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Role of inflammation and oxidative stress in the systemic manifestations of chronic respiratory diseases: lung cancer, chronic obstructive pulmonary disease, and bronchiectasis

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Role of inflammation and oxidative stress in the systemic manifestations of chronic respiratory diseases: lung cancer, chronic obstructive pulmonary disease, and bronchiectasis

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To my parents, my sister, my husband, and my daughter

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LIST OF ABBREVIATIONS

AIDS: Acquired immunodeficiency syndrome

APPs: acute-phase proteins

Argonaute 2: Ago2

CF: cystic fibrosis

COPD: chronic obstructive pulmonary disease

CRP: C-Reactive Protein

CT: computed tomography

DNA: deoxyribonucleic acid

EGFR: epidermal growth factor receptor

GPX: glutathione peroxidase

GSH: glutathione

H. Influenzae: Haemophilus Influenzae

HNE: hydroxy-2-nonenal

H_2O_2: hydrogen peroxide

IL: Interleukin

LC: lung cancer

MCP-1: monocyte chemotactic protein-1

MDA: malondialdehyde

miR: microRNA

miRNA: microRNA

mRNA: messenger RNA

NADPH: nicotinamide adenine dinucleotide phosphate

NSCLC: non-small cell lung cancer

OHdG: hydroxy-2'-deoxyguanosine

OSI: oxidative stress index

oxodG: dihydro-2´-deoxyguanosine

PR: pulmonary rehabilitation

pre-miRNA: precursor miRNA

P. Aeruginosa: Pseudomonas Aeruginosa

RISC: ribonucleic acid-induced silencing complex

RNA: ribonucleic acid

RNS: reactive nitrogen species

ROS: reactive oxygen species

RT-qPCR: quantitative reverse transcription polymerase chain reaction

SCLC: small cell lung cancer

SOD: superoxidase dismutase

TAS: total antioxidant status

TGF-β: transforming growth factor-β

TNF-α: tumor necrosis factor-α

TOS: total oxidative status

UTR: untranslated region

VEGF: vascular endothelial growth factor

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ABSTRACT

ABSTRACT

Background: Chronic respiratory diseases such as lung cancer (LC), chronic obstructive pulmonary disease (COPD), and bronchiectasis affect both lung structures and organs beyond the respiratory system such as bones, muscles, heart, and blood compartment. Systemic manifestations are as relevant as local (respiratory) in chronic respiratory diseases. Several biological mechanisms such as redox imbalance, inflammation, and microRNAs may be involved in the pathogenesis of systemic manifestation in chronic respiratory diseases.

Hypothesis: Oxidative stress, inflammation, and microRNAs may be differentially expressed in the systemic compartment (blood) of patients with different respiratory diseases: COPD, non-CF bronchiectasis, LC with and without COPD.

Objectives: In LC patients and non-LC controls: 1) To evaluate plasma levels of prooxidants, antioxidants, inflammatory markers, and the expression of microRNAs potentially involved in lung carcinogenesis of LC patients with and without COPD and non-LC controls. 2) To explore potential associations between biological and clinical variables of LC patients with and without COPD. In bronchiectasis patients and healthy controls: 1) To analyze plasma levels of inflammatory cells and molecules, prooxidants, and antioxidants of non-CF bronchiectasis patients and healthy controls. 2) To assess the relationships between biological and clinical variables of non-CF bronchiectasis patients.

Methods: Blood samples were obtained from all the study subjects: 1) 32 LC-only patients, 91 LC-COPD patients, and 45 non-LC control subjects, and 2) 30 non-CF bronchiectasis patients and 26 healthy controls. Body composition, lung

function, and blood parameters were analyzed in all study subjects of all the studies. Biological analysis: ELISA was used to assess the oxidative stress and inflammation markers in both studies. qRT-PCR was used to analyze microRNAs in LC patients and non-LC controls.

Results: 1) In LC-only patients compared to non-LC controls, plasma levels of GSH, TEAC, TGF-beta1, and the expression of miR-let7c were significantly increased, while those of miR-451 was significantly decreased. 2) In LC-COPD patients compared to non-LC controls, plasma levels of MDA-protein adducts, TEAC, TGF-beta1 and the expression of miR-let7c were significantly higher, while those of GSH and the expression of miR-451 were significantly declined. 3) In LC-COPD patients, plasma levels of MDA-protein adducts levels were significantly higher, while those of GSH and the expression of miR-210 were significantly declined compared to LC-only patients. 4) In non-CF bronchiectasis patients, plasma MDA-protein adducts, GSH, myeloperoxidase, CRP, ESR, fibrinogen, alpha-1 antitrypsin, IgA, and IgG levels were significantly higher compared to healthy controls.

Conclusions: Systemic oxidative and antioxidative markers such as MDA and GSH are differently expressed in LC patients with COPD, thus implying their contributions to the tumor pathogenesis of these patients. Non-CF bronchiectasis, on the other hand, showed an increased level of oxidant and antioxidant markers analyzed. Systemic inflammation takes place in the plasma of LC patients and non-CF bronchiectasis patients; these findings suggest that inflammation may be involved in the pathogenesis of these chronic respiratory diseases. Plasma miRNAs have been shown to be differently expressed in LC patients, specifically

miR-451, miR-let 7c, and miR-210; which suggests that those miRNAs may be surrogate markers of lung tumorigenesis that could help monitor patients in the clinics.

RESUMEN

RESUMEN

Introducción: Las enfermedades respiratorias crónicas como el cáncer de pulmón (CP), la enfermedad pulmonar obstructiva crónica (EPOC) y las bronquiectasias afectan tanto a las estructuras pulmonares como a órganos fuera del sistema respiratorio, como los huesos, los músculos, el corazón y el compartimento sanguíneo. Las manifestaciones sistémicas son tan relevantes como las locales (respiratorias) en las enfermedades respiratorias crónicas. Varios mecanismos biológicos, como el desequilibrio redox, la inflamación y los microARNs, pueden estar involucrados en la patogenia de las manifestaciones sistémicas en las enfermedades respiratorias crónicas.

Hipótesis: El estrés oxidativo, inflamación, y microARNs pueden expresarse diferencialmente en el compartimento sistémico (sangre) de pacientes con diferentes enfermedades respiratorias: EPOC, bronquiectasias no fibrosis quística (FQ), CP con y sin EPOC.

Objetivos: En pacientes con CP y controles sin CP: 1) Evaluar los niveles plasmáticos de marcadores prooxidantes, antioxidantes y marcadores inflamatorios, y la expresión de microARNs potencialmente implicados en la carcinogénesis pulmonar de pacientes con CP con y sin EPOC, y controles sin CP. 2) Explorar asociaciones potenciales entre variables biológicas y clínicas de pacientes con CP con y sin EPOC. En pacientes con bronquiectasias no FQ y controles sanos: 1) Analizar los niveles plasmáticos de células y moléculas inflamatorias, marcadores prooxidantes y antioxidantes de pacientes con bronquiectasias no FQ y controles sanos. 2) Evaluar las relaciones entre variables biológicas y clínicas de pacientes con bronquiectasias no FQ.

Métodos: Se obtuvieron muestras de sangre de todos los sujetos del estudio: 1) 32 pacientes con CP solo, 91 pacientes con CP-EPOC y 45 sujetos de control sin CP, y 2) 30 pacientes con bronquiectasias no FQ y 26 controles sanos. Se analizaron la composición corporal, la función pulmonar y los parámetros sanguíneos en todos los sujetos de todos los estudios. Análisis biológico: se utilizó ELISA para evaluar el estrés oxidativo y los marcadores de inflamación en ambos estudios. qRT-PCR se utilizó para analizar los microARNs en pacientes con CP y controles sin CP.

Resultados: 1) En los pacientes con CP-solo en comparación con los controles sin CP, los niveles plasmáticos de glutatión reducido (GSH), la capacidad antioxidante equivalente al trolox (TEAC), el factor de crecimiento transformante beta1 (TGF-beta1) y la expresión de microARNs (miR)-let7c aumentaron significativamente, mientras que los de miR-451 disminuyeron significativamente. 2) Pacientes con CP-EPOC en comparación con controles sin CP, los niveles plasmáticos de aductos de proteína malondialdehído (MDA), TEAC, TGF-beta1 y la expresión de miR-let7c fueron significativamente más altos, mientras que los de GSH y de miR-451 disminuyeron significativamente. 3) En pacientes con CP-EPOC, los niveles plasmáticos de MDA fueron significativamente más altos, mientras que los de GSH y de miR-210 disminuyeron significativamente en comparación con los pacientes con CP-solo. 4) En pacientes con bronquiectasias no FQ, los niveles de MDA, GSH, mieloperoxidasa, proteína C reactiva (PCR), velocidad de sedimentación globular (VSG), fibrinógeno, alfa-1 antitripsina, inmunoglobulina (Ig) A e IgG fueron significativamente más altos en comparación con los controles sanos.

Conclusiones: Los marcadores oxidantes y antioxidantes sistémicos como MDA y GSH se expresan de forma diferente en pacientes con CP-EPOC, contribuyendo así a la patogenia tumoral de estos pacientes. Las bronquiectasias no FQ, por otro lado, mostraron un aumento en el nivel de los marcadores oxidantes y antioxidantes analizados. La inflamación sistémica tiene lugar en el plasma de pacientes con CP y pacientes con bronquiectasias no FQ; estos hallazgos sugieren que la inflamación puede estar involucrada en la patogenia de estas enfermedades respiratorias crónicas. Se ha demostrado que los miARNs plasmáticos se expresan de manera diferente en pacientes con CP, específicamente miR-451, miR-let 7c y miR-210; lo que sugiere que esos miARNs pueden ser marcadores cruciales de la tumorigénesis pulmonar que podrían ayudar a monitorear a los pacientes en las clínicas.

1. INTRODUCTION

1. INTRODUCTION

The current thesis is about the systemic manifestations (blood), measured through inflammatory and oxidative stress markers in three highly prevalent chronic respiratory diseases: lung cancer (LC), chronic obstructive pulmonary disease (COPD) and non-cystic fibrosis (CF) bronchiectasis. Furthermore, microRNAs (miRNAs) expression profiles will be studied in LC patients with and without COPD. Control subjects have been analyzed in all the cases.

1.1 Features of chronic respiratory diseases

Chronic respiratory diseases are disorders involving the airways and other structures of the lung, mainly due to the interplay between host and environmental factors (such as cigarette smoke, secondhand smoke, indoor and outdoor air pollutants, allergens and occupational exposures) (1,2). Chronic respiratory diseases are one of the leading causes of morbidity and mortality worldwide, which carry an enormous socio-economic burden, particularly in developing countries (3). According to the Global Burden of Diseases Study 2017, the prevalence cases of chronic respiratory diseases were 545 million (4). Forecasting the years of life lost by 2040 shows a rising deaths toll by Noncommunicable diseases (include chronic respiratory diseases) due to population growth and aging (4–6). In addition to COPD and asthma, chronic respiratory diseases include occupational lung diseases, lung cancer, bronchiectasis, allergic rhino-sinusitis and fibrosis pulmonary (7). Although the trigger factors of each chronic respiratory disease are different, there are still some commonalities, such as lung inflammation and airway remodelling (8,9). In

the current thesis, three major respiratory conditions have been studied: lung cancer, COPD and bronchiectasis.

1.1.1 Lung cancer

Lung cancer (LC) is one of the most commonly diagnosed cancers with a high incidence, morbidity and mortality rate (10). The 5-year overall survival rate of LC is low (11). The late-stage diagnosis of LC is usually due to the absence of early clinical symptoms and the lack of suitable screening programs (12). LC is commonly classified as small cell lung cancer (SCLC), approximately 14% and non-small cell lung cancer (NSCLC), about 85% (11). NSCLC is further categorized into adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and others (11).

Tobacco smoke is the major risk factor for LC and is estimated to cause 90% of all carcinoma diagnoses (13). Ex-smokers still have an increased risk of LC, although tobacco-related risk decreases with cumulative abstinence duration (14). Other factors, including air contamination, ionizing radiation, radon, and occupational exposure (like silica dust, diesel exhaust, tar, soot, and asbestos), are crucial for developing LC (15,16). Genetic factors have also been proposed as the risk factors of LC, but the reason is most likely multifactorial and clear relationships have yet to be fully elucidated (17).

Screening at-risk individuals with low-dose computed tomography (CT) may become an effective strategy (18). However, despite its high sensitivity, the specificity of CT scanning in LC detection tends to be limited. In one study, 74% of participants were identified with lung nodules, while 4% had LC (19). Patients

with indeterminate lung nodules require follow-up studies to monitor growth. The imaging strategy is not enough as it delays the diagnosis, and the cost is high (20). Molecular biomarkers analysis (either in blood, sputum or urine) of high-risk patients may enhance early diagnosis, complete imaging approaches, and have immediate clinical benefit for LC detection. Thus, in the current thesis, we focus on the analysis of plasma biomarkers in LC patients.

1.1.2 Chronic obstructive pulmonary disease (COPD)

COPD is characterized by progressive airflow obstruction and the exposure of tobacco smoke or harmful particles in the airway and lungs of the patient. The estimated worldwide prevalence of COPD is 13.1%, with variations between 11.6% and 13.9% among different continents (21). The World Health Organization predicts that COPD will be the third leading cause of death by 2030 (22). Emphysema (parenchymal destruction) and chronic bronchitis (inflammation and small airway obstruction) are the two main phenotypes of COPD, and most patients have both in combination. The main symptoms of chronic bronchitis are chronic dyspnea, cough, sputum, and fatigue; the symptoms of emphysema include shortness of breath, wheezing y chest tightness (23). Tobacco smoking is the most important factor of COPD. Besides smoking, various causes contribute to COPD development, such as environmental exposure, wood and other fuels for cooking, chemical fume exposures, and genetic factors (like alpha-1 antitrypsin deficiency). Widespread application of spirometry in people with risk factors and respiratory symptoms (such as people exposed to tobacco smoke, occupational dust, biomass fuels and family history of alpha-1 antitrypsin deficiency) is recommended (24). Spirometry is used to avoid misdiagnosis and

assists in assessing the severity of the airflow limitation. Removal of risk factors, treatment with inhaled bronchodilators, together with inhaled corticosteroids and other pharmacological or non-pharmacological agents, can prevent exacerbations and airflow obstructions (25).

1.1.3 Relationships between LC and COPD

The relationships between LC and COPD have been previously reported elsewhere (15,26–33). Both LC and COPD are closely related on the epidemiologic, genetic and pathophysiological basis and share several common risk factors like tobacco smoke, chronic inflammation, gene alterations, and environmental exposures (34).

Cigarette smoking is a common risk factor for LC and COPD (35). Cigarette smoking induces oxidative stress, which increases the activation of the immune system and the inflammatory response, promoting epigenetic changes, leading to both LC and COPD development (30–32,36).

Furthermore, cytokines and growth factors such as tumor necrosis factor-α (TNF-α), Interleukin-10 (IL-10), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and epidermal growth factor receptor (EGFR) have also been shown to promote tumor growth and metastasis in LC patients with underlying respiratory conditions (30,32).

In the current thesis, several molecular mechanisms that may link LC pathogenesis in patients with underlying respiratory diseases have been studied, such as inflammation, oxidative stress and microRNA expression profiles.

1.1.4 Non-cystic fibrosis (CF) bronchiectasis

René Laënnec gave the first description of bronchiectasis in 1816 (37). Later the term bronchiectasis was introduced by Swaine' translation and commentary of Hasse's monograph (38). There are two types of bronchiectasis: CF bronchiectasis and non-CF bronchiectasis (39). CF is a progressive genetic disorder characterized by the production of thick, sticky mucus that can damage the lungs and other organs of the body (40). Non-CF bronchiectasis is characterized by a progressive and permanent dilation of the airways with associated chronic cough, expectoration and dyspnea (41). The underlying causes of non-CF bronchiectasis are established according to Spanish guidelines (42) (table 1).

Table 1. Etiology of non-CF bronchiectasis

Idiopathic	Unknown etiology
Post-infectious	Non-tuberculous mycobacteria Tuberculosis Pneumonia Childhood infections
Associated with chronic respiratory disease	COPD Asthma
Immune deficiencies	Immunoglobulin deficiency Acquired immunodeficiency syndrome (AIDS)
Hypersensitivity	Allergic bronchopulmonary aspergillosis
Genetic disorders	Primary ciliary dyskinesia Young's syndrome Alpha-1 antitrypsin deficiency
Post-inflammatory pneumonitis	Gastroesophageal reflux disease/aspiration
Associated with systemic disorders	Rheumatoid arthritis Lupus Sarcoidosis Recurrent polychondritis Yellow nail's syndrome Panbronchiolitis

Abbreviation: COPD, chronic obstructive pulmonary disease. Adapted from Martínez-García *et al*. 2018 (42).

The pathogenesis of non-CF bronchiectasis is proposed by Cole' vicious circle (43). This hypothesis raises the existence of a vicious circle after the appearance of an initial event, such as infection or primary genetic involvement, which compromises the mucociliary clearance mechanism. This would cause the mucus and bacteria to remain in the bronchial tree for a longer time, with the possibility of producing a selection of the most virulent strains, which would damage the ciliary epithelium (43,44). The resulting chronic inflammatory process would cause difficulty in clearing the bronchial secretions, facilitating recurrent infections and structural damage with the appearance of bronchiectasis, which predisposes to new infections, thus closing the vicious circle (Figure 1).

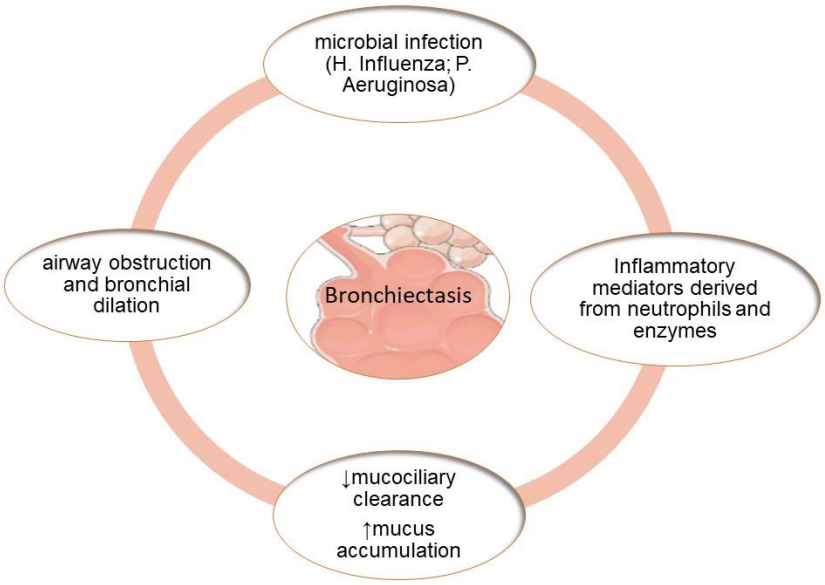

Figure 1. The model describes the pathogenesis of bronchiectasis. Microorganism infections can cause an inflammatory response, which produces a deterioration of mucociliary clearance and retention of airway phlegm, making the airways dilatation and susceptible to infection, which can become persistent. Abbreviations: H. Influenzae, Haemophilus Influenzae; P. Aeruginosa, Pseudomonas Aeruginosa. Adapted from Cole 1986 (43).

Over the last decade, with the wide application of high-resolution tomography, the prevalence of bronchiectasis has been rising, with an estimated incidence of

42 to 566 cases per 100,000 people (42). The prevalence has increased over the past decade, and the disease is more common in older people and females. A recent study reported that high incidence and prevalence of bronchiectasis might be associated with the elderly population (45). Poor lung function is related to severe exacerbations, colonization of *Pseudomonas. aeruginosa*, and increased sputum volumes (46). In addition to exacerbations and daily symptoms, psychological factors such as anger, depression, anxiety and other social issues may worsen the health-related quality of life. For these reasons, target treatment should focus on underlying etiology, airway clearance, prevention of infections and inflammations.

1.2. Systemic manifestations in chronic respiratory diseases

Chronic respiratory diseases affect both lung structures and organs beyond the respiratory system such as bones, muscles, heart and blood compartment. The pathogenesis of these chronic respiratory diseases is caused by mediators and proteins, including oxidants, cytokines, chemokines and growth factors (47). Systemic manifestations are defined as those effects occurring in the whole body rather than in one part (48). Chronic respiratory diseases relate to airway abnormalities and contribute to extrapulmonary systems, including systemic inflammation and oxidative stress, skeletal muscle weakness, cardiovascular compromise, and osteoporosis (49–53). However, whether systemic manifestations are directly and worsened by lung alterations remain to be elucidated. Oxidative stress and inflammation may be related to the mechanisms of the development of systemic effects of chronic respiratory diseases (32,54). A mechanism of the present thesis has been postulated as follows (Figure 2).

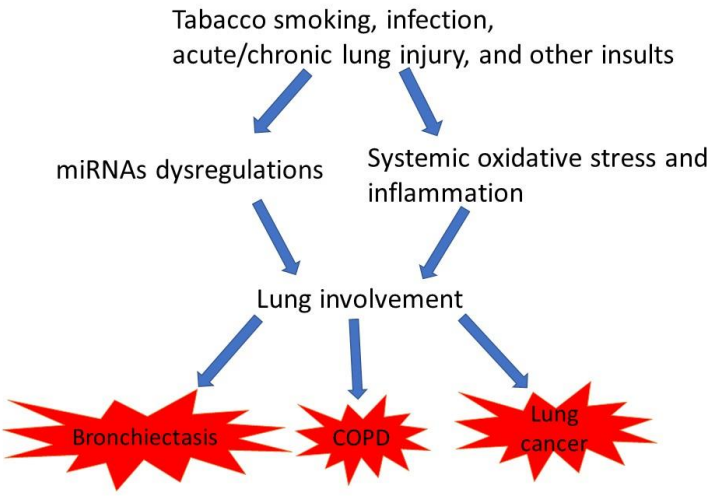

Figure 2. Schema of the postulated mechanism of the current thesis.

1.2.1 Inflammation

Inflammation is the immune system's response to harmful stimuli such as pollutants, pathogens, irritants, microbes and damaged host cells, which is an essential component of various chronic respiratory diseases (55). The inflammatory response is classified as local or systemic. Local inflammation in the lungs plays a significant role in affecting airway remodelling and parenchymal destruction (56). Systemic inflammation results from the release of inflammatory cytokines and activation of the immune system (51). The origin of systemic inflammation in chronic respiratory diseases remains unknown. However, the hypothesis with the most supporting evidence is the "spill-over" of inflammatory mediators from the lung to the circulation, indicating a link between these inflammatory processes (57).

The systemic inflammatory response involves a complex network of many cell types. Neutrophils, monocytes and lymphocytes are the first cells becoming activated in the blood (51). Once inflammation begins, neutrophils are the first

cells that cross the blood vessels to enter an inflamed tissue (58). Circulating monocytes are recruited into the lungs by chemokine factors such as growth-related oncogene-α and monocyte chemotactic protein-1 (MCP-1). Then they differentiate into macrophages and phagocytose pathogens (59). In inflammation, the first step of recruitment of leukocytes in infected tissues is the dilation of blood vessels. The slower blood flow allows leukocytes to interact in large numbers with the endothelial cells lining the blood vessels; afterwards, leukocytes cross the endothelial wall to tissues (60). In the later stage of inflammation, other leukocytes such as eosinophils and lymphocytes also enter the infected site (61).

The production and release of acute-phase proteins (APP), cytokines, chemokines, and growth factors contribute to systemic inflammation (62). APPs comprise the early inflammatory reactions (63). APPs is commonly classified as negative and positive forms: negative APPs are albumin, transthyretin, transferring and transcortin; positive APPs are c protein reactive (CRP), fibrinogen, alpha-1 antitrypsin, ceruloplasmin, ferritin, haptoglobin, and D-dimer protein (64). The circulating inflammatory mediators' response is characterized by an initial release of cytokines like TNF-α and IL-1β and followed by the release of other cytokines (IL-6, IL-8, IL-10, MCP-1) (65). The growth factors such as VEGF and TGF-β have shown to involve in systemic inflammation (32).

As it is still uncertain whether systemic inflammatory markers are a spill-over from peripheral lung, there is great interest in identifying the nature of systemic inflammation because it may help predict clinical outcomes and therapeutic responses.

1.2.1.3 Systemic inflammation in LC

Over the years, it has been increasingly evident that cancer-related inflammation, especially the host systemic inflammation response, is closely related to the development and progression of LC (66–69). Systemic inflammation could impact LC via interaction with the tumor immunological microenvironment (70). The decline of plasma levels TGF-β1 level during radiotherapy was correlated with a better prognosis in NSCLC patients. Moreover, Bruno *et al*. (71) showed overexpression of TGF-β1 in NSCLC patients. Notably, CRP, a systemic inflammatory marker, has been associated with the risk of the development of LC (72). The same research group (73) confirmed that serum CRP was a prognostic marker in advanced and resectable NSCLC. Interestingly, serum levels of IL-6 and IL-8 were significantly greater in LC patients compared with healthy controls, and IL-8 levels have been widely associated with LC risk (74).

1.2.1.4 Systemic inflammation in COPD

Systemic levels of IL-1β, IL-1α, TNF-α, IL-6 and IL-8 were significantly increased in COPD patients than in the healthy subjects (75). In addition, it has been determined that IL-1β and IL-17 were raised in the serum of COPD patients (76). It was shown that patients with COPD had higher CRP serum levels than the control group (77). Importantly, blood levels of VEGF and TGF-β were significantly higher in COPD patients than in healthy controls (32). Furthermore, COPD patients showed a significant increase in blood IL-1β levels compared to healthy controls (32).

1.1.1.5 Systemic inflammation in non-CF bronchiectasis

In bronchiectasis, both infection and inflammation are critical in Cole's “vicious cycle”. Recurrent or persistent infections of airway mucosa may stimulate a progressive neutrophilic inflammation response and host immune defense; which further impair mucociliary clearance and promote airway obstruction, thus resulting in bronchial dilatation, ongoing bacterial infection and persistent inflammation (43). The vicious circle highlights the importance of controlling the inflammatory response. It has been suggested that systemic inflammation may be a phenomenon as the spill-over of local airway inflammation. However, it is impossible to exclude the distant production of inflammatory markers. Systemic inflammation is associated with disease severity and bacterial colonization in non-CF bronchiectasis. Short- and long-term antibiotic treatments reduce systemic inflammation markers (78).

Neutrophils play an essential role in the early phases of inflammation. The increase of total neutrophil levels, elevated levels of CRP, and plasma cytokines have been reported in patients with non-CF bronchiectasis (79,80). Total neutrophil levels are significantly associated with disease severity and bacterial colonization in non-CF bronchiectasis patients (79). Moreover, Chalmers *et al*. demonstrated that neutrophil elastase of airway samples was a potential predictor of the development of bronchiectasis (81). TNF-α is a proinflammatory cytokine produced by monocytes and macrophages (82). It has been reported as a marker of systemic inflammation in stable non-CF bronchiectasis, and high plasma levels of TNF-α are associated with worse disease severity (83). Moreover, serum prealbumin and albumin concentrations correlated significantly

to disease severity, clinical symptoms and health status (84). Higher levels of CPR are associated with the risk of severe exacerbations (85). In addition, elevated serum levels of desmosine are strongly associated with an increased risk of severe exacerbation. The serum desmosine is a promising biomarker of future mortality and cardiovascular risk in bronchiectasis, shown in a study of 433 patients with a median follow-up time of 61.4 months (86). Of note, bronchiectasis patients with primary immunodeficiency have exaggerated IL-17 responses. And elevated serum fibrinogen is associated with worse lung function and *Pseudomonas* colonization (53). Increases in circulating levels of inflammatory cytokines such as IL-6 and IL-10 were described in bronchiectasis patients (87). There is evidence that a faster decline of lung function was associated with increased systemic inflammation in stable bronchiectasis patients (46).

Controlling the inflammatory response may be as important as targeting the bacteria infection. Physiotherapy achieves it by improving mucociliary clearance, antibiotics achieve it by killing bacteria, and the following approach is to control inflammation directly.

1.2.2 Redox imbalance

Oxidative stress is defined as an imbalance between reactive oxygen/nitrogen species and the antioxidant capacity of the organism to respond. This imbalance damages essential biomolecules such as proteins, carbohydrates, lipids, and nucleic acids (88). It alters cellular processes, including the destruction of the cell membrane, blocking the action of major enzymes, affecting cellular respiration and repressing gene expression (89,90).

1.2.2.1 Sources of oxidative stress

The airways are vulnerable to oxidative stress due to their interchange between the body and the environment, exposing them to various oxidants. Air pollutants include tobacco smoke, particulate matter, ozone or nitrogen dioxide from photochemical smog, fossil and biomass fuels, and industrial sources (91). Although the nose and upper respiratory tract effectively clear larger particles, tiny particles can access the lower respiratory tract and further increase airway oxidation and inflammation (14,15). Free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS). Higher levels of ROS/RNS cause cell and tissue injury, resulting in the development of respiratory diseases (92,93). Free radicals are produced as the result of the influence of exogenous factors (cigarette smoke, pollution, ionizing radiation, ultraviolet radiation, particulate matter, pesticides and heavy metals) or endogenous factors (mitochondria, peroxisomes, and other enzymatic systems) (90) (Figure 3).

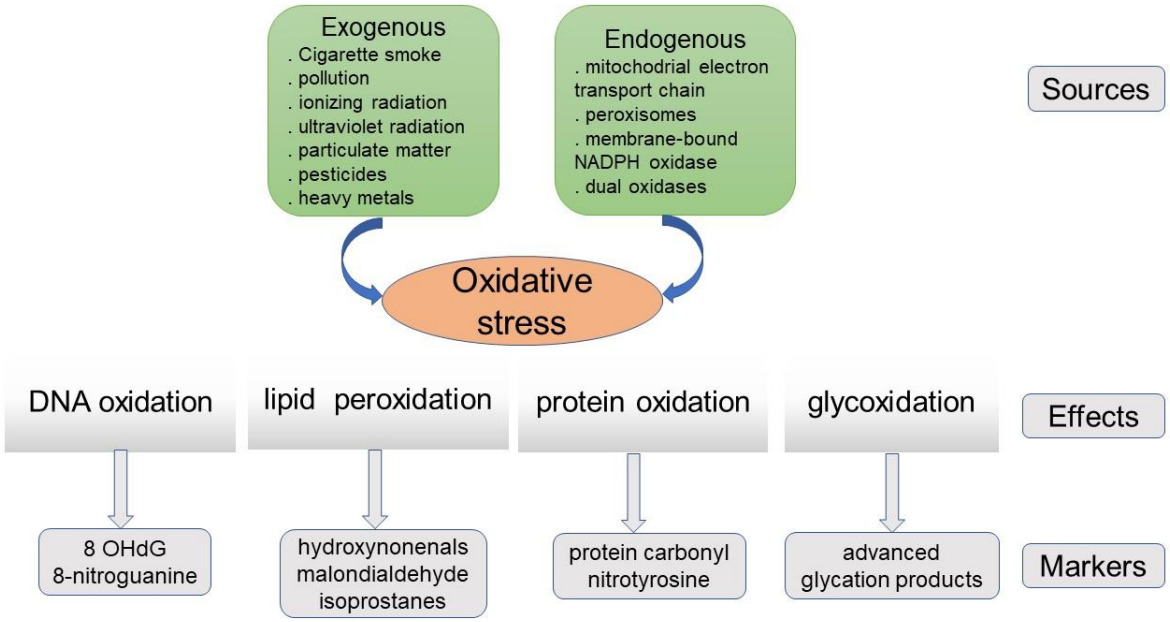

Figure 3. Schematic representation of the sources of oxidative stress and their effects.

Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; DNA, deoxyribonucleic acid. Adapted from Sharifi-Rad *et al.* 2020 (90).

Mitochondria are the main intracellular sources of ROS. The mitochondrial respiratory chain consists of five complexes, namely complexes I, II, III, IV, V (94). The decrease of mitochondrial respiratory chain complex I activity leads to the formation of free radicals, which leads to the productions of superoxide and nitric oxide by neutrophils and macrophages (90). These free radicals also promote the relaxation of vascular smooth muscle, leading to vasodilation and increasing the blood flow to inflammatory sites (95). Currently, oxidative stress is proposed as an inflammation-inducing potential, with the implication in developing chronic pathologies at the systemic level (96).

Oxidative stress can be measured indirectly by detecting/quantifying the levels of mitochondrial DNA oxidation, lipid peroxidation and protein oxidation rather than a direct measurement of ROS. These oxidative stress markers are more persistent than ROS (97,98).

Mitochondrial DNA is subject to oxidative stress-related damage, and is responsible for altered mitochondrial expression and somatic mutations (99). 8-hydroxy-2'-deoxyguanosine (8-OHdG) is an essential marker of DNA oxidative damage (100).

Lipid peroxidation is a metabolic process of oxidative degradation of lipids (101). Malondialdehyde (MDA) is the most common lipid peroxidation product with a high potential for mutagenicity and may generate point mutations in tumor suppressor genes (102). 4 hydroxy-2-nonenal (4-HNE) is the most toxic

secondary product of lipid peroxidation. 4-HNE have been found to be associated with a reduction in cell proliferation (103).

1.2.2.2 Antioxidant defense systems

The human body has integrated antioxidant systems consisting of dietary antioxidants, enzymatic and non-enzymatic antioxidants (104) (Table 2).

Table 2. Enzymatic and non-enzymatic antioxidants classification.

Enzymatic antioxidants	Non-enzymatic antioxidants
Superoxidase dismutase (SOD)	Glutathione reductase
Glutathione peroxidase	Vitamin C
Catalase	Vitamin E
Glutathione transferase	Carotenoids
Thioredoxin	Flavonoids
Peroxiredoxin	Uric acid

Adapted from Birben *et al.* (104).

Enzymatic antioxidant molecules include SODs, catalase and glutathione peroxidase (GPX) (91). The SODs, which play an essential role in ROS metabolism, catalyze the dismutation of superoxide radicals ($O2^{\bullet-}$) into molecular oxygen and hydrogen peroxide (H_2O_2). H_2O_2 is removed by glutathione peroxidase, catalase and peroxiredoxin, thus maintaining the redox homeostasis (105). Low expression of SOD leads to the accumulation of superoxidase radicals and can cause cell apoptosis (106). Over-expression of SOD1 can catalyze H_2O_2 to produce hydroxyl radical, which explains the biological damage related to the increase of ROS production (107,108).

Glutathione peroxidase catalyzes the reduction of lipid hydroperoxides and hydrogen peroxide and removes peroxyl radicals from various peroxides using reduced glutathione (GSH) as an electron donor (109).

Non-enzymatic antioxidant molecules are represented by reduced glutathione (GSH), uric acid, nicotinamide adenine dinucleotide phosphate (NADPH),

vitamins E and C, flavonoids and lipid acid and other antioxidants (91). The reduced GSH is considered one of the most essential ROS scavengers, which plays a critical role in controlling signalling processes and detoxifying some exogenous substances and heavy metals (110).

1.2.2.3 Oxidative stress in LC

Oxidative stress is closely related to carcinogenesis in the form of ROS. In recent years, it has been found that ROS accumulation can induce apoptosis of LC cells, and scavenging ROS in cells can prevent cancer cell death. It is considered that oxidative stress plays a vital role in the occurrence and development of tumors (111).

8-dihydro-2´-deoxyguanosine (8-oxodG) is considered one of the most important markers of DNA oxidation in biological samples. Higher levels of 8-oxodG were found in LC patients compared with healthy subjects (112). Apart from DNA oxidation, enzymes can also oxidize lipids, further increasing the risk of malignancy. MDA is primarily found in body fluid, and it has been shown that serum levels of MDA were higher in LC patients than in the control group (113).

Xiang *et al.* (114) showed that significant differences were found in the serum oxidative stress parameters (total antioxidant status (TAS), total oxidative status (TOS) and oxidative stress index (OSI)) between NSCLC patients and the healthy group. Suggesting that oxidative stress might cause the occurrence of LC.

1.2.2.4 Oxidative stress in COPD

Blood was the most studied as a biological source of oxidative stress due to its non-invasive accessibility. MDA is widely used to monitor oxidative stress. Several studies have revealed that blood concentrations of MDA were significantly higher in COPD patients than in healthy controls (115–117). Interestingly, the reduced GSH levels were significantly lower in patients with COPD than in healthy controls (118). It has been demonstrated that 8-OHdG levels were higher in patients with acute exacerbation of COPD compared to control groups (119). Systemic levels of protein carbonylation were significantly higher in COPD patients compared to non-COPD controls (32).

1.2.2.5 Oxidative stress in non-CF bronchiectasis

An exaggerated neutrophilic involvement in infection is critical in the development of non-CF bronchiectasis (46). The pathogens activate neutrophils, inducing oxidative stress (120,121). Several studies had demonstrated that bronchiectasis patients exhibited high levels of ROS in plasma and exhaled breath condensate (120,122). Olivera *et al*. showed an increase of blood 8-isoprostane levels from baseline at six months in both pulmonary rehabilitation (PR) alone patients and PR with nutritional support patients (123). In another study, plasma biomarkers (catalase activity and 8-isoprostane) were also significantly higher in non-CF bronchiectasis patients compared to control subjects (120). A recent study revealed that plasma carbonylated proteins and superoxide anions levels were significantly higher in bronchiectasis patients than in control subjects (87).

1.2.3 Gene expression of pathways involved in lung tumorigenesis in COPD

Both inflammation and oxidative stress alter the cells' redox status, promoting genomic instability and leading to epigenetic changes (124). Epigenetic regulations refer to a series of biological processes that influence gene expression, including DNA methylation and histone modification and non-coding RNAs (ribonucleic acids) such as microRNAs (miRNAs)-mediated processes (125). The expression and function of microRNA are regulated by many factors and epigenetic networks, including DNA methylation and histone modification mechanisms. In addition, microRNA itself can regulate the key enzymes driving epigenetic modification and have a crucial impact on cell biology (125).

LC is the consequence of multiple genomic alterations that accumulate throughout life (126). Patients with LC and COPD shared similar genetic susceptibility (127). It is generally believed that epigenetic changes play an essential role in lung tumorigenesis by activating oncogenes and/or blocking tumor suppressor genes. Understanding the molecular modifications is of great value for providing a better prognostic molecular signature to develop personalized therapeutic. Genomic medicine would compensate for the LC oncogenesis investigations (128).

1.2.3.1 microRNAs

MiRNAs are small, non-coding, single-stranded RNAs, usually about 19-25 nucleotides. MiRNAs regulate gene expression by either degrading the target messenger RNA or by inhibition of protein translation (129,130). The genome is initially transcribed by RNA polymerase II, generating primary miRNAs and processing them into precursor miRNAs (pre-miRNAs) by enzymes Drosha (131).

Then pre-miRNAs are transferred to the cytoplasm involving Exportin-5, and further processed into miRNAs duplex strands through Dicer nuclease. The selected guide strand becomes the mature miRNA, and loads into the RNA-induced silencing complex (RISC) via argonaute 2 (Ago2). And the passenger strand is degraded (130,132). The RISC complex can target complementary sites in the 3'-untranslated region (3'-UTR), thereby leading to gene silencing by degrading the target messenger RNA (mRNA) or inhibiting mRNA translation into protein (133).

Over the last two decades, miRNAs gained broad scientific interest because they perform essential functions in various processes such as cellular proliferation, differentiation, apoptosis and tumorigenesis (134). Cell proliferation is a natural process in which the number of cells is increased, and is defined as a result of the balance between cell divisions and cell differentiation (110). Several studies have demonstrated that miRNAs regulate cell proliferation (135,136). Cellular differentiation is the process in which cells become specialized as the tissues/organs develops to perform a specific function. miRNAs are a crucial player in regulating cell differentiation (137). Apoptosis is a programmed cell death in which a series of molecular processes in the cell leads to its death. Several miRNAs are involved in the apoptosis pathways (138). These miRNAs regulate the expression of one-third of human genes. This remarkable regulation of gene expression is an independent feature that distinguishes miRNA from other RNAs, and may become a new tumor biomarker and potential therapeutic agent (139).

1.2.3.1.1 miRNAs in LC

As we know, lung biopsies are the golden standard for diagnosing suspicious pulmonary nodules; however, the procedure is challenging to perform or may cause significant complications. Therefore, circulating (plasma, serum and sputum) miRNAs may be novel promising diagnostic biomarkers because of the non-invasive nature of the collection, remarkable stability after multiple freeze-thawed processes or extended storage at room temperature (140,141). microRNAs are detectable quantitatively with simple assays (like quantitative reverse transcription polymerase chain reaction (RT-qPCR)) that are suitable for clinical practice (142).

MicroRNA (miR-21) is an oncogenic miRNA and can regulate the development of lung cancer (143). Plasma miR-21 levels were significantly greater in the early-stage NSCLC patients compared to healthy controls. Besides, the plasma levels of miR-21 of NSCLC patients were significantly declined following surgery (144). Wei *et al*. (145) revealed that plasma miR-21 levels could be a diagnostic biomarker of NSCLC. Moreover, the plasma expression levels of miR-21 in stage III-IV patients were higher than those in stage I-II patients. The present research also revealed that the sensitivity and specificity of plasma miR-21 levels in the diagnosis of NSCLC patients were 76.2% and 70%, respectively.

MiR-451 is one of the most critical miRNAs involved in lung cancer progression (146,147). The low expression level of miR-451 correlated with shorter overall survival of NSCLC patients (148). Besides, the expression of miR-451 in invasive adenocarcinoma was significantly lower than in adenocarcinoma in situ. Lower expression of miR-451 also correlated with poor prognosis for NSCLC (149).

In lung cancer, miR-210 was previously identified to be upregulated in plasma and serum samples (150–153). Shen *et al*. (154) determined that miR-210 (together with miR-126, miR-21 and miR-486-5p) might serve as surrogate plasma markers for NSCLC patients, which could distinguish lung cancer from pulmonary nodules. In addition, it could accurately identify the stage I of lung cancer patients, and would early detect lung cancer in plasma samples. Another study confirmed that serum miR-210 was significantly higher in NSCLC patients than healthy controls, and significant associations were seen between serum miR-210 and clinical stages (155).

Chen *et al*. described ten serum miRNAs (miR-145, miR-221,miR-223, miR-20a miR-24, miR-320, miR-25, miR-199a, miR-222 and miR-152) that could distinguish NSCLC patients from healthy controls (156).

Plasma miRNAs may be important markers in LC screening trials (157). Boeri *et al*. (158) reported that 15 plasma miRNAs expressions could distinguish patients 1-2 years before CT-detection from the control group. Later, the same research group (159) analyzed plasma miRNAs of 69 LC patients and 870 control subjects in two arms. A 24 miRNAs signature classifier for LC detection had a sensitivity of 87% and specificity of 81%for both arms. The present research also revealed that plasma miRNAs could reduce the false-positive results of low-dose CT from 19.6% to 3.7%.

1.2.3.1.2 miRNA in COPD and LC-COPD

Blood miRNAs have been implicated in COPD due to its high stability and easy detection (160). Plasma miRNAs have been suggested as promising biomarkers

of COPD (161). Importantly, miR-126 and miR-29c were upregulated in COPD patients compared to healthy controls (162). Additionally, miR-106b-5p and miR-125a-5p were upregulated in COPD patients compared to control groups (163). Furthermore, miR-106b-5p was found to be negatively correlated with the severity of COPD (163). It has been shown that specific blood miRNA profiles (miR-675, miR-93, miR-513b, miR-1224-3p) could distinguish LC from COPD (164). In COPD patients, mRNAs alterations may precede the progress of LC. In tumor specimens, expression levels of miR-210, miR-let7c were significantly higher in LC-COPD patients than in LC patients (36). In bronchoalveolar lavage samples of LC-COPD patients, 10 miRNAs (miR-15b, miR-132, miR-145, miR-212, miR-223, miR-342-5p, miR-422a, miR-423-5p, miR-425 and miR-486-3p) were upregulated compared with healthy controls (165). To date, the profiles of miRNAs expression levels in the blood of LC-COPD patients have not yet been established. In the current thesis, the expression of blood miRNAs of LC-COPD patients compared to LC-only patients and healthy controls have been analyzed.

2. HYPOTHESIS

2. HYPOTHESIS

Systemic manifestations are as relevant as local (respiratory) manifestations in chronic respiratory diseases. Several biological mechanisms such as redox imbalance, inflammation, and microRNAs may be involved in the pathogenesis of systemic manifestation in chronic respiratory diseases. Our hypothesis was to explore whether oxidative stress, inflammation, microRNAs are differentially expressed in the systemic compartment (blood) of patients with different respiratory diseases: COPD, bronchiectasis, lung cancer with and without COPD. Control subjects have been analyzed in all the cases.

3. OBJECTIVES

3. OBJECTIVES

3.1 Main Objective

To explore oxidative stress, inflammation and microRNAs in the systemic compartment (blood) of patients with COPD, lung cancer with and without COPD, and bronchiectasis compared to control subjects.

3.2 Secondary objectives

1. To evaluate plasma levels of the expression of microRNAs potentially involved in lung carcinogenesis, prooxidants, antioxidants and inflammatory markers of LC patients with and without COPD and non-LC controls. To explore potential associations between biological and clinical variables of LC patients with and without COPD.

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

Study #1. Systemic Profiles of microRNAs, Redox Balance, and Inflammation in Lung Cancer Patients: Influence of COPD.

2. To analyze plasma levels of inflammatory cells and molecules, prooxidants and antioxidants of bronchiectasis patients and healthy controls. To assess the relationships between biological and clinical variables of bronchiectasis patients.

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

Study #2. Do Redox Balance and Inflammatory Events Take Place in Mild Bronchiectasis? A Hint to Clinical Implications.

4. COMPENDIUM OF PUBLICATIONS

4.1 Article 1

Title:

Systemic Profiles of microRNAs, Redox Balance, and Inflammation in Lung Cancer Patients: Influence of COPD.

Authors:

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Systemic Profiles of microRNAs, Redox Balance, and Inflammation in Lung Cancer Patients: Influence of COPD

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Abstract: Lung cancer (LC) risk increases in patients with chronic respiratory diseases (COPD). MicroRNAs and redox imbalance are involved in lung tumorigenesis in COPD patients. Whether systemic alterations of those events may also take place in LC patients remains unknown. Our objectives were to assess the plasma levels of microRNAs, redox balance, and cytokines in LC patients with/without COPD. MicroRNAs (RT-PCR) involved in LC, oxidized DNA, MDA-protein adducts, GSH, TEAC, VEGF, and TGF-beta (ELISA) were quantified in plasma samples from non-LC controls (n = 45), LC-only patients (n = 32), and LC-COPD patients (n = 91). In LC-COPD patients compared to controls and LC-only, MDA-protein adduct levels increased, while those of GSH decreased, and two patterns of plasma microRNA were detected. In both LC patient groups, miR-451 expression was downregulated, while those of microRNA-let7c were upregulated, and levels of TEAC and TGF-beta increased compared to the controls. Correlations were found between clinical and biological variables. A differential expression profile of microRNAs was detected in patients with LC. Moreover, in LC patients with COPD, plasma oxidative stress levels increased, whereas those of GSH declined. Systemic oxidative and antioxidant markers are differentially expressed in LC patients with respiratory diseases, thus implying its contribution to the pathogenesis of tumorigenesis in these patients.

Keywords: LC and COPD; microRNAs; prooxidants and antioxidants; GSH; inflammatory cytokines; associations between clinical and biological variables

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1. Introduction

Lung cancer (LC) is still a leading cause of cancer-related mortality worldwide. Several etiologic factors contribute to LC, among which chronic obstructive pulmonary disease (COPD) is a major contributor [1–4]. In patients with COPD, particularly in emphysema phenotype, LC development was five times greater than in smokers with no COPD [4,5]. Recently, our group and others have demonstrated the implications of relevant biological mechanisms in the increased LC predisposition seen in patients with COPD [6–13].

Adaptation to environmental factors and tumorigenesis are mediated through many different biological events including epigenetics. Epigenetic control of cellular processes includes DNA methylation, histone acetylation and methylation, and chromatin remodeling in tumor development and progression [14]. Furthermore, non-coding single-stranded RNA molecules (microRNAs) have also been shown to regulate the cellular processes

involved in lung tumorigenesis, such as cell proliferation and invasion, apoptosis, angiogenesis, and adaptation to hypoxia [15–17]. Moreover, patterns of microRNA expression may also be used in clinics, given their prognosis value [16,18]. In a previous study from our group [13], expression levels of the microRNAs miR-21, miR-200b, miR-210, and miR-let7c were increased in lung tumor samples from non-small cell LC (NSCLC) patients with COPD compared to those with no COPD. Whether levels of the same microRNAs may be differentially expressed in the plasma of LC patients with and without COPD remains to be seen.

Increased oxidative stress and inflammatory events have also been shown to contribute to the greater predisposition of patients with COPD to develop lung tumors [19]. In this study [19], a differential expression profile of oxidative stress and inflammatory markers was found in the blood and tumors of patients with LC-COPD compared to patients with no COPD. Importantly, increased oxidative stress may also trigger the expression of several microRNAs under pathologic conditions [20].

Several cytokines and growth factors may also mediate lung tumorigenesis in patients with COPD [21,22]. Cellular processes such as apoptosis, repair, and angiogenesis can be mediated through the action of inflammatory molecules [21,22]. Furthermore, vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-beta have also been shown to promote lung tumorigenesis through increased tumor growth and metastasis in patients with underlying respiratory diseases [23–25].

Hence, we hypothesized that a differential expression profile of microRNAs known to be involved in lung tumorigenesis may be identified in the blood compartment of patients with LC and underlying COPD compared to those without COPD. Elucidation of those biological events may add insight into the mechanisms that render COPD patients more prone to develop LC. Moreover, associations with oxidative stress and inflammatory markers were also assessed. Thus, our objectives were that, in plasma samples from LC patients with and without COPD, the following mechanisms were explored: (1) expression levels of microRNAs known to be involved in lung carcinogenesis; (2) redox balance, prooxidant and antioxidant markers; (3) VEGF and TGF-beta 1 protein levels; (4) relationships between clinical and biological variables; and (5) potential associations between expression levels of microRNAs and those of oxidative stress and inflammatory cytokines. A group of non-LC control subjects was also included in the current investigation.

2. Methods

2.1. Study Subjects

This was a hospital-based study in which patients and control subjects were recruited consecutively for 10 years (2009–2019). For the investigation, 168 Caucasian patients were recruited in total. Specifically, 123 patients with NSCLC were recruited from the Lung Cancer Clinic of the Respiratory Medicine Department at Hospital del Mar (Barcelona, Spain). Ninety-one out of the 123 patients had underlying COPD and 32 patients had NSCLC with no COPD. A group of non-tumor control subjects (n = 45) were also recruited for the purpose of the investigation from the COPD Clinics at Hospital del Mar. Therefore, for the purpose of comparisons, the following study groups were established: (1) 45 subjects without LC (32 males, non-LC control group), (2) 32 patients with only LC (15 males, LC-only group), (3) 91 patients with LC and COPD (82 males, LC-COPD group). COPD was defined following existing guidelines [26,27]. Exclusion criteria for all the patients and control subjects included other chronic cardiovascular or respiratory disorders, chronic metabolic diseases, signs of severe bronchial inflammation and/or infection, current or recent invasive mechanical ventilation, chronic oxygen therapy, and poor tolerance and/or collaboration. Approval was obtained from the institutional Ethics Committee on Human Investigation (Hospital del Mar-IMIM, Barcelona, protocol # 2008/3390/I, 4 February 2008, following World Medical Association guidelines (Helsinki Declaration of 2008) for research on human beings. Informed written consent was obtained from all participants.

2.2. Clinical Assessment

Nutritional evaluation included the assessment of body mass index (BMI) and blood analytical parameters in all participants. Lung function parameters were determined in all study subjects following standard procedures [11,13,19]. TNM staging [28,29] was determined only in all patients with LC.

2.3. Blood Samples

In all the study patients and non-LC control subjects, blood samples were obtained from the arm vein after an overnight fasting period. Blood specimens were centrifuged at 1500× *g* for 15 min to collect the plasma samples, which were immediately frozen at −80 °C until further analyses.

2.4. Quantification of microRNAs

RNA isolation. Previously described methodologies were used for this set of experiments [13]. RNA was isolated from 500 microL plasma samples using 500 microL TRIzol reagent (Cat. 15596026, Thermo Fisher Scientific, Waltham, MA, USA). After incubation of the samples at room temperature for 10 min to achieve complete dissociation of nucleoprotein complexes, 200 microL chloroform were added, and samples were then centrifuged at 13,500 rpm at 4 °C for 15 min. The aqueous phase was recovered, and the RNA was precipitated with 600 microL isopropanol. Subsequently, samples were incubated at 4 °C for 30 min and were then cooled down to −20 °C overnight. After thawing the samples at room temperature, they were centrifuged at 13,500 rpm at 4 °C for 10 min, and the supernatant was removed. The remaining pellet was then washed using one mL solution of 75% ethanol to be subsequently centrifuged at 9000 rpm at 4 °C for five minutes. The RNA containing pellet was air-dried for 30 min and was then dissolved in 20 microL RNase-free water. To assess the quality and purity of the isolated RNA, concentrations of total RNA were determined using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

MicroRNA reverse transcription (RT). TaqMan® Advanced miRNA cDNA Synthesis Kit (Cat. A28007, Thermo Fisher Scientific, Waltham, MA, USA) was used to prepare cDNA templates following the manufacturer's instructions. Total RNA isolated samples were manipulated to add a poly (A) tailing on the 3′ position and an adaptor on the 5′ position of the mature microRNAs. Initially, 3 microL Poly (A) reaction mix (0.5 microL Poly (A) buffer, 0.5 microL ATP, 0.3 microL Poly (A) enzyme, and 1.7 microL RNase-free water) was mixed with 2 microL of each sample. The mixture was then incubated in a thermal cycler (Geneamp PCR System 2400, Perkin Elmer, Waltham, MA, USA) to perform the polyadenylation reaction at 37 °C for 45 min. This step was followed by incubation at 65 °C for 10 min to stop the reaction. Immediately, the samples were supplemented with 10 microL ligation reaction mix (3 microL ligase buffer, 4.5 microL PEG 8000, 0.6 microL ligation adaptor, 1.5 microL RNA ligase, and 0.4 microL of RNase-free water) to incorporate the adaptor at the 5′ position. Samples underwent standard cycling at 16 °C for 60 min. MicroRNAs with both poly (A) tail and the adaptor were reverse transcribed (RT) to achieve cDNA. First, modified microRNA samples were mixed with 15 microL RT reaction mix (6 microL RT buffer, 1.2 microL dNTP mix, 1.5 microL universal RT primer, 3 microL RT enzyme mix, and 3.3 microL RNase-free water). Samples were subsequently incubated in the thermal cycler at 42 °C for 15 min to perform the reverse transcription, and finally, they were incubated at 85 °C for 5 min to stop the reaction.

A cDNA amplification step was performed to increase the number of cDNA molecules. Forty-five microL of miR-Amp reaction mix (25 microL miR-Amp master mix, 2.5 microL miR-Amp primer mix, and 17.5 microL RNase-free water) from the synthesis kit were mixed with 5 microL RT reaction product. The amplification reaction consisted of different cycles: enzyme activation at 95 °C for 5 min, denaturation at 95 °C for three seconds, and finally, the extension of the cDNA at 60 °C for 30 s. Denaturation and extension cycles were repeated 14 more times to ensure a sufficient quantity of cDNA. Subsequently, samples

were incubated at 99 °C for 10 min to stop the reaction, and they were finally kept at −80 °C up until the performance of the real-time polymerase chain reaction (PCR) procedures.

Quantitative real time-PCR amplification (qRT-PCR). Real-time PCR was performed using specific primers for the target microRNAs in the study: miR-451, miR-210, miR-126, miR-21, miR-let7c, miR-145, miR-200b, and miR-223 (Table 1). Taqman advanced microRNA 159a assay from *Arabidopsis thaliana* was used as an exogenous control in order to normalize the miRNA amplification. Briefly, five microL of the resulting cDNA samples were mixed with one microL of each specific primer, 10 microL TaqMan fast-advanced master mix (Cat. 4444964, Thermo Fisher Scientific), and four microL RNase-free water. The samples were run in a thermal cycler (QuantStudio system, Thermo Fisher Scientific). The first step was the enzyme activation, achieved at 95 °C for 20 s, which was followed by 40 combined cycles of denaturation (95 °C for one second) and final annealing (60 °C for 20 s). Duplicates from all samples were run, and the average value was calculated for all the study samples. The results obtained from the experiments were collected and analyzed using the ExpressionSuite Software version 1.1 from Applied Biosystems (ThermoFisher Scientific), in which the comparative C_T method ($2^{-\Delta\Delta CT}$) for relative quantification was used [30].

Table 1. MicroRNA assays used for the quantitative analyses of the target genes using real-time PCR.

Assay Name	Assay ID	miRbase Accession Number
hsa-miR-451a	478107_mir	MIMAT0001631
hsa-miR-210-3p	477970_mir	MIMAT0000267
hsa-miR-126-3p	477887_mir	MIMAT0000445
hsa-miR-21-5p	477975_mir	MIMAT0000076
hsa-let7c-5p	478577_mir	MIMAT0000064
hsa-miR-145-5p	477916_mir	MIMAT0000437
hsa-miR-200b-3p	477963_mir	MIMAT0000318
hsa-miR-223-3p	477983_mir	MIMAT0000280
ath-miR159a	478411_mir	MIMAT0000177

Abbreviations: ID, identification; hsa, homo sapiens; miR, microRNA; MIMAT, mature microRNA; ath, arabidopsis thaliana.

2.5. Quantification of Oxidative Stress Markers and Cytokines

In a subset of representative individuals: 40 non-LC control subjects, 19 LC-only patients, and 20 LC-COPD patients, markers of oxidative stress and cytokines were also analyzed in the blood samples.

Oxidatively damaged DNA. Levels of oxidative DNA adduct 8-hydroxy-2-deoxy guanosine (8-OHdG) were measured in plasma using the DNA Damage (8-OHdG) ELISA kit (StressMarq Biosciences $_{INC}$., Victoria, BC, Canada) following the specific manufacturer's instructions and previously described methodologies [13,19]. Briefly, 50 microL of plasma was incubated with 50 microL of antibody per well at room temperature for one hour in a plate cover. After several washes, samples were incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate in the dark at room temperature for 30 min. Immediately afterwards, 100 microL of stop solution was poured into each well. Samples were then shaken from side to side and thoroughly mixed with the solution. After terminating this reaction, the absorbance was read at 450 nm in all the sample wells. A standard curve was always generated with each assay run. Intra-assay coefficients for all the samples ranged from 0.17% to 9.80%. The minimum detectable concentration of DNA in plasma was set to be 0.94 ng/mL (StressMarq Biosciences $_{INC}$, Victoria, BC, Canada).

Malondialdehyde (MDA)-protein adducts. Levels of MDA-protein adducts were measured in plasma using the OxiSelect^{TM} MDA Adduct Competitive ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA) following the specific manufacturer's instructions and previously described methodologies [13,19]. First, an MDA conjugate was coated on an ELISA plate, then 50 microL of plasma specimens were added to the MDA conjugate preabsorbed ELISA plate and incubated at room temperature for 10 min on an orbital shaker. After a

brief incubation, the primary antibody was added and incubated at room temperature for one hour on an orbital shaker. After three washes, samples were incubated with secondary antibody at room temperature for another hour on an orbital shaker. After three washes, the substrate solution was added at room temperature for 20 min, and samples were again shaken on an orbital shaker. Immediately afterwards, 100 microL of the stop solution was poured into each well. Samples were then thoroughly mixed with the solution. After completing this reaction, the absorbances were read at 450 nm. A standard curve was always generated with each assay run. Intra-assay coefficients of variation for all the samples ranged from 0.11% to 9.73%. The minimum detectable concentration of MDA-protein adducts in plasma was set to be 6 pmol/mL (Cell Biolabs, Inc., San Diego, CA, USA).

Reduced glutathione (GSH). GSH was measured in the blood using the Human reduced glutathione (GSH) ELISA Kit (MyBioSource, San Diego, CA, USA) following the specific manufacturer's instructions and previously described methodologies [11,19]. Fifty microL samples were added to every sample well and incubated with horseradish (HRP)-conjugate reagent at 37 °C for 60 min. The plate was covered with a closure plate membrane during the experiment. After four washes, 50 microL chromogen solution A and 50 microL chromogen solution B were added to each well, and samples were then incubated at 37 °C in the dark for 15 min. Finally, 50 microL of the stop solution were poured into each well. The absorbance in each sample was read at 450 nm. Intra-assay coefficients for all the samples ranged from 0.15% to 9.93%. The minimum detectable concentration of GSH in plasma was set to be 1.56 µmol/L (MyBioSource, San Diego, CA, USA).

Plasma levels of Trolox Equivalent Antioxidant Capacity (TEAC). TEAC levels were determined using the OxiSelect^{TM} Trolox Equivalent Antioxidant Capacity (TEAC Assay Kit (ABTS, Cell Biolabs, Inc., San Diego, CA, USA) following the manufacturer's instructions. Twenty-five microL samples were added to the microplate well, and upon addition of 150 microL of the diluted 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) reagent, samples were mixed thoroughly. Samples were then incubated on an orbital shaker for five minutes. Finally, the absorbance was read at 405 nm in all the sample wells. Antioxidant activity was determined by comparison with the Trolox standards. Intra-assay coefficients of variation for all the samples ranged from 0.03% to 9.75%. (Cell Biolabs, Inc., San Diego, CA, USA). The minimum detectable concentration of TEAC in plasma was set to be 250.29 g/mol (Cell Biolabs, Inc., San Diego, CA, USA).

Cytokines. Protein levels of the cytokines TGF-beta 1 and VEGF-A were quantified using specific ELISA kits (RayBiotech, Norcross, GA, USA) for each cytokine following the manufacturer's instructions and previously described methodologies [13,31,32]. All samples were incubated with the specific primary antibodies and were always run together in each assay. Before commencing the assay, samples and reagents were equilibrated to room temperature. Standards (100 microL) were performed as per the manufacturer's instructions. The protocol was followed according to the corresponding manufacturer's instructions for each cytokine. Intra-assay coefficients of variation for all the samples ranged from 0.13% to 9.66% (TFG-beta1) and from 0.13% to 9.93% (VEGF). Absorbances were read at 450 nm in all sample wells. A standard curve was always generated with each assay run. The minimum detectable concentration of TGF-beta 1 was 18 pg/mL (RayBiotech, Norcross, GA, USA). The minimum detectable concentration of VEGF-A was 3.59 pg/mL (RayBiotech, Norcross, GA, USA).

2.6. Statistical Analysis

Normality of the study variables was tested using the Shapiro–Wilk test. Data are expressed as mean and standard deviation (SD) in tables and figures. MicroRNA-451 was selected as the target variable to calculate the sample size. Having a priori unbalanced design, where the LC-COPD group had twice as many patients as the LC group and the control group, a minimum sample of 27 non-LC controls, 27 LC, and 54 LC-COPD patients was required to achieve an 80% statistical power, taking a within groups mean square error equals to 4393. Statistical significance was established at $p < 0.05$. Potential differences of

quantitative variables among the study groups were assessed using the one-way analysis of variance (ANOVA) and Tukey's post hoc analysis to adjust for multiple comparisons. Chi-square test was employed to assess potential differences in categorical variables (smoking history) among the three study groups. Comparisons of the results obtained from the micro-RNA expression analyses within each study group were determined using the Duncan multiple comparison test. Correlations between clinical and biological variables were explored using the Pearson's correlation coefficient. Bivariate analysis was performed to test associations between two variables for the study population. All statistical analyses were performed using the software SPSS 23.0 (SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Clinical Characteristics

Anthropometric variables such as age, body weight, and BMI did not significantly differ among the study subjects (Table 2). The proportions of active smokers were similar in the three study groups (Table 2). However, the proportions of ex-smokers were significantly greater in the LC-COPD group than in the LC-only group and showed a tendency to be higher than in the control subjects ($p = 0.1$, Table 2). The proportions of never smokers were significantly lower in the LC-COPD patients than in the LC-only and the control subjects (Table 2). No significant differences were seen in the number of packs-year among the study groups (Table 2). LC-COPD patients had moderate airway obstruction compared to LC-only and the controls, who had normal lung function parameters (Table 2). LC staging was similar between the two study groups (Table 2). In both groups of patients, nutritional status was preserved, and globular sedimentation velocity (GSV) was increased compared to the controls (Table 2). For both groups of patients, the comorbidities and different treatments are illustrated in Table 2. No significant correlations were found between any of the comorbidities and treatments with the study biological markers.

Table 2. Clinical characteristics of the study patients.

	Non-LC Controls	**LC-Only**	**LC-COPD**
	N = 45	**N = 32**	**N = 91**
Anthropometry			
Age (years)	63 (10)	64 (11)	67 (9)
Male, N/female, N	32/13	15/17	82/9
Body weight (kg)	75 (16)	70 (14)	72 (12)
BMI (kg/m^2)	27 (5)	26 (4)	25 (4)
Smoking history			
Active, N (%)	15 (33)	11 (34)	39 (43)
Ex-smoker, N (%)	17 (38)	9 (28)	48 (53) $^{\#}$
Never smoker, N (%)	13 (29)	12 (38)	4 (4) $^{***,\#\#\#}$
Packs-year	47 (26)	46 (15)	58 (21)
Lung function testing			
FEV_1, % predicted	80 (24)	94 (15) ***	62 (14) $^{***,\#\#\#}$
FEV_1/FVC, % predicted	70 (12)	78 (6) ***	61 (9) $^{***,\#\#\#}$
DL_{CO}, % predicted	81 (22)	82 (19)	68 (19) $^{**,\#\#}$
K_{CO}, % predicted	82 (22)	82 (15)	75 (21)
TNM staging			
Stage 0-II: N (%)	NA	20 (62)	53 (58)
Stage III: N (%)	NA	7 (22)	19 (21)
Stage IV: N (%)	NA	5 (16)	19 (21)

Table 2. *Cont.*

	Non-LC Controls	LC-Only	LC-COPD
	N = 45	**N = 32**	**N = 91**
Blood parameters			
Total leukocytes/μL	8.2 (4.1) × 10^3	10.5 (4.2) × 10^3 *	9.7 (3.6) × 10^3
Total neutrophils/μL	5.3 (2.8) × 10^3	8.3 (4.4) × 10^3 ***	7.2 (3.5) × 10^3 *
Total lymphocytes/μL	2.1 (1.1) × 10^3	1.4 (0.6) × 10^3	1.9 (3.0) × 10^3
Albumin (g/dL)	4.4 (0.6)	4.4 (0.9)	4.0 (0.6) **,#
Total proteins (g/dL)	7.3 (0.8)	7.0 (1.0)	7.1 (0.8)
CRP (mg/dL)	4.1 (8.2)	5.3 (9.4)	5.7 (7.8)
Fibrinogen (mg/dL)	451 (147)	463 (131)	481 (157)
GSV (mm/h)	16.3 (13.9)	27.6 (15.3) *	33.4 (22.0) **
Comorbidities			
Hypertension, N (%)	NA	10 (31.3)	45 (49.5)
Type 2 Diabetes mellitus, N (%)	NA	2 (6.3)	17 (18.7)
Dyslipidemia, N (%)	NA	2 (6.3)	26 (28.6)
Treatments			
Diuretic, N (%)	NA	5 (15.6)	20 (22.2)
Angiotensin converting enzyme inhibitors, N (%)	NA	2 (6.3)	6 (28.6)
Angiotensin-2 receptor blockers, N (%)	NA	2 (6.3)	14 (15.4)
Beta blockers, N (%)	NA	3 (9.4)	5 (5.5)
Calcium channel blockers, N (%)	NA	2 (6.3)	7 (7.7)
HMG-CoA-reductase, N (%)	NA	2 (6.3)	17 (18.7)
Biguanides, N (%)	NA	2 (6.3)	26 (28.6)
LAMA, N (%)	NA	NA	60 (65.9)
LABA, N (%)	NA	NA	30 (33)
Inhaled corticosteroids, N (%)	NA	NA	17 (18.7)

Values are expressed as mean (standard deviation). Abbreviations: COPD, chronic obstructive pulmonary disease; N, number of patients; m, meters; BMI, body mass index; FEV_1, forced expiratory volume in one second; DLco, carbon monoxide transfer; K_{CO}, Krough transfer factor; g, grams; TNM, tumor, nodes, metastasis; NA, not applicable; dL, deciliter; mg, milligrams; CRP, C-reactive protein; GSV, globular sedimentation velocity; LAMA, long-acting muscarinic antagonists; LABA, long-acting beta-agonists; mm, millimeters; h, hour. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between any study group compared to non-LC controls; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ between LC-COPD group compared to LC-only group.

3.2. Differential Pattern of Systemic microRNA Expression in LC Patients

Compared to the non-LC controls, the plasma levels of miR-451 significantly decreased in both groups of patients (Figure 1). Systemic levels of miR-210 significantly declined in LC-COPD compared to LC-only patients (Figure 1). The plasma levels of miR-let7c were significantly greater in LC-only and LC-COPD patients than in the control subjects (Figure 1). Significant associations were detected between plasma microRNA-let7c expression levels and total leukocytes and neutrophil counts (r = 0.467, $p = 0.011$, r = 0.498, and $p = 0.006$, respectively). No significant differences were seen in the plasma expression levels of miR-126, miR-21, miR-145, miR-200b, or miR-223 among the study groups (Figure 1).

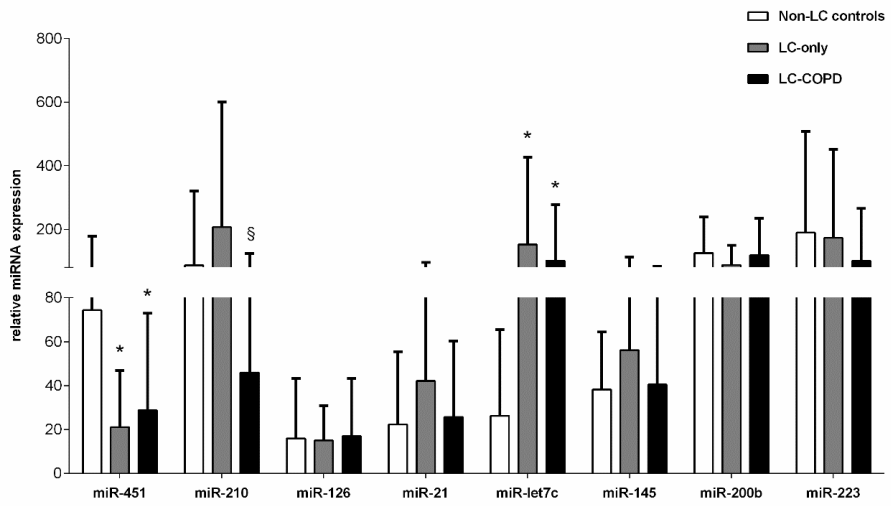

Figure 1. Mean values and standard deviation (relative expression) of microRNA (miR) expression in plasma samples of non-LC controls (white bars), LC-only patients (grey bars), and LC-COPD patients (black bars). Statistical significance: * $p < 0.05$ between either of the two groups of patients with LC and the non-LC control subjects; § $p < 0.05$ for comparisons between LC-only patients and LC-COPD patients. For the sake of clarity, the absence of statistical symbols indicates that no significant differences were found between groups for the different study comparisons.

In the non-LC control group, the patterns of expression of miR-126, miR-21, miR-let7c, and miR-145 were similar, whereas miR-451, miR-210, and miR-200b shared an intermediate pattern and miR-223 followed a different pattern (Figure 2). In the LC-only group, miR-451 and miR-126 followed an identical pattern of expression, while miR-21, miR-let7c, miR-145, miR-200b, and miR-223 followed an intermediate pattern, and miR-210 expressed a different pattern (Figure 2). In the LC-COPD group, plasma expression levels of miR-451, miR-210, miR-126, miR-21, and miR-145 were similar, whereas expression levels of miR-let7c, miR-200b, and miR-223 followed a completely different pattern of expression (Figure 2).

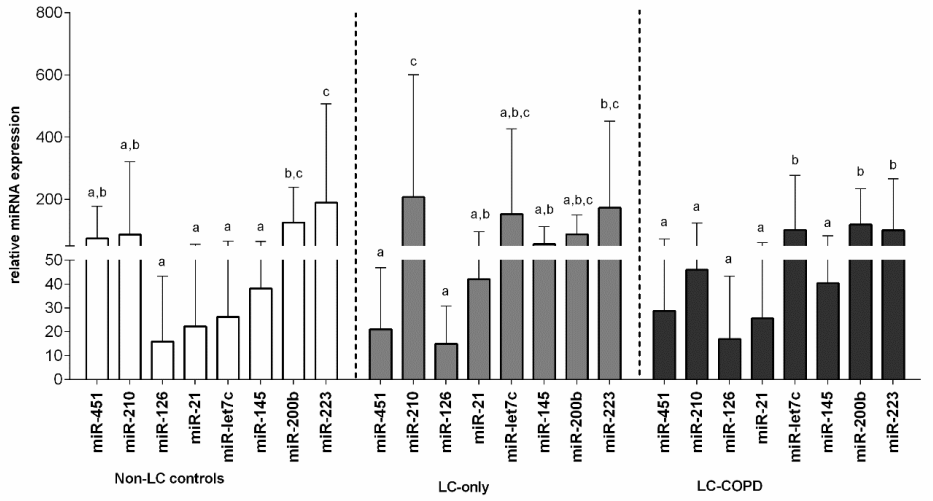

Figure 2. Mean values and standard deviation (relative expression) of microRNA (miR) expression in plasma samples of non-LC controls (white bars), LC-only patients (grey bars), and LC-COPD patients (black bars). Statistical analyses were performed separately for each study group of subjects. The letters a,b,c indicate the statistical significance: the same letter indicate no statistically significant difference among the groups for a given microRNA. In each study group, the expression levels of the study microRNAs did not differ among them if they shared the same letter. Different letters reflect different levels of expression for each study group.

3.3. Redox Balance in LC Patients

3.3.1. Oxidative Stress Markers

Levels of oxidized DNA did not significantly differ between the study groups (Figure 3A). MDA-protein adduct levels were significantly higher in LC-COPD patients compared to LC-only patients and non-LC controls (Figure 3B). No significant differences were seen in MDA-protein adduct levels between LC-only patients and the control subjects (Figure 3B).

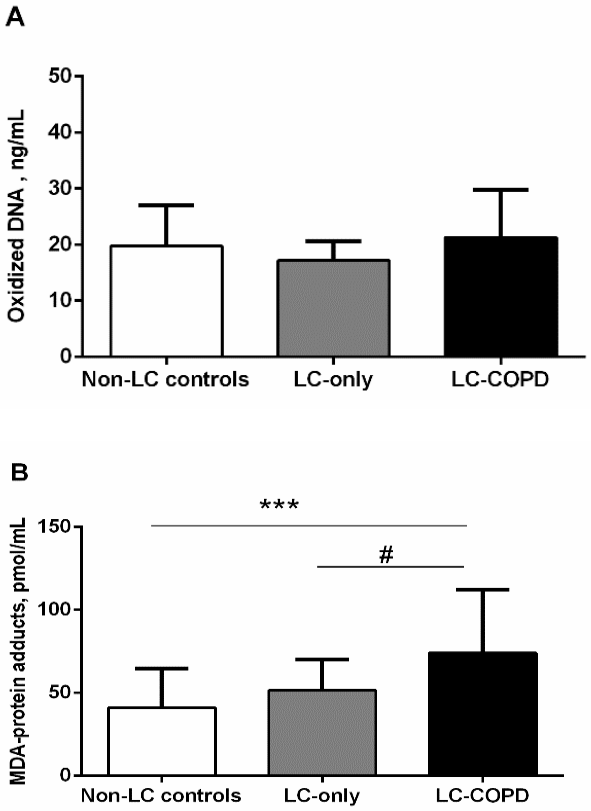

Figure 3. (**A**) Mean values and standard deviation of levels of oxidized DNA (ng/mL) did not differ between the study groups of subjects. (**B**) Mean values and standard deviation of level of MDA-protein adducts (pmol/mL) were significantly higher in LC-COPD patients compared to non-LC control subjects and LC-only patients. Statistical significance is as follows: *** $p < 0.001$ between LC-COPD patients and the non-LC control subjects, # $p < 0.05$ between LC-COPD patients and LC-only patients. The absence of statistical symbols indicates that no significant differences were found between groups for the different study comparisons.

3.3.2. Antioxidants

GSH levels were significantly higher in LC-only patients than in non-LC controls, whereas, in LC-COPD patients, the levels significantly declined compared to LC-only patients and the non-LC controls (Figure 4A). A significant positive correlation (r = 0.513 and $p = 0.001$) was detected between plasma GSH levels and FEV_1/FVC among all the patients. Additionally, among LC-COPD patients, total neutrophil counts were almost inversely associated with GSH plasma levels (r = −0.421 and $p = 0.082$). TEAC levels were significantly greater in both LC-COPD and LC-only patients compared to non-LC controls (Figure 4B). No significant differences were detected in TEAC levels between the two LC patient groups (Figure 4B).

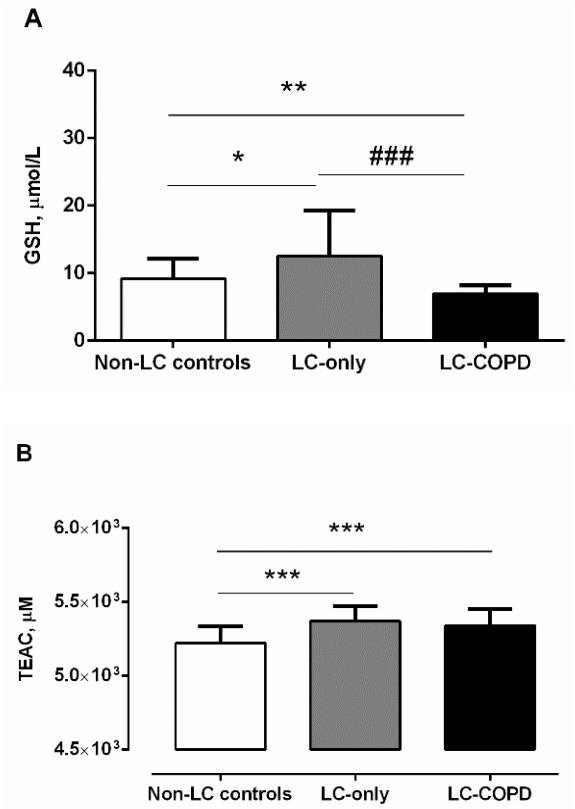

Figure 4. (**A**) Mean values and standard deviation of plasma GSH levels (μmol/L) were significantly higher in LC-only patients than in non-LC patients. However, levels of GSH in LC-COPD patients were significantly lower than in both non-LC controls and LC-only patients. Statistical significance: * $p < 0.05$, ** $p < 0.01$ between either of the two groups of patients with LC and the non-LC control subjects, and ### $p < 0.001$ between LC-COPD and LC-only patients. (**B**) Mean values and standard deviation of plasma TEAC (μM) were significantly higher in both LC-COPD and LC-only patients compared to non-LC controls. Statistical significance is as follows: *** $p < 0.001$ between any group of LC patients and the non-LC controls. The absence of statistical symbols indicates that no significant differences were found between groups for the different study comparisons.

3.4. Cytokines in LC Patients

Levels of VEGF-A did not significantly differ between the study groups (Figure 5A). Importantly, both groups of LC patients, especially those with COPD, exhibited a significant rise in plasma TGF-beta 1 levels compared to the non-LC control subjects (Figure 5B). No significant differences in TGF-beta 1 levels were detected between the two patient groups (Figure 5B). An almost significant positive correlation was observed between plasma TGF-beta 1 and microRNA-let7c expression levels (r = 0.354 and $p = 0.064$). Furthermore, significant inverse associations were observed between plasma TGF-beta 1 levels and DL_{CO} and K_{CO} among all the LC patients (r = −0.379 and $p = 0.027$ and r = −0.608 and $p < 0.001$, respectively).

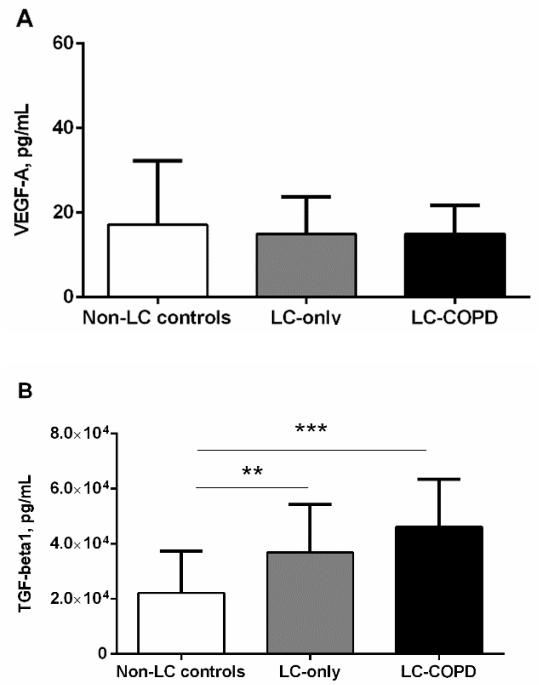

Figure 5. (**A**) Mean values and standard deviation of plasma VEGF-A levels (pg/mL) did not significantly differ between the study groups. (**B**) Mean values and standard deviation of plasma TGF-beta1 were significantly greater in both LC-COPD and LC-only patients compared to non-LC control subjects. Statistical Significance: ** $p < 0.01$, *** $p < 0.001$ between any group of LC patients and the non-LC control subjects. The absence of statistical symbols indicates that no significant differences were encountered between groups for the different study comparisons.

3.5. Bivariate Analysis

The bivariate analysis showed that, in patients with LC-COPD, smoking history, except for current smokers, significantly differed from that reported in the LC patients (Table 3). Additionally, the bivariate analysis demonstrated that lung airflow limitation and diffusion capacity were significantly impaired in patients with LC-COPD compared to LC patients (Table 3). Furthermore, levels of the blood parameters albumin, miR-210, and the antioxidant GSH were significantly reduced in the LC-COPD compared to LC patients, while those of MDA-protein adducts were significantly higher in the former patients than in the latter group (Table 3).

Table 3. Bivariate analysis of clinical and biological variables in patients with LC with and without COPD.

	LC-Only	LC-COPD	*p* Value
Smoking history			
Never smokers, N (%)	12 (38)	4 (4)	<0.001
Ex-smokers, N (%)	9 (28)	48 (53)	0.023
Current smokers, N (%)	11 (34)	39 (43)	0.414
Packs-year, $\bar{x}$ (SD)	46 (15)	58 (21)	0.015
FEV_1, %, $\bar{x}$ (SD)	94 (15)	62 (14)	<0.001
FEV_1/FVC, $\bar{x}$ (SD)	78 (6)	61 (9)	<0.001
DL_{CO}, %, $\bar{x}$ (SD)	82 (19)	68 (19)	0.001
Albumin, $\bar{x}$ (SD)	4.4 (0.9)	4.0 (0.6)	0.016
miR-210, $\bar{x}$ (SD)	207.13 (393.11)	45.95 (78.07)	0.006
MDA-protein adducts, $\bar{x}$ (SD)	51.52 (18.53)	74.20 (37.97)	0.033
GSH, $\bar{x}$ (SD)	12.54 (6.17)	6.97 (1.24)	0.001

Values are expressed as mean (standard deviation). Abbreviations: N, number of patients; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; DLco, carbon monoxide transfer; MDA-protein adducts, malondialdehyde-protein adducts; GSH, reduced glutathione.

4. Discussion

In the current investigation, the most relevant findings were that, in LC-COPD, two different patterns of plasma microRNA expression were observed. Moreover, expression of miR-451 was significantly downregulated in both groups of patients with LC compared to non-LC controls. Plasma protein oxidation levels, as measured by MDA-protein adducts, were increased only in patients with LC-COPD compared to non-LC controls. Nonetheless, systemic levels of the antioxidant GSH significantly declined in LC-COPD patients compared to both LC-only patients and the non-LC control subjects. Conversely, a significant rise in plasma antioxidant TEAC levels was detected in both groups of LC patients compared to the controls. In this study, blood levels of VEGF did not vary across groups, whereas TGF-beta plasma levels significantly increased in both groups of LC patients compared to non-LC controls. The most relevant results obtained in the study are discussed below.

Importantly, systemic levels of microRNA-451 were reduced in both groups of patients with LC, and COPD did not significantly influence those levels. Low levels of the tumor-suppressive miR-451 were associated with poor prognosis in NSCLC patients [33]. Additionally, the tumor suppressor miR-451 was also shown to enhance cisplatin sensitivity via regulation of Mcl-1 expression, suggesting that novel therapeutic targets may be designed thereafter [34,35]. In a previous study from our group [13], levels of miR-451 were also reduced in the tumors of patients with LC, particularly in those with COPD. Hence, miR-451 may be a surrogate of lung tumorigenesis that could help monitor patients in the clinics.

Interestingly, expression levels of miR-let7c were upregulated in plasma samples of both groups of LC patients compared to the non-LC controls. Furthermore, significant associations were found between plasma microRNA-let7c expression levels and inflammatory cell counts, which were increased in both groups of LC patients. These are novel findings that deserve further attention. The results encountered in the current study are in line with those previously reported in the lung tumors of patients with LC, especially in those with COPD [13]. In that study [13], levels of the *k-RAS* gene were also downregulated in the lung tumors of the LC patients with underlying COPD. It would be possible to conclude that, as miR-let7c acts a tumor suppressor in cancer cells, underlying respiratory conditions, such as in COPD, may have induced a positive feedback loop to counterbalance the deleterious effects of cancer biology. In keeping with this, miR-let7c expression levels negatively correlated with metastasis, vascular invasion, and poor survival in NSCLC patients, whose miR-let7c levels were downregulated [36]. Whether patients also had a concomitant respiratory disease was not explored in that study [36]. Moreover, it was also demonstrated that the upregulation of miR-let7c was probably involved in the chemoresistance of lung cancer in patients [37]. Another finding that deserves attention is the significant decrease in miR-210 levels that was observed in the LC-COPD patients compared to LC patients, as confirmed in the bivariate analysis. As miR-210 is upregulated during hypoxia [38], it may be a useful marker of tumor development in patients with underlying COPD. Future research should be targeted at exploring the potential role of miR-210 in the lung predisposition of COPD. Thus, miR-210 may be used as a surrogate marker to monitor LC predisposition in patients with COPD.

Oxidative stress favors carcinogenesis as a result of the processes involved in neoplastic transformation and DNA mutations [22]. Posttranslational modifications induced by oxidative damage of proteins, lipids, and DNA promote the cell viability and growth of cancer cells [12]. In fact, proteins, DNA, and lipids are major targets for the action of oxidants that are not counterbalanced by the tissue antioxidant capacity, leading to the development of oxidative stress [39–41]. Reactive carbonyl derivatives (aldehydes and ketones) are formed by the reaction of oxidants with several amino acid residues (e.g., lysine, proline, and threonine). On the other hand, Michael-addition reactions of lysine, cysteine, or histidine residues with α,ß-unsaturated aldehydes (e.g., malondialdehyde, MDA) may also lead to the formation of reactive carbonyls resulting from the peroxidation of polyun-

saturated fatty acids of the membranes [42–44]. In the current investigation, MDA-protein adduct plasma levels were significantly greater in the LC-COPD patients compared to both non-LC control subjects and the LC-only patients. These findings are in line with those previously reported [11], in which MDA-protein adduct levels were increased in the lung tumors of patients with LC-COPD compared to LC without the underlying respiratory condition. These results were also confirmed in the bivariate analysis. Taken together, these results suggest that COPD per se may induce the rise in systemic oxidative stress levels in LC patients. As systemic oxidative stress levels have also been reported to be increased in patients with only COPD [39,45], this is a likely explanation. Collectively, these results suggest that oxidative stress markers should be detected regularly in the clinics as an early marker of lung tumorigenesis, particularly in patients with chronic respiratory diseases such as COPD.

Powerful antioxidant systems protect cells from oxidatively induced damage. As such, superoxide dismutase isoforms, catalase, and glutathione peroxidases are counted among the most abundant antioxidant enzymes. Non-enzymatic antioxidant systems complement the action of antioxidant enzymes within cells. The most abundant non-protein thiol glutathione is a water-soluble compound, which is widely distributed within tissues. Levels of reduced glutathione (GSH) indicate the redox potential of a tissue. In the current investigation, GSH plasma levels were significantly lower in the LC-COPD patients than in both LC-only patients and the non-LC controls. These results were also confirmed in the bivariate analysis. Additionally, the degree of airway obstruction as measured by FEV_1/FVC significantly correlated with plasma GSH levels, suggesting that patients with greater airflow limitation were those with lower levels of GSH. Moreover, neutrophil counts were inversely associated with GSH in this study. Taken together, these relevant findings reveal that the reduction in redox potential observed in the patients with underlying COPD may predispose them to develop LC. In fact, a recent meta-analysis has put the line forward that GSH plasma levels were reduced in patients with COPD, suggesting that this mechanism is likely involved in the pathogenesis of the chronic airway disease and could also be part of the greater predisposition of these patients to develop LC [45]. Taken together, these findings imply that GSH may be useful for monitoring the lung tumorigenesis process in patients with COPD. In LC patients with no COPD, however, plasma levels of GSH were increased compared to non-LC control subjects. Modifications in redox balance including antioxidant levels were also shown to be part of the pathophysiology of several cancer types, including LC. Interestingly, a significant rise in plasma levels of TEAC were observed in both groups of LC patients. As far as we are concerned, these are novel results, implying that the antioxidant capacity relative to the standard Trolox (vitamin E analog) was increased in response to lung carcinogenesis among all the patients irrespective of COPD.

Inflammatory cytokines such as VEGF and TGF-beta have been demonstrated to participate in the pathophysiology of LC development in patients with chronic respiratory diseases [21,22]. Cell mechanisms such as proliferation and repair, apoptosis, and angiogenesis may be hindered by increased levels of several interleukins and cytokines [21,22]. In the present study, the plasma levels of VEGF did not vary between the study groups, while a significant rise in plasma TGF-beta levels was detected in both groups of LC patients. Importantly, significant inverse associations were observed between plasma TGF-beta levels and diffusion capacity among all the LC patients. These findings suggest that patients with a certain degree of emphysema were those exhibiting greater TGF-beta plasma levels, despite the fact that these levels did not differ between the two patient groups. Indeed, similar findings were previously reported in patients with LC with and without COPD [21,22]. In a recent meta-analysis [46], TGF-beta was shown to help predict the worse prognosis in patients with LC, independently of the presence of underlying respiratory diseases. On this basis, it may be possible to conclude that TGF-beta can be used as a prognosis marker in the follow-up of patients with LC. Nonetheless, TGF-beta does not seem to help predict lung tumorigenesis in patients with underlying COPD in the clinics.

5. Study Limitations

In the current investigation, the number of patients and controls analyzed using ELISA was smaller compared to those used in the microRNA analyses. The objective was to analyze all the samples synchronically within the same plate in order to minimize variability. The most representative subjects in each group, on the basis of the mRNA amplification during RT-PCR experiments, were selected for the purpose of this study. Despite these concerns, the study hypothesis has been confirmed and the sample size calculations were correct in the investigation, as described in the Methods section.

6. Conclusions

A differential expression profile of microRNAs was detected in patients with LC, specifically of miR-451, miR-let7c, and miR-210. Furthermore, in LC patients with COPD, plasma oxidative stress levels (MDA-protein adducts) increased, whereas those of the powerful antioxidant GSH declined. Redox imbalance is differentially expressed in LC patients with underlying respiratory diseases, which reveal its potential implications in the pathogenesis of tumorigenesis in these patients. Decreased levels of the antioxidant GSH may be used as a surrogate biomarker of lung tumorigenesis in patients with chronic respiratory diseases in the clinics. These findings have clinical implications in the management and monitoring of patients with LC, with a special focus on those with underlying COPD.

Author Contributions: Conception and design, E.B., M.G., V.C. and A.S.-F.; patient assessment and recruitment and sample collection, V.C., A.S.-F. and M.A.; molecular biology analyses, M.G., J.T. and E.B.; statistical analyses and data interpretation, X.D., L.Q., M.G. and E.B.; manuscript drafting and intellectual input, E.B., M.G., L.Q. and J.T.; manuscript writing—final version, E.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Hospital del Mar-IMIM (protocol # 2008/3390/I, 4 February 2008).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets are available from the corresponding authors upon reasonable request.

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

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4.2 Article 2

Title:

Do Redox Balance and Inflammatory Events Take Place in Mild Bronchiectasis? A Hint to Clinical Implications

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Do Redox Balance and Inflammatory Events Take Place in Mild Bronchiectasis? A Hint to Clinical Implications

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Abstract: We hypothesized that in mild bronchiectasis patients, increased systemic inflammation and redox imbalance may take place and correlate with clinical parameters. In plasma samples from patients with very mild bronchiectasis, inflammatory cells and molecules and redox balance parameters were analyzed. In the patients, lung function and exercise capacity, nutritional status, bacterial colonization, and radiological extension were assessed. Correlations between biological and clinical variables were determined. Compared to healthy controls, levels of acute phase reactants, neutrophils, IgG, IgA, myeloperoxidase, protein oxidation, and GSH increased and lung function and exercise capacity were mildly reduced. GSH levels were even greater in ex-smoker and *Pseudomona*-colonized patients. Furthermore, radiological extension inversely correlated with airway obstruction and, disease severity, and positively correlated with neutrophil numbers in mild bronchiectasis patients with no nutritional abnormalities. In stable patients with mild bronchiectasis, several important inflammatory and oxidative stress events take place in plasma. These findings suggest that the extension of bronchiectasis probably plays a role in the development of redox imbalance and systemic inflammation in patients with mild bronchiectasis. These results have therapeutic implications in the management of bronchiectasis patients.

Keywords: non-CF bronchiectasis patients; prooxidants; antioxidants; acute phase reactants; myeloperoxidase; reduced glutathione; bronchiectasis severity scores

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1. Introduction

Non-cystic fibrosis (CF) bronchiectasis is a prevalent disease with a burden that is steadily increasing [1]. Bronchiectasis is a chronic respiratory condition characterized by the distortion of the airways, which favors favor the accumulation of lung secretions in patients. In the airways and lungs, bacterial colonization, chronic inflammation, and remodeling take place [2,3]. Acute exacerbations are also common in patients with bronchiectasis, thus, having a negative impact on their quality of life and disease prognosis.

Inflammation and oxidative stress are mechanisms frequently observed in the airways and lungs of patients with bronchiectasis [4,5]. These are relevant mechanisms that underlie the pathophysiology of mucus hypersecretion and accumulation in the lungs and airways [6,7], which lead to further structural alterations in patients [6]. Oxidative stress modifies key cellular structures as a result of the action of oxidants on proteins, DNA, and lipids [8]. Inflammatory molecules through the action of cytokines may amplify the response to several insults in the lungs and airways of patients [9,10]. Most studies have focused on the examination of either antioxidant or prooxidant markers [10]. Increased oxidative stress levels are important players in the pathophysiology of skeletal muscle dysfunction and mass loss and nutritional abnormalities in patients with chronic obstructive pulmonary disease (COPD), especially those with more severe disease [11–14]. Whether oxidative stress may also play a substantial role in the development of systemic manifestations in other chronic respiratory diseases, even at early stages, remains to be answered. In this regard, in patients with mild bronchiectasis, the potential implications of an imbalance between oxidants and antioxidants still needs to be thoroughly analyzed.

Systemic inflammation and oxidative stress have also been reported in patients with chronic respiratory diseases. COPD represents a paramount example of the presence of systemic inflammation and oxidative stress, particularly in patients with severe airflow limitation and emphysema and systemic effects of their respiratory condition (e.g., muscle dysfunction, sarcopenia, and poor exercise tolerance) [8,11–13,15,16]. In patients with mild-to-moderate COPD, the effects of systemic inflammation and/or redox imbalance are barely seen [13,16,17]. Likewise, in patients with bronchiectasis, greater systemic levels of inflammation are directly related to impaired lung function, quality of life, exercise tolerance, and higher scores of disease severity [10,18–21]. Whether patients with very mild bronchiectasis, localized extension, and preserved lung function as measured by several disease scores may also exhibit high levels of systemic inflammation and redox imbalance remains to be elucidated.

On this basis, we hypothesized that the systemic inflammatory profile and redox imbalance between prooxidants and antioxidants would differ in patients with very mild bronchiectasis from a group of healthy subjects. Hence, the study objectives were to thoroughly examine the following features: (1) systemic (blood) inflammatory cells and molecules, (2) systemic levels of prooxidants and antioxidants, (3) lung function and exercise capacity, (4) nutritional status, (5) bacterial colonization, and (6) correlations between clinical and biological variables in a prospective cohort of stable patients with mild bronchiectasis. A group of healthy subjects was also recruited for the purpose of comparisons.

2. Methods

Full details on the different methodologies employed in the investigation are provided in the online Supplementary Materials.

2.1. Study Subjects

This was a prospective, controlled, cross-sectional study, in which 30 patients (7 males) were recruited consecutively from the Multidisciplinary Bronchiectasis Unit of the Respiratory Department at Hospital del Mar (Barcelona, Spain) over the years 2019–2020. Additionally, 26 age- and sex-matched control subjects (9 males) were recruited from the general population (patients' relatives or friends) at Hospital del Mar. In the patients, inclusion criteria were as follows: adults (18 years and over), diagnosis of non-CF bronchiectasis by high-resolution computerized tomography (HRCT) [22,23], no previous exacerbations of the disease at least 4 weeks prior to study entry (range from 2 months to four years). Exclusion criteria for all the patients and control subjects included other chronic cardiovascular or respiratory disorders, chronic metabolic diseases, signs of severe bronchial inflammation and/or infection, current or recent invasive mechanical ventilation, long-term oxygen therapy, and poor collaboration. Most of the patients recruited for the purpose

of the investigation had a mild-to-moderate disease severity on the basis of lung function impairment, disease scoring using several indices, and radiologic extension [22,24–27]. All the patients were stable at the time of study entry. Approval was obtained from the institutional Ethics Committee on Human Investigation (Hospital del Mar-IMIM, Barcelona, Spain, protocol # 2019/8482/1, 14 March 2019) following the World Medical Association guidelines (Declaration of Helsinki, Fortaleza, Brazil, October 2013) for research on human beings. Informed written consent was obtained from all participants.

2.2. Clinical Assessment

Nutritional evaluation included the assessment of body mass index (BMI) and determination of fat-free mass index (FFMI) using bioelectrical impedance [13,17,28–30]. Blood analytical parameters (systemic inflammatory cells and markers) were also obtained in all participants. Lung function parameters were determined in all study subjects following standard procedures [13,17,28–30]. Exercise capacity was assessed through the 6-min walking distance following previous methodologies [31]. Etiology of the non-CF bronchiectasis of all the study patients was also assessed.

2.2.1. Bronchiectasis Severity Scores

The FACED (FEV_1, AGE, Chronic colonization, Extension Dyspnea), EFACED (Exacerbation FACED), and BSI (Bronchiectasis Severity Index) scores were calculated in order to assess the clinical status and disease severity of the bronchiectasis patients according to previous reports [24–26].

2.2.2. Radiological Extension

HRCT-scans were used to evaluate the radiological extension of bronchiectasis in all the study patients. Scores for each patient were calculated by two independent observers following previously established criteria [32,33].

2.2.3. Microbiological Diagnosis

Spontaneous or induced sputum samples were obtained from all the patients. Sputum samples were analyzed in the microbiology laboratory. Conventional semi-qualitative bacterial and fungal cultures were performed. An initial Gram staining was performed in all the samples prior to culturing the sputum if the Murray and Washington criteria were met [34] (Table S1).

2.3. Blood Samples

In all the study patients and control subjects, blood samples were obtained from the arm vein after an overnight fasting period. Blood specimens were centrifuged at 1500 g for 15 min to collect the plasma samples and immediately frozen at −80 °C until further analyses.

2.4. Quantification of Oxidative Stress Markers and Cytokines

Oxidatively damaged DNA. Levels of oxidative DNA adduct 8-hydroxy-2-deoxy guanosine (8-OHdG) were measured in plasma using the DNA Damage (8-OHdG) ELISA kit (StressMarq Biosciences INC., Victoria, BC, Canada) following the specific manufacturer's instructions and previously described methodologies [8,35].

Malondialdehyde (MDA)–protein adducts. Levels of MDA–protein adducts were measured in plasma using the OxiSelect™ MDA Adduct Competitive ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA) following the specific manufacturer's instructions and previously described methodologies [8,35].

Reduced glutathione (GSH). GSH was measured in the blood using the Human Reduced Glutathione (GSH) ELISA Kit (MyBioSource, San Diego, CA, USA) following the specific manufacturer's instructions and previously described methodologies [8,35].

Plasma levels of Trolox Equivalent Antioxidant Capacity (TEAC). TEAC levels were determined using the OxiSelect™ Trolox Equivalent Antioxidant Capacity (TEAC Assay Kit (ABTS, Cell Biolabs, Inc., San Diego, CA, USA) following the manufacturer's instructions. Twenty-five-microliter samples were added to the microplate well, and upon addition of 150 microL of the diluted 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) reagent, samples were mixed thoroughly. Samples were then incubated on an orbital shaker for five minutes. Finally, the absorbance was read at 405 nm in all the sample wells. Antioxidant activity was determined by comparison with the Trolox standards. Intra-assay coefficients of variation for all the samples ranged from 0.39% to 9.96%. (Cell Biolabs, Inc., San Diego, CA, USA). The minimum detectable concentration of TEAC in plasma was set to be 250.29 g/mol (Cell Biolabs, Inc., San Diego, CA, USA).

Quantification of myeloperoxidase in plasma. Plasma levels of myeloperoxidase were measured using the Human Myeloperoxidase ELISA Kit (MyBioSource, San Diego, CA, USA) following the specific manufacturer's instructions and previously described methodologies [8,35].

Quantification of cyclooxygenase in plasma. The Human Cyclooxygenase 2 ELISA Kit (MyBioSource, San Diego, CA, USA) was used following the specific manufacturer's instructions and previously described methodologies [8,35].

2.5. Statistical Analysis

Normality of the study variables was tested using the Shapiro–Wilk test. Data are expressed as mean and standard deviation (SD) in tables and figures. The variables MDA-protein adducts, fibrinogen, and ceruloplasmin were used to calculate sample size. A minimum number of 23 patients and control subjects was required to achieve an 85% statistical power for those variables. Statistical significance was established at $p \leq 0.05$. Potential differences of quantitative variables between the two groups were explored using the Student's *t* test and Mann–Whitney U test (parametric and non-parametric distribution of the variables, respectively). Chi-square test was employed to assess potential differences in categorical variables (smoking history) between the two groups. Potential correlations between clinical, radiological, and biological variables were explored using the Pearson's or the Spearman's correlation coefficients. A Bonferroni-type adjustment was performed to considering the effect of having multiple correlations. In the post hoc analyses, in which the patients were subdivided on the basis of smoking history or pseudomona colonization, Kruskal–Wallis or analysis of variance (ANOVA) tests were used. The software Statistical Package for the Social Sciences (SPSS) 23.0 (SPSS Inc, Chicago, IL, USA) was used for this group of analyses. Correlations are displayed in graphical correlation matrixes, obtained from R package corrplot (https://cran.r-project.org/web/packages/corrplot/index.html, accessed on 21 May 2021), in different colors: blue for positive correlations and red for negative ones. Furthermore, comparisons among groups were also made on the basis of the degree of the disease severity according to the different scores (FACED, EFACED, and BSI), in which the percentages of patients in each category were depicted in histograms using MedCalc statistical software (Penn State University, World Campus, State College, Pennsylvania, PA, USA).

3. Results

3.1. General Clinical Characteristics

Demographic variables are shown in Table 1. The variables age and sex did not significantly differ between the study groups. All the healthy controls were non-smokers. Patients exhibited mild airway obstruction compared to the controls. Lung volumes and diffusion capacity were preserved in bronchiectasis patients. Exercise capacity as measured by the walking test was significantly reduced in the patients compared to the controls. In the patients, radiological extension is also summarized in Table 1.

Table 1. Clinical characteristics in bronchiectasis patients and healthy controls.

	Healthy Controls N = 26	Bronchiectasis Patients N = 30
Clinical characteristics, mean (SD)		
Age, years	61 (11)	66 (12)
Female, N/male, N	17/9	23/7
Smoking history		
Ex-smokers, N (%)	0	9 (30) **
Never smokers, N (%)	26 (100)	21 (70) **
Packs-year, mean (SD)	NA	22 (15)
Lung functional assessment, mean (SD)		
FEV_1, % predicted	100 (13)	76 (25) ***
FVC, % predicted	100 (12)	85 (18) ***
FEV_1/FVC	80 (9)	68 (11) ***
RV, % predicted	NA	151 (36)
TLC, % predicted	NA	102 (16)
RV/TLC	NA	55 (9)
DL_{CO}, % predicted	NA	76 (15)
K_{CO}, % predicted	NA	80 (11)
Exercise capacity, mean (SD)		
6-min walking distance, meters	536 (72)	473 (96) *
Distance, % predicted	107 (12)	98 (17) *
Disease severity		
FACED score, mean (SD)	NA	1.9 (1.3)
Mild, N	NA	20
Moderate, N	NA	8
Severe, N	NA	2
EFACED score, mean (SD)	NA	2.1 (1.5)
Mild, N	NA	25
Moderate, N	NA	5
Severe, N	NA	0
BSI score, mean (SD)	NA	5.5 (3.2)
Mild, N	NA	13
Moderate, N	NA	12
Severe, N	NA	5
Radiological extension, mean (SD)		
Total extension score	NA	8.1 (3.3)
Bronchial dilatation score	NA	1.2 (0.2)
Bronchial wall thickness score	NA	1.3 (0.3)
Global score	NA	10.6 (3.4)

Continuous variables are presented as mean (standard deviation), while categorical variables are presented as the number of patients in each group along with the percentage for the study group. Abbreviations: N, number; NA, not applicable; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; DL_{CO}, carbon monoxide transfer; K_{CO}, Krogh transfer factor. FACED: F, FEV_1, force expiratory volume in the first second; A, Age; C, chronic colonization by Pseudomonas aeruginosa; E, radiological extension; D, dyspnea; EFACED: E, exacerbations with hospitalization in previous year; F, FEV_1, force expiratory volume in the first second; A, Age; C, Chronic colonization by Pseudomonas aeruginosa; E, radiological extension; D, dyspnea; BSI, Bronchiectasis Severity Index. FACED: mild: 0–2, moderate: 3–4, severe: 5–7; EFACED: mild: 0–3, moderate: 4–6, severe: 7–9; BSI: mild: 0–4, moderate: 5–8, severe: ≥ 9. Statistical analyses and significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ between bronchiectasis patients and control subjects.

Nutritional status as determined by BMI and FFMI and blood parameters were within normal ranges for both patients and controls, although albumin and prealbumin were significantly reduced in the patients compared to healthy subjects (Table 2).

Among patients with bronchiectasis, the parameters FEV_1, FEV1/FVC, TLC, and DL_{CO} significantly correlated with blood levels of prealbumin (r = 0.505, $p = 0.004$, r = 0.375, $p = 0.041$, r = 0.451, $p = 0.021$, and r = 0.550, $p = 0.004$, respectively, Figure 1A). Additionally, among the same patients, blood levels of albumin also correlated with DL_{CO} and six-minute walk distance (r = 0.426, $p = 0.030$ and r = 0.484, $p = 0.008$, respectively, Figure 1A).

Inverse significant correlations were found between bronchial wall thickness scores and the lung function parameters FEV_1 and FVC (r = −0.379, $p = 0.039$ and r = −0.371, $p = 0.044$, respectively, Figure 1B), and lymphocyte counts (r = −0.433, $p = 0.017$, Figure 1B), and a positive correlation with neutrophil counts (r = 0.360, $p = 0.050$, Figure 1B). Total global scores positively correlated with immunoglobulin G (IgG) levels (r = 0.447, $p = 0.013$, Figure 1B).

Table 2. Nutritional assessment in bronchiectasis patients and healthy controls.

	Healthy Controls N = 26	Bronchiectasis Patients N = 30
Nutritional parameters, mean (SD)		
BMI (kg/m^2)	27 (4)	25 (4)
FFMI (kg/m^2)	17 (2)	16 (3)
Hemoglobin, g/dL	14.3 (1.3)	13.9 (1.1)
Hematocrit, %	42.9 (3.9)	42.0 (3.7)
Glucose, mg/dL	102.9 (20.6)	94.4 (25.5)
Creatinine, mg/dL	0.8 (0.2)	0.7 (0.3)
Albumin, g/dL	4.6 (0.2)	4.4 (0.3) ***
Total proteins, g/dL	7.3 (0.3)	7.3 (0.4)
Prealbumin, g/dL	26.0 (4.9)	22.1 (5.0) **

Values are presented as mean (standard deviation). Abbreviations: N, number; BMI, body mass index; kg, kilograms; m, meters; FFMI, fat-free mass index; g, grams; dL, deciliter; mg, milligrams. Statistical analyses and significance: **, $p \leq 0.01$; ***, $p \leq 0.001$ between bronchiectasis patients and control subjects.

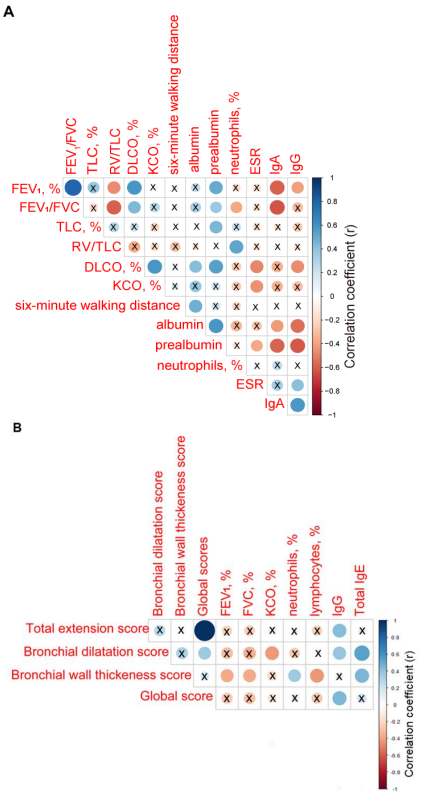

Figure 1. (**A**) Correlation matrix of the clinical and analytical variables. (**B**) Correlation matrix of radiological extension scores and clinical and analytical variables. In both matrices, positive correlations are represented in blue, while negative correlations are represented in red. The intersection within the circle represents p value > 0.05. Color intensity and the size of the circle are proportional to the correlation coefficients, as indicated in the Y axis on the right-hand side of the graph.

3.2. Disease Severity

The use of the severity scores FACED, EFACED, and BSI revealed that the majority of the patients had mild disease (67%, 83% and 43%, respectively, Figure 2A). EFACED and BSI scores negatively correlated with total lymphocytes (r = −0.384, $p = 0.360$ and r = −0.366, $p = 0.047$, Figure 2B). Moreover, BSI score positively correlated with immunoglobulin A (IgA) levels (r = 0.365, $p = 0.047$, Figure 2B). Both FACED and EFACED scores positively correlated with myeloperoxidase (r = 0.530, $p = 0.003$ and r = 0.403, $p = 0.027$, respectively, Figure 2B).

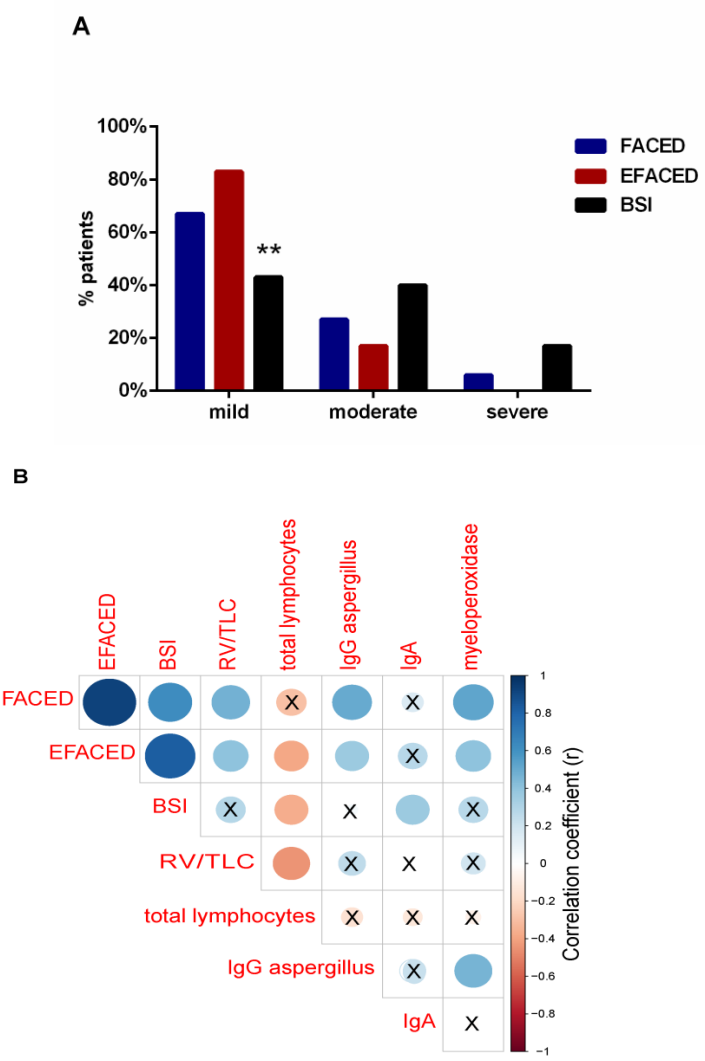

Figure 2. (**A**) Histograms of the proportions of patients according to disease severity: mild, moderate, and severe, according to FACED (blue histograms), EFACED (red histograms), and BSI (black histograms). Statistical significance: ** $p \leq 0.01$ between BSI and EFACED proportions of patients. (**B**) Correlation matrix of the disease severity scores and analytical variables, in which positive correlations are represented in blue, while negative correlations are represented in red. The intersection within the circle represents p value > 0.05. Color intensity and the size of the circle are proportional to the correlation coefficients, as indicated in the Y axis on the right-hand side of the graph.

3.3. Etiology and Microbiology of Bronchiectasis Patients

Most of the patients had post-infectious bronchiectasis, including tuberculosis (Table 3).

Table 3. Etiology of bronchiectasis in the study patients.

	Bronchiectasis Patients N = 30
Etiology	
Post-infectious, N (%)	22 (73)
COPD, N (%)	1 (3)
Unknown etiology, N (%)	7 (24)

Abbreviations: N, number; COPD, Chronic Obstructive Pulmonary Disease.

Moreover, the presence of microorganisms in the sputum of all the patients is listed in Table 4. Approximately one third of the patients had *Pseudomona aeruginosa* in their sputum samples (Table 4).

Table 4. Microbiological status of the study patients.

Patients	Germs	Score
Patient # 1	Haemophilus influenza, S	5
Patient # 2	Moraxella catarrhalis, S	5
Patient # 3	Pseudomona aeruginosa, S	3
Patient # 4	Pseudomona aeruginosa, S	3
Patient # 5	Commensal microbiota, S	5
Patient # 6	Pseudomona aeruginosa, S	5
Patient # 7	Commensal microbiota, S	6
Patient # 8	Pseudomona aeruginosa, S	5
Patient # 9	Pseudomona aeruginosa, S	5
Patient # 10	Commensal microbiota, S	5
Patient # 11	Commensal microbiota, S	5
Patient # 12	Commensal microbiota, S	5
Patient # 13	NSA, I	NA
Patient # 14	Pseudomona aeruginosa, S	5
Patient # 15	Commensal microbiota, S	6
Patient # 16	Pseudomona aeruginosa, S	5
Patient # 17	Commensal microbiota, S	5
Patient # 18	NSA, I	NA
Patient # 19	Commensal microbiota, S	5
Patient # 20	Commensal microbiota, S	5
Patient # 21	Commensal microbiota, S	6
Patient # 22	NC, S	2
Patient # 23	Commensal microbiota, S	3
Patient # 24	NSA, I	NA
Patient # 25	NSA, I	NA
Patient # 26	Commensal microbiota, S	6
Patient # 27	Pseudomona aeruginosa, S	6
Patient # 28	Commensal microbiota, S	6
Patient # 29	Pseudomona aeruginosa, S	6
Patient # 30	Commensal microbiota, S	6

Abbreviations: S, spontaneous; I, induced; NSA, no sputum available; NA, not available; NC, no culture.

3.4. Systemic Oxidative Stress Markers

Prooxidants. Levels of oxidized DNA did not significantly differ between bronchiectasis patients and healthy controls, even when patients were subdivided according to smoking history or pseudomona colonization (Figure 3A and Tables S1 and S2). Plasma MDA–protein adduct levels were significantly higher in bronchiectasis patients, independently of smoking history or pseudomona infection, compared to control subjects (Figure 3B and Tables S1 and S2). In bronchiectasis patients, positive significant correlations were detected between MDA–protein adduct levels and immunoglobulin M (IgM), IgA, and IgG levels (r = 0.499, $p = 0.006$, r = 0.438, $p = 0.017$, and r = 0.416, $p = 0.025$, respectively, Figure 3C).

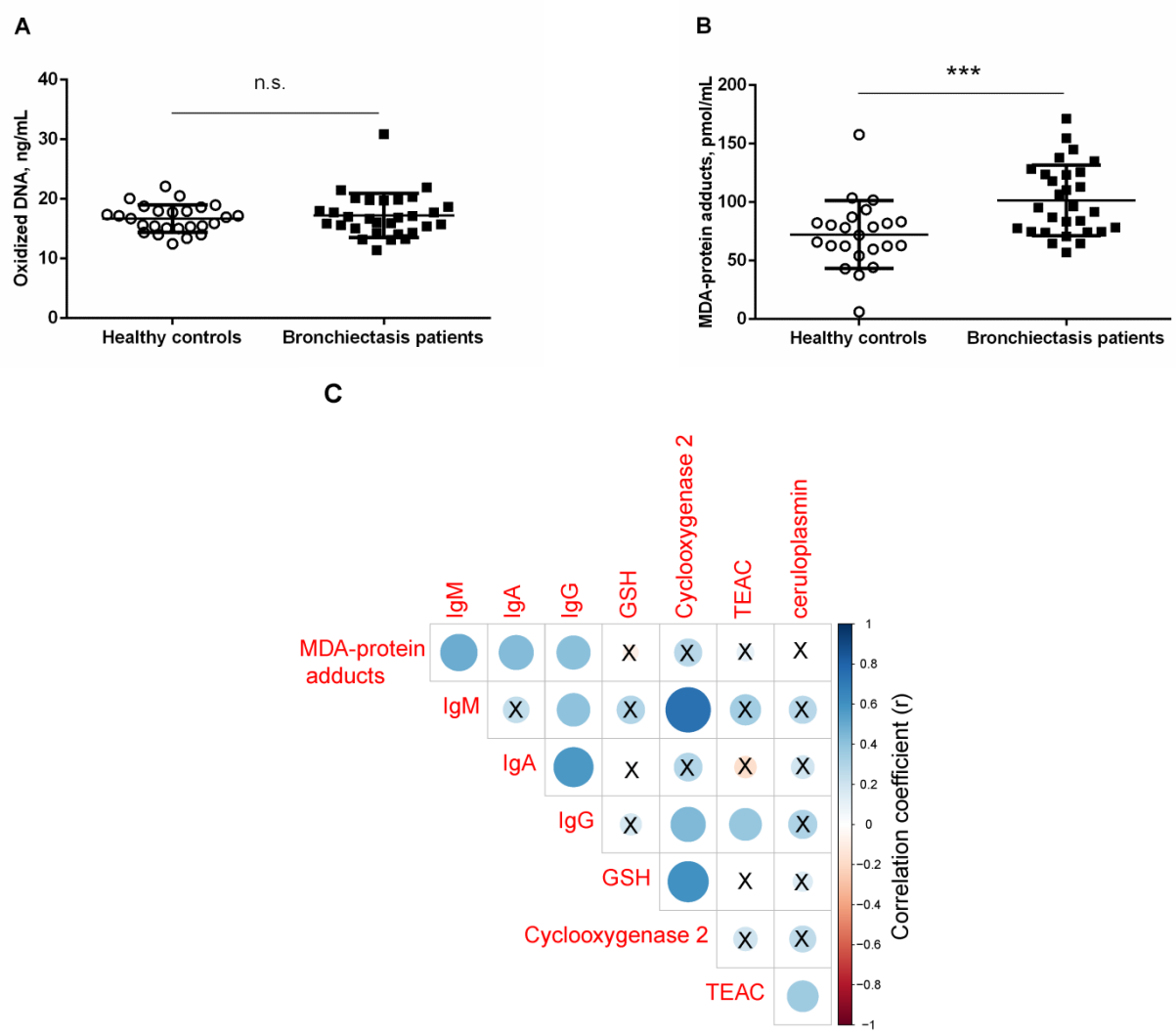

Figure 3. Mean values and standard deviations of plasma (**A**) oxidized DNA levels (ng/mL) and (**B**) MDA–protein adducts (pmol/mL) in healthy controls and bronchiectasis patients. Statistical significance: *** $p \leq 0.001$ between bronchiectasis patients and healthy controls; and n.s., non-significant differences between the two study groups. (**C**) Correlation matrix of the disease among different biological and analytical variables, in which positive correlations are represented in blue, while negative correlations are represented in red. The intersection within the circle represents p value > 0.05. Color intensity and the size of the circle are proportional to the correlation coefficients, as indicated in the Y axis on the right-hand side of the graph.

Antioxidants. Plasma GSH levels were significantly greater in bronchiectasis patients, especially in the ex-smokers and in the pseudomona-colonized patients, than in healthy controls (Figure 4A and Tables S2 and S3). Furthermore, plasma GSH levels were signifi-

cantly higher in ex-smokers and pseudomona-colonized patients than in non-smokers and non-pseudomona patients, respectively (Tables S2 and S3).

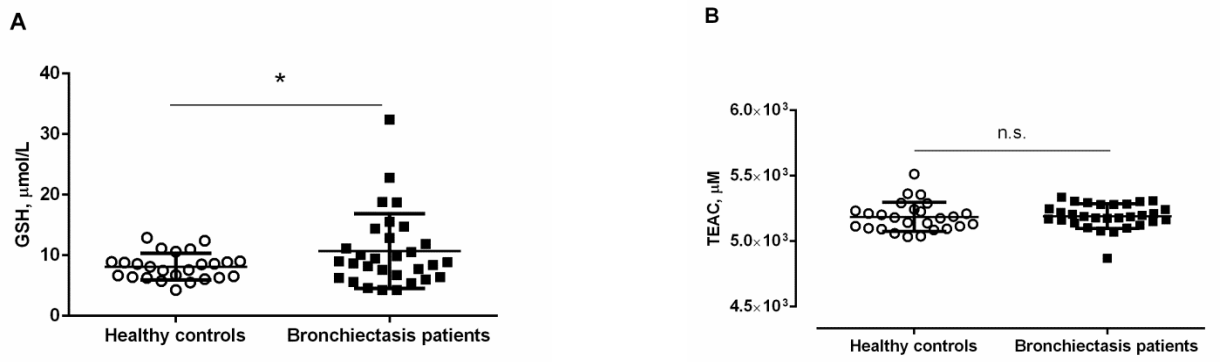

Figure 4. Mean values and standard deviations of plasma (**A**) GSH levels (µmol/L) and (**B**) TEAC levels (µM) in healthy controls and bronchiectasis patients. Statistical significance: * $p \leq 0.05$ between bronchiectasis patients and healthy controls; and n.s., non-significant differences between the two study groups.

No significant differences were detected in plasma TEAC levels between the two study groups, and smoking history or pseudomona colonization did not influence the results (Figure 4B and Tables S2 and S3). Significant positive associations were detected between plasma levels of GSH and cyclooxygenase-2 (r = 0.607, $p < 0.001$, Figure 3C). Significant positive associations were also observed between TEAC levels and either IgG or ceruloplasmin (r = 0.384, $p = 0.036$ and r = 0.358, $p = 0.050$, respectively, Figure 3C).

3.5. Systemic Inflammatory Markers

The percentage of blood neutrophils was significantly greater in the patients compared to healthy controls (Tables 5, S2 and S3). Conversely, total blood lymphocyte counts and the percentage of these cells were significantly lower in the patients than in the healthy controls (Tables 5, S2 and S3). Importantly, levels of the plasma inflammatory parameters c-reactive protein (CRP), erythrocyte sedimentation rate (ESR), fibrinogen, alpha-1 antitrypsin, and ceruloplasmin were significantly higher in bronchiectasis patients, irrespective of smoking history, and particularly non-pseudomona infected patients compared to healthy controls (Tables 5 and S2). Moreover, plasma levels of IgA and IgG were significantly greater in bronchiectasis patients, particularly in those infected by pseudomona, than in healthy controls (Tables 5, S2 and S3).

Among the bronchiectasis patients, positive significant correlations were detected between alpha-1 antitrypsin levels and the percentage of neutrophils, ceruloplasmin, IgM, and IgA (r = 0.441, $p = 0.015$, r = 0.758, $p < 0.001$, r = 0.381, $p = 0.038$, and r = 0.396, $p = 0.030$, respectively, Figure 5A), while a negative correlation was seen between alpha-1 antitrypsin levels and the percentage of lymphocytes (r = −0.393, $p = 0.032$, Figure 5A).

Plasma levels of myeloperoxidase were significantly higher in bronchiectasis patients than in healthy controls (Figure 5B and Table S2). Pseudomona colonization did not significantly influence plasma myeloperoxidase levels among the patients (Table S3). Plasma levels of cyclooxygenase-2 did not significantly differ between bronchiectasis patients and the controls (Figure 5C). However, plasma levels of clyclooxygenase were greater in the ex-smokers than in the non-smoker patients (Table S2).

Plasma levels of myeloperoxidase positively correlated with IgG aspergillus levels (r = 0.460, $p = 0.010$, Figure 5A). Plasma levels of cyclooxygenase-2 also positively correlated with IgM and IgG (r = 0.741, $p < 0.001$ and r = 0.444, $p = 0.014$, Figure 5A), whereas inverse correlations were observed between cyclooxygenase-2 levels and either total leukocyte or neutrophil counts (r = −0.498, $p = 0.005$ and r = −0.474, $p = 0.008$, respectively, Figure 5A).

Table 5. Systemic inflammatory parameters in bronchiectasis patients and healthy controls.

	Healthy Controls N = 26	Bronchiectasis Patients N = 30
Systemic inflammatory parameters, mean (SD)		
Total leukocytes, $\times 10^3/\mu L$	6.3 (1.6)	6.4 (1.6)
Total neutrophils, $\times 10^3/\mu L$	3.9 (1.2)	4.1 (1.4)
Neutrophils, %	57.6 (6.9)	63.2 (7.8) **
Total lymphocytes, $\times 10^3/\mu L$	2.1 (0.6)	1.5 (0.40) ***
Lymphocytes, %	31.7 (6.3)	24.5 (6.2) ***
Total eosinophils, $\times 10^3/\ \mu L$	0.15 (0.12)	0.16 (0.09)
Eosinophils, %	2.3 (1.6)	2.5 (1.4)
Platelets, $\times 10^3/\mu L$	246 (63)	257 (69)
CRP, mg/dL	0.23 (0.4)	0.70 (0.9) *
ESR, mm/h	8 (7)	15 (12) **
Fibrinogen, mg/dL	305 (69)	370 (84) **
Alpha-1 antitrypsin, mg/dL	117.9 (18.3)	132.5 (25.4) *
Ceruloplasmin, mg/dL	22.7 (5.1)	27.0 (5.4) **
IgE, IU/mL	44 (42)	66 (81)
IgG aspergillus, mg/L	26 (23)	37 (35)
IgM, mg/dL	96 (44)	112 (85)
IgA, mg/dL	249 (131)	330 (134) *
IgG, mg/dL	1089 (199)	1273 (384) *

Values are presented as mean (standard deviation). Abbreviations: N, number; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IgE, immunoglobulin E; IgG aspergillus, immunoglobulin G aspergillus; IgM, immunoglobulin M; IgA, immunoglobulin A; IgG, immunoglobulin G; µL, microliter; mg, milligrams; mm, millimeters; h, hour; dL, deciliter; IU, international unit; dL, deciliter. Statistical analyses and significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ between bronchiectasis patients and control subjects.

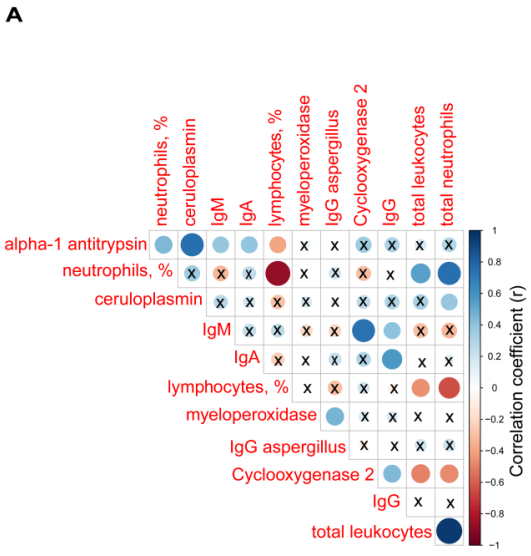

Figure 5. *Cont.*

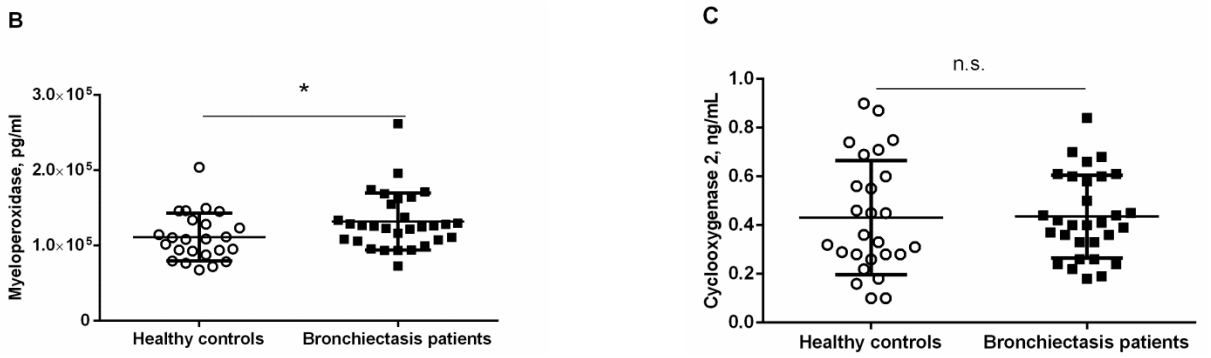

Figure 5. (**A**) Correlation matrix of biological and analytical variables, in which positive correlations are represented in blue, while negative correlations are represented in red. The intersection within the circle represents p value > 0.05. Color intensity and the size of the circle are proportional to the correlation coefficients, as indicated in the Y axis on the right-hand side of the graph. Mean values and standard deviations of plasma (**B**) myeloperoxidase levels (pg/mL) and (**C**) cyclooxygenase levels (ng/mL) in healthy controls and bronchiectasis patients. Statistical significance: * $p \leq 0.05$ between bronchiectasis patients and control subjects and n.s., non-significant differences between the study groups.

4. Discussion

In the current investigation, the most relevant findings were that in stable bronchiectasis patients with relatively mild disease, levels of parameters indicative of systemic inflammation and oxidative stress were significantly increased compared to a population of healthy subjects. Patients exhibited very mild airway obstruction, preserved lung volumes and diffusion capacity, and exercise tolerance was reduced, although within normal ranges, compared to the healthy controls. Acute phase reactant levels along with neutrophil percentages were increased in the stable patients compared to control subjects. These relevant findings are discussed below.

In the current investigation, patients had mild–very mild disease, as demonstrated by the disease severity scores and lung function parameters. The majority of the patients were classified as mild according to BSI and both FACED and EFACED scores, particularly the latter ones. In fact, greater proportions of patients fell into the category of mild disease when using the FACED and EFACED scores than with the BSI classification. Differences between the two types of scales are attributable to the fact that BSI contains more items for each variable [25,26]. Furthermore, patients exhibited mild airway obstruction, and exercise capacity as measured by walking distance was also mildly reduced compared to the control subjects. Nutritional parameters were also within the normal range in this cohort of patients with post-infectious bronchiectasis in most of the cases. These results confirm that the study population met the inclusion criteria for the purpose of the investigation.

Acute exacerbations are associated with increased levels of several systemic inflammatory molecules, namely proinflammatory cytokines, especially in patients with infection by *Pseudomona aeruginosa* [36]. Systemic inflammatory parameters, particularly tumor necrosis factor (TNF)-alpha, fibrinogen, ESR, and CRP, were also significantly higher in patients with mild-to-moderate bronchiectasis compared to healthy controls [19]. Moreover, high plasma levels of TNF-alpha were associated with worse disease severity, as identified by greater computed tomography (CT) scan scores, higher airway obstruction, and colonization by *Pseudomona aeruginosa* [19]. Importantly, in the present study, acute phase reactants (CRP, ESR, fibrinogen, alpha-1 antitrypsin, and ceruloplasmin), neutrophil counts, and myeloperoxidase levels were all significantly increased in this cohort of patients with stable mild bronchiectasis, in whom the prevalence of *Pseudomona aeruginosa* colonization was low (only one third of the patients). Besides, diffusion capacity was negatively associated with ESR among the patients. Importantly, patients with no colonization by *Pseudomona aeruginosa* were those with a significant rise in plasma CRP, ESR, fibrinogen, and ceruloplasmin compared to the controls. In non-smoker patients, levels of ESR and fibrinogen also significantly increased compared to controls. Thus, smoking history did

not influence these results. Collectively, these findings suggest that systemic inflammation is very prominent in bronchiectasis, even in patients with very stable disease (no acute exacerbations in the previous 6–12 months for some of the patients) showing no nutritional abnormalities, active airway infections, or any other systemic manifestations. As far as we are concerned, these are relevant novel findings as patients in previous series exhibited more severe disease and/or reported a history of microbial colonization [19,36]. It should also be mentioned that cyclooxygenase plasma levels did not differ between patients as a whole and healthy controls, implying that this pathway was not involved in the pathophysiology of systemic inflammation associated with bronchiectasis. Nonetheless, a rise in plasma cyclooxygenase levels was detected in the ex-smokers compared to non-smoker patients. These results imply that cigarette smoke exposure may induce cyclooxygenase, as previously stated [37]. Plasma levels of myeloperoxidase were, indeed, higher in the patients than in the controls. As bronchiectasis is a rather neutrophilic disease, as also confirmed in our study, levels of the enzyme myeloperoxidase were expectedly increased in the patients.

In the present study, plasma levels of IgA and IgG were significantly increased in the patients compared to the controls. Patients with colonization by pseudomona were those exhibiting the greatest levels of both IgA and IgG. These results imply that none of the patients had immunodeficiencies in this series, as previously reported in other cohorts [38,39]. Conversely, in another series, high levels of IgA and IgG were inversely correlated with lung function deterioration and disease extension (CT scans) [9]. In that study, however, a control group of patients was not included in the investigation. In the present investigation, levels of IgG and IgA were significantly greater in the patients than in the group of healthy controls. Moreover, inverse correlations were also detected between either IgA or IgG and the degree of airway obstruction (FEV_1, FEV_1/FVC) and diffusion capacity (DL_{CO}). A significant positive correlation was also detected between IgG levels and total global scores of radiological extension. Despite the fact that patients exhibited mild disease (low disease severity scores and preserved lung function), radiological extension may account for the increased levels of all the acute phase reactants and immunoglobulins. Interestingly, inverse correlations were also found between the degree of airway obstruction of the patients and the radiological parameter bronchial wall thickness, as also previously demonstrated in another investigation [40].

Oxidants that escape the antioxidant systems target several cellular structures, among which proteins may be severely affected. Reactive carbonyl derivatives (aldehydes and ketones) result from the reaction of oxidants with several amino acids in the protein side-chains. In addition, Michael addition reactions of other amino acid residues with α,β-unsaturated aldehydes resulting from the peroxidation of polyunsaturated fatty acids also lead to the formation of reactive carbonyls, which can be detected in tissues using specific antibodies [41–43]. Increased levels of MDA–protein adducts have been shown in several compartments of patients with COPD and in lung cancer [11,13,31,44]. In the present study, a rise in plasma MDA–protein adducts was also detected for the first time among patients with stable bronchiectasis. Similarly, an increase in reactive carbonyls and superoxide anion levels was also reported in a cohort of bronchiectasis patients with moderate-to-severe airway obstruction [10]. In this study, however, disease severity was not assessed in the study patients. Significant correlations were observed between plasma MDA–protein adduct levels and IgG, IgM, and IgA, suggesting that disease extension may be associated with levels of oxidative stress.

Cells contain several enzymatic and non-enzymatic antioxidants to fight against the deleterious effects of oxidants. Superoxide dismutase isoforms, catalase, and glutathione peroxidases are abundantly expressed antioxidant enzymes. The action of antioxidant enzymes is complemented by the effects of non-enzymatic antioxidant systems. As such, glutathione, which is a water-soluble compound, is extensively distributed in organs and tissues. Reduced glutathione (GSH) levels are indicative of the redox potential of a tissue. In children with CF (3 years of age), low levels of GSH due to an inherent glutathione

deficiency detected in the airways occurred early in life, which enhanced the oxidative stress response during infections [45].

In the current investigation, however, plasma levels of GSH were increased in the stable non-CF bronchiectasis patients. Such an increase was particularly evident among patients colonized by *Pseudomona aeruginosa* and the ex-smokers. These findings suggest that the rise in GSH was most likely trying to counterbalance the deleterious effects of increased plasma protein oxidation levels, especially among patients with *Pseudomona aeruginosa* colonization. Other markers of oxidative stress also analyzed in the study (oxidized DNA and TEAC) did not seem to play any significant role in this cohort of patients as no significant differences from the healthy controls were observed.

Study Limitations

In the current investigation, a greater number of patients might have been recruited for the purpose of the study. However, sample size calculation showed that a sample of 23 patients was sufficient to reach 85% statistical power. Moreover, the investigation focused mainly on the assessment of patients with mild and very mild disease, in whom a significant rise in plasma inflammatory and oxidative stress balance was already observed. Other series, however, have reported significant changes in patients with more advanced and larger extension bronchiectasis [10,45]. Furthermore, future investigations with a larger cohort of patients will have to address further questions such as whether the profile of bronchial infection including those with no bacterial predominance (normal flora) may determine variations in the systemic patterns of inflammation and redox balance in patients with mild bronchiectasis. Additionally, future research should be devoted to the study of the mechanisms whereby increased oxidative stress and inflammation may precede other systemic manifestations, namely nutritional and muscle status alterations.

5. Conclusions

In stable patients with mild bronchiectasis, several important inflammatory and oxidative stress events take place in plasma. These findings suggest that the extension of bronchiectasis probably plays a role in the development of redox imbalance and inflammation in patients with mild bronchiectasis. Increased inflammation and oxidative stress in bronchiectasis may precipitate the occurrence of additional systemic manifestations in these patients, especially those related to muscle and nutritional abnormalities. These findings have therapeutic implications in the management of bronchiectasis patients.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10194534/s1, Table S1: Criteria for evaluation of the quality of sputum specimens, Table S2: Systemic inflammatory and oxidative stress parameters in bronchiectasis patients according to smoking history, Table S3: Systemic inflammatory and oxidative stress parameters in bronchiectasis patients according to pseudomona aeruginosa colonization.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets are available from the corresponding authors upon reasonable request.

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

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5. OVERALL SUMMARY OF RESULTS

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The overall summary of results of the two studies of the current thesis are described below:

Study #1

In LC-only patients compared to non-LC controls:

- The expression of miR-451 were significantly decreased in LC-only patients compared to non-LC controls.
- The expression of miR-let7c were significantly greater in LC-only patients compared to non-LC controls.
- Plasma levels of GSH, TEAC, and TGF-beta1 were significantly higher in LC-only patients than in non-LC controls.

In LC-COPD patients compared to non-LC controls:

- The expression of miR-451 were significantly decreased in LC-COPD patients compared to non-LC controls.
- The expression of miR-let7c were significantly greater in LC-COPD patients compared to non-LC controls.
- Plasma levels of MDA-protein adducts, TEAC and TGF-beta1 were significantly higher in LC-COPD patients compared to non-LC controls.
- GSH levels were significantly declined in LC-COPD patients compared to non-LC controls.

In LC-COPD patients compared to LC-only patients:

- MDA-protein adduct levels were significantly higher in LC-COPD patients compared to LC-only patients.
- Plasma levels of GSH were significantly declined in LC-COPD patients compared to LC-only patients.

Study #2

In bronchiectasis patients compared to healthy controls:

- Systemic oxidative stress markers:
  - Plasma MDA-protein adduct levels were significantly higher in bronchiectasis patients, independently of smoking history or pseudomonas colonization, compared to healthy controls.
  - Plasma GSH levels were significantly greater in bronchiectasis patients, especially in the ex-smoker and in pseudomonas-colonized patients, than in healthy controls.
- Systemic inflammatory markers:
  - Levels of plasma inflammatory parameters CRP, ESR, fibrinogen, alpha-1 antitrypsin, and ceruloplasmin were significantly higher in bronchiectasis patients, irrespective of smoking history , and particularly non-pseudomonas infected compared to heathy

controls.

- Plasma levels of IgA and IgG were significantly higher in bronchiectasis patients, particularly in those infected by pseudomonas, than in healthy controls.
- Plasma levels of myeloperoxidase were significantly higher in bronchiectasis patients, irrespective of smoking history or pseudomonas colonization, compared to healthy controls.

6. OVERALL SUMMARY OF THE DISCUSSION

6. OVERALL SUMMARY OF THE DISCUSSION

LC, COPD and bronchiectasis are common chronic respiratory diseases. These three diseases affect the lungs and the airways, as well as other organs outside the lungs, the so-called systemic effects. Systemic inflammation, nutritional abnormalities, and skeletal muscle dysfunction are well-known systemic effects in these diseases (49,70,166). There is a growing realization that systemic effects are relevant and may contribute to earlier detection, and management of these diseases. Blood compartments are non-invasive, easy of acquisition and repeat, and cheaply (167), systemic effects on blood components may make the early detection reality. In the current thesis, we focused on the following analyses: 1) oxidative stress in blood of LC patients with and without COPD, and non-CF bronchiectasis patients; 2) inflammation in the blood of LC patients with and without COPD, and non-CF bronchiectasis patients; 3) microRNAs expression profiles in blood of LC patients with and without COPD.

A principle biological mechanism involved in systemic damage is ROS (90). ROS are produced as a result of cellular metabolism, tobacco smoke or pollution. ROS affect cell structures through oxidizing DNA, lipids and proteins (168). An imbalance of oxidant/antioxidant agents in favor of oxidants give rise to oxidative stress (169). Regulation of reducing redox state is crucial for cell viability and organ function (170). The human body responds to ROS with antioxidant systems (including enzymatic and nonenzymatic antioxidants), but in certain cases, it may be insufficient, triggering different pathological processes (171). Markers of oxidant-antioxidant imbalance have been demonstrated in chronic respiratory diseases including LC (32,172), COPD (89,173) and bronchiectasis (87).

Oxidative stress can be determined by measuring the products of both oxidative and antioxidant systems in tissues or body fluids (174). To carry out a global evaluation of oxidative stress, we have determined the levels of oxidized DNA, lipid peroxidation products (MDA), and different antioxidants (GSH and TEAC) in plasma samples. In our study, oxidized DNA levels did not differ between neither LC-only patients nor LC-COPD patients and non-LC controls. Moreover, no significant differences were seen between non-CF bronchiectasis and healthy controls for oxidized DNA levels. We found that levels of MDA-protein adducts were increased in the plasma of LC-COPD patients compared to LC-only patients and non-LC controls. These results were in line with a previous study (31), in which higher levels of MDA-protein adduct were observed in LC-COPD patients compared to LC patients. These findings suggest that COPD may induce a rise in systemic oxidants in LC patients. We also found that plasma levels of MDA-protein adducts were increased in non-CF bronchiectasis patients compared to healthy controls. Similarly, another study showed that MDA levels in exhaled breath condensate of bronchiectasis patients were significantly higher than in the control group (175). Taken together, we can suggest that an increase of MDA levels can be found both in exhaled breath condensate (local) and in the blood (systemic) of bronchiectasis patients. As blood samples can easily repeat, the blood MDA-protein adducts levels may serve as a potential, non-invasive marker in the follow-up of bronchiectasis patients.

Preventive mechanisms against ROS accumulation are antioxidant systems (176). Glutathione is the main non-enzymatic antioxidant and reduced glutathione (GSH) is known as one of the most essential scavengers of ROS (110). We found

that plasma levels of GSH were reduced in LC-COPD patients compared to LC-only patients and non-LC controls. Similar to our findings, a meta-analysis revealed that blood GSH levels were reduced in COPD patients compared to healthy controls (118), suggesting that an impaired antioxidant defenses system plays a prominent role in the pathogenesis of COPD. However, plasma levels of GSH were greater in LC-only patients than in non-LC controls. We also found that plasma levels of GSH were significantly higher in non-CF bronchiectasis compared to healthy controls. As systemic oxidants were increased in non-CF bronchiectasis, the increased plasma levels of antioxidant GSH is most likely to counteract the increased oxidants. Taken together, despite all of these are chronic respiratory diseases, the GSH deregulation may be different, further investigations will be needed. In addition to the aforementioned markers, the TEAC has been used to evaluate the total antioxidant capacity of body fluids (177). In the current thesis, plasma TEAC levels were significantly higher in both groups of LC patients than in non-LC controls. To the best of our knowledge, our investigation would be the first to show that the antioxidant capacity relative to the standard Trolox was increased in response to lung carcinogenesis among the LC patients irrespective of COPD. However, no significant difference was observed in plasma TEAC levels between non-CF bronchiectasis patients and healthy controls, which could suggest that TEAC did not have an important role in non-CF bronchiectasis.

Systemic inflammation through its cellular, molecular effectors plays a significant role in the development and progression in LC, COPD and bronchiectasis (51,83,178). The main source of systemic inflammation was the spillover of

pulmonary inflammatory mediators into the bloodstream or the activation of inflammatory cells in their transit through the pulmonary circulation (57). Neutrophils are the most common circulating leukocytes and are the first line of innate immune defense (179). The accumulated neutrophils in the lungs play an important role in pulmonary failure caused by chronic inflammation, but its mechanism remains unclear (180). High circulation neutrophil counts are linked to disease severity of COPD. In our investigation, plasma levels of neutrophils were higher in both LC patients compared to non-LC controls. Furthermore, the percentage of neutrophils was also significantly increased in bronchiectasis patients compared to healthy controls. Neutrophils recruited to the airways are activated because of the increased granule proteins, such as myeloperoxidase (181). These results are in agreement with our investigation since plasma levels of myeloperoxidase were increased in bronchiectasis patients compared to healthy controls. TGF-beta1 has been demonstrated to recruit and activate neutrophils (182). Serum TGF-beta1 levels have been shown to increase in bronchiectasis patients compared to control subjects (182). A recent study revealed that higher TGF-beta expression predicted a worse prognosis in patients with NCSLC (183). In line with this study, we found that plasma TGF-beta1 levels were greater in both groups of LC patients compared to non-LC controls. Moreover, plasma TGF-beta1 levels correlated inversely with diffusion capacity. These results suggest that patients with a certain degree of emphysema were those exhibiting greater TGF-beta1 plasma levels, despite there was no significant difference between the two study groups. Taken together, TGF-beta1 may be used as a prognosis marker in the follow-up of LC patients.

During the inflammation, the spill-over of cytokines such as IL-6 and TNF-a into the circulation may increase APPs, such as fibrinogen and CRP (184). In our investigation, blood levels of ESR were significantly higher in both LC patients than non-LC controls. Furthermore, the APPs (CRP, ESR, fibrinogen, alpha-1 antitrypsin, and ceruloplasmin) were significantly greater in non-CF bronchiectasis patients than those of healthy controls. The presence of systemic inflammatory markers such as acute phase reactants could arise because of the overflow of local markers in situations of pulmonary inflammation of bronchiectasis patients (83). Taken together, all these findings suggest that systemic inflammation is remarkable in LC and non-CF bronchiectasis.

Cyclooxygenase-2, a known inflammatory target protein, is specially associated with airway inflammation in response to various stimuli (185). Studies have demonstrated overexpression of cyclooxygenase-2 in NSCLC patients (186,187). In our investigation, plasma cyclooxygenase levels did not vary between the non-CF bronchiectasis patients and healthy controls. However, these levels were higher in ex-smokers than in non-smokers. The results are not surprising as cigarette smoke can induce cyclooxygenase expression (188).

In the present thesis, plasma levels of IgA and IgG were significantly increased in the non-CF bronchiectasis patients compared to the controls. Our results are in line with the study of Hill *et al.*, in which higher levels of IgG_1, IgG_2, IgG_3 were observed in bronchiectasis patients compared with the control group (189). Moreover, in the current thesis, inverse correlations were also detected between either IgA or IgG and the degree of airflow obstruction (FEV1, FEV1/FVC) and diffusion capacity (DLCO). Similarly, in another series, IgA and IgG were

correlated inversely with lung function parameters (FEV_1 and FVC) (190). It should also be mentioned that IgG levels correlated positively with total global scores of radiological extensions in the present thesis. Our findings suggest that the radiological extension may account for the increased levels of immunoglobulins. Interestingly, bronchial wall thickness scores correlated inversely with the degree of airway obstruction of bronchiectasis patients in the present thesis. This finding is in agreement with the results demonstrated in another investigation, in which bronchial wall thickness scores negatively correlated with functional parameter values (FEV_1%, FVC% and FEV_1/FVC) (191).

Differential epigenetic mechanisms in LC patients

Oxidative stress and inflammation alter the cells' redox status, promoting genomic instability and leading to epigenetic changes (124). Epigenetic changes mainly include DNA methylation, histone modification, and miRNA-mediated post-transcriptional alterations. The expression and function of miRNAs are regulated by epigenetic factors (including DNA methylation and histone modification) (192). In addition, microRNAs can also regulate epigenetic mechanisms by targeting key enzymes (125). In our study, we have identified plasma miRNA expressions in LC patients. MiRNAs in plasma show high stability and can be protected against endogenous RNAse activity (140), marking them as easy-to-detect markers.

MiR-451 is a tumor suppressive microRNA, lower expression of miR-451 is correlated with a poor prognosis in NSCLC patients (148,149). In our

investigation, plasma expression levels of miR-451 were significantly reduced in both groups of patients with LC compared to non-LC controls, which is consistent with the results of Goto *et al*.(149). As miR-451 strongly suppressed the proliferation of NSCLC cells in vitro by targeting ras-related protein 14 (RAB14) expression (148), plasma levels of miR-451 were expectedly decreased in LC patients. Hence, plasma miR-451 may serve as a novel and noninvasive biomarker for LC patients.

It has been demonstrated that miR-210 expression levels were significantly higher in the tumor tissues of LC patients than in normal control tissues, suggesting that miR-210 may be involved in the progression of LC patients (193). In our investigation, plasma levels of miR-210 were significantly decreased in the LC-COPD patients compared to LC-only patients, which were coincided with the study of Li *et al*. (194), in that study, the expression of miR-210 was lower in the COPD complicating LC group than in the LC group (194). Our findings indicate that miR-210 may serve as a surrogate marker to monitor LC predisposition of COPD.

Interestingly, in the present thesis, plasma expression levels of miR-let7c were significantly higher in both groups of LC patients than in non-LC controls. In a previous study of our group, tumor expression levels of miR-let7c were significantly greater in LC-COPD than in LC patients (36). While its downstream marker *k*-RAS was downregulated in LC-COPD patients than in LC patients. Furthermore, let-7 was found to inhibit RAS expression, and deletion or reduction of let-7 can lead to high RAS expression in lung cancer cells (195).

Taken together, the results obtained in our investigation suggest that miRNAs may contribute to the clinical practice of LC patients, as epigenetic mechanisms may be involved in the pathogenesis of LC, and contribute to progression of LC patients.

7. CONCLUSIONS

7. CONCLUSIONS

1. Systemic oxidative and antioxidative markers such as MDA and GSH are differently expressed in LC patients with COPD, thus implying their contributions to the tumor pathogenesis of these patients. Non-CF bronchiectasis, on the other hand, showed an increased level of oxidant and antioxidant markers analyzed.

2. Systemic inflammation takes place in the plasma of LC patients and non-CF bronchiectasis patients; these findings suggest that inflammation may be involved in the pathogenesis of these chronic respiratory diseases.

3. Plasma miRNAs have been shown to be differently expressed in LC patients, specifically miR-451, miR-let 7c, and miR-210; which suggests that those miRNAs may be surrogate markers of lung tumorigenesis that could help monitor patients in the clinics.

8. FUTURE PERSPECTIVES

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Future research should aim at analyzing markers of oxidative stress and inflammation in the sputum of LC patients with COPD and non-CF bronchiectasis patients. And to study their possible associations with the results obtained from blood samples.

Moreover, as a differential expression profile of blood miRNAs was detected in LC patients, further studies could be performed to explore the mechanism of those miRNAs in the lung specimens (tumor and nontumor) of LC patients. And to assess the possible correlations between the blood miRNAs and lung specimens' miRNAs. Further research should also aim to investigate the expression profile of miRNAs in the blood of non-CF bronchiectasis patients.

9. BIBLIOGRAPHY

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10. APPENDIX

10. APPENDIX

10.1 Scientific collaborations

The investigations of the present thesis were conducted in the Muscle Wasting and Cachexia in Chronic Respiratory and Lung Cancer Research Group, Institute of Medical Research of Hospital del Mar (IMIM)-Hospital del Mar, Barcelona, Spain. The following departments participated in the two publications were the Lung Cancer Clinic of the Respiratory Medicine Department at Hospital del Mar, and the Multidisciplinary Bronchiectasis Unit of the Respiratory Department at Hospital del Mar, Parc de Salut Mar, Barcelona, Spain.

10.2 Other publications

10.2.1 Publication 1

Title:

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Authors:

Liyun Qin, Filipe Gonçalves-Carvalho, Yingchen Xia, Jianhua Zha, Mireia Admetlló, José María Maiques, Sandra Esteban-Cucó, Xavier Duran, Alicia Marín, and **Esther Barreiro**

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Profile of Clinical and Analytical Parameters in Bronchiectasis Patients during the COVID-19 Pandemic: A One-Year Follow-Up Pilot Study

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Abstract: Whether the COVID-19 pandemic may have modified the clinical planning and course in bronchiectasis patients remains to be fully elucidated. We hypothesized that the COVID-19 pandemic may have influenced the management and clinical outcomes of bronchiectasis patients who were followed up for 12 months. In bronchiectasis patients (n = 30, 23 females, 66 years), lung function testing, disease severity [FEV_1, age, colonization, radiological extension, dyspnea (FACED), exacerbation (EFACED)] and dyspnea scores, exacerbation numbers and hospitalizations, body composition, sputum microbiology, and blood analytical biomarkers were determined at baseline and after a one-year follow-up. Compared to baseline (n = 27, three patients dropped out), in bronchiectasis patients, a significant increase in FACED and EFACED scores, number of exacerbations, and erythrocyte sedimentation rate (ESR) was observed, while FEV_1, ceruloplasmin, IgE, IgG, IgG aspergillus, IgM, and IgA significantly decreased. Patients presenting colonization by *Pseudomonas aeruginosa* (PA) remained unchanged (27%) during follow-up. In bronchiectasis patients, FEV_1 declined only after a one-year follow-up along with increased exacerbation numbers and disease severity scores, but not hospitalizations. However, a significant decrease in acute phase-reactants and immunoglobulins was observed at the one-year follow-up compared to baseline. Despite the relatively small cohort, the reported findings suggest that lung function impairment may not rely entirely on the patients' inflammatory status.

Keywords: non-cystic fibrosis bronchiectasis; systemic inflammation and immunoglobulins; nutritional status; lung function; severity scores; immunoglobulins; one-year follow-up

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1. Introduction

Bronchiectasis is a chronic respiratory condition characterized by abnormalities in the airways of the patients that lead to increased sputum production, cough, chest pain, and eventually dyspnea. The etiology varies widely from previous lung infections to genetic diseases that are diagnosed early in childhood [1–3]. Moreover, other chronic respiratory diseases, namely chronic obstructive pulmonary disease (COPD) and asthma, may be associated with bronchiectasis [1,4–7]. The prevalence of bronchiectasis is progressively increasing as the diagnostic tools become more widely available in different clinical settings [1,8–10]. Patients with bronchiectasis are prone to suffer acute exacerbations, which in many cases may require hospitalizations [11–13].

In patients with chronic airway diseases, follow-up studies are of interest to monitor the potential loss of respiratory function and control of symptoms. An eight-year follow-up investigation [14] concluded that female patients were predominant, had persistent symptoms, and a severe loss of lung function was detected. The same investigators also demonstrated [15] that patients with bronchiectasis were colonized by the same bacterium over a five-year follow-up period, and that phenotypic features were associated with different pathogens. A more recent investigation [16] demonstrated that multimorbidity was common in patients with bronchiectasis and negatively influenced survival. Furthermore, the line was also put forward that specific disease scores helped predict mortality and outcomes during the five-year follow-up period in the same study [16]. Whether similar findings can be observed in follow-up studies of shorter duration remains to be studied.

The new human pathogen known as the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was identified for the first time in the province of Wuhan (China) in December 2019 [17,18]. SARS-CoV-2 alters several organs, among which the lungs are the most commonly and severely affected. The coronavirus disease (COVID)-19 has caused an unprecedented pandemic worldwide that started in 2020 [19]. Lifestyle and personal habits have been modified as a result of the widespread pandemic, with strong implications in the management of patients, particularly of those with chronic respiratory diseases. Whether the COVID-19 pandemic has affected the habitual management planning and monitoring of the outpatient clinics in bronchiectasis deserves to be investigated.

On the basis of this, we hypothesized that the COVID-19 pandemic may have influenced the management planning and clinical outcomes of patients with bronchiectasis who were followed up for at least 12 consecutive months in a specialized outpatient clinic. Thus, our objectives were to assess during the COVID-19 pandemic (the year 2020) in patients with non-cystic fibrosis bronchiectasis that were consecutively recruited and followed up for at least 12 months the following clinical outcomes: (1) airway obstruction, disease severity scores, the number of acute exacerbations, and COVID-19 episodes, if any (2) nutritional and inflammatory parameters, and (3) systemic immunoglobulins. All the measurements were conducted at baseline and at the end of the study period in the year 2021.

2. Methods

This was a prospective, follow-up investigation in which 30 patients (7 males) were recruited consecutively from the Multidisciplinary Bronchiectasis Unit of the Respiratory Department at Hospital del Mar (Barcelona, Spain) from 9 July 2019 to 10 March 2020. Twenty-five patients were recruited in the months of July, September, October, November, and December 2019, while five patients were recruited in March 2020. The 25 patients recruited in 2019 were followed up in 2020 up until April 2021 with several month-delays due to the pandemic period. In the first half of 2020, outpatient consultations were cancelled in order to assist all the hospitalized COVID-19 patients, especially those requiring ventilatory support (either non-invasive or invasive). Patients recruited in March 2020 were followed up until May 2021. Thus, all the participants were followed-up during the year 2020 (a period at which the pandemic was more pervasive in our societies). Two patients had concomitant COVID-19 during the follow-up period: in December of the year 2020 and in January of 2021. However, they did not experience any acute bronchiectasis exacerbation as a result of COVID-19.

All the patients participated in three consultations: baseline, 6-month interim, and 12-month visit. Twenty-seven patients (3 patients dropped out, two from 2019 and one from 2020 recruitment periods) were followed up for one year (range from 12 to 18 months) last follow-up visit completed on 4 May 2021). Inclusion criteria were as follows: adults (18 years and over), diagnosis of non-cystic fibrosis (CF) bronchiectasis by high-resolution computerized tomography (HRCT) [1,20], and no previous exacerbations of the disease at least 4 weeks prior to study entry. Exclusion criteria for all the patients included other chronic cardiovascular or respiratory disorders, acute and chronic respiratory failure, chronic metabolic diseases, signs of severe bronchial inflammation and/or infection, current or recent invasive mechanical ventilation, long-term oxygen therapy, and poor collaboration. The majority of the patients recruited for the purpose of the investigation had a mild-to-moderate disease severity according to lung function impairment [21], disease severity scores, and radiological extension [1,22–25]. All the patients were stable: no acute exacerbations in the last four weeks prior to study entry. Approval was obtained from the institutional Ethics Committee on Human Investigation (Hospital del Mar-IMIM, Barcelona, Spain, protocol # 2019/8482/1, 14 March 2019) following the World Medical Association guidelines (Declaration of Helsinki, Fortaleza, Brazil, October 2013) for research on human beings. Informed written consent was obtained from all participants.

2.1. Clinical Assessment at Baseline and at One-Year Follow-Up Time-Points

Body weight and height were measured after a fasting period of at least four hours in all the patients. Moreover, blood samples were also obtained from the arm vein after an overnight fasting period.

The following clinical variables were obtained at baseline and at one-year follow-up time-points: body mass index (BMI), lung function parameters, exercise capacity, dyspnea, number of acute exacerbations/patient/year, hospitalizations for acute exacerbations/patient/year, nutritional status, therapeutic strategies, and systemic inflammatory parameters. Lung function parameters were determined in all study subjects following standard procedures and reference values commonly used in our laboratory [26–30]. Exercise capacity was assessed through the six-minute walking distance following previous methodologies [31]. In order to prevent unnecessary irradiation of the patients, HRCT was only carried out at baseline.

2.2. Bronchiectasis Disease Severity Scores

The FACED score [24] was used for clinical estimation of the patients' status by incorporating variables: forced expiratory volume in one second (FEV_1) [F; cutoff, 50%; 0 or 2 points], age [A; cutoff, 70 years; 0 or 2 points], chronic colonization by *Pseudomonas aeruginosa* [C; yes, 0 or 1 point], radiological extension [E; number of lobes affected; cutoff, two lobes; 0 or 1 point], and dyspnea [D; cutoff, grade 2 on the modified Medical Research Council (mMRC) dyspnea scale; 0 or 1 point]). Severity classification according to FACED scores was as follows: (1) 0–2, mild disease, (2) 3–4, moderate disease, and (3) 5–7, severe disease.

The EFACED score represents [25] (E: exacerbations with hospitalization in the previous year; F: FEV_1; A: age; C: chronic colonization by *Pseudomonas aeruginosa*; E: radiological extension [number of pulmonary lobes affected]; and D: dyspnea). Severity classification according to EFACED scores was as follows: (1) 0–3, mild disease, (2) 4–6, moderate disease, and (3) 7–9, severe disease.

The bronchiectasis severity index (BSI) score [22] (age [maximum value: 6 points], BMI [maximum value: 2 points], FEV_1 [maximum value: 3 points], hospital admission prior to study [maximum value: 5 points], exacerbations prior to the study [maximum value: 2 points], Medical Research Council (MRC) dyspnea scale [maximum value: 3 points], chronic colonization by *Pseudomonas aeruginosa* (PA) [maximum value: 3 points], chronic colonization by other microorganisms [maximum value: 1 points], radiological extension [maximum value: 1 points]). Severity classification according to BSI scores was as follows: (1) 0–4, mild disease, (2) 5–8, moderate disease, and (3) $\geq$9, severe disease.

2.3. Radiological Features and Extension

High-resolution computer tomography (HRCT)-scans were used to evaluate the radiological extension of the bronchiectasis in all the study patients only at baseline. Scores for each patient were calculated by two independent observers following previously established criteria [32,33]. The extent of bronchiectasis (ES) was scored for each lobe as follows: grade 0 = no disease; grade 1 = one or partial bronchopulmonary segment involved; grade 2 = two or more bronchopulmonary segments involved. The lingula was considered a separate lobe in this analysis. The bronchial dilatation (DS) was quantified relative to the adjacent pulmonary arteries as follows: grade 0 = no bronchiectasis; grade 1 = less than twice (200%) diameter of adjacent pulmonary artery (APA); grade 2 = 200–300% diameter of APA; grade 3 = >300% diameter of APA. Bronchial wall thickness (TS) was scored as follows: grade 0 = none; grade 1 = 50% of APA, grade 2 = 50–100% of APA; grade 3 = >100% of APA.

Global scores of both lungs were taken for extension, bronchial dilatation and bronchial wall thickness. The total extent of bronchiectasis (TES) was taken as the sum of the ES for each of the six lobes. The global severity of bronchial dilatation (GDS) was as the "sum of the extent score multiplied by the dilatation score for each lobe", divided by the "total extent score" ($GDS = \sum(ES \times DS)_{1-6}/TES$). Similarly, the global severity of bronchial wall thickness (GWTS) was estimated as the "sum of the extent score multiplied by the thickness score for each lobe" divided by the "total extent score" ($GTS = \sum(ES \times TS)_{1-6}/TES$).

2.4. Microbiological Diagnosis

Spontaneous or induced sputum samples were obtained from all the patients. Sputum samples were analyzed in the microbiology laboratory. Conventional semi-qualitative bacterial and fungal cultures were performed. An initial Gram staining was performed in all the samples prior to culturing the sputum according to the Murray and Washington criteria [34] (Table 1).

Table 1. Criteria for evaluation of the quality of sputum specimens.

Score	Epithelial Cells	Leukocytes	Quality	Culture
1	>25	<10	Very poor	No
2	>25	18–25	Poor	No
3	>25	>25	Dubious	Yes
4	18–25	>25	Sufficient	Yes
5	<10	>25	Good	Yes
6	<25	<25	Uncertain	Yes

Adapted from Murray P.R. et al. See reference [34].

Sputum samples were cultured in Agar Chocolate, Agar Columbia Nalidixic Acid (CNA), Agar MacConkey, and Agar Sabouraud. Bacterial cultures were read at 24 h and 48 h time-points, while those of fungal cultures were read every 24 h for five consecutive days. Antibiotic sensitivity was tested using the microdilution method or disc diffusion following the regulations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [35]. The strains were frozen in two separate freezing tubes at −80 °C.

When available, mycobacteria cultures were also performed in the patients. Upon sample decontamination using the sodium hydroxide (NaOH) method, samples were cultured in a solid medium culture of Lowenstein-Jensen Media (BD BLL™) and a liquid medium BACT/ALERT® (BioMerieux, SA F-69280 Marcy I'Etoile, Lyon, France) or BACTEC™ MGIT™ 960 (BD) for 40 consecutive days.

2.5. Statistical Analysis

The normality of the study variables was tested using the Shapiro–Wilk test. Data are expressed as mean and standard deviation (SD) in tables and figures. A post-hoc power was calculated on the basis of the parameters IgE and IgG for the Paired Sample *t*-Test

applied to check differences from baseline to follow-up measurements. The post-hoc power calculation was 82.94% for the sample size estimated in the study. At baseline, patients were also analyzed separately according to the presence or absence of colonization by PA. Potential differences of quantitative variables between these two groups were assessed using the Student's *t*-test or Mann-Whitney U test for parametric and non-parametric distributions, respectively. A Chi-square test was used to assess potential differences in categorical variables between the PA colonization group and the non-PA colonization group. Potential differences of quantitative variables between the baseline and one-year follow-up time-points were explored using the Paired Samples *t*-Test or Two-Related Samples Tests for parametric and non-parametric distributions. All statistical analyses were performed using the software SPSS 23.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was established at $p \leq 0.05$.

3. Results

3.1. General Characteristics at Baseline

Table 2 illustrates the baseline clinical characteristics of all the patients. Bronchiectasis severity was classified as mild-to-moderate according to FACED, EFACED, and BSI scores (Table 2). Patients predominantly showed a mild-to-moderate airway obstruction (Table 2). Specifically, 15 patients had an FEV_1 predicted greater than 80% (81–124% range), 11 patients had an FEV_1 predicted comprised between 50% and 80% (50–73% range), and four patients had an FEV_1 predicted lower than 50% (37–48% range).

Table 2. Baseline general characteristics of all bronchiectasis patients, and of the two groups of patients.

Anthropometry $\bar{x}$ (SD)	**All Patients N = 30**	**Non-*Pseudomonas aeruginosa* N = 21**	***Pseudomonas aeruginosa* N = 9**
Age, years	66 (12)	69 (11)	61 (12) *
Female, N/male, N	23/7	18/3	5/4
Body weight, kg	64 (16)	63 (18)	66 (11)
Height, cm	158 (10)	157 (11)	162 (8)
BMI (kg/m^2)	25 (4)	25.5 (4.9)	24.9 (2.8)
Etiology			
Post-infectious, N (%)	22 (73)	14 (67)	8 (89)
COPD, N (%)	1 (3)	1 (4)	0 (0)
Unknown etiology, N (%)	7 (24)	6 (29)	1 (11)
Lung function and exercise capacity, $\bar{x}$ (SD)			
FEV1, L	1.78 (0.71)	1.76 (0.71)	1.84 (0.75)
FEV1, % predicted	76 (25)	80 (25)	65 (21)
FVC, L	2.68 (0.92)	2.52 (0.78)	3.05 (1.15)
FVC, % predicted	85 (18)	87.9 (18.5)	78.9 (16.5)
FEV1/FVC	68 (11)	68.6 (11.2)	65.6 (12.3)
6-min walking distance, meters	473 (96)	450 (95)	534 (71) *
Distance, % predicted	98 (17)	96 (16)	105 (19)
Smoking history			
Ex-smokers, N (%)	9 (30)	6 (29)	3 (33)
Never smokers, N (%)	21 (70)	15 (71)	6 (67)
Packs-year,	22 (15)	23 (18)	12 (3)
Disease severity			
FACED score,	1.9 (1.3)	1.95 (1.43)	1.89 (1.05)

Table 2. *Cont.*

Anthropometry $\bar{x}$ (SD)	All Patients N = 30	Non-*Pseudomonas aeruginosa* N = 21	*Pseudomonas aeruginosa* N = 9
Mild, N	20	13	7
Moderate, N	8	6	2
Severe, N	2	2	0
EFACED score,	2.1 (1.5)	2.05 (1.50)	2.33 (1.73)
Mild, N	25	18	7
Moderate, N	5	3	2
Severe, N	0	0	0
BSI score, $\bar{x}$ (SD)	5.5 (3.2)	5.19 (2.62)	6.33 (4.36)
Mild, N	14	10	4
Moderate, N	11	8	3
Severe, N	5	3	2
mMRC score, $\bar{x}$ (SD)	0.63 (0.67)	0.71 (0.46)	0.22 (0.44) *
# exacerbations/patient/year,	0.87 (1.00)	0.86 (0.66)	0.89 (1.62)
# hospitalizations/patient for exacerbations in the previous year,	0.10 (0.31)	0.05 (0.22)	0.22 (0.44)
Nutritional assessment, $\bar{x}$ (SD)			
Hemoglobin, g/dL	13.9 (11.1)	13.7 (1.1)	14.3 (1.2)
Hematocrit, %	42.0 (3.7)	41.4 (3.6)	43.4 (3.8)
Glucose, mg/dL	94.4 (25.5)	98.8 (29.1)	84.1 (8.8)
Creatinine, mg/dL	0.7 (0.3)	0.71 (0.17)	0.79 (0.18)
Albumin, g/dL	4.4 (0.3)	4.4 (0.3)	4.3 (0.3)
Total proteins, g/dL	7.3 (0.4)	7.2 (0.3)	7.5 (0.5)
Prealbumin, g/dL	22.1 (5.0)	22.4 (4.6)	21.4 (6.1)
Radiological extension, $\bar{x}$ (SD)			
Total extension score	8.1 (3.3)	7.1 (3.0)	10.2 (3.0) *
Bronchial dilatation score	1.2 (0.2)	1.2 (0.2)	1.2 (0.2)
Bronchial wall thickness score	1.3 (0.3)	1.3 (0.4)	1.3 (0.3)
Global score	10.6 (3.4)	9.6 (3.2)	12.8 (2.9) *
Treatments			
Bronchodilators, N	23 (77%)	16 (76%)	7 (78%)
Inhaled corticoids, N	10 (33%)	6 (29%)	4 (44%)
Mucolytics, N	2 (7%)	2 (10%)	0
Eradication protocol for PA	9 (30%)	NA	9 (100%)
Respiratory physiotherapy, N	14 (47%)	12 (57%)	2 (22%)

Values are presented as mean (standard deviation). Abbreviations: N, number; BMI, body mass index; kg, kilograms; cm, centimeters; BMI, body mass index; m, meters; COPD, Chronic Obstructive Pulmonary Disease; FEV1, forced expiratory volume in the first second; FVC, forced vital capacity; L, liter; FACED: F, FEV1, forced expiratory volume in the first second; A, Age; C, chronic colonization by *Pseudomonas aeruginosa*; E, radiological extension; D, dyspnea; EFACED: E, exacerbations with hospitalization in previous year; F, FEV1, forced expiratory volume in the first second; A, Age; C, Chronic colonization by *Pseudomonas aeruginosa*; E, radiological extension; D, dyspnea; BSI, Bronchiectasis Severity Index; mMRC, modified medical research council; #, number; g, grams; dL, deciliter; mg, milligrams, PA, *Pseudomonas aeruginosa*, NA, not available. Statistical analyses and significance: *, $p \leq 0.05$ between *Pseudomonas aeruginosa* colonization patients and non-*Pseudomonas aeruginosa* colonization patients.

Most patients were females, and post-infectious sequelae were the most frequent etiological factor in this series (Table 2). At baseline, patients colonized by PA were significantly younger, the walking distance was greater, while the mMRC score was significantly lower than in non-PA patients (Table 2). At baseline, no significant differences were observed in the study nutritional parameters between PA- and non-PA groups of patients (Table 2). PA-colonized patients exhibited a significantly greater extension of their bronchiectasis, as indicated by the total extension and global scores (Table 2). No significant differences in the number of acute exacerbations/patient/year or hospitalizations/patient/year due to exacerbations were seen between PA and non-PA groups of patients in this cohort (Table 2).

Therapy details are also described in Table 2 below. During follow-up, patients with PA followed an eradication protocol based on the use of full doses of quinolones or co-trimoxazole (Stenotrophomonas maltophilia) for at least three consecutive weeks as established in the Spanish guidelines for non-severe bronchiectasis [36].

3.2. Systemic Inflammatory Parameters and Immunoglobulins (Ig) at Baseline

Levels of the systemic inflammatory parameters at baseline for all the patients are shown in Table 3. At baseline, patients with PA colonization showed greater levels of IgG, IgM, and IgA than patients with non-PA colonization (Table 3).

Table 3. Baseline systemic inflammatory parameters of all bronchiectasis patients, and of the two groups of patients.

Systemic Inflammatory Parameters, $\bar{x}$ (SD)	All Patients N = 30	Non-*Pseudomonas aeruginosa* N = 21	*Pseudomonas aeruginosa* N = 9
Total leukocytes, $\times 10^3/\mu L$	6.4 (1.6)	6.4 (1.5)	6.3 (1.9)
Total neutrophils, $\times 10^3/\mu L$	4.1 (1.4)	4.1 (1.3)	4.0 (1.5)
Neutrophils, %	63.2 (7.8)	63.7 (7.7)	62.0 (8.2)
Total lymphocytes, $\times 10^3/\mu L$	1.5 (0.4)	1.5 (0.4)	1.5 (0.4)
Lymphocytes, %	24.5 (6.2)	24.6 (7.0)	24.2 (3.8)
Total eosinophils, $\times 10^3/\mu L$	0.16 (0.09)	0.15 (0.07)	0.20 (0.14)
Eosinophils, %	2.5 (1.4)	2.4 (1.1)	2.9 (2.0)
Platelets, $\times 10^3/\mu L$	257 (69)	254 (63)	265 (86)
CRP, mg/dL	0.70 (0.9)	0.76 (1.07)	0.60 (0.37)
ESR, mm/h	15 (12)	16 (14)	13 (10)
Fibrinogen, mg/dL	370 (84)	370 (89)	369 (75)
Alpha-1 antitrypsin, mg/dL	132.5 (25.4)	132.8 (26.7)	131.7 (23.7)
Ceruloplasmin, mg/dL	27.0 (5.4)	27.2 (6.3)	26.7 (2.9)
IgE, IU/mL	66 (81)	57 (75)	85 (95)
IgG, mg/dL	1273 (384)	1161 (259)	1535 (506) *
IgG aspergillus, mg/L	37 (35)	37 (37)	36 (30)
IgM, mg/dL	112 (85)	83 (42)	150 (94) *
IgA, mg/dL	330 (134)	295 (120)	390 (152) *

Values are presented as mean (standard deviation). Abbreviations: N, number; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IgE, immunoglobulin E; IgG, immunoglobulin G; IgG aspergillus, immunoglobulin G aspergillus; IgM, immunoglobulin M; IgA, immunoglobulin A; IgG, immunoglobulin G; µL, microliter; mg, milligrams; mm, millimeters; h, hour; dL, deciliter; mL, millilitre. Statistical analyses and significance: *, $p \leq 0.05$ between *Pseudomonas aeruginosa* colonization patients and non-*Pseudomonas aeruginosa* colonization patients.

3.3. General Clinical Characteristics at One-Year Follow-Up

Three patients dropped out (two patients recruited in 2019 and one recruited in 2020) from the one-year follow-up part of the study for personal reasons; thus, 27 patients were followed up for 12 months. Compared to baseline, at one-year follow-up, a significant reduction in FEV_1 (70 mL) was observed in the study patients, while the number of exacerbations significantly increased (88%, Figure 1). Indeed, 20 out of 30 patients had at least one exacerbation during the 12-month follow-up period, and 15 patients showed an increased number of exacerbations compared to baseline (Table 4). No significant differences were seen in the rate of FEV_1 decline (either absolute and % predicted values)

at the end of the study period between patients with an increased number of exacerbations during follow-up and those without, n = 20 and n = 7, $p = 0.893$ and $p = 0.912$, respectively). Furthermore, the rate of FEV_1 decline (either absolute and % predicted values) at 12-month time-point did not significantly differ between patients with a former history of frequent exacerbations (two or more in the year prior to study entry) and those without (n = 5 and n = 25, $p = 0.835$, respectively).

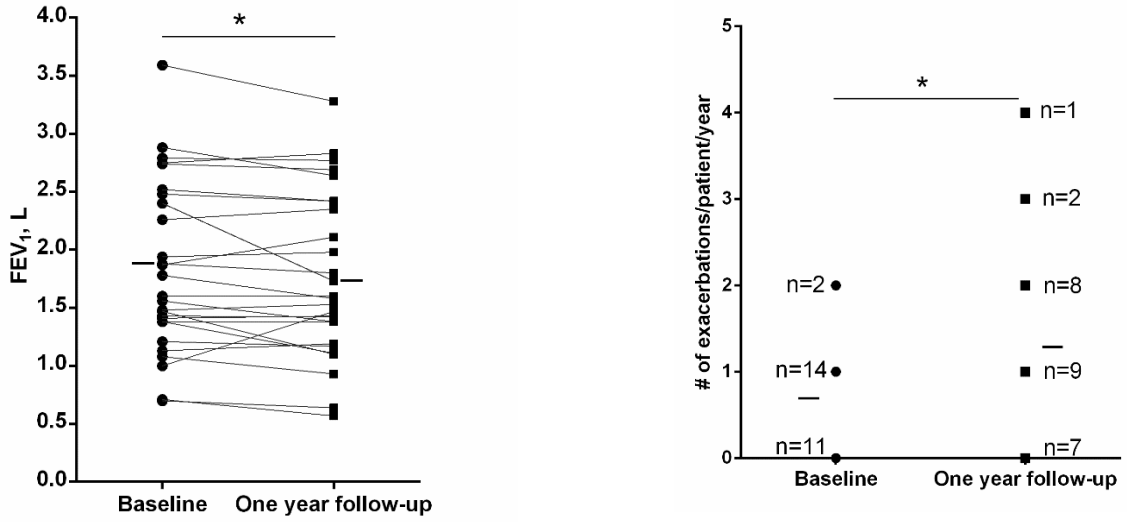

Figure 1. Individual and mean values of FEV_1 lung function parameter and the number of exacerbations/patient/year during the 12-month follow-up period at baseline and at one-year follow-up in bronchiectasis patients. Twenty patients had at least one exacerbation during follow-up. Abbreviations: n, number. Statistical significance is as follows: * $p \leq 0.05$ comparisons between baseline and one-year follow-up time-points.

Table 4. Number of exacerbations and hospitalizations for exacerbations in each patient in the previous year.

Patients	# Exacerbations/Patient/Year		# Hospitalizations/Patient for Exacerbations in the Previous Year	
	Baseline	One-Year Follow-Up	Baseline	One-Year Follow-Up
Patient # 1	1	1	0	0
Patient # 2	1	2	0	0
Patient # 3	0	2	0	0
Patient # 4	1	0	1	0
Patient # 5	1	0	0	0
Patient # 6	1	2	0	0
Patient # 7	0	1	0	0
Patient # 8	0	1	0	0
Patient # 9	0	4	0	0
Patient # 10	2	NA	0	NA
Patient # 11	1	2	0	0
Patient # 12	1	2	0	0
Patient # 13	0	0	0	0
Patient # 14	0	3	0	0
Patient # 15	0	0	0	0

Table 4. *Cont.*

Patients	# Exacerbations/Patient/Year		# Hospitalizations/Patient for Exacerbations in the Previous Year	
	Baseline	One-Year Follow-Up	Baseline	One-Year Follow-Up
Patient # 16	1	1	0	0
Patient # 17	0	2	0	0
Patient # 18	1	0	0	0
Patient # 19	1	1	1	1
Patient # 20	0	1	0	0
Patient # 21	1	1	0	0
Patient # 22	2	0	0	0
Patient # 23	2	3	0	0
Patient # 24	2	NA	0	NA
Patient # 25	1	2	0	1
Patient # 26	1	2	0	0
Patient # 27	3	NA	1	NA
Patient # 28	0	0	0	0
Patient # 29	0	1	0	0
Patient # 30	1	1	0	0

Abbreviations: #, number; NA, not available.

No significant differences were observed between one-year follow-up and baseline time-points in other lung function parameters or exercise capacity (Table 5). No significant differences were observed between the one-year follow-up and baseline time-points of the study nutritional parameters except for hemoglobin, which significantly declined (Table 5). The disease severity scores FACED and EFACED, but not BSI, significantly increased after the one-year follow-up compared to baseline (Figure 2A,B and Table 5, respectively). The rate of FEV_1 decline (absolute and % predicted values) did not show any significant differences between patients with and without a rise in the bronchiectasis severity scores (FACED n = 9 and n = 18, EFACED n = 10 and n = 17, and BSI n = 10 and n = 17, $p = 0.899$, $p = 0.493$, and $p = 0.619$, respectively).

Table 5. General characteristics at one-year follow-up in bronchiectasis patients.

Anthropometry, $\bar{x}$ (SD)	Baseline	One-Year Follow-Up
Age, years	66 (12)	67 (12) ***
Female, N/male, N	20/7	20/7
Body weight, kg	65 (17)	65 (17)
Height, cm	159 (10)	159 (10)
BMI (kg/m^2)	25.4 (4.4)	25.3 (4.5)
Etiology		
Post-infectious, N (%)	20 (74)	20 (74)
COPD, N (%)	0 (0)	0 (0)
Unknown etiology, N (%)	7 (26)	7 (26)
Lung function and exercise capacity, $\bar{x}$ (SD)		
FEV_1, % predicted	76.1 (24.8)	74.4 (25.5)
FVC, L	2.69 (0.95)	2.54 (0.86)

Table 5. *Cont.*

Anthropometry, $\bar{x}$ (SD)	Baseline	One-Year Follow-Up
FVC, % predicted	85 (19)	85 (18)
FEV_1/FVC	68.3 (11.8)	67.4 (12.4)
6-min walking distance, meters	490 (89)	477 (96)
Distance, % predicted	101 (16)	97 (16)
Smoking history		
Ex-smokers, N (%)	7 (26)	7 (26)
Never smokers, N (%)	20 (74)	20 (74)
Packs-year, $\bar{x}$ (SD)	23 (16)	23 (16)
Disease severity		
BSI score, $\bar{x}$ (SD)	5.2 (2.9)	5.7 (2.1)
mMRC score, $\bar{x}$ (SD)	0.67 (0.68)	0.70 (0.78)
Hospitalizations for exacerbations in the previous year, $\bar{x}$ (SD)	0.07 (0.27)	0.07 (0.27)
Nutritional assessment, $\bar{x}$ (SD)		
Hemoglobin, g/dL	14.0 (1.2)	13.5 (1.0) *
Hematocrit, %	42.0 (3.5)	39.2 (10.4)
Glucose, mg/dL	94.3 (26.3)	97.0 (23.8)
Creatinine, mg/dL	0.74 (0.18)	0.75 (0.15)
Albumin, g/dL	4.4 (0.2)	4.4 (0.3)
Total proteins, g/dL	7.3 (0.3)	7.2 (0.4)
Prealbumin, g/dL	22.3 (4.5)	21.8 (4.6)
Radiological extension, $\bar{x}$ (SD)		
Total extension score	8.2 (3.2)	NA
Bronchial dilatation score	1.2 (0.2)	NA
Bronchial wall thickness score	1.3 (0.3)	NA
Global score	10.7 (3.3)	NA

Values are presented as mean (standard deviation). Abbreviations: N, number; BMI, body mass index; kg, kilograms; cm, centimeters; BMI, body mass index; m, meters; COPD, Chronic Obstructive Pulmonary Disease; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; L, liter; FACED: F, FEV_1, force expiratory volume in the first second; A, Age; C, chronic colonization by *Pseudomonas aeruginosa*; E, radiological extension; D, dyspnea; EFACED: E, exacerbations with hospitalization in previous year; F, FEV_1, forced expiratory volume in the first second; A, Age; C, Chronic colonization by *Pseudomonas aeruginosa*; E, radiological extension; D, dyspnea; BSI, Bronchiectasis Severity Index; mMRC, modified medical research council; g, grams; dL, deciliter; mg, milligrams; NA, not available. Statistical analyses and significance: *, $p \leq 0.05$, ***, $p \leq 0.001$ between baseline and one-year follow-up bronchiectasis patients.

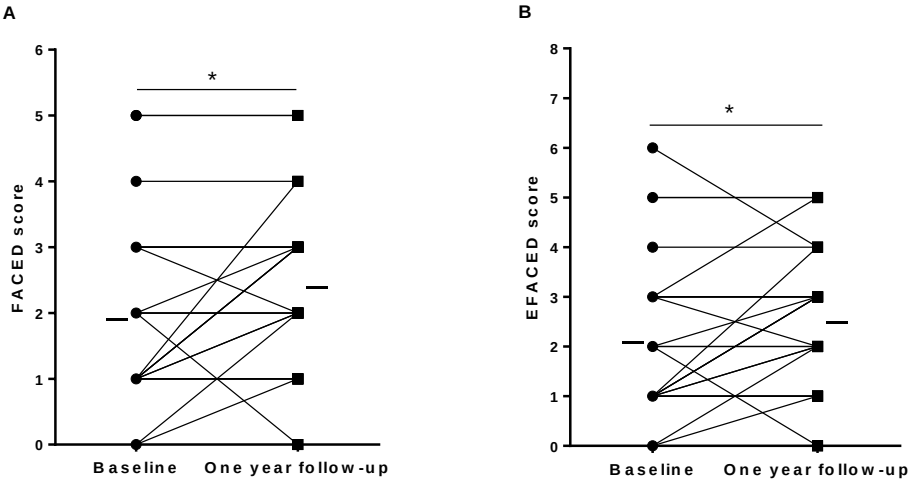

Figure 2. Individual and mean values of (**A**) FACED score and (**B**) EFACED score at baseline and at one-year follow-up in bronchiectasis patients. Statistical significance is as follows: * $p \leq 0.05$ comparisons between baseline and one-year follow-up time-points.

3.4. Systemic Inflammatory Parameters and Immunoglobulins (Ig) at One-Year Follow-Up

A significant rise in the erythrocyte sedimentation rate (ESR) was detected in the patients at one-year follow-up compared to baseline (Figure 3), whereas no differences were observed in the number of leukocytes, neutrophils, lymphocytes, or eosinophils (Table 6). The number of platelets, however, significantly declined at one-year follow-up compared to baseline (Table 6). Blood levels of ceruloplasmin, IgE, IgG, IgG aspergillus, IgM, and IgA significantly declined at one-year follow-up compared to baseline in the bronchiectasis patients (Figures 4 and 5, respectively).

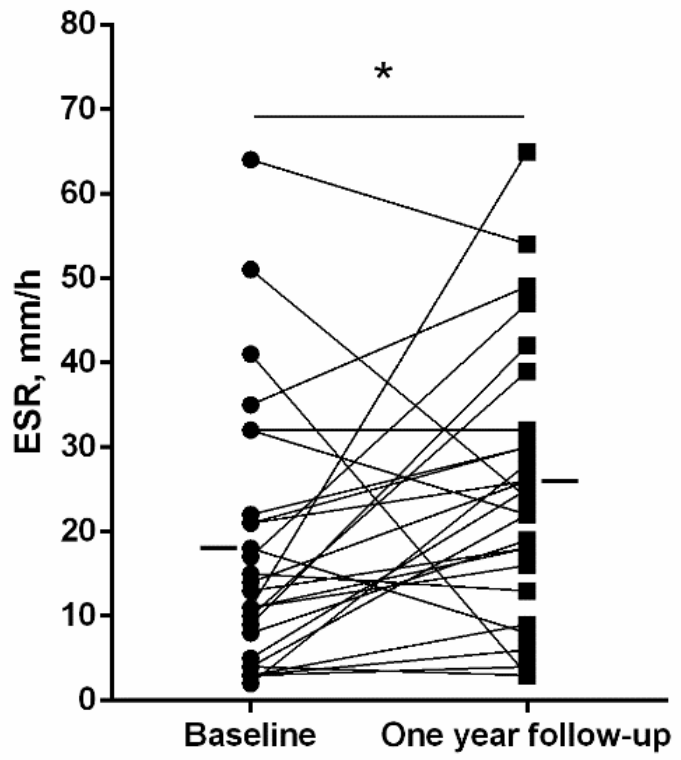

Figure 3. Individual and mean values of levels of ESR at baseline and at one-year follow-up in bronchiectasis patients. Statistical significance is as follows: * $p \leq 0.05$ comparisons between baseline and one-year follow-up time-points.

Table 6. Systemic inflammatory parameters at one-year follow-up in bronchiectasis patients.

Systemic Inflammatory Parameters, $\bar{x}$ (SD)	Baseline	One-Year Follow-Up
Total leukocytes, $\times 10^3/\mu L$	6.3 (1.6)	6.2 (1.5)
Total neutrophils, $\times 10^3/\mu L$	4.1 (1.4)	4.0 (1.2)
Neutrophils, %	63.0 (8.1)	62.5 (8.1)
Total lymphocytes, $\times 10^3/\mu L$	1.5 (0.4)	1.5 (0.5)
Lymphocytes, %	24.6 (6.5)	25.2 (8.6)
Total eosinophils, $\times 10^3/\mu L$	0.17 (0.09)	0.16 (0.08)
Eosinophils, %	2.8 (1.3)	3.0 (1.7)
Platelets, $\times 10^3/\mu L$	262 (70)	241 (56) *
CRP, mg/dL	0.59 (0.63)	0.51 (0.46)
Fibrinogen, mg/dL	379 (62)	396 (70)
Alpha-1 antitrypsin, mg/dL	130.8 (25.3)	131.3 (22.3)

Values are presented as mean (standard deviation). Abbreviations: N, number; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IgE, immunoglobulin E; IgG aspergillus, immunoglobulin G aspergillus; IgM, immunoglobulin M; IgA, immunoglobulin A; IgG, immunoglobulin G; µL, microliter; mg, milligrams; mm, millimeters; h, hour; IU, international unit; dL, deciliter; L, liter. Statistical analyses and significance: *, $p \leq 0.05$ between baseline and one-year follow-up bronchiectasis patients.

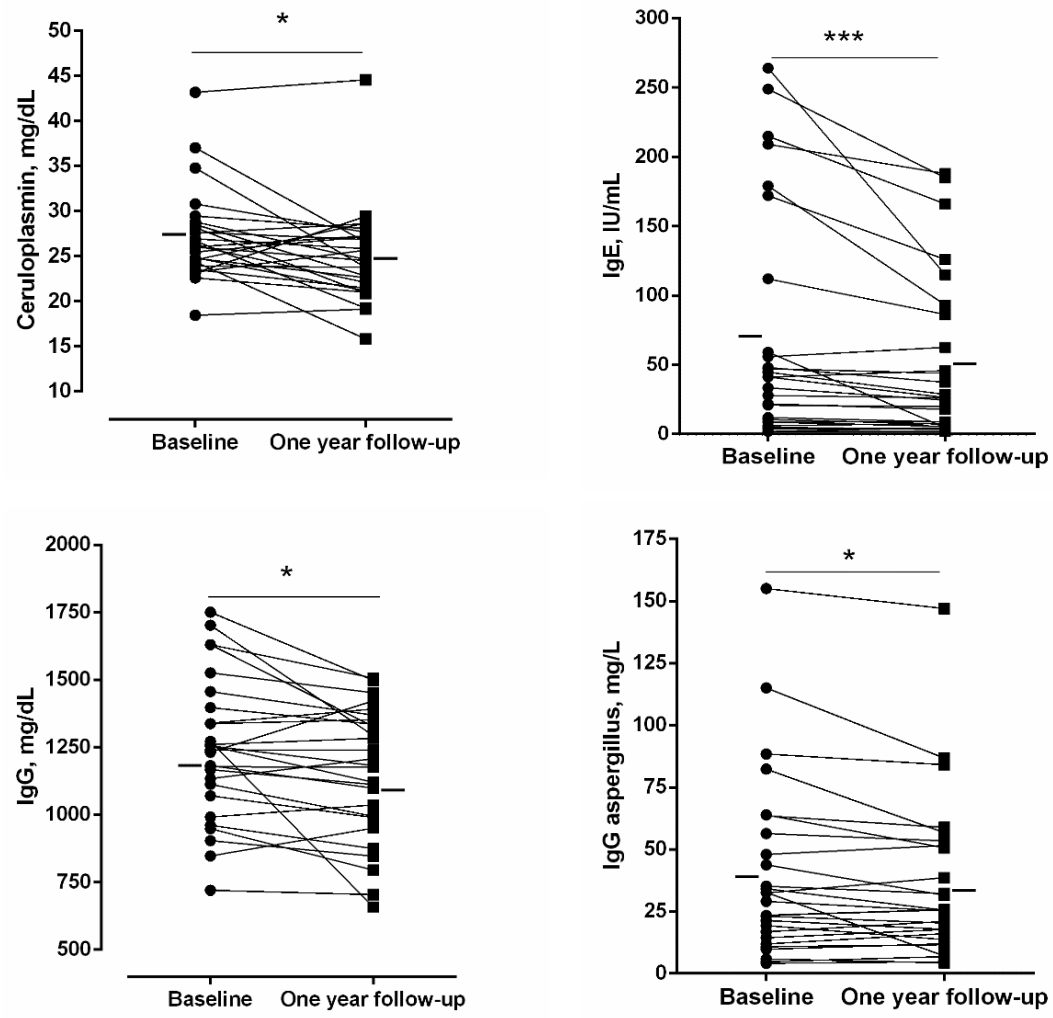

Figure 4. Individual and mean values of levels of ceruloplasmin, IgE, IgG and IgG aspergillus at baseline and at one-year follow-up in bronchiectasis patients. Statistical significance is as follows: * $p \leq 0.05$, *** $p \leq 0.001$ comparisons between baseline and one-year follow-up time-points.

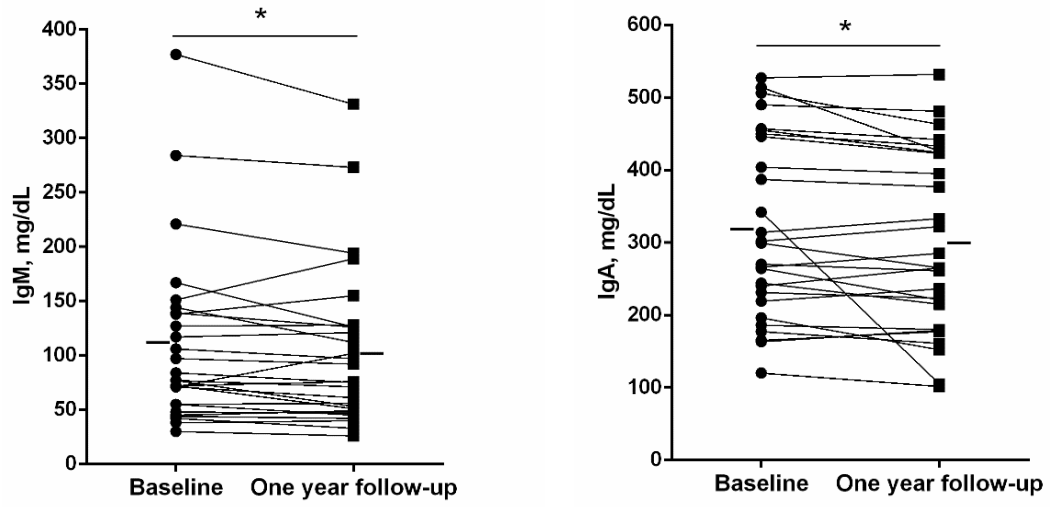

Figure 5. Individual and mean values of levels of IgM and IgA at baseline and at one-year follow-up in bronchiectasis patients. Statistical significance is as follows: * $p \leq 0.05$ comparisons between baseline and one-year follow-up time-points.

3.5. Microbiological Status of the Sputum at Baseline and One-Year Follow-Up

Table 7 illustrates the microbiological results from the sputum cultures of all the patients at baseline and at one-year follow-up. No significant differences were observed in FEV_1 decline (absolute and % predicted values), disease severity scores, or the number of exacerbations/patient/year between patients showing newly acquired colonization and those without ($p > 0.05$ all analyses).

Table 7. Sputum and microbiological status at baseline and at one-year follow-up.

Patients	Baseline		One Year Follow-Up	
	Germs	Score	Germs	Score
Patient # 1	*Haemophilus influenza*, S	5	*Haemophilus influenza*, S	5
Patient # 2	*Moraxella catarrhalis*, S	5	NC, S	1
Patient # 3	*Pseudomonas aeruginosa*, S	3	*Pseudomonas aeruginosa*, S	5
Patient # 4	*Pseudomonas aeruginosa*, S	3	*Pseudomonas aeruginosa*, S	5
Patient # 5	Commensal microbiota, S	5	*Moraxella catarrhalis*, S	4
Patient # 6	*Pseudomonas aeruginosa*, S	5	NC, S	2
Patient # 7	Commensal microbiota, S	6	NSA	NA
Patient # 8	*Pseudomonas aeruginosa*, S	5	*Pseudomonas aeruginosa*, S	3
Patient # 9	*Pseudomonas aeruginosa*, S	5	*Pseudomonas aeruginosa*, S	6
Patient # 10	Commensal microbiota, S	5	NA	NA
Patient # 11	Commensal microbiota, S	5	Commensal microbiota, S	4
Patient # 12	Commensal microbiota, S	5	NC, S	1
Patient # 13	NSA, I	NA	NSA	NA
Patient # 14	*Pseudomonas aeruginosa*, S	5	Commensal microbiota, S	4
Patient # 15	Commensal microbiota, S	6	NC, S	1
Patient # 16	*Pseudomonas aeruginosa*, S	5	NSA	NA
Patient # 17	Commensal microbiota, S	5	*Stenotrophomonas maltophilia*	5
Patient # 18	NSA, I	NA	NSA	NA
Patient # 19	Commensal microbiota, S	5	NSA	NA
Patient # 20	Commensal microbiota, S	5	*Haemophilus influenza*, S	4
Patient # 21	Commensal microbiota, S	6	Commensal microbiota, S	3
Patient # 22	NC, S	2	NC, S	1
Patient # 23	Commensal microbiota, S	3	Commensal microbiota, S	4
Patient # 24	NSA, I	NA	NA	NA
Patient # 25	NSA, I	NA	*Pseudomonas aeruginosa*, S	4
Patient # 26	Commensal microbiota, S	6	NC, S	1
Patient # 27	*Pseudomonas aeruginosa*, S	6	NA	NA
Patient # 28	Commensal microbiota, S	6	*Pseudomonas aeruginosa*, S	3
Patient # 29	*Pseudomonas aeruginosa*, S	6	*Pseudomonas aeruginosa*, S	5
Patient # 30	Commensal microbiota, S	6	NSA	NA

Abbreviations: S, spontaneous; I: induced; NSA, no sputum available; NA, not available; NC, no culture.

4. Discussion

In the current investigation, the most relevant findings were that outpatients with bronchiectasis consecutively recruited from a specialized clinic during the COVID-19 pandemic were predominantly females, exhibited mild airway obstruction and disease

severity as indicated by specific scores, and post-infectious was the commonest etiology in this series of patients. A relatively normal follow-up period was attained despite the pandemic, in which all the participants were followed up for a minimum of 12 months, except for short delays in the consultations as a result of the heavy burden of COVID-19 patients in the first half of the year 2020 and the subsequent reorganization of the clinics that followed. The follow-up period consisted of three different appointments: baseline, six-month, and 12-month visits. Importantly, at one-year follow-up compared to baseline, the patients showed a significant reduction in FEV_1 (70 mL), along with a significant rise in the number of exacerbations per patient, disease severity scores, and in levels of ESR parameter. Nonetheless, levels of the inflammatory parameters ceruloplasmin and the study immunoglobulins were significantly reduced in all the patients at one-year follow-up compared to baseline. These are relevant results that are further discussed below.

A major relevant finding in this study was the significant decline in absolute values of FEV_1 observed among the bronchiectasis patients right after only one-year follow-up with respect to baseline measurements. The 70-mL loss of FEV_1 detected in the patients in the 12-month visit is far greater than that reported to happen under physiological conditions in normal subjects or even in smokers [37]. In this cohort, a loss of 52 mL was calculated in the year prior to study entry. Importantly, an association between the degree of radiological emphysema and small airway disease and FEV_1 decline was also demonstrated in COPD patients, particularly in those with mild-to-moderate disease [38]. In the present study, the 70-mL decline in FEV_1 values at one-year follow-up can only be attributable to the presence of bronchiectasis since none of the patients smoked. In addition, only one patient also had concomitant COPD, and this patient dropped out from the study at month seven. Thus, bronchiectasis per se elicited a significant decline in lung function as early as 12 months of the follow-up period in patients aged 60 to 70 years old. As the loss in lung function was greater under the study period (70 mL) than that observed in the year prior to study entry (52 mL), the mechanisms whereby FEV_1 decline progressively increases in bronchiectasis patients should be fully understood in future investigations.

The number of acute exacerbations, but not those of hospitalizations, increased in the patients at one-year follow-up compared to baseline. In fact, 20 patients experienced an exacerbation during the follow-up period, and 15 out the 30 patients had an increase in the number of acute exacerbations during the 12-month follow-up period. Indeed, acute exacerbations are common in patients with bronchiectasis [11–13,39], which in certain cases may require hospitalizations. Recently, it has been reported that a history of previous hospitalizations, heart failure, and high disease severity scores were associated with a greater risk for hospitalizations in patients with acute exacerbations of bronchiectasis [13]. Furthermore, in the present investigation, FACED and EFACED scores were also significantly greater at one-year follow-up than at baseline in the bronchiectasis patients. Whether disease severity scores may help predict the risk of acute exacerbations and/or hospitalizations in patients with bronchiectasis remains to be fully elucidated. In line with this, it has been proposed that FACED and BSI may not be all that helpful to predict the risk of exacerbations in patients with bronchiectasis [40]. Nevertheless, BSI and FACED scores were very useful markers to predict the five-year mortality in bronchiectasis patients [41].

Interestingly, levels of the parameter ESR were significantly greater after the one-year follow-up than baseline levels in the study patients. ESR is a systemic inflammatory marker that can be used as a prognostic tool in clinical settings of other respiratory diseases [42]. In stable bronchiectasis patients, ESR values did not significantly correlate with disease severity in a retrospective study [43]. Whether ESR may have a prognostic value in bronchiectasis patients warrants further attention.

Ceruloplasmin is a protein synthesized in the liver that carries copper and is also involved in iron metabolism. As an acute phase-reactant, ceruloplasmin levels rise in inflammatory processes [44,45]. In the present study, a small but significant decline (from 27 to 25 mg/dL) in ceruloplasmin levels was observed in bronchiectasis patients at one-year follow-up compared to baseline. The precise biological mechanisms whereby this

inflammatory parameter may be involved in the pathophysiology of bronchiectasis need to be explored in future investigations.

Immunoglobulins, along with B and T cells, are major components of the adaptive immune response in humans. In the current investigation, baseline values of the analyzed immunoglobulins were within the normal range for all the patients. Importantly, at one-year follow-up, the serum values of IgE, IgG, IgG aspergillus, IgM, and IgA significantly declined compared to baseline levels. As indoor and outdoor pollution are potential stimuli of the production of acute phase-reactants and immunoglobulins [46], it is likely that the lockdowns and the reduced outdoor activity experienced during the COVID-19 pandemic may have substantially contributed to the decline in the levels of these markers in the study population. Whether or not the reduction in immunoglobulins and acute phase-reactant levels may influence long-term clinical outcomes and disease prognosis warrants further attention in future investigations.

Study Critique

A potential criticism is the use of a relatively small cohort that has been analyzed in the study. However, outpatients were consecutively recruited in a specialized clinic on the basis of very strict inclusion and exclusion criteria. Moreover, a power calculation was almost 83% in this specific cohort, endorsing the reliability of the study results. Moreover, all the patients were followed up during the COVID-19 pandemic in 2020, when several lockdowns and curfews were applied in society. Despite these measures, non-COVID-19 respiratory outpatients were equally attended in our specialized clinic, and the established visits were highly respected as described in Methods (every six months). All the patients maintained social distancing and were wearing masks at all times (indoors and outdoors) as these measurements were enforced by the local authorities during the pandemic. Only two patients out of 30 had mild COVID-19, without leading to acute exacerbations during follow-up. These results suggest that these preventive measurements safeguarded patients from developing COVID-19.

Patients with PA infection at baseline were significantly younger than those with no PA infection, and the mMRC score was smaller, probably due to the age factor. Moreover, at baseline, patients with PA infection exhibited greater levels of immunoglobulins and larger radiological extension in the HRCT than non-PA patients, while showing no differences in the number of acute exacerbations or hospitalizations in the previous year. Despite the relevance and interest of these findings, caution should be taken as the number of patients in the PA-infected group was relatively small.

Another potential limitation might be the drop-out of three patients during the follow-up period of the study. Nonetheless, the statistical power (83%) was sufficiently high to ensure the validity of the results on the basis of the 27 patients who participated both at baseline and during follow-up. It should also be mentioned that only two patients out of 27 had mild COVID-19 in late 2020 and early 2021, respectively. None of the patients had been vaccinated over the study period, as vaccines against SARS-CoV-2 were not vastly available at that time.

5. Conclusions

In patients with bronchiectasis, a significant decline in FEV_1 was observed only after the one-year follow-up period, along with a rise in the number of acute exacerbations and disease severity scores, but not of hospitalizations. However, a significant decrease in acute phase-reactants and immunoglobulins was observed at one-year follow-up compared to baseline. These findings suggest that the lung function impairment seen in these patients, particularly of the airways, may not rely entirely on their systemic inflammatory status. Identification of the pathophysiological mechanisms leading to substantial lung function impairment in bronchiectasis patients warrants further attention in future research. Despite the relatively small cohort analyzed in this study, the reported findings have clear clinical implications in the management of patients with bronchiectasis.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Hospital of Mar-IMIM (protocol # 2019/8482/1, 14 March 2019).

Informed Consent Statement: All patients signed the informed written consent to participate in the registry. The information remained confidential at all times and no personal information related to any of the participants was introduced in the registry.

Data Availability Statement: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors have none to disclose regarding this study.

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10.2.2 Publication 2

Title:

Respiratory and Peripheral Muscle Weakness and Body Composition Abnormalities in Non-Cystic Fibrosis Bronchiectasis Patients: Gender Differences

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Respiratory and Peripheral Muscle Weakness and Body Composition Abnormalities in Non-Cystic Fibrosis Bronchiectasis Patients: Gender Differences

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Abstract: As demonstrated in COPD, bronchiectasis patients may experience respiratory and peripheral muscle dysfunction. We hypothesized that respiratory and peripheral (upper and lower limbs) muscle function and nutritional status may be more significantly altered in female than in males for identical age and disease severity. In mild-to-moderate bronchiectasis patients (n = 150, 114 females) and 37 controls (n = 37, 21 females), radiological extension, maximal inspiratory and expiratory pressures (MIP and MEP), sniff nasal inspiratory pressure (SNIP), hand grip and quadriceps muscle strengths, body composition, and blood analytical biomarkers were explored. Compared to the controls, in all bronchiectasis patients (males and females), BMI, fat-free mass index (FFMI), fat tissue, upper and lower limb muscle strength, and respiratory muscle strength significantly declined, and FFMI, fat tissue, and quadriceps muscle function were significantly lower in female than male patients. In patients with mild-to-moderate bronchiectasis, respiratory and peripheral muscle function is significantly impaired and only partly related to lung disease status. Quadriceps muscle strength was particularly weakened in the female patients and was negatively associated with their exercise tolerance. Muscle weakness should be therapeutically targeted in bronchiectasis patients. Body composition and peripheral muscle function determination should be part of the comprehensive clinical assessment of these patients.

Keywords: bronchiectasis patients; upper limb muscle function; lower limb muscle function; respiratory muscle function; muscle weakness; differences between male and female patients; radiological extension

1. Introduction

Non-cystic fibrosis (CF) bronchiectasis is a chronic respiratory condition characterized by abnormalities of the airways that facilitate the collection of lung secretions in the patients, which further deteriorates the underlying structure. Remodeling of the airways is frequent in these patients, along with other events, such as chronic inflammation and bacterial

colonization [1,2]. The prevalence of bronchiectasis is progressively increasing as more diagnostic tools become available [3,4].

Extrapulmonary manifestations are common among patients with chronic respiratory diseases, including those with bronchiectasis [5–8]. The analysis of skeletal muscle dysfunction and sarcopenia of patients with chronic obstructive pulmonary disease (COPD) have been a matter of research in multiple previous investigations [9–14]. Peripheral muscle weakness, particularly of the lower limbs, takes place in up to one third of COPD patients, even in those with a mild airway obstruction [10,15–18]. Respiratory muscle dysfunction is also common in COPD patients [19–22]. Several clinical factors are involved in the pathophysiology of skeletal muscle dysfunction and sarcopenia in COPD [9–14]. Inactivity, deconditioning, and nutritional abnormalities, including vitamin D deficiency, are counted among the most relevant contributors to muscle dysfunction and atrophy in these patients [9–14,23]. For instance, acute exacerbations, which are frequent in COPD and bronchiectasis patients, negatively impact on their quality of life and disease prognosis as a result of reduced physical and muscle activity [9–14]. Whether the function of respiratory and limb muscles may be altered in patients with mild-to-moderate bronchiectasis remains to be thoroughly understood.

The study of the potential differences between female and male patients in chronic respiratory disease has gained great attention in biomedical research in the last decade [6]. As such, it has been recently demonstrated that in a large-cohort of bronchiectasis patients, females exhibited a less severe disease along with a better profile of inflammatory biomarkers than the male patients recruited in the same investigation [6]. Disease outcomes may also differ between male and female patients in chronic respiratory patients [24]. Several factors may account for the reported differences observed between female and male chronic respiratory patients. Lung and airway anatomy, chronic infection and inflammation, differences in host defense mechanisms, and environmental factors, including physical activity and nutritional abnormalities, are a few factors that contribute to the gender differences observed in bronchiectasis patients [6,25,26]. In patients with advanced COPD, peripheral muscle dysfunction and damage was significantly more prominent among female patients compared to men [27]. Whether similar findings can be observed in female patients with bronchiectasis remains to be answered.

On this basis, we hypothesized that respiratory and peripheral muscle function, (both upper and lower limbs) and nutritional status (body compartments) may be more significantly altered in female patients than in males for the same age and disease severity. As such, the following objectives were established. In female and male patients with mild-to-moderate bronchiectasis compared to a group of healthy control subjects, parameters assessing respiratory and peripheral muscle function were determined: (1) maximal inspiratory and expiratory pressures (MIP and MEP, respectively), (2) sniff nasal inspiratory pressure (SNIP), (3) hand grip and quadriceps muscle strengths, (4) body composition, (5) blood analytical biomarkers, and (6) correlations between lung function and the extrapulmonary parameters.

2. Methods

2.1. Study Population

Patients with stable non-CF bronchiectasis (n = 150, 114 female) were consecutively recruited from the Bronchiectasis Multidisciplinary Unit at the Hospital del Mar (Barcelona, Spain). Moreover, a group of non-smoker healthy age-matched control subjects (n = 37, 21 females) was also recruited from the general population (patients' relatives). All the patients had a primary diagnosis of bronchiectasis on the basis of high-resolution computerized tomography (HRCT) and published guidelines were followed [4,28,29]. Patients did not have any acute exacerbation at least three months prior to study entry. Habitual medication taken by the patients was maintained throughout the duration of the study: inhaled bronchodilators with and without inhaled corticosteroids, inhaled antibiotics in a few cases, and mucolytics. Patients were consecutively recruited from the Bronchiectasis

Clinic and followed a regular Mediterranean diet, as it is common in this geographical area. Patients were sedentary and were not following any specific exercise training program or going to the gymnasium at the time of study entry. Likewise, healthy control subjects were also sedentary and were not practicing any high-intensity outdoor or indoor exercise program, and they were also following a regular Mediterranean diet.

Exclusion criteria were as follows: acute or chronic respiratory failure [30], COPD [31], other chronic respiratory diseases, including asthma, coronary heart disease, limiting osteoarticular condition, chronic metabolic diseases of any etiology, presence of paraneoplastic syndrome, myopathies, treatment with oral steroids, or other drugs that had potential effects on muscle structure or function.

Nutritional status, lung function, respiratory and peripheral muscle functions, exercise capacity, and blood parameters were determined in both bronchiectasis patients and the control subjects. This was a prospective, cross-sectional study in which patients were recruited for two years (July 2019–June 2021).

2.2. Ethics

The current study was designed following the guidelines of the World Medical Association for Research in Humans (Seventh revision of the Declaration of Helsinki, Fortaleza, Brazil, 2013) [32] and the ethical standards on human experimentation in our institution. The study was approved by the Institutional Ethics Committee on Human Investigation before the start. (Hospital del Mar-IMIM, Barcelona, project number 2019/8955/I). An informed written consent was obtained from both patients and control subjects. Finally, the participation of all participants was confidential and voluntary at all times.

2.3. Bronchiectasis Severity Scores

The FACED (FEV_1, age, chronic colonization, extension, dyspnea), EFACED (exacerbation FACED), and BSI (bronchiectasis severity index) scores were used to assess the disease severity of bronchiectasis patients [33–35].

2.4. Radiological Extension of Bronchiectasis

The radiological extension of bronchiectasis was evaluated by means of HRCT-scans in all the study patients. Scores for each patient were calculated by two independent observers according to previously established criteria [7,36,37]. The extent of bronchiectasis (ES) was scored for each lobe as follows: grade 0 = no disease; grade 1 = one or partial bronchopulmonary segment involved; grade 2 = two or more bronchopulmonary segments involved. The lingula lobe was considered as an independent one in this analysis. The bronchial dilatation (DS) was quantified relative to the adjacent pulmonary arteries as follows: grade 0 = no bronchiectasis; grade 1 = less than twice (200%) diameter of adjacent pulmonary artery (APA); grade 2 = 200–300% diameter of APA; grade 3 $\geq$ 300% diameter of APA. Bronchial wall thickness (TS) was scored as follows: grade 0 = none; grade 1 = 50% of APA, grade 2 = 50–100% of APA; grade 3 $\geq$ 100% of APA.

The CT scans were also scored for parenchymal items in the six lobes: collapse or consolidation, mucus plugging, emphysema, and fibrosis or retraction. Collapse or consolidation: subsegmental collapse or consolidations = 1 and segmental or lobar collapse or consolidations = 2. Mucus plugging was scored as follows: the presence of subsegmental mucus plugging = 1 and in case of the presence of segmental or lobar mucus plugging = 2. Emphysema was recorded as follows: subsegmental emphysema = 1 and segmental or lobar emphysema = 2. Fibrosis or retraction was scored as follows: subsegmental fibrosis or retraction = 1 and segmental, lobar fibrosis, or retraction = 2.

Global scores of both lungs were taken for extension, bronchial dilatation, and bronchial wall thickness. The total extent of bronchiectasis (TES) was taken as the sum of the ES for each of the six lobes. The global severity of bronchial dilatation (GDS) was estimated using a weighted average, calculated as the "sum of the extent score multiplied by the dilatation score for each lobe", divided by the "total extent score" ($GDS = \sum(ES \times DS)_{1-6}/TES$).

Similarly, the global severity of bronchial wall thickness (GWTS) was estimated as the "sum of the extent score multiplied by the thickness score for each lobe" divided by the "total extent score" ($GTS = \sum(ES \times TS)_{1-6}/TES$).

2.5. Nutritional and Body Assessment

Body weight and height were measured after a fasting period of at least four hours in all the patients and healthy controls. Nutritional evaluation included body mass index (BMI), determination of the fat-free mass index (FFMI) using bioelectrical impedance (Bodystat 1500, Bodystat Ltd., Isle of Man, British Isles), and conventional blood markers [12,13]. The following outcomes were measured using bioelectrical impedance: fat-free mass (FFM), FFM index (FFMI), and fat tissue absolute and percentage values.

2.5.1. Lung Function Assessment

Lung function was evaluated through determination of prebronchodilator spirometric values (COVID-19 pandemic period), static lung volumes, and diffusion capacity using standard procedures, equipment, and established reference values [38–41].

2.5.2. Limb Muscle Function

Upper limb muscles—Handgrip strength was evaluated using a specific dynamometer (Jamar 030J1, Chicago, IL, USA). The maximum voluntary contraction of the flexor muscles of the non-dominant hand was assessed. The highest value out of three reproducible maneuvers (<5% variability among them) was accepted as the valid measurement for each subject [42,43]. Reference values from Luna-Heredia et al. [42] were used in the analysis.

Lower limb muscles—In both patients and controls, quadriceps muscle strength was evaluated through the determination of isometric maximum voluntary contraction (QMVC) of the non-dominant lower limb, as formerly described [12,13]. Briefly, an isometric dynamometer (Biopac Systems, Goleta, CA, USA) connected to a digital polygraph (Biopac Systems) was used for these measurements. Individuals had to lie on their back on a stretcher, while the non-dominant ankle was fixed with a strap. Subjects had their lower limbs falling down at 90° from the stretcher. The ankle attached to the strap performed the maneuver to calculate quadriceps muscle strength. The highest value out of three brief reproducible maneuvers (<5% variability among them) was accepted as the QMVC for each subject. Reference values from Seymour et al. [15] were used in the analysis.

2.6. Respiratory Muscle Evaluation

Maximal inspiratory pressure (MIP)—MIP at the mouth was performed from the residual volume (RV). The measurements were taken when participants were in a sitting position. In order to measure the MIP, an occludable oral piece with a small orifice was used to minimize the participation of the buccinator muscles [44]. The oral piece was connected to a pressure manometer (TSD 104, Biopac Systems), whose signal was registered by a digital polygraph (Biopac Systems). The MIP final outcome for each subject was obtained from the highest value out of three reproducible maneuvers (a difference <5% among them), as also previously described [44,45]. Reference values from Araújo et al. [45] were used in the analysis.

Sniff nasal inspiratory pressure (SNIP)—SNIP was also recorded using a pressure transducer connected to a catheter placed in the nostril during the measurement of a SNIP maneuver [45,46]. The subject was instructed to sniff quickly and deeply. The SNIP final outcome for each subject was obtained from the highest value out of ten reproducible maneuvers (a difference < 5% among them), as also previously described [44,45]. Reference values from Araújo et al. [45] were used in the analysis.

Maximal expiratory pressure (MEP)—MEP at the mouth was measured from total lung capacity (TLC). In order to measure the MEP, an occludable oral piece with a small orifice, used to minimize the participation of the buccinator muscles, was connected to a pressure manometer (TSD 104, Biopac Systems), whose signal was recorded by a digital polygraph (Biopac Systems). All the measurements were performed in a sitting position. The MEP final outcome for each subject was obtained from the highest value out of three reproducible maneuvers (a difference <5% among them), as also previously described [44,45]. Reference values from Araújo et al. [45] were used in the analysis.

Exercise Capacity

Exercise capacity was assessed through the measurement of the six-minute walking distance following current guidelines [47–49]. Encouragement was given to all the subjects during the test. Reference values from Enright et al. [49] were used in the analysis.

2.7. Statistical Analysis

The normality of the distribution of the study variables was assessed using the Shapiro–Wilk test. A minimum number of 60 patients and 12 healthy control subjects was required to achieve an 80% statistical power for the target variable (FFMI) assuming a standard deviation of 2.2 and alpha risk of 0.05. The study variables are presented as mean (standard deviation) in figures and tables. Both control subjects and patients were subdivided according to genders (21 and 114 females, control subjects and patients, respectively). The differences between the two groups were assessed using the Student's *t*-test or Mann–Whitney U test. Differences between the control and bronchiectasis patients within each gender group and between gender (only patients) were explored using one-way analysis of variance (ANOVA) or Kruskal–Wallis tests and Tukey's post hoc to correct for multiple comparisons. The Chi-square test was used for the categorical variables (smoking history). In summary, the following comparisons were assessed: (1) bronchiectasis patients and healthy controls as a whole, (2) differences between female and male patients in bronchiectasis patients, (3) differences between female controls and female patients, and (4) differences between male controls and male patients. Potential differences between female and male controls were not assessed in this study, as this was not part of the study hypothesis or objectives. The statistical significance was established as $p < 0.05$. Statistical analysis was performed using the software SPSS version 23 (SPSS Inc, Chicago, IL, USA). Correlations are shown in graphical correlation matrixes for all the patients, obtained from the R package corrplot (https://cran.r-project.org/web/packages/corrplot/index.html, accessed on 15 October 2021), in different colors: blue for positive correlations and red for negative ones.

3. Results

3.1. Clinical Characteristics of the Study Subjects

Tables 1 and 2 illustrate the clinical characteristics of the study population. Age did not differ between patients and control subjects. Healthy controls were non-smokers. BSI and EFACED scores were lower among the male patients compared to female patients (Table 2). Very few patients (12.7%) had chronic colonization by Pseudomonas aeruginosa (PA) and no differences were observed between male and female patients (Tables 1 and 2). Radiological extension of bronchiectasis was greater in male than in female patients (Tables 1 and 2).

Table 1. Clinical characteristics and functional status of bronchiectasis patients and healthy controls.

	Healthy Controls	**Bronchiectasis Patients**
	N = 37	**N = 150**
Age, years, $\bar{x}$ (SD)	62.4 (9.8)	64.6 (13.2)
Disease severity, $\bar{x}$ (SD)		
FACED score	NA	1.67 (1.40)
EFACED score	NA	1.94 (1.64)
BSI score	NA	5.74 (3.42)
Exacerbations in previous year	NA	0.91 (1.11)
Hospitalizations for exacerbations	NA	0.21 (0.66)
Chronic colonization by PA, N (%)	NA	19 (12.7)
Radiological extension, $\bar{x}$ (SD)		
Total extent bronchiectasis score	NA	7.6 (3.6)
Bronchial dilatation score	NA	1.2 (0.3)
Bronchial wall thickness score	NA	1.2 (0.3)
Global severity score	NA	10 (3.5)
Smoking history		
Current smokers, N (%)	0	7 (5)
Ex-smokers, N (%)	0	51 (34)
Never smokers, N (%)	37	92 (61)
Packs-year, $\bar{x}$ (SD)	0	20.5 (16.7)
Lung function, $\bar{x}$ (SD)		
FEV_1, % predicted	99 (12)	74 (22) ***
FVC, % predicted	100 (12)	83 (20) ***
FEV_1/FVC, %	78 (6)	68 (13) ***
DL_{CO}, % predicted	92 (6)	75 (16) ***
K_{CO}, % predicted	88 (13)	78 (14) *
RV, % predicted	107 (8)	147 (35) ***
TLC, % predicted	97 (7)	101 (16)
RV/TLC, %	38 (3)	54 (10) ***
Exercise capacity, $\bar{x}$ (SD)		
6-min walking distance, meters	533 (68)	463 (99) ***
Distance, % predicted	106 (13)	95 (19) ***
Initial oxygen saturation, %	98 (1)	96 (2) ***
Medium oxygen saturation, %	97 (2)	94 (3) ***
Minimum oxygen saturation, %	96 (2)	93 (4) ***
Final oxygen saturation, %	96 (2)	93 (4) ***
Blood parameters, $\bar{x}$ (SD)		
CRP, mg/dL	0.2 (0.1)	0.6 (0.9) ***
ESR, mm/h	5.7 (3.9)	11.0 (9.8) **
Fibrinogen, mg/dL	333.6 (63.7)	393.8 (97.2) ***
Alpha-1 antitrypsin	118.9 (16.9)	135.3 (27.2) **
Hemoglobin, g/dL	14.4 (1.3)	13.9 (1.9) *
Hematocrit, %	43.1 (3.8)	42.2 (4.8)
Creatinine, mg/dL	0.8 (0.2)	0.8 (0.2)
Total proteins, g/dL	7.2 (0.3)	7.1 (0.5)
Albumin, g/dL	4.6 (0.2)	4.4 (0.3) ***
Prealbumin, g/dL	26.3 (4.5)	22.7 (5.2) **

Continuous variables are presented as mean (standard deviation), while categorical variables are presented as the number of patients in each group along with the percentage for the study group. Definition of abbreviations: $\bar{x}$, mean; SD, standard deviation; N, number; NA, not applicable; FACED: F, FEV_1; A, age; C, chronic colonization by *Pseudomonas aeruginosa* (PA); E, radiologic extension; D, dyspnea; BSI, bronchiectasis severity index; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; DLco, carbon monoxide transfer; K_{CO}, Krogh transfer factor; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; g, grams; dL, deciliter; mg, milligrams; mm, millimeters; h, hour. Statistical analyses and significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ between bronchiectasis patients and healthy controls.

Table 2. Clinical characteristics and functional status in bronchiectasis patients and healthy controls according to gender differences.

	Healthy Controls		Bronchiectasis Patients	
	Women	**Men**	**Women**	**Men**
	N = 21	**N = 16**	**N = 114**	**N = 36**
Age, years	63.4 (9.7)	61 (10.1)	65.4 (12.4)	62.0 (15.3)
Disease severity				
BSI	NA	NA	5.98 (3.57)	4.97 (2.80) §
EFACED	NA	NA	2.08 (1.75)	1.5 (1.16) §
FACED	NA	NA	1.76 (1.47)	1.39 (1.10)
Exacerbations in previous year	NA	NA	0.94 (1.17)	0.83 (0.88)
Hospitalization for exacerbations	NA	NA	0.25 (0.73)	0.08 (0.37)
Chronic colonization by PA	NA	NA	14 (12.3)	5 (13.9)
Radiological extension, $\bar{x}$ (SD)				
Total extent of bronchiectasis score	NA	NA	7.3 (3.5)	8.7 (3.5) §
Bronchial dilatation score	NA	NA	1.2 (0.4)	1.1 (0.2)
Bronchial wall thickness score	NA	NA	1.2 (0.3)	1.2 (0.2)
Global severity score	NA	NA	9.7 (3.5)	11 (3.6) §
Smoking history				
Current smokers, N	0	0	6 (5)	1 (3)
Ex-smokers, N	0	0	35 (31)	16 (44)
Never smokers, N	21 (100)	16 (100)	73 (64)	19 (53)
Packs-year,$\bar{x}$ (SD)	0	0	21.3 (15.2)	18.5 (20.6)
Lung function, $\bar{x}$ (SD)				
FEV_1, % predicted	101 (12)	96 (13)	74 (22) ***	74 (23) **
FVC, % predicted	100 (12)	100 (12)	84 (20) **	83 (21) *
FEV_1/FVC, %	80 (5)	75 (7)	67 (13) ***	68 (11)
DL_{CO}, % predicted	88 (8)	94 (4)	74 (16) *	77 (16) *
K_{CO}, % predicted	82 (3)	92 (15)	76 (15)	84 (11) *
RV, % predicted	107 (9)	106 (8)	152 (36) *	132 (28) *§
TLC, % predicted	98 (6)	97 (8)	103 (15)	93 (15) *
RV/TLC, %	39 (1)	38 (4)	57 (10) *	47 (7) ** §§§
Exercise capacity, $\bar{x}$ (SD)				
6-min walking distance, meters	496 (60)	579 (44)	447 (94) *	512 (99) * §§
Distance, % predicted	105 (10)	108 (15)	94 (18) *	97 (23) *
Initial oxygen saturation, %	98 (1)	98 (1)	96 (2) **	96 (2) **
Medium oxygen saturation, %	97 (2)	97 (1)	94 (3) **	94 (3) **
Minimum oxygen saturation, %	96 (2)	97 (2)	92 (4) ***	93 (3) **
Final oxygen saturation, %	96 (2)	97 (2)	92 (4) ***	93 (3) **
Blood parameters, $\bar{x}$ (SD)				
CRP, mg/dL	0.2 (0.1)	0.2 (0.1)	0.6 (0.9) *	0.6 (0.6) *
ESR, mm/h	6.6 (3.6)	4.7 (4.0)	11.3 (9.9) *	10 (9.6) *
Fibrinogen, mg/dL	349.5 (64)	313.8 (59.3)	397.4 (88.6) *	381.8 (122.1) *
Alpha-1 antitrypsin, mg/dL	117.5 (19.0)	121.2 (13.4)	134.3 (24.5) **	138.7 (35.0) *
Hemoglobin, g/dL	13.7 (1.0)	15.2 (1.0)	13.7 (2.1)	14.5 (1.0) * §
Hematocrit, %	41.3 (3.4)	45.6 (2.9)	41.7 (5)	43.9 (3.6) §
Creatinine, mg/dL	0.7 (0.1)	1.0 (0.1)	0.7 (0.1)	1.0 (0.2) §§§
Total proteins, g/dL	7.2 (0.3)	7.2 (0.3)	7.1 (0.4)	7.2 (0.5)
Albumin, g/dL	4.6 (0.2)	4.6 (0.2)	4.4 (0.3) **	4.4 (0.4) *
Prealbumin, g/dL	25.5 (4.7)	27.6 (3.9)	21.8 (4.7) **	25.3 (5.9) §§

Continuous variables are presented as mean (standard deviation), while categorical variables are presented as the number of patients in each group along with the percentage for the study group. Definition of abbreviations: $\bar{x}$, mean; SD, standard deviation; N, number; NA, not applicable; FACED: F, FEV_1; A, age; C, chronic colonization by *Pseudomonas aeruginosa* (PA); E, extension radiologic; D, dyspnea; BSI, bronchiectasis severity index; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; DLco, carbon monoxide transfer; K_{CO}, Krogh transfer factor; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; g, grams; dL, deciliter; mg, milligrams; mm, millimeters; h, hour. Statistical analyses and significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ between healthy and bronchiectasis women or healthy and bronchiectasis men; § $p \leq 0.05$; §§ $p \leq 0.01$; §§§ $p \leq 0.001$ between men and women patients.

Significant positive correlations were detected between FACED, EFACED, and BSI with RV/TLC among all the patients (Figure 1A). Patients as a whole and when divided by gender exhibited mild-to-moderate airway obstruction, reduced diffusion capacity, airway trapping, and decreased exercise capacity compared to the healthy controls (Tables 1 and 2). Additionally, female patients had a greater degree of airway trapping as determined by RV and RV/TLC and worse exercise capacity (distance absolute values) than male patients (Table 2). In the blood compartment, a significant rise in acute phase reactants (CRP, ESR, fibrinogen, and alpha-1 antitrypsin) was observed in the patients compared to control subjects (Table 1). No significant differences in levels of these parameters were observed between male and female patients in this cohort (Table 2). Moreover, levels of hemoglobin, albumin, and prealbumin were mildly reduced in the patients as a whole compared to the controls (Table 1). In the male patients, levels of these parameters were significantly greater than those detected in the female patients (Table 2). Furthermore, significant negative associations were observed between FACED, EFACED, and BSI scores, and creatinine and prealbumin blood parameters (Figure 1A). No significant differences in levels of inflammatory or nutritional parameters were detected when patients were divided according to chronic colonization by PA (data not shown).

3.2. Body Composition

BMI was significantly reduced in the patients compared to the healthy subjects, particularly in the female patients (Figure 1B,C, respectively). No significant differences were observed between male patients and healthy male controls (Figure 1C). A significant reduction in FFM was observed in all the patients compared to healthy controls (Figure 2A). Moreover, FFM was lower in female patients than in healthy females, while no differences were observed in the males (Figure 2B). FFM was greater in the male than in the female patients (Figure 2B). FFMI significantly decreased in the patients as a whole compared to the healthy controls (Figure 2C). Importantly, in female patients, FFMI was also significantly reduced compared to female controls (Figure 2D). Moreover, male patients exhibited a greater FFMI than female patients (Figure 2D). Fat tissue was significantly lower in patients as a whole and in the female patients than in the healthy controls (Figure 3A,B, respectively). The percentage of fat tissue was significantly lower in the male than in the female patients, while no differences were observed in the other groups (Figure 3C,D). In the overall study patients, BMI positively correlated with FFMI, FFM, absolute and percentage fat tissue, and blood creatinine (Figure 3E). Among the study patients, FFM and FFMI parameters were inversely correlated with FACED, EFACED, and BSI, whereas they were positively associated with creatinine blood levels (Figure 3E). No significant differences in the body composition parameters were detected when patients were divided according to chronic colonization by PA (data not shown).

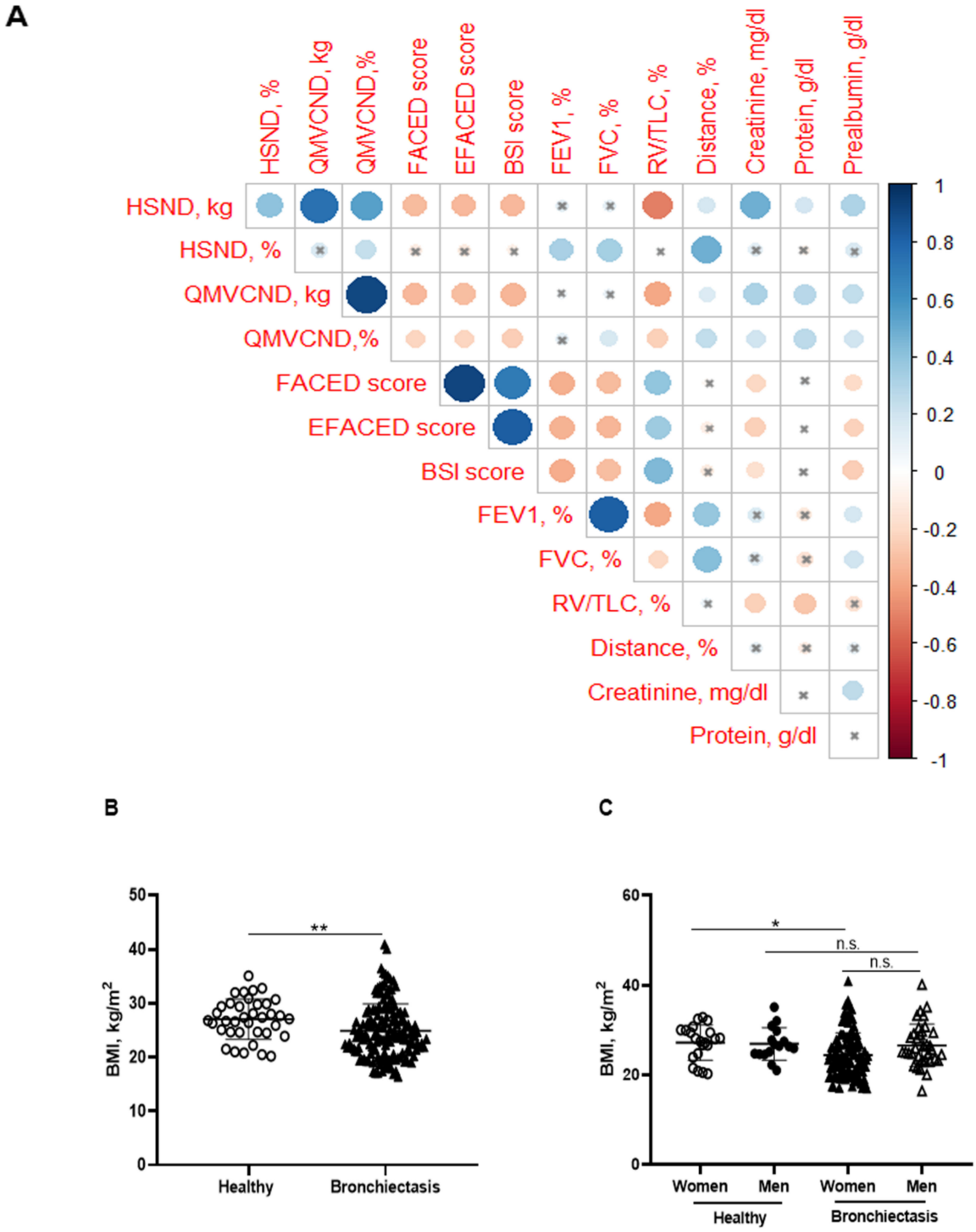

Figure 1. (**A**) Correlation matrix of clinical variables and peripheral muscle strength variables, in which positive correlations are represented in blue, while negative correlations are represented in red. The intersection within the circle represents a p value > 0.05. Color intensity and the size of the circle are proportional to the correlation coefficients, as indicated in the Y axis on the right-hand side of the graph. (**B**) Mean values and standard deviation of body mass index (BMI) (kg/m^2) in healthy controls and bronchiectasis patients. (**C**) Mean values and standard deviation of BMI (kg/m^2) in both female and male healthy controls and bronchiectasis patients. Statistical analyses and significance: ** $p \leq 0.01$ between bronchiectasis patients and healthy controls. * $p \leq 0.05$ between healthy and bronchiectasis women and n.s., non-significant differences between healthy and bronchiectasis men or between men and women patients.

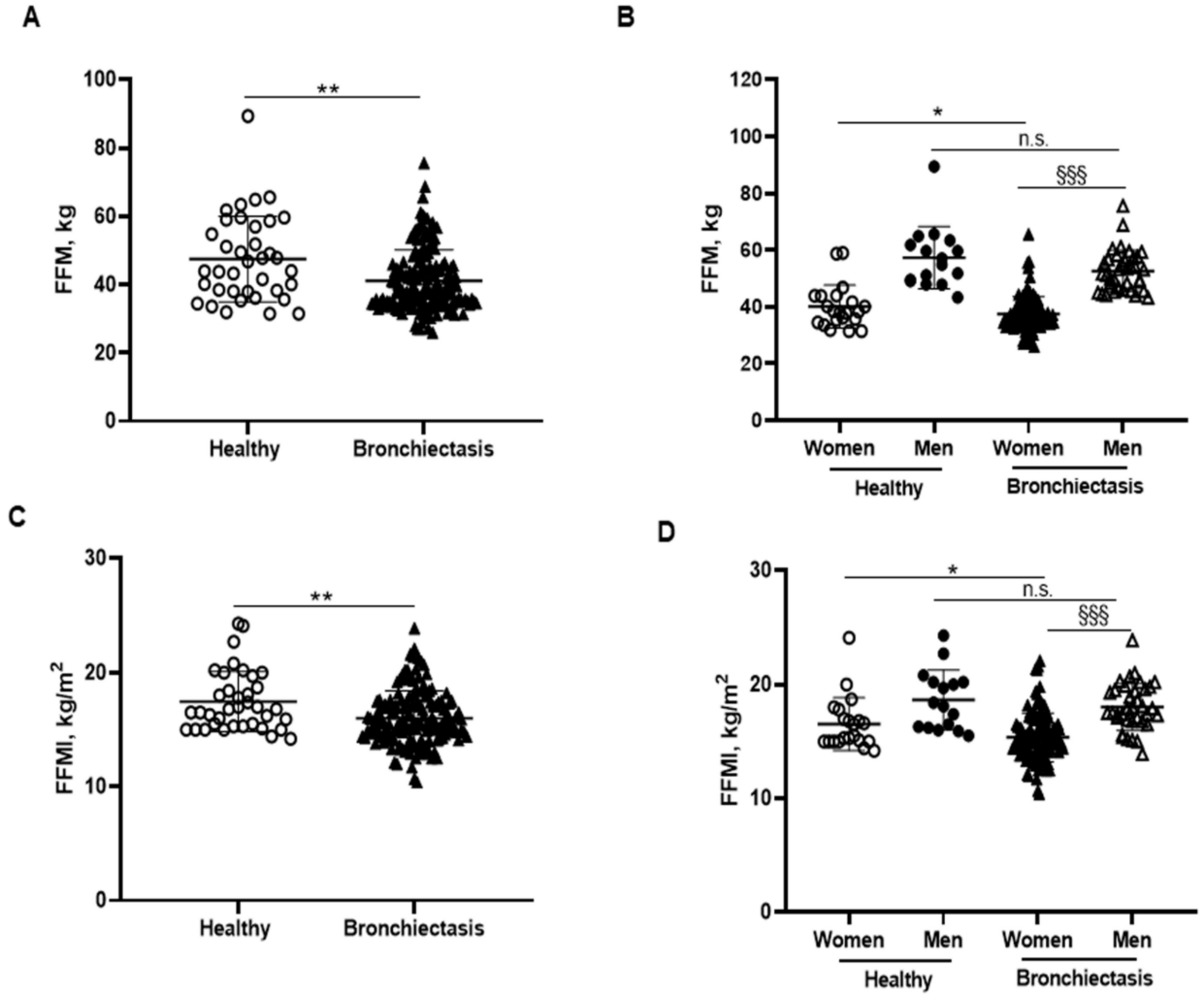

Figure 2. (**A**) Mean values and standard deviation of FFM (kg) in healthy controls and bronchiectasis patients. (**B**) Mean values and standard deviation of FFM (kg) in both female and male healthy controls and bronchiectasis patients. (**C**) Mean values and standard deviation of FFMI (kg/m^2) in healthy controls and bronchiectasis patients. (**D**) Mean values and standard deviation of FFMI (kg/m^2) in both female and male healthy controls and bronchiectasis patients. Statistical analyses and significance: ** $p < 0.01$ between bronchiectasis patients and healthy controls. * $p \leq 0.05$ between healthy and bronchiectasis women; n.s., non-significant differences between healthy and bronchiectasis men; and §§§ $p \leq 0.001$ between men and women patients.

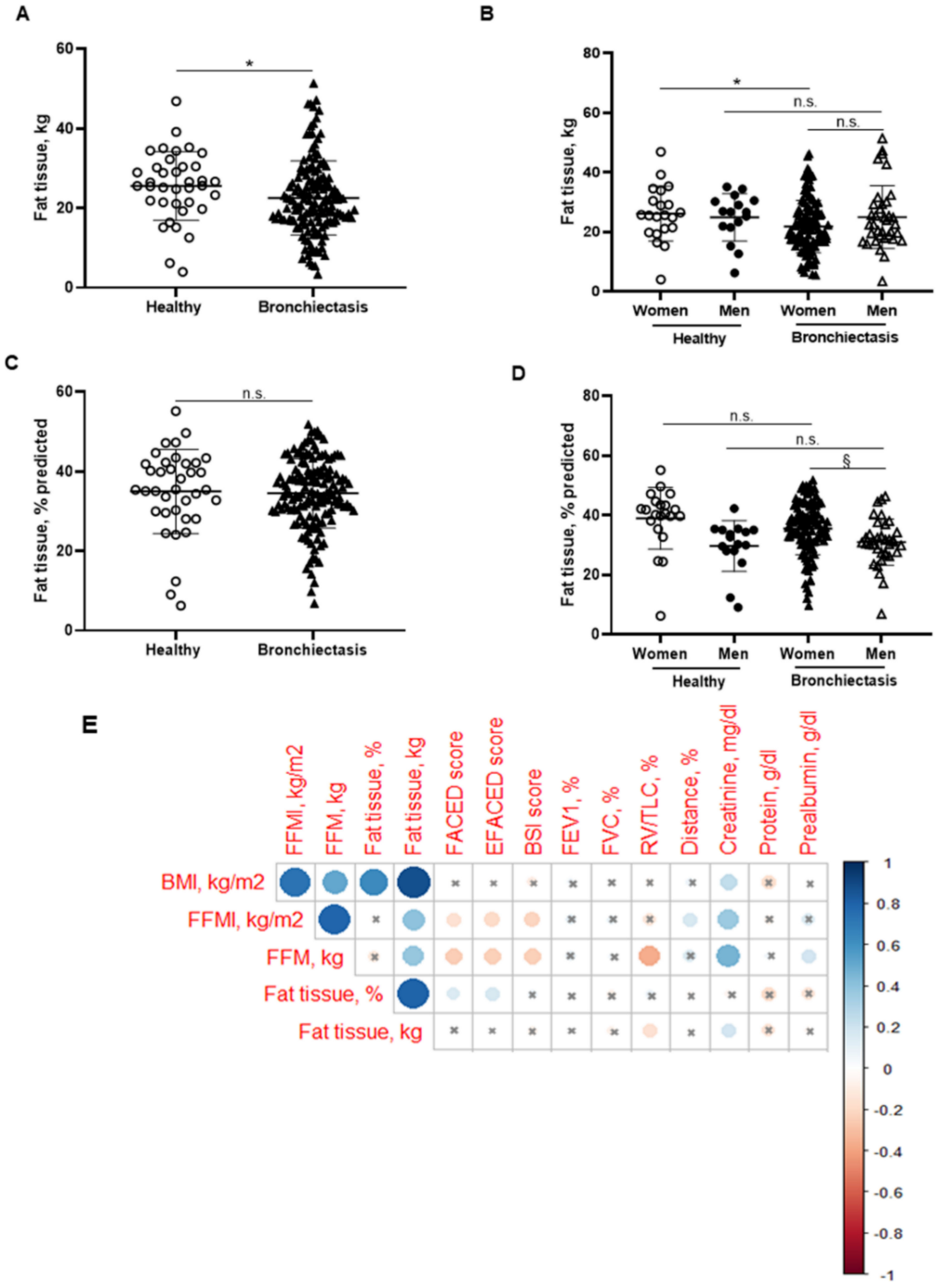

Figure 3. (**A**) Mean values and standard deviation of fat tissue (kg) in healthy controls and bronchiectasis patients. (**B**) Mean values and standard deviation of fat tissue (kg) in both female and male healthy controls and bronchiectasis patients. (**C**) Mean values and standard deviation of fat tissue (% predicted) in healthy controls and bronchiectasis patients. (**D**) Mean values and standard deviation of fat tissue (% predicted) in both female and male healthy controls and bronchiectasis patients. (**E**) Correlation matrix of clinical variables and body composition variables, in which positive correlations are represented in blue, while negative correlations are represented in red. The intersection within the circle represents p value > 0.05. Color intensity and the size of the circle are proportional to the correlation coefficients, as indicated in the Y-axis on the right-hand side of the graph. Statistical analyses and significance: * $p \leq 0.05$ and n.s. (non-significant differences) between bronchiectasis patients and healthy controls. * $p \leq 0.05$ and n.s. between healthy and bronchiectasis women or between healthy and bronchiectasis men; and § $p \leq 0.05$ and n.s. between men and women patients.

3.3. Upper Limb Muscle Strength

Handgrip absolute strength values were significantly reduced in the patients as a whole compared to the healthy controls, particularly among the female patients compared to male patients (Figure 4A,B, respectively). Predicted handgrip strength values were also significantly lower in the patients as a whole than in the controls, while no differences were observed when patients were subdivided according to gender (Figure 4C,D, respectively). Interestingly, handgrip absolute values negatively correlated with FACED, EFACED, and BSI scores and RV/TLC, while it positively correlated with exercise capacity (distance walked), creatinine, protein, and prealbumin blood levels among the overall patients (Figure 1A).

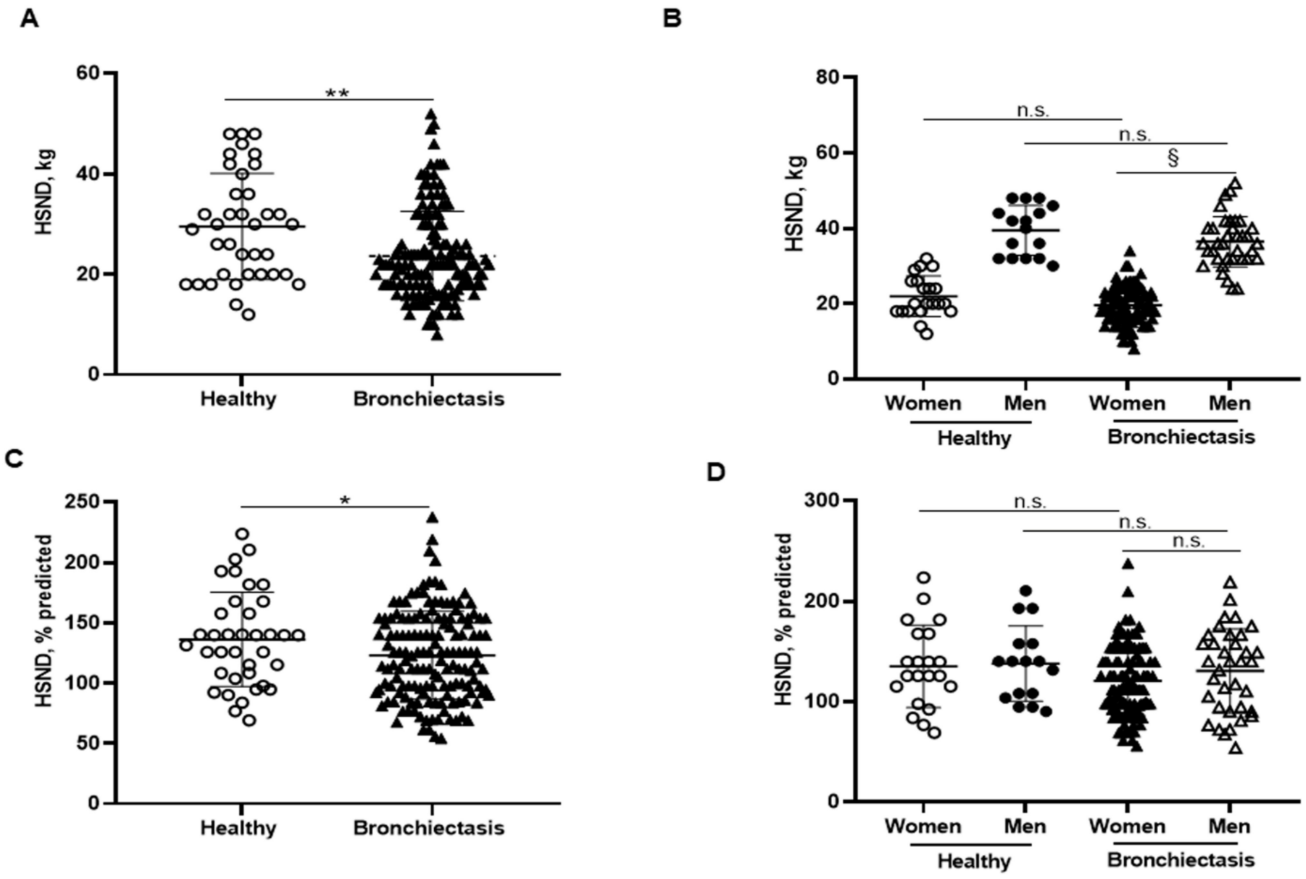

Figure 4. (**A**) Mean values and standard deviation of handgrip strength non-dominant (HSND) (kg) in healthy controls and bronchiectasis patients. (**B**) Mean values and standard deviation of HSND (kg) in both female and male healthy controls and bronchiectasis patients. (**C**) Mean values and standard deviation of HSND (% predicted) in healthy controls and bronchiectasis patients. (**D**) Mean values and standard deviation of HSND (% predicted) in both female and male healthy controls and bronchiectasis patients. Statistical analyses and significance: * $p \leq 0.05$ and ** $p \leq 0.01$ between bronchiectasis patients and healthy controls. n.s., non-significant differences between healthy and bronchiectasis women or between healthy and bronchiectasis men; § $p \leq 0.05$ and n.s. between men and women patients.

3.4. Lower Limb Muscle Strength

Patients as a whole exhibited a significant decline in absolute and predicted values of QMVC compared to healthy controls, which was also confirmed when patients were subdivided into male and female genders (Figure 5A–D, respectively). Additionally, QMVC absolute and predicted values were significantly lower in the female compared to the male patients (Figure 5B,D, respectively). QMVC absolute and predicted values negatively correlated with FACED, EFACED, and BSI scores and RV/TLC, while they positively

correlated with exercise capacity (distance walked), creatinine, protein, and prealbumin blood levels among the overall patients (Figure 1A).

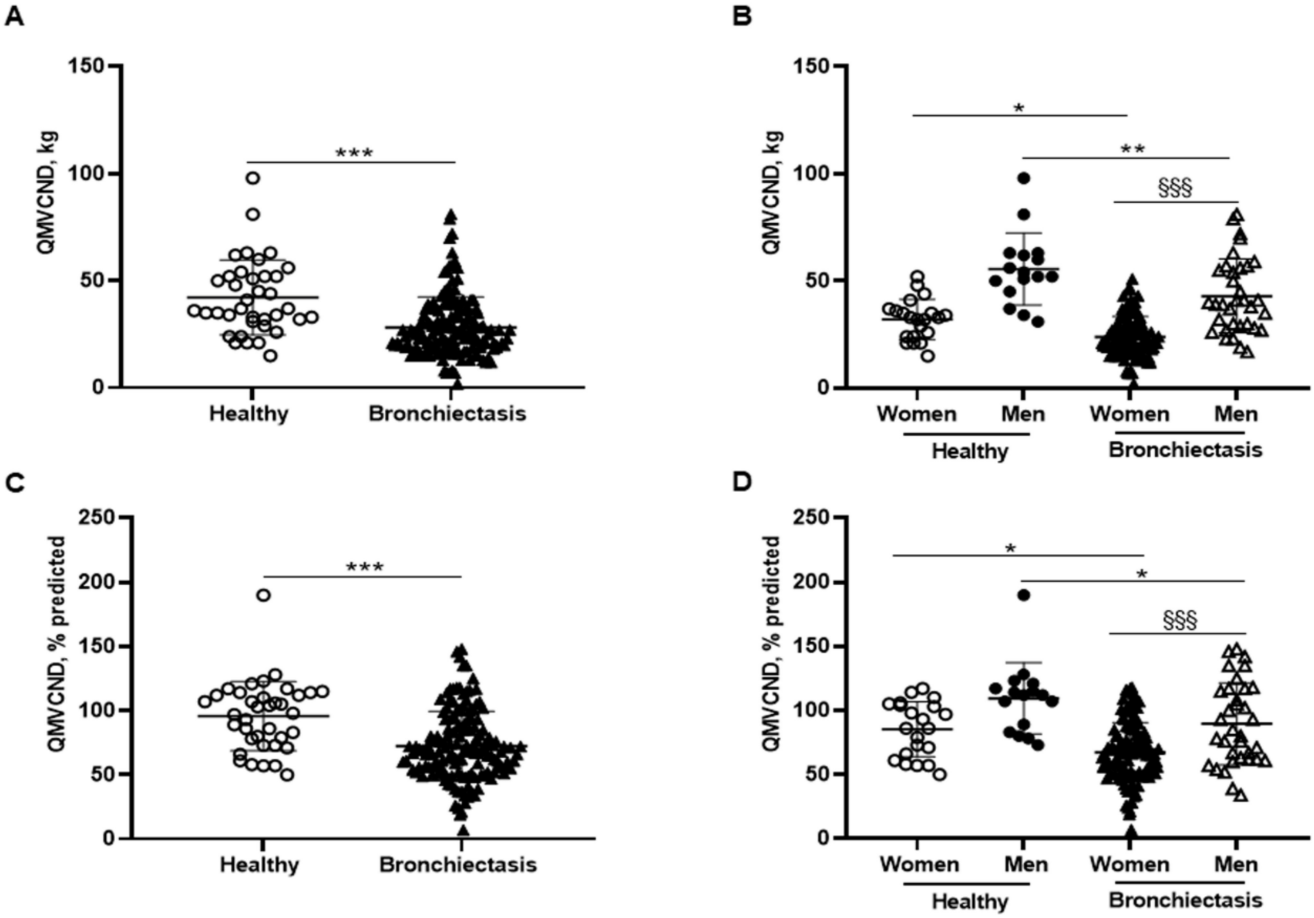

Figure 5. (**A**) Mean values and standard deviation of quadriceps maximal strength during maximum voluntary contraction in the non-dominant leg (QMVC) (kg) in healthy controls and bronchiectasis patients. (**B**) Mean values and standard deviation of QMVC (kg) in both female and male healthy controls and bronchiectasis patients. (**C**) Mean values and standard deviation of QMVC (% predicted) in healthy controls and bronchiectasis patients. (**D**) Mean values and standard deviation of QMVC (% predicted) in both female and male healthy controls and bronchiectasis patients. Statistical analyses and significance: *** $p \leq 0.001$ between bronchiectasis patients and healthy controls. * $p \leq 0.05$; ** $p \leq 0.01$ between healthy and bronchiectasis women or between healthy and bronchiectasis men; §§§ $p \leq 0.001$ between men and women patients.

3.5. Inspiratory and Expiratory Muscle Strength

MIP absolute and predicted values significantly decreased in the patients as a whole compared to the healthy controls and this was also confirmed in the female patients compared to the female controls (Figure 6A–D). In addition, MIP absolute values were significantly greater in the male than in the female patients (Figure 6B). Among the study patients, negative associations were detected between MIP absolute and predicted values and FACED, EFACED, RV/TLC, bronchial dilatation, and wall thickness scores (Figure 6E). SNIP absolute and predicted values were significantly diminished in the patients as a whole and in both male and female patients compared to the respective healthy controls (Figure 7A–D).

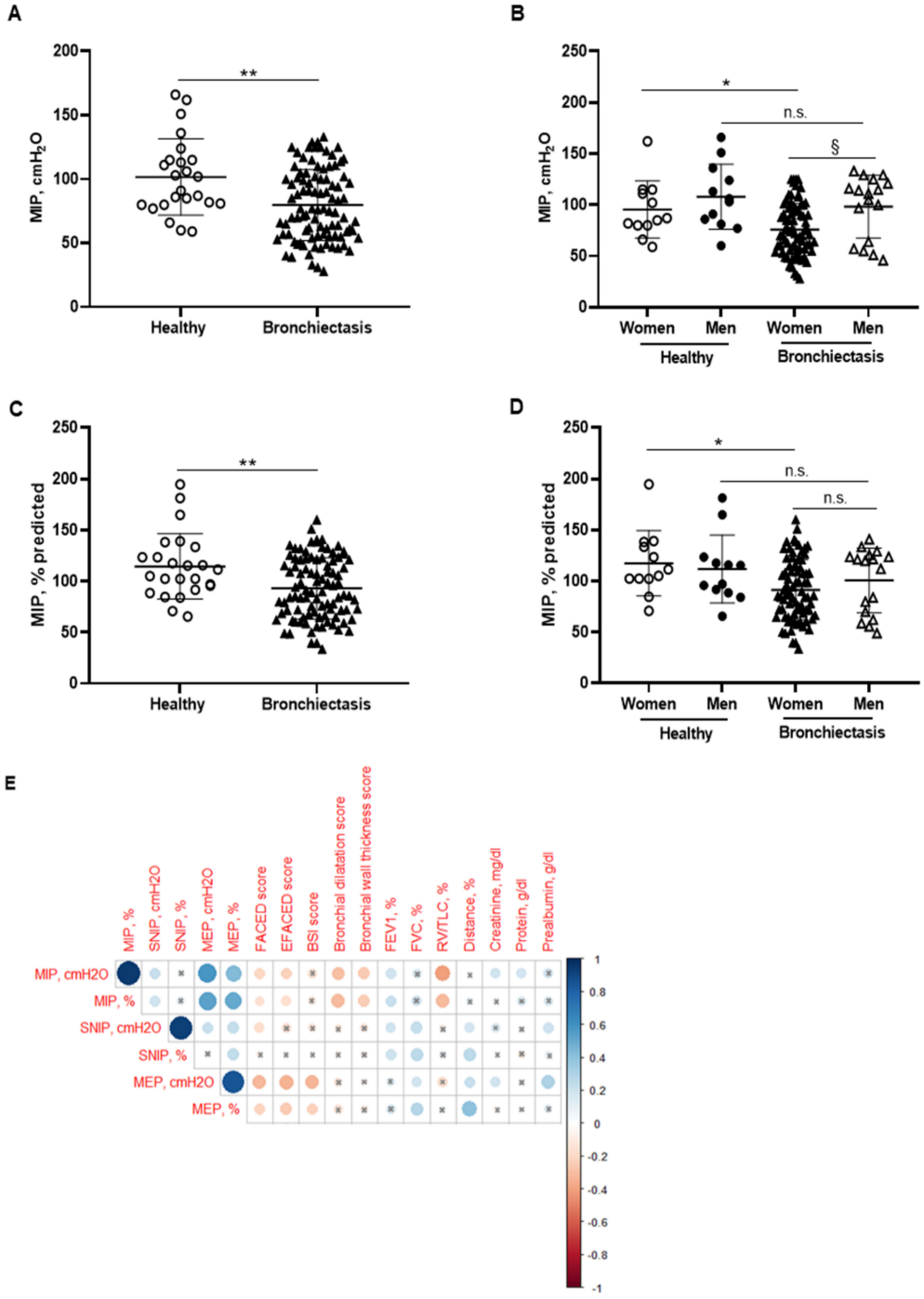

Figure 6. (**A**) Mean values and standard deviation of maximal inspiratory pressure (MIP) (cmH_2O) in healthy controls and bronchiectasis patients. (**B**) Mean values and standard deviation of MIP (cmH_2O) in both female and male healthy controls and bronchiectasis patients. (**C**) Mean values and standard deviation of MIP (% predicted) in healthy controls and bronchiectasis patients. (**D**) Mean values and standard deviation of MIP (% predicted) in both female and male healthy controls and bronchiectasis patients. (**E**) Correlation matrix of clinical variables and respiratory muscle strength variables, in which positive correlations are represented in blue, while negative correlations are represented in red. The intersection within the circle represents p value > 0.05. Color intensity and the size of the circle are proportional to the correlation coefficients, as indicated in the Y-axis on the right-hand side of the graph. Statistical analyses and significance: ** $p \leq 0.01$ between bronchiectasis patients and healthy controls. * $p \leq 0.05$ between healthy and bronchiectasis women and n.s., non-significant differences between healthy and bronchiectasis men; § $p \leq 0.05$ and n.s. between men and women patients.

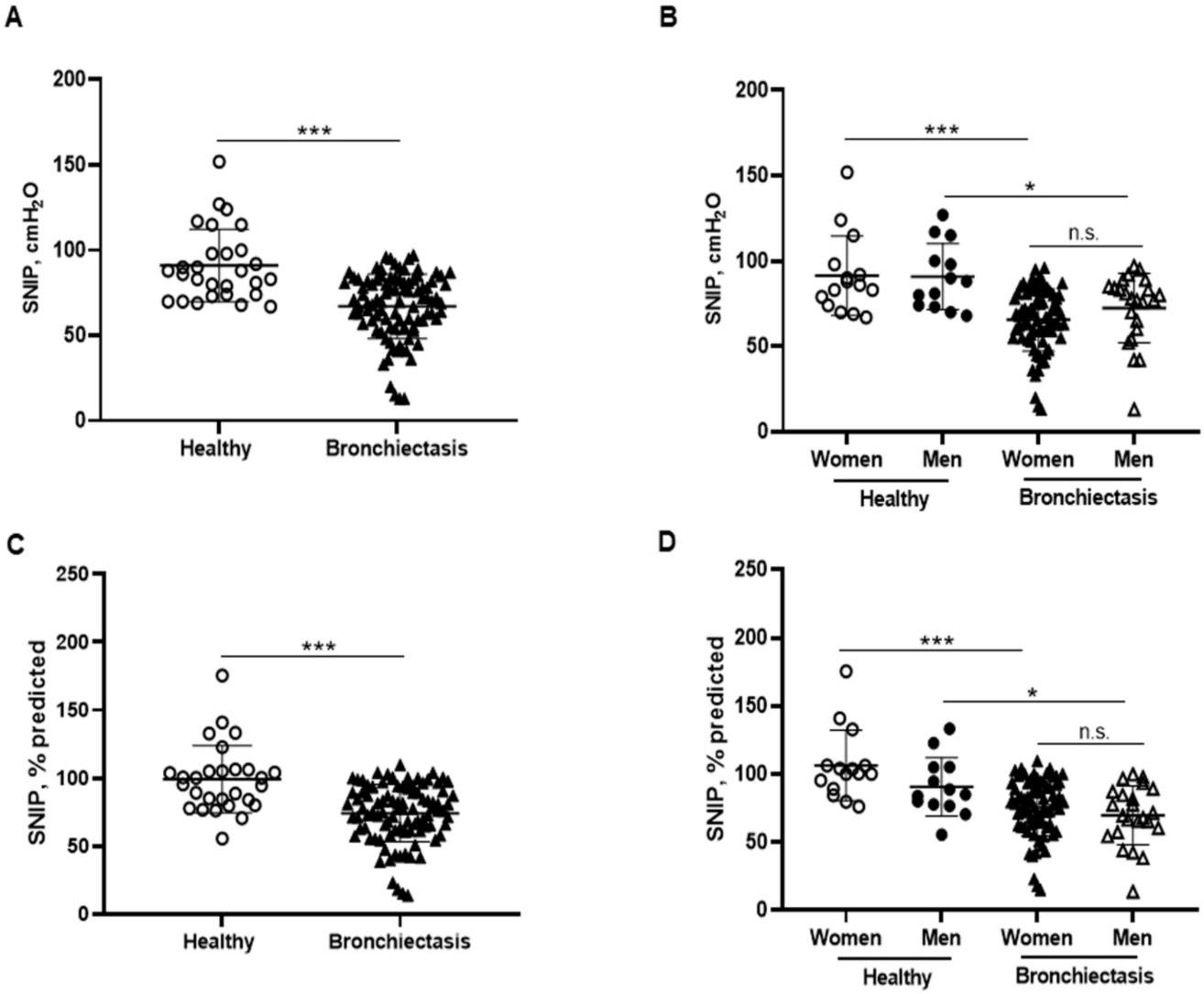

Figure 7. (**A**) Mean values and standard deviation of maximal sniff nasal pressure (SNIP) (cmH_2O) in healthy controls and bronchiectasis patients. (**B**) Mean values and standard deviation of SNIP (cmH_2O) in both female and male healthy controls and bronchiectasis patients. (**C**) Mean values and standard deviation of SNIP (% predicted) in healthy controls and bronchiectasis patients. (**D**) Mean values and standard deviation of SNIP (% predicted) in both female and male healthy controls and bronchiectasis patients. Statistical analyses and significance: *** $p \leq 0.001$ between bronchiectasis patients and healthy controls. *** $p \leq 0.001$ between healthy and bronchiectasis women; * $p \leq 0.05$ between healthy and bronchiectasis men; n.s., non-significant differences between men and women patients.

Absolute and predicted MEP values were significantly lower in the patients as a whole and in both male and female patients than in the respective control subjects (Figure 8A–D). Furthermore, MIP absolute values were significantly increased in the male compared to the female patients (Figure 8B). In the overall study patients, MEP absolute and predicted values were negatively associated with FACED, EFACED, and BSI scores, while they positively correlated with FVC and exercise capacity (distance walked, Figure 6E).

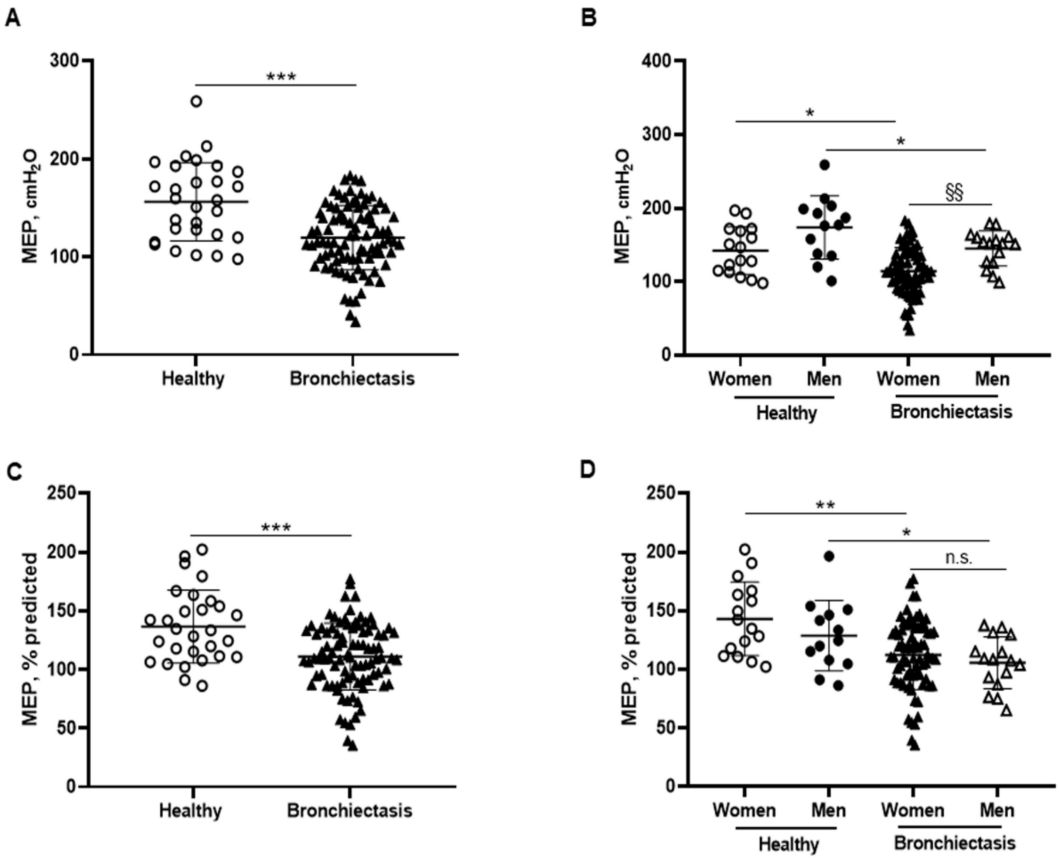

Figure 8. (**A**) Mean values and standard deviation of maximal expiratory pressure (MEP) (cmH_2O) in healthy controls and bronchiectasis patients. (**B**) Mean values and standard deviation of MEP (cmH_2O) in both female and male healthy controls and bronchiectasis patients. (**C**) Mean values and standard deviation of MEP (% predicted) in healthy controls and bronchiectasis patients. (**D**) Mean values and standard deviation of MEP (% predicted) in both female and male healthy controls and bronchiectasis patients. Statistical analyses and significance: *** $p \leq 0.001$ between bronchiectasis patients and healthy controls. * $p \leq 0.05$; ** $p \leq 0.01$ between healthy and bronchiectasis women or between healthy and bronchiectasis men; §§ $p \leq 0.01$ and n.s., non-significant differences between men and women patients.

4. Discussion

Patients with mild-to-moderate bronchiectasis exhibited airflow limitation, a decrease in diffusion capacity along with air trapping, reduced exercise capacity, and a slight increase in blood inflammatory parameters as also previously demonstrated [6,7]. The most relevant findings in the study are discussed below.

Despite body composition parameters being within the normal range (BMI, 24.9 kg/m^2 and FFMI, 16 kg/m^2) among the study patients, they were significantly reduced when compared to a population of healthy subjects (37 recruited for the purpose of the investigation). Importantly, FFM along with BMI and FFMI, were also significantly lower in the patients than in the healthy controls. Importantly, the female patients were those exhibiting the greater reduction in those parameters compared to the healthy female controls. Interestingly, male patients exhibited a significant increase in FFM and FFMI values compared to the female patients. These findings suggest that gender differences exist with regards to the levels of FFM between male and female patients for the same degree of disease severity. Similarly, gender differences have also been reported in several nutritional and inflammatory parameters as well as in BMI between female and male bronchiectasis patients [6,50].

Bronchiectasis patients in this large cohort demonstrated significant inverse associations between body composition parameters (FFM and FFMI), nutritional biomarkers (creatinine and prealbumin), and disease severity as measured by FACED, EFACED, and BSI scores. However, no relationships were observed between nutritional status and lung function parameters among the patients. These results are consistent with previous findings in which no significant associations were demonstrated between lung function and nutritional parameters [51]. Moreover, no differences were observed in body composition or nutritional parameters between patients with chronic colonization by PA (12.7%) and those without, suggesting that this factor did not influence those results in this cohort of patients (data not shown). Importantly, a decline in fat tissue content was observed in all the patients as a whole and particularly among the female patients when the parameter was expressed in kg. No differences were observed between male patients and healthy male subjects in these parameters.

A recent investigation [52] has demonstrated that sedentary behavior and a low number of daily steps predicted the risk of hospitalizations in one year among patients with bronchiectasis compared to patients with a more active lifestyle. In the present study, the distance walked among bronchiectasis patients, both male and females, was also reduced compared to the healthy controls. In addition, total distance walked by female patients was significantly lower than that observed in the male patients, as also previously shown [53]. These results imply that exercise tolerance is an important parameter that should be included in the management of patients with bronchiectasis in clinics.

Skeletal muscle dysfunction is a major systemic manifestation in patients with chronic diseases, including COPD, chronic heart and kidney failure, and cancer [9–14]. Specifically, one third of COPD patients exhibit muscle weakness of the lower limbs irrespective of the degree of the airway obstruction [15]. Despite that COPD patients may present upper and lower limb muscle dysfunction, the latter are most commonly evaluated in clinical settings due to its implications in their exercise tolerance [9–14,54]. Muscle contractile performance in vivo can be identified through the assessment of either muscle strength or resistance, and the former is most widely evaluated in clinics [9–14]. In the current investigation, measurements of upper muscle function demonstrated a significant decline in handgrip strength in the bronchiectasis patients compared to the healthy controls. Strength of the upper limb muscles did not show, however, any significant gender differences.

Muscle weakness of the quadriceps was observed in the bronchiectasis patients compared to the controls. Importantly, a significant decrease in quadriceps muscle strength, absolute and predicted values, was observed in both female and male patients compared to their respective healthy control subjects. Weakness of the upper and lower limb muscles was also observed in bronchiectasis patients of a younger age and severe airway obstruction [55]. Moreover, in the present study, significant positive correlations were observed between quadriceps muscle function (absolute and predicted) and the predicted distance walked in the six-minute walk test. It is also worth mentioning that despite radiological extension being more prominent in male patients, quadricep muscle weakness was significantly greater among the female patients than in the male bronchiectasis patients—both absolute and predicted values. These observations are very relevant and as far as we are concerned these are novel findings that put the line forward that the lower limb muscles of female bronchiectasis patients are more severely affected than those of the males. These results also suggest that quadriceps muscle weakness was independent of the degree of the bronchiectasis radiological extension.

Moreover, from the study results it is also possible to conclude that the upper and lower limb muscles are not equally affected in bronchiectasis patients, the latter being more negatively altered, particularly in the female patients. These findings warrant further attention and future research should aim to identify the pathophysiology of the muscle abnormalities and gender differences within the quadriceps of bronchiectasis patients. Besides, the implications of lower limb muscle weakness towards exercise capacity should also be explored in future investigations. Moreover, similarly to what happens in other

chronic lung diseases, namely COPD and in chronic heart failure patients, pulmonary rehabilitation and particularly exercise training are essential therapeutic strategies to improving muscle function, structure, and performance along with exercise tolerance and quality of life [10,44,56–69]. Hence, it is possible to conclude that pulmonary rehabilitation should be part of the wide spectrum of therapeutic tools currently available in patients with bronchiectasis. As such, pulmonary rehabilitation may be a component of the multidisciplinary therapeutic approach to be applied to specific phenotypes of patients. Future research should be devoted to this specific aspect in the management of patients with bronchiectasis.

In patients with bronchiectasis, inspiratory muscle function, as measured by MIP absolute and predicted values, was reduced, and such a decrease was observed particularly in the female patients compared to the female controls. Furthermore, a significant decline was also observed in SNIP and MEP absolute and predicted values in the patients as a whole and in both male and female patients, as compared to their respective controls. Taken together, these findings reveal that the function of inspiratory and expiratory muscles is significantly altered in bronchiectasis patients. These findings are consistent with previous results, in which a small cohort of bronchiectasis patients also demonstrated a significant decline in MIP and MEP absolute and predicted values compared to healthy controls [70]. The novelty in our study relies on the reported gender differences and on the use of SNIP as a reliable measurement of inspiratory muscle function [44,71]. Importantly, negative associations were also found between either disease severity scores or radiological extension and MIP and MEP parameters, suggesting that patients with greater score values were those with lower respiratory muscle performance.

5. Study Critique

Reference values used in the present study were those published in the literature, in which the phenotypic features of the participants were similar to those of our patients. Moreover, precise reference had to be used for each specific type of the measurements performed in this study. Thus, reference values have been customized to each particular measurement and similarities of the phenotypic characteristics.

Whether the assessment of patients with other concomitant respiratory diseases, such as asthma or COPD, may have yielded similar results should be a matter of research in future investigations. In fact, bronchiectasis has been recently proposed to be one of the most relevant asthma-associated comorbidities [72,73], and the combination of these diseases may worsen clinical outcomes, including muscle dysfunction.

Another potential limitation is related to the lack of use of specific questionnaires to assess physical activity or detailed diet components in the patients and healthy controls. Nonetheless, the participants were all inquired whether they were conducting any outdoor or indoor regular high-intensity physical activity or following any specific exercise training program.

6. Conclusions

In patients with mild-to-moderate bronchiectasis, respiratory and peripheral muscle function is significantly impaired and only partly related to the status of lung disease. Quadricep muscle strength was particularly weakened in female patients and was negatively associated with their exercise tolerance but not with the extent of the bronchiectasis. The results reported herein have clinical implications in the clinical management of these patients. Specific therapeutic strategies targeted to improving muscle mass and performance should be applied to bronchiectasis patients with peripheral muscle weakness. Body composition and peripheral muscle function determination should be part of the comprehensive clinical assessment of these patients.

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intellectual input: E.B. and X.W.; Manuscript writing final version: E.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was designed following the guidelines of the World Medical Association for Research in Humans (Seventh revision of the Declaration of Helsinki, Fortaleza, Brazil, 2013) and the ethical standards on human experimentation in our institution. The study was approved by the Institutional Ethics Committee on Human Investigation before the start. (Hospital del Mar-IMIM, Barcelona, project number 2019/8955/I).

Informed Consent Statement: Informed written consent was obtained from all the patients and the healthy control subjects.

Data Availability Statement: The datasets are available from the corresponding authors upon reasonable request.

Conflicts of Interest: The authors have nothing to disclose regarding this study.

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10.2.3 Publication 3

Title:

B cells and tertiary lymphoid structures influence survival in lung cancer patients with resectable tumors

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

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

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

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

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

1. Introduction

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

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

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

2. Results

2.1. Clinical Characteristics of the Study Patients

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

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

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

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

Table 1. *Cont.*

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

Continuous variables are shown as mean and standard deviation, while categorical variables are described as the number of patients in each group and the percentage in the study group with respect to the total population. Definition of abbreviations: N, number; kg, kilograms; m, meters; BMI, body mass index; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; DL_{CO}, carbon monoxide transfer; K_{CO}, Krogh transfer factor; GOLD: Global Initiative for Chronic Obstructive Pulmonary Disease; NA, not applicable; TNM, tumor, nodes, metastasis; CRP, C-reactive protein; GSV, globular sedimentation velocity; L, liter; COPD, chronic obstructive pulmonary disease. Statistical analyses and significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between LC-COPD patients and LC patients.

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

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

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

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

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

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

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

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

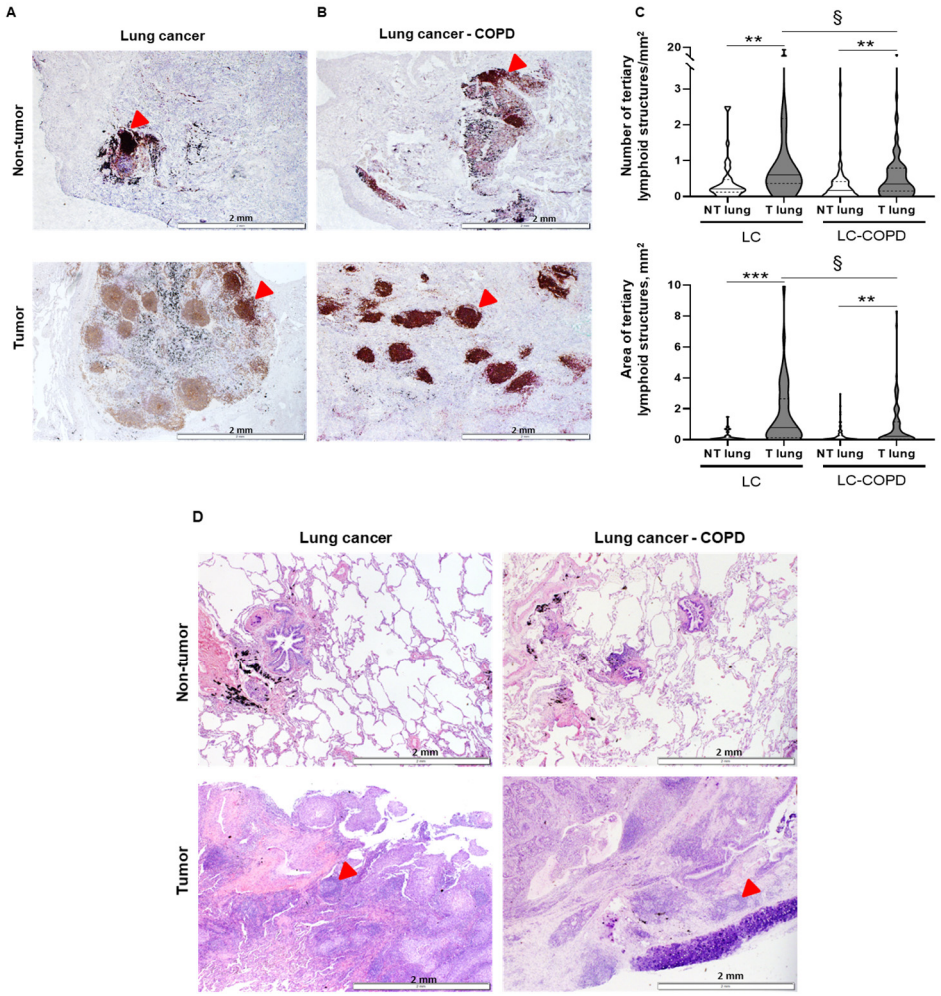

Figure 1. TLSs and germinal centers in tumor and non-tumor lungs of patients. (**A,B**) Representative examples of double immunohistochemical staining for TLSs indicated by red arrows in lung cancer (LC) and LC-COPD patients, respectively. (**C**) Violin plot with median (continuous line) and interquartile range (discontinuous line) of the number of TLSs corrected by area (number/mm^2, top panel) and total area of TLSs (mm^2, bottom panel), respectively. Comparisons were made between the non-tumor (NT) and tumor (T) samples, and the LC and LC-COPD groups of patients. Statistical significance: **, $p \leq 0.01$; ***, $p \leq 0.001$ between tumor and non-tumor lungs in either LC or LC-COPD patients, §, $p \leq 0.05$ in tumor samples between LC and LC-COPD patient groups. (**D**) Representative examples of hematoxylin and eosin staining for the germinal centers contained within the TLSs in LC and LC-COPD patients. Red arrows point towards germinal centers. Definition of abbreviations: TLSs, tertiary lymphoid structures.

2.3. T and B Cell Levels in Lung Samples

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

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

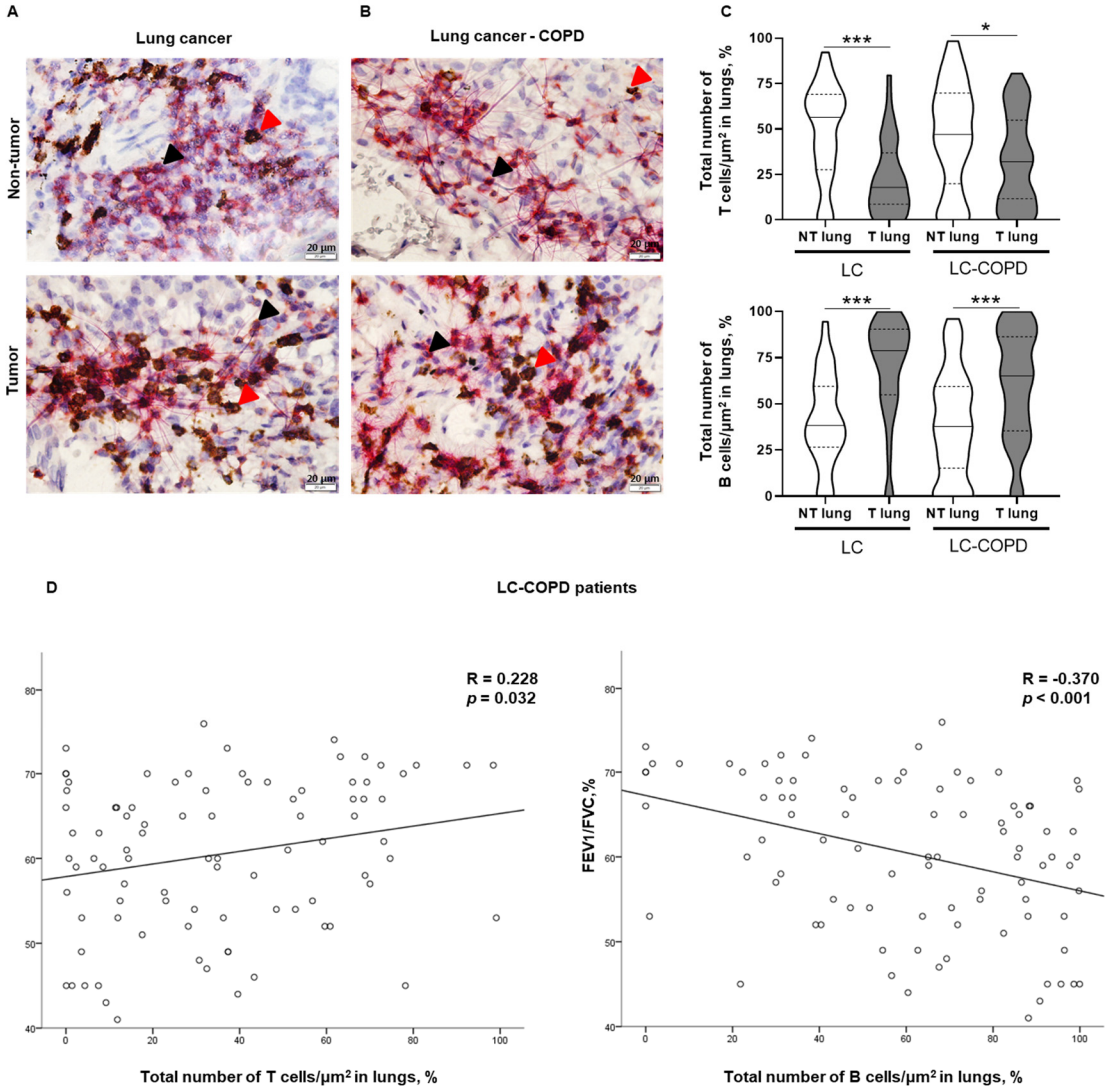

Figure 2. T and B cell counts in tumor and non-tumor lungs of patients and their correlations with lung function parameters. (**A**,**B**) Representative double immunohistochemical staining sections of T and B cells in non-tumor and tumor lung specimens of LC and LC-COPD patients, respectively. T cells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

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

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

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

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

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

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

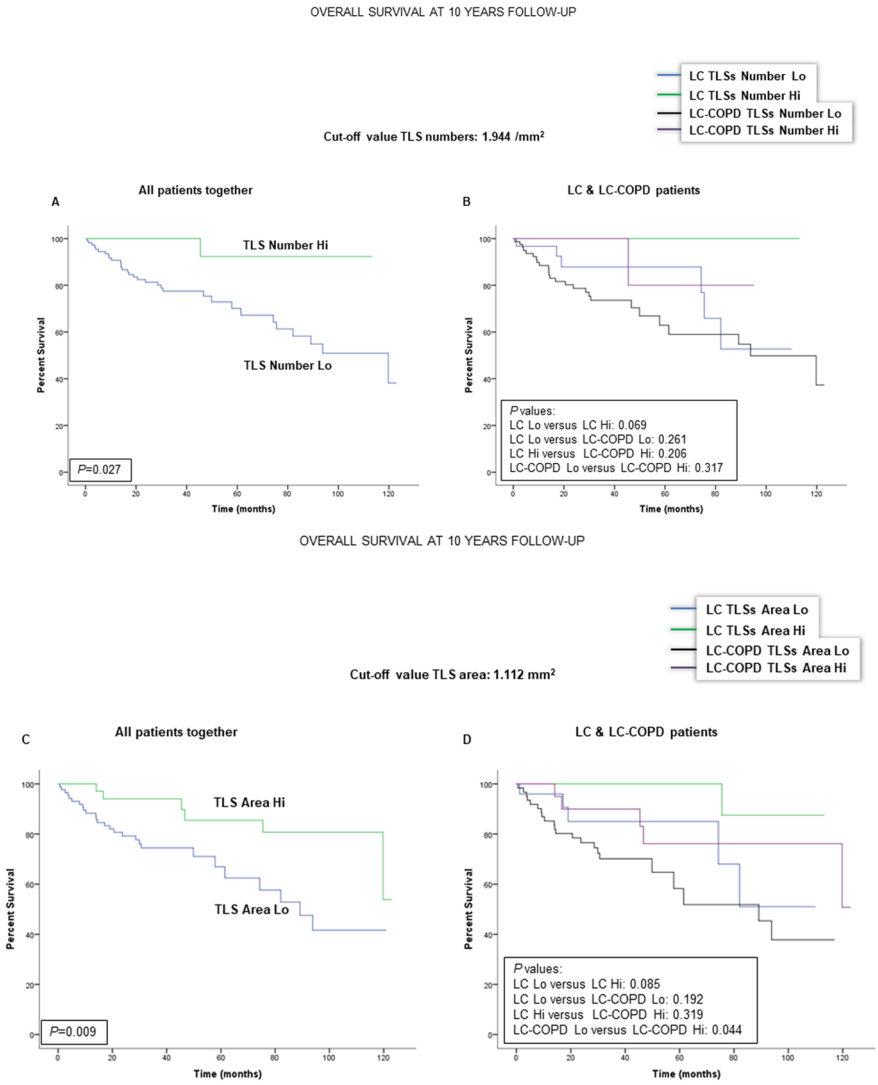

Figure 3. *Cont.*

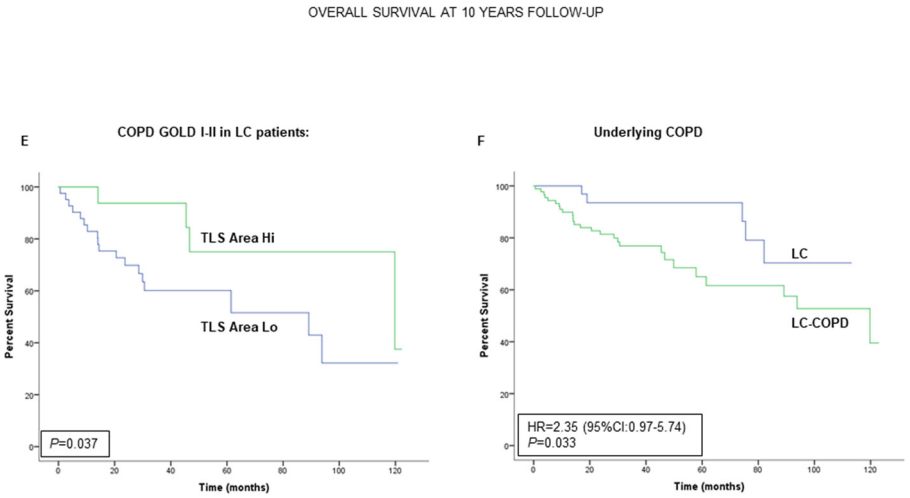

Figure 3. Kaplan-Meier survival curves of the two groups of patients according to TLS levels in tumors. (**A**) Kaplan-Meier survival curves for overall survival (OS) in all patients based on the cut-off value of the number of TLSs (above and below the cut-off value: 1.944/mm^2). Patients with lower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

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

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

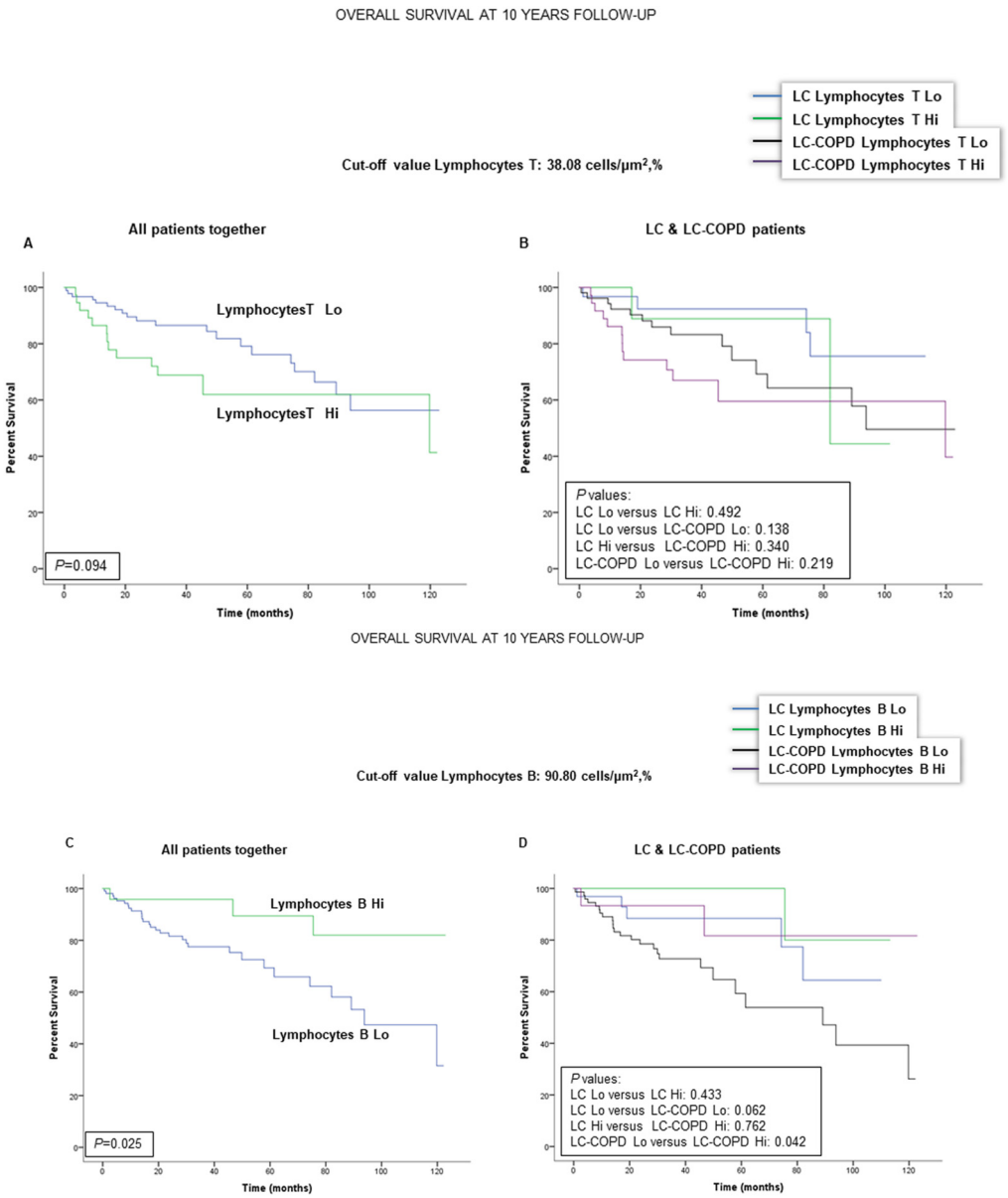

Figure 4. Kaplan-Meier survival curves of the two groups of patients according to T and B cell counts in tumors. (**A**) Kaplan-Meier survival curves for OS in all patients based on the percentage of T cell lymphocytes (above and below the cut-off value: 38.08 cells/µm^2, %). (**B**) Kaplan-Meier survival curves for OS in LC patients with and without COPD based on the percentage of T lymphocytes (above and below the cut-off value: 38.08 cells/µm^2, %). No significant differences were observed in any of the patient groups according to the cut-off value of the percentage of T cells within the TLSs. (**C**) Kaplan-Meier survival curves for OS in all patients based on the percentage of B cell lymphocytes (above and below the cut-off value: 90.80 cells/µm^2, %). Patients with lower proportions of B cells had a significantly worse survival. (**D**) Kaplan-Meier survival curves for OS in LC patients with and without COPD based on the percentage of B lymphocytes (above and below the cut-off value: 90.80 cells/µm^2, %). Smaller proportions of B cells were significantly associated with poorer survival among LC-COPD patients. Definition of abbreviations LC, lung cancer; COPD, chronic obstructive pulmonary disease; Hi, high level (above cut-off value); Lo, low level (below cut-off value).

3. Discussion

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

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

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

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

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

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

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

Study Limitations

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

4. Materials and Methods

4.1. Study Design and Ethics

This is a cross-sectional, prospective study designed following the World Medical Association guidelines (seventh revision of the Declaration of Helsinki, Fortaleza, Brazil, 2013) [36] for research on human beings and approved by the institutional Ethics Committee on Human Investigation (protocol # 2008/3390/I, 4 February, 2008, Hospital del Mar–Instituto Hospital del Mar de Investigaciones Médicas, Barcelona, Spain). All patients invited to participate in the study signed the written informed consent.

Patients were prospectively recruited from the Lung Cancer Clinic of the Lung Cancer Unit at Hospital del Mar (Barcelona, Spain). All of the patients were part of the Lung Cancer Mar Cohort that started in 2008. The last patients were enrolled in March 2018. For this observational study, 133 patients with LC were recruited. Candidates for tumor resection underwent pulmonary surgery prior to administration of any sort of adjuvant therapy. Specimens from the tumor and non-tumor lungs were collected from all the study subjects. Patients were further subdivided post-hoc into two groups on the basis of underlying COPD: 1) 90 patients with LC and COPD (LC-COPD group), and 2) 43 patients with LC without COPD (LC group).

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

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

4.2. Clinical Assessment

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

4.3. Sample Collection and Preservation

Lung samples were obtained from tumors and the surrounding non-tumor parenchyma following standard technical procedures during thoracotomy for the standard care in the treatment of lung tumors. In all patients, the expert pulmonary pathologist selected tumor and non-tumor lung specimens of approximately 10×10 mm^2 area from the fresh samples. Non-tumor specimens were collected as far distal to the tumor margins as possible (average >7 cm). Fragments of both tumor and non-tumor specimens were fixed in formalin and embedded in paraffin blocks until further use.

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

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

All procedures were conducted at room temperature. Hematoxylin counterstaining was performed for two minutes, and slides were mounted for conventional microscopy. Images were taken under a light microscope (Olympus, Series BX50F3, Olympus Optical Co., Hamburg, Germany) coupled with an image-digitizing camera (Pixera Studio, version 1.0.4, Pixera Corporation, Los Gatos, CA, USA). The number of cells and total area (μm^2) were measured in each of the lung specimens (both tumor and non-tumor samples) using the Image J software (National Institute of Health, Maryland, MD, USA).

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

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

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

4.5. Identification of GCs in TLSs of Lung Specimens

In a subgroup of patients (n = 61), the presence of GCs within the TLSs was also specifically evaluated in each lung tumor and non-tumor specimens on three-micrometer sections using hematoxylin and eosin staining by two independent observers [10,46,47]. Images of the stained lung sections (tumor and non-tumor) were captured with a light microscope (Olympus, Series BX50F3, Olympus Optical Co., Hamburg, Germany) coupled with an image-digitizing camera (Pixera Studio, version 1.0.4, Pixera Corporation, Los Gatos, CA, USA). GCs were selected by the presence of two separate topographic zones: 1) one dark-stained area, which was characterized by a dense population of lymphocytes, and 2) a light-stained area, which was characterized by a low-density lymphocyte site. Data are expressed as the number of GCs in all study groups of patients.

4.6. Statistical Analyses

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

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

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

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

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

5. Conclusions

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

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10.2.4 Publication 4

Title:

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

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

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

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

1. Introduction

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

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

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

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

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

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

2. Methods

2.1. Study Design and Ethics

This is a cross-sectional prospective study designed by following the World Medical Association guidelines (Seventh revision of the Declaration of Helsinki, Fortaleza, Brazil, 2013) [36] for research on human beings and approved by the institutional Ethics Committee on Human Investigation (protocol # 2008/3390/I, Hospital del Mar–IMIM, Barcelona, Spain). All patients invited to participate in the study signed the informed written consent.

Patients were prospectively recruited from the Lung Cancer Clinic of the Respiratory Medicine Department at *Hospital del Mar* (Barcelona, Spain). All the patients were part of the *Lung Cancer Mar Cohort*. For this observational study, 53 patients with LC were recruited during the years 2017–2019. Candidates for tumor resection underwent pulmonary surgery prior to administration of any sort of adjuvant therapy. LC diagnosis and staging were established by histological confirmation and classified according to currently available guidelines for the diagnosis and management of LC [37,38]. TNM

(tumor, node, and metastasis) staging was defined as stated in the eighth edition of the Lung Cancer Stage Classification [39]. COPD diagnosis was established as a post-bronchodilator forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) ≤ 0.7, which is not fully reversible by spirometry, according to currently available guidelines for diagnosis and management of COPD [5,40]. Exclusion criteria were as follows: small cell lung cancer (SCLC), chronic cardiovascular disease, restrictive lung disease, metabolic, immune disease, or clot system disorders, signs of severe inflammation and/or bronchial infection (bronchoscopy), current or recent invasive mechanical ventilation, or long-term oxygen therapy.

Specimens from the tumor and non-tumor lungs were collected from all the study subjects. Patients were further subdivided post hoc into two groups on the basis of underlying COPD: (1) 33 patients with LC and COPD (LC–COPD group) and (2) 20 patients with LC without COPD (LC group).

2.2. Clinical Assessment

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

2.3. Sample Collection and Preservation

Lung samples were obtained from tumors and the surrounding non-tumor parenchyma, following standard technical procedures during thoracotomy for the standard care in the treatment of lung tumors. In all patients, the expert pulmonary pathologist selected tumor and non-tumor lung specimens of approximately 10×10 mm^2 area from the fresh samples, as previously validated [7–9]. Non-tumor specimens were collected as far as possible from the distal to the tumor margins (average >7 cm). Fragments of both tumor and non-tumor specimens were fixed in formalin and embedded in paraffin blocks until further use. Another fragment was snap-frozen immediately in liquid nitrogen and stored at −80 °C for the quantification of the cytokine levels.

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

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

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

All procedures were conducted at room temperature. Hematoxylin counterstaining was performed for two minutes, and slides were mounted for conventional microscopy. Images were taken under a light microscope (Olympus, Series BX50F3, Olympus Optical Co., Hamburg, Germany) coupled with an image-digitizing camera (Pixera Studio, version 1.0.4, Pixera Corporation, Los Gatos, CA, USA). The number of cells and total area (μm^2) were measured in each of the lung specimens (both tumor and non-tumor samples), using Image J software (National Institute of Health, Maryland, MD, USA).

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

2.5. Identification of NK Cells in Lung Specimens

NK cells were identified in the tumor and non-tumor lung specimens on three-micrometer sections, using conventional immunohistochemical procedures as previously described [7]. Following deparaffinization, lung cross-sections were immersed in preheated antigen retrieval solution of ethylenediaminetetraacetic acid (EDTA, pH 8, Sigma-Aldrich, St. Louis, MO, USA), incubated at 95 °C for 20 min, and then cooled down to room temperature. Slides were washed over the following steps with phosphate buffer saline (PBS, Sigma-Aldrich). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min. In order to properly identify NK cells in the lung samples (tumor and non-tumor specimens), NKp46 receptor (encoded by the *ncr*1 gene) was measured by using a specific primary antibody, as also previously reported [42,43]. Thus, primary antibody incubation with anti-natural Cytotoxicity Triggering Receptor 1 (anti-NCR1 protein antibody, Abcam, Cambridge, UK) was performed for one hour. Slides were then incubated with biotinylated universal secondary antibody for 30 min, followed by another 30 min incubation with HRP-streptavidin and diaminobenzidine for five minutes (kit LSAB+HRP Dako Cytomation Inc., Carpinteria, CA, USA) as a substrate. Hematoxylin counterstaining was performed, and slides were dehydrated and mounted for conventional microscopy. Images of the stained lung sections (tumor and non-tumor) were captured with a light microscope (Olympus, Series BX50F3, Olympus Optical Co., Hamburg, Germany) coupled with an image-digitizing camera (Pixera Studio, version 1.0.4, Pixera Corporation, Los Gatos, CA, USA). In addition, NCR1-positively-stained cells were counted in the tumor and non-tumor lung specimens of all the patients. The area of the lungs in which NK cells were identified (μm^2) was also measured in both tumor and non-tumor specimens, using Image J software (National Institutes of Health, USA). Data are shown as the percentage of NK cells in the measured area in both tumor and non-tumor lung specimens (% NK cells/$\mu m^2 \times 100$).

2.6. Quantification of Cytokines in Lung Tissue

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

2.7. Statistical Analyses

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

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

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

Continuous variables are presented as mean and standard deviation, while categorical variables are presented as the number of patients in each group and the percentage in the study group with respect to the total population. Definition of abbreviations: N, number; kg, kilograms; m, meters; BMI, body mass index; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; DL_{CO}, carbon monoxide transfer; K_{CO}, Krogh transfer factor; GOLD: Global initiative for chronic Obstructive Lung Disease; NA, not applicable; TNM, tumor, nodes, metastasis; CRP, C-reactive protein; GSV, globular sedimentation velocity; L, liter. Statistical analyses and significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between lung cancer–Chronic Obstructive Pulmonary Disease (LC–COPD) patients and LC patients.

3. Results

3.1. Clinical Characteristics

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

number of pack/year was significantly greater in LC–COPD patients compared to LC patients, while the number of never smokers was significantly greater in the latter group (Table 1). The lung functional parameters FEV_1, FEV_1/FVC, DL_{CO}, and K_{CO} in LC–COPD patients were significantly lower than in LC patients (Table 1). Most of the patients with COPD were in GOLD I and II stages (90%). TMN staging or histological subtypes did not significantly differ between the two groups. The number of patients with adjuvant treatment following thoracotomy did not differ between the two study groups. In LC–COPD compared to LC patients, the levels of total leucocytes and neutrophils were significantly increased while levels of albumin significantly decreased. Total proteins, fibrinogen, C-reactive protein (CRP), globular sedimentation velocity (GSV), and body weight loss did not differ between LC–COPD and LC patients.

3.2. Treg and NK Cells in Lung Specimens

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

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

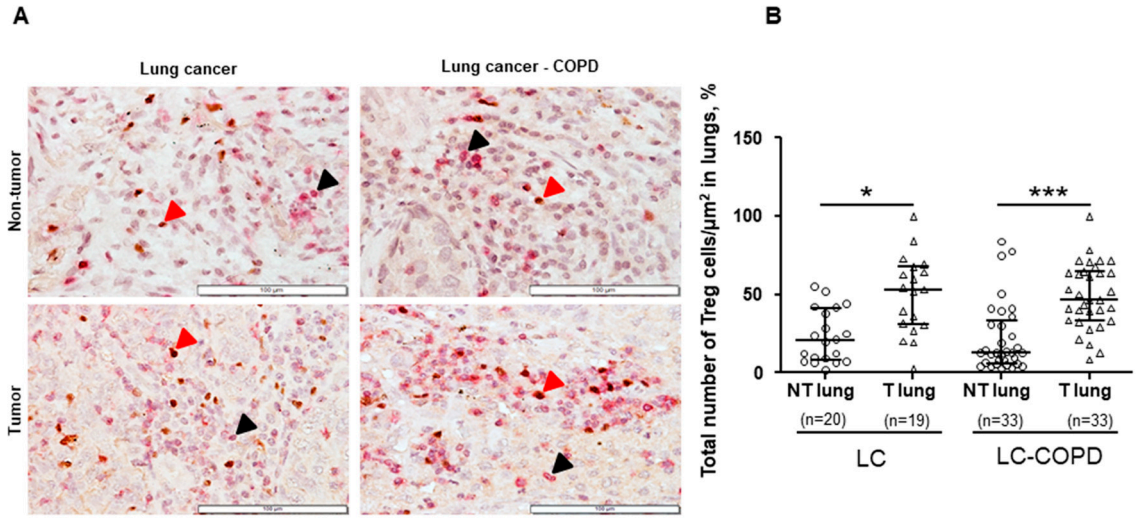

Figure 1. (**A**) Representative examples of double immunohistochemical staining for Treg cells (CD3-FOXP3 positively stained T cells) in LC and LC–COPD patients, respectively. All types of T cells (CD3+) are stained in only in red (black arrow), while Treg cells (CD3+-FOXP3+) are specifically stained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

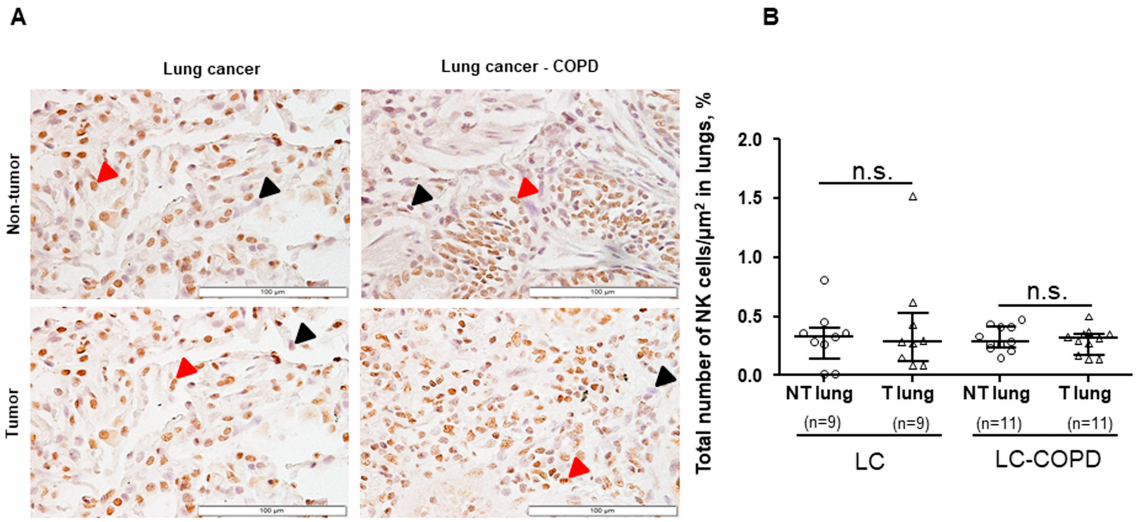

Figure 2. (**A**) Representative examples of double immunohistochemical staining for NK cells (NCR1+) in LC and LC–COPD patients, respectively. Black arrows point toward NK cells negatively stained in blue with hematoxylin, and red arrows point toward NK cells (NCR1+) stained in brown. (**B**) Median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

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

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

3.3. IgG and IgA Secreting Plasma Cells in Lung Specimens

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

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

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

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

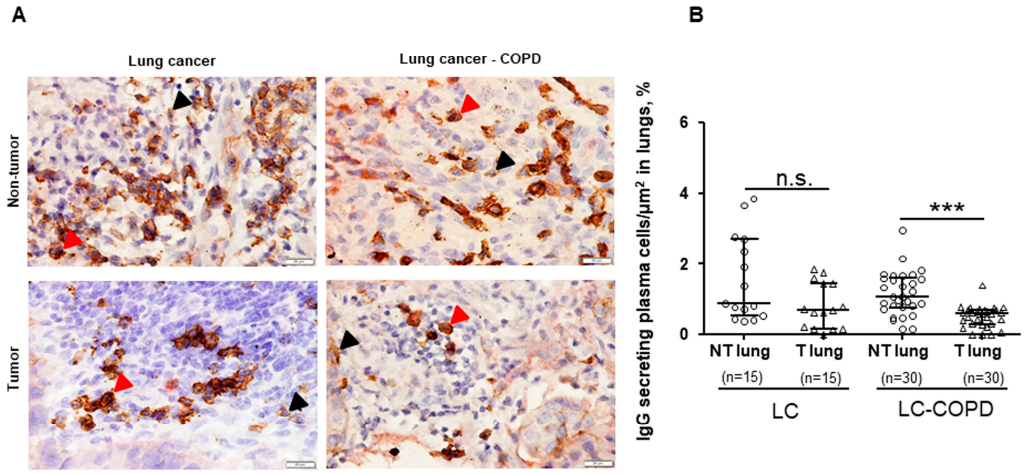

Figure 3. (**A**) Representative examples of double immunohistochemical staining for IgG-secreting plasma cells (CD138-IgG positively stained plasma cells) in LC and LC–COPD patients, respectively. All types of plasma cells (CD138+) are stained only in brown (black arrow), while IgG-secreting plasma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

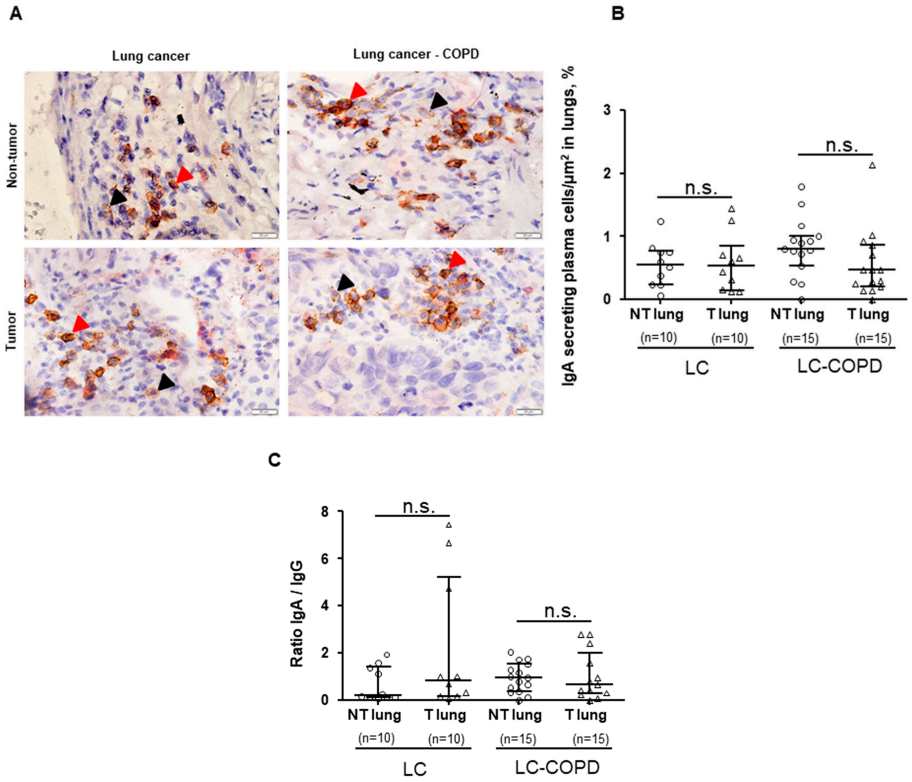

Figure 4. (**A**) Representative examples of double immunohistochemical staining for IgA-secreting plasma cells (CD138-IgA positively stained plasma cells) in LC and LC–COPD patients, respectively. All types of plasma cells (CD138+) are stained only in brown (black arrow), while IgA-secreting plasma cells (CD138+IgA+) are specifically stained with both brown and red. (**B**) Median and interquartile

ranges between 75th and 25th percentiles of number of IgG-secreting plasma cells in the total measured area. Black-stained regions within the lungs correspond to anthracosis. (**C**) Median and interquartile ranges between 75th and 25th percentiles of IgA/IgG ratio in LC and LC–COPD patients, respectively. Comparisons were made between the non-tumor (NT) and tumor (T) samples and the LC and LC–COPD groups. For technical reasons, the number of patients in each group or type of samples (tumor and non-tumor) may differ. Statistical significance: n.s. No significance between tumor and non-tumor lungs in either LC or LC–COPD patients. Definition of abbreviations: LC, lung cancer; COPD, chronic obstructive pulmonary disease; CD, cluster of differentiation; Ig, immunoglobulin.

3.4. Cytokines Levels in Lung Specimens

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

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

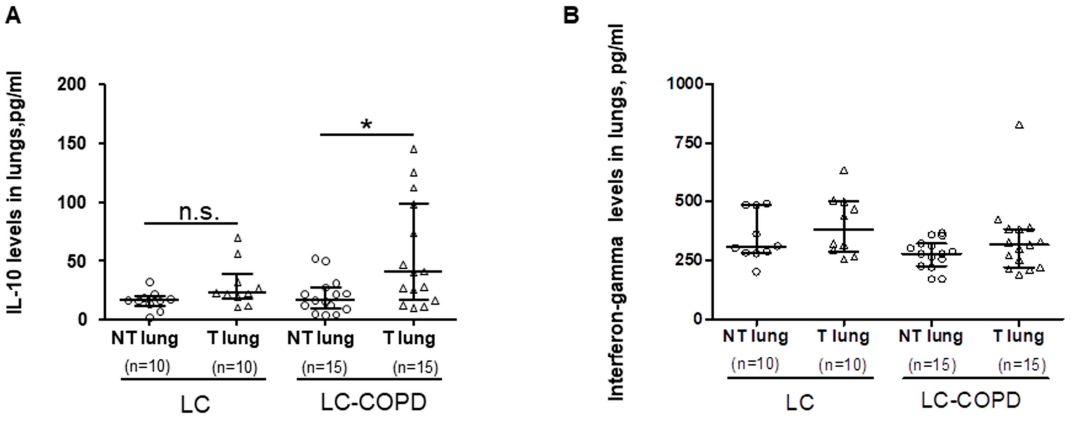

Figure 5. (**A**) Mean values and SD of number IL-10 levels by ELISA in LC and LC–COPD patients, respectively. (**B**) Median and interquartile ranges between 75th and 25th percentiles of number of interferon-gamma levels by ELISA in LC and LC–COPD patients, respectively. Comparisons were made between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

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

Levels of IL-10 significantly increased in the tumors compared to non-tumor specimens only in LC–COPD patients, whereas no significant differences were found in interferon-gamma levels between tumor and non-tumor lung specimens in either LC or LC–COPD patients (Figure 5A,B). A subanalysis conducted only in patients with lung adenocarcinoma revealed identical results for this set of experiments

4. Discussion

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

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

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

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

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

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

5. Conclusions

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

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

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10.3 Communications

10.3.1 Communication 1

Title:

Immunomodulation with monoclonal antibodies of PARP expression and activity in lung tumors of mice

Authors:

J Tang, J Yélamos López, C Ampurdanés, X Wang, **L Qin**, and **E Barreiro**

Journal:

Arch Bronconeumol 2019; 55 (Especial Congreso 2): 18.

Impact Factor :

4.872 (2020), Quartile: Q2

IMMUNOMODULATION WITH MONOCLONAL ANTIBODIES OF PARP EXPRESSION AND ACTIVITY IN LUNG TUMORS OF MICE

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Hospital del Mar, Barcelona, Spain.

Introduction: Poly-ADP ribose polymerase plays crucial roles in DNA repair and other cell functions. Immunomodulators have proven to reduce tumor burden. PARP activity may modify the immune system response. We hypothesized that immunomodulators may modify the expression and activity of PARP-1 and PARP-2 enzymes in lung tumors of mice. Objectives: To evaluate the selective expression of PARP-1 and PARP2 and PARP activity in lung tumors of mice treated with immunomodulators (anti-CD-137, anti-CTLA-4, anti-PD-1, and anti-CD-19). Methods: Three groups of wild-type BALB/C mice were established (N = 9/group): non-tumor control mice, tumor-bearing mice, and treated mice. Lung tumors (LP07 adenocarcinoma) were harvested from the lungs of mice treated with a cocktail of immunomodulators after one month. In lung tumor and non-tumor specimens, PARP-1 and PARP-2 expression (immunoblotting) and active PARP polymers (immunohistochemistry) were assessed with selective monoclonal antibodies in all mice. Results: 1) PARP expression and activity in tumors compared to non-tumor lungs: Whereas PARP-1 expression was significantly lower in the tumors (both treated and non-treated mice) than in the non-tumor lung specimens, PARP-2 did not significantly differ among the groups. PARP activity did not significantly differ between tumors and non-tumor lungs. 2) PARP expression and activity in tumors between treated and non-treated mice: Whereas PARP-1 and PARP-2 expression did not significantly differ between tumors specimens in either treated or non-treated mice, immunomodulators elicited a significant rise in PARP activity in tumors of treated animals compared to non-treated mice. Conclusions: In lung tumors of BALB/C mice, PARP-1 and PARP-2 were differentially expressed compared to non-tumor lungs. Treatment with the immunomodulators induced a rise in PARP activity in the tumors of the treated mice. These results suggest that PARP may have interfered with the immune profile of the tumor microenvironment. Future studies should be devoted to elucidating the specific biological mechanisms of PARP in response to immunomodulators.

10.3.2 Communication 2

Title:

Immunological Events and Survival in Lung Cancer Patients with COPD

Authors:

E. Barreiro, J. Tang, X Wang, **Q Liyun**, V Curull, M. Mateu-Jiménez, D. Ramis-Cabrer, L Pijuan, X Duran, A Rodríguez-Fuster, R Aguiló

Journal:

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

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PMID: 32877328

Impact Factor:

21.405 (2020), Quartile: Q1

Immunological Events and Survival in Lung Cancer Patients with COPD

E. Barreiro[1], J. Tang[1], X. Wang[1], Q. Liyun[1], V. Curull[2], M. Mateu-Jimenez[1], D. Ramis-Cabrer[1], L. Pijuan[3], X. Duran[4], A. Rodriguez-Fuster[5], R. Aguilo[5]; [1]Pulmonology Department-Muscle and Lung Cancer Research Group, IMIM-Hospital Del Mar, Barcelona, Spain, [2]Hosp Del Mar, Barcelona, Spain, [3]Pathology Department, IMIM-Hospital Del Mar, Barcelona, Spain, [4]Scientific and Technical Department, IMIM-Hospital Del Mar, BARCELONA, Spain, [5]Thoracic Surgery-Muscle and Lung Cancer Research Group, IMIM-Hospital Del Mar, BARCELONA, Spain.

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

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

Am J Respir Crit Care Med 2020;201:A7566

Internet address: www.atsjournals.org Online Abstracts Issue

10.3.3 Communication 3

Title:

Immunological Events and Survival in Lung Cancer Patients with COPD

Authors:

J Tang, X Wang, **L Qin**, V Curull, M Mateu-Jiménez, D Ramis-Cabrer, L Pijuan, X Duran, A Rodríguez-Fuster, R Aguiló Espases, and **E Barreiro**.

Journal:

Eur Respir J 2020; 56: 1650; DOI**:** 10.1183/13993003.congress-2020.1650.

Impact Factor:

16.671 (2020), Quartile: Q1

Immunological Events and Survival in Lung Cancer Patients with COPD

Jun Tang, Xuejie Wang, Liyun Qin, Victor Curull, Merce Mateu-Jiménez, Daniel Ramis-Cabrer, Lara Pijuan, Alberto Rodríguez-Fuster, Rafael Aguiló, Esther Barreiro Portela.

European Respiratory Journal 2020 56: 1650; DOI: 10.1183/13993003.congress-2020.1650

Abstract

Immune microenvironment plays a role in the development of lung cancer (LC). We hypothesized that immune profile of B and T cells may differ in tumors of LC patients with and without COPD and may also influence survival.

Objectives: 1) To analyze levels of tertiary lymphoid structures (TLSs), B and T cells in tumor and non-tumor (control samples) lung specimens of LC patients with/without COPD and 2) to analyze the influence of those biological markers in the patients´10-year survival. TLSs (numbers and area), B (CD20), and T (CD3) cells were identified in both tumor and non-tumor specimens (thoracotomy) from 90 LC-COPD patients and 43 LC-only patients (immunohistochemistry). Survival was analyzed in all 133 patients. Immune profile in tumors of LC-COPD versus LC: The number of TLSs significantly decreased in tumors of LC-COPD compared to LC patients. No significant differences were observed in tumors between LC-COPD and LC for B or T cells. In tumors compared to non-tumor specimens, a significant rise in TLSs was observed in LC (numbers and area) and LC-COPD (area), T cell counts declined in tumors of LC, while B cell counts increased in tumors of both LC and LC-COPD patients.

Survival: In LC-COPD patients: lower numbers of TLSs and greater numbers of B cells were associated with worse survival. In LC patients: lower levels of T cells were associated with longer survival rates. TLSs, B cells and T cells are differentially expressed in tumors of LC-COPD from that in LC-only patients.

Method: FIS 18/00075 & CIBERES (FEDER, ISC-III), SEPAR 2018, unrestricted research grant from Menarini SA 2018.

10.3.4 Communication 4

Title:

Skeletal muscle dysfunction and body composition alterations in non-cystic fibrosis bronchiectasis patients: gender differences

Authors:

X Wang, A Balaña-Corberó, J Martínez-Llorens, **L Qin**, M Admetlló, E Hernández-Leal, X Duran, A Sancho-Muñoz, and **E Barreiro**

Journal:

European Respiratory Journal 2020; 56: 1836; DOI**:** 10.1183/13993003.congress-2020.1836.

Impact Factor:

16.671 (2020), Quartile: Q1

Skeletal Muscle Dysfunction and Body Composition Alterations in Non-Cystic Fibrosis Bronchiectasis Patients: Gender Differences

Xuejie Wang, Ana Balañá Corberó, Juana Martínez Llorens, Liyun Qin, Mireia AdmetIIó, Esmeralda Hernández Leal, Xavier Duran, Antonio Sancho Muñoz, Esther Barreiro Portela

European Respiratory Journal 2020 56:1836;DIO: 10.1183/13993003.congress-2020.1836

Abstract

Muscle dysfunction and nutritional abnormalities are common manifestations in chronic respiratory diseases. Whether patients with non-cystic fibrosis bronchiectasis (NCFB) may experience alterations in muscle mass and performance remains unanswered.

Objectives: To assess muscle mass, function, nutritional parameters, and exercise tolerance in NCFB patients. Body weight and composition (muscle mass, bioimpedance), blood nutritional parameters, lung function (spirometry, lung volumes, and diffusion capacity, muscle function (dynamometry, upper and lower limb muscle groups, handgrip and QMVC, respectively), and exercise capacity were evaluated in 101 NCFB patients that were prospectively and consecutively recruited in the Bronchiectasis Multidisciplinary Unit at Hospital del Mar (years 2018-2019, Barcelona). Seventy-seven female patients were recruited (24 males). All patients had mild-to-moderate airway obstruction, air trapping, and reduced diffusion capacity. Compared to male patients, female patients were 10 years older, muscle mass was lower, fatty tissue was greater, muscle strength of both upper and lower limbs was significantly reduced, and exercise capacity was decreased. When patients were subdivided according to disease severity (FACED, females and males), patients with greater FACED scores exhibited a significant reduction in body weight, muscle strength (upper and lower extremities), exercise capacity, and lung function. Nutritional abnormalities and reduced muscle mass and function are very prominent in female NCFB patients, especially in the more severe patients.
Method: CIBERES (ISCIII), unrestricted grant from Menarini SA, and SEPAR 2018.

10.3.5 Communication 5

Title:

Alteraciones musculares y nutricionales en pacientes con bronquiectasias no fibrosis quística: diferencias entre hombres y mujeres

Authors: X. Wang Wang, A. Balañá Corberó, J. Martínez Llorens, N. Rovira Ribalta, **L. Qin Qin**, J. Tang Tang, M. Ademetlló Papiol, X. Durán Jordà, A. Sancho Muñoz y **E. Barreiro Portela**

Journal:

Arch Bronconeumol 2020; 56 (Especial Congreso 1): 322.

Impact Factor:

4.872 (2020), Quartile: Q2

ALTERACIONES MUSCULARES Y NUTRICIONALES EN PACIENTES CON BRONQUIECTASIAS NO FIBROSIS QUÍSTICA: DIFERENCIAS ENTRE HOMBRES Y MUJERES

X. Wang Wang[1], A. Balañá Corberó[1], J. Martínez Llorens[1], N. Rovira Ribalta[1], L. Qin Qin[1], J. Tang Tang[1], M. Ademetlló Papiol[1], X. Durán Jordà[2], A. Sancho Muñoz[1] y E. Barreiro Portela[1] [1]Servicio de Neumología, Grupo de Investigación en Desgaste Muscular y Caquexia en Enfermedades Crónicas Respiratorias y Cáncer de Pulmón, Hospital del Mar-IMIM, CIBERES, UAB, UPF, Barcelona, España. [2]Servicios Científico-Técnicos, Hospital del Mar-IMIM, CIBERES, UAB, UPF, Barcelona, España.

Introducción: La disfunción muscular y las alteraciones nutricionales, que son frecuentes en las enfermedades crónicas respiratorias, inciden negativamente en la calidad de vida de los pacientes. Nuestra hipótesis fue demostrar la existencia de alteraciones nutricionales y musculares en pacientes con bronquiectasias no fibrosis quística (FQ) y evaluar las diferencias entre mujeres y hombres.

Material y métodos: En una cohorte (años 2018-2019) de pacientes con bronquiectasias no FQ (77 mujeres) procedentes del dispensario monográfico del Hospital del Mar se evaluaron los siguientes parámetros: peso y composición corporal, fuerza de los músculos de las extremidades superiores e inferiores (dinamometría específica), test de marcha 6 minutos, función pulmonar (espirometría, difusión y volúmenes) y parámetros analíticos de inflamación sistémica y nutricionales.

Resultados: Todos los pacientes presentaron una obstrucción leve-moderada al flujo aéreo, atrapamiento aéreo y alteraciones en la difusión. Respecto de los pacientes varones, las mujeres eran 10 años mayores, el índice de masa magra (FFMI, kg/m2) fue menor, contenido graso fue mayor, la fuerza muscular de las extremidades superiores e inferiores fue menor (50% reducción) y la capacidad de ejercicio fue también menor. La subdivisión de los pacientes según los criterios de gravedad (FACED score) para ambos sexos, aquellos con los índices más elevados, presentaron una mayor disminución de su peso corporal, fuerza muscular de ambos grupos de extremidades, capacidad de ejercicio y de su función pulmonar.

Conclusiones: La disfunción muscular y las alteraciones nutricionales son frecuentes e importantes en las mujeres con bronquiectasias no FQ respecto de los varones. Dichas alteraciones resultaron más evidentes en los pacientes más graves según el índice FACED. Estos hallazgos y sus causas deberán estudiarse más a fondo en estudios posteriores. Financiado por CIBERES (ISC-III) y SEPAR 2018.

10.3.6 Communication 6

Title:

Perfil inmunológico y supervivencia en pacientes con càncer de pulmón: papel de la EPOC

Authors:

J Tang, X Wang, **L Qin**, V Curull, M Mateu-Jiménez, D Ramis-Cabrer, L Pijuan, X Duran, A Rodríguez-Fuster, R Aguiló Espases, **E Barreiro**

Journal:

Arch Bronconeumol 2020; 56 (Especial Congreso 1): 245.

Impact Factor:

4.872 (2020), Quartile: Q2

PERFIL INMUNOLÓGICO Y SUPERVIVENCIA EN PACIENTES CON CÁNCER DE PULMÓN: PAPEL DE LA EPOC

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Introducción: El microambiente inmunológico juega un papel importante en el desarrollo del cáncer de pulmón (CP). El perfil inmunológico (células B y T) en tumores pulmonares puede diferir entre pacientes con y sin EPOC, y puede influir en su supervivencia. Nuestra hipótesis fue la de demostrar que las estructuras terciarias linfoides (ETLs) y su composición celular (B y T) difieren en los tumores de los pacientes con y sin EPOC, así como en su supervivencia (10 años seguimiento).

Métodos: En biopsias tumorales y no tumorales (toracotomía) de pacientes con EPOC (CP-EPOC, N = 90) y sin EPOC (CP, N = 43) se cuan-tificaron los parámetros: número y área de ETLs y células B y T (doble tinción inmunohistoquímica). Los pacientes fueron clínicamente evaluados y se evaluó la supervivencia a los 10 años.

Resultados: Perfil inmune en los tumores de CP-EPOC versus CP: El número de ETLs fue menor en los tumores de CP-EPOC que en pacientes CP, sin hallarse diferencias estadísticamente significativas entre ambos grupos de enfermos para los contajes de células B o T. En los tumores versus no tumor, se observó un aumento significativo de ETLs en pacientes CP (número y área) y en pacientes CP-EPOC (área). Supervivencia: En pacientes CP-EPOC: un menor número de ETLs y un mayor número de células B, se asoció a mejor supervivencia. En pacientes CP: un menor número de células T resultó en una mejor supervivencia. Todos los pacientes juntos: un número menor de células T y de ETLs y un número mayor de células B se asociaron a una mejor supervivencia.

Conclusiones: En los tumores de pacientes CP-EPOC versus CP, se encontró una expresión diferenciada de células B yT y de ETLs, cuyos papeles específicos en la tumorigénesis deberán ser analizados en el futuro.

Financiado por FIS 18/00075 & CIBERES (FEDER, ISC-III), SEPAR 2018, Menarini SA 2018.

10.3.7 Communication 7

Title:

Systemic inflammation in patients with stable non-cystic fibrosis bronchiectasis

Authors:

L. Qin, M. Guitart, M. Alvarado-Miranda, X. Wang and **E. Barreiro**

Journal:

Arch Bronconeumol 2020; 56 (Supl Congr 2): 27

Impact Factor:

4.872 (2020), Quartile: Q2

SYSTEMIC INFLAMMATION IN PATIENTS WITH STABLE NON-CYSTIC FIBROSIS BRONCHIECTASIS

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Introduction: Non-cystic fibrosis bronchiectasis is a chronic airway disease with various etiologies and severities. Systemic inflammation is involved in the pathophysiology during acute exacerbations. Whether systemic inflammation is predominant in patients with stable non-CF bronchiectasis remains to be elucidated. Objectives: We aimed to evaluate whether systemic inflammation takes place in patients with stable non-CF bronchiectasis with no acute exacerbation within at least three months prior to study entry.

Methods: This was a prospective investigation in which 30 patients with clinically stable non-CF bronchiectasis and 20 age- and sex-matched healthy controls were recruited. Systemic inflammatory parameters: CRP, GSV, fibrinogen, ceruloplasmin, alpha-1 antitrypsin, IgA, and IgG; blood nutritional parameters: albumin and prealbumin; and baseline lung function: spirometry, lung volumes, and diffusion capacity were assessed in both patients and healthy controls. Sputum samples were collected in patients with bronchiectasis for microorganism culture. Statistical differences of all the study variables between the two groups were explored using the Student´s t-test. Correlations between physiological/clinical and biological variables were examined using Pearson's parametric test.

Results: In patients with stable non-CF bronchiectasis compared to the healthy controls, patients with non-CF bronchiectasis, exhibited mild-to-moderate airway obstruction, systemic levels of CRP, GSV, fibrinogen, ceruloplasmin, IgA and IgG were significantly greater, while those of albumin and prealbumin were significantly lower in the patients. Levels of ceruloplasmin were shown to positively correlate with systemic markers such as CRP, GSV, and alpha-1 antitrypsin, and to negatively correlate with albumin. Prealbumin levels were positively correlated with lung function parameters, while IgA and IgG were negatively associated with FEV1. Pseudomonas aeruginosa colonization was identified in 36% of the patients in this series.

Conclusions: In patients with stable non-CF bronchiectasis, systemic inflammation takes place independently of previous acute exacerbations. Funding: FIS18/00075(FEDER), CIBERES, unrestricted grant from Menarini SA, and SEPAR 2020

10.3.8 Communication 8

Title:

Skeletal muscle dysfunction and body composition alterations in non-cystic fibrosis bronchiectasis patients: gender differences

Authors:

X Wang, A Balaña-Corberó, J Martínez-Llorens, **L Qin**, M Admetlló, E Hernández-Leal, X Duran, A Sancho-Muñoz, **E Barreiro**.

Journal:

Arch Bronconeumol 2020; 56 (Supl Congr 2): 25

Impact Factor:

4.872 (2020), Quartile: Q2

SKELETAL MUSCLE DYSFUNCTION AND BODY COMPOSITION ALTERATIONS IN NON-CYSTIC FIBROSIS BRONCHIECTASIS PATIENTS: GENDER DIFFERENCES

X. Wang[1], A. Balaña-Corberó[1], J. Martínez-Llorens[1], L. Qin[1], M. Admetlló[1], E. Hernández Leal[2], X. Duran[3], A. Sancho-Muñoz[1]and E. Barreiro[1] [1]Pulmonology Department, Scientific and Technical Department, Hospital del Mar-IMIM, CIBERES, UAB, UPF, Barcelona, Spain. [2]Hospital del Mar, Barcelona, Spain. [3]Scientific, Statistics, and Technical Department, Hospital del Mar-IMIM, Mar Health Park, Barcelona, Spain.

Introduction: Muscle dysfunction and nutritional abnormalities are common manifestations in chronic respiratory diseases. Whether patients with non-cystic fibrosis bronchiectasis (NCFB) may experience alterations in muscle mass and performance remains unanswered. Objectives: To assess muscle mass, function, nutritional parameters, and exercise tolerance in NCFB patients. Methods: Body weight and composition (muscle mass, bioimpedance), blood nutritional parameters, lung function (spirometry, lung volumes, and diffusion capacity), peripheral muscle function (dynamometry, upper and lower limb muscle groups, handgrip and QMVC, respectively), respiratory muscle function, and exercise capacity were evaluated in 131 NCFB patients that were prospectively and consecutively recruited in the Bronchiectasis Multidisciplinary Unit at Hospital del Mar (years 2018-2020, Barcelona).

Results: Ninety-eight female patients were recruited (33 males). All patients had mild-to-moderate airway obstruction and air trapping. Compared to male patients, female patients were older, muscle strength of both upper and lower limbs was significantly reduced, and exercise capacity was decreased. When patients were subdivided according to disease severity (FACED, females and males), patients with greater FACED scores exhibited a significant reduction in body weight, muscle strength (upper and lower extremities), exercise capacity, and lung function.

Conclusions: Nutritional abnormalities and reduced muscle function are very prominent in female NCFB patients as opposed to males. Moreover, these features were especially present in the more severe patients according to FACED scores. The study of the systemic manifestations in patients with bronchiectasis warrant further attention in the near-future. Funding: CIBERES (ISCIII), unrestricted grant from Menarini SA, and SEPAR 2020

10.3.9 Communication 9

Title:

PARP activity and expression in response to DNA damage in mice with lung cancer-induced cachexia

Authors:

J. Tang, X. Wang, **L. Qin**, C. Ampurdanés, X. Duran, L. Pijuan, J. Yélamos, **E. Barreiro**

Journal:

Eur Respir J 2021; 58: PA1117; DOI: 10.1183/13993003.congress-2021.

Impact Factor:

16.671 (2020), Quartile: Q1

PARP activity and expression in response to DNA damage in mice with lung cancer-induced cachexia

JUN TANG[1], Xuejie Wang[1], Liyun Qin[1], Coral Ampurdanés[2], Xavier Duran[3], Lara Pijuan[4], José Yélamos[5], Esther Barreiro[1] [1]Pulmonology Department, Lung Cancer and Muscle Research Group, Hospital del Mar-IMIM, Parc de Salut Mar, Health and Experimental Sciences Department (CEXS), Universitat Pompeu Fabra (UPF), Department of Medicine, Universitat Autònoma de Barcelona, Parc de Recerca Biomèdica de Barcelona (PRBB), Barcelona, Barcelona, Spain. [2] Cancer Research Program, Hospital del Mar Medical Research Institute (IMIM)-Hospital del Mar, Barcelona, Barcelona, Spain. [3]Scientific, Statistics, and Technical Department, Hospital del Mar-IMIM, Parc de Salut Mar, Barcelona, Barcelona, Spain. [4]Pathology Department, Hospital del Mar-IMIM, Parc de Salut Mar, Barcelona, Barcelona, Spain. [5]Cancer Research Program, Pathology Department, Hospital del Mar Medical Research Institute (IMIM)-Hospital del Mar, Barcelona, Spain.

Abstract
Lung cancer (LC) is a major leading cause of death worldwide and cachexia is a frequent comorbidity. Poly (ADP ribose) polymerases (PARP)-1 and PARP-2 are important players in cancer regulating chromatin structure. PARP activity is also involved in muscle mass maintenance and metabolism. We hypothesized that in a mouse preclinical model of LC-induced cachexia, DNA damage is increased along with PARP activity and expression. We aimed to evaluate: 1) body weight and muscle function, 2) DNA damage, 3) PARP activity levels and 4) PARP-1 and PARP-2 protein expression in lung tumor samples of BALB/c cachectic mice. Lung tumors (subcutaneous inoculation of 4×105 LP07 adenocarcinoma cells) were harvested from the lungs of wild-type BALB/c mice: non-tumor control mice and tumor-bearing mice (N=9/group). DNA damage (immunohistochemistry), PARP activity (PARP colorimetric assay kit assay), and PARP-1 and PARP-2 expression (immunoblotting) were identified in lung tumor samples, and body and muscle function were also assessed. Compared to non-tumor control lungs, in lung tumor samples, DNA damage increased, while PARP activity and PARP-1 protein expression decreased. Limb muscle function significantly impaired in lung cancer cachexia mice. In lung tumors of wild type mice in this experimental model of LC cachexia, PARP activity along with PARP-1 expression decreased, while greater DNA damage levels were observed. These results have potential clinical implications as PARP inhibitors are currently in use for the treatment of breast and ovarian cancer types, in which cachexia and muscle dysfunction are very prominent.

Funding: FIS 18/00075 (FEDER, ISC-III), CIBERES (ISC-III), SEPAR 2018 & 2020.

10.3.10 Communication 10

Title:

Systemic inflammation in patients with stable non-cystic fibrosis bronchiectasis

Authors:

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SYSTEMIC INFLAMMATION IN PATIENTS WITH STABLE NON-CYSTIC FIBROSIS BRONCHIECTASIS

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Background: Non-cystic fibrosis bronchiectasis is a chronic airway disease with various etiologies and severities. Systemic inflammation is involved in the pathophysiology during acute exacerbations. Whether systemic inflammation is predominant in patients with stable non-CF bronchiectasis remains to be elucidated.

Objectives: We aimed to evaluate whether systemic inflammation takes place in patients with stable non-CF bronchiectasis with no acute exacerbation within at least three months before study entry.

Methods: This was a prospective investigation in which 30 patients with clinically stable non-CF bronchiectasis and 26 age- and sex-matched healthy controls were recruited. Systemic inflammatory parameters: CRP, GSV, fibrinogen, ceruloplasmin, alpha-1 antitrypsin, IgA, and IgG; blood nutritional parameters: albumin and prealbumin; and baseline lung function: spirometry and diffusion capacity were assessed in both patients and healthy controls.

Results: Compared to the healthy controls, patients with non-CF bronchiectasis exhibited mild-to-moderate airway obstruction, systemic levels of CRP, GSV, fibrinogen, ceruloplasmin, IgA and IgG were significantly greater, while those of albumin and prealbumin were markedly lower. Levels of ceruloplasmin were shown to positively correlate with systemic markers such as CRP, GSV and alpha-1 antitrypsin, and to negatively correlate with albumin. Prealbumin levels were positively correlated with lung function parameters, while IgA and IgG were negatively associated with FEV1.

Conclusions: In patients with stable non-CF bronchiectasis, systemic inflammation takes place independently of previous acute exacerbations.

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10.3.11 Communication 11

Title:

Loss of muscle mass and function and nutritional abnormalities in patients with bronchiectasis

Authors:

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LOSS OF MUSCLE MASS AND FUNCTION AND NUTRITIONAL ABNORMALITIES IN PATIENTS WITH BRONCHIECTASIS

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Background: Muscle dysfunction and nutritional abnormalities are common manifestations in chronic respiratory diseases. Whether patients with non-cystic fibrosis bronchiectasis (NCFB) may experience alterations in muscle mass and performance remains unanswered.

Objectives: To assess muscle mass, function, nutritional parameters and exercise tolerance in NCFB patients.

Methods: Body weight and composition (muscle mass, bioimpedance), blood nutritional parameters, lung function (spirometry, and diffusion capacity), peripheral and respiratory muscle function and exercise capacity were evaluated in 142 NCFB patients that were prospectively and consecutively recruited in the Bronchiectasis Multidisciplinary Unit at Hospital del Mar (years 2018-2021, Barcelona).

Results: One hundred and six female patients were recruited (36 males). All patients had mild-to-moderate airway obstruction, air trapping, and reduced diffusion capacity. Compared to male patients, female patients were older, muscle mass was lower, fatty tissue was greater, creatinine and prealbumin were lower, muscle strength of both upper and lower limbs was significantly reduced, and exercise capacity was decreased. When patients were subdivided according to disease severity (FACED, females and males), patients with greater FACED scores exhibited a significant reduction in body weight, muscle strength (upper and lower extremities), exercise capacity and lung function.

Conclusions: Nutritional abnormalities and reduced muscle function are very prominent in female NCFB patients as opposed to males. Moreover, these features were especially present in the more severe patients according to FACED scores.

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10.3.12 Communication 12

Title:

Inflamación sistémica en pacientes estables con bronquiectasias no fibrosis quística

Authors:

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INFLAMACIÓN SISTÉMICA EN PACIENTES ESTABLES CON BRONQUIECTASIAS NO FIBROSIS QUÍSTICA

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Introducción: Las bronquiectasias no debidas a fibrosis quística (FQ) son una enfermedad crónica de las vías respiratorias con diversas etiologías y gravedades. La inflamación sistémica está involucrada en la fisiopatología de las bronquiectasias durante las exacerbaciones agudas. Queda por dilucidar si la inflamación sistémica es predominante en pacientes estables con bronquiectasias no debidas a FQ. Nuestro objetivo fue evaluar si existe inflamación sistémica en pacientes estables con bronquiectasias no debidas a FQ sin exacerbación aguda durante al menos tres meses antes del reclutamiento al estudio. Material y métodos: Se reclutaron 30 pacientes clínicamente estables con bronquiectasias no debidas a FQ y 26 controles sanos de la misma edad y sexo. Parámetros inflamatorios sistémicos: PCR, VSG, fibrinógeno, ceruloplasmina, alfa-1 antitripsina, IgA e IgG; parámetros nutricionales: albúmina y prealbúmina; y función pulmonar basal: se evaluaron la espirometría, los volúmenes pulmonares y la capacidad de difusión tanto en pacientes como en controles sanos. Se recogieron muestras de esputo en pacientes con bronquiectasias para cultivo de microorganismos. Las diferencias estadísticas de todas las variables entre los dos grupos se exploraron mediante la prueba t de Student. Se analizaron las correlaciones entre las variables fisiológicas/clínicas y biológicas mediante coeficiente de correlación de Pearson.

Resultados: En comparación con los controles sanos, los pacientes estables con bronquiectasias no debidas a FQ presentaban obstrucciones leves a moderadas de las vías respiratorias, los niveles sistémicos de PCR, VSG, fibrinógeno, ceruloplasmina, IgA e IgG se encontraban significativamente aumentados, mientras que los de albúmina y prealbúmina se encontraban significativamente disminuidos en los pacientes. Se demostraron que los niveles de ceruloplasmina se correlacionaban positivamente con los marcadores sistémicos como PCR, VSG y alfa-1 antitripsina, y se correlacionaban negativamente con la albúmina. Los niveles de prealbúmina se correlacionaron positivamente con los parámetros de función pulmonar, mientras que IgA e IgG se asociaron negativamente con el FEV1. Se identificó que el 30% de los pacientes tuvieron colonización por Pseudomonas aeruginosa.

Conclusiones: En pacientes con bronquiectasias estables no a debidas FQ, la inflamación sistémica se produce independientemente de las exacerbaciones agudas previas.

10.4 Finding sources

The two studies included in the current thesis have been founded by:
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