Universidade de Aveiro Departamento de Ciências Médicas
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The impact of Chronic Obstructive Pulmonary Disease on iNKT lymphocytes

O impacto da Doença Pulmonar Obstrutiva Crónica nos linfócitos iNKT

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários a obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Maria de Fátima Matos Almeida Henriques Macedo, Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro e da Doutora Alda Sofia Pires de Dias Marques, Professora Adjunta da Escola Superior de Saúde da Universidade de Aveiro.

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palavras-chave

células iNKT; doença pulmonar obstrutiva crónica; reabilitação respiratória; fenótipo; citotoxicidade.

resumo

A doença pulmonar obstrutiva crónica (DPOC) é uma das doenças inflamatórias mais comuns das vias aéreas e uma das principais causas de morbidade e mortalidade em todo o mundo. A doença é caracterizada por uma limitação persistente do fluxo aéreo, geralmente progressiva. As respostas inflamatórias crónicas e imunes desempenham papéis fundamentais no desenvolvimento e progressão da DPOC. A inflamação é uma resposta protetora normal, mas na DPOC esta inflamação é amplificada. Várias células inflamatórias, seus mediadores e enzimas participam na resposta inflamatória na DPOC. A reabilitação respiratória é um componente fundamental da gestão da doença pulmonar obstrutiva crónica. Ela é projetada para melhorar a condição física e psicológica de pessoas com doenças respiratórias crónicas e para promover a adesão a longo prazo do comportamento que melhora a saúde. O objetivo principal deste trabalho foi contribuir para a compreensão do papel das células iNKT na patologia da DPOC. Além disso, também pretendemos explorar o efeito da reabilitação respiratória nas células iNKT em pacientes com DPOC. Análises clínicas e imunológicas foram feitas em pacientes com DPOC (n=7), pacientes com DPOC que realizaram reabilitação respiratória (n=4) e controlos saudáveis com idade e género idêntico aos doentes (n=14). Os participantes foram estudados duas vezes, com um intervalo de 12 semanas. Foram estudados os seguintes parâmetros clínicos: índice de massa corporal, percentagem de massa gorda, função pulmonar, força dos músculos respiratórios, força muscular isométrica, teste das cinco repetições sentar-levantar, teste de avaliação da DPOC e questionário da dispneia. As células iNKT foram estudadas em termos de percentagem, fenótipo, citotoxicidade e produção de citocinas. Não foi observada nenhuma alteração na percentagem de células iNKT, seus subconjuntos e produção de citocinas no sangue periférico de pacientes com DPOC em comparação com os controlos. Os nossos resultados sugeriram que as células iNKT de pacientes com DPOC podem ter uma diminuição na ativação precoce pela redução da expressão do CD69. Foi também sugerida uma redução na capacidade citotóxica em doentes. A reabilitação respiratória não pareceu afetar a redução da expressão do CD69, mas pareceu contribuir para o aumento da citotoxicidade das células iNKT e desempenhar um papel na melhoria do defeito citotóxico. Este é o primeiro estudo que conduziu uma extensa análise de correlações entre variáveis clínicas para a DPOC e variáveis imunológicas. Os resultados das nossas correlações indicaram que algumas células podem estar associadas a uma melhoria no estado de saúde do paciente e outras com o agravamento da DPOC. Este foi um estudo exploratório, e mais investigações sobre este tema são necessárias para fortalecer as conclusões.

keywords iNKT cells; chronic obstructive pulmonary disease; pulmonary rehabilitation; phenotype; cytotoxicity.

abstract Chronic obstructive pulmonary disease (COPD) is one of the most common inflammatory diseases of the airways and a leading cause of morbidity and mortality worldwide. The disease is characterized by a persistent airflow limitation, generally progressive. Chronic inflammatory and immune responses play key roles in the development and progression of COPD. The inflammation is a normal protective response, but in COPD this inflammation is amplified. Several inflammatory cells, their mediators and enzymes participate in the inflammatory response in COPD. Pulmonary rehabilitation is a core component of chronic obstructive pulmonary disease management. It is designed to improve the physical and psychological condition of people with chronic respiratory disease and to promote the long-term adherence of health-enhancing behavior. The main aim of this work was to contribute for understanding the role of iNKT cells in COPD pathology. In addition we also aimed to explore the effect of pulmonary rehabilitation on the iNKT cells in patients with COPD. Clinical and immunological analysis were done in patients with COPD (n=7), patients with COPD performing pulmonary rehabilitation (n=4) and age- and gender-matched healthy controls (n=14). Participants were studied twice, with an interval of 12 weeks. The following clinical parameters were studied: body mass index, body fat percentage, pulmonary function, respiratory muscle strength, quadriceps muscle strength, five time seat to stand, COPD assessment test and modified medical research council. The iNKT cells were studied in terms of percentage, phenotype, cytotoxicity and cytokine production. No alteration in percentage of iNKT cells, their subsets and cytokine production were observed in the peripheral blood of patients with COPD in comparison with controls. Our results suggested that iNKT cells from patients with COPD might have a decrease in early activation by reduction of CD69 expression. A reduction in cytotoxic capacity in patients was also suggested. Pulmonary rehabilitation did not seem to affect the reduction of CD69 expression, but seemed to contribute to the increase in iNKT cell cytotoxicity and might have a role in improving the cytotoxic defect. This is the first study that conducted an extensive correlation analysis between clinical variables for COPD and immunological variables. Findings from our correlations indicated that some cells might be associated with an improvement in the health condition of the patient, and others with the worsening of COPD. This was an exploratory study, and further research on this topic is warranted to strengthen conclusions.

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α-GalCer	α-Galactosylceramide
ACK	Ammonium-Chloride-Potassium
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BFP	Body fat percentage
BMI	Body mass index
BSA	Bovine serum albumin
CAT	COPD assessment test
CCQ	COPD control questionnaire
CHP	Cumen hydroperoxide
COPD	Chronic obstructive pulmonary disease
COPD-PR	Chronic obstructive pulmonary disease – pulmonary rehabilitation
CRQ	Chronic respiratory questionnaire
DC	Dendritic cell
DN	Double-negative
EDTA	Ethylenediamine tetraacetic acid
ESSUA	*Escola Superior de Saúde da Universidade de Aveiro*
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
iFBS	Inactivated fetal bovine serum
IFN-γ	Interferon gamma
IL	Interleukin
iNKT	Invariant natural killer T
I3S	*Instituto de Investigação e Inovação em Saúde*
Lab3R	Respiratory Rehabilitation and Research Laboratory
MEP	Maximal expiratory pressure
MHC	Major histocompatibility complex
MIP	Maximal inspiratory pressure

mMRC	Modified British Medical Research Council
NK	Natural killer
NKT	Natural killer T
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PMA	Phorbol 12-myristate 13-acetate
QMS	Quadriceps muscle strength
SGRQ	St. George's respiratory questionnaire
TCR	T cell receptor
TH	T-helper
TNFα	Tumor necrosis factor α
Treg	Regulatory T
5TSS	Five time seat to stand

1. Introduction

1.1. Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is one of the most common inflammatory diseases of the airways [1] and a leading cause of morbidity and mortality worldwide [2]. It is a chronic adult disease [3], affecting approximately 14% of individuals aged 65 years old or older, independently of exposure history, and it is responsible for 19.9% of adults hospitalizations aged 65–75 years old [4].

The disease is defined as a preventable and treatable condition and can be characterized by persistent airflow limitation that is not fully reversible [5], [6]. The airflow limitation is generally progressive and associated with an enhanced chronic inflammatory response in the airways due to exposure of the lungs to noxious particles or gases [5], mainly caused by cigarette smoking [7]. Although tobacco is the most known and studied risk factor of COPD, there are other factors that can cause and contribute to the disease, such as genetic, ageing, gender, growth and development of the lung, environmental pollutants, occupational dust, vapours and fumes, asthma, chronic bronchitis, infections and socioeconomic status (poverty) [5], [8]. The best documented genetic risk factor linked to COPD is a severe hereditary deficiency of α1-antitrypsin, a major circulating inhibitory of serine proteases. Low concentrations of this enzyme, particularly in combination with smoking or other environmental exposures, increases the risk of parenchymal destruction leading to COPD. This hereditary deficiency is present in 1–3% of patients with the disease [9].

Pathology and pathogenesis of chronic obstructive pulmonary disease

Even though COPD is recognized to have important systemic manifestations, such as cardiovascular events, systemic inflammation and alterations of metabolism, which may contribute to the disease severity in individual patients, its principal pathological changes occur in the lungs [5], [10], [11].

The inflammation is a normal protective response to the inhaled toxins, but in COPD this inflammation in the lungs, particularly in the small airways is amplified. Generally, the inflammation mediated by T cells and structural changes in the airways tend to increase with the severity of disease. This inflammation persists for years, even after smoking cessation, through unknown mechanisms, although it is believed that autoantigens and persistent microorganisms may play a role [5], [12], [13]. Lung inflammation is further

modified by two other processes involved in the COPD pathogenesis: the oxidative stress and an excess of proteases (Figure 1) [5], [13]. Infections of the respiratory tract by viruses and bacteria also contribute to amplify the lung inflammation and the pathogenesis of COPD [14], [15].

COPD is characterized by a specific pattern of inflammation involving increased numbers of macrophages, neutrophils, B and T lymphocytes (mostly $CD8^+$, but also $CD4^+$) in the lungs [5], [12], [16]. These inflammatory cells release inflammatory mediators and enzymes, which interact with structural cells in the airways, pulmonary vasculature and lung parenchyma [5], [17]. The increased neutrophil number can result in an increased release of proteinases and oxidants, leading to imbalances that induce lung destruction [12], [18]. Macrophages secrete several inflammatory mediators, including growth factors, such as transforming growth factor-β, which stimulate fibroblast proliferation causing fibrosis in the small airways; proinflammatory cytokines that increase inflammation, and chemokines, which attract circulating cells into the lungs perpetuating the inflamation. Additionally, macrophages promote the recruitment and activation of monocytes and T lymphocytes [1], [18]. The role of T cells in COPD pathogenesis is not fully understood [19], however, it is known that T lymphocytes are increased in the lung parenchyma and airways, with an increase in CD8:CD4 ratio. CD8 cells may be cytotoxic [12], because of its potential to secrete tumor necrosis factor, granzymes and perforins, and to activate the Fas–Fas ligand apoptotic pathway, which leads to alveolar wall destruction [20]. B lymphocytes, important agents in the acquired immune system, are also increased in pathogenesis of the COPD, maybe as a response to chronic infection of the airways [12], [18], [21]. Inflammatory response is enhanced and maintained due to pro-inflammatory mediators, such as: leukotriene B4, interleukin-8, tumor necrosis factor α (TNFα), macrophage inflammatory protein-1α released by inflammatory and structural cells, attracting more inflammatory cells and creating a positive inflammatory loop [10]. In conclusion, COPD is developed by the actions of both innate and acquired immune responses [10], [22].

In COPD the oxidative metabolism is over-activated, because of oxidants from cigarette smoke and internal production of oxidants by phagocytes, such as neutrophils and macrophages, which are involved in bronchial inflammation [18], [23], [24].

Proteases activity is regulated by production and release of antiproteases [18], however, in COPD there is an imbalance of proteases and antiproteases, which is the consequence of increase production/activity of proteases and inactivation/reduce production of antiproteases [12]. This imbalance is caused by cigarette smoke and inflammation itself, which leads inflammatory cells to secrete proteases and inhibit the activity of several antiproteases, such as α_1-antitrypsin, by oxidative stress [12], [18].

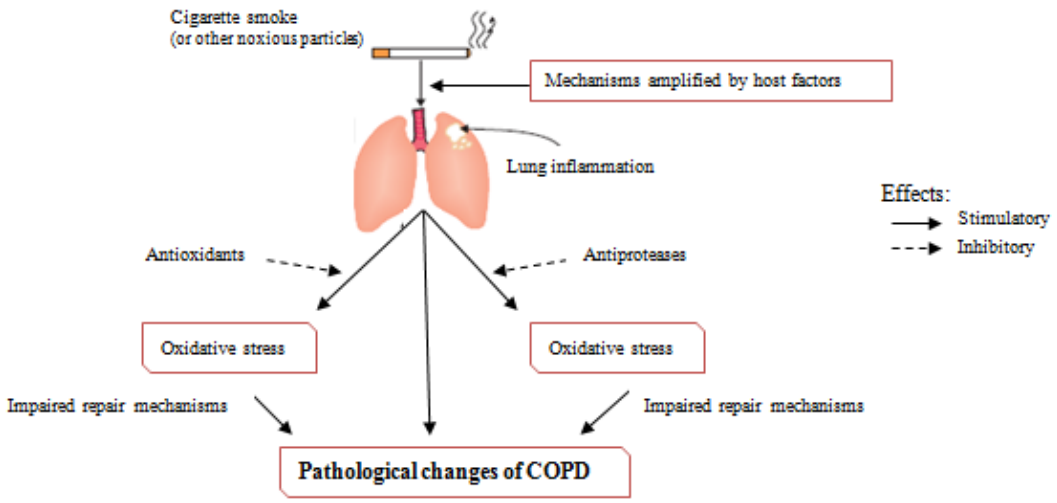

Figure 1. The mechanism of COPD pathogenesis. Dashed bars represent inhibitory effects. Adapted by MacNee [12].

Pathophysiology of chronic obstructive pulmonary disease

The underlying pathogenic mechanisms of COPD lead to pathological changes, which result in physiological alterations, such as airflow limitation and air trapping, gas exchange abnormalities, mucous hypersecretion and ciliary dysfunction, pulmonary hypertension, exacerbations, and systemic effects [5], [12].

Airflow obstruction (Figure 2) is induced by inflammation, fibrosis and luminal exudates in small airways, which are correlated with the reduction of Forced Expiratory Volume in one second (FEV_1) and FEV_1/FVC (Forced Vital Capacity) ratio, and possibly with the accelerated decline in FEV_1, typical of the disease [12], [17]. The peripheral airway obstruction progressively traps air during expiration leading to hyperinflation. Loss of the lung elastic recoil, by destruction of alveolar walls, and the destruction of alveolar support, also are involved in the airflow obstruction [12]. The hyperinflation reduces

inspiratory capacity and can occur when the lung is at rest, called static hyperinflation or during exercise, denominated by dynamic hyperinflation [12], [25]. In patients with COPD, hyperinflation contributes to dyspnea, i.e., a subjective experience of breathing discomfort that consists of qualitatively distinct sensations that vary in intensity, and limitation of exercise capacity, characteristics of the disease [26].

The anatomical changes found in patients with COPD, such as severe obstruction and ventilatory muscle impairment, cause reduced ventilation, leading to gas exchange abnormalities. These abnormalities result in arterial hypoxemia with or without hypercapnia. As the disease progresses, the gas transfer for oxygen and carbon dioxide becomes worse [12], [27].

Hypersecretion results from the metaplasia, increased number of goblet cells and enlarged size of bronchial submucosal glands as a consequence of chronic airway irritation, caused by cigarette smoke and other noxious agents and gases [5], [12], [28]. Squamous metaplasia of epithelial cells induce ciliary dysfunction that results in an abnormal mucociliary escalator and difficulty in expectorating [12].

In patients with COPD, exacerbations of the disease are frequent and are caused by infection with bacteria or/and viruses, air pollution, alterations of ambient temperature or unknown factors. Exacerbations, i.e., "a worsening of the patient's respiratory symptoms that is beyond normal day-to-day variation and leads to change in medication", adopted by Gold 2016 [5]; are characterized by an increase inflammation (increase of the neutrophils number and in some mild exacerbations, increase of eosinophils number). The physiologic changes typical of COPD become more severe in exacerbations, with an increased hyperinflation and airflow limitation, difficulties in pulmonary gas exchange because of increased inequality between ventilation and perfusion which can result in hypoxemia, mucous hypersecretion, oedema and bronchoconstriction [5], [12], [29]. Other diseases, like thromboembolism, pneumonia and acute cardiac failure can aggravate or mimic COPD exacerbations [5].

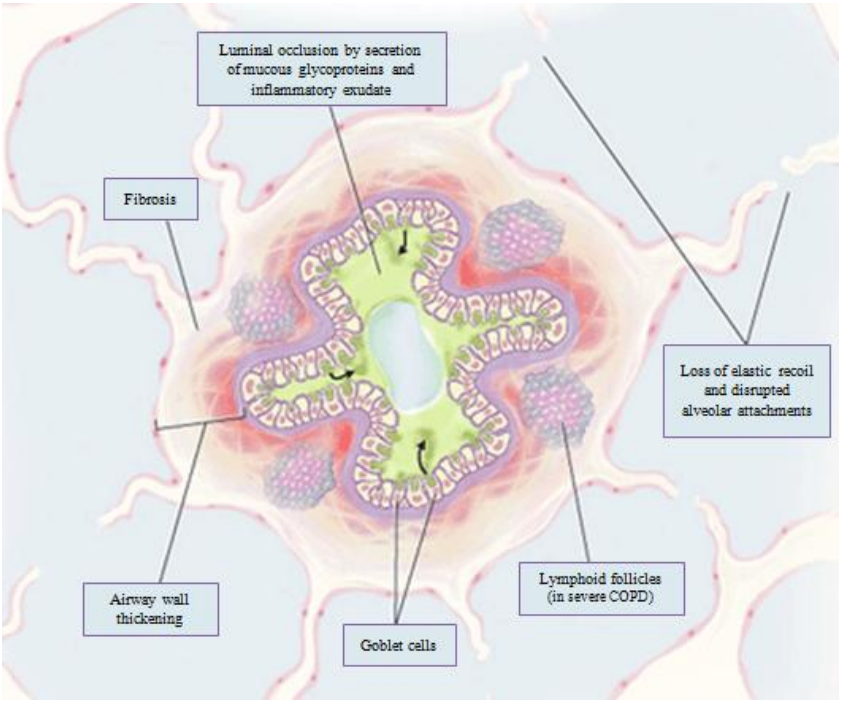

Figure 2. Pathological changes that are underlying to small airway obstruction in COPD. Adapted by Barnes [30].

Symptoms and diagnosis of chronic obstructive pulmonary disease

Predominant symptom in COPD is the persistent and slowly progressive breathlessness (dyspnea) with efforts [1], [31]. Nevertheless, other symptoms are also typical of the disease, such as cough, sputum production, wheezing, and chest tightness. COPD symptoms can be variable from day-to-day, and the patient does not need to display the set of symptoms to have the disease [5], [31], [32]. In severe and very severe COPD, symptoms like fatigue, weight loss and anorexia are frequent [5], [33] and can also be a sign of other diseases, including tuberculosis and lung cancer [5]. Depression and anxiety are also commonly present in patients with the disease [34].

A clinical diagnosis of COPD should be done if the patient is older than 40 years old and displays the following criteria: typical symptoms of the disease (such as, progressive and persistent dyspnea, chronic cough or sputum production), family history of COPD and, exposure history to risk factors for the disease (e.g., cigarette smoke and noxious particles or gases) [5]. Lung function measured with spirometry is the most accurate diagnostic test to evaluate patients who have clinic context of COPD [35]. Through FEV_1 and FVC, which are the key lung function variables, it is possible to classify the stage and severity of airflow limitation in COPD (Table 1). According to the GOLD classification, COPD is

diagnosed to a patient who has the FEV_1/FVC ratio less than 70% [5]. In order to minimize variability, spirometry ought to be performed after the administration of a short-acting inhaled bronchodilator in an appropriate dose [5].

Table 1. Classification of severity of airflow limitation in COPD, based on postbronchodilator FEV_1. Adapted from GOLD [5].

Stage and Severity	Definition
I - Mild	FEV_1/FVC < 0.70; $FEV_1 \geq 80\%$ predicted
II - Moderate	FEV_1/FVC < 0.70; $50\% \leq FEV_1 < 80\%$ predicted
III - Severe	FEV_1/FVC < 0.70; $30\% \leq FEV_1 < 50\%$ predicted
IV - Very Severe	FEV_1/FVC < 0.70; $30\% < FEV_1$ predicted

A good diagnosis of COPD should not be based only on the disease severity measured by lung function, but also in the evaluation of current level of patient's symptoms, exacerbation risk and presence of comorbidities [5]. Thus, it is important to consider other measurements, such as Modified British Medical Research Council (mMRC) questionnaire, which is a simple measure of breathlessness and adequate for assessment of symptoms, COPD Assessment Test (CAT), and COPD Control Questionnaire (CCQ), which evaluate the health status and the clinical control in patients and number of exacerbations and hospitalizations per year [5].

Other tests, like Chronic Respiratory Questionnaire (CRQ) [36] and St. George's Respiratory Questionnaire (SGRQ) [37] are more accurate to measure the quality of life and the health status of patients with COPD, but are also too time consuming to be used in routine clinical practice [5].

Several additional investigations can be considered as part of the diagnosis and assessment of the disease, including imaging (chest X-ray and computed tomography, Figure 3), lung volumes and diffusing capacity by body plethysmography or helium dilution lung volume measurement, oximetry and arterial blood gas measurement, α-1 antitrypsin deficiency screening (serum concentration <15-20% of the normal value is highly suggestive of this deficiency) and exercise testing (e.g., walking tests and cycle or treadmill ergometry test) [5].

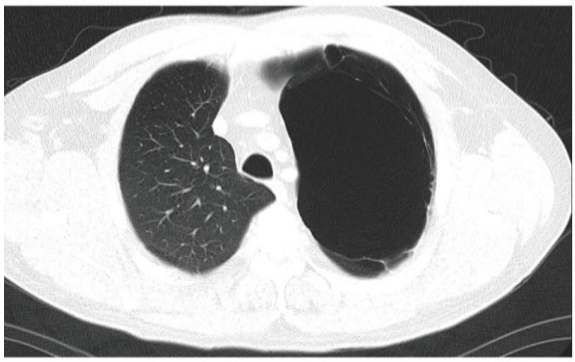

Figure 3. Computed tomography of chest that shows a parenchymal pulmonary destruction with a loss of alveolar attachments in left lobe of a patient with COPD. Adapted by Desai and Steiner [38].

Therapeutic options for chronic obstructive pulmonary disease

Although COPD does not have cure, it can be controlled. The main goal of the treatment focuses in delaying the disease progression. If therapy is appropriate, it can reduce the symptoms, improve health status, as well as exercise tolerance [5].

In patients who smoke, the smoking cessation must be the first step of any therapy, because it is the intervention most capable to influence the COPD natural history [5].

Secondly, patients can use pharmacologic therapy to reduce symptoms and improve their general health status. Pharmacologic therapy includes medications like bronchodilators, vaccines, antibiotics, mucolytic and antioxidant agents, immunoregulators, antitussives, vasodilators, narcotics, between others. However, the most usual pharmacologic therapy for patients with COPD is the treatment with bronchodilator medications [5]. Bronchodilator medications increase the FEV_1 or change other spirometric variables, normally by modifying airway smooth muscle tone, thereby promoting the widening of the airways and reduction of dynamic hyperinflation at rest and during exercise with improvements in exercise endurance time [5], [39], [40]. The bronchodilators often used by patients with COPD are $beta_2$-agonists, anticholinergics, methylxanthines, corticosteroids and phosphodiesterase-4 inhibitors [5].

Genetic augmentation therapy, more precisely α-1 antitrypsin augmentation therapy should be applied only in young patients with severe hereditary α-1 antitrypsin deficiency and established parenchymal destruction [5].

Besides pharmacologic therapeutic options, there is a non-pharmacologic therapy to patients with COPD, called pulmonary rehabilitation, which is considered a core component of COPD management [5], [41].

Other treatments, such as oxygen therapy and ventilatory support can also be applied under specific conditions of disease with clinical benefits to patients with COPD. In more severe cases, surgery or even lung transplantation may be considered [5].

1.1.1. Pulmonary rehabilitation as a core component of chronic obstructive pulmonary disease management

Pulmonary rehabilitation is designed to improve the physical and psychological condition of people with chronic respiratory disease and to promote the long-term adherence of health-enhancing behaviors. So, pulmonary rehabilitation is a comprehensive intervention based on a thorough patient assessment followed by patient-tailored therapies, which include, but are not limited to, exercise training, education, and behavior change. This intervention is implemented by an interdisciplinary team, including physicians, physiotherapists, respiratory therapists, nurses, psychologists, exercise physiologists, behavioral specialist, occupational therapists, nutritionists, and social workers, between others [42]. The aims of pulmonary rehabilitation include reducing symptom burden, maximizing exercise performance, promoting autonomy, increasing physical and emotional participation in daily activities, improving health-related quality of life, and effecting long-term health-enhancing behavior change [42], [43]. Rehabilitation therapy does not directly improve lung mechanics or gas exchange, but optimizes the function of other body systems, minimizing the effect of lung dysfunction [41]. An effective rehabilitation program has to be extended by a minimum length of 6 weeks. However, the longer the patient performs the program, more effective will be the results [5], [44].

Pulmonary rehabilitation programs vary considerably in their components, but generally include patient assessment, exercise training, education, psychosocial support and nutritional counselling [5], [42], [43]. The exercise component of pulmonary rehabilitation increases inspiratory volume and reduces dynamic hyperinflation (occurs when heavy exercise training decreases the ventilatory requirement and respiratory rate, prolonging the time allowed for expiration) resulting in a mitigation of dyspnea when the person is performing tasks [41]. Exercise also increases skeletal-muscle function by induce changes in muscle biochemistry, increasing aerobic function of the muscles of ambulation, delaying fatigue and resulting in increased exercise tolerance [41], [42], [45]. Depression and anxiety are also reduced maybe as a result of increased exercise capacity and

participation in daily activities (Figure 4) [41]. Various modes of training are required for improvements in cardiorespiratory endurance, strength, and/or flexibility. In general the training can include endurance training, interval training, resistance training, neuromuscular electrical stimulation, and respiratory muscle training [42]. The educational component of this intervention focuses on collaborative self-management and behavior change, by providing information and knowledge concerning the disease; building skills, like goal setting, problem solving and decision making; developing action plans that allow individuals to better recognize and manage the disease and focuses on modifying nutritional intake and smoking patterns; adhering to medication and regular exercise; and using effective breathing techniques and energy-saving strategies [42], [45].

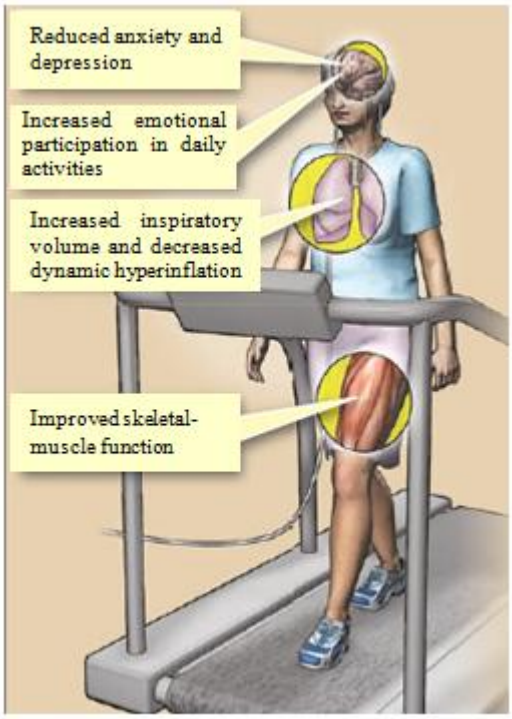

Figure 4. Effects of exercise training as part of a pulmonary rehabilitation for patients with COPD. Adapted by Casaburi et al. [41].

1.2. The immune system in chronic obstructive pulmonary disease

Chronic inflammatory and immune responses play key roles in the development and progression of COPD [46]. The inflammation is a normal protective response, nevertheless, in COPD this inflammation is amplified [12]. Several inflammatory cells, their mediators and enzymes participate in the inflammatory response in COPD, by interact with structural cells in the airways, lung parenchyma and pulmonary vasculature [5], [16],

[46]. Some of the cells that participate in the pathogenesis of COPD are B cells and T cells [3]. $CD8^+$ T cells have been implicated in COPD pathogenesis mainly indeed their numbers in lung parenchyma and small airways correlate inversely with FEV_1, which is the clinical measure most frequently used to define COPD severity [47]. Currently, natural killer (NK) cells and natural killer T (NKT) cells have gained increasing attention. NK and NKT cells are two types of killer cells that have the ability to directly kill target cells [3]. NK cells are less specific lymphocytes, being their activation controlled by a balance of signals from stimulatory receptors and inhibitory receptors [48]–[50]. NK cells are a lymphoid population defined by not express CD3 and include surface expression markers, like CD56, in humans and NK1.1, in mouse. Human NK cells can thus be defined phenotypically as $CD3^-CD56^+$ and murine NK cells as $CD3^-NK1.1^+$ [51], [52].

But contrary to NK cells, NKT cells express a T cell receptor generated by somatic DNA rearrangement, which is a hallmark of acquired immune system cells. Thus, by sharing characteristics of both NK and T cells, NKT cells have a unique role in bridging innate and acquired immunity [53]. NKT cells recognize glycolipid antigens presented by the non-polymorphic major histocompatibility complex class I-like molecule CD1d [54], [55]. When a glycolipid antigen binds to CD1d, the antigen/CD1d complex binds to the TCR of NKT cells and the recognition occurs [56]. After recognizing glycolipids, NKT cells are stimulated to secrete large amounts of cytokines and chemokines, which regulate their movement and activation [57]. NKT cells have been linked to the regulation of immune responses in a broad range of diseases, like autoimmunity, inflammation, infectious diseases and cancer [54], [57]. NKT cells are divided into two subsets based upon differences in TCR characteristics [58]; Type I NKT cells, or invariant NKT (iNKT) cells, which express a TCR composed of an invariable α chain associated with a limited repertoire of β chains, recognizing glycolipid antigens, and type II NKT cells, which express an unbiased TCR repertoire with variable α and β chains, recognizing a range of hydrophobic antigens, including sulfatide and lysophosphatidylcholine [59].

Invariant NKT cells are the best-known and studied subset of CD1d-restricted cells.

1.2.1. Invariant natural killer T cells

Invariant natural killer T cells express a TCR with an invariant α-chain, Vα14-Jα18 in mice and Vα24-Jα18 in humans, paired with certain TCRβ chains, Vβ8.2, Vβ7 or Vβ2

in mice and Vβ11 in humans (Figure 5) [57], [60]. The most widely acknowledged iNKT cell antigen is the glycolipid α-galactosylceramide (α-GalCer), which is a synthetic form of a chemical purified from the marine sponge *Agelas mauritianus* [61]. This lipid is also used for the identification of iNKT cells, through its loading on CD1d tetramers [62]. Nevertheless, there are other glycolipid antigens to iNKT cells, such as bacterial and mammalian glycolipids [56], [63], [64]. The monoclonal antibody 6B11, a combination of an anti-Vα24 antibody with an anti-Vβ11 antibody, and CD1d tetramers loaded with PBS57 are also used to identify iNKT cells by binding to their semi-invariant TCR. However, some researchers do not use any of the specific reagents referred above to identify iNKT cells, using instead CD3 monoclonal antibody in combination with CD56 or CD16 monoclonal antibody. For this reason, this cells can not be called iNKT cells, so they are named NKT-like cells [60], [65], [66].

iNKT exhibit an activated or memory phenotype by constitutively expressing a variety of markers, including CD44, CD69 and CD122 [53]. They are widely distributed into the body and can be found in the liver, spleen, peripheral blood, lung, bone marrow, fat tissue, gastrointestinal tract and skin [67], [68]. Humans usually have a low frequency of iNKT cells, ranging from ~0.01% to 1%, amongst peripheral blood T cells, as well as an increased variability between individuals in comparison with mice, which makes the study of iNKT cells in humans very challenging [69]–[71].

Invariant NKT cells express diverse surface receptors. Some of them, such as CD161 (NK1.1 in mouse), are common to NKT and NK cells. Others, such as CD4 and CD8 are also found in other T cells [53].

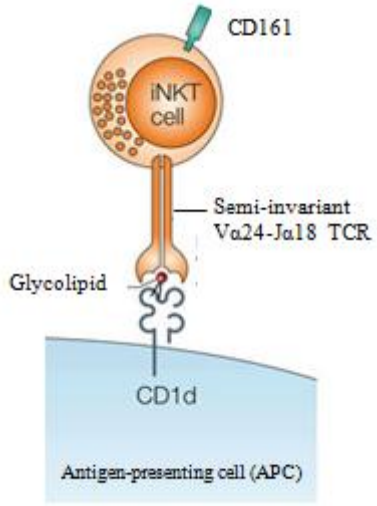

Figure 5. Overall scheme of iNKT cell activation mediated by the semi-invariant TCR recognition of glycolipids bound to CD1d at the surface of antigen presenting cells. Adapted by Kaer [72].

In humans, three different subsets of iNKT cells can be defined according to CD4 and CD8 expression: $CD4^+$, $CD4^-CD8^-$ or also called double-negative (DN) and $CD8^+$. However, in mice, iNKT cells are mostly $CD4^+$ or DN [73], [74]. Whereby the absence of $CD8^+$ iNKT cells suggested that these cells could be deleted in the thymus, but was later shown that $CD8^+$ iNKT cells exist in mice, although at a quite low percentage [75]. The percentage of CD4/CD8 iNKT cell subsets is very variable among individuals, i.e., $CD4^+$ and DN iNKT cells are the most common subsets in humans, with $CD4^+$ varying between 17-53%, DN between 19-63% and $CD8^+$, which is the less common subset, representing only 5-25% of the iNKT cell population [74], [76]–[84]. These frequencies are altered in a variety of conditions [68].

After activation, iNKT cells rapidly secrete large amounts of T-helper 1 (Th1), Th2, and/or Th17-specific cytokines and exhibit cytolytic activities. $CD4^+$ iNKT cells release Th1 cytokines, including interferon-γ (IFN-γ) and TNF-α, and TH2 cytokines, such as interleukin-4 (IL-4), IL-13 and IL-10. In contrast DN and $CD8^+$ iNKT cells mainly produce TH1 cytokines and display cytotoxic activity [56], [60], [85]. Efficient production of cytokines, mostly IL-4, is important to regulate the immune system by inhibition of TH1 and induction of TH2 response [86]. The capacity of iNKT cells to rapidly produce copious amounts of cytokines upon antigenic stimulation, endows these cells with potent immunomodulatory activities [53]. Thus, cytokines, chemokines and surface molecules expressed by iNKT cells deeply influence many other cell types [67], as represented in

Figure 6. iNKT cells have the capacity to activate NK cells, macrophages, T cells and promote their differentiation, B cells leading to antibody production and memory responses, and dendritic cells that express CD1d establishing a bidirectional activation. Furthermore, iNKT cells promote neutrophil recruitment and regulate their supressive activities, as well as suppress regulatory T (Treg) cells [67], [87].

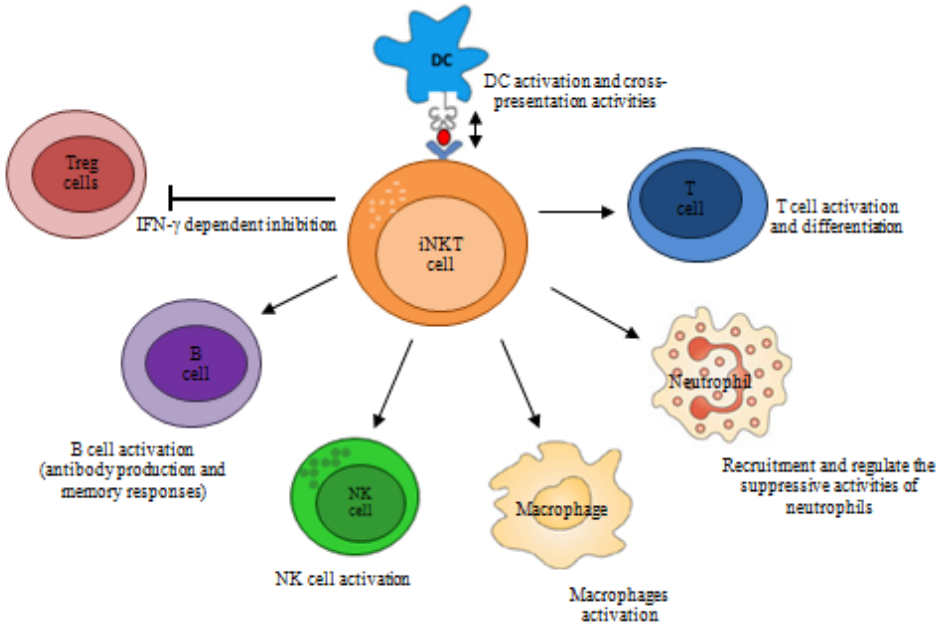

Figure 6. Interactions between iNKT cells and other types of immune cells. DC – dendritic cell. Adapted by Lawrenczyk et al. [87].

iNKT cells develop in the thymus and most of them leave the thymus in an immature stage and fulfill their terminal maturation in the periphery [69]. The development of the iNKT cells begins with the expression of the invariable TCR, which induce the differentiation pathway of these cells. Invariant NKT cells are positively selected by the interaction of the TCR with CD1d molecules expressed by thymocytes [88]. iNKT cell maturation in mice is composed by four stages. The first stage (stage 0) is defined by a population of $CD24^{+}CD44^{-}NK1.1^{-}$ cells. Subsequently, in stage 1, CD24 is downregulation and the cells start to proliferate, maintaining low expression of CD44 and NK1.1 ($CD24^{-}CD44^{-}NK1.1^{-}$). While still proliferating, CD44 is upregulated ($CD24^{-}CD44^{+}NK1.1^{-}$) in stage 2. In addition, at this stage some cells downregulate CD4, constituting the DN iNKT cell lineage. The last maturation stage (stage 3) is characterized

by the upregulation of NK1.1 and other NK cell lineage markers ($CD24^{-}CD44^{+}NK1.1^{+}$), which generally occurs after migration to the periphery [59], [67], [88]–[90].

As in mice, iNKT cell maturation process in humans is also completed at the periphery, in which cells increase the expression of CD161 (human equivalent to NK1.1). The earliest detectable NKT cell precursors in humans are $CD4^{+}$ and $CD161^{-}$ cells, whereby $CD4^{-}$ and $CD161^{+}$ NKT cells arise at later developmental stages [59]. iNKT cells are potently activated when they encounter APCs with exogenous or endogenous antigens loaded in CD1d molecules. Depending on the type of antigen, three different pathways can lead to iNKT cell activation process [67], [91]. Direct pathway, in which the antigen is initially internalized and loaded in CD1d molecules or directly loaded in the CD1 molecules present at the cell surface, resulting in CD1d-lipid complex. This complex present at the surface of the APC interacts with the TCR of the iNKT cell, inducing its activation (Figure 7A). Direct + indirect pathway is characterized by binding of some antigens to Toll-like receptors present at the APC surface, starting a cascade of events that promotes the loading of endogenous antigens in CD1d molecules and IL-12 secretion. This process results in a double activation of iNKT cells by IL-12 and by CD1d interaction with TCR (Figure 7B). In the indirect pathway, there is no TCR mediated recognition of the antigen, i.e., the antigen only promotes IL-12 secretion by APCs (Figure 7C) [91].

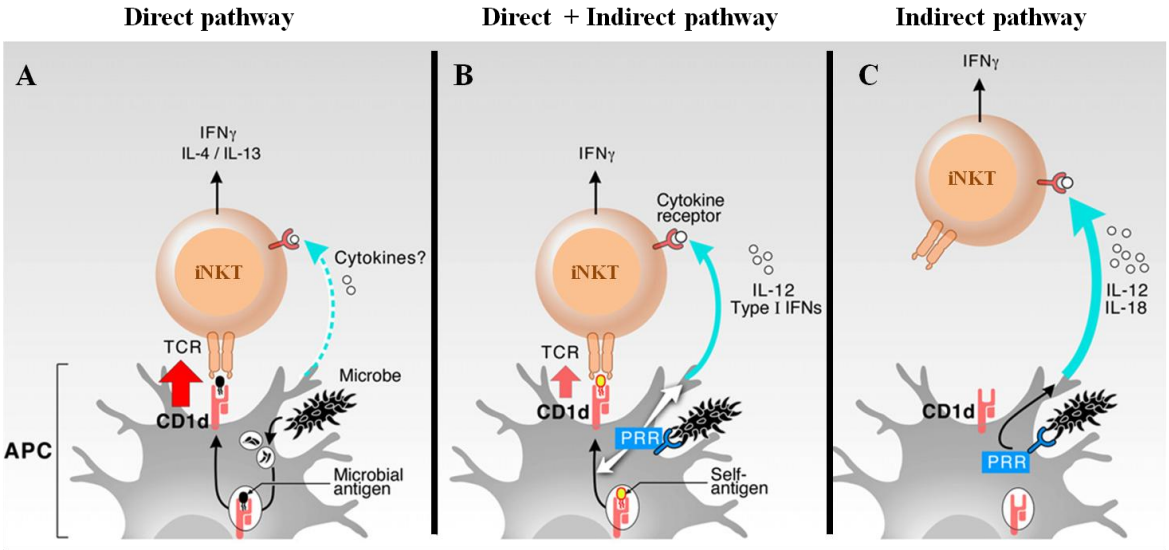

Figure 7. Different pathways leading to iNKT cell activation during infection. A – Direct pathway; B – Direct + Indirect pathway; C – Indirect pathway. Adapted by Brigl and Brenner [91].

1.2.2. Cytotoxic markers in lymphocytes

A pathway used by cytotoxic T lymphocytes ($CD8^+$), NK and NKT cells to destroy pathogenic cells occurs by exocytosis of granule components towards the target cell, delivering a lethal hit of cytolytic molecules [65], [92], [93]. Cigarette smoke succeeds in damaging the physical barrier of mucosal epithelium and the innate immune system, and it induces effector mechanisms of the immune system, which are particularly cytotoxic to the patient with COPD [94]. Many cells have the potential to cause the damage seen in the airways of patients with COPD, including three distinct classes of human killer cells referred above, i.e., $CD8^+$ T cells, NK cells and NKT cells [95], [96]. NK and NKT cells can be a potent source of cytokines and cytotoxic molecules, and an increase in their number or improper effector function could contribute to COPD pathology [47]. While cytotoxic $CD8^+$ T cells have been widely studied in COPD, the role of cytotoxicity of NK and NKT cells is attracting increased attention [3].

Granzyme B, CD107a and CD158b are some molecules used to monitor the cytotoxic response of lymphocytes [65], [92], [93].

Granzyme B

Granzymes are a family of serine-proteases, which have their catalytic activity dependent on a serine residue at the active site. In humans, five granzymes with differing substrate specificity have been identified. However, Granzyme B has the strongest pro-apoptotic activity of all granzymes. Through its caspase-like ability to cleave substrates at aspartic acid residues, Granzyme B activates pro-caspases, thus inducing apoptosis initiating [97]. $CD8^+$ T lymphocytes and NK cells use the exocytosis of Granzyme B granules, stored in the cytoplasm of the effector cell, to cause targeted cell apoptosis [98], [99]. Granzyme B is also expressed at high levels in activated iNKT cells [100], [101]. Granzymes could play an important role in the lung tissue destruction witnessed in COPD and contribute to the pathogenesis of the disease. This destruction may occur from either direct cell-cell interactions or exogenously present granzyme [99].

Peripheral blood of patients with COPD presents an increased frequency of T lymphocytes, NK and NKT-like cells expressing Granzyme B [102], [103]. In addition, the proportion of NK and NKT-like cells are increased in bronchoalveolar lavage fluid (BALF) in COPD, associated with increased NK cytotoxicity and increased expression of

Granzyme B by both cell types [103]. It was also found a significant correlation between Granzyme B expression in BALF-derived T-cells and apoptosis of bronchial epithelial cells [102]. Therefore, granzyme-mediated apoptosis can be a mechanism of lung injury in COPD [102]. However, other study found that the proportion of NK and NKT-like cells in peripheral blood from smokers with COPD was reduced, with defective cytotoxic effector cell function and with reduced proportions expressing Granzyme B [99].

CD107a

CD107a is a lysosomal transmembrane protein, highly glycosylated, with a 40-kDa backbone and 17 predicted N-glycosylation sites, as well as 9 O-glycosylation sites. The intralysosomal part of CD107a contains two highly N-glycosylated domains, which are separated by a proline and serine-rich hinge, highly O-glycosylated [104], [105].

The expression of CD107a on the cell surface has been reported as a marker of cytotoxic degranulation in $CD8^+$ T lymphocytes and NK cells [106], [107]. This cytotoxic marker was shown to be upregulated on the cell surface after stimulation. CD107a is a sensitive marker of NK cell functional activity [107]. It is also known that activated iNKT cells have an upregulation of CD107a expression [108]. In the presence of cigarette smoke, associated with most cases of COPD, the upregulation of CD107a on iNKT cells is inhibited [108]. Cigarette smoke also inhibits CD107a expression in NK cells without affecting other NK cell markers [109].

CD158b

NK cell receptors can be divided into both activating receptors and inhibitory receptors, such as CD158b. CD158b belongs to the family of killer cell immunoglobulin-like receptors [110] and contains long cytoplasmic regions [65], [111] with pairs of immunoreceptor tyrosine-based inhibitory motifs that release inhibitory signals [111]. The presence of these receptors is not exclusive to NK cells, since NKT cells also express these similar receptors [112]. Inhibitory receptors discriminate malignant from healthy cells by surveying the surface expression of self-MHC class I molecules, thereby preventing the attack of NK and NKT cells against healthy cells [3], [110]. In patients with COPD, the expression of CD158b on NK and NKT-like cells is increased [3]. Besides that, there are

evidences that the frequency of CD158b in NK cells is negatively correlated with FEV_1% prediction and FEV_1/FVC [3].

1.3. iNKT cells in the context of chronic obstructive pulmonary disease

COPD pathology is associated with innate and acquired inflammatory immune responses [17]. iNKT cells may play a role in the pathogenesis of chronic airway disease, but there are few studies done in COPD (Table 2). A decreased frequency of iNKT cells in peripheral blood of patients with stable COPD when they stained the cells with 6B11 monoclonal antibody comparing with healthy controls was observed, however, when the iNKT cells were stained with α-GalCer-loaded CD1d-tetramer, no significant alterations were found [113]. On the contrary, according to other investigation, patients with COPD present increased iNKT cell frequency in the peripheral blood when compared to healthy subjects [114]. Moreover, a mouse model of COPD induced by chronic cigarette smoke exposure showed a rise in activated iNKT cell numbers in the lung. The contribution of these cells to disease pathology is highlighted by experiments in $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice, which lack all NKT cells and or specifically lack iNKT cells respectively. These animals have suppressed several features of COPD, such as inflammation and parenchymal destruction [114]. In addition, repeated intranasal administration of the iNKT cell agonist α-GalCer in mice leads to high levels of IL-4 and features of COPD, such as airway inflammation with significant increases in infiltration of macrophages and $CD8^+$ T cells, as well as the proinflammatory cytokines IL-6 and TNF-α [115]. These mice also show mucus production, pulmonary fibrosis and parenchymal destruction with loss of alveolar attachments and decrease of elastic recoil. Neutralization of IL-4 reduces parenchymal destruction induced by α-GalCer, which indicates the importance of iNKT cells in the pathogenesis of COPD by an IL-4 dependent mechanism [115]. Further, other study concluded that mice infected with Sendai virus develop chronic lung disease and airway hypersensitivity similar to COPD in humans and such chronic manifestation was dependent IL-13 from macrophages and iNKT cells [116]. The frequency of iNKT cells analyzed in induced sputum, airway biopsy and bronchoalveolar lavage do not have significant changes between patients with COPD and healthy people [117].

Table 2. Summary of iNKT cells studies in chronic obstructive pulmonary disease.

Species / Type of sample	Results	References
Human / peripheral blood	Patients with stable COPD display lower frequency of iNKT cells in the peripheral blood than controls when the cells were stained with the 6B11 monoclonal antibody	[113]
Human / peripheral blood	No significant differences in frequency of iNKT cells in the peripheral blood of patients with stable COPD comparing with controls, when the cells were stained with α-GalCer-loaded CD1d-tetramer	[113]
Human / peripheral blood	Patients with COPD display higher frequency of iNKT cells in peripheral blood than controls	[114]
Mice / intranasal	Repeated intranasal administration of the iNKT cell agonist α-GalCer leads to high levels of IL-4 and features of COPD	[115]
Mice / lung tissue	Mice infected with Sendai virus develop chronic lung disease and airway hypersensitivity similar to COPD in humans and such chronic manifestation was dependent IL-13 from macrophages and iNKT cells	[116]
Human / induced sputum; airway biopsy; bronchoalveolar lavage	No significant differences in frequency of iNKT cells in induced sputum, airway biopsy and bronchoalveolar lavage between patients with COPD and controls	[117]

2. Aims

The aims of this work were:

- to study the invariant natural killer T (iNKT) cells in patients with chronic obstructive pulmonary disease (COPD);
- to investigate the effect of pulmonary rehabilitation on the iNKT cells in patients with COPD.

To achieve these aims we studied the iNKT cells in terms of percentage, phenotype, cytotoxicity and cytokine production in three groups, i.e., patients with COPD, patients with COPD who performed a pulmonary rehabilitation (COPD-PR), and age- and gender-matched healthy controls. In addition we analyzed clinical parameters, relevant for respiratory function, in the 3 groups of participants to integrate the immunological and clinical data.

3. Materials and Methods

3.1. Ethics

Ethical approval was previously obtained from the Ethics and Deontology Committee at the University of Aveiro, Portugal (Appendix I).

3.2. Design and recruitment

A pre-post exploratory study design was conducted, based on collection of sociodemographic data, anthropometric measurements, subjective and objective measures of clinical evaluation, including blood sample collection from patients with chronic obstructive pulmonary disease. Three different groups of participants were recruited; group 1 was composed of four patients with COPD who performed pulmonary rehabilitation (COPD-PR), group 2 included seven patients with COPD who never underwent pulmonary rehabilitation (COPD) and group 3 included fourteen age- and gender-matched healthy controls (control). The COPD-PR group was recruited through the pulmonary rehabilitation program conducted at Respiratory Rehabilitation and Research Laboratory (Lab3R), School of Health Sciences (ESSUA), University of Aveiro. Patients with COPD were identified by the pulmonologists of the *Centro Hospitalar Baixo Vouga* and physicians from the *Agrupamento dos Centros de Saúde do Baixo Vouga*. The controls were recruited from Castelo de Paiva, and included healthy volunteers, and healthy people attending to social institutions, i.e., *Santa Casa da Misericórdia de Castelo de Paiva*, Castelo de Paiva Day Care Center, Senior University of Castelo de Paiva and *Associação de Solidariedade Social de Souselo.* Permission was granted by the directors of these institutions. The study was explained and detailed information was provided (Annex I) by the researcher (master student) to interested participants and written informed consent was obtained before any data collection (Annex II). Healthy volunteers were eligible if they: had > 40 years old; presenting spirometric values within the expected for their age, height and sex and normal clinical history, and were able to provide informed consent to participate in the study. Patients with COPD were eligible if they: had > 40 years old, diagnosis of COPD according to the guidelines defined by the Global Initiative for Chronic Obstructive Lung Disease [5], and were able to provide informed consent to participate in the study. Participants were excluded if they: had experienced an acute respiratory infection in the month prior to collection of data, present musculoskeletal impairment (e.g. scoliosis, limb amputation), cardiorespiratory, additional to the diagnosis of COPD (e.g.

asthma; heart failure), neurologic (e.g. sclerosis, neuromuscular disease) or cognitive impairment (e.g. dementias), and were bedridden or dependent on a wheelchair. To test the effects of the pulmonary rehabilitation in the COPD-PR group, two data collections were needed, one at baseline, before patients starting the pulmonary rehabilitation (called pre) and another after 12 weeks, in post-pulmonary rehabilitation (named post). Moments of data collection for the other two groups were similar to facilitate comparisons between groups (Figure 8).

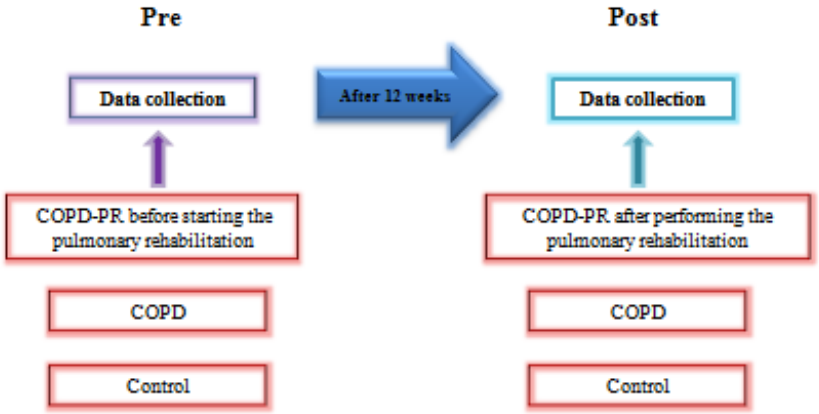

Figure 8. Overall design of the study.

3.3. Pulmonary rehabilitation

The pulmonary rehabilitation attended by the COPD-PR group included exercise training (twice a week from 60 minutes each session) and psychosocial support and education (once every other week for 60 minutes), over a period of 12 weeks.

Exercise training

The exercise training was supervised by a physiotherapist. Sessions of exercise training were composed of: i) A warm-up and a cool-down period, which included range-of-motion, stretching, low-intensity aerobic exercises and breathing techniques (5–10 min); ii) Endurance training (walking or cycling) at 60–80% of the average speed achieved during the 6-min walk test (15–20 min). The training intensity was adjusted according to the participant's symptoms on the modified Borg scale, with a rating of 4–6 on perceived dyspnea/fatigue indicating adequate training intensity; iii) Strength training including 7 exercises (2–3 sets of 10 repetitions) of the major upper and lower limb muscle groups

using free weights and ankle weights (15–20 min). The amount of weight was 50–85% of the 1-repetition maximum. The training progression was based on the two-for-two rule (load was increased when 2 additional repetitions could be performed on 2 consecutive sessions) and on the participant's symptoms (modified Borg scale 4–6); iv) Balance training was based on proprioceptive activities with static and dynamic exercises using upright positions (10 min).

Psychosocial support and education

The psychosocial support aimed to help the family to manage the emotional demands of living with COPD, facilitate the communication within the family and with health/social services, and develop a sense of family identity, improving its cohesion. The education intended to provide information about COPD, increase the skills of the family in order to adjust and manage the disease, and promote adherence to therapy and healthy lifestyles.

Psychosocial support and education was conducted by a multidisciplinary team, including physiotherapist, nurse, gerontologist, psychologist, and clinician. The professionals assumed the role of facilitators by supporting participants in their doubts, encouraging them to share experiences, normalizing emotions, and assuming an empathic attitude. Several didactic methods were used in the sessions, such as group discussions, home tasks, role playing, and brainstorming.

3.4. Outcome measures

A structured questionnaire based on the International Classification of Functional, Health and Disability checklist [118] was used to collect sociodemographic, anthropometric, and objective and subjective clinical data from all participants of the study. Sociodemographic data included age, sex, academic qualifications, marital status and current occupation. General clinical data, smoking habits, physical activity, vital signs and oxygen saturation were collected.

Anthropometric data involved weight and height measurements to calculate the body mass index (BMI) and the body fat percentage (BFP). Dyspnea and fatigue was assessed using the modified Borg scale [119] (see Annex III), activities limitation resulting from dyspnea was assessed with the modified British Medical Research Council (mMRC) questionnaire [120] (see Annex III) and lung function was assessed with a portable

spirometer (MicroLab 3500, CareFusion Corporation, Kent, UK), according to the international guidelines [121]. Quality of life was assessed using the self-reported COPD Assessment Test (CAT) [122] (see Annex III). Participants performed maximal knee extension efforts on dominant leg, during 6 seconds and quadriceps muscle strength (QMS) was measured using a hand-held dynamometer [7] (microFET2TM, Hoggan Health Industries, USA). Respiratory muscle strength, i.e., maximal static inspiratory (MIP) and expiratory mouth pressures (MEP) were measured using a portable mouth pressure meter (MicroRPM™, CareFusion Corporation, USA) [123]. To evaluate the lower limb muscle function and balance, the five-repetition sit-to-stand (5TSS) test was performed [124]. This test measures the time taken to stand five times from a sitting position (using a chair) as rapidly as possible, with the upper limbs crossed on the chest. Participants performed three repetitions of all practical tests, with at least 1 min rest between each repetition.

These tests have been widely applied in patients with COPD and healthy subjects, in order to compare clinical state, limitations in daily life and quality of life among patients and healthy people [5], [123]–[131].

3.5. Blood sample collection - Immunological analysis by characterization of peripheral blood iNKT cells

Blood samples of the COPD and COPD-PR groups were collected by a nurse in the clinical area of the School of Health Sciences, at the University of Aveiro and blood samples of the controls were collected at the *Policlínica de Castelo de Paiva*, *Santa Casa da Misericórdia de Castelo de Paiva*, and *Associação de Solidariedade Social de Souselo*. A nurse collected from each patient 20mL of blood in ethylenediamine tetraacetic acid (EDTA) containing tubes which were sent to the *Instituto de Investigação e Inovação em Saúde* (i3S) to be processed.

3.5.1. Peripheral blood mononuclear cells isolation

Once the blood samples arrived at the lab, peripheral blood mononuclear cells **(**PBMCs) were isolated using Histopaque-1077® (Sigma-Aldrich, St. Louis, MO, USA) by density gradient centrifugation, under sterile conditions. The blood was carefully layered on the same quantity of Histopaque-1077® and centrifuged at 400xg for 30 min at room temperature (RT), without brake. After centrifugation it was possible to distinguish

different layers. PBMCs were located in a ring between the plasma and Histopaque-1077® (Figure 9). Firstly, the plasma was aspirated to a new falcon tube and centrifuged at 2500 rpm for 30 min at 4ºC. Upon plasma centrifugation, the supernatant was stored into a new falcon tube at -20ºC. Secondly, PBMCs were collected and washed once by adding 10mL of phosphate buffered saline 1x (PBS 1x, see appendix II) and centrifuged at 250xg for 10 min at RT. After this centrifugation the PBMCs still contained remaining erythrocytes, which were lysed by incubating cells for 10 min with 10mL of ACK lysis solution (see appendix II). The cells were then washed again by adding 10mL of PBS 1x and centrifuged at 250xg for 10 min at RT. The supernatant was discarded and lastly, cells were washed once more with 10mL of PBS 1x and counted using Trypan Blue to determine PBMCs viability. Cells were diluted in necessary amount of Trypan Blue for each sample and were counted in a cell counting chamber (haemocytometer) using an inverted microscope.

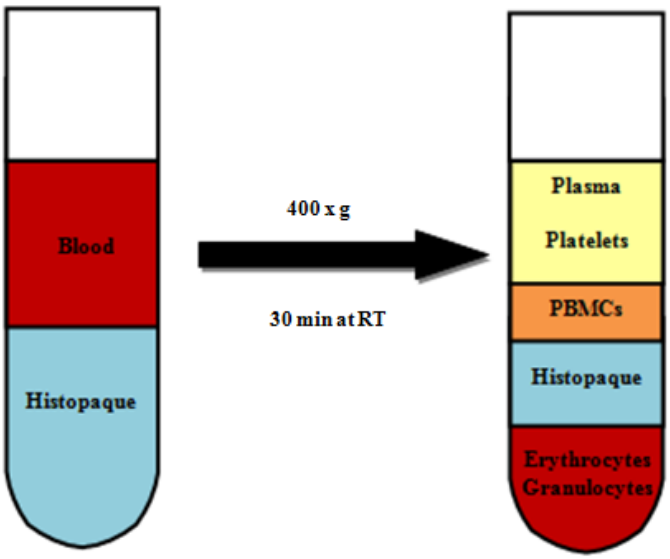

Figure 9. Representative figure of PBMCs isolation using Histopaque-1077®.

PBMCs were frozen in liquid nitrogen for posterior use (all samples of pre: controls, COPD and COPD-PR, and four of post: 3 COPD and 1 COPD-PR) or used immediately (remaining samples) for flow cytometry analysis and/or iNKT cell expansion and stimulation. The samples were frozen because the study was still being prepared and all the antibodies needed for experimental work were not available, as well as, by the fact that I was still in training, in order to learn the techniques used in this laboratory work. Samples of the three studied groups were analyzed by flow cytometry, including iNKT

cells percentage, phenotypic and cytotoxic profiles and only the remaining cells of COPD group and controls were used to perform the iNKT cell expansion, stimulation, and analysis of their cytokine production profile and cytotoxicity by flow cytometry. In the second blood collection (post) all samples of the three groups were used to analyze percentage, phenotype and cytotoxicity of iNKT cells by flow cytometry (Figure 10) so no expansion and subsequent analyzes was done.

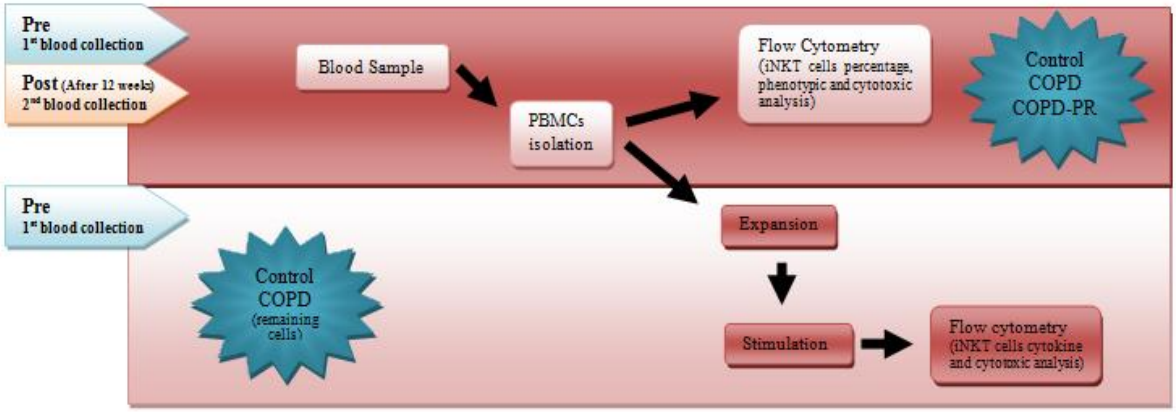

Figure 10. Global scheme of the processing of blood samples.

Thawing PBMCs

Cells which were preserved in liquid nitrogen were later thawed, but no more than 2 cryovials at a time. The cells were melted in a 37ºC water bath and 1mL of RPMI 10%iFBS (see appendix II) it was slowly added. The cells were then quickly transferred to a falcon tube containing 9mL of RPMI 10%iFBS and the empty vial was washed with RPMI 10%iFBS, transferring remaining cells to the falcon tube, which was centrifuged at 1500 rpm, 5 min at RTº. Upon centrifugation, the cells were resuspended in 5mL of RPMI 10%iFBS with 50µg/mL of DNAse and incubated for 15 min at 37ºC, in order to avoid cell clumping. The cells were centrifuged and resuspended once more in 5mL of RPMI 10%iFBS to finally count the cells using Trypan Blue.

3.5.2. iNKT cells percentage, phenotype and cytotoxic analysis by flow cytometry

Extracellular staining (The entire process was made at 4ºC, including centrifugations)

Up to $1x10^6$ PBMCs were stained per well in a round-bottomed 96-well plate and centrifuged at 1200 rpm, for 2 min. The supernatant was rejected and cells were resuspended in the vortex and washed with 200µl of

PBS/2%FBS/1%PenStrep/0.01%NaN_3 (flow cytometry solution, see appendix II). Again the plate was centrifuged, the supernatant was rejected and the cells were once more resuspended in the vortex and stained with 25µl of the antibody/tetramer mix diluted in flow cytometry solution for 20 min, at 4ºC, in the dark. After incubation, the cells that will be stained intracellularly were washed twice by adding 100-200µl of flow cytometry solution, centrifuged at 1200 rpm for 2 min, the supernatant were rejected and the cells were resuspended in the vortex. The remaining cells were washed once more and fixed by adding 200µl of PBS 1%formaldehyde (see appendix II) and transferred to FACS tubes (Falcon, BD Bioscience) also containing 200µl of PBS 1%formaldehyde. PBMCs were kept at 4ºC in the dark until their acquisition on the flow cytometer.

Intracellular staining (The entire process was made at RT, including centrifugations)

Upon extracellular staining and two washings, cells were fixed by incubating with PBS 2%formaldehyde for 10 min, at RT, in the dark. Then, cells were centrifuged at 1200 rpm for 2 min, at RT, the supernatant was discarded and PBMCS were permeabilized by incubating with 0.5%Saponin (Sigma-Aldrich, St. Louis, MO, USA) diluted in flow cytometry solution (see appendix II), for 5 min, in the dark. After incubation, cells were centrifuged again at 1200 rpm for 2 min, supernatant was discarded and PBMCs were resuspended in 25µl of fluorochrome-labeled antibody diluted in flow cytometry solution with 0.5%saponin. After 30 min of incubation, at RT, in the dark, cells were washed twice with 0.5%saponin diluted in flow cytometry solution and once just with flow cytometry solution. The supernatant was always discarded and the cells were resuspended in 200µl of PBS 1x and transferred to FACS tubes also with 200µl of PBS 1x.

3.5.3. iNKT cell expansion, stimulation and analysis of their cytokine production profile

Remaining cells from the flow cytometry staining were used in iNKT cell expansion by culturing $1x10^6$ PBMCs per well in 2mL of RPMI 10%iFBS medium with 100ng/mL of α-GalCer (Avanti polar lipids, Alabaster, AL, USA) at a concentration $0.5x10^6$cells/mL in a 24-well plate. At day 1 of culture, 50U/mL of recombinant human IL-2 (kindly provided by the National Cancer Institute, NCI, USA) was added to each well. On days 11-14 of expansion, cells were collected, counted and stimulated by culturing

$1x10^6$ cells/well in a round-bottomed 96-well plate in 200µl of RPMI 10%iFBS with 25ng/mL Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA), 1µg/mL Ionomycin (Sigma-Aldrich, St. Louis, MO, USA) and 10µg/mL Brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) for 5h before staining with cytokine-specific monoclonal antibodies. Cells for the compensation necessary to the acquisition on the flow cytometer were not stimulated.

3.5.4. iNKT cell cytokine production profile and cytotoxic analysis by flow cytometry

After expansion and stimulation cells were stained as described previously in point 3.5.2; however the used staining mixtures were different.

Antibodies used in flow cytometry

All the used antibodies in flow cytometry are listed in Table 3. The human CD1d tetramer used was loaded with PBS57 and labelled with the fluorochrome phycoerythrin (PE), from the National Institute of Health tetramer core facility.

All samples were acquired in a BD FACS Canto™ II flow cytometer, using the BD FACSDiva™ software (BD Biosciences, San Diego, CA, USA). Flow cytometry analyses were performed using the FlowJo software (FlowJo LLC, Ashland, OR, USA).

Table 3. Antibodies used in flow cytometry.

Antibody	Clone	Fluorochrome	Brand
Anti-human CD3	OKT3	PerCPCy5.5	eBioscience
Anti-human CD56	MEM-188	FITC	eBioscience
Anti-human CD158b	DX27	PECy7	Biolegend
Anti-human CD69	FN50	APC	eBioscience
Anti-human CD8	RPA-T8	APCeFluor780	eBioscience
Anti-human CD161	HP-3G10	eFluor450	eBioscience
Anti-human CD4	OKT4	bv510	Biolegend
Anti-human CD107a	H4A3	PECy7	Biolegend
Anti-human Granzyme B	GB11	Alexa647	Biolegend
Anti-human IFNgamma	4S.B3	PECy7	eBioscience
Anti-human IL-4	8D4-8	APC	eBioscience
Isotype Control (Mouse IgG1 K)	P3.6.2.8.1	PECy7	eBioscience
Isotype Control (Mouse IgG1 K)	P3.6.2.8.1	APC	eBioscience

PerCPCy5.5 – Peridinin chlorphyll protein conjugated with Cy5.5; FITC – Fluorescein Isothiocyanate; PECy7 – Phycoerythrin conjugated with Cy7; APC – Allophycocyanin; APCeFluor 780 – Allophycocyanin conjugated with eFluor 780; bv510 – Brilliant Violet 510; Alexa647 – Alexa Fluor®

3.6. Statistical analysis

A code was assigned to each participant and the data was treated anonymously. Statistical analysis was performed using SPSS Statistics 22 (IBM Corporation, Armonk, New York) and graphs were plotted in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). The normality of data distribution was investigated with Shapiro-Wilk test and level of significance considered was set at $p<0.05$.

Outcome measures analysis

Descriptive statistics were used to describe the sample using mean and standard deviation for continuous data (i.e., age), median and interquartile range for ordinal (i.e., CAT and mMRC) and continuous non-normally distributed data (i.e., BMI, BFP, pulmonary function, MIP, MEP, QMS and 5TSS) and frequencies and percentages for categorical data (i.e., gender, academic qualification, marital status, occupation, smoking

habits and spirometric classification of COPD). Kruskal-Wallis test was used to compare ordinal/continuous non-normally distributed data among groups. When significant differences were observed between groups, Mann-Whitney U test was used for multiple comparisons. Mann-Whitney U test was also used to compare QMS between COPD and COPD-PR groups. Chi-Square test was used for categorical measures.

Immunological analysis

Mean and standard deviation values were calculated and statistical significance was assessed by Unpaired T-test (for continuous normally distributed data) or Mann-Whitney U and Wilcoxon matched-pairs signed rank tests (for continuous non-normally distributed data). Unpaired T-test and Mann-Whitney U test was used to compare data between groups and Wilcoxon matched-pairs signed rank test was used to compare data from patients of COPD-PR between pre and post-pulmonary rehabilitation.

Correlations analysis

Correlations between clinical (i.e., MIP, MEP, 5TSS and pulmonary function) and immunological variables (i.e., T lymphocytes, iNKT cells, NK cells and cytotoxic markers) were performed using Spearman's non-parametric rank correlation coefficient. The choice of these clinical variables for performed correlations is justified because they are the best measures addressed in this study, which characterize the COPD. The strength of the correlations was assessed using the criteria suggested by Mukaka et al. [132], (i.e., 0-0.30 negligible correlation; 0.31-0.50 poor correlation; 0.51-0.70 moderate correlation; 0.71-0.90 high correlation and 0.91-1 very high correlation).

4. Results

4.1. Sample characterization

Table 4 shows the characteristics of all participants. Eleven patients with chronic obstructive pulmonary disease and fourteen healthy age- and gender-matched controls were included in this study. However, in the group of patients with COPD, we had two distinct groups; i.e., COPD group (n=7) composed by patients that were never treated with pulmonary rehabilitation, and the COPD-PR group (n=4), composed by patients that were naïve at the start of the study but performed pulmonary rehabilitation during 12 weeks during the course of the study. The control group was composed by nine males and five females and had a mean age of 64.2 years old (standard deviation of ± 9.6 years); the COPD group was composed by five males and two females and had a mean age of 62 years old (standard deviation of ± 12.7 years); and the COPD-PR group was composed by three males and one female and had a mean age of 65.3 years old (standard deviation of ± 4.3 years). From the 1st to the 2nd clinical assessment, we had two dropouts (one participant of the COPD group and one of the control group), thus twenty-three participants completed the clinical assessments. No significant differences in terms of age, gender, academic qualifications, marital status, occupation, smoking habits, body mass index (BMI) and body fat percentage (BFP) were found between the studied groups (Table 4). Lung function parameters, i.e., forced expiratory volume in one second (FEV_1) predicted and ratio of forced expiratory volume in one second and forced vital capacity (FEV_1/FVC, Tiffeneau index) were significantly different ($p<0.05$) from both groups of patients with COPD and age- and gender-matched controls, who displayed higher median values, as well as, higher values of interquartile range. The spirometric classification of COPD presented no significant difference between the two groups of patients.

Table 4. Sociodemographic and anthropometrical characterization of the studied groups.

Characteristics	Control (n=14)	COPD (n=7)	COPD-PR (n=4)	p[a] value
Age (years)	64.2 ± 9.6	62.0 ± 12.7	65.3 ± 4.3	0.849
Gender, n (%)				
Male	9 (64.3)	5 (71.4)	3 (75)	0.897
Female	5 (35.7)	2 (28.6)	1 (25)	
Academic qualifications, n (%)				
No qualifications	0	0	0	0.052
1st Basic Education Cycle	5 (35.7)	3 (42.9)	1 (25)	
2nd Basic Education Cycle	2 (14.3)	1 (14.3)	0	
3rd Basic Education Cycle	1(7.1)	2 (28.6)	1 (25)	
High school	3 (21.4)	0	0	
Medium course	0	0	2 (50)	
University degree	3 (21.4)	0	0	
Marital status, n (%)				
Married/Cohabiting	9 (64.3)	4 (57.1)	3 (75)	0.906
Widowed	2 (14.3)	1 (14.3)	0	
Separated/Divorced	1 (7.1)	1 (14.3)	1 (25)	
Single	2 (14.3)	1 (14.3)	0	
Occupation, n (%)				
Employed	5 (35.7)	2 (28.6)	0	0.373
Retired	9 (64.3)	5 (71.4)	4 (100)	
Unemployed	0	0	0	
Smoking habits, n (%)				
Never	6 (42.9)	2 (28.6)	1 (25)	0.668
Former	6 (42.9)	2 (28.6)	2 (50)	
Current	2 (14.3)	3 (42.9)	1 (25)	
BMI (kg/m^2)	24.3 [22.1 to 27]	25.8 [22.1 to 27.8]	29.8 [27.7 to n/a]	0.053
BFP (%)	28.9 [20 to 34.3]	28.1 [20.4 to 34.3]	33.1 [26.1 to n/a]	0.602
Pulmonary function				
FEV_1 predicted (%)	97.5 [91.5 to 116.5]†	66 [36 to 78]	75 [73 to 79.3]	0.000*
FVC predicted (%)	101 [90 to 118.8]	87 [74 to 97]	102 [87 to 106.5]	0.055
FEV_1/FVC	77 [74 to 80.5]†	64 [38 to 67]	57 [55 to 69.8]	0.001*
Spirometric classification of COPD, n (%)				
GOLD A (Mild)	---	4 (57.1)	3 (75)	0.309
GOLD B (Moderate)	---	0	1 (25)	
GOLD C (Severe)	---	2 (28.6)	0	
GOLD D (Very severe)	---	1 (14.3)	0	

[a] Kruskal-Wallis test used for continuous non-normally distributed measures; Chi-Square test used for categorical measures.

COPD: Chronic Obstructive Pulmonary Disease; COPD-PR: Chronic Obstructive Pulmonary Disease-Pulmonary Rehabilitation; BMI: Body Mass Index; BFP: Body Fat Percentage; FEV_1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity; FEV_1/FVC: Tiffeneau index; GOLD: Global Initiative for Chronic Obstructive Lung Disease; n/a: not applicable.

Data are presented as Mean values ± Standard deviation or Median values [interquartile range] unless otherwise indicated. *p <0.05. † significantly different from COPD and COPD-PR.

4.2. Effect of pulmonary rehabilitation on clinical variables in patients with chronic obstructive pulmonary disease

Results from the clinical variables obtained in the three studied groups, in the 1st clinical assessment (baseline/pre) and the 2nd clinical assessment (post-pulmonary rehabilitation) are shown in table 5. Only COPD Assessment Test (CAT) presented significant changes ($p<0.05$), when compared COPD and COPD-PR groups to healthy controls. In the control group this clinical measure did not present differences between the 1st/pre and the 2nd/post clinical assessment, while the COPD and COPD-PR groups presented different values with time. No other significant changes were observed between groups.

Table 5. Clinical variables for chronic obstructive pulmonary disease in the studied groups taking into account the time (pre and post).

Clinical Measures	Control		COPD		COPD-PR		Mean difference for control	Mean difference for COPD	Mean difference for COPD-PR	p value
	Pre	Post	Pre	Post	Pre	Post				
QMS (Kgf)	---	---	20.7 [16.5 to 22]	15.5 [10 to 21.7]	21.7 [21 to n/a]	13.1 [10.6 to 16.4]	---	-3.2 [-9.8 to 3.1]	-7.6 [-8.9 to n/a]	0.364 [a]
MIP (cmH_2O)	84 [73 to 93.5]	85 [64 to 93]	68 [44 to 82]	92 [73.8 to 105.3]	94 [58 to 103.8]	75 [48.8 to 114.8]	1 [-7 to 12.5]	14.5 [8.8 to 37.8]	-1.5 [-33 to 17.3]	0.074
MEP (cmH_2O)	122 [87.8 to 132.3]	114 [93.5 to 142]	128 [86 to 172]	149 [95.8 to 164]	108 [104.3 to 148.5]	123 [82 to 132.5]	-4 [-13 to 11.5]	6.5 [-7.3 to 19.5]	-11 [-40 to 28.3]	0.595
5TSS (s)	6.5 [5.3 to 7.2]	6.2 [5 to 8.2]	6.6 [5.3 to 8.9]	7.9 [5.3 to 8.9]	6.1 [4.3 to 10.1]	6.7 [5.1 to 7.9]	0.1 [-0.1 to 0,8]	0.4 [-1.1 to 1.9]	0.6 [-2.8 to 1.5]	0.772
CAT	0 [0 to 1.5]	0 [0 to 1.5]	8 [4 to 9]	6.5 [4.5 to 12.7]	8.5 [4.3 to 16.5]	11.5 [5.3 to 20]	0 [-0.5 to 0]†	2.5 [-3.8 to 7.5]	2 [1 to 4.5]	0.044*
mMRC	0 [0 to 0]	0 [0 to 0]	1 [1 to 1]	1 [0 to 1.3]	1 [0.3 to 1.8]	1 [0.3 to 1.8]	0 [0 to 0]	-0.5 [-1 to 0.3]	0 [0 to 0]	0.192
Pulmonary function										
FEV_1 predicted (%)	97.5 [91.5 to 116.5]	100 [89 to 118]	66 [36 to 78]	65 [40.8 to85]	65 [40.8 to 85]	75 [73 to 79.3]	1 [-5 to 4]	-1.5 [-7.3 to 5.5]	-14.5 [-24.5 to -3.8]	0.111
FVC predicted (%)	101 [90 to 118.8]	108 [102 to 123.5]	87 [74 to 97]	84 [79 to 101]	102 [87 to 106.5]	96 [89 to 99.3]	3 [-6 to 9]	1 [-5.3 to 5.3]	-6.5 [-14 to 9.3]	0.510
FEV_1/FVC	77 [74 to 80.5]	76 [73.5 to 79.5]	64 [38 to 67]	62.5 [40.8 to 65]	57 [55.5 to 69.8]	57 [49 to 65]	-1 [-4.5 to 2]	-0.5 [-5.8 to 3]	-5 [-13.3 to 7]	0.765

Note: Comparisons were performed using Kruskal-Wallis test (continuous non-normally distributed measures), unless otherwise stated.

[a] Mann-Whitney U test used for comparisons between mean difference for COPD and COPD-PR.

COPD: Chronic Obstructive Pulmonary Disease; COPD-PR: Chronic Obstructive Pulmonary Disease-Pulmonary Rehabilitation; QMS: Quadriceps Muscle Strength; MIP: Maximal Inspiratory Pressure; MEP: Maximal Expiratory Pressure; 5TSS: Five Time Seat to Stand; CAT: COPD Assessment Test; mMRC: modified Medical Research Council; FEV_1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity; FEV_1/FVC: Tiffeneau index; n/a: not applicable.

Data are presented as Median value [interquartile range]. *p <0.05. †significantly different from COPD and COPD-PR.

4.3. Immunological analysis by characterization of peripheral blood iNKT cells in patients with chronic obstructive pulmonary disease

To study the iNKT cells in the peripheral blood of patients with chronic obstructive pulmonary disease (COPD), PBMCs were stained with an antibody anti-human CD3 and the human PBS57-loaded CD1d tetramer and analyzed by flow cytometry. After defining the lymphocyte gate in the side scatter and forward scatter plot, T cells ($CD3^+$) were selected and the percentage of CD1d-PBS57 tetramer positive cells (iNKT cells) within this gate was determined (Figure 11).

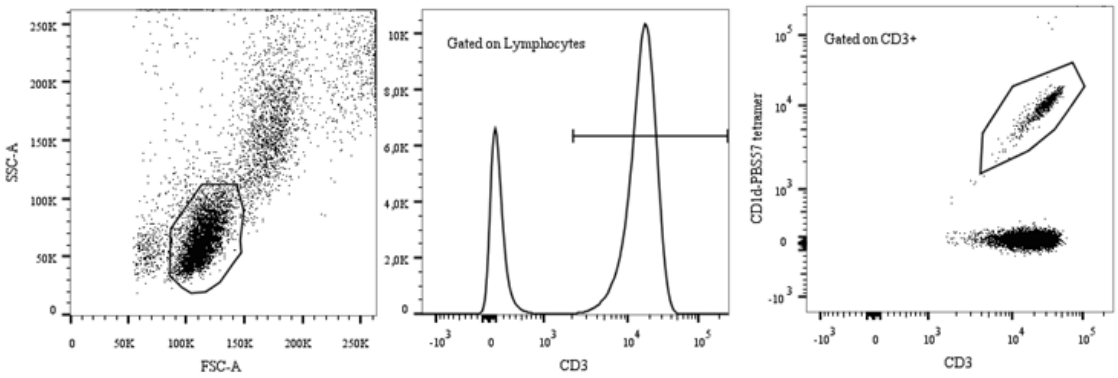

Figure 11. Gating strategy used for the analysis of the percentage of iNKT cells by flow cytometry.

In humans, the iNKT cell population can be divided in three subsets according to the expression of CD8 and CD4 molecules: $CD4^-CD8^+$ ($CD8^+$), $CD4^+CD8^-$ ($CD4^+$), and $CD4^-CD8^-$ (DN) [73], [74]. Figure 12 shows a representative example of the gating strategy used to define the CD8/CD4/DN iNKT subsets.

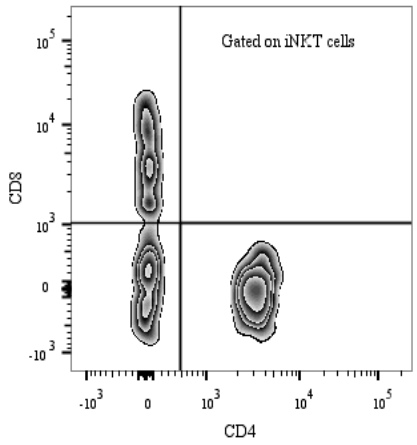

Figure 12. Gating strategy used for the analysis of iNKT CD4/CD8/DN cell subsets percentage, determined by flow cytometry.

Besides CD4 and CD8 expression, iNKT cells also express a variety of molecular markers, such as CD161, CD56 and CD69 (Figure 13). The expression of these markers by iNKT cells was also analyzed in patients with COPD.

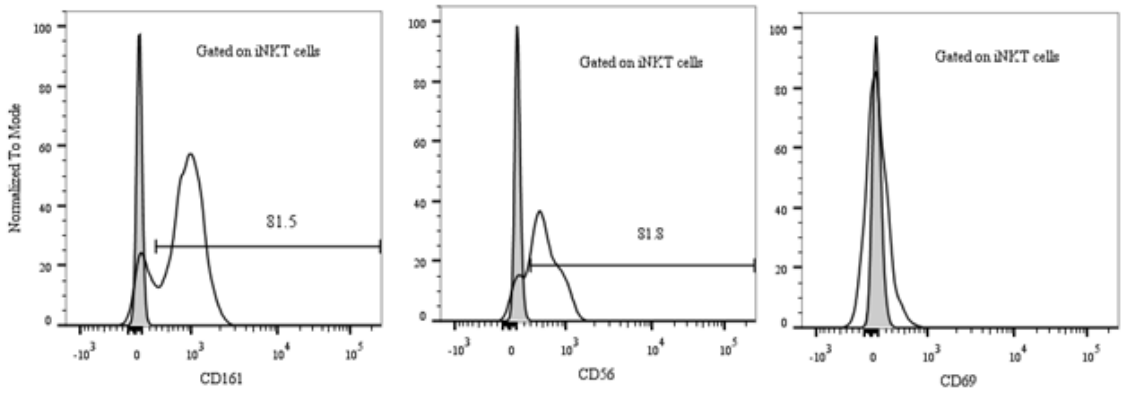

Figure 13. Gating strategy used to analyze the expression of CD161, CD56 and CD69 on iNKT cells by flow cytometry. Tinted line corresponds to unstained and black line to stained sample.

In non-activated iNKT cells, the expression of CD69 is low, and it is not possible to distinguish a positive and a negative population. Therefore, mean fluorescence intensity (MFI) was used to measure CD69 expression on iNKT cells.

To study the cytotoxicity of iNKT cells, the following markers were used: CD158b, CD107a and Granzyme B. The gating strategy to analyze these markers is shown in Figure 14.

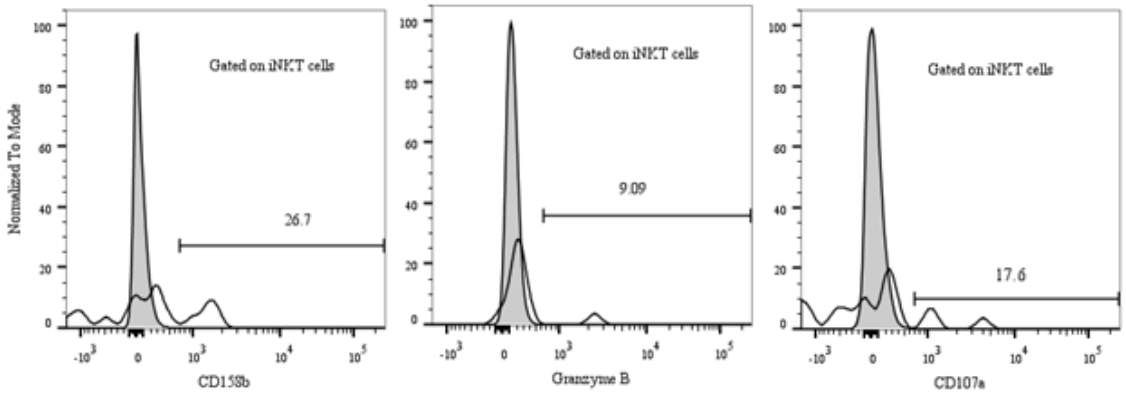

Figure 14. Gating strategy used for the analysis of cytotoxic markers in iNKT cells, determined by flow cytometry. Tinted line corresponds to unstained and black line to stained sample.

We also analyze NK cells ($CD3^{-}CD56^{+}$), as well as their expression of CD158b (Figure 15).

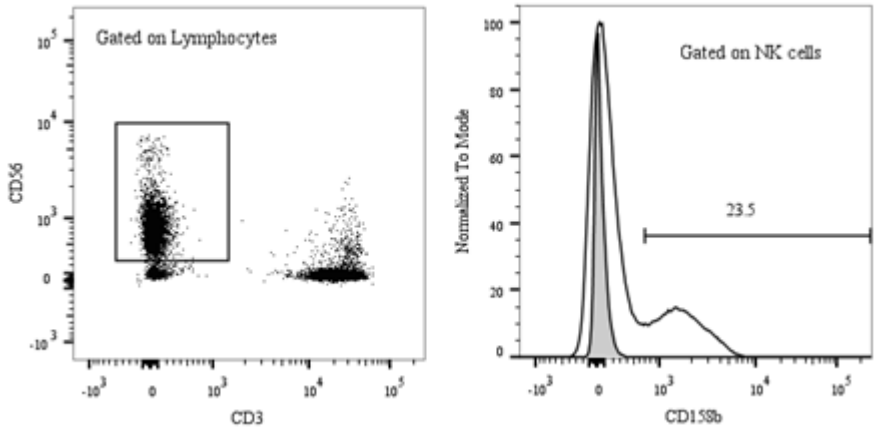

Figure 15. Gating strategy used for the analysis of the percentage of NK cells and the expression of CD158b on these cells, determined by flow cytometry. NK cells were identified among peripheral lymphocytes and defined based on the expression of CD56 and the lack of CD3. Tinted line corresponds to unstained and black line to stained sample.

4.3.1. Analysis of iNKT cells in chronic obstructive pulmonary disease

The *ex vivo* blood iNKT cell percentage, iNKT subsets and markers of cytotoxicity were analyzed in patients with COPD (n=11) and age- and gender-matched controls (n=14) by flow cytometry. Since in the 1st blood collection, none of the patients with COPD had started pulmonary rehabilitation, we analyzed the COPD and COPD-PR groups as a single group. To address the function of iNKT cells, their capacity to produce cytokines and to express cytotoxic markers after stimulation was also analyzed.

The percentage of iNKT cells did not seem to modify in chronic obstructive pulmonary disease patients' peripheral blood

As expected, we found a high variation in the percentage of iNKT cells in the peripheral blood of subjects (Table 6 and Figure 16). However, no significant differences were observed in the percentage of iNKT cells, when patients with COPD were compared to age- and gender-matched controls (Figure 16). NK cell percentage was also determined and no significant differences were found (Table 6).

Table 6. Statistical results of T lymphocyte, iNKT cell subsets and NK cells in patients with chronic obstructive pulmonary disease and age- and gender-matched controls.

%[a,b] / MFI[c]	Control (n=14)	COPD (n=11)	p value
iNKT[a]	0.06±0.06	0.89±2.76	0.600[d]
iNKT CD4^{+}[b]	39.58±24.62	34.73±28.66	0.653[e]
iNKT CD8^{+}[b]	16.53±16.67	27.43±16.99	0.122[e]
iNKT DN[b]	42.67±19.23	32.23±16.66	0.167[e]
iNKT CD161^{+}[b]	81.61±81.61	66.65±28.34	0.124[d]
iNKT CD56^{+}[b]	13.92±21.02	18.61±17.75	0.250[d]
iNKT CD69^{+}[c]	215.89±149.31	160.41±152.06	0.046[d]*
CD4^{+}[a]	64.71±10.89	63.42±8.41	0.749[e]
CD8^{+}[a]	30.21±9.68	29.46±7.05	0.833[e]
NK[a]	17.41±9.43	22.36±11.37	0.246[e]

[a] Percentage among lymphocytes; [b] Percentage among iNKT cells; [c] mean fluorescence intensity; [d] Mann-Whitney U test; [e] Unpaired T-test.

COPD: Chronic Obstructive Pulmonary Disease; iNKT: invariant Natural Killer T cell; NK: Natural Killer cell. Data are presented as Mean values ± Standard deviation; *p <0.05.

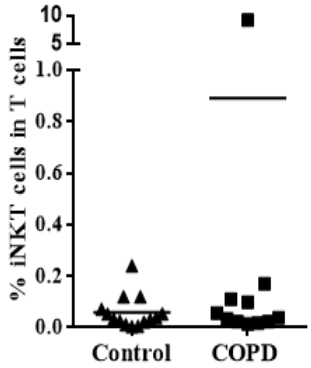

Figure 16. iNKT cells percentage among T lymphocytes in the peripheral blood of age- and gender-matched controls and patients with COPD. iNKT cells were identified in the gate of T cells (CD3^{+}) as positive for CD1d-PBS57 tetramer, by flow cytometry. Horizontal bars represent means.

Patients with COPD did not seem to present major alterations in **iNKT cell subsets:** only a decrease in the expression of the activation marker CD69 in iNKT cells

It has been shown that iNKT cells are a heterogeneous population composed by functionally distinct subsets. The frequencies of iNKT CD8^{+}, CD4^{+} and DN cells in

patients with COPD and controls are represented in Figure 17. Despite the absence of statistically significant alterations in these three iNKT cell subsets between patients with COPD and age- and gender-matched controls, there was a tendency for higher iNKT $CD8^+$ cells percentage in the COPD group. Conversely, iNKT DN cells in patients tended to have lower percentages than controls (Figure 17).

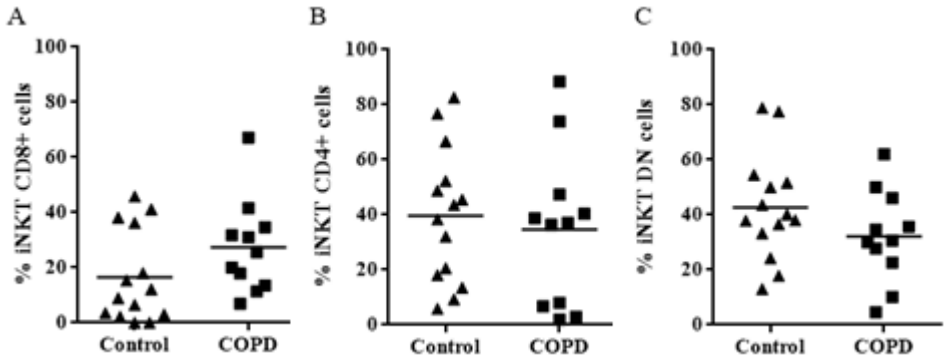

Figure 17. Percentage of iNKT cell subsets among peripheral blood iNKT cells from age- and gender-matched controls and patients with COPD. A, B, C – iNKT $CD8^+$ (A) or $CD4^+$ (B) or DN (C) cells percentage.

As a control, we also analyzed the percentage of total $CD4^+$ and $CD8^+$ T cells (Table 6). Like for iNKT cells, no significant alterations were observed when patients with COPD were compared to controls (Table 6).

The analysis of the percentage of iNKT cells expressing CD161 and CD56 did not present statistically significant differences (Table 6).

CD69 is known to be constitutively expressed in iNKT cells. We analyzed the expression of this activation marker on iNKT cells and we found that patients with COPD presented a lower expression ($p<0.05$), as determined by, mean fluorescence intensity of this molecule (Figure 18).

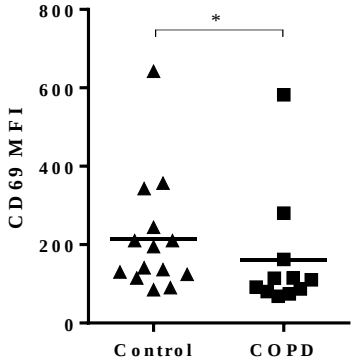

Figure 18. Mean fluorescence intensity of CD69 on iNKT cells in the peripheral blood of age- and gender-matched controls and patients with COPD. Horizontal bars represent means. Mann-Whitney U test was used to determine statistical significance *p<0.05 among controls and patients with COPD.

Expression of **cytotoxic markers on iNKT cells** in patients with COPD: decrease in the iNKT DN cell subset expressing CD107a

The cytotoxic capacity of iNKT cells was also inferred by the study of different cytotoxic markers. The expression of CD158b, CD107a and Granzyme B in total $CD8^+$ T cells and iNKT cells was analyzed. $CD8^+$ T cells did not present significant differences in the expression of cytotoxic markers (data not shown). As shown in Figure 19, we verified that the percentage of iNKT cells expressing CD158b, CD107a and Granzyme B did not display statistically significant differences between COPD group and age- and gender-matched controls. Nevertheless, patients with COPD tended to have a lower percentage of $CD107a^+$ iNKT cells than controls. In both studied groups, Granzyme B expression was very low (Figure 19).

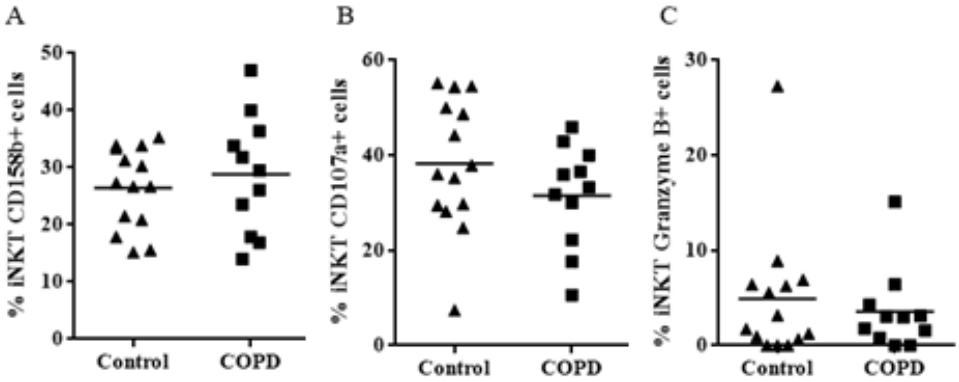

Figure 19. Percentage of iNKT cells expressing cytotoxic markers in the peripheral blood of age- and gender-matched controls and patients with COPD. A, B, C – Percentage of iNKT cells expressing CD158b (A) or CD107a (B) or Granzyme B (C). Horizontal bars represent means.

These cytotoxic markers were also analyzed in iNKT CD4/CD8/DN cell subsets. Only in the iNKT DN cell subset there was a statistically significant decrease ($p<0.05$) in the percentage of cells expressing CD107a in patients with COPD when compared to controls (Figure 20).

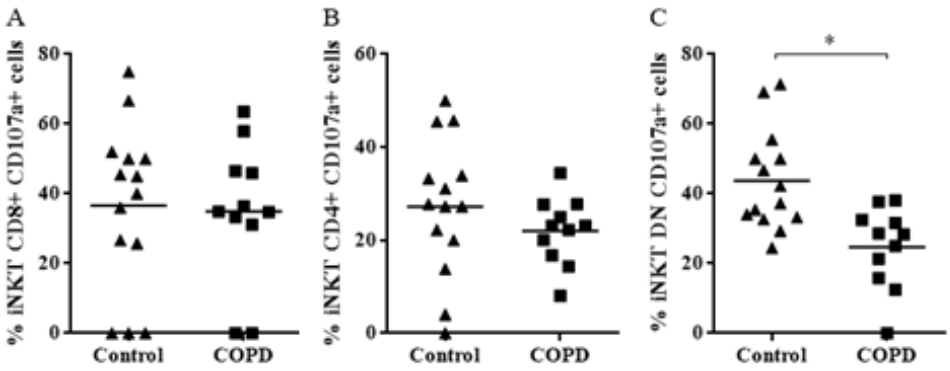

Figure 20. Percentage of iNKT cell subset expressing CD107a in the peripheral blood of age- and gender-matched controls and patients with COPD. A, B, C – Percentage of iNKT $CD8^+$ (A) or $CD4^+$ (B) or DN (C) cells expressing CD107a. Horizontal bars represent means. Unpaired T-test was used to determine statistical significance *$p<0.05$ among controls and patients with COPD.

We also verified the expression of CD158b in NK cells, but no significant alterations were found between patients and healthy controls (data not shown).

No significant alterations were observed in **cytokine production by iNKT cells** in patients with COPD

Due to the low number of iNKT cells in the peripheral blood, testing cytokine production *ex vivo* would require a large amount of blood. Thereby, iNKT cells from PBMCs were expanded, by culturing them for 11-14 days in the presence of α-GalCer and IL-2. The expanded cells were then stimulated with PMA/Ionomycin in the presence of Brefeldin A and the amount of IFN-γ or/and IL-4 producing cells was determined by flow cytometry (Figure 21).

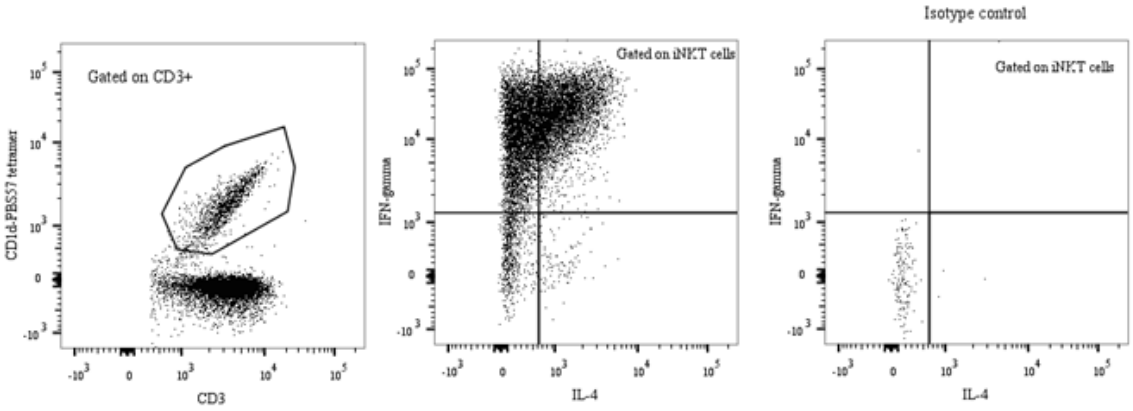

Figure 21. Gating strategy used to determine the percentage of iNKT cells producing cytokines after stimulation with PMA/Ionomycin in the presence of Brefeldin A.

Patients with COPD presented no significant alterations in the percentage of iNKT cells producing cytokines, although a tendency for a reduction of IL-4 production is present (Figure 22).

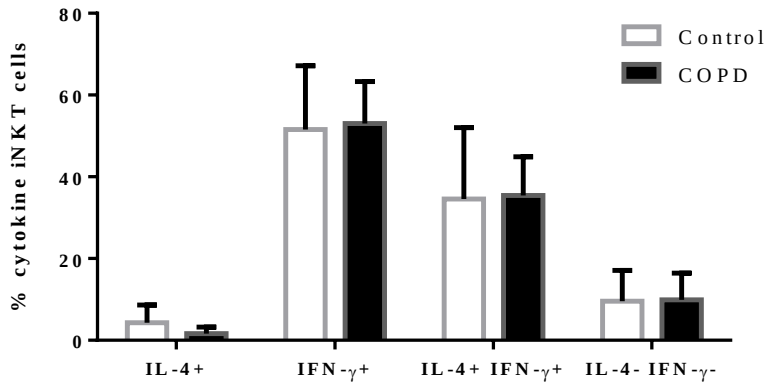

Figure 22. Response of iNKT cells from patients with COPD to PMA/Ionomycin/Brefeldin A stimulation. Expanded iNKT cells were stimulated with PMA+Ionomycin in the presence of Brefeldin A for 5h. The percentage of cells producing IL-4 or/and IFN-γ in age- and gender-matched controls and patients with COPD was determined by flow cytometry. Experiments performed with thawed PBMCs. Results are represented as Mean values ± Standard deviation of 6 patients with COPD and 10 age- and gender-matched controls.

No significant alterations were observed in the **cytotoxicity of iNKT cells after stimulation** in patients with COPD

An important focus of this study was iNKT cell cytotoxicity. Since Granzyme B is known to be upregulated after stimulation, the percentage of Granzyme B in expanded and stimulated iNKT cells was determined by flow cytometry. We found no significant

alterations in percentage of iNKT cells expressing Granzyme B, however, a tendency for a reduction was observed in patients with COPD (Figure 23).

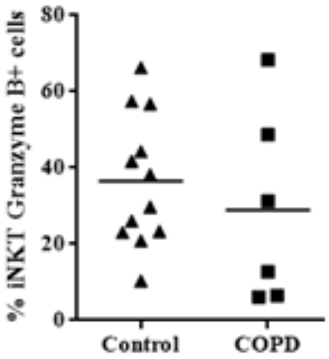

Figure 23. Response of iNKT cells from patients with COPD to PMA/Ionomycin/Brefeldin A stimulation. Expanded iNKT cells were stimulated with PMA+Ionomycin in the presence of Brefeldin A for 5h. Percentage of iNKT cells expressing Granzyme B. Experiments performed with thawed PBMCs. Horizontal bars represent means.

4.3.2. Effect of pulmonary rehabilitation on iNKT cells in patients with chronic obstructive pulmonary disease

To determine the effect of pulmonary rehabilitation of patients with COPD in iNKT cells, two analyses were conducted: baseline/pre (1st blood collection) and post-pulmonary rehabilitation (2nd blood collection). Three distinct groups of participants were defined, i.e., patients with COPD who performed pulmonary rehabilitation (COPD-PR), patients with COPD that were never treated with pulmonary rehabilitation (COPD) and age- and gender-matched controls. We had one dropout from the 1st to the 2nd blood collections (the same patient with COPD who did not complete the clinical assessments), thus a total of twenty-four participants completed the study.

The percentage of iNKT cells was not altered in the peripheral blood of patients with chronic obstructive pulmonary disease after the pulmonary rehabilitation

To access the effect of pulmonary rehabilitation on the iNKT cells of patients with COPD, we compared the percentage of iNKT cells between COPD and COPD-PR groups in post-pulmonary rehabilitation (Figure 24A). No statistically significant alterations were observed between groups. In addition we compared the percentage of iNKT cells within

the COPD-PR group before and after pulmonary rehabilitation. Similarly, no significant differences were observed (Figure 24B).

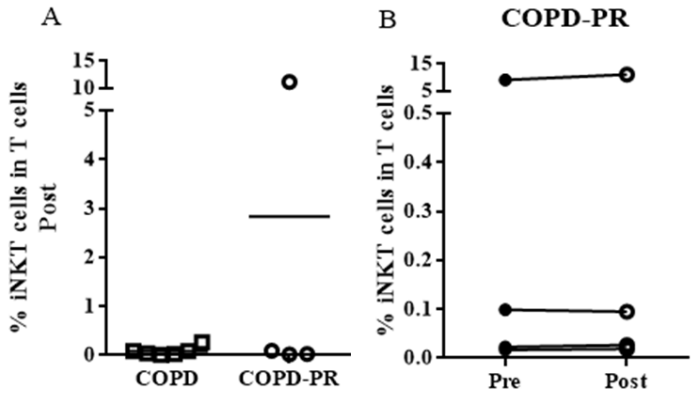

Figure 24. Effect of pulmonary rehabilitation on the percentage of iNKT cells among T lymphocytes in the peripheral blood. A – iNKT cells percentage in COPD and COPD-PR groups in the post analysis; B – iNKT cell percentage in patients from the COPD-PR group, before (pre) and after (post) performing pulmonary rehabilitation. iNKT cells were identified in the gate of T cells ($CD3^+$) as positive for CD1d-PBS57 tetramer, by flow cytometry. Horizontal bars represent means.

No significant alterations were observed in the **iNKT cell subsets** in patients with COPD after the pulmonary rehabilitation

We also analyzed the effect of pulmonary rehabilitation in iNKT cell subsets. An absence of significant alterations in the percentage of iNKT cell subsets between the two groups was observed, as well as between pre and post-pulmonary rehabilitation in COPD-PR group (Figure 25). However, patients in COPD-PR group presented a tendency for a reduction in the percentage of iNKT $CD8^+$ cells (Figure 25D), which did not reach statistical significance.

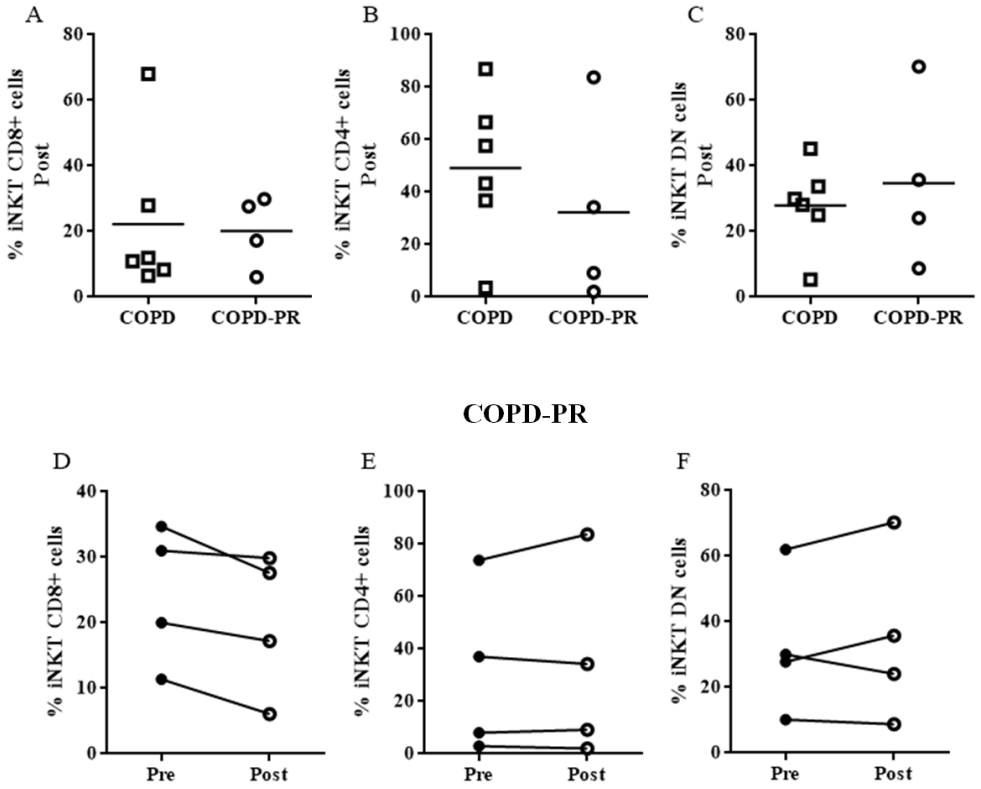

Figure 25. Effect of pulmonary rehabilitation on the percentage of iNKT cell subsets in the peripheral blood. A, B, C – Percentage of iNKT $CD8^+$ (A), or $CD4^+$ (B), or DN (C) cells in COPD and COPD-PR groups in the post analysis; D, E, F – Percentage of iNKT $CD8^+$ (D), or $CD4^+$ (E), or DN (F) cells in patients of the COPD-PR group, before (pre) and after (post) performing pulmonary rehabilitation. Horizontal bars represent means.

Similarly, no statistically significant changes were observed in the percentage of iNKT cells expressing CD56 and CD161, between COPD and COPD-PR groups (Figure 26A and B), neither before nor after pulmonary rehabilitation of the COPD-PR group (Figure 26C and D). Nevertheless, patients who performed pulmonary rehabilitation tended to have higher a percentage of iNKT $CD161^+$ cells and a lower percentage of iNKT $CD56^+$ cells (Figure 26C and D, respectively).

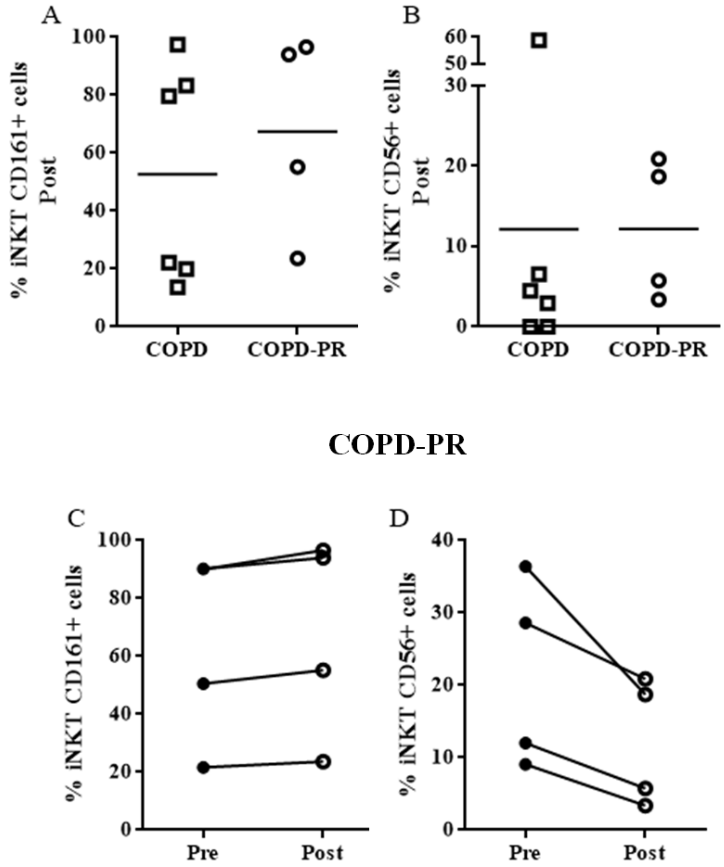

Figure 26. Effect of pulmonary rehabilitation on the percentage of $CD161^+$ and $CD56^+$ iNKT cells in the peripheral blood. A, B – Percentage of iNKT cells expressing CD161 (A) or CD56 (B) in COPD and COPD-PR groups in the post analysis; C, D – Percentage of iNKT cells expressing CD161 (C) or CD56 (D) in COPD-PR group, before (pre) and after (post) performing pulmonary rehabilitation. Horizontal bars represent means.

Pulmonary rehabilitation did not seem to affect the CD69 decrease observed in patients with COPD

Since CD69 expression was decreased in patients with COPD, we wanted to see if pulmonary rehabilitation could improve this defect. We found no statistically significant differences between patients from the COPD-PR group and the COPD group (Figure 27A), neither between pre and post-pulmonary rehabilitation in patients from the COPD-PR group (Figure 27B).

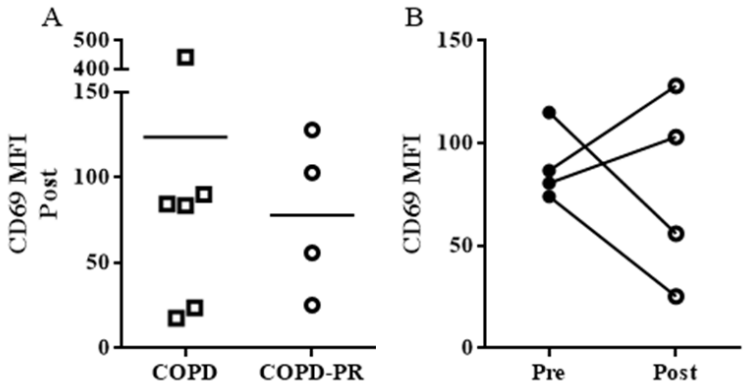

Figure 27. Effect of pulmonary rehabilitation on the mean fluorescence intensity of CD69 on iNKT cells in the peripheral blood. A – Mean fluorescence intensity of iNKT cells expressing CD69 in COPD and COPD-PR groups in the post analysis; B – Mean fluorescence intensity of iNKT cells expressing CD69 in COPD-PR group, before (pre) and after (post) performing pulmonary rehabilitation. Horizontal bars represent means.

Expression of **cytotoxic markers on iNKT cells** in patients with COPD after pulmonary rehabilitation: increase in the percentage of iNKT cells expressing Granzyme B but did not seem to affect $CD107a^+$ iNKT DN decrease

The impact of pulmonary rehabilitation in the expression of cytotoxic markers expressed by iNKT cells was also analyzed. As shown in Figure 28A and C, no statistically significant changes were found in percentage of iNKT cells expressing CD158b between COPD and COPD-PR groups, neither between pre and post-pulmonary rehabilitation in the COPD-PR group.

Regarding the percentage of iNKT cells expressing Granzyme B, a statistically significant increase ($p<0.05$) was observed in patients of the COPD-PR group in comparison with patients with COPD who never underwent pulmonary rehabilitation (Figure 28B). This was also a tendency when the same patients were analyzed before and after they had performed pulmonary rehabilitation (Figure 28D).

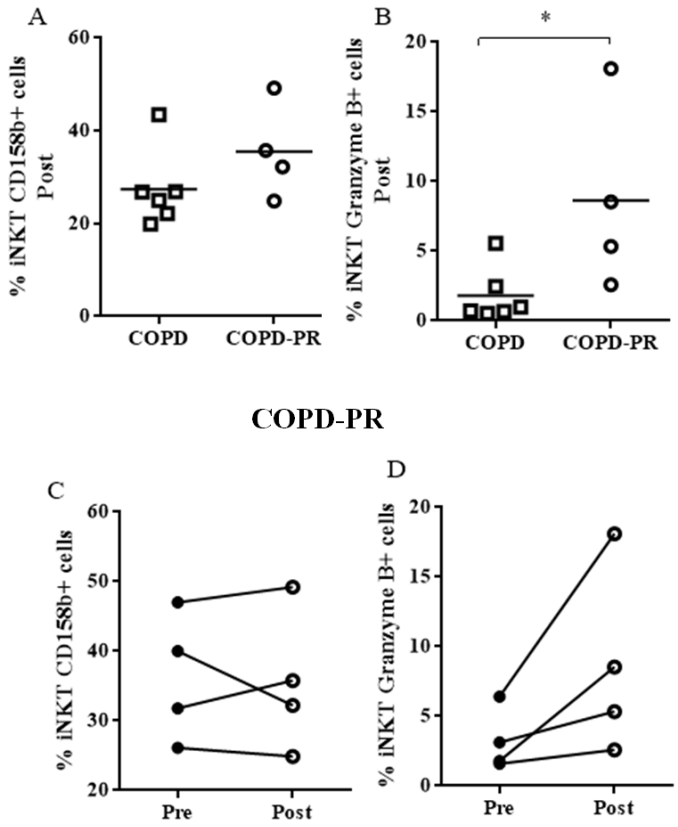

Figure 28. Effect of pulmonary rehabilitation on the percentage of iNKT cells cytotoxic markers in the peripheral blood. A, B – Percentage of iNKT cells expressing CD158b (A) or Granzyme B (B) in COPD and COPD-PR groups in the post analysis; C, D – Percentage of iNKT cells expressing CD158b (C) or Granzyme B (D) in the COPD-PR group, before (pre) and after (post) performing pulmonary rehabilitation. Horizontal bars represent means. Mann-Whitney U test was used to determine statistical significance *$p<0.05$ among COPD and COPD-PR groups.

When we analyzed CD107a expression in the baseline/pre, we found a tendency for a decrease of CD107a expression in iNKT cells, which was significant for the iNKT DN subset, in patients with COPD. When we compared the COPD and COPD-PR groups in the post-pulmonary rehabilitation, no significant alterations were observed in total iNKT cells (Figure 29A). In relation to percentage of iNKT cells expressing CD107a in COPD-PR group before and after pulmonary rehabilitation, no significant changes were detected, but we could observe that 3 out of the 4 patients of this group had a tendency to increase CD107a expression in iNKT cells (Figure 29B), after pulmonary rehabilitation.

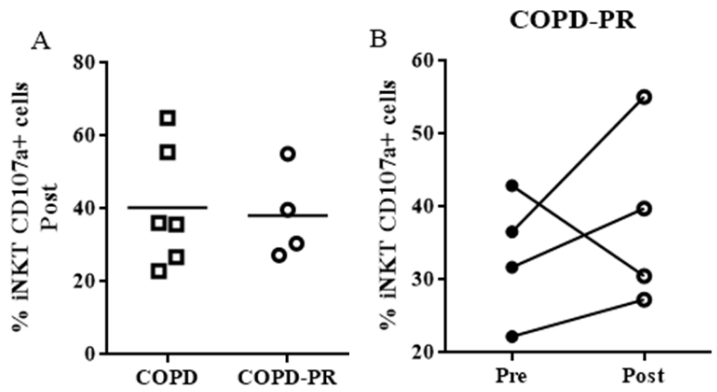

Figure 29. Effect of pulmonary rehabilitation on the percentage of iNKT cells expressing CD107a in the peripheral blood. A – Percentage of iNKT cells expressing CD107a in the COPD and COPD-PR groups in the post analysis; B – Percentage of iNKT cells expressing CD107a in patients from the COPD-PR group, before (pre) and after (post) performing pulmonary rehabilitation. Horizontal bars represent means.

The analysis of cytotoxic markers in iNKT cell CD4/CD8/DN subsets did not reveal significant alterations in patients with COPD after pulmonary rehabilitation (data not shown).

4.4. Correlations between clinical variables for chronic obstructive pulmonary disease and immunological variables

For this section we used the clinical and immunological data obtained from the baseline/pre, and thus COPD and COPD-PR groups were treated as single group of patients with COPD (n=11). The control group was composed by 14 subjects. Tables S1 to S4 in appendix III have the p values of performed correlations.

The percentage of NK cells was positively correlated with MIP in patients with COPD

We started by performing a correlation analysis between lymphocyte subsets (NK, iNKT, $CD4^+$ and $CD8^+$ T cells) and clinical variables. The percentage of $CD4^+$ and $CD8^+$ T lymphocytes and of iNKT cells did not display any correlation with studied clinical variables in patients with COPD (Appendix III – Table S2). However, there was a moderate positive correlation (r_s=0.609, p=0.047) between NK cells and maximal inspiratory pressure (MIP) in patients with COPD (Figure 30B), which did not occur in healthy controls (Figure 30A and appendix III – Table S1).

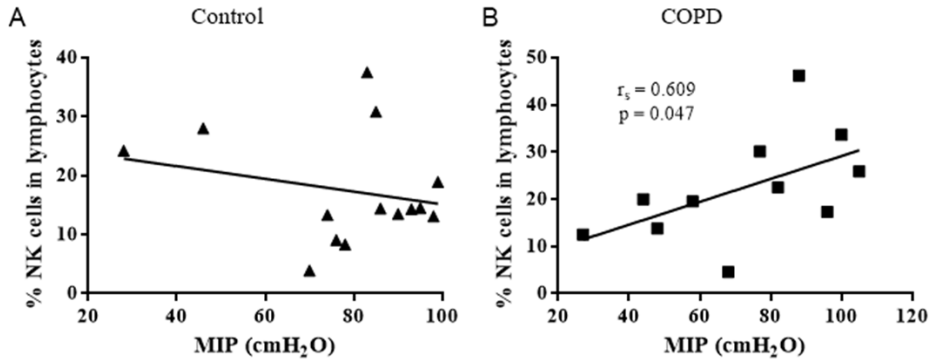

Figure 30. Correlation between percentage of NK cells and values (cmH_2O) of MIP in age- and gender-matched controls (A) and patients with COPD (B). r_s: Spearman's rank correlation coefficient. Correlation is significant when $p<0.05$ (two-tailed).

<u>iNKT DN cells was correlated with MIP and MEP in patients with COPD</u>

We have also performed a correlation analysis between the percentage of iNKT cell subsets and clinical parameters. In patients with COPD, the percentage of iNKT DN cells displayed negative correlations with the measures of respiratory muscle strength: a moderate negative correlation (r_s=-0.691, p=0.019) between iNKT DN cells and maximal inspiratory pressure (MIP, Figure 31B), and a high negative correlation (r_s=-0.770, p=0.006) between iNKT DN cells and maximal expiratory pressure (MEP, Figure 31D). No significant correlation was found in age- and gender-matched controls for these parameters (Figure 31A and C, and appendix III – Table S1).

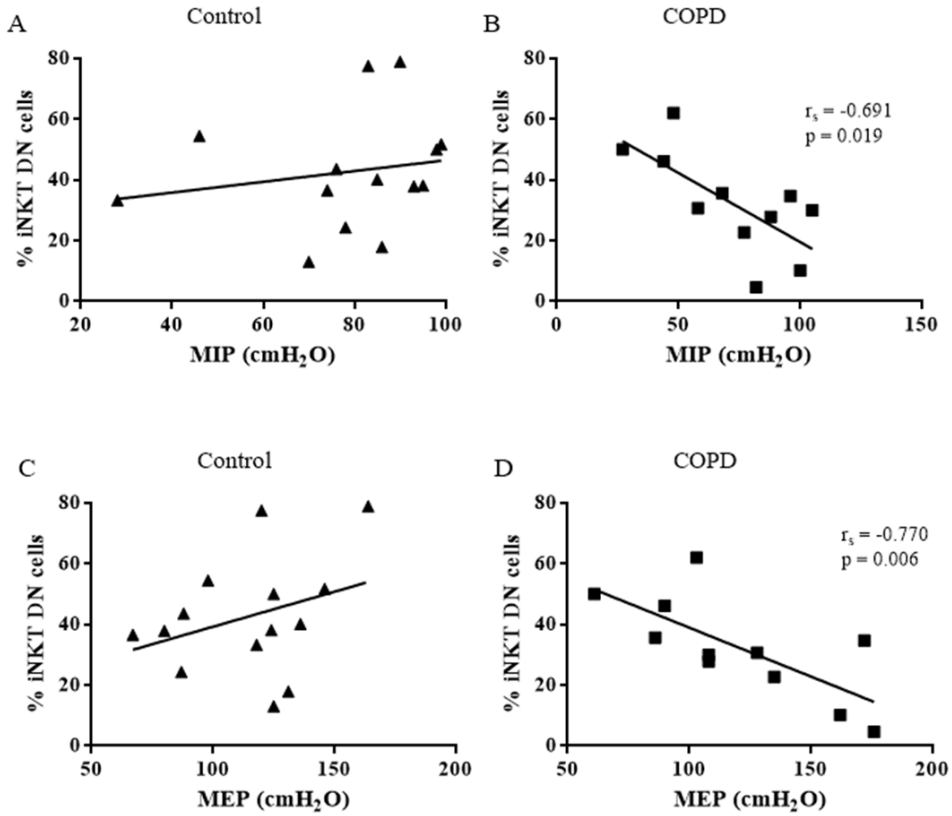

Figure 31. Correlation between percentage of iNKT DN cells and values (cmH_2O) of respiratory muscle strength in age- and gender-matched controls and patients with COPD. A, B – Correlation between percentage of iNKT DN cells and values (cmH_2O) of MIP in age- and gender-matched controls (A) and patients with COPD (B); C, D – Correlation between percentage of iNKT DN cells and values (cmH_2O) of MEP in age- and gender-matched controls (C) and patients with COPD (D). r_s: Spearman's rank correlation coefficient. B – Correlation is significant when $p<0.05$ (two-tailed); D – Correlation is significant at the 0.01 level (two-tailed).

The activation marker CD69 was negatively correlated with FEV_1

CD69 expression on iNKT cells showed a moderate negative correlation (r_s=-0.699, p=0.017) with predicted forced expiratory volume in one second (FEV_1) in patients with COPD (Figure 32B). Age- and gender-matched controls did not show a significant correlation between these two parameters (Figure 32A and appendix III - Table S1).

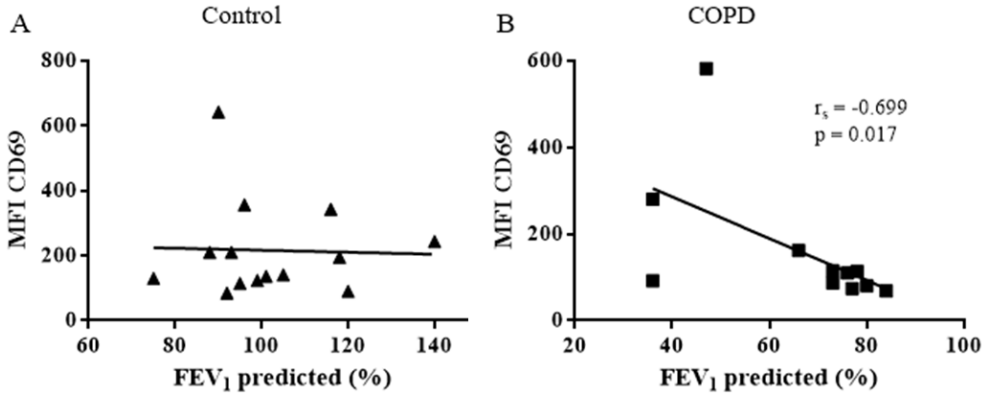

Figure 32. Correlation between mean fluorescence intensity of CD69 in iNKT cells and the percentage of predicted FEV_1 in age- and gender-matched controls (A) and patients with COPD (B). r_s: Spearman's rank correlation coefficient. Correlation is significant when $p<0.05$ (two-tailed).

Cytotoxic markers displayed correlations with different clinical variables in patients with COPD

We performed an extensive correlation analysis between clinical variables and cytotoxic markers (Tables S3 and S4 in appendix III).

The percentage of $CD8^+$ T cells expressing CD158b displayed a high negative correlation (r_s=-0.727, p=0.011) with MIP in patients with COPD (Figure 33B), but not in controls (Figure 33A, Appendix III – Table S3).

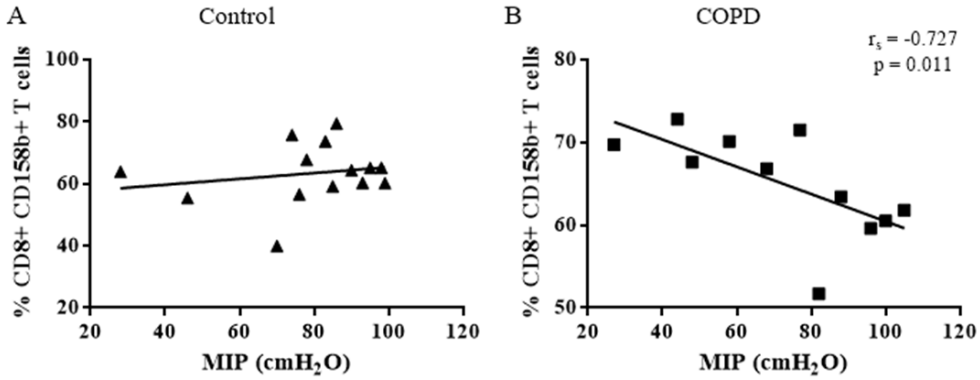

Figure 33. Correlation between percentage of $CD8^+$ T cells expressing CD158b and values (cmH_2O) of MIP in age- and gender-matched controls (A) and patients with COPD (B). r_s: Spearman's rank correlation coefficient. Correlation is significant when $p<0.05$ (two-tailed).

Patients with COPD also had a high positive correlation (r_s=0.788, p=0.004) between the percentage of iNKT cells expressing CD158b and the percentage of predicted forced vital capacity (FVC, Figure 34B). This correlation was not present in controls (Figure 34A and appendix III – Table S3).

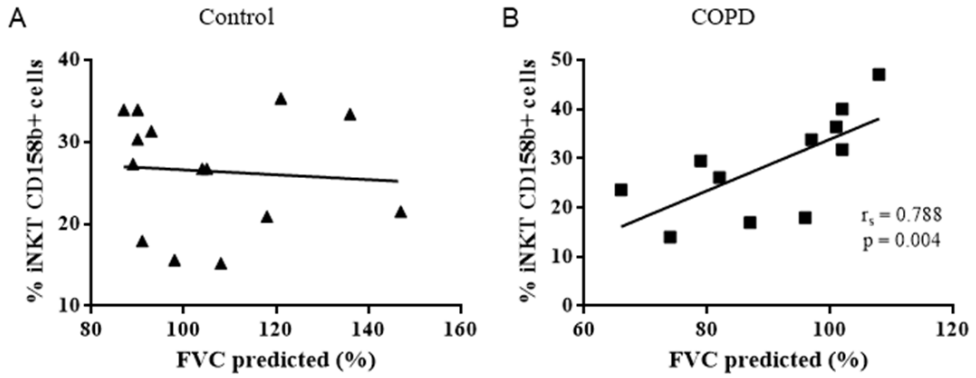

Figure 34. Correlation between percentage of iNKT cells expressing CD158b and percentage of predicted FVC in age- and gender-matched controls (A) and patients with COPD (B). r_s: Spearman's rank correlation coefficient. Correlation is significant at the 0.01 level (two-tailed).

Furthermore, iNKT $CD8^+$ cells expressing CD158b displayed a moderate negative correlation (r_s=-0.671, p=0.024) with ratio of forced expiratory volume in one second and forced vital capacity (FEV_1/FVC) in patients with COPD (Figure 35B). Once again, age- and gender-matched controls did not present significant correlations with these parameters (Figure 35A and appendix III – Table S3).

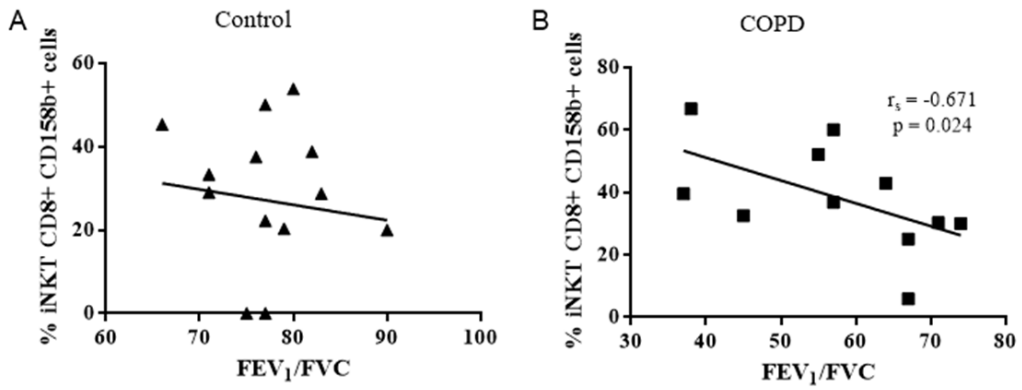

Figure 35. Correlation between percentage of iNKT $CD8^+$ cells expressing CD158b and FEV_1/FVC ratio in age- and gender-matched controls (A) and patients with COPD (B). r_s: Spearman's rank correlation coefficient. Correlation is significant when $p<0.05$ (two-tailed).

Conversely, a moderate negative correlation (r_s=-0.694, p=0.018) between the percentage of $CD8^+$ T cells expressing Granzyme B and the percentage of predicted FEV_1 was present in patients with COPD (Figure 36B), but not in control subjects (Figure 36A, appendix III – Table S3).

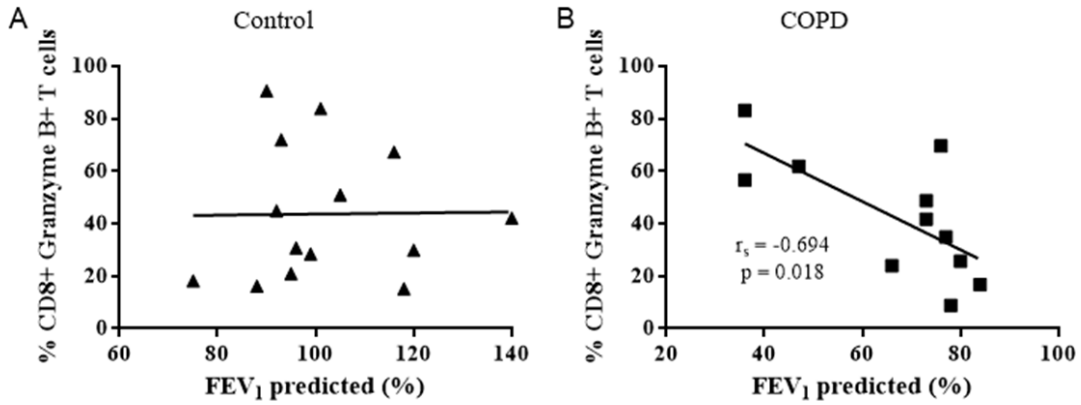

Figure 36. Correlation between percentage of $CD8^+$ T cells expressing Granzyme B and percentage of predicted FEV_1 in age- and gender-matched controls (A) and patients with COPD (B). r_s: Spearman's rank correlation coefficient. Correlation is significant when p<0.05 (two-tailed).

A correlation between FEV_1/FVC ratio and the percentage of iNKT cells expressing Granzyme B was also established in patients with COPD (Figure 37B). This was a high negative correlation (r_s=-0.730, p=0.011). No significant correlation was shown in age- and gender-matched controls (Figure 37A and appendix III – Table S3).

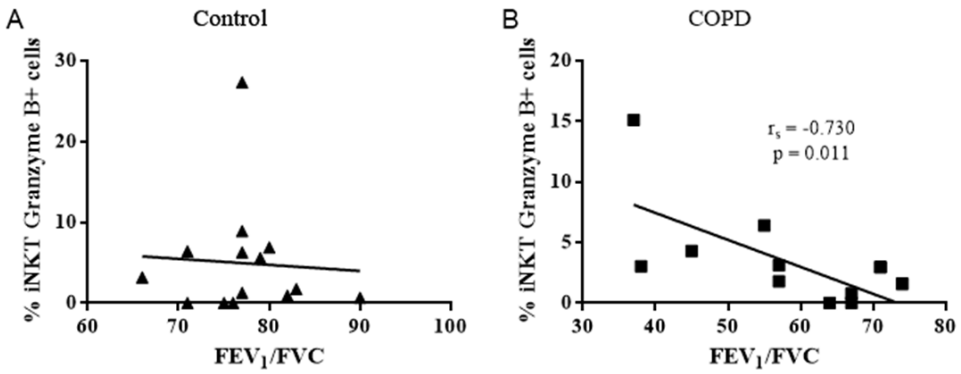

Figure 37. Correlation between percentage of iNKT cells expressing Granzyme B and FEV_1/FVC ratio in age- and gender-matched controls (A) and patients with COPD (B). r_s: Spearman's rank correlation coefficient. Correlation is significant when p<0.05 (two-tailed).

Interestingly, the percentage of iNKT DN cells expressing Granzyme B was also negatively correlated (r_s=-0.682, p=0.021) with this parameter in patients with COPD, although at a moderate level (Figure 38B). In controls this correlation was not significant (Figure 38A and appendix III – Table S3).

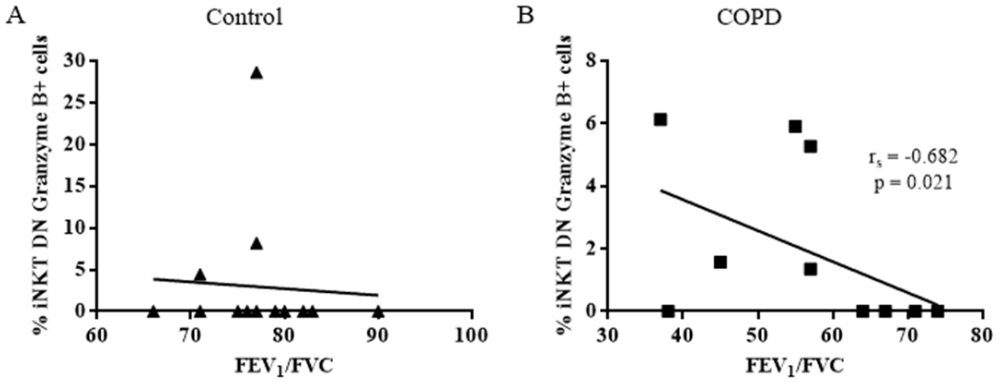

Figure 38. Correlation between the percentage of iNKT DN cells expressing Granzyme B and FEV_1/FVC ratio in age- and gender-matched controls (A) and patients with COPD (B). r_s: Spearman's rank correlation coefficient. Correlation is significant when $p<0.05$ (two-tailed).

5. Discussion

5.1. Effect of pulmonary rehabilitation on clinical measures for chronic obstructive pulmonary disease

In patients with COPD, pulmonary rehabilitation has clearly demonstrated to reduce symptoms (e.g., dyspnea), improve muscle force and activity, promote autonomy and participation in daily activities, improve health-related quality of life and reduce healthcare utilization [42], [43], [133]. In this study, only COPD Assessment Test (CAT) presented significant changes between groups, i.e., in the age- and gender-matched controls this clinical measure did not present differences between the 1st and the 2nd clinical assessment, while patients with COPD with and without pulmonary rehabilitation presented different values over time. This result revealed that healthy subjects presented a better quality of life than the patients, as predicted. No other significant changes were observed. The absence of significant improvements in patients who underwent pulmonary rehabilitation may be justified by the fact that only four patients adhered to this treatment, and thus a small number of subjects was analyzed.

5.2. Analysis of iNKT cells in chronic obstructive pulmonary disease

In this study, no differences were found in iNKT cell percentage in the peripheral blood of healthy controls in comparison with patients with COPD. As expected, we found a high variation in the percentage of iNKT cells in the peripheral blood of both groups. Previous studies have described conflicting results. A previous study (n_{COPD}=10) found a decreased frequency of iNKT cells in peripheral blood of patients with stable COPD when they stained the cells with 6B11 monoclonal antibody, however, when the iNKT cells were stained with α-GalCer-loaded CD1d-tetramer, no significant alterations were found between groups, according to our results [113]. Conersely, an increase of iNKT cell frequency in the peripheral blood of patients with COPD was observed (n_{COPD}=28) [114]. In another study, frequency of iNKT cells were analyzed in induced sputum, airway biopsy and bronchoalveolar lavage, but no significant differences were found between controls and patients with COPD (n_{COPD}=5) [117].

We found no significant differences in the percentage of NK cells when patients with COPD were compared to age- and gender-matched controls. This is in accordance with two previous studies (n_{COPD}=56) [134]; (n_{COPD}=30 ex-smokers; n_{COPD}=41 current smokers) [103], but contrary to what was observed in other study, which described a decrease in

relative number of NK cells in the peripheral blood (n_{COPD}=11) [99]. In induced sputum and in airways of patients with COPD, the percentage of NK cells are increased (n_{COPD}=11) [96]; (n_{COPD}=41) [103]. No difference was found in the number of NK cells in lung parenchyma (n_{COPD}=6 non-smokers; n_{COPD}=5 smokers without parenchymal destruction; n_{COPD}=10 smokers with parenchymal destruction) [20].

Regarding the iNKT cell subsets, patients with COPD did not present significant differences in any subset in comparison with healthy subjects. Studies of the iNKT cell subsets in COPD are scarce. When the researchers studied the $CD4^+$ iNKT cells stained with α-GalCer-loaded CD1d-tetramer no significant differences in the frequencies between patients with COPD and healthy control subjects were observed, in accordance to our results [113].

As a control, we also analyzed the percentage of total $CD4^+$ and $CD8^+$ T cells and likewise no significant alterations were observed when patients with COPD were compared to controls. In accordance with our results, other study also did not obtain significant differences in the number of $CD8^+$ T cells in the peripheral blood of patients with COPD comparing with controls, while the number of $CD4^+$ T cells was reduced in patients [3]. In the airways and in lung parenchyma of patients with COPD, both $CD4^+$ and $CD8^+$ T cells are increased [12]. Additionally, the percentage of $CD8^+$ T cells further increases with COPD progression [17]. Increased $CD8^+$ T cells were also observed in repeatedly α-GalCer administered mice, which produce similar pathological hallmarks of COPD [115].

Importantly, a decrease in the expression of CD69 in patients with COPD was found. CD69 is a surface marker of early stage lymphocyte activation [135]. Our results can indicate that COPD interferes in some way with the early activation of iNKT cells. A study which investigated the potential role of iNKT cells in a mice model of chronic exposure to cigarette smoke mimicking COPD symptoms, and in an acute model of lipid peroxidation using cumen hydroperoxide (CHP) as the trigger, concluded that exposure to CHP led to an enhanced CD69 expression on iNKT cells [114].

In this study, we also analyzed for the first time the expression of cytotoxic markers (CD158b, CD107a and Granzyme B) in iNKT cells, in the context of COPD. It is know that CD158b is a inhibitory receptor that is altered in NK cells in patients with COPD [3]. However, we did not found alterations in this marker in patients with COPD. Granzyme B

is the most common granzyme and an effector of cytotoxicity, activating pro-caspases, thus inducing apoptosis initiating [97]. It is also known that Granzyme B is altered in $CD8^+$ T cells and NK cells in patients with COPD [99], [102], [103]. In this study, we found no alterations in Granzyme B expression in iNKT cells at the basal level. However, upon stimulation, a tendency for a decrease in the expression of Granzyme B by iNKT cells was found. This might indicate a reduction in the cytotoxic capacity of iNKT cells from the peripheral blood of patients with COPD. Finally, CD107a is a degranulation marker, which is upregulated in iNKT cells after stimulation and it is defective in cells that are exposed to cigarette smoke [107]–[109]. We found a tendency for a decrease in expression of CD107a in total iNKT cells from patients with COPD, which did not reach statistical significance. Interestingly, a significant reduction in CD107a expression was observed in iNKT DN cells. This is accordance with what was expected, since COPD is mainly caused by cigarette smoke. It may also indicate that iNKT cells from patients with COPD are less prone to release cytotoxic granules.

It is known that cytokines play a key role in orchestrating the chronic inflammation of COPD by recruiting, activating, and promoting the survival of multiple inflammatory cells in the respiratory tract [1]. In this study, we demonstrated that, upon stimulation, cytokine production (IL-4 and IFN-γ) by iNKT cells from peripheral blood is not altered in COPD. To our knowledge, this is the first study analyzing the production of cytokines by iNKT cells in COPD, however it was previously demonstrated that the production of IFN-γ and IL-4 by $CD8^+$ T cells is increased in bronchoalveolar lavage [136]. Likewise, the percentage of IFN-γ^+ $CD8^+$ T cells in peripheral blood is increased in stable patients with COPD when compared to healthy never-smoking controls [137].

Overall, we performed an extensive analysis of iNKT cells from the peripheral blood of patients with COPD. We found that these cells seem to be less activated, as determined by CD69 expression. Furthermore, our results suggest that iNKT cells from patients with COPD may have a decrease in cytotoxic capacity. It would be important to increase the number of patients analyzed to take definitive conclusions.

5.3. Effect of pulmonary rehabilitation on iNKT cells in patients with chronic obstructive pulmonary disease

This part of the study was limited by the low number of patients that performed pulmonary rehabilitation (n=4). We did not found significant differences in the percentage of iNKT cells, neither in their subsets after pulmonary rehabilitation in patients of the COPD-PR group and in comparison with patients with COPD who never undergone this treatment. Furthermore, the reduction in CD69 expression observed in patients with COPD did not seem to be altered by pulmonary rehabilitation. On the contrary, we found a significant increase in the percentage of iNKT cells expressing Granzyme B in patients of the COPD-PR group in comparison with patients with COPD who never performed pulmonary rehabilitation, and this was also a tendency when the same patients were analyzed before and after they had performed pulmonary rehabilitation. Although pulmonary rehabilitation did not seem to affect the decrease in $CD107a^+$ iNKT DN cells, 3 out of the 4 patients of the COPD-PR group had a tendency to increase CD107a expression in iNKT cells after the treatment, which can indicate that pulmonary rehabilitation can improve this parameter. These results suggest that pulmonary rehabilitation may contribute to the increase in iNKT cell cytotoxicity and may have a role in improving the cytotoxic defect. It would be important to increase the number of patients analyzed, who underwent the pulmonary rehabilitation to improve the conclusions.

5.4. Correlations between clinical variables for chronic obstructive pulmonary disease and immunological variables

In our investigation, we found a positive correlation between NK cells and maximal inspiratory pressure (MIP) in patients with COPD, indicating that these two variables are directly proportional. Furthermore, iNKT DN cells displayed negative correlations with the measures of respiratory muscle strength: MIP and maximal expiratory pressure (MEP).

CD69 expression on iNKT cells showed a negative correlation with predicted forced expiratory volume in one second (FEV_1) in patients with COPD. So, expression of CD69 in iNKT cells seems to increase with pulmonary function decline. This was not expected, since we found that patients with COPD had a decrease in CD69 expression in iNKT cells.

Regarding to cytotoxic markers, a negative correlation between the percentage of $CD8^+$ T cells expressing CD158b and MIP was observed in patients, indicating that these two

variables are inversely proportional. We also found a positive correlation between the percentage of iNKT cells expressing CD158b and the percentage of predicted forced vital capacity (FVC) in patients with COPD. This might indicate that CD158b expression is associated with an improvement in the health condition of the patient. Furthermore, iNKT $CD8^+$ cells expressing CD158b displayed a moderate negative correlation with FEV_1/FVC ratio in patients with COPD. It was possible verified that when this inhibitory receptor increases in iNKT $CD8^+$ cells, the pulmonary function gets worse, indicating that the frequency of $CD158b^+$ iNKT $CD8^+$ cells reflects the severity of COPD to a certain extent. A negative correlation between the percentage of $CD8^+$ T cells expressing Granzyme B in the peripheral blood and the percentage of predicted FEV_1 was found in patients with COPD. This is in accordance with a previous investigation, which also found a negative correlation between $CD8^+$ T cells expressing Granzyme B and predicted FEV_1 in the lungs, demonstrating that $CD8^+$ T cells display increased transcripts for Granzyme B with worsening pulmonary function [138]. Lastly, the percentage of total iNKT and of DN iNKT cells expressing Granzyme B displayed a negative correlation with FEV_1/FVC ratio in patients with COPD. A significant correlation between Granzyme B expression in BAL-derived T-cells and apoptosis of bronchial epithelial cells was found [102]. The idea that apoptosis of lung structural cells might contribute to parenchymal destruction has gained increasing support from basic and clinical data [139], [140].

Since the immunological investigations addressed to the chronic obstructive pulmonary disease are very few, these new data about the correlations of clinical health and lymphocytes can promote new studies in this disease, including more patients.

We had some limitations in the performance of this project, such as: only a few patients with the inclusion criteria and that accepted participate in the study; the poor adherence by the patients with COPD to the pulmonary rehabilitation; and the freeze of some blood samples, like referred in the part of the materials and methods.

Our findings open paths to new investigations about the cytotoxic markers role in COPD, as well as the effect of pulmonary rehabilitation on the immune system, to enhance our knowledge on the pathology and pathogenesis of this chronic disease which is a major cause of chronic morbidity and mortality throughout the world.

6. Conclusion

We analyzed the iNKT cells in patients with COPD. No alteration in percentage of iNKT cells, their subsets (CD4/CD8/DN) and cytokine production (IL-4 and IFN-γ) in the peripheral blood of patients with COPD was found in comparison with age- and gender-matched controls. Importantly, iNKT cells seem to be less activated, as determined by reduction of CD69 expression. Here we analyzed for the first time the expression of cytotoxic markers (CD158b, CD107a and Granzyme B) in iNKT cells and their subsets, in the context of COPD. Our results suggest that iNKT cells from patients with COPD may have a decrease in cytotoxic capacity, by a tendency for a decrease in the percentage of Granzyme B^+ iNKT cells upon stimulation, further a tendency for a decrease in expression of CD107a in total iNKT cells, followed by a significant reduction in CD107a expression in iNKT DN cells. It would be important to increase the number of patients analyzed to strengthen our conclusions. It would also be interesting analyze the iNKT cells in the airways, induced sputum or lung tissue of patients with COPD, because they are the source of inflammation of this chronic disease.

Relatively to the effect of pulmonary rehabilitation on iNKT cells in patients with COPD, no differences were observed in the percentage of iNKT cells, neither in their subsets after pulmonary rehabilitation. The reduction in CD69 expression observed in patients with COPD did not seem to be altered. Our results about iNKT cells expressing Granzyme B and CD107a suggest that pulmonary rehabilitation may contribute to the increase in iNKT cell cytotoxicity and may have a role in improving the cytotoxic defect. It would be necessary to increase the number of patients analyzed, who underwent pulmonary rehabilitation to be able to conclude about the effects of such intervention on immunological state.

An extensive correlation analysis between clinical measures for COPD and immunological measures it was performed for the first time. It was found positive correlations in patients with COPD between percentage of NK cells and MIP, and iNKT cells expressing CD158b and the percentage of predicted FVC. This might indicate that these cells are associated with an improvement in the health condition of the patient. Several negative correlations were also found between percentage of iNKT DN cells and measures of respiratory muscle strength (MIP and MEP), iNKT cells expressing CD69 and FEV_1, $CD8^+$ T cells expressing CD158b and MIP, iNKT $CD8^+$ cells expressing CD158b and FEV_1/FVC ratio, $CD8^+$ T cells expressing Granzyme B and FEV_1, and iNKT and of

DN iNKT cells expressing Granzyme B and FEV_1/FVC ratio. This might indicate that these cells are associated with worsening of COPD. These correlations are the beginning of in-deep studies about this chronic disease, promoting further investigations and maybe new treatments.

The studies about iNKT cells addressed to the COPD are scarce and those that exist have described conflicting results. So, our investigation open paths to new studies about the role of iNKT cells, their cytotoxicity and cytokine production in the pathology and pathogenesis of COPD, as well as, the effect of pulmonary rehabilitation in immune system of these patients. This investigation work is a comprehensive study about COPD, enriched by the combination of clinical and laboratory data collection, allowing me to acquire knowledge in the field of pulmonary physiotherapy and immunology.

7. References

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Appendix I

Ethics' approval

universidade de aveiro

CONSELHO DE ÉTICA

Parecer nº: 10/2015.

Requerente: Doutora Maria de Fátima Macedo.

Título do Projeto: "Efeitos da reabilitação respiratória nos linfócitos iNKT em pessoas com doença pulmonar obstrutiva crónica".

Orientadoras: Doutora Maria de Fátima Macedo (DCM) e Doutora Alda Marques (ESSUA).

Equipa de Investigação: Doutora Maria de Fátima Macedo (DCM), Doutora Alda Marques (ESSUA) e Tânia Marante (Estudante de Mestrado).

Relator: Doutor Armando J. Pinho.

Relatores Adjuntos: António J. A. Nogueira, António Rocha Andrade, Jorge Carvalho Arroteia, Paula Cristina M. S. Pereira.

I. Relatório

O processo encontra-se devidamente instruído contendo elementos essenciais relativos a:

- Caracterização do projeto;
- Equipa de investigação;
- Bibliografia;
- Apêndices:

1. Declaração de apoio do Diretor do Departamento de Ciência Médicas (DCM) da Universidade de Aveiro e Diretor do iBiMED;
2. Declaração de apoio do Diretor do Instituto de Biologia Molecular e Celular - IBMC;
3. Instrumento de recolha de dados;
4. Folhas de informação ao participante;
5. Formulário de consentimento informado.

II. Parecer

A. Fundamentação:

1. A proposta relativa ao projeto apresenta-se devidamente fundamentada, sendo inteligível e exaustiva no que respeita aos seus objetivos, metodologia, indicação de estudos semelhantes e bibliografia.
2. A amostra está bem definida e os procedimentos a seguir, nomeadamente no que respeita às exigências científicas dos instrumentos de avaliação, procedimentos de medida e tratamento de resultados estão desenvolvidos.
3. Os riscos associados ao desenvolvimento do projeto estão considerados.
4. De acordo com acima referido, a proposta respeita os princípios de ética neste tipo de investigação na medida em que:

4.1. O estudo salvaguarda o consentimento informado dos participantes, com pelo menos 40 anos de idade e anteriormente à recolha de dados;

4.2. O estudo salvaguarda a participação voluntária da participação;

4.3. O estudo é devidamente acompanhado pela equipa de investigação;

4.4. Os riscos associados à participação no estudo não são superiores aos riscos associados a uma colheita de sangue para análises clínicas;

4.5. Os dados recolhidos no projeto são analisados pela equipa de investigação, mantendo-se confidenciais e anónimos, sob a responsabilidade das coordenadoras do estudo;

4.6. Os dados são armazenados por um período de cinco anos e depois destruídos.

B. Sugestões de aperfeiçoamento

Não há.

C. Conclusão

De acordo com o anteriormente assinalado e com os princípios seguidos pelo CED é emitido o seguinte parecer:

A Comissão Permanente do Conselho de Ética, constituída pelos ora Relatores, após apreciação conjunta da documentação recebida e atendendo a que os procedimentos descritos no estudo de investigação apresentado:

(a) asseguram a não utilização de qualquer método invasivo para além de recolhas de sangue idênticas à utilizada em análises clínicas de rotina;

(b) asseguram que os participantes, com 40 ou mais anos, serão oportunamente informados e esclarecidos sobre as condições em que vão decorrer as observações e recolha de dados, de modo a ser obtido o consentimento informado;

(c) garantem que os dados recolhidos serão tratados de maneira a permanecerem confidenciais e anónimos,

Considera, por unanimidade, que merece parecer favorável a realização do projeto em análise.

Os Relatores:

D. Decisão

Submetido ao CED o parecer da sua Comissão Permanente, este Conselho, em sua reunião plenária de 13 de Abril de 2016, por entender que ficam salvaguardadas as exigências éticas e os princípios da justiça e da autonomia e bem estar dos participantes concorda por unanimidade com o mesmo em razão do que o ratifica e dá

CONSELHO DE ÉTICA

parecer favorável à realização do projeto "Efeitos da reabilitação respiratória nos linfócitos iNKT em pessoas com doença pulmonar obstrutiva crónica".

Aveiro, 13 de Abril de 2016

Conselho de ética e Deontologia da Universidade de Aveiro.

Presidente: _____

Vice - Presidente: _____

Secretário: _____

Appendix II

Composition of the solutions (Materials and Methods)

ACK lysis solution

For a final volume of 500mL, add 4.15g of ammonium chloride (NH_4Cl) and 0.5g of potassium bicarbonate ($KHCO_3$) to H_2O. Adjust pH to 7.2.

Culture medium - RPMI 10%iFBS:

- 500mL RPMI 1640 GlutaMAX (Invitrogen);
- 5mL of non essential aminoacids (NEA, Invitrogen);
- 5mL of kanamycin (K, Invitrogen);
- 5mL of sodium pyruvate (SP, Invitrogen);
- 50mL (10%) of inactivated fetal bovine serum (iFBS, Invitrogen).

Flow cytometry solution (PBS 2%FBS 1%PenStrep 0.01%NaN_3)

For a solution final volume of 500mL add 10mL of fetal bovine serum (FBS), 5mL of PenStrep, 0.05g of sodium azide (NaN_3) and 50mL of PBS 10x to H_2O.

Flow cytometry solution with Saponin (PBS 2%FBS 1%PenStrep 0.01%NaN_3 0.5%Saponin)

For 100mL of solution add 0.5g of saponin to PBS 2%FBS 1%PenStrep 0.01%NaN_3.

PBS 10x

For a total volume of 1000mL add 80g of sodium chloride (NaCl), 2g of potassium chloride (KCl), 6.09g of sodium phosphate dibasic (Na_2HPO_4) and 2g of potassium dihydrogen phosphate (KH_2PO_4) to H_2O. Adjust pH to 7.3.

PBS 1x

For 500mL, add 50mL of PBS 10x to 450mL of H2O.

PBS 1%formaldehyde

For a final volume of 4mL add 250µL of 16%formaldehyde to 3.75mL of PBS 1x.

PBS 2%formaldehyde

For a final volume of 4mL add 500µL of 16% formaldehyde to 3.5mL of PBS 1x.

Appendix III

Correlation tables (Results)

Table S 1. Spearman's rank correlations between clinical variables and T lymphocytes, iNKT cell subsets and NK cells in age- and gender-matched controls.

Control n = 14		**$CD4^+$**	**$CD8^+$**	**iNKT**	**iNKT $CD161^+$**	**iNKT $CD56^+$**	**iNKT $CD69^+$**	**iNKT $CD8^+$**	**iNKT $CD4^+$**	**iNK T DN**	**NK**
MIP (cmH_2O)	r_s	-0.134	0.222	0.486	0.284	-0.327	-0.264	0.532	-0.481	0.323	0.037
	p	0.648	0.446	0.078	0.326	0.253	0.362	0.050	0.081	0.260	0.899
MEP (cmH_2O)	r_s	0.037	-0.004	0.352	0.244	-0.227	-0.214	-0.337	0.066	0.295	0.224
	p	0.899	0.988	0.217	0.400	0.436	0.463	0.239	0.823	0.306	0.441
5TSS (s)	r_s	-0.020	-0.029	0.035	0.169	0.253	0.306	-0.169	0.376	-0.152	0.552*
	p	0.946	0.923	0.905	0.563	0.383	0.288	0.563	0.185	0.605	0.041
FEV_1 predicted (%)	r_s	-0.257	0.314	0.180	0.002	-0.077	0.013	0.411	-0.486	0.305	-0.336
	p	0.375	0.274	0.537	0.994	0.794	0.964	0.144	0.078	0.288	0.240
FVC predicted (%)	r_s	-0.055	0.057	0.209	-0.026	-0.090	0.034	0.267	-0.407	0.264	-0.332
	p	0.852	0.846	0.473	0.929	0.759	0.908	0.357	0.149	0.362	0.246
FEV_1/FVC	r_s	-0.434	0.452	-0.291	0.007	0.205	0.119	0.428	-0.178	-0.049	-0.060
	p	0.121	0.105	0.313	0.982	0.483	0.685	0.127	0.543	0.868	0.838

COPD: Chronic Obstructive Pulmonary Disease; MIP: Maximal Inspiratory Pressure; MEP: Maximal Expiratory Pressure; 5TSS: Five Time Seat to Stand; FEV_1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity; FEV_1/FVC: Tiffeneau index; iNKT: invariant Natural Killer T cell; NK: Natural Killer cell; r_s: Spearman's rank correlation coefficient.

*Correlation is significant when $p<0.05$ (two-tailed).

Table S 2. Spearman's rank correlations between clinical variables and T lymphocytes, iNKT cell subsets and NK cells in patients with chronic obstructive pulmonary disease.

COPD n = 11		$CD4^+$	$CD8^+$	iNKT	iNKT $CD161^+$	iNKT $CD56^+$	iNKT $CD69^+$	iNKT $CD8^+$	iNKT $CD4^+$	iNKT DN	NK
MIP (cmH_2O)	r_s	-0.073	0.055	-0.542	-0.191	-0.209	-0.291	-0.518	0.436	-0.691*	0.609*
	p	0.832	0.873	0.085	0.574	0.537	0.385	0.102	0.180	0.019	0.047
MEP (cmH_2O)	r_s	0.055	-0.191	-0.260	-0.351	-0.214	-0.401	-0.483	0.510	-0.770**	0.515
	p	0.873	0.573	0.440	0.290	0.527	0.222	0.132	0.109	0.006	0.105
5TSS (s)	r_s	0.055	-0.182	0.492	0.127	0.327	0.282	0.391	-0.527	0.318	-0.336
	p	0.873	0.593	0.124	0.709	0.326	0.401	0.235	0.096	0.340	0.312
FEV_1 predicted (%)	r_s	-0.452	0.429	-0.201	-0.283	0.119	-0.699*	-0.050	0.027	0.046	0.256
	p	0.163	0.188	0.553	0.399	0.728	0.017	0.883	0.936	0.894	0.448
FVC predicted (%)	r_s	-0.118	0.000	0.123	0.178	0.588	-0.337	0.319	-0.474	0.068	0.342
	p	0.729	1.000	0.718	0.601	0.057	0.311	0.339	0.141	0.842	0.304
FEV_1/FVC	r_s	-0.311	0.498	-0.540	-0.365	-0.493	-0.342	-0.489	0.534	-0.050	0.123
	p	0.353	0.119	0.086	0.269	0.123	0.303	0.127	0.090	0.883	0.718

COPD: Chronic Obstructive Pulmonary Disease; MIP: Maximal Inspiratory Pressure; MEP: Maximal Expiratory Pressure; 5TSS: Five Time Seat to Stand; FEV_1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity; FEV_1/FVC: Tiffeneau index; iNKT: invariant Natural Killer T cell; NK: Natural Killer cell; r_s: Spearman's rank correlation coefficient.

*Correlation is significant when $p<0.05$ (two-tailed); **Correlation is significant at the 0.01 level (two-tailed).

Table S 3. Spearman's rank correlations between clinical variables and cytotoxic markers of T lymphocytes, iNKT cell subsets and NK cells in age- and gender-matched controls.

Control n = 14		**CD8 CD158b$^+$**	**CD8 CD107a$^+$**	**CD8 Granzyme B$^+$**	**iNKT CD158b$^+$**	**iNKT CD107a$^+$**	**iNKT Granzyme B$^+$**	**iNKT CD8 CD158b$^+$**	**iNKT CD8 CD107a$^+$**	**iNKT CD8 Granzyme B$^+$**	**iNKT CD4 CD158b$^+$**	**iNKT CD4 CD107a$^+$**	**iNKT CD4 Granzyme B$^+$**	**iNKT DN CD158b$^+$**	**iNKT DN CD107a$^+$**	**iNKT DN Granzyme B$^+$**	**NK CD158b$^+$**
MIP (cmH_2O)	**r_s**	0.242	-0.147	-0.187	0.357	-0.116	0.073	0.678**	0.526	0.319	-0.007	-0.255	0.315	-0.117	-0.394	0.028	-0.191
	p	0.404	0.615	0.523	0.210	0.692	0.805	0.008	0.053	0.266	0.982	0.379	0.273	0.691	0.164	0.926	0.512
MEP (cmH_2O)	**r_s**	-0.118	-0.183	-0.132	0.079	-0.048	0.011	0.272	0.205	0.209	-0.004	0.086	0.415	0.114	0.045	0.279	-0.117
	p	0.688	0.532	0.653	0.787	0.869	0.970	0.347	0.483	0.472	0.988	0.770	0.140	0.699	0.878	0.335	0.691
5TSS (s)	**r_s**	-0.075	0.059	0.538*	-0.126	0.007	0.316	-0.174	-0.309	0.350	0.222	0.502	0.013	-0.233	-0.154	0.358	-0.466
	p	0.799	0.840	0.047	0.669	0.982	0.272	0.551	0.282	0.220	0.446	0.068	0.965	0.422	0.599	0.209	0.093
FEV_1 predicted (%)	**r_s**	-0.209	-0.354	0.046	-0.108	-0.160	0.055	-0.073	-0.139	-0.262	-0.222	-0.414	-0.018	-0.225	-0.317	-0.162	-0.147
	p	0.473	0.215	0.876	0.713	0.584	0.851	0.805	0.635	0.366	0.446	0.142	0.951	0.440	0.270	0.580	0.615
FVC predicted (%)	**r_s**	-0.425	-0.546*	-0.132	-0.217	-0.244	0.024	-0.128	-0.212	-0.240	-0.271	-0.419	-0.010	-0.157	-0.269	-0.138	-0.042
	p	0.130	0.044	0.653	0.456	0.400	0.934	0.662	0.466	0.409	0.349	0.136	0.972	0.593	0.353	0.639	0.887
FEV_1/FVC	**r_s**	0.160	-0.018	0.254	-0.047	0.033	0.101	-0.087	-0.123	-0.346	-0.151	-0.249	0.008	-0.292	-0.121	-0.186	-0.102
	p	0.584	0.952	0.382	0.874	0.910	0.732	0.767	0.675	0.226	0.606	0.390	0.979	0.311	0.679	0.525	0.727

COPD: Chronic Obstructive Pulmonary Disease; MIP: Maximal Inspiratory Pressure; MEP: Maximal Expiratory Pressure; 5TSS: Five Time Seat to Stand; FEV_1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity; FEV_1/FVC: Tiffeneau index; iNKT: invariant Natural Killer T cell; NK: Natural Killer cell; r_s: Spearman's rank correlation coefficient.

*Correlation is significant when $p<0.05$ (two-tailed); **Correlation is significant at the 0.01 level (two-tailed).

Table S 4. Spearman's rank correlations between clinical variables and cytotoxic markers of T lymphocytes, iNKT cell subsets and NK cells in patients with chronic obstructive pulmonary disease.

COPD n = 11		CD8 $CD158b^+$	CD8 $CD107a^+$	CD8 Granzyme B^+	iNKT $CD158b^+$	iNKT $CD107a^+$	iNKT Granzyme B^+	iNKT CD8 $CD158b^+$	iNKT CD8 $CD107a^+$	iNKT CD8 Granzyme B^+	iNKT CD4 $CD158b^+$	iNKT CD4 $CD107a^+$	iNKT CD4 Granzyme B^+	iNKT DN $CD158b^+$	iNKT DN $CD107a^+$	iNKT DN Granzyme B^+	NK $CD158b^+$
MIP (cmH_2O)	r_s	-0.727*	-0.500	-0.455	0.100	-0.173	-0.328	0.091	-0.014	-0.140	0.101	-0.055	-0.027	0.136	-0.518	-0.223	-0.118
	p	0.011	0.117	0.160	0.770	0.612	0.325	0.790	0.968	0.682	0.768	0.873	0.937	0.689	0.102	0.510	0.729
MEP (cmH_2O)	r_s	-0.588	-0.260	-0.360	-0.205	-0.433	-0.247	0.164	-0.425	0.226	-0.193	-0.132	0.047	-0.419	-0.351	-0.323	0.349
	p	0.057	0.441	0.277	0.545	0.184	0.465	0.630	0.193	0.504	0.569	0.698	0.890	0.199	0.290	0.333	0.292
5TSS (s)	r_s	0.355	0.127	0.573	0.118	0.382	0.501	0.427	0.278	0.461	-0.275	-0.528	0.067	-0.091	0.236	0.590	-0.036
	p	0.285	0.709	0.066	0.729	0.247	0.116	0.190	0.408	0.154	0.413	0.095	0.844	0.790	0.484	0.056	0.915
FEV_1 predicted (%)	r_s	0.155	0.356	-0.694*	0.393	-0.224	-0.526	-0.201	-0.327	-0.493	0.553	0.339	-0.278	0.224	-0.142	-0.212	-0.185
	p	0.649	0.282	0.018	0.232	0.508	0.096	0.554	0.326	0.123	0.078	0.308	0.408	0.508	0.678	0.532	0.585
FVC predicted (%)	r_s	0.150	-0.114	-0.342	0.788**	0.278	0.082	0.319	-0.009	-0.277	0.570	0.288	0.318	0.601	-0.123	0.487	-0.066
	p	0.659	0.739	0.304	0.004	0.408	0.810	0.339	0.979	0.409	0.067	0.391	0.341	0.050	0.719	0.129	0.847
FEV_1/FVC	r_s	-0.091	0.192	-0.530	-0.146	-0.374	-0.730*	-0.671*	-0.101	-0.516	0.134	0.300	-0.440	0.105	-0.187	-0.682*	-0.359
	p	0.789	0.572	0.094	0.668	0.257	0.011	0.024	0.768	0.104	0.695	0.370	0.175	0.759	0.581	0.021	0.278

COPD: Chronic Obstructive Pulmonary Disease; MIP: Maximal Inspiratory Pressure; MEP: Maximal Expiratory Pressure; 5TSS: Five Time Seat to Stand; FEV_1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity; FEV_1/FVC: Tiffeneau index; iNKT: invariant Natural Killer T cell; NK: Natural Killer cell; r_s: Spearman's rank correlation coefficient.

*Correlation is significant when $p<0.05$ (two-tailed); **Correlation is significant at the 0.01 level (two-tailed).

Annex I

Information sheet

Folha de informação ao participante

O Sr./Sra. está a ser convidado/a para participar no estudo de investigação clínica intitulado: "Efeitos da Reabilitação Respiratória nos Linfócitos iNKT em pessoas com Doença Pulmonar Obstrutiva Crónica". Mas, antes de decidir, é importante que compreenda porque é que a investigação está a ser realizada e o que é que a mesma envolve. Por favor, leia a informação com atenção e discuta a sua participação com outros, se assim o entender. Se houver algo que não esteja claro para si ou necessitar de informação adicional, por favor contacte os investigadores (contactos no final deste documento). Use o tempo que precisar para decidir se deseja ou não participar. Muito obrigado desde já por ler a informação.

Muito obrigado desde já por ler a informação.

Qual é o propósito do estudo?

Este estudo visa contribuir para a compreensão da forma como a reabilitação respiratória melhora a condição clinica do paciente com a Doença Pulmonar Obstrutiva Crónica, através do estudo de um determinado tipo de células do sistema imunitário, designadas Linfócitos iNKT. A DPOC é uma condição de saúde que se caracteriza por uma limitação do débito aéreo, geralmente progressiva e com reduzida reversibilidade. A sua origem está normalmente associada a uma resposta inflamatória anómala dos pulmões, à inalação de partículas ou gases nocivos. É ainda desconhecido o efeito da reabilitação respiratória neste tipo específico de células do sistema imunitário. Assim, os resultados deste estudo irão potencialmente contribuir para uma melhor compreensão da atividade celular imunitária estimulada pelo tratamento através da reabilitação respiratória da DPOC e futuros possíveis tratamentos para esta doença, de modo a contribuir para a qualidade de vida dos doentes. Para que seja possível alcançar estes objetivos vimos então solicitar a sua participação neste estudo que será realizado na Universidade de Aveiro.

Porque é que fui escolhido?

Foi escolhido/a porque é uma pessoa saudável ou com doença pulmonar obstrutiva crónica. Para o estudo, precisamos de dados de aproximadamente 20 pessoas, com uma condição clínica semelhante à sua, que aceitem participar.

Tenho de participar?

A decisão de participar, ou não, é completamente sua. Se decidir participar vai-lhe ser pedido que assine um formulário de consentimento informado mas, é totalmente livre de desistir antes ou durante a recolha dos dados, sem que para tal tenha de dar qualquer justificação. A decisão de desistir ou de não participar, não afetará a qualidade dos serviços de saúde que lhe são prestados agora ou no futuro.

O que me acontecerá caso decida participar?

Após receber o consentimento informado devidamente assinado, será feita uma avaliação do seu estado de saúde geral. Primeiro, serão gravados os sons dos seus pulmões durante aproximadamente 20 segundos, com um microfone ligado a um computador portátil. Seguidamente, ser-lhe-á medido o peso e a altura e avaliada a força dos seus músculos da respiração e a capacidade respiratória, através de dois testes que consistem em inspirar e soprar para um equipamento. Depois, será avaliada a força dos seus músculos da coxa, pedindo-lhe quando está sentado que faça força procurando esticar a sua perna contra a mão de uma pessoa durante 6 segundos, e a sua tolerância ao exercício através de um teste de sentar e levantar de uma cadeira cinco vezes. Ser-lhe-á também avaliada a sua expetoração e saliva, medida a quantidade de oxigénio no seu sangue, a sua frequência cardíaca, respiratória e tensão arterial. Ser-lhe-á também pedido que responda a um questionário para avaliar o seu nível de atividade física e que classifique a sua falta de ar, expetoração e tosse numa escala com diferentes graus. Por último ser-lhe-á colhido 20 mL de sangue por profissionais especializados e pedido que forneça expetoração para um frasco (este último passo apenas se aplica a doentes com DPOC; participantes saudáveis não fornecerão expetoração). Aos doentes com DPOC que ainda não tenham iniciado a reabilitação respiratória esta planeado fazer 2 colheitas de sangue e de expetoração (uma inicial e outra 12 semanas após o inicio da reabilitação) e nos indivíduos saudáveis e nos indivíduos com DPOC que não estão a fazer reabilitação respiratória, esta planeada também a realização de duas colheitas com um intervalo de 12 semanas. A iniciativa de fazer a primeira colheita, não implica a segunda, o paciente é totalmente livre de desistir de participar neste estudo a qualquer momento. A aplicação do protocolo terá a duração de aproximadamente 30 minutos. A dor e desconforto da colheita de sangue é a mesma de uma colheita de sangue para análises clínicas.

Quais são os efeitos secundários, desvantagens e riscos se eu resolver participar?

Os riscos serão os mesmos associados a uma colheita de sangue para análises clínicas.

Quais são os possíveis benefícios se eu resolver participar?

Não existem benefícios diretos de participar no estudo. No entanto, a informação obtida neste estudo poderá ajudar a melhorar o diagnóstico e monitorização da DPOC, uma doença crónica que afeta cerca de 800.000 portugueses.

A minha participação será confidencial?

Toda a informação recolhida no decurso do estudo será mantida estritamente confidencial. Os dados recolhidos serão salvaguardados com um código e palavra-passe, para que ninguém o/a possa identificar. Apenas os investigadores do projeto terão acesso aos seus dados.

O que acontecerá aos resultados do estudo?

Os resultados do estudo serão analisados e incorporados numa Dissertação de Mestrado e alguns serão publicados em Jornais Científicos. No entanto, em nenhum momento o Sr./Sra. será identificado/a. Se gostar de obter uma cópia de qualquer relatório ou publicação, por favor diga ao investigador com quem contactar.

Quem é que está a organizar e a financiar o estudo?

O estudo decorre em parceria entre a Universidade de Aveiro e o Instituto de Biologia Molecular e Celular do Porto em colaboração com o Centro Hospitalar do Baixo Vouga. Este estudo não é financiado por nenhuma entidade.

Quem fez a revisão do estudo?

O estudo foi revisto e aprovado pela Comissão de Ética do Centro Hospitalar do Baixo Vouga e da Universidade de Aveiro.

Contactos para mais informações sobre o estudo

Prof. Fátima Macedo, Prof. Alda Marques

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Annex II

Informed consent

Termo de Consentimento Livre e Esclarecido

Título do Projeto: Efeitos da Reabilitação Respiratória nos Linfócitos iNKT em pessoas com Doença Pulmonar Obstrutiva Crónica.

Nome da Investigadora Responsável: Prof. Maria de Fátima Macedo

Outros Investigadores do projeto: Prof. Alda Marques e Dr.ª Tânia Marante

Por favor leia e assinale com uma cruz (X) os quadrados seguintes.

1. Eu confirmo que percebi a informação que me foi dada e tive a oportunidade de questionar e de me esclarecer. ☐

2. Eu percebo que a minha participação é voluntária e que sou livre de desistir, em qualquer altura, sem dar nenhuma explicação, sem que isso afete qualquer serviço de saúde que me é prestado. ☐

3. Eu concordo que as recolhas de dados sejam fotografadas com o objetivo de ajudar no planeamento de futuros estudos e publicação em Revistas Científicas ou Congressos na área. ☐

4. Eu compreendo que os dados recolhidos durante o estudo são confidenciais e que só que só as 3 investigadoras do projeto terão acesso a eles. Portanto, dou autorização para que os mesmos tenham acesso a esses dados. ☐

5. Eu compreendo que os dados recolhidos durante o estudo podem ser utilizados para publicação em Revistas Científicas e usados noutras investigações, sem que haja qualquer quebra de confidencialidade. Portanto, dou autorização para a utilização dos dados para esses fins. ☐

_____	_____	_____
Nome da pessoa	Data	Assinatura

_____	_____	_____
Nome da Investigadora	Data	Assinatura

Annex III

Modified Borg scale

Modified British Medical Research Council questionnaire

COPD Assessment Test

Escala de Borg Modificada

0	Nenhuma
0.5	Muito, muito leve (só notável)
1	Muito leve
2	Leve
3	Moderada
4	Um pouco forte
5	Forte
6	
7	Muito forte
8	
9	
10	Muito, muito forte (máxima)

[119] (Borg, 1998)

BORG, G. (1998) Borg's perceived exertion pain scale. *Champaign. IL: Human Kinetics*.

Questionário de Dispneia

(Medical Research Council (MRC) Dyspnoea Questionnaire)

Por favor, marque com uma cruz (**X**) o quadrado correspondente à afirmação que melhor descreve a sua sensação de falta de ar.

GRAU 1 **Sem problemas de falta de ar excepto em caso de exercício físico intenso.** *"Só sinto falta de ar em caso de exercício físico intenso".*	
GRAU 2 **Falta de fôlego em caso de pressa ou ao percorrer um piso ligeiramente inclinado.** *"Fico com falta de ar ao apressar-me ou ao percorrer um piso ligeiramente inclinado".*	
GRAU 3 **Andar mais devagar que as restantes pessoas devido a falta de fôlego, ou necessidade de parar para respirar quando anda no seu passo normal.** *"Eu ando mais devagar que as restantes pessoas devido à falta de ar, ou tenho de parar para respirar quando ando no meu passo normal".*	
GRAU 4 **Paragens para respirar de 100 em 100 metros ou após andar alguns minutos seguidos.** *"Eu paro para respirar depois de andar 100 metros ou passado alguns minutos".*	
GRAU 5 **Demasiado cansado(a) ou sem fôlego para sair de casa, vestir ou despir.** *"Estou sem fôlego para sair de casa".*	

[120] DGS DGdS. Orientações Técnicas sobre Reabilitação Respiratória na Doença Pulmonar Obstrutiva Crónica (DPOC). In: Doença DdSdPeCd, editor. Lisboa2009 (adaptado).

Teste de Avaliação da DPOC – CAT

Este questionário irá ajudá-lo a si e ao seu profissional de saúde a medir o impacto que a DPOC (Doença Pulmonar Obstrutiva Crónica) está a ter no seu bem-estar e no seu quotidiano. As suas respostas e a pontuação do teste podem ser utilizadas por si e pelo seu profissional de saúde para ajudar a melhorar a gestão da sua DPOC e a obter o máximo benefício do tratamento. Para cada um dos pontos a seguir, assinale com um (X) o quadrado que melhor o descreve presentemente. Certifique-se que seleciona apenas uma resposta para cada pergunta.

Por exemplo: Estou muito feliz 0 1 2 3 4 5 Estou muito triste

Nunca tenho tosse	0	1	2	3	4	5	**Estou sempre a tossir**
Não tenho nenhuma expetoração (catarro) no peito	0	1	2	3	4	5	**O meu peito está cheio de expetoração (catarro)**
Não sinto nenhum aperto no peito	0	1	2	3	4	5	**Sinto um grande aperto no peito**
Não sinto falta de ar ao subir uma ladeira ou um lance de escadas	0	1	2	3	4	5	**Quando subo uma ladeira ou um lance de escadas sinto bastante falta de ar**
Não sinto nenhuma limitação nas minhas atividades em casa	0	1	2	3	4	5	**Sinto-me muito limitado nas minhas atividades em casa**
Sinto-me confiante para sair de casa, apesar da minha doença pulmonar	0	1	2	3	4	5	**Não me sinto nada confiante para sair de casa, por causa da minha doença pulmonar**
Durmo profundamente	0	1	2	3	4	5	**Não durmo profundamente devido à minha doença pulmonar**
Tenho muita energia	0	1	2	3	4	5	**Não tenho nenhuma energia**

Fonte: [122] George, F. H. M. (2013). Diagnóstico e Tratamento da Doença Pulmonar Obstrutiva Crónica.: Direção Geral de Saúde.

Muito obrigado pela sua colaboração.