice with 1 × PBS and incubated with 50 μL Strep-HRP for one hour at room temperature. After four additional washes, wells were incubated in the dark with 50 μL TACS-Sapphire ^{TM} colorimetric substrates for fifteen minutes at room temperature. Lastly, the enzyme reactions were stopped by adding 50 μL of 0.2mol of hydrochloric acid per well. Absorbance was read in a microplate reader at 450 nm. Intra-assay coefficients of variation for PARP activity level were less than 4% and inter-assay coefficients of variation were less than 5%.

4.4.3. PARP Expression in Human Lungs Using Enzyme Linked Immunosorbent Assay

PARP-1 and PARP-2 expression levels in tumor samples and non-tumor samples of human lungs were quantified using ELISA (MyBioSource, Inc., San Diego, CA, USA). All procedures were performed following the manufacturer's instructions and previous studies [10]. The amount of total protein levels in lung homogenates were quantified using NanoDrop ND1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) with duplicates of each sample. Before starting the assay, reagents and samples were naturally warmed to room temperature. A standard curve was always run with each assay along with the samples. An identical volume of samples (40 μL) and biotinylated human PARP-1 and PARP-2

antibodies (10 µL) were added to the pre-coated wells with the correspondent PARP-1 and PARP-2 antibodies in duplicate. Subsequently, 50 µL streptavidin-HRP secondary antibodies were loaded into all wells and plates were incubated on an orbital micro-plate at 37 °C for one hour. Then, all wells were washed five times for 45 s and incubated for another ten minutes with substrate solutions at 37 °C in the dark. Finally, 50 µL stop solution was added to each well to end the enzyme reaction. Optical densities in each well were immediately detected in a microplate reader set to 450 nm. Intra-assay coefficients of variation were less than 8% and inter-assay coefficients of variation were less than 10% for both PARP-1 and PARP-2 levels.

4.5. Statistical Analysis

The normality of the study variables was tested using the Shapiro-Wilk test. Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test, 13 subjects were necessary in each group to recognize a minimum difference of 0.015 units in the mean of the variable PARP activity as statistically significant. The common deviation was assumed to be 0.011. Finally, as 15 patients were recruited in each group, by taking the results of the one-way analysis of variance for the variable PARP activity group means, that is, 0.0149, 0.0210, 0.0136, 0.0248, a variance error = 9.75×10^{-5} and a sample size = 15 (balanced groups) we obtained a power of 83.90%.

Qualitative variables are represented as total numbers and percentages with respect to total values, while quantitative variables are reported as the mean and standard deviations. Differences in physiological variables in clinical parameters between LC and LC-COPD groups of patients were analyzed using Student's T-test. Differences between patient groups (LC and LC-COPD) and types of samples (tumor and non-tumor) were analyzed using Kruskal–Wallis equality-of-populations rank test followed by Dunn's Pairwise Comparison or one-way ANOVA and Tukey post hoc to adjust for multiple comparisons of the biological variables. Statistical significance was established at $p \leq 0.05$. Physiological and clinical variables are shown in tables, while biological variables are in figures that use violin plots or histograms according to the normality of the variables. All the statistical analyses of the study were conducted using the software Statistical Package for the Social Science (SPSS, version 23, SPSS Inc., Chicago, IL, USA).

5. Conclusions

In lung tumors of patients with underlying COPD, an overactivation of PARP enzyme was observed along with increased DNA damage levels. A decline in PARP-1 and PARP-2 protein expression was seen in lung tumors irrespective of COPD. Other phenotypic features (airway obstruction) beyond cancer may account for the increase in DNA damage and PARP overactivation seen in the tumors of patients with underlying COPD. These findings warrant special attention when designing specific therapeutic strategies that may include PARP inhibitors in the treatment of patients with NSCLC as COPD may render these patients more prone to benefit from those therapies.

Author Contributions: Study conception and design: E.B., J.Y., V.C., J.T.; Patient assessment and recruitment and sample collection: J.T., V.C., A.R.-F., R.A., L.P.; Pathological diagnosis and tumor identification: L.P.; Molecular biology analyses: J.T., X.W., C.A.; Statistical analyses and data interpretation: J.T., X.D., E.B.; manuscript drafting and intellectual input: E.B., J.T., J.Y.; manuscript writing final version: E.B. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by FIS 18/00075 (FEDER, ISC-III) & CIBERES (ISC-III), SEPAR 2018& SEPAR 2020.

Conflicts of Interest: The authors declare no conflict of interest.

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5. SUMMARY OF THE MAIN FINDINGS

5. SUMMARY OF THE MAIN FINDINGS

Studies #1, #2, #4 and #5

In lung tumors of LC-COPD VS LC:

Both number/area and area of TLSs, germinal centers significantly decreased in LC-COPD patients.

Lung tumor VS Non-tumor Lungs in LC-COPD patients:

- Immune profile:
  - TLSs, B cells, germinal centers, Tregs levels were significantly higher in lung tumors
  - T cells and plasma secreting cells IgG levels were significantly lower in lung tumors
  - *Associations with patients´ 10-year survival:*
    - ✓ Greater area and number of TLSs and higher proportion of B cells in lung tumors were associated with longer survival rates in all patients together
    - ✓ Greater area of TLSs and higher proportion of B cells in lung tumors were associated with better survival rates only in LC-COPD patients
- Cytokines:
  - IL-10 significantly increased in lung tumors
- Stroma components:
  - CAFs levels significantly increased while normal fibroblasts levels significantly decreased in lung tumors
  - Endothelial cell marker (CD31) decreased in tumors only in LC patients but not in LC-COPD patients
- PARP:
  - DNA damage and PARP activity levels significantly increased in lung tumors only in LC-COPD patients

- PARP-1 and PARP-2 expression levels significantly decreased in lung tumors

Study #3

LC-treated mice VS LC-bearing mice:

- Final body weight and body weight gain with/without tumor significantly increased, while tumor weight and area significantly decreased in LC-treated mice
- Tumor proliferation significantly decreased while apoptotic cell percentage significantly increased in LC-treated mice
- CD3, CD4 and CD8 cell levels significantly increase in LC-treated mice
- Oxidative stress, antioxidant enzyme, apoptosis, autophagy, signaling markers NF-κB and sirtuin-1 increased in LC-treated mice

6. DISCUSSION

6. DISCUSSION

In the present thesis, we investigated whether COPD influences the underlying biological mechanisms of the tumor microenvironment, DNA damage, and repair which may predispose COPD patients to develop lung tumors. Also, we studied whether a combined immune therapy affects tumor progression through several cell biological mechanisms beyond the immune regulation in mice with experimental lung adenocarcinoma. Our results showed that in patients, the presence of underlying COPD appears to play a role in tumor immune profile and influence patients´ overall survival, it also affects tumor stroma components, DNA damage and repair processes. Moreover, in the experimental model of lung cancer mice, we found that the treatment with a combination of monoclonal antibodies reduced tumor burden regulating cell processes such as oxidative stress, apoptosis, and autophagy.

Our first finding was that TLSs and B cells levels in lung tumors were associated with better prognosis in lung cancer patients. Moreover, the presence of underlying COPD induced a differential immune profile in lung tumor and also influenced patients´ overall survival.

TLSs are transient lymphoid structures that contain a T cell-rich zone and a B cell-rich area that are formed in chronic inflammation, infection, or tumor sites. In several cancer types, TLSs and B cells are demonstrated to correlate with longer survival and improved immunotherapy response (133–135). In line with these studies, we found that higher levels of both the number and area of TLSs and B cell counts in lung tumors were associated with better overall survival in all patients together. Likewise, Germain et al. demonstrated that higher density of B cells in TLSs was associated with better survival in both early and advanced stages NSCLC patients treated with chemotherapy (111).

The immune features and the impact on patients' survival linked to TLSs and B cells in NSCLC with underlying COPD are unknown. In the current thesis, firstly, we found that B cells and T cells densities did not differ according to the presence of COPD. The possible explanation of these results is that the recruitment of immune cells to the tumor sites probably depends on tumor mutational burden (TMB), thus weakening the influence of the underlying COPD. Similarly, we neither found

differences of other immune cell types such as T reg, NKs, IgG, and IgA secreting plasma cells in lung tumors according to COPD status. Higher TMB was associated with higher $CD8^+$ T cell infiltration in ovarian cancer (241), KRAS-mutant lung carcinoma subsets display different immune patterns (242) could support this suggestion.

Nevertheless, both the number and area of TLSs and the GC counts were lower in lung tumors of COPD patients. The reason for this difference is unclear, but there are some possible explanations. Firstly, TLSs and GC formation may be affected in lung tumors with underlying COPD conditions. CXCL 13 is a key chemokine to induce TLS formation and it is expressed by different cell types such as follicular dendritic cells, B cells, etc. Then CXCL13 likely binds to reticular fibers to mediate its function (243). The principal component of reticular fibers type III collagen was also reported to limit tumor metastasis in lung cancer and breast cancer (244,245). Importantly, type III collagen was demonstrated to be degraded in the emphysematous phenotype of COPD (246) which may support the hypothesis that the formation of TLS and GC is impaired in COPD conditions. Furthermore, Silina et al. found that both chemotherapy and corticosteroids impaired GC formation in TLSs and abolished the protective effects of TLSs in lung squamous cell carcinoma patients (247). In the present thesis, all lung samples were collected before the patients had received any chemotherapy or radiotherapy. However, the use of corticoids before surgery was not identified in the study patients.

We also found that greater area of TLSs and higher levels of B cell counts were associated with better prognosis in lung cancer patients with underlying COPD but not in those without this entity. Evidence demonstrates that in chronic inflammation and cancer conditions, the infiltrating T cell function is impaired by the expression of immune-checkpoints such as PD-1, PD-L1, CTLA-4, and TIM-3, namely T cell exhaustion. Importantly, Biton et al. (248) found that the tumor-infiltrating T lymphocytes were more exhausted in lung tumors of patients with underlying COPD compared to lung cancer-only patients. In the second cohort of the same study, lung cancer patients with underlying COPD also benefited more from an anti-PD-1 inhibitor (nivolumab) with significantly longer progression-free survival than lung cancer only patients (248). In another retrospective study, Mark et al. (249) also demonstrated that the presence of COPD in lung cancer patients was associated with improved response to immune checkpoint inhibitors (anti-PD-1 or anti-PD-L1) therapy. Taken

together, T cells may be severely exhausted in lung tumors of COPD patients and TLSs and B cells may be the immune subtype generating anti-tumor immunity in this circumstance.

Our second main finding was that in the tumor stroma, the levels of CAFs were higher while normal fibroblasts were lower irrespectively of the presence of COPD. Endothelial cell marker CD31 level was lower in lung tumors compared to non-tumor lung specimens only in lung cancer patients but not in those with underlying COPD. And the extracellular matrix marker Type I collagen level did not differ between the two groups of patients.

CAFs, the principal cellular component of tumor stroma promotes tumor growth and metastasis in different cancer types. In lung cancer, a previous study also showed higher levels of α-SMA expressed CAFs in lung tumors compared to tumor-free lungs (250). The specific marker of CAFs α-SMA in stromal fibroblast was also associated with poor prognosis in patients with early-stage of NSCLC after surgery (251). In line with these studies, we found higher levels of CAFs and lower levels of normal fibroblasts in lung tumors compared to non-tumor lung specimens. Up to now, the impact of COPD on tumor stroma is unclear. In chronic obstructive conditions, α-SMA expressed myofibroblasts seem to be increased which may contribute to airway remodeling (252,253). However, we found that the underlying COPD did not influence CAFs expression in tumors of NSCLC patients. This result suggests that the role of CAFs in lung cancer patients with underlying COPD may not be a simple overlay and CAFs seem not to accelerate tumor invasion and progression in COPD patients.

CAFs are also an important source of extracellular components, they secrete principally types I, III, V collagens, and fibronectin. Meanwhile, CAFs can also modulate ECM homeostasis secreting degrading products MMPs and LOC-protein. Apart from CAFs, cancer cells are also shown to produce endogenously Type I collagen (254). In both lung cancer and COPD conditions, the ECM component Type I collagen dysregulation was associated with disease progression and poor survival (254,255). Moreover, a previous study showed that the degradation of Type I collagen did not differ in the serum of lung cancer and COPD patients (256). In our study, we did not find any difference in Type I collagen levels in lung tumors compared to non-tumor lungs. Taken together, these results indicate that COPD may not influence the density of ECM in NSCLC patients.

Another important characteristic of the tumor ECM is the architecture which depends on the collagen alignment, the pore size, and stiffness. These aspects of ECM have been demonstrated to facilitate cancer cell escape and favor tumor metastasis (257). These aspects are not considered in the abovementioned studies, and maybe key mechanisms of tumor progression and metastasis addition to the density of the ECM components. Future studies should take these aspects into account when analyzing ECM components.

Blood vessels are important for cancer cell growth applying oxygen and nutrients. Endothelial cells make up the endothelium of the blood vessels and can be evaluated by Platelet Endothelial Cell Adhesion Molecule-1 (PECAM), also namely CD31. The marker CD31 has been proven to appropriately measure the intratumoral microvessels density in lung cancer. However, the role of CD31 in cancer remains debatable. Mohamed et al. found that high expression of CD31 was associated with poor prognosis in colorectal patients (258). Rask et al. found no correlation of CD31 with survival in patients with ovarian cancer (190). On the contrary, a high level of CD31 was associated with better prognosis in patients with pancreatic ductal cancer (191). In NSCLC, a previous study also showed that high density of intratumoral microvessels identified by CD31 and CD 34 was correlated with better treatment responses might due to the better delivery of the specific immune cells and drugs to the tumor niche (192). In the current thesis, we found that the levels of CD31 significantly reduced in lung tumors only in lung cancer patients but not in those with the underlying COPD condition. These findings may suggest that intratumoral microvessels density measured by CD31 may obtain prognostic value in lung cancer patients with and without COPD.

Also, we tried to evaluate the involved biological mechanisms of immune therapy on lung tumor progression. We found that in a mouse model with experimental lung adenocarcinoma, the treatment of the combination of four monoclonal antibodies anti-PD-1, anti-CTLA4, anti-CD137, and anti-CD19 successfully reduced tumor burden along with the elicitation of oxidative stress, apoptosis, and autophagy in tumor cells.

So far, four PD-1 inhibitors pembrolizumab, nivolumab, atezolizumab, and durvalumab are approved in lung cancer. However, only the combination of nivolumab and ipilimumab, a PD-1 inhibitor, and a CTLA-4 inhibitor reversing the inhibited immune system has been approved in NSCLC patients. Dai et al. (259)

firstly demonstrated that the combination of anti-PD-1, anti-CTLA-4, anti-CD137, and anti-CD19 injected intratumorally completely reduced tumor burden along with long-term survival in mice with melanoma and lung carcinoma.

The second finding of Dai´s study was that the combination of four monoclonal antibodies showed a significantly better response than the combination of three of them including anti-PD-1, anti-CTLA-4, and anti-CD137 (259). These may suggest the potential implication of the combined normalization and enhancement strategy of the immune system may be better than the approved combination of anti-PD-1 and anti-CTLA-4 in lung cancer treatment. In line with this study, we found significantly reduced tumor burden with lower tumor weight and area, and lower cell proliferation rates in the treated mice. The complete tumor regression was not achieved as Dai's study. This possibly due to that we administrated the treatment intraperitoneally to mimic the conventional medication administration way. Also for ethical standards of our center, we had to sacrifice the mice before day 30 which may also limit the effect of the treatment.

Dai et al. (259) also found that the treatment of the combination of the four monoclonal antibodies successfully shifted the immune tumor microenvironment from Th2 to Th1 immune profile. Accordingly, we also found a rise of T cells indicating a boost of the immune system in the tumor of the treated mice group. Beyond the immune system, other biological mechanisms also have been demonstrated to regulate immunotherapy response in our study.

Cancer cells are characteristic of the presence of high levels of reactive oxygen species (ROS), and they can keep the redox balance with their high antioxidant capacity and survive. This redox balance in cancer cells can be broken by increased levels of ROS when passing the threshold and cause cancer cell death.

Many anticancer drugs are based on inducing ROS levels. Chemotherapy and radiotherapy also generate oxidative stress in cancer cells. During chemotherapy and radiotherapy, the increased ROS can cause cancer cell death inducing apoptosis, necroptosis, and autophagy along with activation of the NF-kB signaling pathway (260,261)**.** Accordingly, in our mice model treated with the combination of monoclonal antibodies, we found a rise of oxidative stress identified by increased levels of protein tyrosine nitration and total MDA-protein adducts along with increased level of antioxidant enzyme SOD1 in these mice. In line with previous studies, we also found

higher levels of apoptosis with increased levels of TUNEL-positive nuclei and Bax protein. Autophagy was also increased with higher levels of LC3B protein along with higher activation of the NF-kB and sirtuin-1 in the treated mice. These results suggest that oxidative stress modulates the immune response. T cell regulation in the condition of ROS in tumors may directly support this hypothesis (260).

Antioxidant capacity in cancer is a double-edged sword. On the one hand, they can counteract the effect of oxidant products and keep the oxidative homeostasis in cells. However, the high antioxidant activity also links with tumor metastasis and drug resistance. Piskounova et al. (260) reported that in melanoma while oxidative stress limited metastasis, the anti-oxidant promoted tumor progression and extension. Zhang et al. (262) reported decreased production of ROS and high levels of resistance gene production in colorectal cancer cell lines. In NSCLC cell lines, Silva et al. found antioxidant enzyme levels influence cisplatin resistance (263).

Taken together, this combination of monoclonal antibodies we used significantly reduced lung adenocarcinoma burden with increased oxidative stress level alongside higher levels of downstream apoptosis and autophagy. The increased level of antioxidant products may suggest the investigation on the combination of immunotherapy with prooxidants and/or antioxidants-inhibitors in lung cancer treatment.

The last main finding of the current thesis was that DNA damage and PARP activity levels were higher in lung tumors compared to non-tumor control lungs only in COPD patients along with decreased PARP-1 and PARP-2 expression.

Cigarette smoke and other toxic stimuli can damage DNA and subsequently produce mutations and epimutations which contribute to carcinogenesis and tumor progression. The DNA repair enzyme PARP, especially PARP-1 and PARP-2 are activated to reverse these damages. Cancer cells also take advantage of these enzymes to survive. PARP enzymes are also demonstrated to involve in other cell processes. PARP promotes inflammation regulating the NF-kB pathway (264). In a Parp-1(-/-) and Parp-2(-/-) mice with experimental lung adenocarcinoma, authors reported lower tumor burden along with increased levels of oxidative stress, apoptosis, and autophagy compared to the wide-type control mice (265). PARP also involves in immune cell regulation inhibiting Treg regeneration, PARP can also regulate T cell function regulating TGF-β receptors expression (266).

Previous studies found that PARP is differentially expressed in cancers. Ossovskaya et al. found that PARP-1 mRNA is upregulated in NSCLC and in other cancers including breast cancer, ovarian cancer, endothelium cancer, skin cancer but not in prostate adrenal, colon carcinoma (267). In pancreatic cancer, Bosch et al. reported that PARP-1 was highly expressed in acinar cells in normal and cancer acinar cells but almost null in normal and cancer ductal cells (268). Notably, Zaremba et al. (269) found no significant correlation between PARP activity and PARP-1 protein level in a panel of 19 human cancer cell lines suggesting that PARP activity depends on posttranslational modification and/or endogenous activation or repression. Bianchi et al. (270) also highlighted that PAR expression, a marker of PARP activity, but not PARP expression was correlated with PARP inhibitor sensitivity in cervical cancer cell lines.

In line with these studies, we found that both PARP-1 and PARP-2 protein levels were lower (the decrease ranged from 44% to 52%) but not null in lung tumors compared to non-tumor lungs of patients irrespectively of COPD status. Contrarily, PARP activity was higher in lung tumors of patients with underlying COPD. Importantly, PARP as a highly sensitive sensor is rapidly activated during DNA damage is well documented. Keeping with this, we also found a rise in DNA damage along with PARP overactivation in lung tumors of patients with underlying COPD but not in those without this pathology.

So far, four PARP inhibitors are approved in advanced and breast cancer patients with BRCA mutation with improved survival rates. However, the use of PARP inhibitors in lung cancer is not satisfactory, especially in NSCLC. Despite numerous clinical trials of PARP inhibitors are underwent in NSCLC patients, only two studies published their results. One is a phase II study conducted by Ramalingam et al. (271), they found a modest difference in patients' progression-free survival of NSCLC patients treated with a PARP inhibitor veliparib compared with the combination of carboplatin and paclitaxel but the difference was not statistically significant. In another recent phase II trial of 182 EGFR mutant NSCLC patients, treatment of the combination of gefitinib and PARP inhibitor olaparib did not show beneficial effects compared to gefinitib alone (236). Notably, none of these studies have distinguished patients with the presence of underlying COPD. Taken into account our results, a more personalized therapeutic therapy should be considered when designing the therapeutic strategies of PARP inhibitors for the treatments of NSCLC patients.

7. CONCLUSION

7. CONCLUSION

1. Tumor immune microenvironment, stroma components, and PARP are differentially expressed in lung tumors of lung cancer patients with underlying COPD.

2. The reduction in TLS and GC formation, the rise in DNA damage and PARP overactivation probably contribute to the greater susceptibility of COPD patients to develop lung tumors.

3. In mice treated with the combination of monoclonal antibodies, increased levels of oxidative stress along with activated apoptosis and autophagy may be part of the mechanisms whereby immunotherapy may reduce tumor burden.

4. In conclusion, the presence of COPD should be considered when designing therapeutic strategies for lung cancer including immunotherapy as well as PARP inhibition.

8. FUTURE PERSPECTIVES

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The following hypotheses should be explored in the near future:

1. The specific contribution of the immune system to tumor development in patients with COPD, especially TLSs and B cells as they were shown to elicit beneficial effects. The underlying biological mechanisms that lead to decreased levels of TLSs and GCs formation in lung cancer-COPD patients deserve further attention.

2. Future studies should elucidate the specific role of CD31 in lung cancer patients with underlying COPD, specifically this potential predictive should be explored in larger cohorts of lung cancer patients with and without COPD.

3. Studies on tumor stroma should also include the analysis of the architecture of the stroma components in order to obtain a panoramic view.

4. The physical and chemical signals elicited from tumor stroma should be investigated in lung tumors of patients with underlying COPD.

5. Clinical trials based on the use of PARP inhibitors should be conducted in NSCLC patients with and without underlying COPD to ensure whether lung cancer patients with COPD could benefit more from PARP inhibitors alone or in combination with other conventional anti-cancer therapies.

6. Future studies should explore the combination of immunomodulators along with the use of prooxidants and/or antioxidants-inhibitors as a potential therapeutic strategy for lung cancer treatment.

7. A mouse model for lung cancer and COPD overlap is needed in order to analyze the associations of these two highly prevalent lung diseases.

9. BIBLIOGRAPHY

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10. APPENDIX

10. APPENDIX

10.1 Other publication

Title:

Preoperative body weight and albumin predict survival in patients with resectable lung neoplasms: role of COPD

Authors:

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Journal:

Archivos de Bronconeumología. 2021 Jan;57(1):51-60.

DOI: 10.1016/j.arbres.2020.07.021.

PMID: 32877328

Impact Factor:

4.957 (2019), Quartile: Q1

Arch Bronconeumol. 2020;xxx(xx):xxx–xxx

Sociedad Española de Neumología y Cirugía Torácica SEPAR

ARCHIVOS DE Bronconeumología

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Original Article

Preoperative Body Weight and Albumin Predict Survival in Patients With Resectable Lung Neoplasms: Role of COPD

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ARTICLE INFO

Article history:
Received 14 March 2020
Accepted 17 July 2020
Available online xxx

Keywords:
Lung cancer
COPD
Nutritional status
Blood albumin
Blood total proteins
Overall survival

ABSTRACT

Introduction: The impact of preoperative nutritional status on survival in lung cancer (LC) patients with underlying chronic obstructive pulmonary disease (COPD) is still unclear. We hypothesized that presurgical nutritional assessment may differentially predict mortality in patients with resectable LC with moderate COPD and relatively well-preserved nutritional status.
Methods: Nutritional assessment [body mass index (BMI), blood parameters including albumin and protein levels, and body weight loss], and other clinical parameters [cigarette smoking (CS) history, LC staging and histological subtypes, COPD severity, lung function, and adjuvant therapy] were evaluated in 125 patients from the LC Mar Prospective Cohort: 87 LC-COPD patients and 38 LC patients without COPD before thoracotomy. Ten-year overall survival (OS) was analyzed in all patients.
Results: Prior to thoracotomy, in LC-COPD patients compared to LC, BMI and albumin declined relatively, low levels of the parameters BMI, albumin, and total proteins were associated with poorer 10-year survival, especially in the LC-COPD. CS burden also correlated with impaired survival. COPD per se worsened the prognosis in LC patients.
Conclusions: In the present cohort of LC patients with resectable tumors and relatively well-preserved nutritional status, the parameters BMI and blood albumin and protein levels measured prior to thoracotomy predicted OS, especially in those with COPD. These are clinically relevant findings, since values of those nutritional parameters were within the normal ranges in the majority of the analyzed patients. A thorough nutritional preoperative assessment should be included in the study of patients with resectable LC, particularly in those with chronic airway obstruction.

El peso corporal preoperatorio y la albúmina predicen la supervivencia en pacientes con neoplasias de pulmón resecables: el papel de la EPOC

Palabras clave:
Cáncer de pulmón
EPOC
Estado nutricional
Albúmina en sangre
Proteínas totales en sangre
Supervivencia global

RESUMEN

Introducción: El impacto del estado nutricional preoperatorio en la supervivencia en pacientes con cáncer de pulmón (CP) y enfermedad pulmonar obstructiva crónica (EPOC) subyacente aún no está claro. Planteamos la hipótesis de que la evaluación nutricional prequirúrgica puede predecir diferencialmente la mortalidad en pacientes con CP resecable y EPOC moderada, y un estado nutricional relativamente bien conservado.
Métodos: Se evaluaron el estado nutricional (índice de masa corporal [IMC], parámetros sanguíneos que incluyeron los niveles de albúmina y proteínas y pérdida de peso corporal) y otros parámetros clínicos (antecedentes de tabaquismo, estadificación del CP y el subtipo histológico, gravedad de la EPOC, función

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https://doi.org/10.1016/j.arbres.2020.07.021
0300-2896/

Please cite this article in press as: Tang J, et al. Preoperative Body Weight and Albumin Predict Survival in Patients With Resectable Lung Neoplasms: Role of COPD. Arch Bronconeumol. 2020. https://doi.org/10.1016/j.arbres.2020.07.021

pulmonar y terapia adyuvante) en 125 pacientes de la cohorte prospectiva de CP del Hospital del Mar: 87 pacientes con EPOC-CP y 38 pacientes con CP sin EPOC antes de la toracotomía. Se analizó la supervivencia global (SG) a 10 años en todos los pacientes.

Resultados: Antes de la toracotomía, en los pacientes con EPOC-CP, el IMC y la albúmina disminuyeron en comparación con los del grupo de CP; los niveles bajos de los parámetros IMC, albúmina y proteínas totales se asociaron con menor supervivencia a 10 años, especialmente en los EPOC-CP. La carga tabáquica también se correlacionó con una disminución en la supervivencia. La EPOC empeoró *per se* el pronóstico en pacientes con CP.

Conclusiones: En la presente cohorte de pacientes con CP resecable y estado nutricional relativamente bien conservado, el IMC y los niveles de albúmina y proteínas en sangre medidos antes de la toracotomía predijeron la SG, especialmente en aquellos con EPOC. Estos son hallazgos clínicamente relevantes, ya que los valores de esos parámetros nutricionales estaban dentro de los rangos normales en la mayoría de los pacientes analizados. Se debe incluir una evaluación nutricional preoperatoria exhaustiva en el estudio de pacientes con CP resecable, particularmente en aquellos con obstrucción pulmonar crónica.

Introduction

Lung cancer (LC) is a major cause of mortality among different cancer types worldwide.[1–5] Several etiologic factors including underlying respiratory diseases have been reported in the literature.[6–9] Chronic obstructive pulmonary disease (COPD) has been consistently demonstrated to predispose patients to cancer development.[6–9] COPD is also a high prevalent condition worldwide and is associated with many different types of comorbidities.[3,10,11] Skeletal muscle dysfunction and alterations in body composition and nutritional abnormalities are counted among the most clinically relevant ones as they impact disease prognosis and survival in COPD patients.[12]

In LC and COPD patients, it has been well-established that body weight loss and altered nutritional parameters as disease progresses have a negative impact in their survival. Recently, it has been demonstrated that in patients with resected LC, the level of body mass index (BMI) before the surgery predicted the overall survival (OS) of the patients that were followed up for several years.[13] In other cancer types,[14] a 5% reduction in BMI significantly increased the risk of mortality, especially in the elderly. In another recent investigation,[15] baseline body weight loss and albumin had a negative impact on prognosis in patients with LC treated with immunotherapy. Moreover, other investigations have also reported that nutritional abnormalities predicted survival in LC patients with advanced stages,[16] impaired clinical outcomes,[17] and postoperative complications.[18,19] Whether nutritional status may also predict OS in LC patients with underlying respiratory conditions, namely COPD and preserved body composition remain to be elucidated.

On this basis, we hypothesized that clinical parameters defining nutrition prior to surgery may differentially predict mortality in patients with resectable LC with moderate COPD and relatively well-preserved nutritional status. Accordingly, the following objectives were established. In a prospective cohort of LC patients with and without COPD the associations between preserved preoperative nutritional status (BMI, albumin, and total protein levels) and OS were analyzed. All patients were followed up to a period of ten years.

Methods

Study design and ethics

This is a prospective study designed following the World Medical Association guidelines (Seventh revision of the Declaration of Helsinki, Fortaleza, Brazil, 2013)[20] for research on human beings and approved by the institutional Ethics Committee on Human Investigation (*Hospital del Mar–IMIM*, Barcelona, Spain). All patients invited to participate in the study signed the written informed consent.

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* that started in 2008. For this observational study, 125 patients with LC were recruited. Patients were further subdivided post hoc into two groups on the basis of underlying COPD: (1) 87 patients with LC and COPD (LC-COPD group) and (2) 38 patients with LC without COPD (LC group). These patients were simultaneously participating in another investigation aimed to explore the associations between the immune microenvironment (B cells and tertiary lymphoid structures) in the lung tumor specimens and OS of the patients (submitted to another journal).

Study patients

LC diagnosis and staging were established by histological confirmation and classified according to currently available guidelines for the diagnosis and management of LC.[21,22] TNM (tumor, node, and metastasis) staging was defined as stated in the 8th edition Lung Cancer Stage Classification.[23] In all cases, pre-operative staging was performed using chest and upper abdomen Computed Tomography (CT) scan and Fluoro-deoxy-glucose positron emission tomography/computed tomography (PET) body-scan. When suspected mediastinal lymph-node involvement, a fiberoptic bronchoscopy with endo-bronchial ultra-sound (EBUS) and trans-tracheal biopsy of the suspected nodes were performed. In case of negative results, a surgical exploration of the mediastinum: cervical video-assisted mediastinal lymphadenectomy (VAMLA) and/or anterior mediastinotomy were performed, the latter depending on the location of the suspected nodes. Notwithstanding, in all surgical cases, intraoperative systematic hilar and mediastinal lymphadenectomy (at least, ipsilateral paratracheal, subcarinal, and ipsilateral pulmonary ligament) was performed as previously recommended.[24,25] Standard clinical guidelines were used to establish the selection of patients and contraindications for thoracic surgery as previously described.[26] Decisions on the best therapeutic approach were always made during the weekly meetings of the Multidisciplinary Lung Cancer Committee. Lung tumor resections were applied using classical thoracotomy for all the patients in this study.

In the current investigation, exclusion criteria for the participants were established as follows: small cell lung cancer (SCLC), severe malnutrition status, chronic cardiovascular disease, metabolic or clot system disorders, signs of severe inflammation and/or bronchial infection (bronchoscopy), current or recent

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invasive mechanical ventilation, or long-term oxygen therapy. The presence/absence of these diseases was confirmed using standard clinical tests: exercise capacity electrocardiogram, clinical examination, blood tests, bronchoscopy and echocardiography.

Clinical assessment

In all patients, lung function parameters were assessed following standard procedures. Diagnosis and severity of patients with COPD were determined according to current guidelines.[10] Preoperative nutritional evaluation included the assessment of body mass index (BMI) and nutritional blood parameters from all patients. All the parameters were quantified prior to thoracotomy for their lung neoplasm. Moreover, body weight loss over the previous year to study entry was also quantified in all the study patients. Body weight loss during follow-up was not quantified routinely in patients of this study.

Statistical analyses

The normality of the study variables was examined using the Shapiro–Wilk test. Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test, 33 subjects were necessary in the LC group and 66 in the LC-COPD group to identify a statistically significant difference greater than or equal to 3 units in mean of BMI variable. The common standard deviation was assumed as 5. The number of patients in each group was calculated using GRANMO (IMIM, Barcelona). Moreover, taking each variable categorized into two groups, estimated power for two-sample comparisons of survivor functions Log-rank test was applied using the Freedman method. Accepting an alpha risk of 0.05 in a two-sided test with 87 and 38 patients in each group (post hoc subdivision), the statistical power was 85% (albumin), BMI (76%), and 100% (FEV_1).

For the descriptive analysis of clinical parameters, qualitative variables were described as frequencies (number and percentage) and quantitative variables as mean and standard deviation. Differences in clinical parameters between LC and LC-COPD were assessed using Student's *T*-test or Mann–Whitney *U* tests for parametric and non-parametric variables, respectively. Chi-square test was used to assess differences between the two groups for the categorical variables.

OS was defined as the time from the date of diagnosis of LC to the date of death from this disease or the last follow-up which was completed in December 2018. The median follow-up duration was 37.7 months (P25 = 20.4 months, P75 = 60.5 months). Patients were followed for a minimum of one year and up to maximum period of 10 years. Patients who did not died of lung cancer were excluded in the investigation. Two patients who were lost during follow-up were not included in the OS analyses. Threshold analysis was carried out for each continuous variable to determine the best cut-off point as predictor of OS, which was the endpoint in the study. The cut-off point was defined using the web-based software Cutoff Finder,[27] which has also been previously used in other studies.[28,29] For each variable, we identified the threshold level at which a log-rank test allowed segregation of patients into groups with better and worse survival. Kaplan–Meier survival curves were performed for each dichotomized variable (below versus above cutoff values, described as Lo and Hi) and log-rank test *p*-value was estimated.

Univariate and multivariate Cox regression models were used to study OS among all LC patients. Those variables with *p*-value smaller than 0.1 were entered into the multivariate analyses. The proportional hazard assumption, checked by examining Schoenfeld residuals (for overall model and variable by variable), was not violated. Statistical significance was established at $p \leq 0.05$. All statistical analyses were carried out using the software Stata/MP 15 (StataCorp LLC, Texas, USA).

Results

Clinical characteristics

No significant differences were seen in age between the two study groups (Table 1). In this prospective study, the number of LC-COPD patients was greater than patients in the LC group (Table 1). BMI, although within normal ranges, was significantly lower in LC-COPD than in LC patients (Table 1). The number of male patients among the LC-COPD group was significantly higher than in LC group (Table 1). Smoking history including packs-year was significantly worse in the LC-COPD patients than in LC group (Table 1). In LC-COPD patients, the lung functional parameters FEV_1, FEV_1/FVC, DL_{CO} and K_{CO} were significantly reduced compared to LC patients (Table 1). COPD patients were mostly in GOLD I and II stages (91%). TNM staging or histological subtypes did not significantly differ between the two groups. The proportions of patients that received adjuvant therapy did not significantly differ between the two study groups (Table 1).

Total leukocyte, neutrophil and lymphocyte counts were significantly greater in LC-COPD patients (Table 1). However, albumin levels were significantly reduced in LC-COPD patients (Table 1). No significant differences were shown in levels of total proteins, fibrinogen, C-reactive protein (CRP), globular sedimentation velocity (GSV) between LC-COPD and LC patients (Table 1). Body weight loss did not significantly differ between the study groups (Table 1). In LC-COPD group of patients, only four patients (2 current smokers and 2 ex-smokers) experienced the greatest weight loss range (6–10 kg) within the previous 12 months before study entry (Table 1). In LC group, only one smoker patient exhibited a great range of weight loss (Table 1).

Preoperative nutritional variables, cigarette smoking, and OS in LC and LC-COPD patients

When all patients were analyzed together, a lower level of BMI (cut-off: 21.5 kg/m^2) was associated with a poorer 10-year survival (Fig. 1A). When patients were subdivided according to the presence of COPD, a significant worse ten-year survival was observed in LC-COPD patients with a lower degree of BMI (Fig. 1B). Similar results were observed when only ex-smokers and never-smokers were analyzed independently (Fig. S1A and S1B). As to the levels of albumin, when all patients were analyzed together, a lower level of albumin (cut-off: 3.55 g/dL) was associated with a poorer 10-year survival (Fig. 2A). When patients were subdivided according to the presence of COPD, a significant worse survival was observed in LC-COPD patients with lower levels of albumin (Fig. 2B). Similar findings were also obtained when non-smokers (never smokers and ex-smokers) were analyzed separately (Fig. S2A and S2B). The patients' 10-year survival was almost significantly reduced in patients with lower levels of total proteins (cut-off: 5.65/dL) when patients were analyzed altogether ($p = 0.055$) and in the group of LC-COPD ($p = 0.075$, Fig. 3A and B, respectively). Non-significant differences were observed in OS when non-smokers (never-smokers and ex-smokers) were analyzed independently (Fig. S3A and S3B). Cigarette smoking was also analyzed in the study. When all patients were analyzed together higher levels of cigarette smoking burden (cut-off: 48 packs-year) were associated with a poorer 10-year survival (Fig. 4A). Moreover, when patients were subdivided according to the presence of COPD, an almost significant worse survival ($p = 0.090$) was observed in LC-COPD patients with a greater cigarette smoking burden (Fig. 4B).

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Table 1
Clinical and functional characteristics of the study patients.

Anthropometric variables	Lung cancer ($N=38$)	Lung cancer-COPD ($N=87$)
Age, years	64 (12)	67 (8)
Male, N/Female, N	15/23	75/12***
BMI, kg/m²	27.5 (3)	25.5 (4)*
Smoking history		
Current: *N*, %	12, 31	41, 47.1
Ex-smoker: *N*, %	7, 19	43, 49.4**
Never smoker: *N*, %	19, 50	3, 3.5***
Pack-years	17 (22)	57 (25)***
Lung function parameters		
FEV_1, %	89 (11)	66 (15)***
FEV_1/FVC	75 (5)	61 (9)***
DLco, %	85 (14)	67 (18)***
Kco, %	85 (12)	69 (17)***
GOLD stage		
GOLD stage I: *N*, %	NA	18, 21
GOLD stage II: *N*, %	NA	61, 70
GOLD stage III: *N*, %	NA	8, 9
TNM staging		
Stage 0–II: *N*, %	32, 84	71, 81.6
Stage III: *N*, %	6, 16	12, 13.8
Stage IV: *N*, %	0, 0	4, 4.6
Adjuvant therapy		
With adjuvant therapy: *N*, %	11, 28	41, 53
Without adjuvant therapy: *N*, %	27, 71	46, 47
Histological diagnosis		
Squamous cell carcinoma: *N*, %	3, 8	16, 18
Adenocarcinoma: *N*, %	30, 79	65, 75
Others: *N*, %	5, 13	6, 7
Blood parameters		
Total leucocytes/μL	$7.41\ (2.51) \times 10^3$	$9.22\ (2.96) \times 10^3$**
Total neutrophils/μL	$4.83\ (2.54) \times 10^3$	$6.03\ (2.64) \times 10^3$*
Total lymphocytes/μL	$1.77\ (0.76) \times 10^3$	$2.34\ (1.63) \times 10^3$*
Albumin (g/dL)	4.3 (0.4)	4.1 (0.6)*
Total proteins (g/dL)	7.0 (0.5)	6.8 (0.8)
Fibrinogen (mg/dL)	431 (127)	453 (156)
CRP (mg/dL)	6.0 (8.2)	7.8 (13.5)
GSV (mm/h)	27 (14)	26 (16)
Body weight loss, kg		
0, *N*, %	36, 94.7	80, 92.0
1–5, *N*, %	1, 2.6	3, 3.4
6–10, *N*, %	1, 2.6	4, 4.6

Continuous variables are presented as mean and standard deviation while categorical variables are presented as the number of patients in each group and the percentage in the study group with respect to the total population. Definition of abbreviations: *N*, number; kg, kilograms; m, meters; 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.
* $p<0.05$.
** $p<0.01$.
*** $p<0.001$ between LC-COPD patients and LC patients.

Interestingly, no significant differences were seen in OS when non-smoker patients were analyzed separately (Fig. S4A and S4B). In the study cohort, the presence of underlying COPD per se was also significantly associated with a lower 10-year survival as shown in Fig. 5. Consistently, no significant differences were seen when non-smokers were analyzed independently (Fig. S5).

Preoperative lung function parameters and OS in LC and LC-COPD patients

When all patients were analyzed together, a lower level of FEV_1 (cut-off: 82.5%) was associated with a poorer 10-year survival (Fig. 6A). When patients were subdivided according to the presence of COPD, no significant differences in ten-year survival were observed in any of the subgroups (Fig. 6B). No significant differences were seen when non-smokers (never smokers and ex-smokers) were analyzed independently (Fig. S6A and S6B). When all patients were analyzed together, a lower level of DL_{CO} (cut-off: 83.5%) was not significantly associated with a poorer 10-year survival (Fig. 7A). When patients were subdivided according to the presence of COPD, no significant differences were observed in any of the subgroups (Fig. 7B). No significant differences were seen when non-smokers (never smokers and ex-smokers) were analyzed separately (Fig. S7A and S7B).

Potential associations of adjuvant therapy with survival in LC and LC-COPD patients

Administration of adjuvant therapy did not significantly influence the 10-year survival in any of the study groups of patients (Fig. 8). Consistently, no significant differences were seen when

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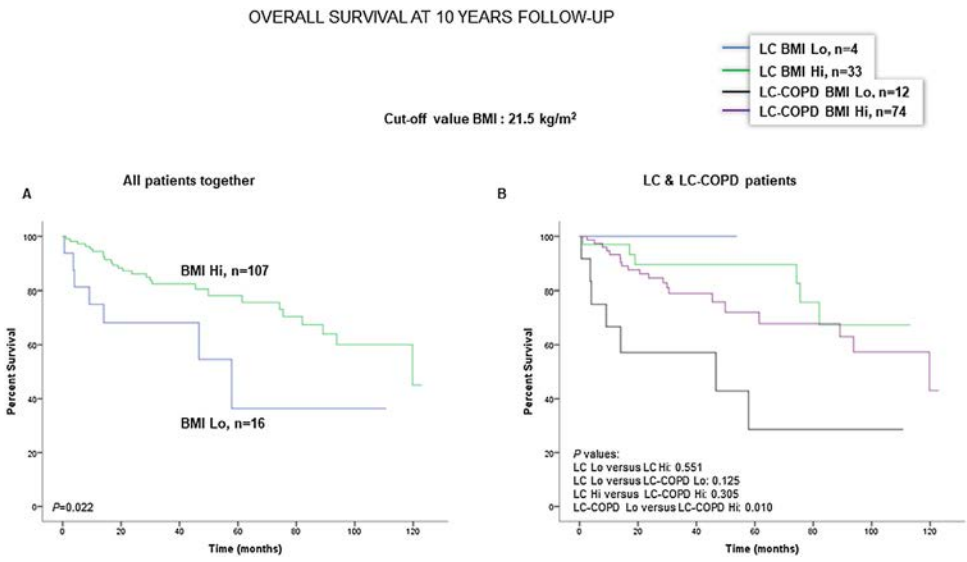

Fig. 1. (A) Kaplan–Meier survival curves for OS in all patients based on the cut-off value of the BMI (above and below the cut-off value: 21.5 kg/m^2). (B) Kaplan–Meier survival curves for OS in LC patients with and without COPD based on the cut-off value of the BMI (above and below the cut-off value: 21.5 kg/m^2). This information was not available in two patients. Definition of abbreviations: BMI, body mass index; LC, lung cancer; COPD, chronic obstructive pulmonary disease; Hi, high level (above cut-off value); Lo, low level (below cut-off value).

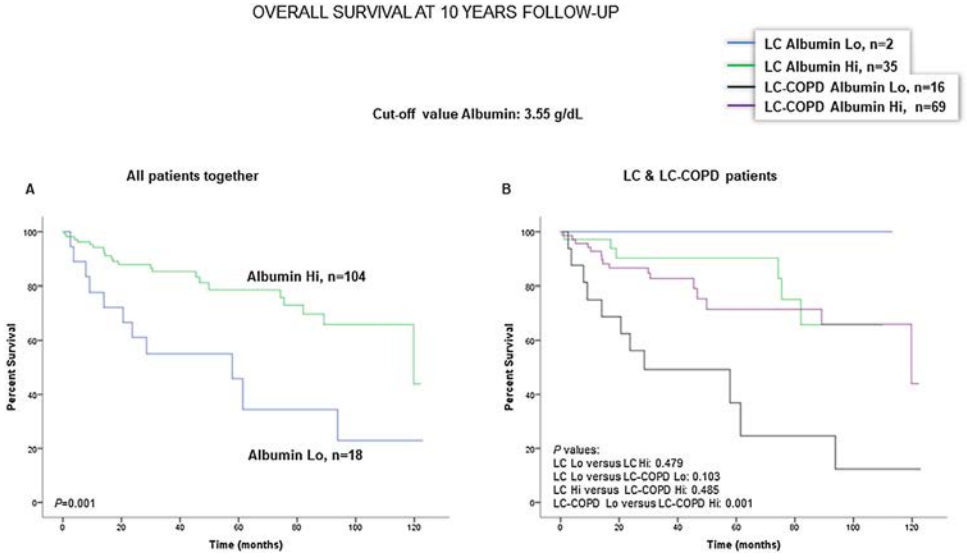

Fig. 2. (A) Kaplan–Meier survival curves for OS in all patients based on the cut-off value of the albumin level (above and below the cut-off value: 3.55 g/dL) in blood. (B) Kaplan–Meier survival curves for OS in LC patients with and without COPD based on the cut-off value of the albumin level (above and below the cut-off value: 3.55 g/dL) in blood. This information was not available in three patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; Hi, high level (above cut-off value); Lo, low level (below cut-off value).

non-smokers (never-smokers and ex-smokers) were analyzed separately (Fig. S8).

Univariate and multivariate analyses

The univariate analysis showed that smoking history (HR = 1.01, $p = 0.02$), and COPD (HR = 2.41, $p = 0.04$) predicted a higher mortality risk, while BMI (HR = 0.92, $p = 0.05$), FEV_1 (HR = 0.98, $p = 0.06$), albumin (HR = 0.51, $p = 0.02$), and total protein levels (HR = 0.68, $p = 0.04$) were associated with a lower mortality risk among all the LC patients (Table 2). Furthermore, the multivariate Cox proportional hazard regression analysis showed that BMI (HR = 0.89, $p = 0.03$) was an independent prognostic factor for OS among all the LC patients (Table 2).

Discussion

In the current investigation, the main findings were that the preoperative nutritional variables BMI and albumin and total protein levels in LC patients with relatively well-preserved nutritional status predicted mortality throughout a 10-year follow-up period. Furthermore, in LC patients with underlying COPD, lower levels of those nutritional parameters, especially BMI and albumin, were associated with a poorer survival. These results lead to the concept that presurgical nutritional status, even if within normal ranges, may predict long-term survival in patients with resectable lung neoplasms, particularly in those with underlying mild-to-moderate COPD (GOLD stages I and II). Collectively, these are relevant clinical findings, which were observed in patients with resectable LC, thus implying that in more advanced stages of LC, the associations

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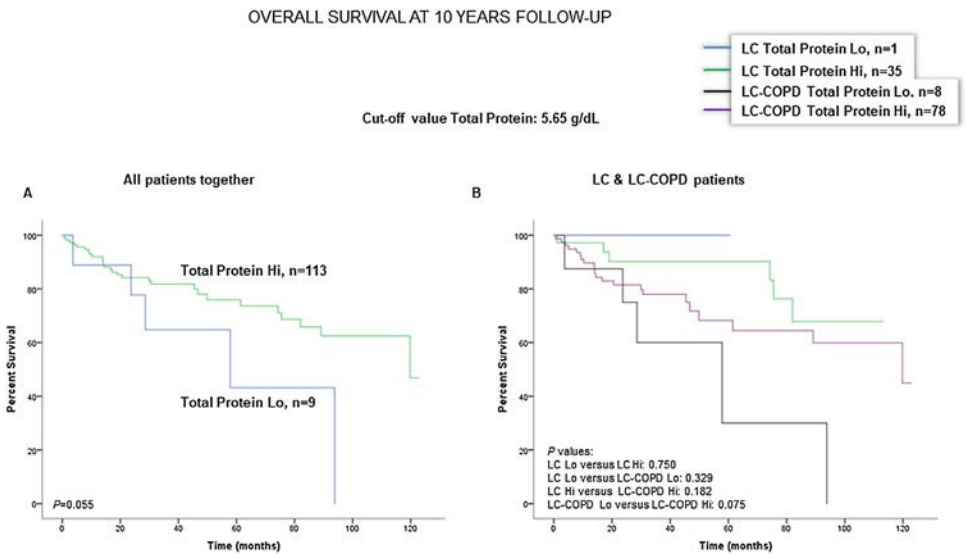

Fig. 3. (A) Kaplan–Meier survival curves for OS in all patients based on the cut-off value of the total protein level (above and below the cut-off value: 5.65 g/dL) in blood. (B) Kaplan–Meier survival curves for OS in LC patients with and without COPD based on the cut-off value of protein levels (above and below the cut-off value: 5.65 g/dL) in blood. This information was not available in three patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; Hi, high level (above cut-off value); Lo, low level (below cut-off value).

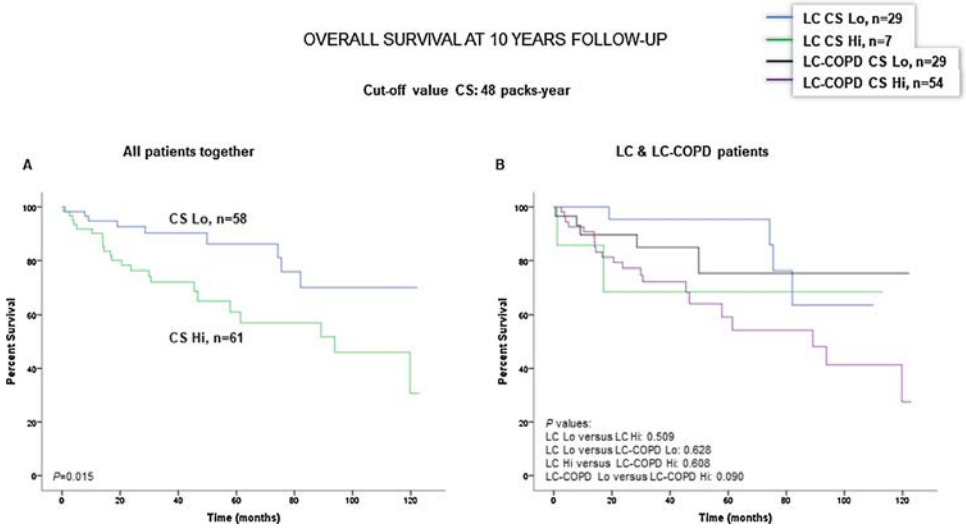

Fig. 4. (A) Kaplan–Meier survival curves for OS in all patients based on the cut-off value of cigarette smoking burden (above and below the cut-off value: 48 packs-year). (B) Kaplan–Meier survival curves for OS in LC patients with and without COPD based on the cut-off value of cigarette smoking burden (above and below the cut-off value: 48 packs-year). This information was not available in six patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; CS, cigarette smoking; Hi, high level (above cut-off value); Lo, low level (below cut-off value).

Table 2
Univariate and multivariate analyses with overall survival in all patients.

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*p*-Value	HR (95%CI)	*p*-Value
BMI, kg/m^2	0.92 (0.85–1.00)	0.05	0.89 (0.81–0.99)	**0.03**
Packs-year	1.01 (1.00–1.03)	0.02	1.01 (0.99–1.03)	0.18
FEV_1	0.98 (0.96–1.00)	0.06	0.99 (0.95–1.02)	0.43
FEV_1/FVC %	0.97 (0.94–1.01)	0.10	1.02 (0.96–1.08)	0.51
Albumin (g/dL)	0.51 (0.30–0.88)	0.02	0.74 (0.32–1.70)	0.48
Total proteins (g/dL)	0.68 (0.46–0.99)	0.04	0.93 (0.55–1.55)	0.77
COPD	2.41 (0.99–5.87)	0.04	1.03 (0.30–3.58)	0.96
Adjuvant therapy	1.51 (0.76–3.00)	0.20		

Definition of abbreviations: BMI, body mass index; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity.

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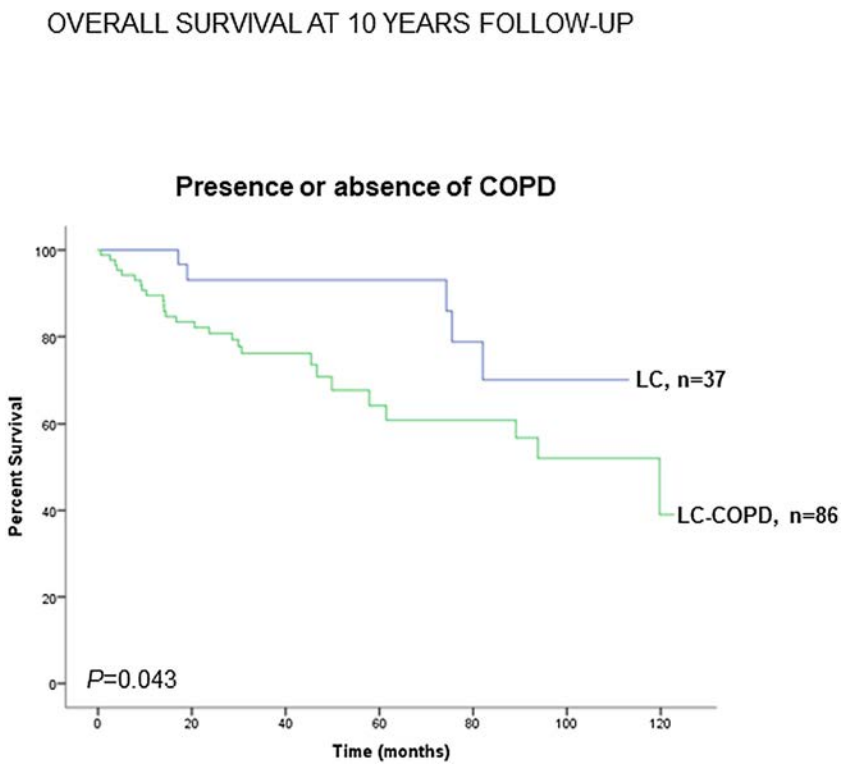

Fig. 5. Kaplan–Meier survival curves for OS in LC patients according to the presence of underlying COPD. This information was not available in two patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease.

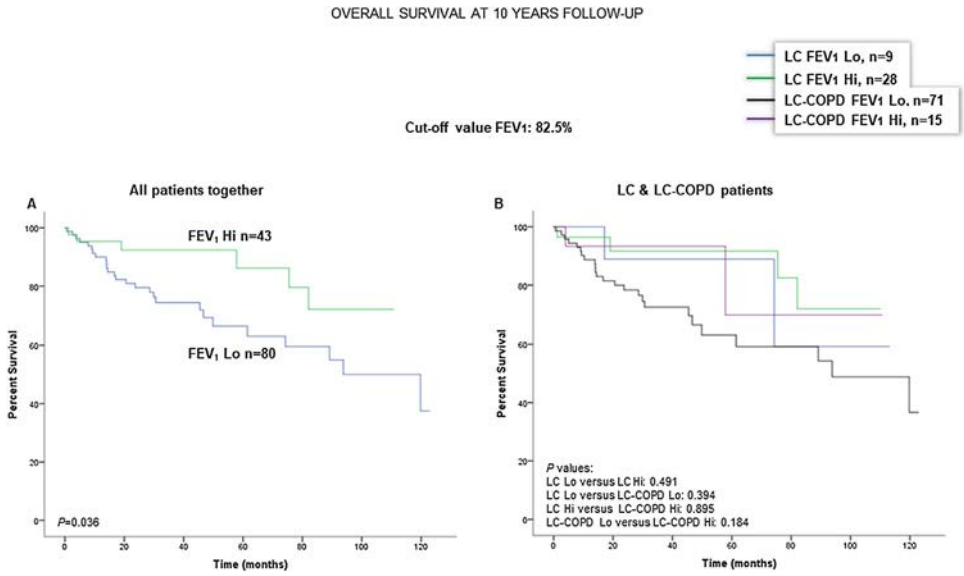

Fig. 6. (A) Kaplan–Meier survival curves for OS in all patients based on the cut-off value of FEV_1 (above and below the cut-off value: 82.5%). (B) Kaplan–Meier survival curves for OS in LC patients with and without COPD based on the cut-off value of FEV_1 (above and below the cut-off value: 82.5%). This information was not available in two patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in one second; Hi, high level (above cut-off value); Lo, low level (below cut-off value).

between nutritional abnormalities and OS might be even more blatant. Nonetheless, a recent investigation,[13] demonstrated that BMI, weight loss, and sarcopenia had a negative impact on survival of patients with resected lung tumors, independently of their tumor stage.

In patients with advanced stage LC, multivariate analyses demonstrated that BMI $\geq$ 21 kg/m^2 was a favorable predictor of survival, whereas inflammatory markers and age were associated with poorer survival.[16] Malnutrition (BMI and albumin) was prevalent in patients with advanced LC stages and in general was associated with impaired clinical outcomes.[17] The prevalence of postoperative complications was also associated with BMI in LC patients who underwent surgery for their neoplasm.[18,19] In LC patients with underlying emphysema or fibrosis albumin was shown to predict OS at five years of follow-up.[30]

Other reports have also demonstrated the implications between nutritional parameters and long-term survival among patients with LC. For instance, a reduction in more than 5% BMI significantly

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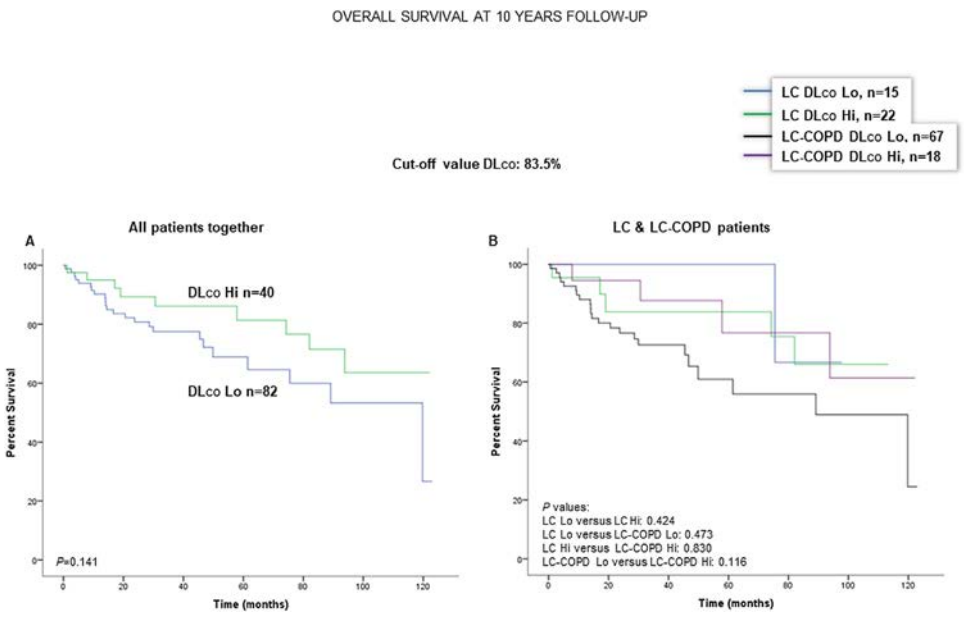

Fig. 7. (A) Kaplan–Meier survival curves for OS in all patients based on the cut-off value of DL_{CO} (above and below the cut-off value: 83.5%). (B) Kaplan–Meier survival curves for OS in LC patients with and without COPD based on the cut-off value of DL_{CO} (above and below the cut-off value: 83.5%). This information was not available in three patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; DL_{CO}, transfer factor of the lung for carbon monoxide; Hi, high level (above cut-off value); Lo, low level (below cut-off value).

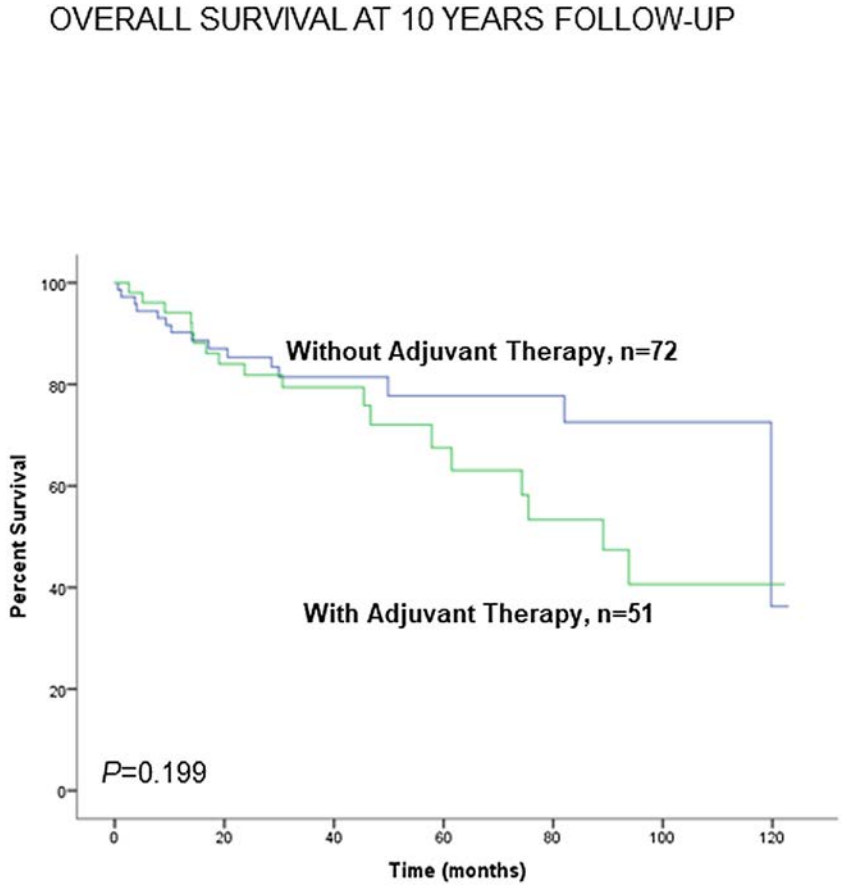

Fig. 8. Kaplan–Meier survival curves for OS in all patients based on the presence or absence of administration of adjuvant therapy to all patients together (LC and LC-COPD). This information was not available in two patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease.

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increased all-cause mortality in a large cohort (81,388 cases) of patients with different cancer types including lung cancer.[14] In another study,[15] weight loss and low albumin levels also exerted a negative impact on disease survival even in patients receiving immunotherapy. In a meta-analysis,[31] healthy life style habits were also associated with better prognosis for several cancer types including LC.

In the current investigation, the study patients with LC were further subdivided according to the presence of underlying COPD. Associations between several nutritional parameters measured prior to surgery and OS were found in all patients as a group, but particularly in those with COPD. These are novel findings that put the line forward that preoperative nutritional status, even if well-preserved, is a clinical feature that warrants attention in LC patients, particularly in those with airway obstruction. Importantly, variables such as BMI, albumin, and total protein levels on the one hand, and packs-year, FEV_1, and the presence of COPD were independently associated with OS in the univariate analyses. Nonetheless, in the multivariate analyses, BMI was the only variable that kept the prognosis value among all the study patients. This is a novel finding that is in line with previous studies in which BMI was also associated with postoperative complications following surgery.[18,19] Another novelty in the current investigation was that patients were followed up to a period of 10 years as opposed to studies where patients were followed up for shorter periods of time were retrospective.[16–19,30]

Importantly, despite the significant reduction in BMI and in the blood parameter albumin seen in the LC-COPD patients, their levels remained within the normal range for the most of the patients in both groups. These are relevant observations, since they could be ignored in standard clinical settings, while they have proved to have a prognostic value when patients were followed up for several years. This is even more important, given that the parameters were measured at baseline, prior to undergoing a major surgical procedure for the curative treatment of their lung neoplasms. To sum up, although BMI was within normal ranges or even high in the majority of the patients analyzed in this cohort, we believe that it should be carefully evaluated and considered as an important clinical, prognostic parameter in patients who have to undergo surgical procedures and when designing original research studies.

In general, LC patients with baseline impairment in body weight and/or muscle mass are the targets for nutritional support and follow-up. Commonly, patients with nutritional depletion are those exhibiting more advanced stages of their tumors including those with LC. It is likely that tumors induce a hypercatabolic state that renders patients more susceptible to experience weight loss and eventually to cachectic states.[32] Nonetheless, baseline poor nutritional status and altered body weight and composition may, in turn, elicit deficiencies of the immune system activity that may favor the progress of tumor growth and development.[33,34] Therefore, it is important that nutritional abnormalities and body weight loss, even though of small magnitude, are identified and diagnosed rapidly in clinical setting of LC patients, particularly in those with underlying respiratory conditions such as COPD. Furthermore, COPD per se was also shown to worsen disease prognosis among all patients with LC, suggesting that an additional chronic respiratory condition impaired OS in this cohort of patients. In the present investigation, however, COPD patients with LC did not experience significant differences in the variable weight loss quantified prior to surgery with respect to LC patients with no COPD. These findings reveal that BMI per se is a strong predictor of the LC patients' survival irrespective of whether they experienced significant body weight loss prior to surgery.

Importantly, cigarette smoking above 48 packs-year was significantly associated with a worse prognosis among all patients with LC in the present cohort. Moreover, in LC with COPD an almost significant association with a worse OS was also observed. These are clinically relevant findings that are in accordance with recent results,[35] in which cigarette smoking burden significantly correlated with survival in patients with lung adenocarcinoma. Nonetheless, in the multivariate analysis the influence of CS was lost. This is in line with the lack of differences in the subanalyses.

Study limitations

In this cohort of LC patients with relatively well-preserved nutritional status, international nutritional classifications have not been used as only very few patients would have fell into the most severe categories. Furthermore, several parameters indispensable for the classification of the patients into several groups of nutritional abnormalities were not available for all of them.[36,37]

Another limitation might be the surgical procedure (thoracotomy versus VATS) used to resecting the lung tumors in the study patients. Nevertheless, we believe that this has had no significant effect on either BMI or albumin prior to surgery or on the long-term survival of the patients as also demonstrated previously.[38,39]

In the present investigation, no specific scales of comorbidity were used prior to patient recruitment.[40] Nonetheless, very selective inclusion and exclusion criteria were established before patient recruitment. As described in methods, despite these limitations, we believe that the current investigation sheds light into the relevance of preoperative nutritional status, even if relatively well-preserved, in patients with resectable lung tumors and in particular in those with underlying COPD. The current results will serve as the basis for the design of multicenter studies to analyze the prognosis value of other variables using similar approaches in the near-future.

Conclusions

In the present cohort of LC patients with resectable tumors and well-preserved nutritional status, the parameters BMI and blood albumin and protein levels measured at baseline prior to thoracotomy predicted OS, especially in those with underlying COPD. These are clinically relevant findings, since values of those nutritional parameters were within the normal ranges in the majority of the analyzed patients. A thorough nutritional preoperative assessment should be included in the study of patients with resectable LC, particularly in those with chronic airway obstruction.

Authors' contributions

Study conception and design: EB, VC; Patient assessment and recruitment: JT, VC, DRC; Surgical procedures and staging: ARF, RA; Statistical analyses and data interpretation: XD, JT, DRC, VC, EB; manuscript drafting and intellectual input: EB, JT; manuscript writing final version: EB.

Sources of funding

This study has been supported by FIS 18/00075 & CIBERES (FEDER, *Instituto de Salud Carlos III*, Spain), SEPAR 2018, and unrestricted research grant from Menarini SA 2018.

Conflict of interest

None.

Acknowledgments

The authors are thankful to Ms Mireia Admetlló for her help with the patient clinical assessment.

Please cite this article in press as: Tang J, et al. Preoperative Body Weight and Albumin Predict Survival in Patients With Resectable Lung Neoplasms: Role of COPD. Arch Bronconeumol. 2020. https://doi.org/10.1016/j.arbres.2020.07.021

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.arbres.2020.07.021.

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Please cite this article in press as: Tang J, et al. Preoperative Body Weight and Albumin Predict Survival in Patients With Resectable Lung Neoplasms: Role of COPD. Arch Bronconeumol. 2020. https://doi.org/10.1016/j.arbres.2020.07.021

10.2 Communication

Title:

Immunological Events and Survival in Lung Cancer Patients with COPD

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Journal:

American Journal of Respiratory and Critical Care Medicine. 2020;201:A7566

https://doi.org/10.1164/ajrccm-conference.2020.201.1_MeetingAbstracts.A7566

PMID: 32877328

Impact Factor:

17.452 (2019), Quartile: Q1

Immunological Events and Survival in Lung Cancer Patients with COPD

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Rationale: Lung cancer (LC) is highly prevalent in our societies and COPD is a risk factor. Immune microenvironment plays a role in the development of lung cancer (LC). We hypothesized that immune profile of B and T cells may differ in tumors of LC patients with and without COPD and may also influence the patients' survival. Objectives: 1) To analyze levels of tertiary lymphoid structures (TLSs), B and T cells in tumor and non-tumor (control samples) lung specimens of LC patients with/without COPD and 2) to analyze the influence of those biological markers in the patients´10-year survival. Methods: TLSs (numbers and area), B (CD20), and T (CD3) cells were identified in both tumor and non-tumor specimens (thoracotomy) from 90 LC-COPD patients and 43 LC-only patients (immunohistochemistry, double staining with specific antibodies). Survival (Kaplan-Meier curves) was analyzed in all 133 patients. Results: Immune profile in tumors of LC-COPD versus LC: The number of TLSs significantly decreased in tumors of LC-COPD compared to LC patients. No significant differences were observed in tumors between LC-COPD and LC for B or T cells. In tumors compared to non-tumor specimens, a significant rise in TLSs was observed in LC (numbers and area) and LC-COPD (area), T cell counts declined in tumors of LC, while B cell counts increased in tumors of both LC and LC-COPD patients. Survival: In LC-COPD patients: lower numbers of TLSs (cut-off: 0.9672) and greater numbers of B cells (cut-off: 85.18) were associated with longer survival rates. In LC patients: lower levels of T cells (cut-off: 8.607) were associated with longer survival rates. All patients together: lower numbers of T cells (cut-off: 8.554) and TLSs (cut-off: 0.9176) and greater numbers of B cells (cut-off: 91.45) were associated with longer survival rates. Conclusions: TLSs, B cells and T cells are differentially expressed in tumors of LC-COPD from that in LC-only patients. Further analyses are required to identify the specific role of TLSs in LC development in patients with COPD.

This abstract is funded by: FIS 18/00075 & CIBERES (FEDER, ISC-III), SEPAR 2018, unrestricted research grant from Menarini SA 2018

10.4 Funding

The five studies included in the current thesis have been funded by:

CIBERES (Instituto de Salud Carlos III, Spain), FIS 14/00713 (FEDER), FIS 18/00075 (FEDER), SEPAR 2016, SEPAR 2018, SEPAR 2020, FUCAP 2016, Unrestricted grant from Menarini SA 2018 (Spain), Glaxo Smith Kline SA (Spain) 2018, PT13/0010/0005 (FEDER), and the “Xarxa de Bancs de tumors sponsored by Pla Director d’Oncologia de Catalunya (XBTC)”, Catalan Government.