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PhD THESIS

BRONCHOALVEOLAR LAVAGE: A TOOL FOR STUDYING INFLAMMATION AND THE MICROBIOME IN RESPIRATORY PATHOLOGY

– A B S T R A C T –

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INTRODUCTION

The global incidence of lung cancer diagnoses and fatalities due to the disease is on the rise. It stands as the foremost cause of cancer-related death in both men and women, and it holds the highest prevalence among all types of cancer. Despite the availability of numerous therapeutic approaches, the five-year survival rate for lung cancer patients hovers around 15%. A significant proportion of lung cancer cases are detected at an advanced stage, contributing to a bleak long-term prognosis, primarily because there is a shortage of noninvasive clinical tests for early screening and diagnosis. Consequently, it becomes imperative to identify specific biomarkers to expedite accurate diagnosis.

Several studies have highlighted the substantial role played by chronic inflammation in the carcinogenesis process. This holds true not only in the early stages of malignancy but also in later phases, including malignant conversion, invasion, and metastasis. Both the innate and acquired immune responses underscore the functional link between inflammation and cancer. Inflammatory cells and tumor cells alike release cytokines and chemokines, proteins that play pivotal roles in enhancing cellular activity and the humoral system.

Inflammatory cytokines such as IFN-gamma, IL-1b, IL-2, IL-4, IL-6, IL-10, IL-12p70, and TNF-alpha have been shown to play roles in the immune response associated with the progression of cancer.

Given the intricate interplay between lung cancer and the body's inflammatory and immune responses, there is significant potential in exploring specific cytokines present in bronchoalveolar lavage fluid (BALF). Consequently, this study's primary goal is to evaluate the diagnostic and prognostic significance of selected cytokines in BALF. Through the analysis of their presence and concentrations, our aim is to determine whether these particular cytokines can reliably serve as indicators of the presence, stage, or progression of lung cancer.

The lung microbiota constitutes a intricate ecosystem encompassing various microorganisms, including bacteria, fungi, and viruses. These microorganisms are believed to influence pulmonary homeostasis and contribute to pathological processes. Dysbiosis, characterized by an imbalance in the composition of the lung microbiota, has been implicated in the onset and progression of diverse pulmonary

conditions, encompassing lung cancer and COPD. Exploring holdronchial microbiota in individuals afflicted with lung cancer and benign pulmonary disorders holds promise for uncovering potential biomarkers for early detection and identifying therapeutic targets for tailored interventions.

It's worth noting that hospitalization and invasive diagnostic procedures can significantly distress patients, leading to the emergence of anxiety and depression. The hospital environment, marked by unfamiliar surroundings, loss of personal autonomy, and apprehension about the unknown, can trigger psychological symptoms and exacerbate pre-existing mental health issues.

In culmination, the confluence of these three research domains — cytokine dynamics, bronchial microbiota, and the implications of invasive diagnostics — presents a holistic perspective on pulmonary health. As this thesis unfolds, it endeavors to weave together these elements, drawing upon their interdependencies and their collective significance in shaping our understanding of respiratory diseases.

GENERAL PART

The respiratory system is a critical component of human physiology, and its health is essential for overall well-being. The lungs, in particular, play a pivotal role in ensuring adequate oxygenation for the entire body. Diseases that affect the lungs can significantly impact quality of life and overall health outcomes. Among these, lung cancer and chronic lung diseases stand out due to their prevalence and clinical significance.

Lung cancer, a malignant transformation of lung tissue cells, is the leading cause of cancer-related deaths worldwide. It primarily originates from the epithelial cells of the lungs and can be classified into two main types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for about 85% of all lung cancer cases and includes subtypes such as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Risk factors for lung cancer include smoking, exposure to radon gas, asbestos, and certain other environmental pollutants.

Interstitial lung diseases (ILD) are represented by a vast, heterogeneous group of distinct diseases that affect the lung parenchyma through inflammation and fibrosis. This category of lung diseases includes entities such as idiopathic pulmonary fibrosis, hypersensitivity pneumonitis or organizing pneumonitis. Patients with ILD complain of non-specific symptoms, such as dry cough, progressive dyspnea and chest discomfort, which can lead to a delay in establishing the diagnosis.

Lung diseases, given their diverse etiologies and manifestations, require a spectrum of diagnostic tests. These range from non-invasive procedures like chest roentgenograms (X-rays) and pulmonary function tests (PFTs) to more invasive measures like biopsies and bronchoscopy. Each modality offers specific insights into lung health and potential pathologies.

As for lung cancer, it remains a significant challenge to the healthcare system as an increasing number of individuals are diagnosed and die from this disease. It is imperative to identify some biomarkers to facilitate establishing the diagnosis of lung cancer as early as possible. Chronic inflammation is recognized as an essential factor in the process of carcinogenesis, influencing various stages of cancer development, including malignant transformation, invasion and metastasis.

Understanding the role of cytokines in the context of lung cancer has the potential to provide valuable information for the identification of new markers for diagnosis and prognosis, as well as for the development of targeted therapies. There are also studies that have shown that dysbiosis of the lung microbiota is involved in the pathogenesis and evolution of various respiratory diseases, including lung cancer.

Further research is essential to uncover the precise roles of cytokines and lung microbiota in lung cancer with the aim of identifying new biomarkers that may contribute to diagnosis, prognosis prediction and treatment efficacy assessment in these patients.

SPECIAL PART

1. SERUM AND BRONCHOALVEOLAR LAVAGE FLUID LEVELS OF CYTOKINES IN PATIENTS WITH LUNG CANCER AND CHRONIC LUNG DISEASE: A PROSPECTIVE COMPARATIVE STUDY.

Several studies have highlighted the role played by chronic inflammation in the process of carcinogenesis. This is true not only in the early stages of malignancy, but also in later stages, including the stage of malignant conversion, invasion and metastasis. Both innate and acquired immune responses underscore the functional link between inflammation and cancer. Inflammatory cells and tumor cells both release cytokines and chemokines, proteins that play essential roles in enhancing cellular activity and the humoral system.

There is existing research demonstrating the potential of detecting certain cytokines from bronchoalveolar lavage fluid or blood in the differential diagnosis of lung cancer. Inflammatory cytokines, including IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, and TNF-α, have been implicated in the immune response associated with cancer progression. IFN-gamma, primarily produced by NK cells and T cells, plays a crucial role in enhancing antigen presentation and the cytotoxic activity of T cells. IL-1β, categorized as a pro-inflammatory cytokine, is recognized for promoting angiogenesis and tumor cell invasiveness. Similarly, IL-2 is a cytokine secreted by T cells in response to antigen stimulation, playing a critical role in T cell proliferation and NK cell activity. Other interleukins may promote tumor growth by inhibiting effector T-cell function and enhancing regulatory T-cell function.

It is postulated that TNF-α, IFN-γ, TGF-β, VEGF, and various interleukins are among the most critical cytokines involved in the development of lung cancer and have the potential to serve as diagnostic, prognostic, and treatment response markers. Consequently, the research objectives were expanded to include an examination of the relationship between the levels of inflammatory proteins, namely IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12p70, in both blood and bronchoalveolar lavage fluid (BALF). Furthermore, the study aims to compare these protein levels between patients diagnosed with lung cancer and those with benign lung diseases.

This study involved 33 patients diagnosed with lung cancer and 33 patients with benign lung conditions as a control group. The diagnosis of lung cancer was confirmed through histopathological and immunohistochemical examination of bronchial biopsy samples. The average age of the lung cancer patients was 62.7 years, while the control group had an average age of 58.2 years, with no statistically significant age difference. However, the lung cancer group had a significantly lower mean body mass index (BMI) compared to the control group, and their distribution of BMI categories also differed significantly. More lung cancer patients were male, but this gender difference was not statistically significant. Smoking history was more prevalent among lung cancer patients, with a significantly higher median pack-year smoking history.

Examination of clinical data showed that cough was common in both groups, with no significant difference in type. Thoracic pain was significantly more common in lung cancer patients, while hemoptysis, fever, and fatigue showed no significant differences. Weight loss was significantly more prevalent among lung cancer patients. Dyspnea and its severity, measured using the Modified Medical Research Council (mMRC) dyspnea scores, were comparable between the groups. Anorexia was absent in lung cancer patients but present in the control group, while wheezing and stridor were significantly more frequent in controls. The duration of symptom onset was significantly shorter in lung cancer cases, and they had a higher Charlson Comorbidity Index (CCI) score.

Lung function studies revealed significant differences in spirometry patterns between the two groups, with lung cancer patients showing a higher prevalence of obstructive and mixed patterns. The degree of respiratory dysfunction based on forced expiratory volume in 1 s (FEV1) was similar between the groups. Laboratory analysis of serum markers showed significantly higher levels of inflammatory markers (CRP, ESR, leucocytes, neutrophils, IL-1, IL-6, and ferritin) in lung cancer patients compared to controls at both the initial assessment and one month after treatment. Bronchial lavage analysis revealed significant differences in inflammatory markers (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, TNF-α, and IFN-γ) between lung cancer patients and controls at the initial assessment and one month after treatment. Cytokine levels in bronchoalveolar lavage fluid correlated significantly, with notable associations between several cytokines. Serum and lavage cytokines also showed significant correlations.

	IFN-γ	IL-1b	IL-2	IL-4	IL-6	IL-10	IL-12p70	TNF-α
IFN-γ	1	0.285	0.418	0.199	0.157	0.122	0.526	0.199
IL-1b	0.285	1	0.461	0.181	0.394	0.086	0.309	0.228
IL-2	0.418	0.461	1	0.293	0.261	0.335	0.502	0.286
IL-4	0.199	0.181	0.293	1	0.215	0.307	0.461	0.394
IL-6	0.157	0.394	0.261	0.215	1	0.241	0.197	0.203
IL-10	0.122	0.086	0.335	0.307	0.241	1	0.277	0.355
IL-12p70	0.526	0.309	0.502	0.461	0.197	0.277	1	0.182
TNF-α	0.199	0.228	0.286	0.394	0.203	0.355	0.182	1

Figure 1 – Correlation matrix of cytokines from bronchoalveolar lavage fluid.

In summary, this study has highlighted significant differences and associations in clinical parameters, serum markers, and inflammatory markers found in bronchoalveolar lavage fluid between patients diagnosed with lung cancer and those with benign lung diseases. These findings emphasize the importance of understanding the distinct inflammatory profiles associated with these conditions, as they hold the potential to guide the development of targeted therapies or diagnostic strategies. Notably, the study revealed that cancer-specific cytokines in lavage fluid exhibited earlier, and more pronounced increases compared to their levels in peripheral blood. The strongest correlations were observed between serum IL-6 and lavage fluid IL-6, as well as between serum IL-1 and BALF IL-1β. These results could have meaningful implications for clinical practice, potentially aiding in the diagnosis and prognosis of lung cancer. However, it is essential to conduct further research to validate these findings and explore their broader clinical implications, including the diagnostic and prognostic value of these cytokines in lung cancer management..

2. THE ASSOCIATION OF IFN-Γ, TNF-A, AND INTERLEUKINS IN BRONCHOALVEOLAR LAVAGE FLUID WITH LUNG CANCER: A PROSPECTIVE ANALYSIS

Current research endeavors are centered on elucidating the role of cytokines in lung cancer and identifying potential biomarkers that can be employed for diagnosis, prognosis assessment, and the evaluation of therapy responses. Numerous studies have showcased the potential usefulness of detecting specific cytokines, including interleukins, tumor necrosis factors, and tumor growth factors, in bronchoalveolar lavage fluid or blood samples to differentially diagnose lung cancer and monitor cancer cell proliferation.

The primary objective of this study is to evaluate the diagnostic and prognostic significance of the following cytokines in BALF: IFN-gamma, IL-1b, IL-2, IL-4, IL-6, IL-10, IL-12p70 and TNF-alpha . By analyzing their concentration, our aim is to determine whether these specific cytokines can reliably serve as indicators of the presence, stage or progression of lung cancer.

Therefore, with the availability of accurate diagnostic and prognostic methods, clinicians can tailor treatments to individual patient needs more effectively. Hence, the third objective of this study is to investigate how the identified cytokines and their respective concentrations can influence clinical management decisions for lung cancer patients. By establishing a clear link between cytokine profiles and patient outcomes, this study aims to contribute to the development of more precisely targeted therapeutic strategies, ensuring that those grappling with this devastating disease receive the most optimal treatment plans available.

In this study, 33 participants were categorized into two groups based on their bronchoalveolar lavage fluid (BALF) characteristics: 22 had inflammatory BALF, while 11 had non-inflammatory BALF. The average age in the inflammatory group was 60.3 years, while in the non-inflammatory group, it was 59.2 years, with no statistically significant age difference. The mean body mass index (BMI) was similar between the two groups. Gender distribution showed a higher percentage of males in the inflammatory group, but this difference was not statistically significant. Smoking history and the average pack-years of smoking were comparable between

the groups. The Modified Medical Research Council (mMRC) dyspnea score and Charlson Comorbidity Index (CCI) score did not significantly differ between the two groups. Respiratory dysfunction measured by FEV1 was also similar.

Diagnostic studies revealed no significant difference in tumor location between the groups, but metastasis was more common in the inflammatory group. Tumor size showed no significant difference. In the inflammatory group, Small Cell Lung Cancer (SCLC) histology was more common, while in the non-inflammatory group, Adenoid Cystic Carcinoma (ACC) was prevalent. Immunohistochemistry results did not significantly differ between the groups.

Bronchoalveolar lavage fluid (BALF) analysis at the time of diagnosis showed significant differences between the inflammatory and non-inflammatory groups for several inflammatory markers, including IFN-gamma, IL-1b, IL-2, IL-6, IL-10, IL-12p70, and TNF-alpha. These differences persisted during follow-up, indicating that these markers can differentiate between lung cancer patients with inflammatory and non-inflammatory BALF.

Receiver operating characteristic (ROC) plot analysis revealed that IL-12p70 had the highest AUC value, followed by IL-2, IL-6, IL-4, TNF-alpha, IL-10, IL-1b, and IFN-gamma, indicating their significant association with lung cancer risk. Sensitivity and specificity percentages showed that IL-6 had the highest sensitivity, while IL-1b had the highest specificity.

Regression analysis with adjusted factors for the inflammatory markers indicated that IL-6 exhibited the highest odds ratio, suggesting that patients with higher IL-6 levels had a significantly increased risk of lung cancer. IL-12p70 also showed a significantly increased risk, as did IL-2 and IL-1b, albeit to a lesser extent. IL-10 had a modest association with lung cancer risk, while TNF-alpha did not significantly impact risk. These findings provide valuable insights into the associations between specific inflammatory markers and the risk of lung cancer, potentially aiding in the early diagnosis and risk assessment of this disease.

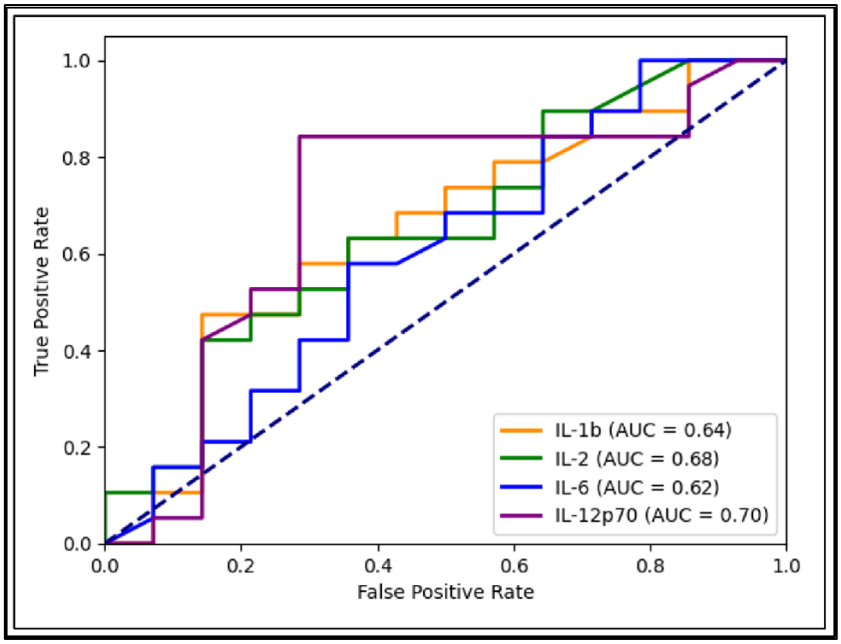

Figure 2 – ROC plot analysis.

In summary, this study found that patients with inflammatory bronchoalveolar lavage fluid (BALF) cytology had elevated levels of specific inflammatory markers, including IFN-gamma, IL-1b, IL-2, IL-6, IL-10, and IL-12p70, compared to those with non-inflammatory BALF and the control group. Follow-up analysis also demonstrated that some of these markers, such as IFN-gamma, IL-1b, IL-2, IL-4, and IL-6, maintained higher levels over time. The analysis of receiver operating characteristic (ROC) plots revealed that IL-1b, IL-2, IL-6, IL-10, IL-12p70, and TNF-alpha were significantly associated with lung cancer, although their ability to discriminate between lung cancer and other conditions was relatively modest.

Moreover, regression analysis indicated that patients with lung cancer had significantly higher odds of having elevated levels of IL-1b, IL-2, IL-6, and IL-12p70 above certain threshold values. However, it's important to note that further research with larger sample sizes and longer follow-up periods is necessary to fully explore the implications of these inflammatory cytokines found in BALF. Such investigations can help clarify the potential utility of these inflammatory markers in the diagnosis, prognosis, and management of lung cancer.

3. BRONCHIAL MICROBIOTA AND THE STRESS ASSOCIATED WITH INVASIVE DIAGNOSTIC TESTS IN LUNG CANCER VS. BENIGN PULMONARY DISEASES: A CROSS-SECTIONAL STUDY.

Recent advancements in microbiome research have amplified our recognition of the potential role played by the lung microbiota in respiratory health and diseas. The lung microbiota constitutes a intricate ecosystem encompassing various microorganisms, including bacteria, fungi, and viruses. These microorganisms are believed to influence pulmonary homeostasis and contribute to pathological processes. Dysbiosis, characterized by an imbalance in the composition of the lung microbiota, has been implicated in the onset and progression of diverse pulmonary conditions, encompassing lung cancer and COPD. Exploring holdronchial microbiota in individuals afflicted with lung cancer and benign pulmonary disorders holds promise for uncovering potential biomarkers for early detection and identifying therapeutic targets for tailored interventions.

Furthermore, a mounting body of evidence suggests the existence of the gut-lung axis, a two-way communication network connecting the gut and lung microbiomes, which could have an impact on stress and anxiety levels experienced by patients. For instance, stress-induced modifications in the gut microbiota have been observed to influence the lung microbiota, and conversely, changes in the lung microbiota can reciprocally affect the gut, potentially influencing the development of pulmonary diseases and the psychological well-being of individuals. Consequently, unraveling the interplay between the bronchial microbiota and patients' stress levels holds the potential to yield valuable insights for devising strategies to alleviate anxiety and enhance the overall quality of life for individuals undergoing invasive lung procedures. Furthermore, patients grappling with lung cancer and chronic lung conditions may experience heightened levels of anxiety and depression due to the uncertainty surrounding their prognosis and the potential impact of their illness on their daily lives.

The primary objective of this study is to perform a comparative analysis of the bronchial microbiota in individuals undergoing bronchoscopy, specifically patients with lung cancer and those with benign pulmonary diseases. This analysis aims to shed light on the potential role of the bronchial microbiota in the development of these diseases and to explore potential connections between microbiota composition

and the psychological well-being of patients. Additionally, this study aims to evaluate the stress levels experienced by individuals undergoing invasive lung tests, including patients with lung cancer and those with benign pulmonary conditions. By examining the prevalence of anxiety and depression in these patient groups, we aim to gain a deeper understanding of the psychological impact of invasive diagnostic procedures and the hospital environment on their mental health. This knowledge will be crucial in developing targeted interventions aimed at alleviating anxiety and enhancing the overall well-being of patients during their hospitalization and beyond.

In this study, researchers conducted a comprehensive analysis of two groups of patients: the malignant group with lung cancer and the benign group with chronic lung disease. The cohort included 33 patients in each group, and their characteristics were examined, including demographics, BMI, and smoking history. The study found that there was no statistically significant age difference between the malignant and benign groups. However, the malignant group had a significantly lower mean BMI compared to the benign group. The distribution of gender varied between the groups, with a higher proportion of males in the malignant group, although this difference was not statistically significant. Smoking habits showed a higher percentage of current or ex-smokers in the malignant group, but this disparity was not statistically significant. Notably, there was a significant difference in pack-year smoking history, indicating that individuals in the malignant group had a more extensive history of smoking.

Both groups had a comparable percentage of individuals reporting exposure to respiratory hazards, and this difference was not statistically significant. In terms of clinical data, the study found similar rates of coughing and dry cough between the groups. Thoracic pain was significantly more frequently reported in the malignant group, while other symptoms such as hemoptysis, fever, and dyspnea did not exhibit statistically significant differences. Weight loss was significantly more common in the malignant group, whereas anorexia was exclusively observed in the benign group. Fatigue was reported by both groups, with no statistically significant difference. Wheezing and stridor were more prevalent in the benign group.

The spirometry investigation results revealed a significant difference in the distribution of patterns, with the malignant group exhibiting higher prevalence of obstructive and mixed patterns, while the benign group had a higher prevalence of restrictive patterns. However, there was no significant difference between the groups

in terms of the degree of respiratory dysfunction measured by FEV1. Additionally, bronchoalveolar lavage fluid analysis showed significant differences in the presence of various microorganisms between the malignant and benign groups, including Acinetobacter spp., Candida spp., and Parainfluenza virus. Cytology results also indicated significant differences in the presence of atypical cells, tumoral cells, lymphocytes, and eosinophils.

The study further assessed the psychological well-being of patients using scales and surveys. At both the initial and follow-up time points, the malignant group exhibited significantly lower ECOG performance status, lower Karnofsky score, higher GAD-7 score (indicating more severe anxiety symptoms), and higher PHQ-9 score (indicating more severe depression symptoms) compared to the benign group. Similarly, the Hospital Anxiety and Depression Scale (HADS) questionnaire results at the time of diagnosis showed no significant difference in anxiety scores between the groups, but the malignant group had significantly higher depression scores. At follow-up, there were no significant differences in anxiety or depression scores between the group.

In summary, this study has shed light on the disparities in bronchial microbiota between patients with lung cancer and those with benign pulmonary diseases who underwent bronchoscopy. Notably, Candida spp. were more prevalent in the benign group, while Acinetobacter spp. and Parainfluenza virus were exclusively found in the malignant group. Additionally, patients with lung cancer exhibited heightened stress levels, more severe anxiety, and greater depression symptoms compared to their benign counterparts, and these distinctions persisted during follow-up assessments. It is worth noting that the malignant group displayed significantly lower ECOG and Karnofsky scores, indicating a poorer performance status. These findings underscore the necessity for further investigation into the role of bronchial microbiota in the development and progression of lung cancer, as well as the influence of stress, anxiety, and depression on patient outcomes in the context of invasive lung tests.

PERSONAL CONTRIBUTION

In conclusion, we can say that the objectives of the scientific research were achieved because we demonstrated the following:

- Dosing cytokines from BALF in order to quantify lung inflammation determined by various respiratory pathologies, with the establishment of reference intervals for these investigations.
- Comparison of plasma and BALF interleukins in patients with lung cancer vs. those with benign lung pathology, evaluating the interrelationship between local lung inflammation and systemic inflammation.
- The variability of the analyzed cytokines between the studied groups indicates the potential of their use as diagnostic and prognostic biomarkers in different pathologies, such as the lung cancer studied in the present paper.
- Studying the lung microbiota, highlighting the predominance of certain microorganisms in lung cancer compared to benign pathology, confirms the involvement of the microbiome in the pathogenesis of various diseases.
- The results obtained from the evaluation of the mental state and the performance status show us the psychological burden associated with the diagnoses with a reserved prognosis.

FUTURE RESEARCH DIRECTIONS

Although this PhD thesis includes a rigorous and complex research process, there are future research directions we wish to pursue:

- Narrowing the profile of study cytokines in lung cancer and their dosage both in BALF and in serum on larger groups of patients in order to validate the most reliable dosage method and their utility in the diagnosis and management of the disease.

- Deepening the understanding of the role of the lung microbiota in the pathogenesis of lung cancer with the aim of identifying the usefulness of the microbiome in the development of new targeted therapies.
- The deepening of the psychological stress factors associated with the communication of a serious diagnosis and the need to apply invasive diagnostic investigations, with the implementation of psychological counseling programs for these patients.