Mechanisms and pathways involved in lung tumor development in patients with chronic respiratory conditions

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ABSTRACT

Chronic respiratory diseases, especially chronic obstructive pulmonary disease (COPD), and several molecular mechanisms may predispose to lung cancer (LC) development. **Hypothesis**: We hypothesized that different biological mechanisms such as oxidative stress, inflammatory events and epigenetic alterations may alter key cellular processes that are strongly involved in tumor initiation and progression in COPD patients. **Objectives**: In tumor and non-tumor lungs and in blood, to explore potential differences between LC patients with and without COPD, in several biological mechanisms that underlie lung tumor development. To evaluate the different profile of these molecular mechanisms between tumor and non-tumor lungs in either LC or LC-COPD patients. **Methods**: In lung specimens (tumor and non-tumor), oxidative and nitrosative stress markers, antioxidant systems, Th1 and Th2 cytokines, M1 and M2 macrophages, epigenetic events and downstream biomarkers were determined in LC patients with and without COPD. Redox balance markers and Th1 and Th2 cytokines were also evaluated in the blood compartment of LC patients with and without COPD. **Results**: In tumor lungs and in the blood of LC patients with COPD, an increased oxidative and nitrosative stress was observed, and an upregulation of the Th1 inflammatory response. Expression of specific microRNAs, DNA methylation levels and downstream biomarkers were altered in the lung tumors of LC patients with COPD, which in turn, promoted an increase in cell proliferation, invasion and angiogenesis. In the tumor lungs of LC patients with and without COPD, redox and nitrosative imbalance was higher, Th1 and Th2 cytokines were greater and epigenetic events and downstream biomarkers were altered. **Conclusions**: A different expression profile of several molecular mechanisms, involved in tumor development, exist in lung tumors and in blood of LC patients with COPD, which may predispose COPD patients to a higher risk of developing LC.

RESUMEN

Las enfermedades crónicas respiratorias, y en especial la enfermedad pulmonar obstructiva crónica (EPOC), así como diversos mecanismos moleculares, podrían ser factores de predisposición al desarrollo de cáncer de pulmón (CP). **Hipótesis**: La hipótesis de trabajo fue que diferentes mecanismos biológicos como el estrés oxidativo, los procesos inflamatorios y las modificaciones epigenéticas, podrían alterar diversos procesos celulares involucrados en el inicio y en la progresión tumoral en pacientes con EPOC. **Objetivos**: En tejido pulmonar (tumoral y no tumoral) y en sangre, explorar las diferencias potenciales entre pacientes con CP con y sin EPOC, en diversos mecanismos biológicos que subyacen el desarrollo del tumor pulmonar. Evaluar el perfil diferente de estos mecanismos moleculares entre el pulmón tumoral y no tumoral, tanto en pacientes con CP como en pacientes con CP y EPOC. **Métodos**: Se determinaron marcadores de estrés oxidativo y nitrosativo, sistemas antioxidantes, citosinas Th1 y Th2, eventos epigenéticos y sus biomarcadores efectores, en el pulmón tumoral y no tumoral de pacientes con CP, con y sin EPOC. También se evaluó el estrés oxidativo y nitrosativo, así como las citosinas Th1 y Th2, en la sangre de pacientes con CP, con y sin EPOC. **Resultados**: En el tumor pulmonar y en la sangre de los pacientes con CP y EPOC, se observó un aumento del estrés oxidativo y nitrosativo, así como un incremento de la respuesta inflamatoria Th1. La expresión de microRNAs específicos, los niveles de metilación del ADN, y los biomarcadores efectores se vieron alterados en el tumor pulmonar de pacientes con CP y EPOC, lo que a su vez promovió en estos pacientes un aumento de la proliferación celular, la invasión y la angiogénesis. En el tumor pulmonar de los pacientes con CP, con y sin EPOC, se observó un aumento del estrés oxidativo y nitrosativo, un incremento de las citosinas Th1 y Th2, así como alteraciones en los eventos epigenéticos y en los niveles de los biomarcadores efectores. **Conclusiones**: En los tumores pulmonares y en la sangre de los pacientes con CP y EPOC, existe un perfil de expresión diferente de diversos mecanismos moleculares implicados en el desarrollo tumoral, lo que podría predisponer a los pacientes con EPOC a un mayor riesgo de desarrollar CP.

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PREFACE

Scientific collaborations

In the present thesis, the investigations have been carried out in the Muscle Wasting and Cachexia in Chronic Respiratory Diseases and Lung Cancer Research group, Institute of Medical Research of Hospital del Mar (IMIM)-Hospital del Mar, Barcelona, Spain. In addition, the three studies have been conducted with the collaboration of the Pulmonology Department, the Thoracic Surgery Department and the Pathology Department at Hospital del Mar, Parc de Salut Mar, Barcelona, Spain.

Publications

Some of the studies that are included in the present thesis have been published in international journals:

Study #1

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Mol Med. 2016 Jan; 22:85-98

Study #2

M Mateu-Jimenez, V Curull, L Pijuan, A Sánchez-Font, A Rodríguez-Fuster, R Aguiló, J Gea, E Barreiro. Systemic and tumor Th1 and Th2 inflamamtory profile and macrophages in lung cancer: influence of underlying chronic respiratory disease.
J. Thorac Oncol. 2016 Oct; 2: 235-248

Study #3

M Mateu-Jimenez, V Curull, A Sánchez-Font, L Pijuan, A Rodríguez-Fuster, R Aguiló, J Gea, E Barreiro. Profile of epigenetic mechanisms in lung tumors of patients with underlying chronic respiratory conditions.
Clinical Epigenetics, *in revision*

Communications

Some of the results that are included in the current thesis, and those other that are from other investigations developed in the PhD period, have been showed in form of either poster or oral communication in different national conferences.

1. M Mateu-Jiménez, V Curull, A Sánchez-Font, A Rodríguez-Fuster, R Aguiló, L Pijuan, J Gea, E Barreiro**.** Epigenetic regulation in lung cancer patients with and without COPD.
XI CIBERES Conferences (Abstract book, page 55), Madrid, Spain, September 2016.

2. M Mateu-Jiménez, A Sánchez-Font, L Pijuan, A Rodríguez-Fuster, J Gea, V Curull, E Barreiro. Eventos inflamatorios en la predisposición al cáncer de pulmón en pacientes con EPOC.
VIII CIBERES Conferences (Abstract book, page 31), Valladolid, Spain, October 2015.

3. M Mateu-Jiménez, A Sánchez-Font, L Pijuan, A Rodríguez-Fuster, J Gea, V Curull, E Barreiro. Mecanismos biológicos en la predisposición al cáncer de pulmón en pacientes con EPOC.
VII CIBERES Conferences, (Abstract book, page 22), Valladolid, Spain, October 2014.

4. M Mateu-Jiménez**,** A Sánchez-Font, L Pijuan, A Rodríguez-Fuster, J Gea, V Curull, E Barreiro. Mechanisms and pathways involved in lung tumor development in patients with chronic respiratory conditions.
Cancer seminars at *Hospital del Mar-IMIM*, *Parc de Salut Mar,* Barcelona Biomedical Research Park (PRBB), Barcelona, Spain, June, 2014.

5. M Mateu-Jiménez, A Chacón, M Vilà-Ubach, C Fermoselle, A Urtreger, Elisa D Bal de Kier Joffé, E Barreiro. Nuevas dianas terapéuticas en el cáncer de pulmón: inhibición de los sistemas MAPK, NF-ĸB, proteasoma y estrés oxidativo en un modelo murino.

VI CIBERES Conferences (Abstract book, page 18), Bunyola, Mallorca, Illes Balears, Spain*,* October 2013.

6. M Mateu-Jiménez, C Fermoselle, A Urtreger, Elisa D Bal de Kier Joffé, E Barreiro. New therapeutic targets in lung cancer: inhibition of MAPK, NFkB, proteasome pathways and oxidative stress in a murine model.
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ABBREVIATIONS

AP-1: activator protein-1

BMI: body mass index

CDKN1A: cyclin-dependent kinase inhibitor 1A

CDKN2A: cyclin-dependent kinase inhibitor 2A

COPD: chronic obstructive pulmonary disease

CRK: v-crk avian sarcoma virus CT10 oncogene homolog 17

CRP: c-reactive protein

DLco: carbon monoxide transfer

DNA: deoxyribonucleic acid

EFNA3: ephrin-A3

EGFL7: EGF-like-domain, multiple 7

EGFR: epidermal growth factor

ETS1: v-ets avian erythroblastosis virus E26 oncogene homolog 1

FEV1: forced expiratory volume in the first second

FGFRL1: fibro blast growth factor receptor-like 1

FVC: forced vital capacity

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GSH: glutathione

GSV: globular sedimentation velocity

g1: multi-exonic gene assay may detect genomic DNA if presenting the sample

HDAC: histone deacetylase

HAT: histone acetyltransferase 18

HIF-α: hypoxia inducible factor-1α

HOXA1: homeobox A1

Abbreviations

Hs: homo sapiens

ID: identification

INF: interferon

IL: interleukin

KCO: krogh transfer factor

KDa: kilodaltons

Kg: kilogram

KRAS: kirsten rat sarcoma viral oncogene homolog 11

L: liter

LC: lung cancer

m: meters

MARCKS: myristoylated alanine-rich protein kinase C substrate

MDA: malondialdehyde

MIF: macrophage migration inhibitory factor

miR: microRNA

miRNA: microRNA

MW: molecular weight

M1: macrophages type 1

m1: multi-exonic gene assay does not detect genomic DNA

M2: macrophages type 2

NF-kB: nuclear factor kB

NT: nitrotyrosine

N: number

NM: mRNA RefSeq database category

Nrf-2: NF-E2 related factor-2

PDCD4: programmed cell death 4

Pred: predicted

PTEN, phosphatase and tensin homolog 10

P53: tumor protein p53

P63: tumor protein p63

RAB14: RAB14, member RAS oncogene family

ROS: Reactive Oxigen Species

RNS: Reactive Nitrogen Species

SIRT1: sirtuin 1

SNAIL1: snail family zinc finger 1

SOD: superoxide dismutase

SPRY2: sprouty homolog 2

STAT3: signal transducer and activator of transcription 3

TGF-beta: tumor growth factor-beta

TNM: tumor, nodes, metastasis

TOM1: target of myb1

TPM1: tropomyosin 1 (alpha)

VEFG: vascular endothelial factor growth

ZEB2: zinc finger E-box binding homeobox 2

INTRODUCTION

The current thesis is about the main biological mechanisms that are involved in lung cancer (LC) development in patients with chronic obstructive pulmonary disease (COPD). Both LC and COPD pathogenesis have a strong global burden in the population and both diseases are considered one of the major causes of mortality worldwide (1;2).

Briefly, LC is characterized by an increase in cell growth, angiogenesis promotion and anti-apoptosis, whereas the main features of COPD are cell death, destruction of the airway walls and anti-angiogenesis. However, both diseases have the same main etiological factor, which is cigarette smoking (CS), and they may also share several molecular mechanisms (3;4). Concretely, CS promotes oxidative and nitrosative stress, which induce the activation of the inflammatory response, leading to epigenetic changes, which may promote LC and COPD pathogenesis (3;4) Moreover, it has been demonstrated that COPD is an independent risk factor for the development of lung carcinogenesis (5).

In this section of the thesis the following topics are explained in detail: the main structures of the lung, the major common features of COPD and LC, the epidemiology of lung carcinogenesis in COPD patients, and the biological processes that may explain the association between the two diseases.

1. The lungs

The lungs are two soft, spongy and elastic organs that are situated inside the rib cage on each side of the mediastinum and are separated by the heart, vessels and other structures (6;7). Both lungs have a conical form, are covered with visceral pleura and are located freely in their corresponding pleural cavity. The main function of the lungs is gas exchange, which occurs between the air and blood by diffusion through the lung tissue (6;7). Gas exchange consists on transferring oxygen from the inspired air into the blood flow, as well as to eliminate waste gases (such as dioxides of carbon) from the bloodstream into the expired air (6;8).

1.1 Structure and anatomy

Each lung is formed by sections that are called lobes. The right lung is larger than the left, and is composed by three lobes: the upper, the middle and the lower lobes. However, the left lung is only constituted by two lobes: the upper and the lower, because it shares space with the heart (7;8).

The trachea is divided into two breathing tubes that are called the main stem bronchi, which lead into the right and left lungs. The main bronchi are subdivided successively into smaller bronchi and then into thinner tubes called bronchioles, that terminate in one or more respiratory bronchioles (7;8). Every respiratory bronchiole divides into tiny air sacs that are called alveolar sacs, which consist of several alveoli that are covered by blood capillaries (6;7). Alveoli are the main place where gas exchange takes place, inspired oxygen is diffused into the bloodstream in order to be liberated in the tissues as a substrate, and carbon dioxide is transferred into the atmosphere (7;8). The lungs are formed by different specialized cells in order to do its function (9). Hence, the airways, bronchi and alveolar walls are constituted by pulmonary epithelial cells and by mesenchymal cells that give structural support producing extracellular matrix. Furthermore, the vasculature of the lungs consists of different cells types such as endothelial, smooth muscle, and fibroblasts (9). In addition, the lung also contains resident phagocytic cells such as macrophages and inflammatory cells.

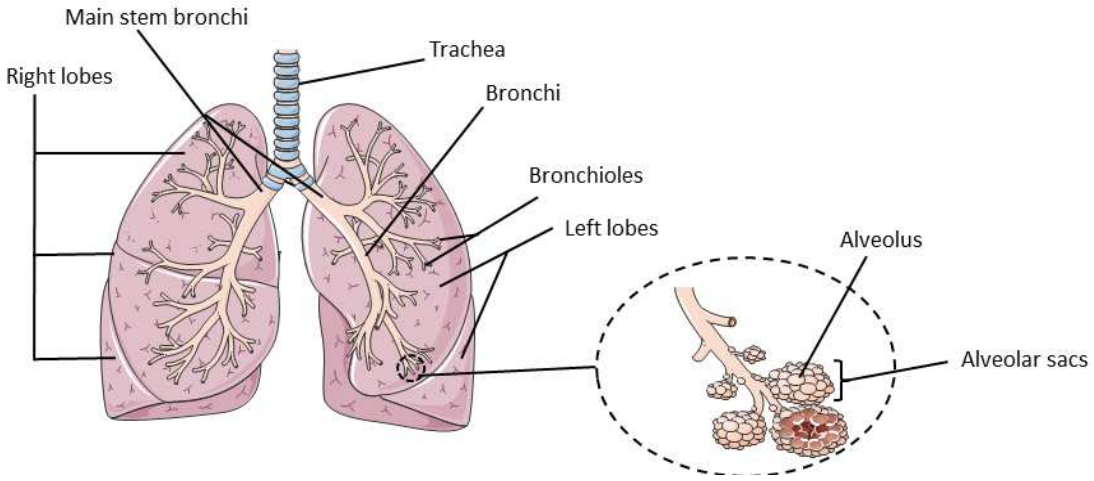

Figure 1. Structure and anatomy of the lung. Figure was produced using Servier Medical Art (http:\\www.servier.com)

1.2 Lung function assessment

The main lung function parameters that are used in order to evaluate and monitor the progression of respiratory diseases are: forced expiratory volume in the first second (FEV1), forced vital capacity (FVC), FEV1/FVC ratio, carbon monoxide transfer (DL_{CO}) and the krough transfer factor (K_{CO}) (8;10). Briefly, FEV1 is the volume of air liberated at the end of the first second; FVC is the volume of air delivered during an expiration after a full inspiration; TV is the volume of air inspired or expired with each normal rest; DLCO is the carbon monoxide uptake from a single inspiration, normally in 10 seconds and K_{CO} is the diffusing capacity of the lung per unit volume, corrected by alveolar volume (8;10).

2. Chronic obstructive pulmonary disease (COPD) and lung cancer (LC)

2.1 COPD

COPD is a lung disease that is characterised by irreversible or partially reversible airflow limitation, which is normally progressive and it is associated with an abnormal inflammatory response of the lung to harmful gases or particles (11;12). The main clinical manifestations of COPD are chronic bronchitis (inflammation and remodelling of the large-airway) and emphysema (breakdown of lung tissue), whereas the main symptoms are chronic cough, sputum, dyspnoea and weigh of loss (4;11).

CS is the major cause of COPD pathogenesis, which has been reported in numerous studies (4;13). However, other less important risk factors for COPD development that includes air pollution and genetic factors such as alpha1-antitrypsin deficiency, or several candidate genes that are associated with the development of COPD (14-16). Nowadays, COPD is one of the major causes of morbidity and mortality worldwide, and the burden of this disease is expected to increase in the next years (1;13). Concretely, it is predicted that COPD will become the third leading cause of death by 2030 (12).

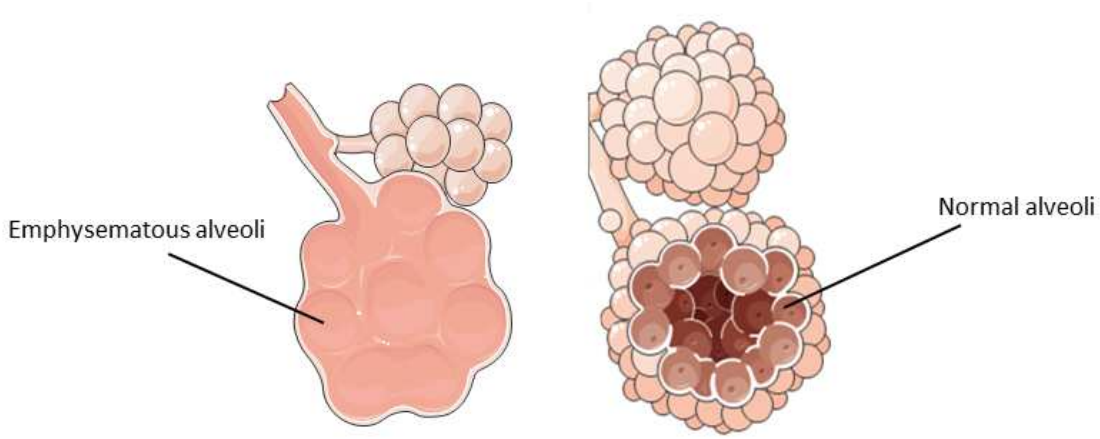

Figure 2. Alveoli with emphysema (alveolar membrane breakdown) from a COPD patient *versus* normal alveoli. Figure was produced using Servier Medical Art (http:\\www.servier.com)

2.1.1 COPD classification

There are many guidelines in order to classify COPD severity. One of the most common used is the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (17) which is a global document that provides recommendations on diagnosis and management of COPD (4;18). Spirometry is essential in order to diagnose COPD, and airflow obstruction has been established when the ratio of the spirometry parameters FEV1/FVC is below 70% of the predicted value. GOLD classification is based on the evaluation of symptoms, and exacerbation risk, and distinguishes between four categories of patients with COPD (4;19;20):

- Group A: with less symptoms and with 0-1 exacerbation per year.

- Group B: with more symptoms and 0-1 exacerbations per year.

- Group C: with less symptoms and more or equal than 2 exacerbations per year.

- Group D: with more symptoms and more or equal than 2 exacerbations per year.

However, in order to assess COPD severity, there are other systems that are based in both clinical and spirometry parameters, such as the Spanish guideline of COPD (GESEPOC), the European Respiratory Society/American Thoracic Society technical standards (ERS/ATS), the guideline of National

Institute for Health and Care Excellence (NICE) and the guideline updated from the American College of physicians (ACP)/American college of chest physicians (ACCP)/ATS and ERS (21;22)

2.2 Lung cancer

Lung cancer (LC) is a malignancy which usually originates in the tissues of the lungs or in the cells that line the airways (23). This disease is one of the main causes of cancer deaths in the entire world and it is rarely curable, because when the diagnosis is made, LC is often in an advanced stage and treatment options are limited (3;24). Therefore, LC has a survival rate less than 16% in the 5 years (24;25). The main common symptoms of LC are cough, dyspnea, hemoptysis and weight loss. In addition, patients can have other complications such as bone, spinal cord or brain metastasis as well as superior vena cava (SVC) syndrome (26;27). Those symptoms can be the cause of the main location of the cancer, can be due to the cancer metastasis, or could also be clinical manifestations of the different comorbidities associated to LC such as arterial hypertension, cardiac disease, peripheral vascular disease or diabetes (26;27).

CS is the most important risk factor for LC development, which has been demonstrated in several studies, being associated cigarette smoke with more than 90% of all LC cases (28-30). However, there are other minor etiological factors for LC such as asbestos or radon exposure and genetic factors, for instance mutations in many proto-oncogenes and tumor suppressor genes that are involved in cell proliferation, cell invasion, cell differentiation and tumor growth such as epidermal growth factor (*EGFR*), mitogen activated protein kinase kinase (*MEK*) and ki-ras2 kirsten rat sarcoma viral oncogene homolog (*K-RAS*)(31-33).

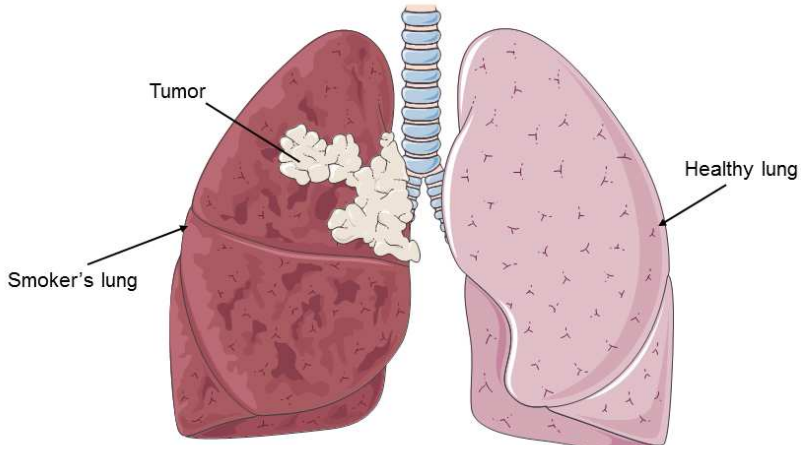

Figure 3. A smoker's lung with a tumor *versus* a healthy lung. Figure was produced using Servier Medical Art (http:\\www.servier.com)

2.2.1 Histological classification and staging

Histological classification of LC is important in order to determine the management of the patients with LC and to predict the prognosis of the disease (34). The two major histological classifications for LC are small-cell lung cancer (SCLC), which includes the 15% of all LC cases, and non-small cell lung cancer (NSCLC), that accounts for an 85% of all LC cases (35;36). SCLC is derived from cells that present neuroendocrine characteristics and it is characterised by rapid growth rate, spread to separate sites and early dissemination of the lymph node (35). NSCLC is divided into 3 pathologic subtypes: adenocarcinoma (usually found in the peripheral parts of the lung), squamous cell carcinoma (normally found near the main bronchi) and large cell carcinoma (found in any part of the lung), that account for approximately a 40%, 20% and 3%, respectively, of all LC cases (23;37).

The evaluation of LC staging uses the tumor-node-metastasis (TNM) classification. The 7th edition of TNM is followed nowadays in clinical practice in order to asses LC staging, althought the 8th edition has been published, this new system will be implemented in January 1, 2018 (38;39). The T descriptor shows the extent of spread of the primary tumor, that involves tumor size, invasion of adjacent structures, endobronchial location, distance from the carina, and the presence of satellite nodules. The N component is the nodal descriptor, which describes how is involved the regional lymph node. Finally,

the M component describes the absence or presence of metastasis (the common metastatic sites in LC are liver, bones, adrenals and brain) (36;38;40;41).

TNM	Stage group
T1a-T1b N0 M0	IA
T2a N0 M0	IB
T1a-T2a N1 M0 T2b N0 M0	IIA
T2b N1 M0 T3 N0 M0	IIB
T1a-T3 N2 M0 T3 N1 M0 T4 N0-N1 M0	IIIA
T1-T4 N3 M0 T4 N2 M0	IIIB
M1a-b	IV

Table 1. TNM staging classification of LC (38;39)

2.3 COPD as a risk factor for LC development

2.3.1 Cigarette smoking as a major cause of COPD and LC

CS is the main etiological factor of both COPD and LC development. It contains different elements and free radicals that cause direct damage in the airways, promoting a response to CS that consists on the liberation of endogenous intracellular molecules and several molecular signals (4;13). These signals are identified by different receptors and an inflammatory response that is not developed specifically, promotes lung destruction (emphysema) and important systemic effects that can induce chronic bronchitis, and lead to COPD

development (4;13;42). In addition, CS also stimulates the proliferation of globet cells, that create mucus into the respiratory tract, promoting an excessive mucus production (hypermucus secretion), that is related to COPD severity (8;43). CS is also strongly associated with the inducement of LC by two ways, among others. Firstly, the carcinogenic components that contains tobacco induce mutations in several important tumor suppressor genes, such as *P53*, that are involved in cell cycle deregulation and carcinogenesis (44). Secondly, the N-nitroso-compounds, one of the main groups of chemicals that are present in tobacco, are potent carcinogens and have high ability to promote LC development (28;45). Therefore, quitting smoking has been demonstrated to have favourable effects in both diseases, as smoking cessation has produced a slower reduction of lung function in COPD patients and has declined the rate of mortality in both LC and COPD patients (46;47).

2.3.2 Epidemiology of COPD and LC

Although COPD and LC share the main etiologic factor, these two diseases seem to be opposite: whereas COPD is associated with matrix destruction, epithelial cell death and anti-angiogenesis, LC is characterised by cell proliferation, lack of apoptosis and promotion of angiogenesis (3;11). Nevertheless, there is a high prevalence of LC in COPD patients and several types of studies such as epidemiological, LC screening studies, population-based cohort studies and interventional trials have demonstrated a strong association between both diseases (48-50). Importantly, COPD has been shown to be an independent risk factor for LC in several studies (48-50). For instance, in an epidemiological study it was found that COPD prevalence in patients with LC was 50% whereas it was an 8% in control subjects, and that it was independent of smoking exposure, sex and age (5).

In the late 1980's many matched case-control and epidemiological studies started to show that the presence of airway obstruction in current smokers was correlated with an increase in LC incidence and that this was a better indicator for LC than the age or the smoking history (51;52). Concretely, it was found that the probability of developing LC in 10 years was 8.8% in those

patients with airway obstruction whereas it was a 2% for patients with normal pulmonary function (51). It was also demonstrated that the risk of LC development was higher when the degree of airway obstruction was increased (51;52). More recently, it has been reported that a slightly reduction in FEV1 was a significant good predictor of increased LC risk, especially in women (53;54). Additionally, it has been found that FEV1 and FEV1/FVC could be significant good markers of LC prognosis, even when controlling the study for smoking history (51).

In addition to airway obstruction, it has been shown that emphysema and chronic bronchitis could also be considered as independent risk factors for LC development (5;55;56). Several studies have observed that there is an increased frequency of LC in patients with emphysema, being higher this frequency in patients with both emphysema and airway obstruction (55;57). It has been also reported that patients with chronic bronchitis exhibited an increased risk for LC development (56). Moreover, it was found that the fact of having emphysema combined with chronic bronchitis is a prognostic predictor of LC patient's death (5;58).

Hence, the different features of COPD: airway obstruction, emphysema and chronic bronchitis, have been associated with LC development (5;52;55). This shows that COPD patients have more susceptibility to having LC, as it has been observed in different investigations that only the presence of COPD *per se*, increases the incidence of suffering from LC (5;57). Concretely, numerous epidemiological studies have reported an increased risk between 2 to 6 times of developing LC in smokers with COPD compared to smokers without this disease (57;59;60). Even in a study with a large amount of never-smoker subjects, the presence of COPD promoted an increase of 2.4 fold the risk of suffering from LC (58).

2.3.3 COPD severity and LC

It is known that the severity of COPD has an influence on LC frequency, but the results obtained in several investigations seem to be controversial. On the one

hand, it has been shown that compared to smokers without airflow limitation, severe COPD patients who are active smokers, have a higher risk of developing LC than mild and moderate COPD (58;61). However, on the other hand, other studies have found that LC incidence decreased from I to IV GOLD stages of COPD, having less incidence of having LC patients at stage IV than those in stage I (62;63). Furthermore, it has been evidenced that the severity of emphysema could predict the location of the tumor in an early stage of LC patients (64).

In addition, the characteristics of LC are different in patients that have COPD compared to those without this disease (58). It has been observed that smokers with COPD normally develop squamous cell carcinoma, although adenocarcinoma subtype is increasing the incidence in light smokers and females (57;65). It has been hypothesized that this tendency could be due to the change in the composition of cigarettes that may have less content of tar and nicotine. Then, smokers have to compensate it with smoking more cigarettes and with a deeper inhalation, which can result in a change in the histological type of the LC, which also affects differently depending on the gender (23).

Additionally, it has been shown that the histology subtype of LC developed also depends on COPD severity (58). Therefore, it has been reported that the most frequent histology type in patients with GOLD stage I is adenocarcinoma, whereas the most common histology type of GOLD stage II and III is squamous cell carcinoma (62). It is known that one of the major causes of death in patients with mild to moderate COPD is LC, and that those patients with LC and COPD have a worse prognosis and quality of life than patients without this disease (66;67). In fact, it has been found that suffering from COPD is strongly associated with LC mortality, as patients with LC and COPD have a lower five-year survival rate compared to patients with only LC (58;68). Additionally, it has been showed that an increased severity of COPD is correlated with a higher risk of LC deaths (69). Then, an early diagnosis of COPD is very important in order to screen the population with higher risk of having LC, which would increase the survival of those patients (70).

2.4 Potential biological mechanisms commonly expressed in both LC and COPD

As above mentioned, LC and COPD share the main etiologic risk factor (CS) and there is high evidence that they could also have analogous pathogenic pathways (48;71). CS induces oxidative and nitrosative stress, which in turn increases the activation of the immune system and the inflammatory response, promoting epigenetic changes, which could lead to both COPD and LC development (72;73). Then, oxidative and nitrosative stress, chronic inflammation, and epigenetic modifications can be important causes of the increased cell proliferation, cell adhesion, epithelial mesenchymal transition and angiogenesis that is seen in LC patients, and the elevated apoptosis and lung damage that characterizes COPD pathogenesis (72;73). In addition, tumor stroma and activated fibroblasts could have an important role in inducing LC in COPD patients (74). In the current thesis, several molecular mechanisms that could link LC pathogenesis in patients with COPD have been studied such as oxidative and nitrosative stress, inflammation, epigenetic events and downstream regulator genes. A brief description of these mechanisms is explained below.

2.4.1 Oxidative and nitrosative stress

CS contains free radicals such as reactive oxygen and nitrogen species (ROS and RNS, respectively) that include species such as superoxide anion (O_2^-), peroxides (H_2O_2), hydroxyl radicals (OH^-) and peroxynitrites ($ONOO^-$) (75;76). In normal conditions, the cell maintains a balance between the formation of ROS or RNS and its removal by different pathways and antioxidant systems such as superoxide dismutase I (Cu-ZnSOD), superoxide dismutase II (MnSOD) and catalase (73;77). However, when ROS or RNS are produced in high levels, the antioxidant capacity of the tissue is exceeded, oxidative and nitrosative stress occurs (78). Both processes promote the accumulation of cell and DNA damage, which if it is not repaired correctly results in DNA mutations (79). Moreover, oxidative stress induced the transition from the G1 to the M

phase of the cell cycle, promoting an increase of cell proliferation (79;80). Then, this fact can elevate the epithelial mesenchymal (EMT) transition in the lung tissue from normal to carcinogenesis in smokers with COPD (81). Additionally, ROS and RNS can promote the degradation of tumor suppressor proteins, driving to an increased cell division and a lower apoptosis and DNA repair, inducing cancer development and progression (73). Furthermore, RNS can also elevate the levels of vascular endothelial growth factor (VEGF), augmenting angiogenesis and leading to tumor growth and cell invasion (82).

Protein carbonylation, 3-nytrotirosine formation (NT) and the final products of lipidic peroxidation such as malondialdehyde (MDA) or 4-hydroxy-2-nonenal (HNE), are important markers of oxidative and nitrosative stress (83-85). Several investigations have showed that oxidative and nitrosative stress have an important role in the development of both LC and COPD. For instance, it has been found an increase of oxidative and nitrosative stress in the blood of LC patients compared to controls (86). Moreover, it has also been found that levels of nitrosative stress were greater in the tumor lesions than in the non-tumor parenchyma of LC patients without COPD (79). In addition, it has been observed that structural and functional proteins are more nitrated and carbonylated in the normal airways of LC patients compared to controls, especially in those who have COPD (79;87). Furthermore, oxidative stress levels have been shown to be greater in the airways of LC-COPD than in LC patients (87). Therefore, oxidative and nitrosative stress are one of the mechanisms proposed to explain the association between COPD and LC although it is not fully understood at a molecular level (88;89).

2.4.2 Inflammation

The inflammatory response is a complex network of many different cells types and mediators (90). COPD pathogenesis is characterized by chronic inflammation, which in turn creates a local microenvironment that promotes malignant transformation and potentiates cancer progression (11;91). Then, chronic inflammation and the release of several interleukins (IL) by cells from the immune system, contributes to the lung carcinogenesis that usually occurs

in COPD patients (87;92). In the same manner, there are many inflammatory diseases that have been found to promote the development of other cancers (93-95). For instance, Barret esophagus has been linked to esophageal cancer, chronic pancreatitis has been associated with the development of cancer in pancreas, and patients with inflammatory bowel disease have been found to have a higher risk of having colon cancer (93-95).

CS promotes the expression of several cytokines that lead to an increase of the inflammatory response and that can inhibit apoptosis, induce angiogenesis, cell differentiation and also can suppress the mechanisms of cell repair (87;92). Inflammation in the airways contributes to alterations in the bronchial epithelium and promotes lung damage, leading to COPD, and resulting in an increase of cell division in order to maintain cell homeostasis (96). Therefore, the increased cell division and the chronic mitogenesis, augments the probability of DNA damage, with the consequent DNA mutations that can promote carcinogenesis (97). In line with this, it has been evidenced that chronic inflammation in the lower respiratory airway, can induce the development of cancer (49;98). In addition, many investigations have shown that systemic levels of different interleukins are higher in COPD patients, especially during exacerbations and with advanced disease (99-102). In the same way, circulating and local cytokines as well as growth factors have been associated with LC, being greater its levels than in control subjects (87;103).

Hence, chronic inflammation can promote neoplasia, tumor growth and metastasis, and it also has an important role in COPD pathogenesis (104;105).

2.4.2.1 Th1 and Th2 responses

T helper 1 lymphocytes (Th1 lymphocytes) release IL-2, interferon-gamma and TNF-alpha, which are responsible for cellular immunity and promote antitumor effects (106;107). However, T helper 2 lymphocytes (Th2 lymphocytes) predominantly produce IL-4 and IL-10, which potientate the inhibition of the immune system and favour tumor development (106;107). It has been reported that in LC and other malignancies such as breast or gastric cancer, Th1 and

Th2 balance is altered in the peripheral blood, with maintenance of the body to a Th2-dominant phenotype (108-110). This dominance of Th2 cytokines prevents the patient from destroying the tumor cells that have survived for instance to an incomplete resection, promoting a tumor relapse (105). Several studies have found that LC patients with higher systemic Th1 cytokines had a better prognosis than those with lower levels, increasing this tendency in patients in stage II or III (105;111). Additionally, in the airways and in the blood of COPD patients it has been found an increase of T cells secreting interferon-gamma, suggesting a greater Th1 response in these patients (89;112).

VEGF has an important role in the regulation of angiogenesis, which is increased in LC, and its expression is regulated by several growth factors such as transforming growth factor-beta (TGF-beta) and epidermal growth factor (EGFR). It has been observed that VEGF levels are augmented in the blood of LC patients and that they are associated with an increase of LC deaths (113). Interestingly, VEGF has been directly correlated with the angiogenic and aggressive potential of the tumor in LC patients (114).

Macrophages are important phagocytic immune effector cells that are formed through differentiation of monocytes (115;116). Macrophages can have different phenotypes depending on the microenvironment, which has an important role in LC and COPD pathogenesis (115;117). There are two different states of activation for macrophages: M1 macrophages, which are stimulated by IFN-gamma or TNF-alpha and secrete high levels of Th1 cytokines; and M2 macrophages that are induced by IL-4 or IL-13 and produce Th2 cytokines (116;118). M1 subtype act in the initial states of tumorigenesis, having antitumor effects, while M2 macrophages are thought to be in established tumors, promoting tumor development and angiogenesis (118-120). Therefore, it has been observed that in the tumor islets of NSCLC patients macrophages are predominantly of the M2 subtype (118;120;121). Interestingly, M1 macrophage density in tumor islets and stroma has been positively associated with survival time in LC patients whereas M2-polarized subtype has been associated with poor prognosis (118;120-123).

There is also high evidence that a transcriptional shift toward an M2 gene profile occur in smokers with COPD as well as a down-regulation of M1 genes (124-126). In addition, there is a partially change in macrophage polarization to an M1 phenotype with smoking cessation in COPD patients (125). Hence, contribution of M1 and M2 in LC and COPD development should be better defined, as both populations exist in these diseases (120;122;126).

2.4.3 Epigenetic mechanisms

Both oxidative stress and inflammation alter the redox status of the cells, which promote the destabilization of the genome and also lead to epigenetic changes (72). Epigenetic is described as the heritable changes in gene function that occur without alterations in the nucleotide sequence (72;127). These changes can be transmitted from one generation to the next (mitotic inheritance) and between generations of species (meiotic inheritance). The heritable material of eukaryotic cells is packaged in the form of chromatin (72;128). The main functional unit of chromatin is the nucleosome, which is formed by a core that consists of a complex of eight histone proteins (two each of histones H2A, H2B, H3 and H4) and a 146 nucleotide pairs long of double stranded DNA (128;129). There are two main regions in chromatin: heterochromatin, that is highly condensed, which reduces the accessibility of the transcription factors to the DNA, so it replicates late and contains inactive genes; and euchromatin, which is few condensed and contains the majority of the active genes because transcription can be activated easily (129).

Epigenetic mechanisms can be divided into the following groups: noncoding RNAs such as microRNAs (miRNAs), covalent and non-covalent histone modifications and DNA methylation (130;131) These modifications are controlled by chromatin-modifying enzymes that alter chromatin structure or by the recruitment of several DNA interacting proteins with enzymatic activities that modify the chromatin (72;129). Chromatin modifications, due to epigenetic alterations, tend to alter the transcription of different genes that affect to important cellular processes involved in COPD and LC development such as apoptosis, cell proliferation and differentiation, DNA repair and angiogenesis. In

addition, there is high evidence that epigenetic modifications are involved in the process of tissue stem cell differentiation to malignant cells, promoting tumor initiation and progression (131). Then, several studies have reported that epigenetic alterations play an important role in both LC and COPD pathogenesis (130;131).

2.4.3.1 MicroRNAs

MicroRNAs (miRNAs, miRs) are small, non-coding RNA molecules (from 19 to 25 nucleotides long) that regulate gene expression at transcriptional and post-transcriptional level (132;133). The creation of mature miRNAs is a process with different stages (134;135). Firstly, miRNAs genes are transcribed in the nucleus, which are called primary miRs (pri-miRs). Then, a RNAse III enzyme (Drosha) splits pri-miRs into precursors, called pre-miRs, that are transported to cytoplasm and cut by another RNAse III enzyme (Dicer) to form a miRNA duplex. Finally, this duplex is transformed into a mature miRNA, which is incorporated into an RNA-induced silencing complex (RISC) (132;134;135). MiRNAs bind to the complementary sequence in the 3'-untranslated region (3'-UTR) of the target mRNAs and cause mRNA degradation or translational repression, so miRNAs are strongly involved in gene silencing (72;132;135).

Only one miRNA is capable to target different transcripts and can regulate different processes involved in LC and COPD development such as cell proliferation, inflammation, apoptosis, cell differentiation and cell migration (132-134). Moreover, microRNAs have a key role in the development of adaptative and innate immune responses, that are also related with the pathogenesis of both diseases (136). Then, several miRNAs have been involved as either tumor suppressor genes or oncogenes in many different tumor types (133-135).

There is increasing evidence that deregulation of the expression of many miRNAs is associated with tumor progression and survival, which has been found in many cancers, including LC (137-139). Moreover, different studies support that miRNAS could be potential biomarkers of LC, as it has been shown

that circulating miRNAs can differentiate malignant from non-malignant tissue (140;141). Furthermore, several studies have reported that an up-regulation or a down-regulation of different miRNAs could be one of the causes of LC development (142;143).

The most consistently up-regulated miRNAs in both blood and lung specimens of LC patients are miR-21 and miR-210, which have an important role in promoting cancer (138;139;143). MiR-21 has been shown to contribute to tumor growth and invasion, being associated with a worse prognosis of LC patients (142;144). Furthermore, miR-21 targets many genes associated with malignancy such as the tumor suppressor genes phosphatase and tensin homologue (*PTEN*) and tropomysosin alpha-1 (*TPM1*) as well as the programmed cell death 4 (*PDCD4*) (143). MiR-210 has been shown to be involved in tumor initiation and in the promotion of an hypoxic phenotype, which is associated with a poor survival of LC patients (145;146). In addition, there is strong evidence that miR-210 increases radioresistance in human LC cells by targeting hypoxia-inducible factor 1 (*HIF-1*) (145;146).

Oppositely, miR-let7 family, miR-451, and miR30a have been found to be down-regulated in LC patients (141;147;148). MiR-Let7 family has an important role in regulating cell proliferation and differentiation during development in different species, so it could act as a tumor suppressor gene (149;150). Moreover, reduced levels of miR-let7c are associated with metastasis, cell invasion and advanced TNM stages, due to its interaction with *RAS* genes (148;151). MiR-451 acts as a tumor suppressor gene in LC, inhibiting ras-related protein 14 (RAB14), and its down-regulated expression has been correlated with tumor differentiation, proliferation and lymph-node metastasis in LC patients (147;152). There is high evidence that miR-30a also acts as a tumor suppressor gene, targeting Snail Family Zinc Finger 1 (*SNAIL-1*) and inhibiting epithelial mesenquimal transition. In addition, reduced levels of miR30a promotes cell invasion and migration (153;154). The down-regulation of these three miRNAs (miR-let7, miR-451 and miR-30a) is associated with shorter survival of LC patients (147;150).

Furthermore, it has been reported that in COPD also occurs an alteration of several miRNAs (139;155;156). It has been found that there is a different pattern of miRNA expression between smokers and non-smokers, and that cigarette smoke promotes a down-regulation of several miRNA expression (157;158). This fact has been also demonstrated in mouse and rat lungs exposed to tobacco smoke, in which was predominantly observed a down-regulation of miRNAs in both models (158;159). Furthermore, several studies have demonstrated that many miRNAs are deregulated in lung tissue of COPD patients compared to healthy subjects (139;155-157). There is strong evidence that several miRNAs are expressed differentially in plasma from LC compared to COPD patients, which could be useful as a diagnostic tool, in order to distinguish between LC and COPD patients (160;161).

2.4.3.2 Histone modifications

Histones are alkaline proteins which are extremely conserved and that can be post-transcriptionally modified, usually at the basic aminoacid lysine (K) and arginine (R) residues of their C-terminal tails (129). Histone tails can be phosporylated, methylated, acetylated, ubiquitinated, sumolyated and ADP-ribosylated, by the corresponding histone modifying enzymes (162;163). Both histone's core and their modifications have an important role in nuclear scaffolding, controlling the interaction of DNA, RNA polymerase and other transcription factors in order to regulate gene expression (162;164;165).

CS promotes alterations in histone proteins, that have been shown to occur very early in the process of oncogenesis (166). One of the major modifications is histone acetylation, controlled by histone acetylatransferases (HATs), which promotes changes in the chromatin structure that are associated with the increase of gene transcription (164;165). On the contrary, histone deacetylation, by histone deacetylases (HDACs), removes the acetyl groups and induces the repression of gene transcription (164;165).

It has been suggested that deregulation of HDACs and HATs can promote LC and COPD, as a decreased HDAC activity induces an elevated

transcription of several proinflamamtory genes that can promote COPD and carcinogenesis, and an over-expression of HDACs leads to a lower transcription of many tumor suppressor genes, promoting lung neoplasia (167;168). Therefore, in many tumor types it has been observed augmented levels of HDACs and decreased activity of HATs (169;170). Additionally, it has been evidenced that LC patients with an elevated acetylation in their lungs show better survival prognosis (171;172).

It has been demonstrated that CS causes hyperacetylation of histones and decrease histone deacetylase activity in lungs of smokers and in mice exposed to tobacco smoke (167;173;174). Therefore, it has been revealed that in the lungs and in the airways of COPD patients, there is an increase in histone acetylation, which is associated with the promoter region of many inflammatory genes that play a key role in COPD inflammation (175;176). Moreover, the reduction of HDAC activity such as HDAC-2 has been found in COPD patients and it is correlated with COPD severity and with the smoking exposure levels (167). Therefore, both hyper and hypoacetylation seem to have an important role in the development and progression of LC and COPD.

2.4.3.3 DNA methylation

Methylation is a post replicative epigenetic modification of DNA that in vertebrates occurs in the CpG dinucleotide, and it is very important in order to regulate gene expression (177;178). Moreover, methylation has a role in DNA strand separation and can result in errors in chromosome segregation (179;180). It is known that altered DNA methylation is related with cancer and other diseases such as COPD, because it is associated with gene silencing (181;182). In human cancers it is frequently found a general hypomethylation in the entire genome, but there are also specific sites of hypermethylation (181;182). CS, which is the major cause of both LC and COPD, is one of the main modifiers of DNA methylation, which affects to the transcription of several genes involved in key cellular processes that affect to the development of both diseases (183;184). Several studies have shown that tobacco smoke can alter

DNA methylation patterns and that the methylation of different genes can be correlated with the smoking index (185-187).

Evidence shows that when lung tumor lesions and non-tumor specimens are compared in LC patients, differentially methylated genes exist between them (188;189). Moreover, different tumor suppressor genes that are involved in LC development have been identified to be inactivated by an abnormal methylation in these genes or in the corresponding promoters (189;190). Furthermore, the methylation pattern of several genes has been correlated with LC patient's outcome, normally being associated an increase of gene methylation with an early recurrence of LC (191-193).

It has been reported that an altered pattern of DNA methylation is associated with the presence of COPD as well as with the severity of the disease (184;194). Moreover, it has been demonstrated that DNA methylation occurs widely in the small airways of COPD patients and it is related with an aberrant expression of important genes and pathways involved in COPD pathology (184;194;195). In addition, there are several genes that are differentially methylated in patients with LC depending if they have or not COPD. For instance, it has been shown that the mithocondrial transcription factor-A (*MTFA*) gene expression is lower, due to its promoter methylation, in lung tissue of LC patients with COPD compared to only LC patients (196;197).

2.4.4 Regulation of cell growth and proliferation

2.4.4.1 Autophagy

Autophagy is a dynamic process that provides a turnover of proteins and cytoplasmic organelles through a degradation pathway that is dependent on the lysosome. It mainly consists in four steps: 1) development of an isolation membrane; 2) formation of an autophagosome with encapsulated cargo; 3) fusion of the autophagosome to the lysosome; and 4) the digestion of lysosomal contents by the degradative phase (198). Autophagy is involved in important biological processes such as cell differentiation, immunity and development (199). It is induced rapidly by stress, providing a protective mechanism in order

to maintain cell function during early stages of diseases (198;199). However, in advanced stages of disease it could contribute to the pathogenic development by an adaptation to cellular stress. Therefore, autophagy can induce both protective and deleterious processes, depending on the environmental conditions, the specific stimuli and cell type (200). Important regulators of autophagy pathway are the microtubule associated-protein 1 light chain 3 (LC3), the bcl-2 interacting protein-1 (beclin-1) and the nucleoporin p62 (p62) (198).

Autophagy has several roles in the promotion of carcinogenesis, because it causes cell survival in starvation conditions through the recycling of intracellular nutrients, and it also can be a tumor suppressor, as defects in autophagy promote oxidative stress, tissue damage and inflammation (201). Several studies have revealed an increase of autophagy in many solid tumors such as LC (201;202). There is strong evidence that the activation of autophagic proteins is associated with epithelial cell apoptosis and inflammation, due to cigarette smoke, which has an important role COPD pathogenesis (203). Then, it has been reported that in COPD patients there is an overexpression of autophagic regulators and in consequence, an increase of autophagy (203;204).

2.4.4.2 Apoptosis

Apoptosis, also called programmed cell death, is a process whereby damaged or redundant cells are eliminated by certain stimuli. It is very important in order to maintain tissue homeostasis and development in multicellular organisms (205). In brief, in apoptosis there can be observed three types of biochemical changes: activation of caspases, DNA and protein breakdown, and membrane changes in order to be recognised by phagocytic cells (206). Apoptosis is associated with the removal of malignant cells, hyperplasia and tumor progression (207;208). When there is unbalance between anti-apoptotic (such as BCL-2) and pro-apoptotic (such as BAX) proteins, the affected cells have a dysregulated apoptosis. Therefore, resistance to apoptosis play an important

role in several cancers, such as LC, as it allows the survival of cells that accumulate oncogenic events (206;208).

Disorders in apoptosis are also related to tissue injury and inflammation that has observed in different diseases such as COPD (209). Moreover, there is high evidence that apoptosis of alveolar endothelial cells is associated with emphysema (210). In this regard, several studies have revealed an increase of apoptosis in lung tissue and sputum of COPD patients (210-212). Therefore, apoptosis seems to be involved in LC and COPD pathogenesis.

2.4.4.3 Cell proliferation and cell adhesion

Cell proliferation is a process in which the number of cells is increased, as a result of a balance between cell division and apoptosis or cell differentiation. Organisms need a continuous cell proliferation in order to develop and maintain during a long time, but it is also necessary the existence of different mechanisms that suppress those cells that present a deregulated growth or that have mutations (213). When these mechanisms do not work, malignant transformation of the cells occurs. There are many molecules, cytokines and microRNAs that control cell proliferation and that have been observed to be deregulated in LC (214;215). Therefore, it is known that cell proliferation is increased in the majority of the tumors, including LC, being characterized by uncontrolled cell proliferation and low levels of apoptosis (213). However, on the contrary, COPD patients are characterized by high levels of cell death and matrix destruction (209).

In order to execute many essential processes of the cells, it exists a controlled interaction between cells and the extracellular matrix that occur by the action of many molecules such as cell adhesion molecules (216). These molecules have a key role in inducing cell proliferation, migration and differentiation and in normal tissue are well-regulated (217). However, when these molecules have an abnormal expression, the changes in the interactions between cell-cell and cell-matrix can promote tumor development and metastasis (218). Additionally, cell adhesion molecules also have a role in

COPD pathogenesis, as they control the recruitment of leucocytes in the cell wall and its migration to the inflamed tissues, inducing the inflammatory response that characterizes COPD (219;220). Therefore, in LC and COPD patients it has been observed a downregulation or an overexpression of several cell adhesion molecules which also is related with a worse prognosis of the patients (217;221).

2.4.5 Tumor microenvironment

2.4.5.1 Epithelial mesenchymal transition

The epithelial mesenchymal transition (EMT) is a process in which epithelial cells transform to invasive, migratory and motile mesenchymal cells. It is involved in embryogenesis and organ development but it also has a key role in promoting cancer initiation and progression (222;223). During EMT cells loss the cell-cell adhesions, acquire resistance to apoptosis, suffer alterations in the cell-surface proteins and the cytoskeletal proteins reorganize its expression (222;223). EMT is regulated by several signaling pathways and transcription factors that induce a reduction in the epithelial markers and increases the expression of mesenchymal markers that promote the capacity of migration and invasion to cancer cells and its spread to other sites by the blood (223). Several studies have observed that in LC patients there is a high expression of EMT markers such as SNAIL-1, E-cadherin and hypoxia inducible factor-1 (HIF-1) (224). In addition, it has been suggested that it exists a correlation between the presence of EMT markers and a worse prognosis of LC patients (222). Moreover, it has been reported that miR-200 inhibits EMT by targeting the *ZEB1* and *ZEB2* genes, and low levels of miR-200 has been associated with higher metastasis in LC patients (225). Interestingly, EMT also contributes to the fibrosis of small airways and promotes changes in the extracellular matrix, which both have and important role in COPD pathogenesis (226). In addition, it has been observed that there are increased levels of EMT markers in the lungs of COPD patients compared to controls (227). Then, it has been evidenced that EMT associated with angiogenesis could be an important link in LC development in COPD patients (226;228).

HYPOTHESIS

The general hypothesis of the current thesis was to identify the profile of clinical, tumor characteristics, and underlying biological mechanisms that may differ in patients with LC with and without COPD. Indeed, the main question was to identify features that may render patients with chronic respiratory diseases more prone to develop lung tumors, as earlier demonstrated in many epidemiological studies. A more specific hypothesis was that several biological mechanisms previously shown to underlie tumor initiation and progression, such as oxidative stress and inflammation may alter key cellular processes namely cell proliferation and adhesion, angiogenesis, autophagy and apoptosis through epigenetic regulation. The epigenetic mechanisms analysed in the current thesis were: microRNAs expression of those shown to be involved in LC, histone acetylation, and DNA methylation. The general overview of the thesis hypothesis is schematically represented in the figure below:

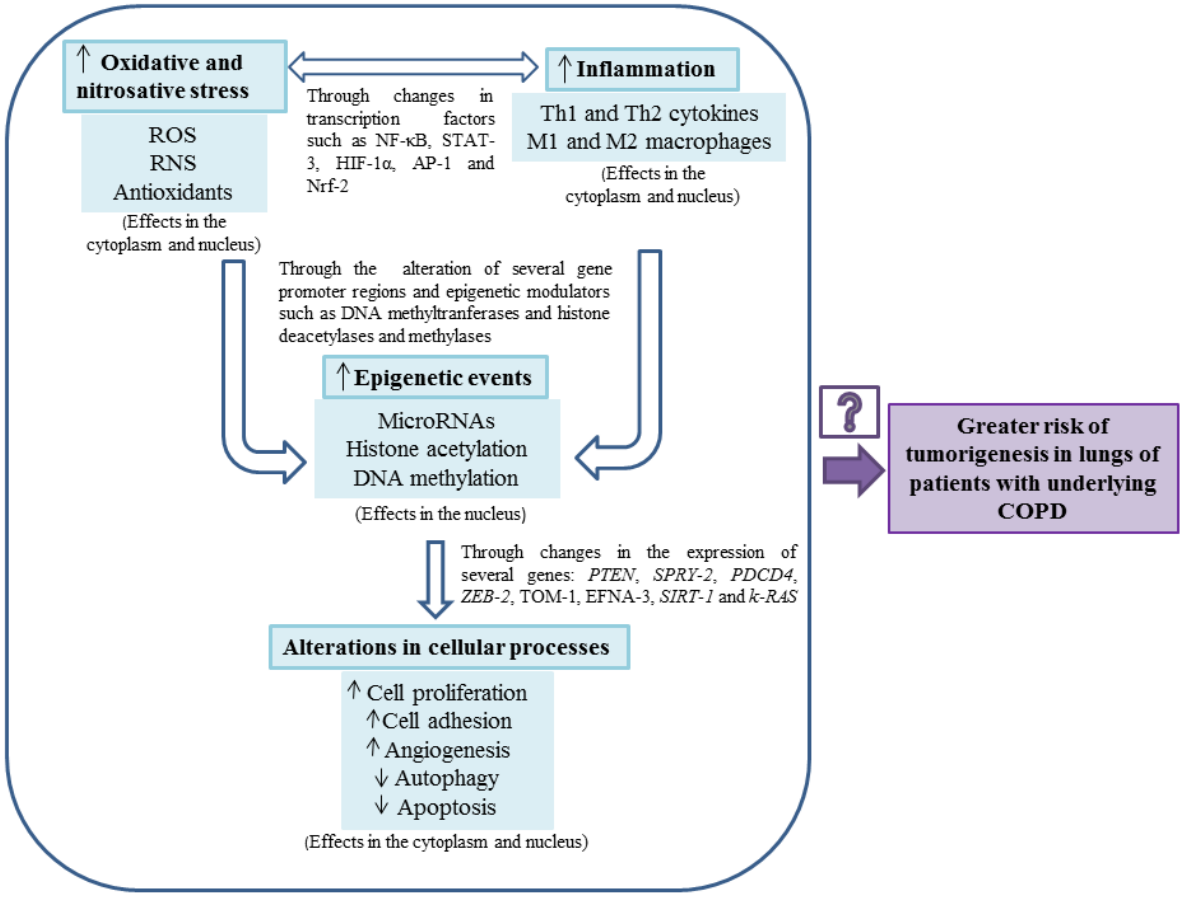

Figure 4. Schematic representation of the thesis hypothesis.

OBJECTIVES

According to the study hypothesis, the current thesis has been divided into three major studies, distributed as corresponding chapters. The specific objectives of each study are briefly summarized below:

1. Study #1: Redox imbalance in lung cancer patients with underlying chronic respiratory conditions

In LC patients with and without COPD:

1) To evaluate patient characteristics including clinical, physiological, functional and nutritional parameters

- In lungs (tumor and non-tumor) and in blood compartment:

2) To explore levels of oxidative and nitrosative stress markers and antioxidant systems
3) To assess the influence of smoking history in the expression levels of markers of redox balance in LC patients with COPD

Published article: M Mateu-Jimenez, A Sánchez-Font, A Rodríguez-Fuster, R Aguiló, L Pijuan, C Fermoselle, J Gea, V Curull, E Barreiro. Redox imbalance in lung cancer of patients with underlying chronic respiratory conditions. *Mol Med*. 2016 Jan; 22:85-98

2. Study #2: Systemic and tumor Th1 and Th2 inflammatory profile and macrophages in lung cancer: influence of underlying chronic respiratory disease

In LC patients with and without COPD:

1) To assess the characteristics of the patients including clinical, physiological, functional and nutritional parameters

- In lungs (tumor and non-tumor) and blood compartment:

2) To determine protein levels of the cytokines of Th1 (IL-2, TNF-α and IFN-gamma) and Th2 (IL-4, IL-10, TFG-beta and VEGF) responses
3) To evaluate the influence of smoking history in the expression levels of Th1 and Th2 cytokines in LC patients with COPD

- In lungs (tumor and non-tumor):

4) To determine levels of EGFR protein expression
5) To identify the numbers of the two types of macrophages: M1, which have been associated with antitumor responses and extended patient's survival, and M2, which have been related with tumor initiation and progression
6) To explore the patients' survival according to the number of M1 and M2 macrophages

Published article: M Mateu-Jimenez, V Curull, L Pijuan, A Sánchez-Font, A Rodríguez-Fuster, R Aguiló, J Gea, E Barreiro. Systemic and tumor Th1 and Th2 inflammatory profile and macrophages in lung cancer: influence of underlying chronic respiratory disease. *J. Thorac Oncol.* 2017 Feb; 2: 235-248

3. Study #3: Profile of epigenetic mechanisms in lung tumors of patients with underlying respsiratory conditions

In LC patients with and without COPD:

1) To study patient characteristics including clinical, physiological, functional and nutritional parameters

- In lungs (tumor and non-tumor):

2) To explore the expression of epigenetic regulation through the following mechanisms:
    - o MiRNAs potentially involved in lung carcinogenesis
    - o Histone acetylation
    - o DNA methylation
3) To evaluate the expression of markers of cellular processes potentially regulated by the target miRNAs:
    - o Cell proliferation and invasion
    - o Cell adhesion and migration
    - o Cell differentiation and epithelial mesenchymal transition
    - o Angiogenesis
    - o Autophagy
    - o Apoptosis

In revision article: M Mateu-Jimenez, V Curull, A Sánchez-Font, L Pijuan, A Rodríguez-Fuster, R Aguiló, J Gea, E Barreiro. Profile of epigenetic mechanisms in lung tumors of patients with underlying chronic respiratory conditions, *Clinical Epigenetics*, in revision

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METHODS

The different methodologies that have been used in the three studies are described in the table below. Detailed information on the different methods used in each study is described in the corresponding online data supplement of each published study.

Numbers of study subjects according to the type of samples		
	Blood samples	
	LC patients	LC-COPD patients
Study #1	N=21	N=59 LC-COPD, moderate smokers, N = 24 LC-COPD, heavy smokers, N=35
Study #2	N=20	N=60 LC-COPD, moderate smokers, N = 25 LC-COPD, heavy smokers, N=35
Study #3	-	-
	Lung specimens (tumor and non-tumor)	
	LC patients	LC-COPD patients
Study #1	N=12	N=23 LC-COPD, moderate smokers, N = 8 LC-COPD, heavy smokers, N=15
Study #2	N=20	N=20 LC-COPD, moderate smokers, N = 9 LC-COPD, heavy smokers, N=11
Study #3	N=20	N=20

Variables obtained from the study patients	
Anthropometric variables	Age, gender and body mass index (BMI)
Smoking history	Current smoker, ex-smoker, never smoker and number of packs-year

Study #1, #2 and #3	**Lung function testing**	Forced expiratory volume in one second (FEV1), FEV1/FVC (forced vital capacity), carbon monoxide transfer (DL_{CO}) and Krogh transfer factor (K_{CO})
	TNM staging	Stages: IA, IB, IIA IIB, IIIA, IIIB and IV
	Histological diagnosis	Squamous cell carcinoma, adenocarcinoma or other types of carcinoma
	Blood analytical parameters	Total leucocytes, total neutrophils, total lymphocytes, albumin content, total protein content, fibrinogen, C reactive protein (CRP), globular sedimentation velocity (GSV), and ceruloplasmin content
	Body weight loss	0 kg, 1-4 kg, 5-8kg and 9-12kg

Laboratory techniques

Study #1	**Immunoblotting of 1D electrophoresis**	In tumor and non-tumor lungs: Oxidative and nitrosative stress markers: protein carbonylation levels, malondialdehide-protein adducts, total protein nitration levels Antioxidant enzymes SOD-1, SOD-2 and catalase levels
	Two-D electrophoresis and silver staining	Identification of carbonylated proteins in lung tumor and non-tumor specimens
	Mass spectrometry	Identification of carbonylated proteins in lung tumor and non-tumor specimens
	Enzyme-linked immunosorbent assay (ELISA)	Plasma levels of the oxidative stress markers protein carbonylation, nytrotirosine and the antioxidant glutathione (GSSG/GSH)

	Lucigenin-derived chemiluminiscence	Measurement of superoxide anion radicals in blood
Study #2	**Enzyme-linked immunosorbent assay (ELISA)**	Plasma and lung (tumor and non-tumor) levels of TNF-alpha, VEGF, IL-2, IL-10, IFN-gamma, TGF-beta, IL-4 and EGFR (the latter only in lung specimens)
	Double-staining immunohistochemistry and optical microscopy	Counts of M1 and M2 macrophages in tumor and non-tumor lung parenchyma
Study #3	**RNA extraction using trizol technique**	Lung (tumor and non-tumor) RNA isolation
	Taqman based qPCR reactions (real-time PCR)	MicroRNA lung expression: miR-126, miR-21, miR-210, miR-Let7c, miR-200b, miR-30a-30p, miR-451, miR-Let7a and miR-155 Gene expression in the lungs (tumor and non-tumor): *PTEN*, *MARCKs*, *TPM-1*, *PDCD4*, *SPRY-2*, *ETS-1*, *ZEB-2*, *EGFL-7*, *TOM-1*, *CRK*, *MIF*, *RAB-14*, *FGFRL-1*, *EFNA-3*, *CDKN1A*, *CDKN2A*, *SNAIL-1*, *P53*, *P63* and *K-RAS*
	QIAmp DNA Mini Kit	DNA isolation from the lungs (tumor and non-tumor)
	Enzyme-linked immunosorbent assay (ELISA)	Quantification of DNA methylated levels in the lungs (tumor and non-tumor)
	Immunoblotting of 1D electrophoresis	Quantification of protein levels in tumor and non-tumor lungs: HDAC2, LC3, BAX, BCL-2, fibulin-2, fibulin-3, fibulin-5 and angiopoietin-2
	Immunohistochemistry and optical microscopy	Cell proliferation levels (ki-67-stained nuclei) in the lungs (tumor and non-tumor)

Statistical analyses		
	Clinical variables	**Biological variables**
Study #1	Student's *T- test* (quantitative variables) Chi-squared test (qualitative variables)	Blood parameters: Student's *T-test* Lung (tumor and non-tumor) parameters: ANOVA and *Tukey's* post hoc test Receiver operating characteristic (ROC) curves
Study #2	Student's *T- test* (quantitative variables) Chi-squared test (qualitative variables)	Blood parameters: Student's *T-test* Lung (tumor and non-tumor) parameters: ANOVA and *Tukey's* post hoc test Kaplan-Meier survival curves
Study #3	Student's *T- test* (quantitative variables) Chi-squared test (qualitative variables)	Lung (tumor and non-tumor) parameters: ANOVA and *Tukey's* post hoc test

RESULTS

Summary of main findings study #1, #2 and #3

In the current thesis it was found that in LC patients with COPD compared to those without this disease, there was an increase in the proportion of smokers (both current and ex-smokers), a raise in the number of packs smoked per year and an increase in weight loss.

In addition, it was observed that expression levels of protein oxidation, antioxidant systems and Th2 cytokines were greater in the tumor lesions of LC patients with COPD than in the lung tumors of patients without COPD. An increase in the expression levels of microRNAs and DNA methylation in the tumor lesions of LC patients with COPD compared to the tumors of those LC patients without any respiratory condition, was also shown. These epigenetic alterations, promoted the downregulation of different genes that induced changes in several target pathways such as increased cell proliferation, invasion and angiogenesis, and decreased cell adhesion, migration and differentiation in the tumor lungs of LC patients with COPD compared to those without COPD.

In the tumor specimens compared to the surrounding non-tumor lungs of both groups of patients, it was observed a characteristic pattern of regulation in the expression of the antioxidant systems that consisted in increased protein levels of SOD-2 and decreased expression levels of catalase. Moreover, in the tumor lungs compared to the non-tumor specimens of LC patients with and without COPD, it was found that the expression of several microRNAs was altered. These changes in epigenetic regulation induced a decrease of several genes that altered several cellular processes such as increased cell proliferation, invasion and angiogenesis and decreased apoptosis levels in the tumors compared to the non-tumor lungs of both groups of patients.

Systemic levels of oxidative and nitrosative stress as well as protein levels of Th1 cytokines were increased in LC patients with COPD compared to those without any respiratory condition. Taken together, the results obtained in the current thesis may have potential implications at the time of identifying biomarkers that will allow an early diagnosis of LC, as well as to identify potentially new therapeutic targets for LC treatment.

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DISCUSSION

Clinical and functional characteristics of the study subjects

Many epidemiological investigations (5;229;230) have revealed that the number of LC patients with a chronic respiratory condition is higher than the number of LC patients without one. This has been confirmed in the three studies of the current thesis, as the number of LC patients with COPD was threefold greater than LC patients without this disease. Although it is well known that LC is mainly caused by CS, around 10-25% of LC is developed in non-smokers, who are usually women with adenocarcinoma histology type (231). It has been shown that LC could also originate as a result of passive smoking, exposure to radon, genetic or dietary factors (232). This fact was observed in the three studies, as the proportion of those who had never smoked was inexistent in LC patients with COPD, whereas the proportion of current or ex-smokers was higher among patients with LC and COPD than in LC patients. This suggests that lung carcinogenesis in COPD patients could be mostly caused by CS.

It is widely known that COPD disease is characterized by a strong airflow limitation, and as expected, in the present thesis a decrease in lung function parameters of LC patients with COPD compared to LC has been observed. Additionally, it is also known that COPD is associated with body mass loss, and that this is more prominent if it is accompanied by lung carcinogenesis, as it is also an important comorbidity observed in LC patients (233;234). In line with this, in all the studies of the present thesis an increase in body weight loss in LC patients with COPD compared to those without this disease was observed.

Potential biological mechanisms involved in LC and COPD

Redox balance in tumor and non-tumor lungs

Oxidative stress has been established as an important factor of several cancers and human diseases, including LC and COPD (81). In fact, several investigations have reported that oxidative stress levels were increased in LC and COPD patients compared to control subjects (87;235;236). It is known that oxidative stress and reactive oxygen species (ROS) promote the accumulation

of damaged cells and DNA and that they also increase cell proliferation and epithelial mesenchymal transition, which could induce lung carcinogenesis development and progression in COPD patients (79). Additionally, ROS can induce lipid peroxidation, which releases an important oxidative stress marker, malondhyaldeyde (MDA), that was shown to have a strong mutagenic potential and to be carcinogenic in mice, as tumors can develop in a short period of time after MDA administration (237). Importantly, in a previous study from our group, it was shown that protein levels of MDA protein adducts were higher in the normal bronchial epithelium of LC-COPD than in LC patients (87). The results obtained in the current thesis are in line with this, as levels of MDA protein adducts were found to be increased in the tumor and non-tumor lungs of LC patients with COPD compared to those LC patients without it. Interestingly, this increase of the main product of lipid peroxidation was not influenced by smoking history, because there were no differences in MDA protein levels between heavy and moderate smokers in the subgroup of LC patients with COPD. This finding suggests that the increase in oxidative stress levels in LC patients with COPD, may be due to having COPD and could be a strong contributor to lung carcinogenesis development in COPD patients.

Additionally, in the present thesis it was shown that several important proteins such as actin, vimentin or enolase were carbonylated in the tumor and non-tumor lungs in both groups of patients. These findings are supported by a previous investigation, in which it was found that proteins were more carbonylated in the normal epithelium of LC and LC-COPD than in control subjects (87). Protein carbonylation is a selective process, which usually alters the function of the proteins (238), and in the present study, most of the proteins that were found to be carbonylated were functional, structure and regulator proteins. Then, the alteration of proteins that are important for the cell function could be involved in the promotion of lung carcinogenesis and COPD. For instance, it has been demonstrated that the carbonylation of specific proteins in the lungs leads to the development of emphysema in several mice models (238;239).

Nitrosative stress has also been established as an important cause of LC and COPD development, and an increase of nitrosative stress markers in LC

and COPD patients compared to controls has been demonstrated (86). In the present study, protein nitration levels were higher in the non-tumors of LC patients with COPD compared to LC patients without it, which could suggest that the increase in protein nitration in the lungs could only be due to the presence of COPD, regardless of having tumor cells or not. In a previous study, it was observed that levels of nitrosative stress were greater in the tumor lesions than in the non-tumor lungs of LC patients (79). In keeping with this, in the current thesis an increase in total protein tyrosine nitration levels in tumors compared to non-tumors was found only in LC patients, suggesting that tyrosine nitration could contribute to the formation of the lung tumors in LC patients without COPD. In fact, it has been reported that tyrosine nitration is involved in protein dysfunction and the degradation of several proteins that strongly participate in tumor initiation and progression (240;241). Indeed, a study reported that nitrotyrosine levels were increased in smokers with COPD compared to smokers without this disease, which could mean that CS does not have an influence in the increase of protein nitration, and that this increase is mainly due to the presence of COPD (242). This finding is in agreement with the results obtained in the present study, as there were no differences in protein nitration levels between heavy and moderate smokers in the LC-COPD subgroup of patients.

Several studies have hypothesized that antioxidants may protect against cancer, as they neutralize ROS, and it seemed that the presence of high levels of antioxidants in cells and animal models decreased DNA damage, which is associated with cancer development (243). Nevertheless, it has recently been shown that supplementing the diet with antioxidants in a mice model of LC, increased tumor progression and reduced the survival of the mice (244). Additionally, in another recent study it was observed that antioxidants increased the number of melanoma metastasis in an experimental mice model (245)**.** Therefore, it has been suggested that benefits from antioxidants depend on the biological context (243). As an example, SOD2 is an antioxidant that has been found to be a tumor suppressor in the initial stages of a tumor, but once the tumor progresses it overexpresses and acts as an oncogene (246;247). Interestingly, in the current thesis an increase in SOD2 levels in the tumors compared to non-tumor lungs was found in both groups of patients, being more

prominent this increase in those patients with COPD that were heavy smokers. These findings suggest that SOD2 could be involved in lung tumor development, cell proliferation and cell survival, especially in patients with COPD, and that this increase in SOD2 levels could mostly be caused by cigarette smoke exposure. Futhermore, a report showed that protein levels of SOD2 were higher in the lungs of NSCLC compared to controls, and that those were also greater in malignant than in non-malignant tissue (248). In fact, several studies have demonstrated that an upregulation of SOD2 increases both metastasis and the invasiveness of the tumor (246;247).

In contrast, in the current study no differences were observed in the antioxidant SOD-1 levels between tumor and non-tumor specimens in any of the two groups of patients. However, SOD-1 levels were increased in tumor and non-tumor lungs of LC patients with COPD compared to LC patients without underlying COPD, with no differences between patients with different exposures to tobacco smoke. These findings suggest that the increase in the antioxidant SOD-1 in the lungs could be due to the fact of having a chronic respiratory condition and that it is independent of lung carcinogenesis and cigarette smoke. In fact, a study reported that SOD-1 expression was correlated with COPD severity in the small airways, having greater levels in severe than in mild COPD patients (249). On the other hand, in the current thesis it was also shown that protein levels of the antioxidant catalase were lower in tumors than in non-tumor parenchyma in both groups of patients, especially in those patients with COPD that were more exposed to cigarette smoke. In fact, it has been reported that an overexpression of catalase inhibited metastasis in mice models, whereas the blocking of catalase caused spontaneous tumors to appear in mice models (250;251). Similar to our findings, several studies have observed a decrease in mRNA and protein levels of catalase in the tumors of LC patients without COPD (77). Additionally, it has been demonstrated that catalase levels were lower in COPD patients with moderate disease than those with mild disease, which could explain the decline observed also in patients with LC and COPD (252). These results suggest that probably due to an excess of prooxidants, the antioxidant capacity of catalase is depleted, which seems to predispose to lung carcinogenesis, independently of

having COPD or not, and especially in those COPD patients more exposed to cigarette smoke.

Systemic Redox Balance

Apart from its local effects in the lungs, it has been demonstrated that systemic oxidative and nitrosative stress levels are also altered in both LC and COPD patients (87;253). Several studies have shown that levels of oxidative and nitrosative stress were increased in the blood of patients with LC with and without COPD, and that those are directly correlated with advanced stages of LC (87;253). In addition, other studies have reported that patients with only COPD also showed higher levels of several oxidative stress markers such as MDA and protein carbonylation, as well as higher levels of nitrosative stress (235;254). These studies suggest that oxidative and nitrosative stress levels could be more elevated in those patients with both diseases, although there is a lack of investigations that take into account the presence of COPD or not in LC patients. Therefore, in the current thesis it was found that systemic levels of superoxide anion, protein carbonylation and nitrotyrosine were increased in those patients with LC and COPD compared to patients with only LC, confirming that oxidative and nitrosative stress levels were raised in those patients with both diseases. Importantly, these findings were not influenced by CS, as there were no differences between heavy and moderate smokers in the subgroup of LC patients with COPD, suggesting that the increase of oxidative and nitrosative stress in LC patients may only be due to the presence of an underlying chronic respiratory disease.

Glutathione transferases, an important antioxidant system, seems to play a role in cancer progression, as a decrease in the ratio of reduced glutathione (GSH)/glutathione disulphide (GSSG) has been shown to promote an increase of oxidative stress and cancer promotion (255;256). Then, a study showed that glutathione levels were decreased in the blood of LC patients compared to control subjects (253). GSH also plays an important role in COPD patients, as it neutralizes the free radicals released by the activated inflammatory cells, and lower levels of GSH have been found in COPD patients (257). Nevertheless, in the current investigation no differences were found between LC patients with

and without COPD in systemic levels of oxidized glutathione (GSSG/GSH), and smoking history did not influence the results in those LC patients with COPD.

Taken together, all these findings could suggest that an increase in systemic protein oxidation, carbonylation and nitration, along with superoxide anion formation, and an increase in the antioxidant SOD1 in the lungs, may play an important role in lung carcinogenesis development in patients with COPD.

Th1/Th2 inflammatory response in tumor and non-tumor lungs

Oxidative stress can activate several genes and mediators that induce different signal cascades and changes in transcription factors that lead to the expression of many inflammatory cytokines that could promote chronic inflammation (258). Additionally, inflammatory cells can also release soluble mediators such as cytokines, which recruit more inflammatory cells and produce ROS, inducing oxidative stress (258). Chronic inflammation is the main etiological factor which is supposed to play a role in LC development in patients with COPD, and in this inflammatory response the participation of lymphocytes and macrophages is key (87;92). It is known that two types of $CD4^+$ T lymphocytes exist: Th1 and Th2, and that both are strongly involved in tumor development and progression (108-110). Th1 lymphocytes directly destroy tumor cells releasing TNF-α, IL-2 and IFN-gamma cytokines, whereas Th2 cells produce IL-4 and IL-10, which facilitate tumor progression (106;107). It has been demonstrated that alterations in the Th1/Th2 balance, towards a Th2 dominant pattern, occur in several types of malignancies, including LC (108-110). Moreover, it has been reported that the dominance of Th2 cytokines could promote a relapse of the tumor after a lung tumor resection (105).

Interestingly, in the current investigation it was found that levels of VEGF, TGF-beta and IL-10 were increased in the tumors of LC patients with underlying COPD compared to those without it. Although COPD is a disease with a Th1 dominant pattern, studies have also reported an increase of several Th2 cytokines in COPD patients (259). Similar to our findings, different studies have shown higher levels of TGF-beta in the small airways of patients with COPD, which in turn inhibited Th1 response and favored Th2 cytokines (260;261).

Additionally, the fact that in the current thesis COPD patients had a lung tumor, could have contributed to the increase in Th2 cytokines in the lungs of those patients with LC and COPD compared to patients with only LC. However, non significant differences were observed in the profile of the Th1 cytokines IFN-gamma, TNF-alpha or IL-2 in the tumors between LC patients with and without COPD, which could suggest that proinflammatory cytokines have not had an important role, probably due to the increase in the Th2 response that occured in the lung tumors. Interestingly, the results obtained in the lungs were not influenced by the smoking history of those patients with LC and COPD, suggesting that the increase of Th2 cytokines protein levels in the lungs may be a mechanism of lung tumor development in patients with underlying respiratory conditions.

Additionally, in the current thesis it was observed that levels of TNF-alpha, IL-2, TGF-beta and IL-10 were greater in the tumors than in the non-tumor lungs of LC patients with and without COPD. Then, these findings suggest that lung tumor formation could be due to an increase of the Th2 cytokine IL-10 and to the increased levels of TGF-beta, which has been also reported to contribute to the Th2 response (262). Additionally, it also seems that the increase in the proinflammatory pattern, characteristic of the Th1 cytokines, also contributes to the development of the tumor. In agreement with these findings, several studies have shown that levels of Th2 cytokines were higher in the lungs of LC patients than in control subjects (263). In addition, it has also been reported that a proinflammatory environment seems to contribute to tumor initiation and promotion (264). Importantly, the increase in both types of cytokines was not influenced by CS, as there were no differences in the levels of the different cytokines between heavy and moderate smokers in the subgroup of LC patients with COPD.

Systemic Th1/Th2 inflammatory response

In the present thesis it was observed that systemic levels of IL-2, TNF-α, TGF-beta and IL-10 were greater, whereas those of VEGF and IL-4 were lower in LC patients with COPD than in those without underlying COPD. Both VEGF and TGF-beta tend to promote a release of the Th2 cytokines IL-4 and IL-10, so

these findings suggest that Th2 response could be countered in patients with both diseases, as TGF-beta and IL-10 levels where increased, while VEGF and IL-4 levels were reduced. Several studies have shown an increase in systemic Th2 cytokines in NSCLC patients, whereas it has also been observed a raise in Th1 cytokines in COPD patients, although these studies always compared patients with healthy controls and they did not take into account the presence of COPD or not in LC patients (105;265;266). Then, in the current thesis it was found that Th1 response was increased in those patients with LC and COPD compared to those LC patients without any underlying respiratory disease. Therefore, chronic inflammation that occurs in COPD patients with LC seems to promote a systemic Th1 response, which may prevent lung tumor development to those patients with COPD. Supporting this, several studies have found that an increase of the Th1 response seems to be related with a better outcome for patients with several types of malignancies (267;268).

M1 and M2 macrophages in tumor and non-tumor lungs

Similar to the dichotomy of Th1 and Th2 cytokines, a double polarization in macrophages also exists, which plays a key role in the inflammatory events that occur in COPD and LC diseases. Depending on the cytokines that they release, macrophages can be activated to an M1 or an M2 phenotype (116;118). M1 macrophages secrete proinflammatory (Th1) cytokines and have anti-tumor properties, whereas M2 macrophages secrete anti-inflammatory (Th2) cytokines and promote tumor growth and cell survival (116;118). In the same line, in the current thesis it was found that the numbers of M2 macrophages were greater, whereas those of M1 were lower in the tumor lesions than in the non-tumors lungs of both groups of patients. Additionally, the ratio of M1/M2 macrophages was also lower in the tumors than in the non-tumor specimens in LC patients with and without COPD. In agreement with these findings, several studies have observed that levels of M2 cells were greater in malignant than in non-malignant lung tissue of NSCLC patients (269;270). Interestingly, in the present thesis it was also found that the ratio of M1 to M2 macrophages was increased in the tumors of LC patients with COPD compared to those without any underlying respiratory condition. These findings show a major contribution of M1 macrophages in the tumors of those patients with

COPD, which could indicate a better outcome for them. In fact, several investigations have revealed that increased numbers of M1 macrophages in the tumor lungs are positively associated with extended survival of LC patients, although LC patients were not evaluated separately by the presence of COPD or not (118;120;271). Nevertheless, in the current study numbers of M1 and M2 macrophages or M1/M2 ratio was not correlated with patient's survival. These results could be due to the fact that the number of lung tumors available for the study was relatively small in order to obtain significant correlations in survival analyses, as the samples were obtained from patients that underwent thoracotomy for their lung tumor resection.

Taken together, all these findings suggest that in those patients with LC and COPD exist an increase of the proinflammatory response, showed by a dominance of the Th1 cytokines and M1 macrophages in the blood and in the lung tumors, respectively, which may protect COPD patients from lung tumor growth and progression.

Epidermal growth factor receptor in tumor and non-tumor lungs

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase, which regulates several signaling pathways that control cell proliferation. In fact, EGFR seems to induce tumorigenesis, as it promotes cell division and suppresses apoptosis (272). Mutations in the *EGFR* gene have been observed in NSCLC patients, and it has been reported that tyrosine kinase (TK) inhibitors seem to be more effective in those NSCLC patients with somatic mutations in the EGFR TK domain, although it has been reported only in those with the adenocarcinoma subtype (273-275). Several studies have shown that EGFR is upregulated in many malignancies, including NSCLC, and that its expression is associated with a worse prognosis of the patients and an increase in the number of metastasis (272;276;277). In agreement with these studies, in the present thesis it was found that EGFR levels were increased in tumor lesions than in non-tumor specimens in both groups of patients. However, no differences were found in EGFR levels between LC patients with and without COPD in either tumor or non-tumor lungs. Taken together, these results

suggest that EGFR contributes to lung tumor formation independently of having or not a respiratory chronic disease.

Differential epigenetic mechanisms and downstream regulation in the tumor lesions of COPD patients

Both oxidative stress and inflammation alter the redox status of the cells, which promote the destabilization of the genome and also lead to epigenetic changes (72). Epigenetic modifications such as histone alterations, DNA methylation and microRNAs regulation, play an important role in LC initiation and progression (278). In the current thesis, it was reported that expression levels of miR-21, miR-200b, miR-210 and miR-let7c were increased in the tumor lesions of LC patients with COPD compared to those without any respiratory condition. Importantly, expression levels of the corresponding downstream genes that regulate those microRNAs (*PDCD4*, *TPM-1*, *MARCKs*, *PTEN*, *SPRY-2*, *ETS-1*, *ZEB-2*, *FGFRL-1*, *EFNA-3* and *k-RAS*) were decreased in the tumor lungs of LC patients with COPD compared to those without this disease. These findings could suggest that the upregulation of several microRNA's, and the reduced levels of its downstream target genes, may promote alterations in several important cellular processes such as cell proliferation and invasion, angiogenesis and apoptosis, that may promote tumor development and progression in those patients with COPD.

In fact, several studies have found that an upregulation of miR-21 in tumor lungs and in cells from NSCLC patients, promoted cell proliferation and invasion through the downregulation of its downstream genes *PTEN* and *PDCD4*, supporting the results obtained in the present study (143;279;280). Moreover, another study reported that miR-21 expression levels were greater in the mononuclear cells of COPD patients than in control subjects, so miR-21 also seems to play a strong role in CODP development. (281). In addition, it has also been demonstrated that an increase of the miR-200 family promotes tumor metastasis in NSCLC cell lines by decreasing several genes involved in the EMT process (282). In line with this, in the current thesis it was observed that the expression of the EMT markers SNAIL-1 and ZEB-2 were downregulated in the tumors of patients with LC and COPD compared to those without COPD, suggesting that EMT may not be involved in LC development in COPD patients,

but it may increase tumor metastasis. In fact, a study reported that ZEB family was more expressed in metastatic than in primary lung tumors (283). In keeping with the results reported in the current investigation, studies have found that hypoxia inducible miR-210 was overexpressed in most solid tumors, and it was also observed that an increase of miR-210 expression promoted angiogenesis and metastasis by downregulating *EFNA-3* and *FGFRL-1* levels (146;284). Interestingly, an investigation demonstrated that miR-210 was upregulated in lung tumors that corresponded to LC patients with late stages of the disease, suggesting that miR-210 contributes to lung tumor progression, which may be more relevant in those patients with COPD (285). With regards to miR-let7c, studies have reported that reduced levels of miR-let7c in LC patients seem to be associated with metastasis and cell invasion due to its interaction with *RAS* genes, although in the current thesis miR-let7c levels were higher in LC patients with COPD than in those without it (148;151). Those controversial results obtained compared with the literature, could be due to the fact that in those investigations it was not taken into account the presence of COPD in LC patients, so it could suggest that COPD may promote a slight reduction of the cancer cell effects in those patients.

Many studies have identified that DNA methylation of several genes involved in a variety of cellular processes is associated with both LC and COPD diseases (184;286). Interestingly, in the current thesis it was reported that DNA methylation levels were increased in the tumor lesions of LC patients with COPD compared to those without any underlying respiratory condition. In agreement with the results obtained, many studies have revealed that several genes were more methylated in the lung tumors of COPD patients than in those without underlying COPD (287;288). Therefore, DNA methylation seems to be a strong contributor to the development of LC in COPD patients.

In the current thesis, it was found that expression levels of the tumor suppressor gene *P53* were reduced in the lung tumors of LC patients with COPD compared to those without this disease, suggesting that loss of *P53* may be involved in LC development in COPD patients. In fact, several investigations have demonstrated that inactivation of *P53*, by either deletions or mutations, leads to alterations in the activation of downstream genes that regulate cell cycle and apoptosis (289). Moreover, it has been reported that altered levels of

the P53 protein promoted lung carcinogenesis and it was associated with a worse prognosis of the patients (290;291). In addition, studies have revealed that alterations in *P53* gene were related with an increased resistance to chemotherapy and radiation in LC patients (292;293). Therefore, the suppression of *P53* may play an important role in lung carcinogenesis in COPD patients.

Taken together, all these results suggest that an upregulation of several microRNAs and DNA methylation, as well as a decreased expression of different downstream genes and tumor suppressor genes, promote alterations in many cellular pathways such as increased cell proliferation, invasion and angiogenesis and decreased apoptosis, which may predispose COPD patients to develop lung carcinogenesis.

Differential epigenetic mechanisms and downstream regulation in the non-tumor lungs of COPD patients

In the present thesis, expression levels of the different epigenetic markers and target genes in the non-tumor lungs were also analyzed, and it was found that miR-200b and miR-126 expression was more elevated in the non-tumor specimens of LC patients with COPD compared to those without any respiratory disease. In fact, studies have demonstrated that many miRNAs were differentially expressed in smokers with COPD compared to those smokers without it, suggesting that COPD *per se* seems to promote an alteration in microRNAs pattern (157). In addition, a recent study has reported that several microRNAs, including miR-126, are essential for COPD development and that those could also be associated with both staging and prognosis of the disease (294). It is widely known that alterations in histone modifications, as well as in DNA methylation, are strongly involved in both LC and COPD development and progression (295). In the present investigation, it was found that DNA methylation levels were increased in the non-tumor specimens of LC patients with COPD compared to those without underlying COPD. These results may imply that an alteration in the epigenetic pattern characterized by an upregulation of several microRNAs and methylated genes, occurs in COPD patients independently of the presence of tumor cells.

In the current thesis, it was also found that expression levels of the tumor suppressor genes *CDKN2A* and *P63* were greater in the non-tumor specimens of LC patients with COPD than in those without this disease. Although several studies have reported that *CDKN2A* and *P63* tend to be inactivated in lung tumors, and that increased levels of both markers are associated with better prognosis of LC patients, the results obtained in the current investigation could be explained by the fact that in the non-tumor specimens there are no tumor cells (296-298). Therefore, it could be suggested that some tumor suppressor genes may protect patients with COPD from lung tumor development. In the present thesis it was found that *SIRT-1* levels were higher in the non-tumor lungs of LC patients with COPD than in those without underlying COPD. Previously published results have reported that a reduction in *SIRT-1* levels was associated with apoptosis and lung tumor suppression, and additionally, other studies have observed that an upregulation of SIRT-1 was correlated with tumor promotion and poor prognosis of LC patients (299-302). However, several studies have also reported that *SIRT-1* expression was decreased in COPD patients compared to controls (303;304)**.** These controversial results obtained in the current thesis regarding to SIRT-1 expression, could be due to the lack of studies that analyze this marker in LC patients taking into account the presence of CODP or not, and also due to the fact that in this case the samples explored are non-tumor specimens that do not have cancer cells.

Taken together, the results obtained suggest that despite not having tumor cells, in LC patients with COPD, several epigenetic and downstream gene modifications also exist that could alter different cellular processes involved in lung tumor development such as cell proliferation and invasion.

Differential epigenetic mechanisms and downstream regulation in tumor lesions in LC patients with and without COPD

In the present thesis, it was found that in lung tumors compared to non-tumor specimens, expression levels of miR-451 and miR-126 were reduced in LC patients with COPD, while in both groups of patients those of miR-30a-30p were also reduced, and those of miR-210 were increased. In keeping with these findings, several investigations have reported that miR-451, miR-126 and miR-30a-30p downregulation is associated with an increase in cell proliferation and

metastasis in lung cancer cells (214;305;306). Additionally, it has also been found that miR-210 is overexpressed in the lung tumors of LC patients in later stages, suggesting that miR-210 may be strongly involved in lung tumor progression (285). Compared to non-tumor lungs, levels of miR-let7c were lower in tumor lesions of LC patients without COPD, whereas a rise of miR-let7c expression was found in the tumor lungs of those LC patients with COPD. Although studies have shown that reduced levels of miR-let7c in LC patients were associated with metastasis and cell invasion, the upregulation of miR-let7c observed in those patients with LC and COPD could be due to the fact that in those studies the presence of COPD was not taken into account, and suggests that underlying COPD may attenuate the effect of the tumor cells in those patients. Therefore, it seems that the deregulation in the expression of specific microRNAs contribute to lung tumor formation in LC patients with and without underlying COPD.

Expression levels of the downstream target genes *PTEN*, *MARCKS*, *FGFRL-1*, *SNAIL-1*, *P63* and *k-RAS* were lower in tumor lesions than in the non-tumor lungs only in patients with LC and COPD. On the other hand, expression levels of the downstream targets *TPM-1*, *TOM-1 CRK*, fibulin-2, *MIF* and *EFNA-3* were more augmented in tumor lesions than in the non-tumor lungs in patients with only LC. In addition, in the current thesis it was also reported that in the tumor lesions of both groups of patients, levels of the proliferation marker ki-67, the anti-apoptotic protein BCL-2, and the autophagy markers P62 and LC3B were greater than in the non-tumors, whereas expression levels of the inhibitor of cell growth *CDKN1A* were decreased. In fact, several studies have reported increased cell proliferation and invasion, as well as reduced levels of apoptosis in patients with lung tumors (307;308). However, on the contrary, in COPD patients lower levels of cell proliferation and higher expression of apoptotic markers have usually been found (210). The different results obtained in the current study are due to the fact that findings obtained in those investigations were in COPD patients without LC. Therefore, the alterations observed in the different biological mechanisms in both groups of patients, may be caused by having a lung tumor.

Taken together, the results obtained suggest that epigenetic modifications promote changes in the expression of several downstream targets, which in turn lead to alterations in cellular processes such as an increased cell proliferation, angiogenesis, autophagy, cell migration and adhesion, and reduced apoptosis, which contribute to lung tumor formation and progression in both groups of patients.

The results obtained in the current investigation could contribute to the clinical practice in LC patients, as epigenetic mechanisms may be used as potential biomarkers of LC diagnosis and prognosis, and they also could be targeted by different drugs so as to inhibit several pathways that contribute to cancer development. However, further studies with an increased number of patients, are needed in order to better understand epigenetic events and their downstream alterations in the different biological mechanisms involved in tumor growth and progression.

CONCLUSIONS

Oxidative and nitrosative stress, inflammatory profile and epigenetic regulation have been found to be differentially expressed in the lung tumors and in the blood of LC patients with underlying COPD. It seems that redox and nitric oxide imbalance and an increase of the Th1 inflammatory response, together with an upregulation of several microRNAs and DNA methylation, may predispose COPD patients to a higher risk of developing LC. Those epigenetic modifications promoted alterations in downstream targets and cellular processes such as increased cell proliferation, invasion and angiogenesis, which also contribute to lung tumor formation and progression in COPD patients.

Taken together, the findings obtained in the current thesis may contribute to establish potential new biomarkers for the early diagnosis of LC in patients with chronic respiratory conditions.

FUTURE PERSPECTIVES

Future research should aim to explore the potential contribution of the immune system to LC development in patients with underlying COPD, as Th1 and Th2 inflammatory responses are strongly influenced by the release of several cytokines by the immune cells. Therefore, exploring the profile of T and B cells, dendritic cells, neutrophils and NK cells, could help to determine disease progression, and to design immunotherapeutic strategies for LC treatment.

Moreover, future studies should also focus on investigating microRNAs expression and DNA methylation in the blood compartment of LC patients with underlying COPD, as blood extraction is a technique with no invasion, that could help to establish a panel of non-invasive biomarkers for LC diagnose in patients with chronic respiratory conditions. As several microRNAs have been found to be upregulated in the tumor lungs of LC-COPD patients, future studies could be based on the development of a mouse model with induced LC and COPD, in which the expression of those microRNAs could be inhibited with antisense oligonucleotides or with pharmacological agents, in order to assess if those could be used as potential therapeutic targets for LC treatment.

Finally, further research based on prospective screening studies, on a high number of LC patients, with and without COPD, should be followed up for many years. This is needed in order to identify biomarkers with high predictive value for LC development and progression in patients with underlying COPD.

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ADDENDUM

Apart from the scientific publications that are part of the current thesis, during the years that I have been working in the Muscle Wasting and Cachexia in Chronic Respiratory Diseases and Lung Cancer group, under the supervision of Dr. Esther Barreiro, I have had the opportunity to participate in other studies that have been also published in international journals:

A Chacón-Cabrera, **M Mateu-Jiménez**, K Langohr, C Fermoselle, E García-Arumí, AL Andreu, J Yélamos, **E Barreiro**. Muscle protein catabolism and phenotype in respiratory and limb muscles in PARP-1 and PARP-2 mice with lung cancer caquexia.
J Cell Physiol. 2017 Dec;232(12):3744-3761

M Mateu-Jiménez, C Fermoselle, F Rojo, J Mateu, R Peña. A J Urtreger, M J. Diament, E D Bal de Kier Joffé, L Pijuan, A Garcia de Herreros, **E Barreiro**. Pharmacological approaches in an experimental model of non-small cell lung cancer: effects on tumor biology.
Curr Pharm Des. 2016; 22(34):5300-5310

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A Chacon-Cabrera, C Fermoselle, AJ Urtreger, **M Mateu-Jimenez**, M Sandri, MJ Diament, ED Bal de Kier Joffé, **E Barreiro**. Pharmacological strategies in lung cancer-induced cachexia: effects on muscle proteolysis, autophagy, structure, and weakness.
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E Barreiro, C Fermoselle, **M Mateu-Jimenez**, A Sánchez-Font, L Pijuan, J Gea, **V Curull**. Oxidative stress and inflammation in the normal airways and blood of patients with lung cancer and COPD.
Free Radic Biol Med. 2013 Dec; 65: 859-871