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Effects of pulmonary rehabilitation on immune mediators detected in the saliva of people with COPD

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

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palavras-chave	Doença pulmonar obstructiva crónica, DPOC, reabilitação respiratória, IL-8, IL-6, TNF-α, IL-10, IgA secretada, medidas clínicas.

resumo

Enquadramento: A doença pulmonar obstrutiva crónica (DPOC) é a terceira causa de morte no mundo. É caracterizada por uma obstrução persistente e progressiva das vias aéreas, associada a uma resposta inflamatória crónica nas vias aéreas e pulmões. Estas pessoas apresentam níveis alterados de citocinas pró / anti-inflamatórias e de IgA secretada (IgAS). A reabilitação respiratória (RR) é a intervenção terapêutica mais eficaz na melhoria dos sintomas, da tolerância ao exercício, da funcionalidade, força muscular e qualidade de vida de pessoas com DPOC, mas o seu impacto nos níveis dos mediadores inflamatórios e IgAS destas pessoas não é, ainda, consensual.

Objetivo: Comparar os níveis de mediadores inflamatórios (TNF-α, IL-6, IL-8, IL-10) e IgAS na saliva de pessoas com DPOC com os de pessoas saudáveis. Avaliar o impacto da RR nesses mediadores e na IgAS e correlacionar os seus níveis com dados clínicos.

Métodos: As pessoas com DPOC foram recrutadas nos cuidados de saúde primários e participaram num programa de RR comunitário duas vezes por semana durante três meses. Os dados sociodemográficos (idade, sexo), antropométricos e clínicos (função pulmonar- FEV_1pp, teste de marcha dos 6 minutos-TM6M, força muscular dos quadricípetes e *handgrip*, funcionalidade-teste de sentar e levantar 1-min) foram recolhidos três meses antes, imediatamente antes, imediatamente após e três meses após a RR. As amostras de saliva das pessoas com DPOC foram recolhidas todos os meses e armazenadas a -80ºC. As pessoas saudáveis foram recrutadas de academias séniores e comunidade envolvente e avaliadas uma vez. Os mediadores inflamatórios e a IgAS foram quantificados nas amostras de saliva através do método ELISA.

Resultados: Vinte pessoas com DPOC (n=14, 70% do sexo masculino; 71 [67.3-76] anos; FEV_1pp= 48.2± 16.4) e onze pessoas saudáveis (n=6, 54.5% do sexo masculino; 73 [68-74] anos; FEV_1pp= 109.6±27.6) participaram no estudo. Não foram encontradas diferenças significativas nos níveis dos mediadores inflamatórios e IgAS entre pessoas com DPOC e saudáveis. A IL-10 não foi detetado na maioria das amostras de saliva. Na última sessão de RR, foi observado um aumento significativo de IL-6. Foi observada uma tendência de aumento na IgAS durante a RR. Antes da RR, foi observada uma correlação significativa positiva entre o TNF-α e a força muscular do quadricípete nos doentes responsivos (p=0.0026; r=0.9580), e entre a IL-6 e o TM6M em todos os doentes (p=0.0191; r=0.6222). Após a RR, foi observada uma correlação significativa positiva entre a IL-6 e a força muscular do quadricípete (p=0.0463; r=0.5269). Foram encontradas correlações significativas (0.0025≤ p ≥ 0.0438; -0.8009 ≤ r ≥ 0.9539) entre a IgAS e o teste de sentar e levantar 1-min, FEV_1pp, a força *handgrip*, TM6M e a força muscular dos quadricípetes em diferentes momentos do estudo.

Conclusão: Este estudo sugere que a RR leva a um aumento significativo nos níveis de IL-6 e a uma alteração, ainda que não significativa, nos níveis de IgAS. Além disso, a IL-6, o TNF-α, mas principalmente a IgAS parecem correlacionar-se com parâmetros clínicos de pessoas com DPOC.

Mais estudos são necessários para confirmar algumas tendências observadas e aumentar o conhecimento sobre a evolução da resposta inflamatória durante RR.

keywords

Chronic obstructive pulmonary disease, COPD, pulmonary rehabilitation, IL-8, IL-6, TNF-α, IL-10, secretory IgA, clinical outcomes.

abstract

Background: Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the world. It is characterised by persistent and progressive airway obstruction associated with a chronic inflammatory response in the airways and lungs. These patients have altered levels of pro / anti-inflammatory cytokines and of secretory IgA (SIgA). Pulmonary rehabilitation (PR) is the most effective therapeutic intervention in improving the daily symptoms, exercise tolerance, functionality, muscle strength and quality of life of people with COPD, but its impact of on the levels of inflammatory mediators and SIgA is not consensual.

Aim: To compare the levels of inflammatory mediators (TNF-α, IL-6, IL-8 and IL-10) and SIgA in saliva samples from people with COPD and from healthy people. To evaluate the impact of PR on these mediators and SIgA and correlate their levels with clinical data.

Methods: People with COPD were recruited from primary health care and enrolled in a community PR programme twice a week during three months. Sociodemographic (age, sex), anthropometric (height, weight) and clinical data (lung function- FEV_1pp, six minute walking test- 6MWT, quadriceps and handgrip muscle strength, functionality- 1 min sit-to-stand) were collected three months before, immediately before, immediately after and three months after PR. Saliva samples of people with COPD were collected every month and stored at -80 ° C. Healthy people were recruited from senior academies and surrounding community, and evaluated once. Inflammatory mediators and SIgA were quantified by ELISA.

Results: Twenty people with COPD (n=14, 70% male; 71 [67.3-76] years; FEV_1pp= 48.2±16.4) and eleven healthy people (n=6, 54.5% male; 73 [68-74] years; FEV_1pp=109.6±27.6) participated in the study. No significant differences were found in the levels of analysed cytokines and SIgA between patients with COPD and healthy people. IL-10 was not detected in most samples. In the last PR session, a significant increase in IL-6 was observed. It was observed a trend towards an increase of SIgA during PR. Before PR we found a significant positive correlation between TNF-α and quadriceps muscle strength in responder patients (p=0.0026; r=0.9580) and between IL-6 and 6MWT in all patients (p=0.0191; r=0.6222). After PR a significant positive correlation between IL-6 and quadriceps muscle strength (p=0.0463; r=0.5269) was observed. Significant correlations were found (0.0025≤ p ≥ 0.0438; -0.8009 ≤ r ≥ 0.9539) between SIgA and FEV_1pp, handgrip strength, 1 minute STS, 6MWT and quadriceps muscle strength at different times of the study.

Conclusion: This study suggest that PR leads to a significant increase in IL-6 and to a variation of SIgA levels, although not significant. Furthermore, IL-6, TNF-α, but mostly SIgA seem to correlate with clinical parameters of people with COPD.

Further studies are needed to confirm some trends observed and to increase our knowledge on the variation of the inflammatory response during PR.

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Abbreviations

6MWT - Six minute walking test

ABTS - 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)

BAL - bronchoalveolar lavage

BMI - Body mass index

BSA - Bovine serum albumin

COPD – Chronic obstructive pulmonary disease

DAMP - Danger-associated molecular patterns

EBC - Exhaled breath condensate

ELISA - Enzyme-Linked Immunosorbent Assay

ESSUA - *Escola Superior de Saúde da Universidade de Aveiro*

FEV_1pp - Forced expiratory volume in one second percent predicted

FVCpp - Forced expiratory volume in one second percentage predicted

ICS - Inhaled corticosteroids

GOLD - Global initiative for chronic obstructive lung disease

IL - Interleukin

LABA - Long-acting beta-agonist

LAMA - Long-acting muscarinic antagonist

LLD – Lower limit of detection

MCID - Minimal clinically important difference

MMP9 - Matrix metallopeptidase 9

mMRC - Modified British Medical Research Council

PBS - Phosphate-buffered saline

PR - Pulmonary rehabilitation

PRR - Pattern-recognition receptors

SABA - Short-acting beta-agonist

SAMA - Short-acting muscarinic antagonist

SIgA - Secretory Immunoglobulin A

STS - Sit-to-stand

TGF-β - Transforming growth factor-β

TLR - Toll-like receptors

TMB - Tetramethylbenzidine

TNF-α - Tumor necrosis factor α

1. Introduction

1. INTRODUCTION

1.1. Definition, diagnosis and assessment of chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) represents a major health-care burden (1). It is defined as a "common, preventable and treatable disease that is characterised by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases" (2). COPD is a heterogeneous and complex disease, since it has a number of intrapulmonary and extrapulmonary components whose dynamic interactions over time are not linear and not all of these components are present in all individuals at a given time-point (3).

Remodeling and narrowing of small airways and destruction of the lung parenchyma are two major pathologic processes responsible for the progressive limitation of airflow in COPD (Figure 1). Chronic inflammation in pulmonary periphery is responsible for these pathological changes (4).
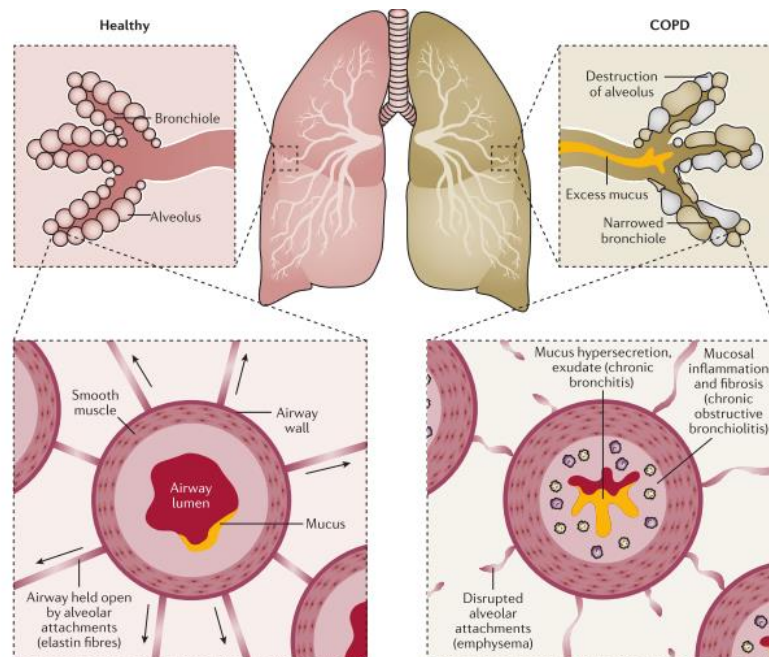
Figure 1- Airways in healthy people and in people with chronic obstructive pulmonary disease (COPD). In COPD, airflow limitation is caused by chronic bronchitis (with mucus hypersecretion and small airway chronic inflammation) and emphysema. Adapted from (5).

The diagnosis involves a comprehensive assessment of COPD, i.e., level of airway obstruction, impact of disease on patient's health status, and symptoms/risk of future events (such as exacerbations, hospital admissions, or death) (2). The level of airway obstruction is assessed with spirometry, and is indicative of COPD when, post-bronchodilator, the ratio of the Forced Expiratory Volume in One Second (FEV_1)/Forced Vital Capacity (FVC) is inferior to 0.70. Values obtained in FEV_1 percentage predicted (FEV_1pp) classify the severity of airway obstruction into four levels (GOLD 1, 2, 3 and 4) (2). The impact of the disease on each patient requires the symptomatic assessment and risk of exacerbations. Therefore, after spirometry, patients should have their breathlessness during activities assessed with the Modified British Medical Research Council (mMRC) Questionnaire or undergo a comprehensive assessment of their symptoms with the COPD Assessment Test (CAT), a multidimensional measure of health status impairment in COPD (2). Finally, their history of moderate and severe (including prior hospitalizations) exacerbations should be registered. The combination of these data identify patients as "ABCD" according to their symptom burden and risk of exacerbation (Figure 2) (2).
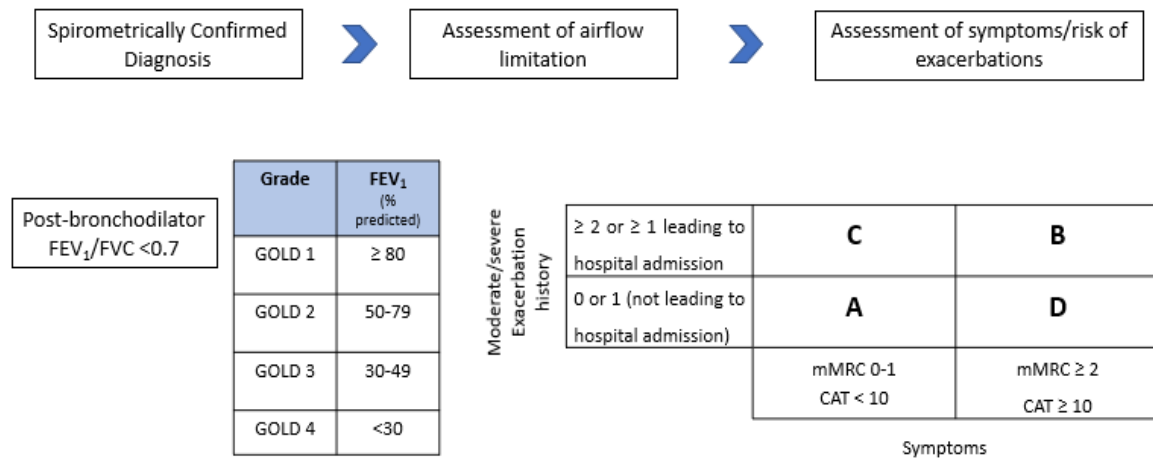
Figure 2- The refined ABC assessment tool. Adapted from (2).
Abbreviations: mMRC- Modified British Medical Research Council; CAT- COPD Assessment Test

1.2. Epidemiology

COPD is the third cause of death in the world (6) affecting 384 million people worldwide with around three million deaths annually (2). The estimated prevalence of COPD in Portugal is about 800000, about 14.2% for Portuguese over 40 years old (7). In the last 5 years the diagnosis of COPD increased 241% (8). In 2016, COPD was responsible for 2791 deaths, 20.7% of all deaths recorded caused by respiratory diseases (7).

1.3. Clinical manifestation

Symptoms of patients with COPD include dyspnoea, chronic cough (productive or unproductive), sputum production, fatigue, wheezing and chest tightness (2). While in mild COPD, patients experience breathlessness only when they perform high energy activities, with progression of the disease, they also begin to feel breathlessness during normal daily activities (9). Acute exacerbations are common in people with COPD (2 to 3 per year) (2). These events are defined as an acute worsening of respiratory symptoms that result in additional therapy, which are the main responsible for patient's clinical deterioration (2,10).

Patients with COPD are also simultaneously affected by other diseases, called comorbidities, that can occur at any stage of the disease (11). About 80% of patients with COPD are likely to have at least one comorbidity such as cardiovascular disease, skeletal muscle dysfunction, metabolic syndrome, osteoporosis, depression, anxiety and lung cancer (2,12).

1.4. Causes and risk factors

Tobacco smoking is the main cause of COPD worldwide (2). It is also known to exist a genetic risk factor in a small proportion of the world's population, i.e., deficiency of α-1 antitrypsin, a major circulating inhibitor of serine proteases (2). Gender is also considered a risk factor. COPD in women is distinct from men with respect to phenotype, symptom burden and comorbidities (13). Currently smoking females with COPD have more severe obstruction than male smokers, despite similar exposure to cigarettes (14). Although COPD

has a higher prevalence in men than in women, women are more predisposed to develop chronic bronchitis, have more dyspnea, and suffer more frequently from coexistent anxiety or depression than men (13,15). Aging is also a risk factor for COPD. Increasing aging of the airways and parenchyma mimic some of the structural changes associated with COPD (2). Long-term cigarette smoke exposure can progressively deplete cells of their defense and repair proceedings, driving cells towards apoptosis, senescence or stem cell exhaustion (16).

There are other important risk factors for developing COPD such as exposure to smoke from biomass fuel, socioeconomic status, asthma, airway hyper-reactivity, chronic bronchitis and infections (2).

1.5. Treatment

COPD treatment includes pharmacological and non-pharmacological therapy (Figure 3) (2). Pharmacological therapy is used to reduce symptoms, reduce the frequency and severity of exacerbations, and improve exercise tolerance and health status (2). Pharmacological treatments should be complemented by appropriate non-pharmacological interventions. Smoking cessation has the greatest capacity to influence the natural history of COPD, however, the inflammatory and structural changes in the airways increase with disease severity and persist after smoking cessation (2,17). Pulmonary rehabilitation is a grade A evidence intervention and is one of the most cost-effective non-pharmacological interventions for patients with COPD. It is part of the integrated care considered essential to manage COPD (2).
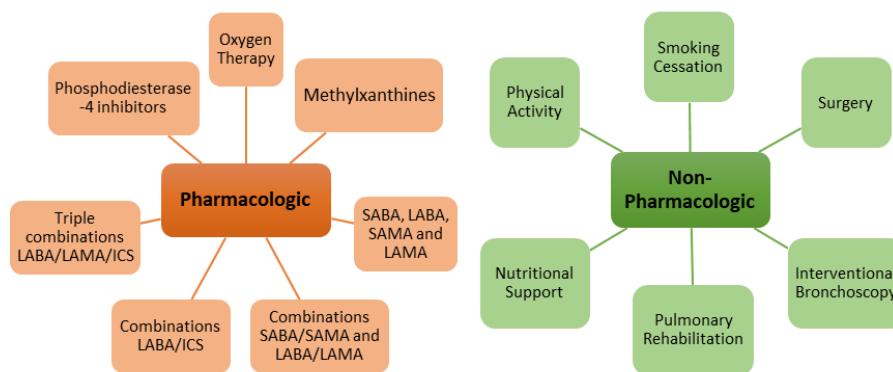
Figure 3- Pharmacological and non-pharmacological treatment in chronic obstructive pulmonary disease (2). **Abbreviations**: SABA- beta$_2$-agonist with short acting; LABA- beta$_2$-agonist with long acting; SAMA- anticholinergics with shorth acting; LAMA- anticholinergics with long acting; ICS- Corticosteroids.

1.6. Inflammatory response in chronic obstructive pulmonary disease

Progressive and persistent airflow limitation in patients with COPD is associated with a chronic inflammatory response in the airways and lungs to noxious particles or gases (2). Besides that, COPD is also associated with systemic inflammation (18). However, the origin of the systemic inflammation present in patients with COPD remains poorly understood (19), but one of the most widely accepted hypothesis is the spill-over of inflammatory mediators from the lung to the blood, which may underlie and potentiate comorbidities (4,20).

Injury of airway epithelial cells by cigarette smoke and other irritants inhaled into the respiratory tract triggers a non-specific inflammatory response that leads to the release of endogenous intracellular molecules or danger-associated molecular patterns (DAMPs). These signals are identified by pattern-recognition receptors (PRR) such as Toll-like receptors (TLR), which results in the release of cytokines (21). Macrophages and epithelial cells in patients with COPD can be activated by cigarette smoke and release inflammatory mediators (such as TNF-α, IL-6 and IL-8), contributing to a more pro-inflammatory milieu (18,22). Then, inflammatory cells, such as neutrophils and monocytes (which differentiate into macrophages in the lungs), are recruited to the site of inflammation to orchestrate the innate immune response (5,23). The infiltration of the bronchial wall by these inflammatory cells leads to an increase in the tissue volume of the wall which contributes to small airflow obstruction (24). Inflammatory cells attracted to the injured site release proteases, such as

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elastase and matrix metallopeptidase 9 (MMP9), which results in mucus hypersecretion, elastin degradation and emphysema. Epithelial cells and macrophages also release transforming growth factor-β (TGF-β), which triggers fibroblast proliferation for tissue remodeling (25). Neutrophils are a potent source of proteases, particularly neutrophil elastase, which leads to emphysema development, while also releasing IL-8, a pro-inflammatory cytokine (24). Activation of adaptive immunity occurs later in the course of the disease and leads to increased numbers of T lymphocytes and B lymphocytes in the lungs (5). Lymphocytes, specifically T cells, are found in the lung parenchyma and airways, where they are the major source of cytokines (24).

Overall, the inflammatory response plays a central role in the development of pathological changes in the airways and lung parenchyma such as the promotion of mucus hypersecretion, proteolysis and narrowing of small airways, which, in turn, lead to clinical phenotypes of chronic bronchitis, airway obstruction and emphysema (22,26).

1.6.1.   Analysis of the inflammatory response

Assessment of inflammation of airway diseases can be performed by a diversity of techniques, involving analysis of induced sputum, measurement of biomarkers in the in the exhaled air, exhaled breath condensate (EBC), bronchoalveolar lavage (BAL) and transbronchial biopsy (27). These methods present advantages and disadvantages which will now be briefly discussed. Previous studies have used blood samples to analyse the inflammatory profile of patients with COPD, and suggested that active inflammation marked by increased serum levels of TNF-α, IL-6 and IL-8 was associated with progression of COPD (28,29). However, the blood test may not describe the exact inflammatory state of the lungs, but rather a possible leakage from it to the blood, which may be affected by other comorbidities (18). Moreover, this method requires a specialised professional for blood collection, and causes discomfort to the patient.

Bronchial biopsies have been useful for recording the expression of inflammatory proteins in patients with COPD (30). However, this method is too invasive, so it is inappropriate for repeated measurements that are necessary for routine assessment (30).

Over the last few years, there has been an increasing trend for assessing inflammatory biomarkers on airway diseases on samples collected by noninvasive techniques such as induced sputum, exhaled air, and EBC (22). In studies that require multiples collections of samples it is important to use non-invasive samples as they do not cause discomfort and facilitate patients' adherence to sample collection protocols, particularly in longitudinal studies. The use of saliva as biological specimens for the measurement of diagnostic biomarkers is also becoming increasingly popular due to its non-invasive nature (31).

Saliva is a clinically informative fluid that contains adaptive and innate immune components and multiple biomarkers, which make it useful for multiplexed assays (31,32). Based on the anatomical proximity between the oral cavity and the airways we expect that the inflammatory profile of the airways can be reflected in the inflammatory profile of the mucosa in the oral cavity, since several salivary biomarkers have been associated with the diagnosis, prevention and treatment of various diseases, regardless of their origin (33).

Nevertheless, the use of saliva to study inflammation in respiratory diseases is still uncommon, even though its usefulness is already supported by some studies (34,35). It has actually been suggested that saliva is an appropriate specimen for biomarker assessment of the disease activity in COPD (34). However, it is also important to note that there may be some disadvantages of its use; e.g., the inflammatory profile of saliva can be affected by oral diseases, namely by dental caries (36), gingival inflammation (37), or by oral squamous cell carcinoma (38). Nevertheless, the use of saliva is still rare in the study of COPD, and further investigation is still needed which is the purpose of this dissertation.

1.6.2. Inflammatory mediators

The levels of many different inflammatory mediators, such as cytokines, released by inflammatory cells are increased in the lungs of patients with COPD, which maintain inflammation and recruit circulating cells into the lungs (5). Cytokines can be classified as pro- or anti-inflammatory. Pro-inflammatory cytokines are involved in the up-regulation of inflammatory reactions, while anti-inflammatory cytokines are responsible for controlling the pro-inflammatory cytokine response (39). A variety of inflammatory cytokines have their expression altered in BAL, serum and sputum of patients with COPD, and appear to

amplify inflammation (23,29). Some of these mediators and their expression in patients with COPD are shown in Table 1. The concentration of TNF-α is higher in sputum, BAL and serum of patients with COPD than in people without the disease (29,40). Serum TNF-α levels are significantly higher in severe than in mild and moderate COPD and there is a significant negative correlation between BAL TNF-α levels and FEV_1/FVC (29). Many cells, including epithelial cells, macrophages, T lymphocytes, and airway smooth muscle cells, have the capacity to secrete TNF-α, when activated (23). This cytokine has relevant inflammatory effects to COPD, resulting in activation of destruction of lung parenchyma through the release of proteinases and stimulation of mucus secretion (41).

IL-6 and IL-8 are higher in serum and BAL of patients with COPD than in people without the disease with a significant negative correlation between BAL levels and FEV_1/FVC (29). Furthermore, frequent exacerbations correlate with higher sputum IL-6 and IL-8 levels (42). IL-6 is produced by bronchial epithelium, macrophages and other cells at the site of inflammation in response to environmental stress such as smoking, while IL-8 sources include monocytes, tissue and alveolar macrophages, pulmonary epithelium and smooth muscles cells of the airway (29). When increased, the action of these cytokines contributes to the establishment of pulmonary emphysema (22).

IL-10 is a potent anti-inflammatory cytokine that is released from monocytes and alveolar macrophages in response to inflammatory stimuli (41). It has inhibitory effects on the inflammatory development of COPD. Decreased serum levels of IL-10 are negatively related to smoking, GOLD grade, mMRC score, and clinical history of patients, but positively correlated with the pulmonary ventilation function of patients (43).

Table 1- Studies reporting serum/plasma/BAL/Saliva/EBC levels of inflammatory markers in chronic obstructive pulmonary disease.

	Serum	Plasma	BAL	Sputum	Saliva	EBC
TNF-α	↑(28,29) ↓(34)	=(44)	↑(29)	↑(40,45,46)	↓(34)	
IL-6	↑(29)	=(44)	↑(29)	↑(34,45)		↑(47)
IL-8	↑(28,29,48)		↑(29) =(34)	↑(34,40,45,48–52)	=(34)	
IL-10	↓(43)			↓(53)		
IL-18	↑(54)	↑(44)		↑(46)		
IL-17	↑(43,55)					
IL-35	↓(43)					
TGF-β				↑(48)		
LTB4				↑(48)		
IL-1β	↑(55,56)					

↑: increase in patients with COPD compared to healthy people. ↓: decrease in patients with COPD compared to healthy people. =: no significant difference between patients with COPD and healthy people.
Abbreviations: BAL- Bronchoalveolar lavage; EBC- Exhaled breath condensate; IL- Interleukin; TGF- transforming growth factor; LTB4- Leukotriene B4

1.6.3. Secretory Immunoglobulin A

In COPD, of the 80% of exacerbations with an infectious cause, approximately half have a bacterial origin (57). Secretory Immunoglobulin A (SIgA) is the predominant immunoglobulin in secretions of the mucosal immune system. It is a first-line defense mechanism of the upper and lower conducting airways against foreign antigens and microorganisms. Its main function is to neutralize bacteria, by interfering in their motility or competing for sites of epithelial adhesion (58). Secretion of SIgA depends on the polymeric immunoglobulin receptor (pIgR), which transports dimeric IgA from the basolateral surface of the epithelium to the apical side. Then, the secretory component of pIgR is cleaved off forming SIgA which is released to mucosal surfaces (Figure 4) (59). Patients with COPD have an increase of IgA synthesis in the lungs. However, they do not

have increased secretory IgA levels, probably due to a reduced pIgR-mediated transport of IgA across the epithelium (60). Insufficient delivery of SIgA to the surface of individual small airways compromises protection of the lungs through SIgA, as it might allow colonizing bacteria to cross the epithelial barrier and drive persistent inflammation (61). SIgA deficiency correlates with severity of airflow obstruction in patients with COPD (61). Indeed, SIgA-deficient small airways are characterised by structurally abnormal epithelium, frequent bacterial invasion across the mucosal surface and accumulation of inflammatory cells (61).
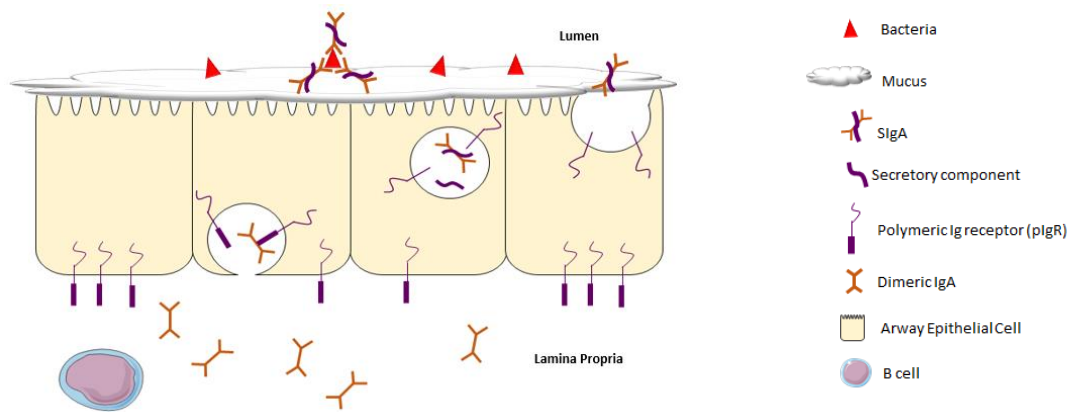
Figure 4- Transport of dimeric IgA from the lamina propria across the epithelial barrier, by pIgR, realising SIgA to the mucosal surface to neutralization of bacteria. Adapted from (59).

1.7. Pulmonary Rehabilitation and inflammation

Pulmonary rehabilitation (PR) is defined as "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, 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" (62). The goals of pulmonary rehabilitation include minimizing symptom burden, maximizing exercise performance, promoting autonomy, increasing participation in everyday activities, enhancing (health-related) quality of life, and effecting long-term health-enhancing behavior change (62). Independently of disease stage and complexity, the benefits of PR to

patients with COPD have been widely demonstrated (63). PR has been shown to be the most effective therapeutic strategy to improve daily symptoms, shortness of breath, health status, exercise tolerance, muscle strength, social function, depression, vitality and overall improvement in quality of life (63,64). Exercise is a core component of PR (63). Regular exercise can attenuate chronic inflammation by inducing systemic increase of cytokines with anti-inflammatory properties (65). It is correlated with a reduction on the levels of proinflammatory cytokines in sputum, which may indicate a protective effect of exercise in patients with COPD (66). Furthermore, the muscle strength and muscle cross-sectional area improvements observed during PR, suggest that exercise training has an anabolic effect, which might be due to diminished inflammation, as inflammation seems to hinder anabolism and stimulate catabolism (67). Thus, a beneficial effect on inflammation status of patients with COPD can be expected from undertaking PR. To our knowledge, few studies have investigated the effects of PR on the inflammatory profile of patients with COPD, focusing mainly on systemic inflammation. Some authors did not find modification on plasma/serum TNF-α and IL-6 levels (68), but a decrease on plasma IL-8 levels, after PR, has been observed (69). Moreover, a recent study reported that 5 in 7 patients had an increase of plasma TNF-α levels at the end of a 6 months PR program (70). Thus, the effect of PR on inflammation status of patients with COPD is not completely understood, especially local inflammation, which needs to be more investigated, since it plays a large and crucial role in the development of COPD (22).

Although it is known that patients with COPD have an SIgA deficit, which compromises protection against bacterial respiratory infections (61), the effect of PR on SIgA levels in patients with COPD is yet unknown. A recent study has shown that regular moderate exercise increases salivary SIgA levels in elderly people (71). Therefore, a benefic effect of PR on pulmonary immune function through the increase of SIgA, can be expected.

1.8. Objectives

The effect of PR on inflammation status of patients with COPD is not well established. It is necessary to fully understand the effect of this therapy on the immune status of patients and how the effectiveness of PR in the clinical profile is related to pulmonary inflammation.

If PR can remodel the inflammatory status of patients with COPD this might represent a significant advance on personalized management.

Thus, the aims of this dissertation were:

- To compare the levels of inflammatory mediators and SIgA in saliva of patients with COPD and healthy subjects.
- To evaluate the impact of pulmonary rehabilitation on levels of these inflammatory mediators and SIgA in saliva of patients with COPD and compare them with clinical data.

In the present study, saliva samples were used, due to the simplicity in its collection and analysis. The inflammatory mediators chosen for this study were TNF-α, IL-6, IL-8, and IL-10.

2. Methods

2. METHODS

This study was integrated in two larger studies:

- "PRISMA - Pulmonary Rehabilitation: a response for patients with COPD in an InduStrialized environment and its implication on lung MicrobiotA", funded by LabEx DRIIHM, International Observatory Hommes-Milieux of Estarreja, looking at the effects of pulmonary rehabilitation to better manage the disease trajectory namely acute exacerbations and on the airway microbiome and inflammation, in an industrialized area.

- "PRIME - Pulmonary Rehabilitation Innovation and Microbiota in Exacerbations of COPD", funded by Programa Operacional de Competitividade e Internacionalização – POCI through Fundo Europeu de Desenvolvimento Regional - FEDER (POCI-01-0145-FEDER-028806) and Fundação para a Ciência e Tecnologia (PTDC/SAU-SER/28806/2017).

2.1. Ethics

Ethical approval was previously obtained from Centro Hospitalar do Baixo Vouga, Agrupamento dos Centros de Saúde do Baixo Vouga (Annex I) and National Data Protection permission (Annex II). Prior to any data collection, written informed consents were collected from patients. The strict confidentiality and anonymity of all data collected was ensured.

2.2. Design and participants

Patients with COPD were recruited from Centro Hospitalar do Baixo Vouga and Agrupamento dos Centros de Saúde do Baixo Vouga. Patients were eligible if diagnosed with COPD according to GOLD criteria (2) and presented in a stable state. Patients were excluded if they presented: history of an acute cardiac/respiratory condition within the previous month; significant cardiac, musculoskeletal or neuromuscular diseases that impaired their participation in the PR programme; signs of cognitive impairment and history of neoplasic or immunological disease. During routine appointments, clinicians informed eligible patients about the study. Only patients who showed interest to participate were included in the study and approached by the researcher who clarified any doubts and collected the

informed consents. All eligible patients were offered community-based PR (at primary health care centres or at the Respiratory Research and Rehabilitation Laboratory – Lab3R, School of Health Sciences (ESSUA), University of Aveiro) for 12 weeks, with exercise training twice a week and psychoeducational sessions once every other week. Detailed description of the intervention delivered can be found elsewhere (72–74).

Healthy people with sixty years old or more were recruited from senior academies and surrounding community. These participants were excluded if they have had an acute respiratory disease in the previous month, had a significant cardiorespiratory, musculoskeletal, neurological and/or psychiatric disorder, that could interfere or limit their participation in the study or in data collection and, showed signs of substances abuse. After the identification of eligible participants, patients who showed interest to participate were included in the study and approached by the researcher who clarified any doubts and collected the informed consents. Healthy people matched for gender, age and body mass index to patients with COPD, were included in this study.

2.3. Data collection

Sociodemographic (i.e., sex, age, education, marital status, occupation), anthropometric (i.e., weight, height, body mass index) and clinical (i.e., comorbidities, medication, long term oxygen therapy, noninvasive mechanical ventilation, smoking habits) data were first collected with a structured questionnaire.

Then, additional clinical data were collected:

- Lung function was assessed with spirometry (FEV_1, FEV_1pp, FVC, FVCpp, FEV_1/FVC) (75). Minimal clinically important difference (MCID) for FEV_1 is 100 ml (76).

- Peripheral muscle strength (quadriceps) and handgrip muscle strength were measured with dynamometers (77). There is no MCID stablished for these clinical data. Therefore, a MCID above 6% was used as previously recommended (78).

- Functionality was assessed with 1 minute sit-to-stand (1 minute STS) test. In this test, patients sit and stand from a chair without using their arms for support as many times as possible for 1 minute period or as long as they feel comfortable (79).

The number of repetitions is registered. Minimal clinically important difference for 1 minute sit-to-stand is 3 repetitions (80).

- Exercise tolerance was assessed with the six minute walking test (6MWT). Subjects were instructed to walk at a 30 meter corridor, while attempting to cover as much distance as possible in 6 min (79). Minimal clinically important difference for 6MWT is 30 meters (81).

Patients with COPD were comprehensively assessed with this protocol at T0 (baseline – month 1), T1 (3 months after T0 and prior to the pulmonary rehabilitation programme – month 3), T2 (immediately after the PR programme – month 6) and at T3 (3 months after the PR programme – month 9). Additionally, saliva samples were collected, to a small recipient, during the data collection protocol, every month from each participant. The different timepoints of data collection and the respective measurements collected in each timepoint are summarised in Figure 5. Healthy people were assessed only once, and their saliva samples were also collected.
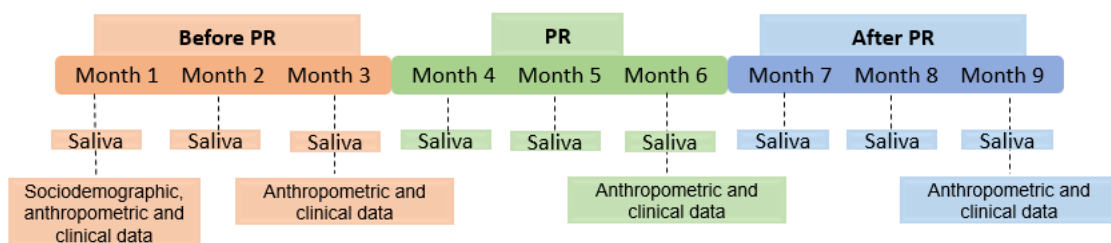
Figure 5- Chronology of saliva and clinical data collection of patients with chronic obstructive pulmonary disease.

2.4. Sample processing

Upon collection all saliva samples were kept at 4 °C for a maximum of 36 hours followed by storage at -80 °C. Before analysis, saliva samples were thawed at room temperature and centrifuged for 10 min at 10000 g at 4 °C. Supernatants were collected and aliquots were frozen at -80 °C (Figure 6).
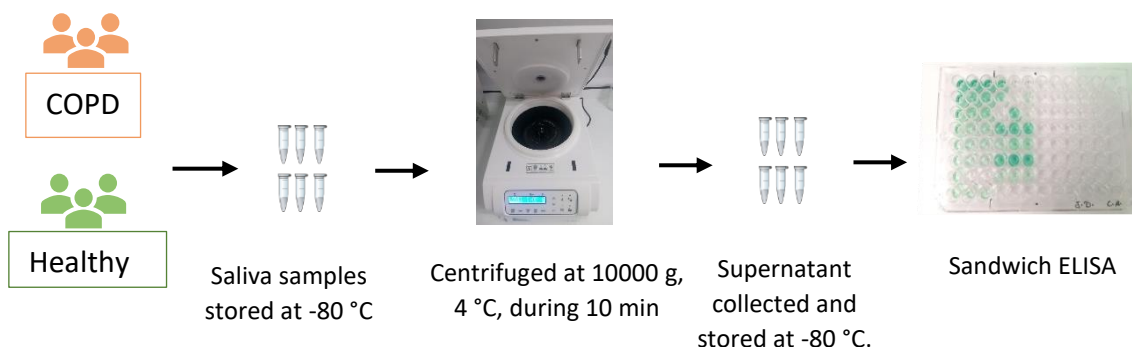
Figure 6 - General scheme of processing and analysis of saliva samples.

2.5. Cytokine quantification

The levels of TNF-α, IL-6, IL-8 and IL-10 were quantified by ELISA with Development Kits from Peprotech according to manufacturer's instructions. Plates with half area from CORNING were used.

Firstly, plates were covered with 50 µl of capture antibody diluted in PBS (at 1 µg/ml for anti-human TNF-α antibody; 0.5 µg/ml for anti-human IL-6; 0.125 µg/ml for anti-human IL-8; 1 µg/ml for anti-human IL-10). The plate was incubated overnight on a shaker with very low stirring at room temperature. Wells were washed 4 times with 150 µl washing buffer per well. Then, 150 µl of Block Buffer were added to each well and the plate was incubated for 1 hour at room temperature. The plate was washed 4 times and 50 µl of standard recombinant protein diluted in diluent (from 2000 pg/ml to 0 for TNF-α and IL-6, from 150 pg/ml to 0 for IL-8, from 3000 pg/ml to 0 for IL-10) or sample were added to each well. The plate was incubated for 2 hours and washed 4 times. A biotinylated detection antibody diluted in diluent (at 0.15 µg/ml for anti-human TNF-α; at 0.1 µg/ml for anti-human IL-6; at 0.25 µg/ml for anti-human IL-8; at 0.5 µg/ml for anti-human IL-10) was added and the plate was incubated for 2 hours before washing and aspirated 4 more times. For TNF-α, IL-6 and IL-8 quantification assays, Streptavidin-HRP at 0.05 µg/ml was added to each well. The plate was incubated for 30 minutes and washed 4 times. For IL-10 quantification, Avidin-HRP diluted at 1:2000 was added to each well before washing. Then, for color development, 50µl of Substrate Tetramethylbenzidine (TMB) for TNF-α, IL-6 and IL-8 assays and 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) for IL-10 assay were added to each

well and the plate was incubated for 20 minutes under dark conditions. For TNF-α, IL-6 and IL-8 assays, 50 µl of 1M HCl Stop Solution were added to each well. Finally, the plate was placed in an ELISA plate reader, for color development monitor, at 450 nm with wavelength correction set at 620 nm. For IL-10 assay, after the incubation with ABTS substrate, the color development was immediately monitored at 405 nm with wavelength correction set at 650 nm.

Constitution of solutions:

- PBS: dilute 10×PBS to 1×PBS, pH 7.20 in sterile water.
- Wash buffer: 0.05% Tween-20 in PBS.
- Block Buffer: 1% BSA in PBS.
- Diluent: 0.05% Tween-20, 0.1% BSA in PBS.

Several experiments were performed for each cytokine and the lower limit of detection (LLD) of each cytokine was not the same in all experiments.

2.6. Secretory IgA quantification

The concentration of secretory IgA (Human) was quantified with a commercially available ELISA Kit from Abnova. Briefly, 190 µl of red EIA buffer were added to the wells allocated for saliva. Then, 100 µl of calibrators and control serum were added into allocated wells, as well as 10 µl of saliva, previously diluted in EIA buffer (5 µl of saliva + 500 µl of EIA buffer). The plate was incubated 90 min at 37 °C and washed 3 times with 250 µl washing buffer per well. After this, 100 µl of Conjugate were allocated into the wells, and the plate was incubated for 30 min at 37 °C before washing 5 times. Then 100 µl of substrate solution were dispensed into the wells and the plate was incubated 10-20 min at room temperature (18-25 °C). 100 µl of Stop Solution were dispensed into the wells and color development was measured at 450 nm.

Constitution of solutions:

- Six calibrators: 0; 2; 20; 40; 100; 400 µg/ml. Human secretory IgA diluted in tris buffered BSA solution, preservative – 0.01% Bronidox L, 0.01% 2-Methyl-4-isothiazolin-3-one-hydrochloride; also contains bright blue dye

- Control serum: Dilution of preselected human serum, with high content of secretory IgA with BSA solution; preservative – 0.01% Bronidox L, 0.01% 2-Methyl4-isothiazolin-3-one-hydrochloride, colourless.

- Conjugate: Aqueous solution of murine monoclonal to human IgA alfa chain coupled with horseradish peroxidase diluted on phosphate buffered solution with casein from bovine milk and detergent (Tween-20), contains 0.1% phenol as preservative and bright red dye.

- Red EIA buffer: Phosphate buffered saline with casein from bovine milk and detergent (Tween-20), contains 0.1% phenol as preservative; contains red dye

- EIA buffer: Phosphate buffered saline with casein from bovine milk and detergent (Tween-20), contains 0.1% phenol as preservative and blue dye.

- Washing solution concentrate 26x: Aqueous solution of sodium chloride and detergent (Tween 20), contains proClin300 as a preservative

- Stop solution: 5.0% vol/vol solution of sulphuric acid.

The LLD for SIgA was 2 μg/ml.

2.7. Statistical analysis

Data were analysed using GraphPad Prism, version 8.0.2 (GraphPad Software, San Diego, CA), and IBM SPSS Statistics, version 24.0 (IBM Corp., Armonk, NY). Descriptive statistics were first used to describe the sample. Continuous data (age, BMI, height, weight, lung function) were described as mean ± standard deviation or median [interquartile range] and categorical data (gender, education, marital status, occupation, smoking status, GOLD stage, GOLD ABCD classification, long term oxygen therapy, noninvasive mechanical ventilation, medication, Charlson comorbidity index- CCI) were described as frequencies and percentages. Qui-square test or Fisher's exact test, if n<5 expected in >20% of cells, was used to compare categorical data, and continuous data were compared using Unpaired t test or Mann-Whitney test if normality test was or was not assumed, respectively (82). The Normality of the data was assessed based on Shapiro-Wilk test (82).

In the analysis of the results the following time points were used:

- Before PR: in which data collected from month 3 was used. When it was not possible to use this month, we used month 2 or 1.

- Last PR session: in which data collected from month 6 was used.

- After PR: in which data collected from month 9 was used.

Unpaired t test was performed on parametric data, and Mann-Whitney test on nonparametric data, to evaluate the difference in cytokines and SIgA between healthy people and patients with COPD before PR (82).

Comparisons between before PR and the last PR session were performed using Paired t test on parametric data and Wilcoxon test on nonparametric data (82).

Patients were divided in responders and non-responders for each clinical parameter (FEV_1, 6MWT, 1-minute STS, quadricep muscle strength, handgrip strength). They were considered responders to a given clinical parameter if they showed an improvement larger than or equal to the established MCID. As explained previously, as there is no MCID established for the handgrip muscle strength and quadriceps muscle strength, patients were classified as responders if T1-T0/T0>6%, as recommended (78).

For the purpose of this dissertation, due to time constrains, comparisons of cytokines and SIgA levels between responders and non-responders, before and during PR (months 3 to 6), were performed only for the clinical parameter with more responder patients. Unpaired t test and Mann-Whitney test were used in this analysis if normality test was or was not assumed, respectively (82).

Correlations between cytokines and clinical data were performed by Pearson or Spearman tests if normality test was or was not assumed, respectively (82). A p-value <0.05 was considered significant.

3. Results

3. RESULTS

Preliminary data of this work have been presented in the V Post-Graduation Symposium in Biomedicine (Appendix III).

3.1. Characterisation of the sample

The present study included 31 participants: 20 patients with COPD (mostly males, n=14, 70%), and 11 age- gender- and BMI-matched healthy people (n=6, 54.5% males). Patients with COPD had a median age of 71 (interquartile range: 67.3-76), presented an average BMI of 25.9 ± 4.7kg/m^2, a FEV_1pp of 48.2 ± 16.4 and most of them were former smokers (n=13) and grade B of GOLD (n=10). Healthy people had a median age of 73 (interquartile range: 68-74), a BMI of 25.9 ± 2.9kg/m^2, a FEV_1pp of 109.6 ± 27.6 and most of them never smoked (n=10). Table 2 presents the sample characteristics.

Table 2- Baseline characteristics of participants.

Characteristics	COPD (n= 20)	Healthy (n= 11)	P-value
Sex, Male n (%)	14 (70 %)	6 (54.5%)	0.390
Age (years)	71 [67.3-76]	73 [68-74]	0.9755
BMI (Kg/m^2)	25.9 ± 4.7	25.9 ± 2.9	0.9977
Weight (Kg)	70 [60-77]	65 [60.5-86.5]	0.7525
Height (m)	1.7 ± 0.1	1.63 ± 0.10	0.3763
Education, n (%)			0.682
Did not attend	1 (5%)	0 (0%)	
Basic education	15 (75%)	9 (81.8%)	
Secondary	1 (5%)	2 (18.2%)	
Intermediate education	1 (5%)	0 (0%)	
High education	2 (10%)	0 (0%)	
Marital status, n (%)			0.783
Married	15 (75%)	10 (90.9%)	
Widow	2 (10%)	1 (9.1%)	
Divorced	3 (15%)	0 (0%)	
Occupation, n (%)			1.000
Employed	1 (5%)	1 (9.1%)	
Housekeeper	1 (5%)	0 (0%)	
Retired	17 (85%)	10 (90.9%)	
Unemployed	1 (5%)	0 (0%)	
Smoking status, n (%)			0.002*

Current	1(5%)	0 (0%)	
Former	13 (65%)	1 (9.1%)	
Never	6 (30%)	10 (90.9%)	
Pack/years	23 [0-46]	0 [0-0]	0.0006*
Lung function			
FEV_1	1.2 ± 0.5	2.4 ± 0.7	<0.0001*
FEV_1pp	48.2 ± 16.4	109.6 ± 27.6	<0.0001*
FVC	2.4 ± 0.8	2.9 ± 0.8	0.1141
FVCpp	76.3 ± 16.7	104.8 ± 31.0	0.0138*
FEV_1/FVC	49.6 ± 14.6	83.6 ± 6.9	<0.0001*
GOLD Stage, n (%)			
Mild (I)	1 (5%)	----------------------	
Moderate (II)	6 (30%)	----------------------	
Severe (III)	12 (60%)	----------------------	
Very Severe (IV)	1 (5%)	----------------------	
GOLD ABCD, n (%)			
A	3 (15%)	----------------------	
B	10 (50%)	----------------------	
C	1 (5%)	----------------------	
D	6 (30%)	----------------------	
Long term oxygen therapy, n (%)			
Yes	4 (20%)	----------------------	
No	16 (80%)	----------------------	
Noninvasive mechanical ventilation, n (%)			
Yes	0 (0%)	----------------------	
No	20 (100%)	----------------------	
Medication, n (%)			
Antibiotics	0 (0%)	----------------------	
Bronchodilators		----------------------	
SABA	2 (10%)	----------------------	
SAMA	2 (10%)	----------------------	
SABA/SAMA combination	0 (0%)	----------------------	
LABA	5 (25%)	----------------------	
LAMA	7 (35%)	----------------------	
LABA/LAMA combination	4 (20%)	----------------------	
ICS	2 (10%)	----------------------	
ICS/LABA combination	7 (35%)	----------------------	
Xanthines	3 (15%)	----------------------	

LTRA	2 (10%)	---------------------	
Expectorants	4 (20%)	---------------------	
Not informed	1 (5%)	---------------------	
CCI, n (%)			0.002*
Mild (1-2)	0 (0%)	3 (27.3%)	
Moderate (3-4)	11 (55%)	8 (42.1%)	
Severe (≥5)	9 (45%)	0 (0%)	

Values are presented as mean±standard deviation or median [interquartile range], unless otherwise stated.
* $p<0.05$

Abbreviations: BMI- body mass index; GOLD- global initiative for chronic obstructive lung disease; FEV_1pp- forced expiratory volume in one second percentage predicted; FVCpp- forced vital capacity percentage predicted; SABA- short-acting beta agonists; SAMA- short-acting muscarinic-antagonist; LABA- long-acting beta-agonists; LAMA- long-acting muscarinic antagonists; ICS- inhaled corticosteroids; LTRA- leukotriene receptor antagonist; CCI- Charlson comorbidity index.

3.2. Immune mediators in chronic obstructive pulmonary disease and healthy people

The concentration of cytokines (IL-6, TNF-α, IL-8) and SIgA in saliva samples from patients with COPD (from a month prior to PR) was compared to the concentration of these cytokines measured in saliva samples from age- gender- and BMI-matched healthy people (Figure 7). A large variability on the concentration of TNF-α was observed in all participants, with six patients with COPD showing no detectable levels of this cytokine before PR. IL-6 was not detected in most of the samples on both groups: only two healthy people and four patients with COPD had detectable levels of IL-6, before PR, and the values were very different in each individual. Mean levels of IL-8 were very similar between patients with COPD and healthy people and no trend was observed. Levels of IL-10 were not detected in most of the saliva samples collected during the 9 months of the study (data not shown). In total, only 26 (20%) samples of patients with COPD had detectable levels of IL-10. Furthermore, in healthy people this cytokine was not detected in any of the saliva samples. Therefore, comparisons between COPD and healthy people in this cytokine were not possible. No significant differences were observed between patients with COPD and healthy people for any of the analysed cytokines. Levels of SIgA showed a large variability among participants of each group. Although not significant, a trend for a SIgA decrease in patients with COPD was observed.
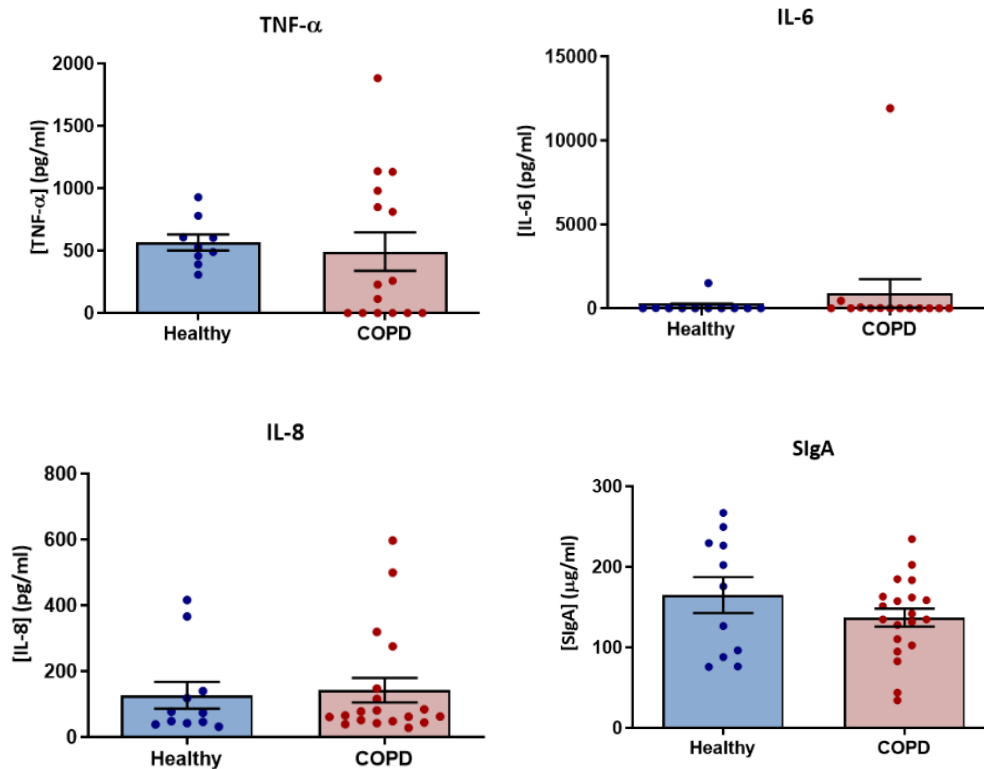
Figure 7- Concentration of TNF-α, IL-6, IL-8 and SIgA in saliva samples of patients with chronic obstructive pulmonary disease and healthy people. TNF-α: (Healthy n=9, COPD n=15) Mann-Whitney test, p=0.3660. IL-6: (Healthy n=11, COPD n=14) Mann-Whitney, p=0.5704. IL-8: (Healthy n=11, COPD n=19) Mann-Whitney test, p=0.7031. SIgA: (Healthy n=11, COPD n=20) Unpaired t test, p=0.2168. Data are presented as mean±standard error of mean.

3.3. Impact of pulmonary rehabilitation on clinical data and secretion of immune mediators.

3.3.1. Impact of pulmonary rehabilitation on clinical data

Twenty patients with COPD participated in the PR programme with an adherence rate higher than 60%. Table 3 shows clinical data measured at T1 (prior PR) and T2 (post PR). Significant improvements on functionality - 1 min STS (pre: 22 [18-29]; post: 25 [20-35]; p=0.0096) were observed (Table 3).

Table 3- Clinical results prior-post PR.

	n	Prior PR	Post PR	P-value
Lung Function				
FEV$_1$	20	1.1 [0.9-1.3]	1.1 [0.8-1.3]	0.3075
FEV$_1$pp	20	50.8 ± 19.7	46.5 ± 14.8	0.1123
FVC	20	2.4 ± 0.7	2.2 ± 0.7	0.1147
FVCpp	20	76.5 ± 18.1	70.5 ± 15.6	0.0451*
FEV$_1$/FVC	20	51.1 ± 13.7	52.9 ± 16.1	0.4481
Peripheral Muscle Strength				
Quadriceps	19	30.0 ± 8.8	32.1 ± 7.7	0.3183
Handgrip	20	32.8 ± 8.3	30.5 ± 10.8	0.0778
Exercise tolerance				
6MWT	20	384.6 ± 119.5	421.3 ± 117.8	0.0631
Functionality				
1 min STS	19	22 [18-29]	25 [20-35]	0.0096*

Values are presented as mean±standard deviation or median [interquartile range]. * p<0.05
Abbreviations: PR- Pulmonary rehabilitation; FEV$_1$pp- Forced Expiratory Volume in One Second percent predicted; FVCpp- Forced Vital Capacity percent predicted; 6MWT- 6 minute walking test: 1-min STS- 1-minute sit-to-stand.

3.3.2.   Impact of pulmonary rehabilitation on the levels of cytokines in saliva

The concentration of selected cytokines in saliva samples before PR and in the last PR session is shown in Figure 8. Five patients (45.5%) showed an increase of TNF-α in the last PR session compared to before PR and four (36%) showed a decrease. Moreover, two patients had no detectable levels of TNF-α before PR and in the last PR session. No significant differences were observed in TNF-α levels between these two groups. Levels of IL-6 were significantly higher in the last PR session than before PR. In the last PR session, 6 (50%) patients with COPD showed higher levels of IL-6, 5 patients had no changes (undetectable levels before and in the last PR session) and only 1 patient had a decrease of IL-6 levels. Levels of IL-8 were very similar between before PR and the last PR session in most patients and no significant differences were observed.
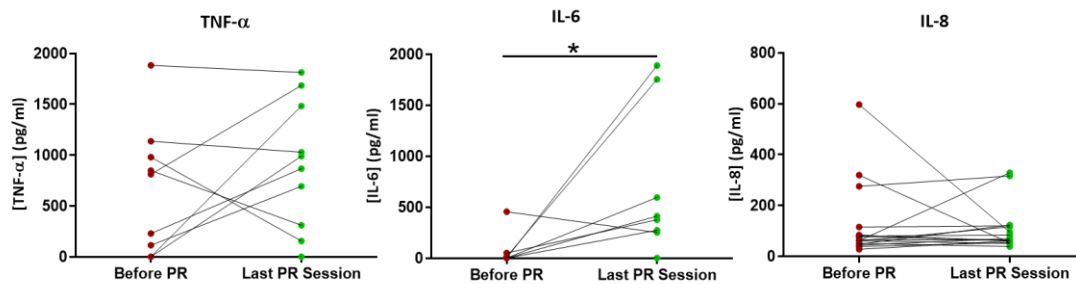
Figure 8 - Levels of TNF-α, IL-6 and IL-8 in saliva samples obtained before (month 2 or 3) and in the last pulmonary rehabilitation session (month 6) of patients with chronic obstructive pulmonary disease. TNF-α: n=11; Wilcoxon test, p= 0.2500. IL-6: n= 12; Wilcoxon test, p= 0.0313. IL-8: n=16; Wilcoxon test, p= 0.5282.*: p<0.05.

3.3.3. Impact of pulmonary rehabilitation on secretory IgA

Although not statistically significant, a trend towards an increase of SIgA mean levels was observed in the last PR session compared to before PR (Figure 9). Figure 10 shows the longitudinal trend of an increase in SIgA mean levels since the beginning of PR (month 4) until two months after finishing PR (month 8). At month 9, the mean levels of SIgA decreased abruptly.
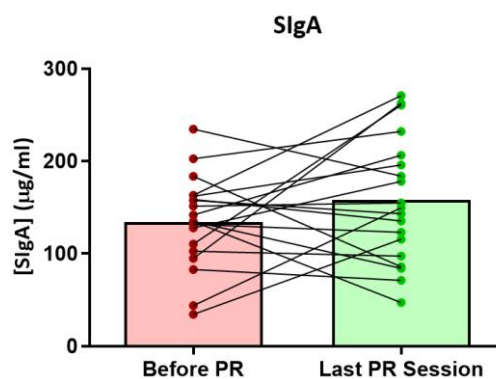

Figure 9 - Levels of SIgA before (month 1 or 3) and in the last pulmonary rehabilitation session (month 6) of patients with chronic obstructive pulmonary disease (n=19). Paired t test; p= 0.1920. Data are presented as mean.
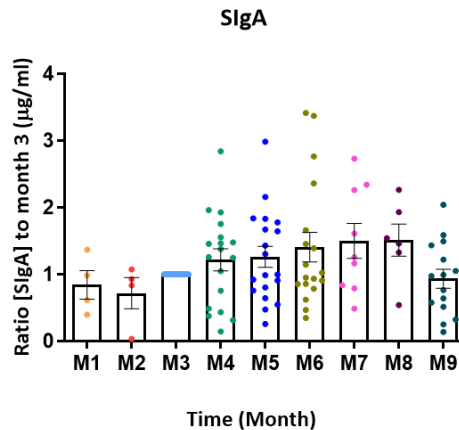
Figure 10- Secretory IgA levels found in saliva during the 9 months of the study: three months prior (M1-M3), three months during (M4-M6) and three months after (M7-M9) pulmonary rehabilitation. Each column represents the mean values normalized to the level measured at month 3. n of M1; M2; M3; M4; M5; M6; M7; M8; M9: 4; 4; 19; 18; 19; 18; 9; 6; 15. Data are presented as mean±standard error of the mean.

3.4. Association between clinical data and immune mediators

As 1-minute STS was the clinical parameter with most responder patients, figures 11, 12, 16 and 17 show the comparison of immune mediators between responder and non-responder patients of that clinical variable.

3.4.1. Correlations between clinical data and cytokines

The levels of immune mediators were compared between responder and non-responder patients, as defined with the values measured for 1-minute STS. Figure 11 shows the levels of cytokines found in saliva samples collected before PR in responder and non-responder patients. TNF-α levels exhibited a large variability in responder patients, while most of the non-responder patients had no detectable levels of TNF-α. No significant differences between TNF-α levels of responder and non-responder patients were found. IL-6 was only detected in two responder patients, and no significant differences were observed between the groups. The levels of IL-8 were very similar between both groups and again no significant differences were found.
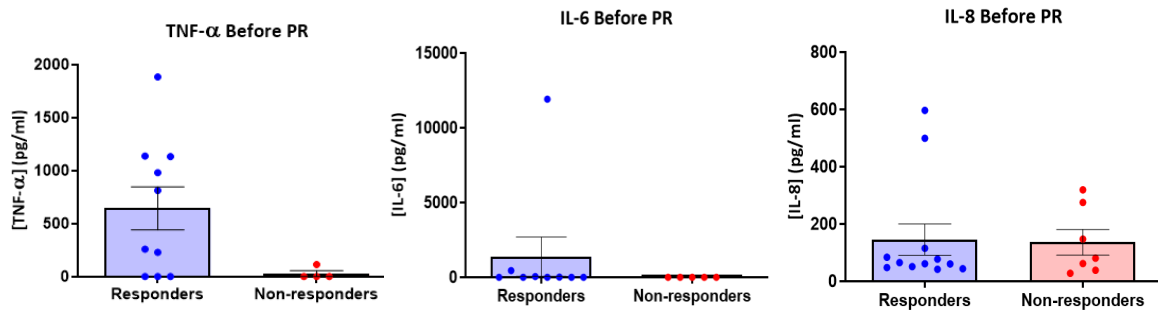
Figure 11- Levels of TNF-α, IL-6 and IL-8 in responder and non-responder patients before pulmonary rehabilitation. TNF-α: n responders= 10, n non-responders= 4, Mann-Whitney test, p= 0.0719. IL-6: n responders= 9, n non-responders= 5, Mann-Whitney test, p= 0.1983. IL-8: n responders= 12, n non-responder= 7, Mann-Whitney test, p=0.9018. Data are presented as mean±standard error of the mean.

Figure 12 shows the variation of cytokines between month 3 (before PR) and month 6 (last PR session) in responder and non-responder patients. TNF-α presented a larger variability in responders than in non-responders to PR and it was only possible to calculate the variation of TNF-α for two non-responder patients due to missing values. Responder and non-responder patients had similar levels of IL-6 and IL-8. No significant differences were found in cytokines variation between responder and non-responder patients.
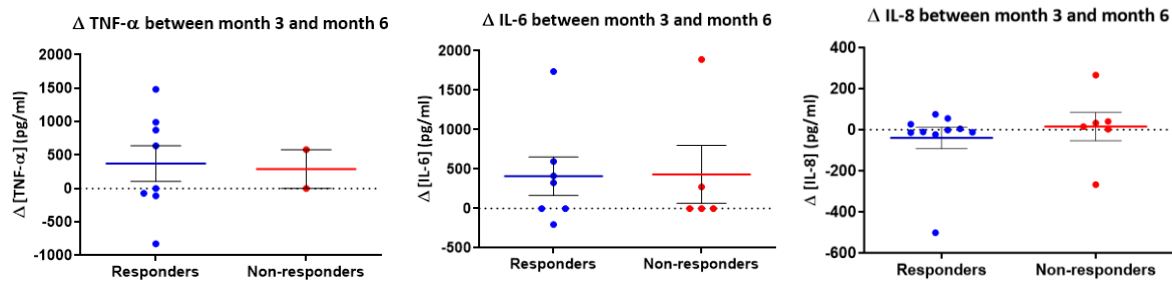

Figure 12- Variation of cytokines between month 3 and month 6 in responder and non-responder patients. TNF-α: n responders= 8, n non-responders= 2; Mann-Whitney test, p=0.9333. IL-6: n responders= 7, n non-responders= 5; Mann-Whitney test, p=0.8737. IL-8: n responders= 10, n non-responders= 6; Mann-Whitney test, p= 0.3132. Data are present as mean±standard error of the mean.

Correlations between immune mediators and clinical data were analysed. Only two significant correlations were observed in all patients between clinical data and cytokines. Before PR, a significant, moderate and positive correlation between IL-6 and 6MWT (p=0.0191; r=0.6222) was found (Figure 13). After PR, there was a significant, moderate and positive correlation between IL-6 and quadriceps muscle strength (p=0.0463; r=0.5269) (Figure 14). No other significant correlations were observed for this cytokine (Appendix I). A significant, strong and positive correlation between TNF-α and quadriceps muscle strength was observed in responder patients (p=0.0026; r=0.9580), before PR (Figure 15). No other significant correlations were observed for TNF-α in responder or non-responder patients (Appendix I).

No significant correlations were found between IL-8 and clinical data. Moreover, correlations between the variation of cytokines and the variation of clinical data, along the months 3 to 6, were also analysed, but no significant correlations were found.

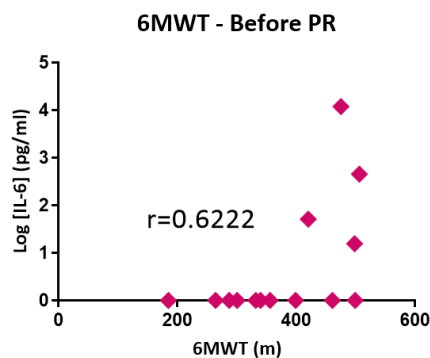**Figure 13-** Correlation between the 6-minute walk test and IL-6, before pulmonary rehabilitation. A significant, positive and moderate correlation was observed (n=14; Spearman test: p=0.0191; r=0.6222).
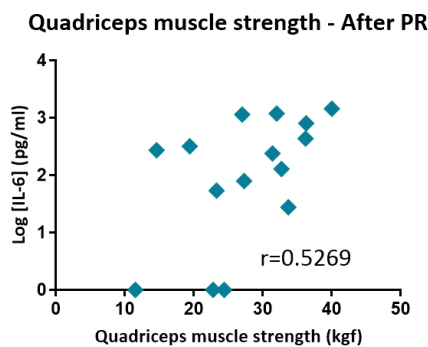

Figure 14- Correlation between quadriceps muscle strength and IL-6, after pulmonary rehabilitation. A significant, positive and moderate correlation was observed (n=15; Spearman test: p=0.0463; r=0.5269).
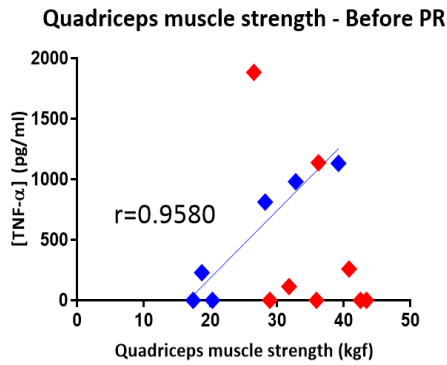
Figure 15- Correlation between the quadriceps muscle strength and TNF-α in responder (n= 6; blue) and non-responder patients (n= 8; red), before pulmonary rehabilitation. A significant, positive and strong correlation was observed in responder patients (Pearson test: p=0.0026; r=0.9580).

3.4.2. Correlations between clinical data and SIgA

Levels of SIgA, before PR and between months 3 and 6, were very similar between responder and non-responder patients (Figure 16 and Figure 17). Both groups showed a large variability of SIgA and no significant differences were found.
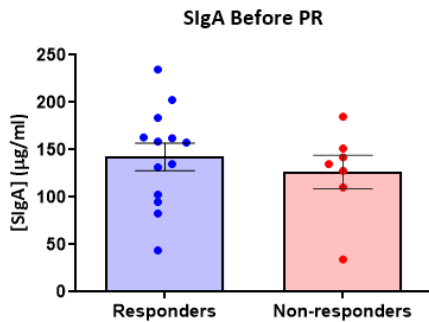
Figure 16– Levels of SIgA before pulmonary rehabilitation between responder (n=13) and non-responder (n=7) patients with chronic obstructive pulmonary disease. Unpaired t test: p=0.5053. Data are presented as mean± standard error of the mean.
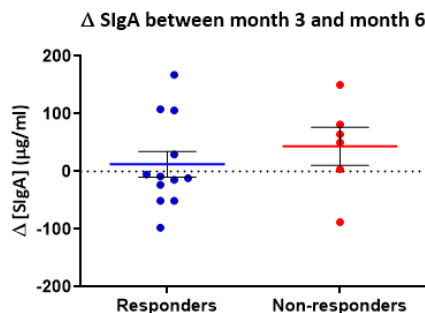
Figure 17– Variation of SIgA between month 3 and 6 in responder (n=12) and non-responder (n=6) patients. Unpaired t test, p=0.4346. Data are presented as mean±standard error of the mean.

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SIgA was the immune mediator with more significant correlations with clinical data. Before PR, a significant, negative and moderate correlation was found between 1 minute sit-to-stand and SIgA (p=0.0205; r=-0.5136) (Figure 18). In the last PR session (month 6), significant correlations were found between SIgA and FEV_1pp (p=0.0358; r=0.4839), positive and moderate; and between SIgA and handgrip strength (p=0.0205; r= -0.5268), negative and moderate (Figure 19). After PR, a significant, negative and moderate correlation between SIgA and 6MWT (p=0.0187; r=-0.5794) was observed (Figure 20). No other significant correlations were found (Appendix II).

Figure 18- Correlation between SIgA and 1-minute sit-to-stand test, before pulmonary rehabilitation. A significant, negative and moderate correlation (n= 20; Spearman test: p=0.0205; r=-0.5136) was observed.


Figure 19- Correlation between SIgA and FEV_1pp or handgrip strength, in the last session of pulmonary rehabilitation. Significant, positive and moderate correlation between FEV_1pp and SIgA (n= 19; Pearson test: p=0.0358; r=0.4839), and significant, negative and moderate correlation between SIgA and handgrip muscle strength (n= 19; Pearson test: p= 0.0205; r= -0.5268) were found.
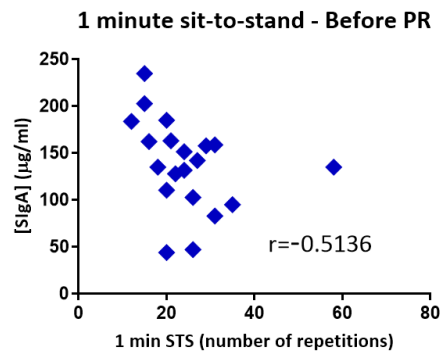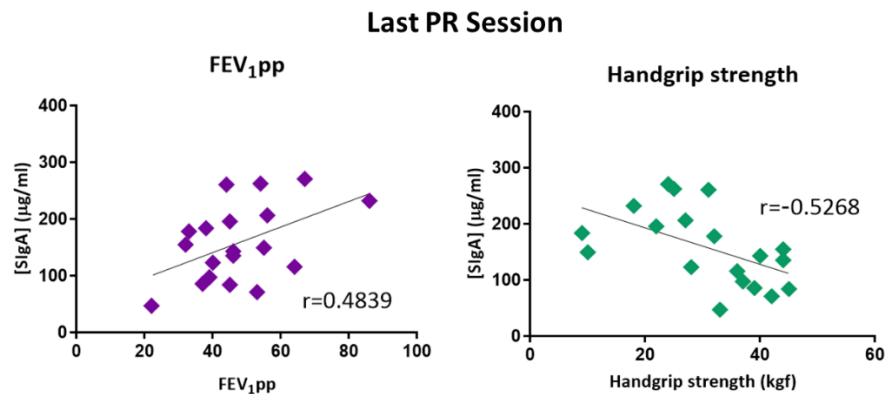
6MWT - After PR
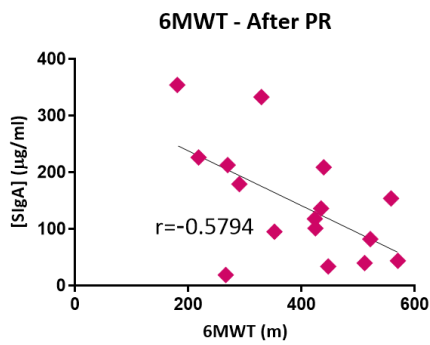
r=-0.5794

Figure 20- Correlation between SIgA and 6-minute walk test (6MWT), after pulmonary rehabilitation. A significant, negative and moderate correlation between SIgA and 6MWT (n= 16; Pearson test: p=0.0187; r=-0.5794) was found.

Some correlations were also found between clinical data and SIgA when analysing responder and non-responder patients independently. Before PR, none of these groups showed a significant correlation between SIgA and clinical data. However, in the last PR session there was a significant, negative and moderate correlation between SIgA and handgrip strength in non-responder patients (p= 0.0438; r= -0.5660) (Figure 21).

Furthermore, there were significant, negative and strong correlations between 1-minute STS (p=0.0096; r=-0.7374) or 6MWT (p=0.0095; r=-0.8009) and the concentration of SIgA in samples collected from responder patients after PR (Figure 22). No other significant correlations were found (Appendix II).

Handgrip strength - Last PR Session

r=-0.5660
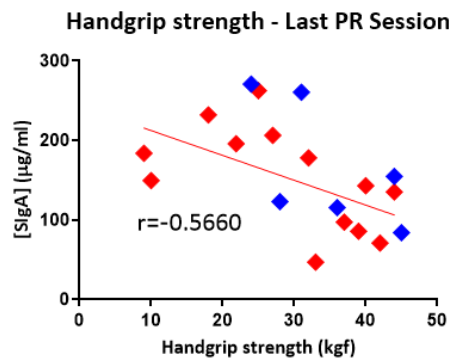
Figure 21- Correlation between SIgA and handgrip strength, in responder (blue) and non-responder (red) patients, in the last session of pulmonary rehabilitation. A significant, moderate and positive correlation between SIgA and handgrip strength was observed in non-responder patients (n= 13; Pearson test: p= 0.0438; r= -0.5660).
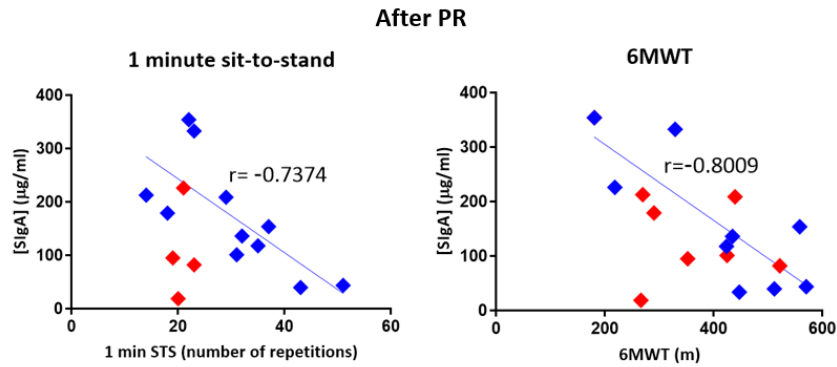
Figure 22- Correlation between SIgA and 6-minute walk test (6MWT) or 1-minute sit-to-stand test (1-min STS), in responder (blue) and non-responder (red) patients, after pulmonary rehabilitation. A significant correlation between SIgA and 1-min STS (n=11; Pearson test: p= 0.0096; r= -0.7374), and 6MWT (n= 9; Pearson test: p= 0.0095; r= -0.8009) were observed in responder patients.

The presence of significant correlations between SIgA and clinical data in the last PR session led us to wonder if the production of SIgA during PR was somewhat related to the initial values (prior to PR) of some clinical data. In order to test this hypothesis, we tested the correlation between the variation of SIgA and the initial values in the clinical data at month 3. Significant correlations with FEV_1pp and handgrip muscle strength were found. A positive and moderate correlation was observed with FEV_1pp (p=0.0025; r=0.6678) (Figure 23) and a negative and moderate correlation was observed with handgrip muscle strength (p=0.0096; r=-0.5925) (Figure 24). It was found that 77.8% of the patients who had an increase in SIgA levels between month 3 and month 6 had a $FEV_1>50pp$ and a handgrip muscle strength lower than 30 kilogram-force (Kgf) at month 3. On the other hand, 88.9% and 77.8% of the patients who had a decrease of SIgA had a $FEV_1<50pp$ and a handgrip strength higher than 30 kgf, respectively. No other significant correlations were found. Therefore, changes in SIgA levels seemed to be associated with patients initial lung function and upper limb muscle strength values.
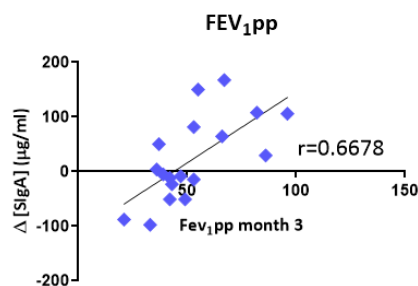
Figure 23- Correlation between the variation of SIgA between month 3 and 6 and FEV_1pp at month 3. A significant, moderate and strong correlation (n= 18; Pearson test: p=0.0025; r=0.6678) was observed.
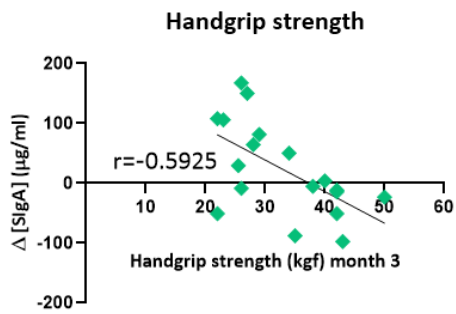
Figure 24- Correlation between the variation of SIgA between month 3 and 6 and handgrip strength at month 3. A significant, negative and moderate correlation (n=18; Pearson test: p=0.0096; r=-0.5925) was observed.

Significant correlations were found between the variations of SIgA and other clinical data, along the months 3 to 6, i.e., a significant, positive and strong correlation with handgrip muscle strength (p= 0.0031; r= 0.9539) (Figure 25); and a significant, moderate and negative correlation with the 1 minute STS (p=0.0398, r= -0.6073) (Figure 26) in responder patients. No other significant correlations were found.
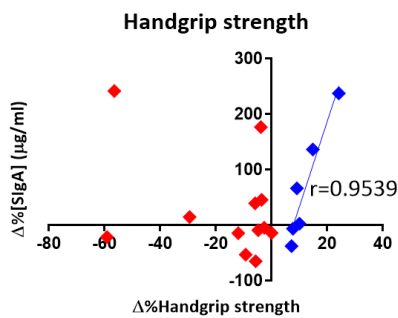
Figure 25- Correlation between the variation of SIgA and the variation of handgrip strength (month 6 - month 3) in responder (blue; n= 6) and non-responder (red; n=12) patients. A significant positive and strong correlation was observed (Pearson test: p= 0.0031; r= 0.9539) in responder patients.
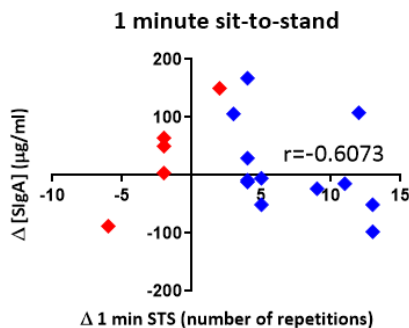
Figure 26- Correlation between the variation of SIgA and the variation of 1 minute sit-to-stand (month 6 - month 3) in responder (n= 12; blue) and non-responder (n= 5; red) patients. A significant negative and moderate correlation was observed (Spearman test: p= 0.0398; r= -0.6073) in responder patients.

4. Discussion

4. DISCUSSION

The present study aimed to compare the concentration of selected cytokines (TNF-α, IL-6, IL-8, IL-10) and SIgA between samples of saliva from patients with COPD and samples from healthy people and evaluate the effects of PR on these immune mediators. Moreover, correlations between the levels of immune mediators and clinical data were also explored. Although significant differences in the concentrations of the analysed inflammatory mediators or SIgA were not observed between samples from patients with COPD and healthy people, the levels of IL-6 showed a significant increase in the last PR session and the levels of SIgA often correlated with clinical data at several time points in patients with COPD.

Inflammatory mediators in chronic obstructive pulmonary disease and healthy people

Controversy exists in the literature regarding the levels of TNF-α, IL-6 and IL-8 between healthy people and patients with COPD. No significant differences between those two groups were observed in systemic levels of TNF-α (68), similarly to our findings. However, TNF-α has been found frequently increased in serum, sputum and BAL of patients with COPD (28,29,40,45,46), but also decreased in serum and saliva of these patients (34). Here, salivary IL-6 and IL-8 levels were also not significantly different between patients with COPD and healthy people, similarly to other studies in BAL, salivary (34) and plasma samples (44). However, a significant increase of IL-6 and IL-8 in sputum (34,51), serum and BAL (29) samples of patients with COPD has been reported. These cytokines are involved in COPD development and have relevant inflammatory effects during COPD: contribute to emphysema development and to stimulation of mucus secretion (22,41,83), therefore higher levels of these cytokines in patients with COPD were expected. This controversy, present in the literature and with no definite findings in our study either, might be explained by the relatively small sample sizes commonly integrated.

In the present study, IL-10 was not detected in most of the saliva samples from patients with COPD or healthy people. In agreement with our results, an analysis of saliva samples from healthy adolescent girls found that 64% of samples had levels of IL-10 below the lower limit of detection (84). However, other studies based on stress-reactivity (85) and gingivitis

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(86) were able to detect IL-10 in saliva samples. It seems therefore important to repeat the analysis of the present study in a larger cohort. Ideally, in future research, analysis in saliva should be compared with analysis in blood since IL-10 has been detected in blood samples of patients with COPD (43,87).

Although not statistically significant, a trend for lower levels of SIgA in patients with COPD than in healthy people was observed. This finding corroborates with the lower levels of SIgA detected in BAL of patients with COPD (88). Reduced levels of SIgA may compromise protection against respiratory bacterial infections and contribute to start an inflammatory response driving to the development of anomalies inherent to COPD (61). However, since no significant differences were found, further studies are needed to confirm our results, namely with larger samples.

Effects of pulmonary rehabilitation on inflammatory mediators

In the last PR session, the levels of TNF-α were not significantly different from before PR, which is in agreement with previous studies with plasma/serum samples (68,89). Thus, our results support previous findings that PR does not affect TNF-α levels. However, due to the small cohort used in this study, further research with larger samples is recommended to confirm our results.

Previous studies suggest that exercise can improve local and systemic inflammation in patients with COPD, since they reported a decrease of IL-8 in sputum, after 3 weeks of physiotherapy (52), and in plasma, after a home-based pulmonary rehabilitation programme (69). However, our results do not support such findings but suggest that PR does not have an impact on salivary IL-8 levels, since no significant modifications were observed in the last PR session. However, this finding warrants confirmation.

In the present study, salivary levels of IL-6 were significantly higher in the last PR session, in patients with COPD. However, previous studies reported no modification on IL-6 systemic levels after PR (68,89). On the other hand, an increase of IL-6 in plasma after exercise in patients with COPD (68) and, in saliva, after exercise in young males (90), has been reported. It is therefore likely that the observed increase of IL-6 in the last PR session was due to physical exercise stimulation (68), since most of saliva samples were collected

immediately after the exercise component of the PR programme. During exercise, muscle contraction stimulates the release of IL-6 from the skeletal muscle, which leads to an anti-inflammatory cascade by inhibiting the release of pro-inflammatory cytokines and triggering the release of IL-10, an anti-inflammatory cytokine (91). A significant correlation between systemic and salivary levels of IL-6 has been observed (32), although others did not observed such significant correlation (92–94). Thus, it is still unclear if the observed increase of IL-6, mostly regarded as a pro-inflammatory cytokine involved in the development of pulmonary emphysema (22), indicates a worsening of the inflammatory condition or if it is indicative of an increase of IL-6 levels, derived from skeletal muscle, with anti-inflammatory properties (91). Therefore, this analysis should be repeated with samples collected immediately before PR sessions.

A trend for an increase of SIgA, in the last PR session, and a constant increase of this mediator since the beginning of PR (in month 4) until 2 months after finishing PR (month 8), suggests that the impact of PR on SIgA levels is due to the exercise and persist until two months after PR finishes. These trends are concordant with the salivary SIgA increase previously observed by others after 12 months of exercise (71) or moderate daily physical activity (95) in elderly people. Although not significant, the results of the present study suggest that PR might have a positive impact on SIgA levels.

Correlations between clinical data and immune mediators

The comparison of selected immune mediators between responder and non-responder patients did not show significant differences, suggesting that the concentration of cytokines and SIgA detected in saliva samples of patients with COPD is not associated with their responsiveness to PR. However, this analysis was performed only for 1-min STS and so, future studies with larger samples should repeat this analysis with more clinical parameters. Besides that, some significant correlations were observed between clinical data and selected cytokines or SIgA. Higher levels of IL-6 and TNF-α in elderly people are usually associated to the occurrence of sarcopenia (96), an age-related decrease of muscle mass and muscle strength (97). Moreover, higher plasma concentrations of TNF-α were already associated with lower muscle mass and lower muscle strength in well-functioning

older people (98). Surprisingly, our results show that, before PR, there is a positive association between salivary levels of TNF-α and quadriceps muscle strength in responder patients. Probably, TNF-α detected in saliva is not associated to systemic TNF-α, involved in muscle wasting (96,98), as previously demonstrated (32). Thus, our results suggest that, first, salivary levels of TNF-α can be related to quadriceps muscle strength and, second, patients with a positive association between salivary levels of TNF-α and quadriceps muscle strength, before PR, are more likely to have an improvement of quadricep strength above 6% with PR, which means having a clinical relevant improvement.

Significant positive correlations between IL-6 and 6MWT or quadriceps muscle strength, were observed, before and after PR, respectively. In contrast to our results, plasma levels of IL-6 have been associated with reduced muscle mass and strength, namely quadriceps strength, and exercise capacity in well-functioning elderly people with or without obstructive lung disease (98,99). This way, while systemic IL-6 has been found to be related to muscle waste (98,99), our results suggest that salivary levels are related to muscle strength and exercise tolerance. Probably, saliva does not reflect systemic IL-6 responses, as reported by previous studies (92–94). Thus, further studies in saliva and blood samples are recommended, with larger samples, in order to confirm the observed correlations and clarify if different correlations are actually found in salivary and systemic levels of IL-6 and TNF-α.

SIgA was the immune mediator mostly correlated with the clinical data. Our results suggest that, regardless of the occurrence of PR and improvement on some clinical parameters, salivary SIgA is inversely associated with functionality, exercise tolerance and handgrip strength. These results were not expected since people with great physical condition are usually associated with an active lifestyle and we know that regular moderate exercise leads to the increase of salivary SIgA in elderly (71,95). Probably, the levels of SIgA were affected by other factors, such as circadian rhythm (100), psychological (101) and physical stress (102), or by salivary flow rate (102), which could have influenced our results. Further studies with larger samples and these factors controlled, should be performed. Moreover, we observed that both patients with handgrip responsiveness and patients with low handgrip strength, before PR, were more likely to have an increase on SIgA levels with PR.

SIgA and FEV_1pp were found positively associated, in the last PR session, suggesting that higher levels of SIgA are associated with an improvement of airway obstruction. In fact, SIgA deficiency, in BAL, was already associated with the severity of airway obstruction (88). Moreover, our results suggest that patients with higher FEV_1pp, before PR, are more likely to have an increase of SIgA during PR. Thus, patients with a less severe disease are more able to have a positive impact from PR on SIgA levels and so, more able to have a higher mucosal immune protection against respiratory bacterial infections during PR (61).

Future research, in larger samples and with optimised protocols, are needed to confirm and to better understand these correlations and go further in discovering how immune mediators are related to clinical outcomes.

4.1. Limitations

The present study has some limitations which need to be acknowledged. Firstly, a relatively small cohort was analysed, and it was not always possible to analyse all time points in all patients due to missing data. In the future, a larger sample might contribute to clarify some of the trends observed. Secondly, most but not all saliva samples, during the months of PR, were collected immediately after PR sessions, and the acute effects to physical exercise might have influenced some of the results obtained. In the future it is recommended that saliva samples are collected immediately before PR session starting. Additionally, for the majority of patients, it was only possible to collect one saliva sample before PR. This has somewhat impaired our understanding of the variability of cytokine levels without PR influence. Moreover, we are not sure if the mediators analysed in this study are the most suitable for this research or if there are other relevant ones that we did not analyse. Furthermore, there are still some uncertainties regarding the use of saliva samples to reflect the inflammatory condition of the patient.

5. Conclusion

5. CONCLUSION

In conclusion, the concentration of selected inflammatory mediators and SIgA present in saliva samples did not differ between healthy people and patients with COPD. Levels of IL-6 increased significantly in the last PR session and SIgA tends to increase during and up to two months following PR. IL-6 levels were positively associated with exercise tolerance and quadricep muscle strength before and after PR, respectively. Additionally, TNF-α was positively associated with quadriceps muscle strength before PR, but only in responder patients. Furthermore, patients with COPD with less severe airway obstruction, before PR, seem to have a higher predisposition to benefit from PR on SIgA levels. Several correlations were found between SIgA and clinical parameters related to lung function, functionality, peripheral muscle strength and exercise tolerance at different time points of this study. This suggest that SIgA detected in saliva may be an informative mediator about the clinical condition of patients with COPD. However, more studies are needed, namely with a larger cohort to confirm some trends and correlations observed in this study and go further in discovering the variation of the inflammatory response during PR. SIgA seems to be a promising informative mediator to explore in future studies.

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Annex I – Ethics' approval

CENTRO HOSPITALAR DO BAIXO VOUGA, E.P.E. / AVEIRO

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Pessoa Colectiva nº 510 123 210

Exma. Senhora

Dra. Alda Sofia Pires de Dias Marques
Escola Superior de Saúde
Universidade de Aveiro
Agras do Crasto - Campus Universitário de
Santiago
3810-193 Aveiro

| S/ Ref.ª | S/ Comunicação de | N/ Ref.ª 777638 | Aveiro, 22.03.2017 |

ASSUNTO: Resposta ao V/ Pedido de confirmação para a realização de estudo no CHBV, E.P.E.

Em resposta à V/ solicitação subordinada ao tema "GENIAL – Marcadores genéticos e clínicos na trajetória da DPOC", vimos, pelo presente, informar que por deliberação do Conselho de Administração, nesta data, se encontra autorizado o pedido formulado.

Nesse sentido, solicitamos a V. Exa se digne enviar um relatório final ao Serviço de Investigação e Formação do CHBV, E.P.E.

Com os melhores cumprimentos,

A Diretora do Serviço de Investigação e Formação

(Dra. Joana Guimarães)

Na resposta indicar o número e as referências deste documento. Em cada ofício tratar só de um assunto.

CHBV – 347

Annex II – National Data Protection approval

Autorização n.º 8828/ 2016

Universidade de Aveiro , NIPC 501461108, notificou à Comissão Nacional de Protecção de Dados (CNPD) um tratamento de dados pessoais com a finalidade de realizar um Estudo Clínico com Intervenção, denominado GENIAL - Marcadores Genéticos e Clínicos na Trajetória da DPOC .

Existe justificação específica, validada pela Comissão de Ética Competente (CEC), para o tratamento do dado pessoal raça/etnia.

O participante é identificado por um código especificamente criado para este estudo, constituído de modo a não permitir a imediata identificação do titular dos dados; designadamente, não são utilizados códigos que coincidam com os números de identificação, iniciais do nome, data de nascimento, número de telefone, ou resultem de uma composição simples desse tipo de dados. A chave da codificação só é conhecida do(s) investigador(es).

É recolhido o consentimento expresso do participante ou do seu representante legal.

A informação é recolhida diretamente do titular e indiretamente do processo clínico.

As eventuais transmissões de informação são efetuadas por referência ao código do participante, sendo, nessa medida, anónimas para o destinatário.

A CNPD já se pronunciou na Deliberação n.º 1704/2015 sobre o enquadramento legal, os fundamentos de legitimidade, os princípios aplicáveis para o correto cumprimento da Lei n.º 67/98, de 26 de outubro, alterada pela Lei n.º 103/2015, de 24 de agosto, doravante LPD, bem como sobre as condições e limites aplicáveis ao tratamento de dados efetuados para a finalidade de investigação clínica.

No caso em apreço, o tratamento objeto da notificação enquadra-se no âmbito daquela deliberação e o responsável declara expressamente que cumpre os limites e condições aplicáveis por força da LPD e da Lei n.º 21/2014, de 16 de abril, alterada pela Lei n.º 73/2015, de 27 de junho – Lei da Investigação Clínica –, explicitados na Deliberação n.º 1704/2015.

O fundamento de legitimidade é o consentimento do titular.

A informação tratada é recolhida de forma lícita, para finalidade determinada, explícita e legitima e não é excessiva – cf. alíneas a), b) e c) do n.º 1 do artigo 5.º da LPD.

Assim, nos termos das disposições conjugadas do n.º 2 do artigo 7.º, da alínea a) do n.º 1 do artigo 28.º e do artigo 30.º da LPD, bem como do n.º 3 do artigo 1.º e do n.º 9 do artigo 16.º ambos da Lei de Investigação Clínica, com as condições e limites explicitados na Deliberação da CNPD n.º 1704/2015, que aqui se dão por reproduzidos, autoriza-se o presente tratamento de dados pessoais nos seguintes termos:

Responsável – Universidade de Aveiro

Finalidade – Estudo Clínico com Intervenção, denominado GENIAL - Marcadores Genéticos e Clínicos na Trajetória da DPOC

Categoria de dados pessoais tratados – Código do participante; idade/data de nascimento; género; raça/etnia; dados antropométricos; sinais vitais; dados da história clínica; dados dados de exame físico; dados de meios complementares de diagnóstico; medicação prévia concomitante; genéticos; dados de cuidadores/acompanhantes (apenas os relacionados com as necessidades do participante); dados de qualidade de vida/efeitos psicológicos

Exercício do direito de acesso – Através dos investigadores, presencialmente

Comunicações, interconexões e fluxos transfronteiriços de dados pessoais identificáveis no destinatário – Não existem

Prazo máximo de conservação dos dados – A chave que produziu o código que permite a identificação indireta do titular dos dados deve ser eliminada 5 anos após o fim do estudo.

Da LPD e da Lei de Investigação Clínica, nos termos e condições fixados na presente Autorização e desenvolvidos na Deliberação da CNPD n.º 1704/2015, resultam obrigações que o responsável tem de cumprir. Destas deve dar conhecimento a todos os que intervenham no tratamento de dados pessoais.
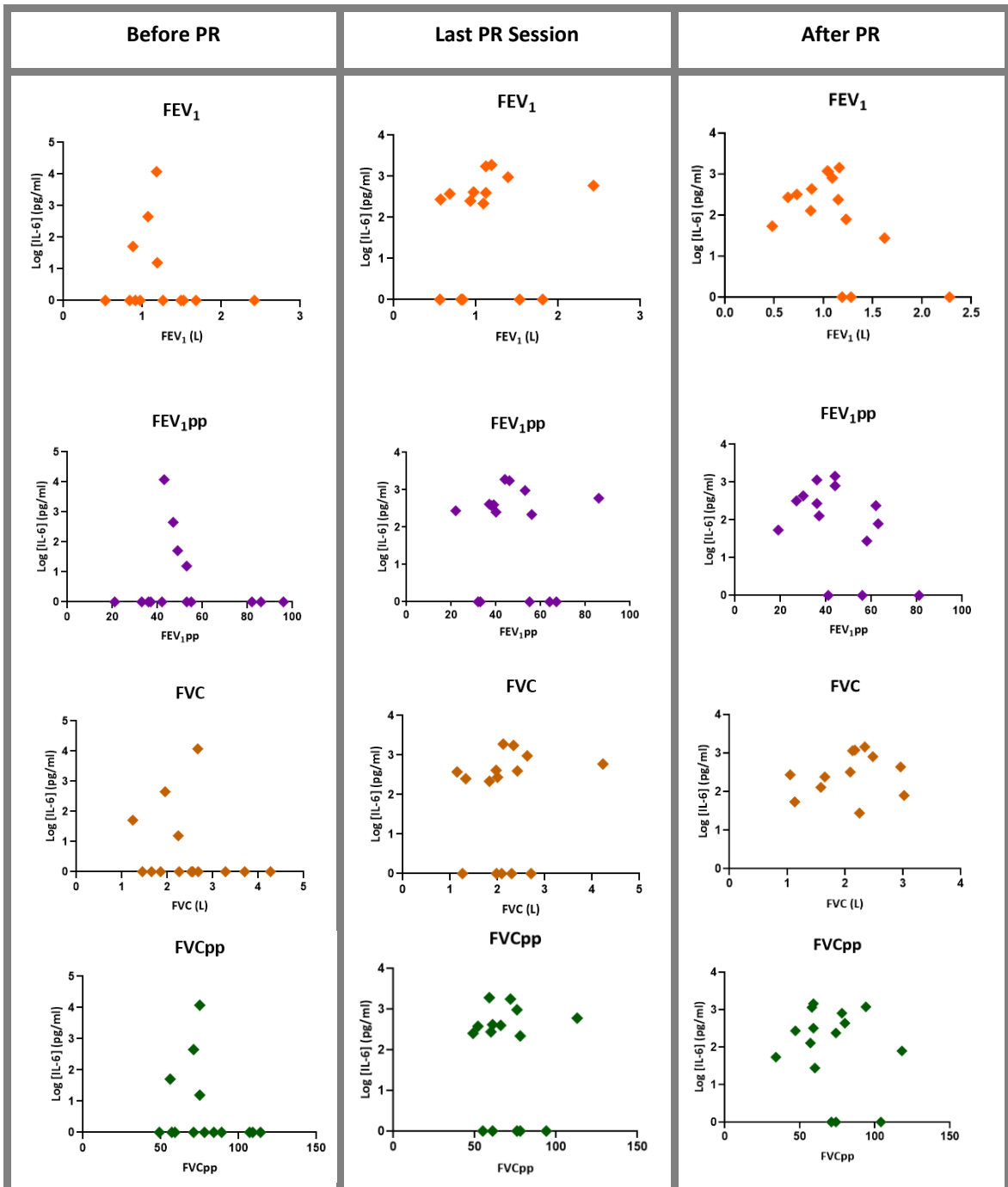
Lisboa, 23-08-2016

A Presidente

Filipa Calvão

Appendix I – Correlations between clinical data and TNF-α or IL-6 (Results)

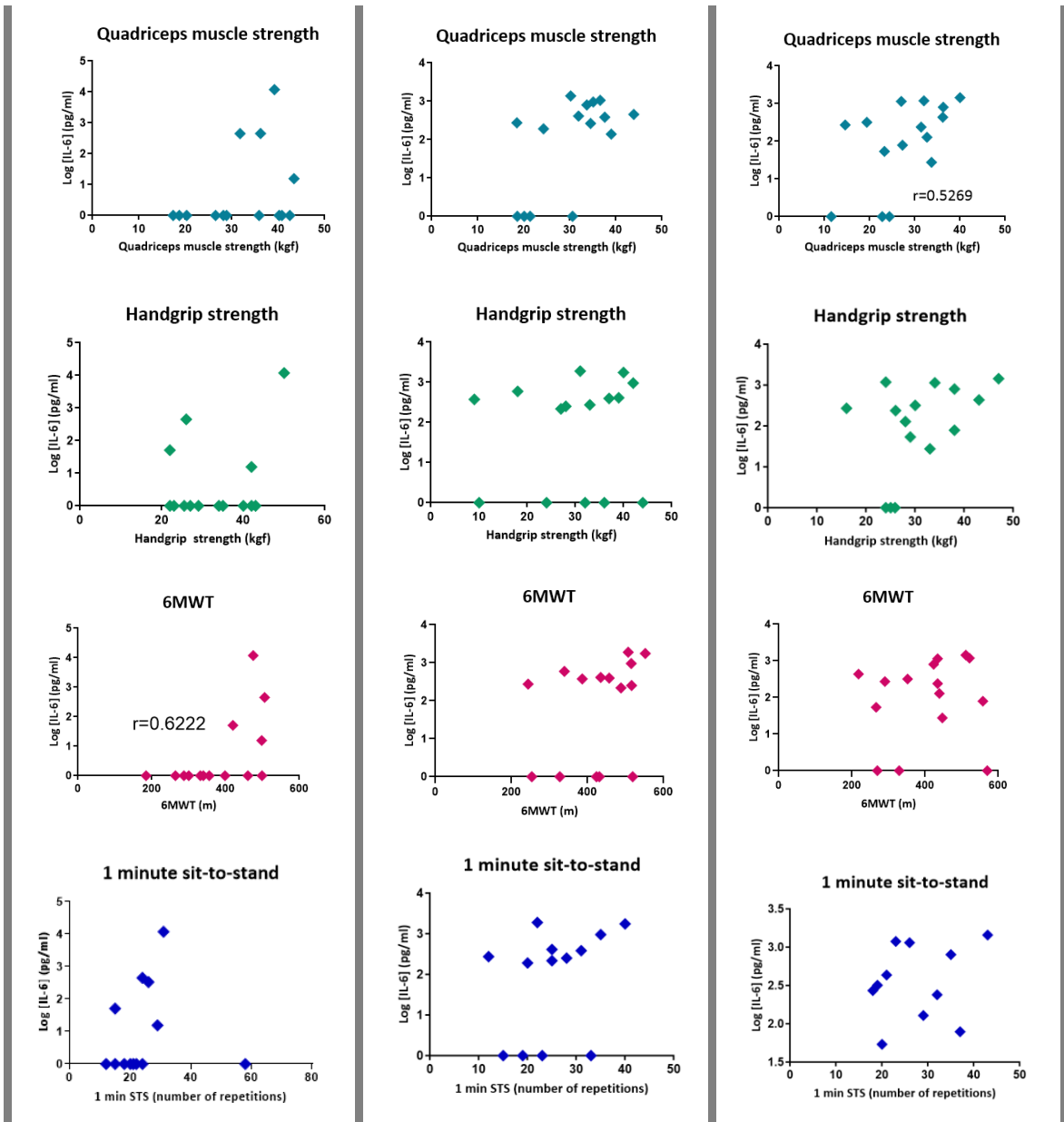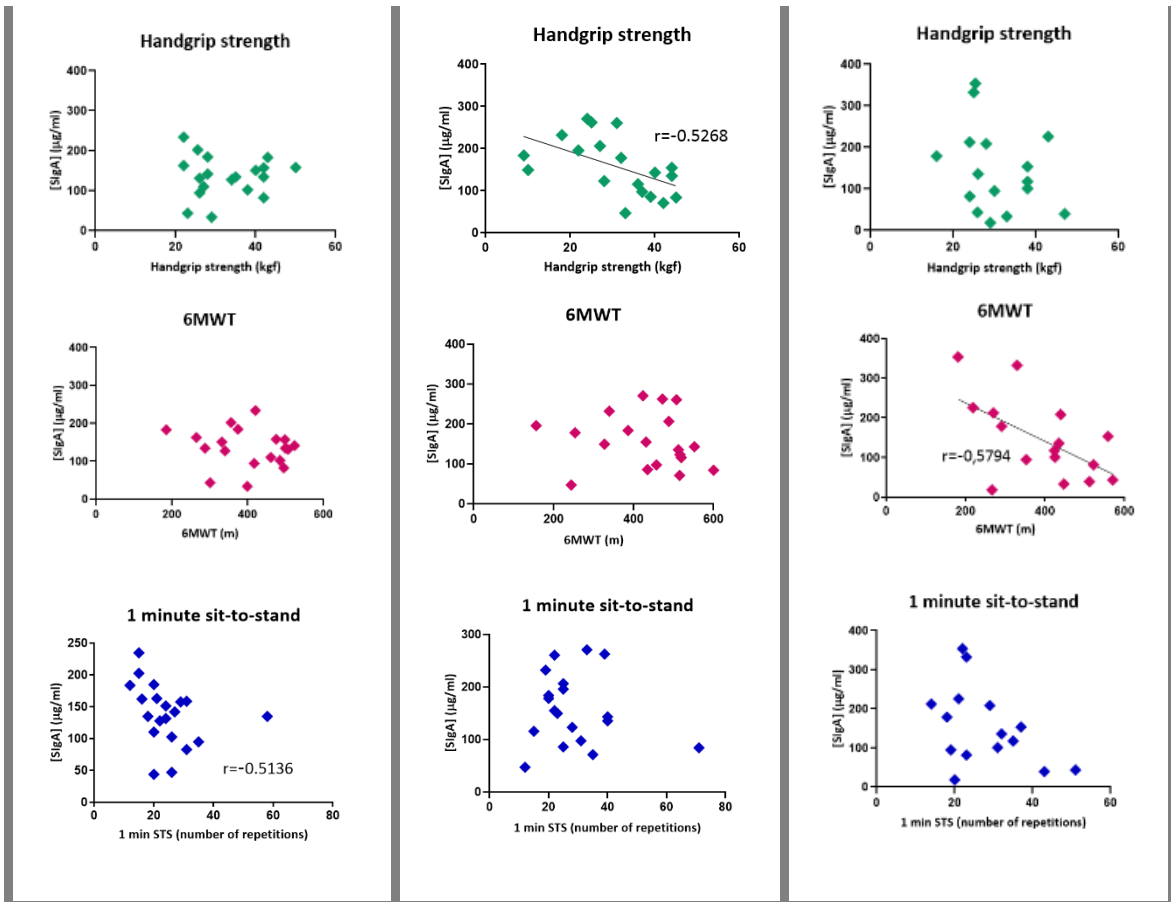| Before PR	Last PR Session	After PR

Figure Supplemental 1- Correlations between clinical data and IL-6 in patients with chronic obstructive pulmonary disease. Before pulmonary rehabilitation, a significant, positive and moderate correlation was found between the IL-6 and the 6-minute walk test (n=14; Spearman test: p=0.0191; r=0.6222). After pulmonary rehabilitation, there was a significant positive and moderate correlation between IL-6 and quadriceps muscle strength (n=15; Spearman test: p=0.0463; r=0.5269). No other significant correlations were found.
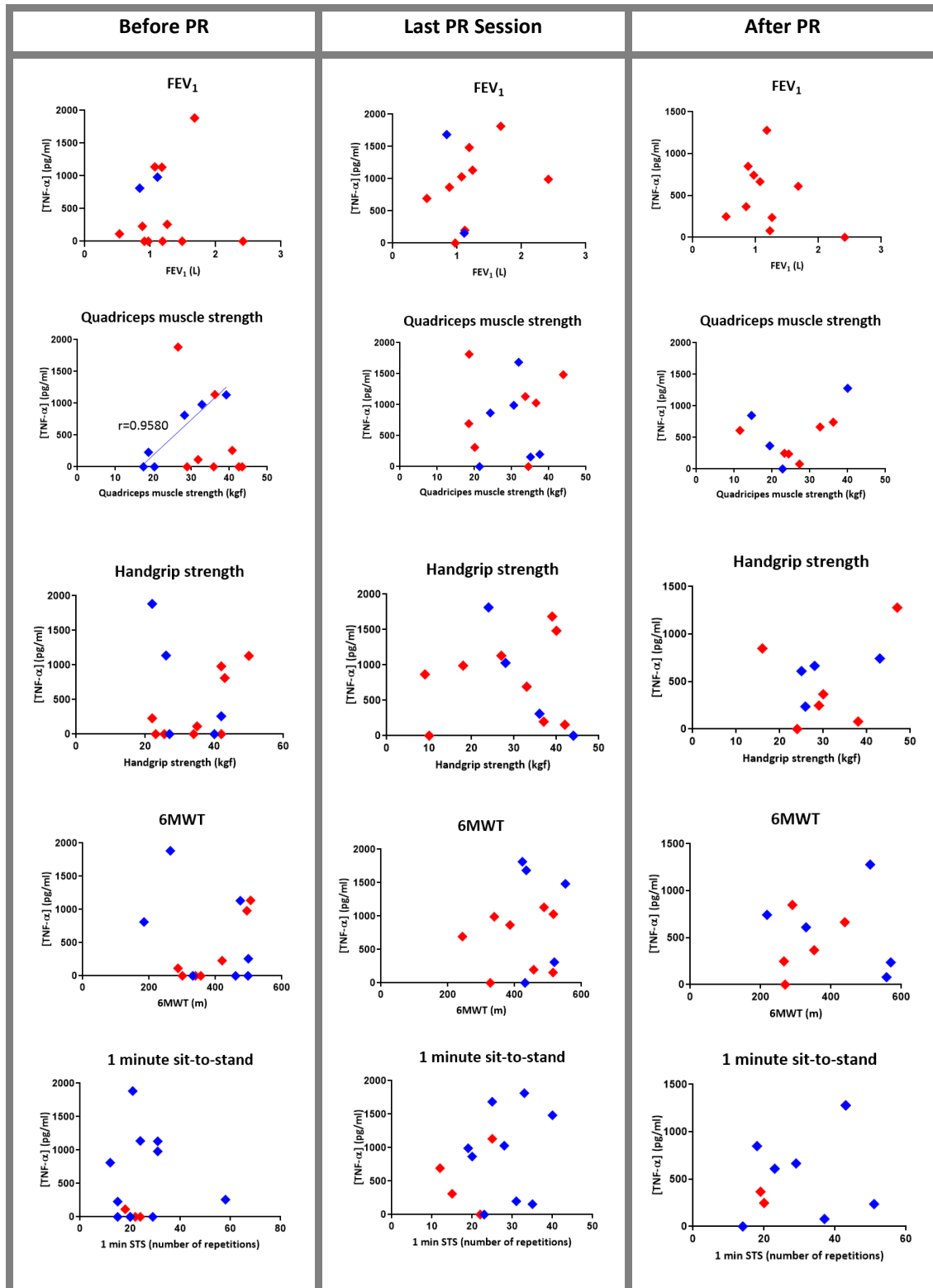
Figure Supplemental 2- Correlations between clinical data and TNF-α in responder (blue) and non-responder (red) patients. Before pulmonary rehabilitation a positive, significant and strong correlation between TNF-α and quadriceps muscle strength was observed in responder patients (n=6; Pearson test: p=0.0026; r=0.9580).

Appendix II – Correlations between clinical data and Secretory IgA (Results)

Before PR	Last PR Session	After PR
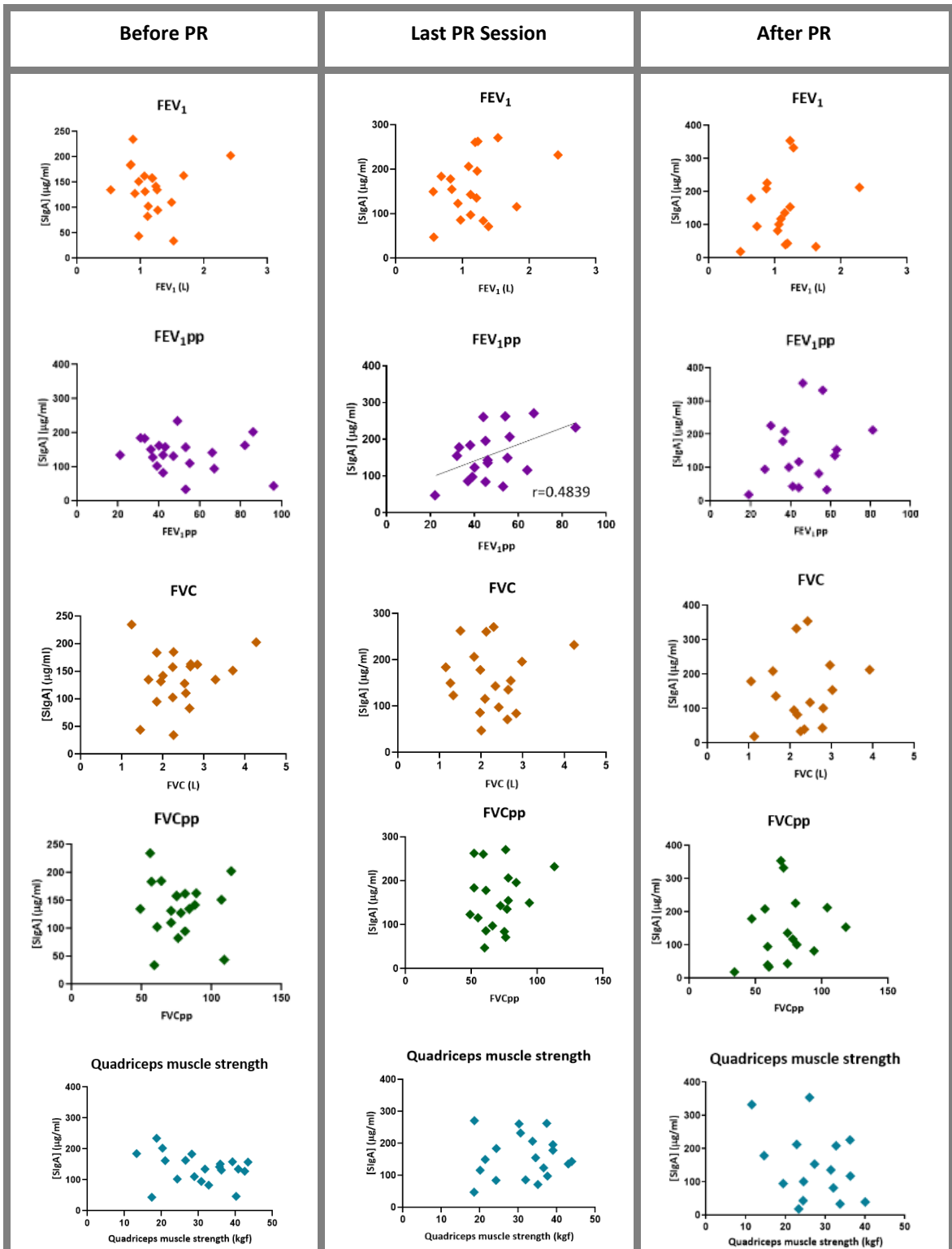
Figure Supplemental 3- Correlations between SIgA and clinical data in patients with chronic obstructive pulmonary disease. Before pulmonary rehabilitation, a significant, negative and moderate correlation between SIgA and 1-minute sit-to-stand (n=19; Spearman test: p=0.0205; r=-0.5136) was found. In the last session of pulmonary rehabilitation, there was a significant, positive and moderate correlation between FEV_1pp and SIgA (n=19; Pearson test: p=0.0358; r=0.4839), and a significant, negative and moderate correlation between SIgA and handgrip muscle strength (n=19; Pearson test: p= 0.0205; r= -0.5268). After pulmonary rehabilitation there was a significant, negative and moderate correlation between SIgA and 6-minute walk test (n=16; Pearson test: p=0.0187; r=-0.5794).
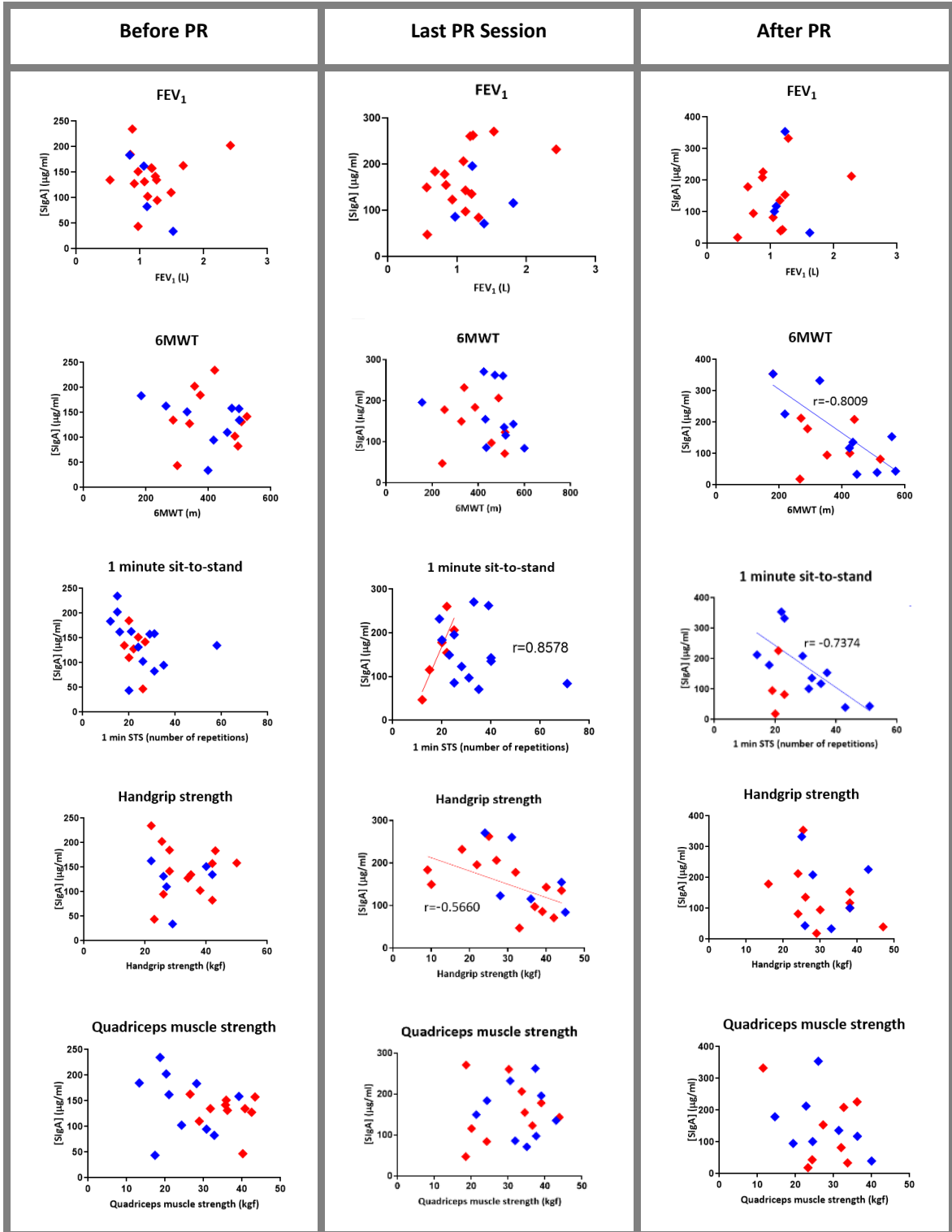
Figure Supplemental 4- Correlations between SIgA and clinical data in responder (blue) and non-responder (red) patients with chronic obstructive pulmonary disease. In the last session of pulmonary rehabilitation significant, strong and positive correlation between SIgA and 1-minute sit-to-stand (1-min STS) (n=6; Pearson test: p=0.0289; r=0.8578), and significant, moderate and negative correlation between SIgA and handgrip strength (n= 13; Pearson test: p= 0.0438; r= -0.5660) were observed in non-responder patients. After pulmonary rehabilitation, significant, negative and strong correlations between SIgA and 1-min STS (n=11; Pearson test: p= 0.0096; r= -0.7374), and 6-minute walking test (n=9; Pearson test: p= 0.0095; r= -0.8009) were observed.

Appendix III – Scientific outputs under this dissertation

APPENDIX III – SCIENTIFIC OUTPUTS UNDER THIS DISSERTATION

Joana Dias, Sara Miranda, Ana Helena Tavares, Célia Freitas, Ana Catarina Sousa, Carla Valente, Ana Sousa, Alda Marques, Catarina Almeida "Pulmonary rehabilitation on inflammatory mediators and SIgA in patients with COPD" V Post-graduation symposium in biomedicine, 27th June 2019, Aveiro, Portugal

Pulmonary rehabilitation on inflammatory mediators and SIgA in patients with COPD

Joana Dias[1*], Sara Miranda[2,3], Ana Helena Tavares[3,4], Célia Freitas[2,3], Ana Catarina Sousa[6,7,8], Carla Valente[9], Ana Sousa[1,3], Alda Marques[2,3], Catarina Almeida[1,3]

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Background

• Patients with chronic obstructive pulmonary disease (COPD) have a deregulated immune response and show altered levels of pro/anti-inflammatory cytokines and of secretory IgA (SIgA), a first-line airway defense mechanism in the lungs.

• Pulmonary rehabilitation (PR) leads to physiological and psychosocial improvements in COPD, but its impact on levels of inflammatory mediators remains unclear.

Aim

This study aimed to:

• Compare the levels of inflammatory mediators (TNF-α, IL-6 and IL-8) and SIgA in saliva of patients with COPD and healthy subjects.

• Evaluate the impact of PR on these mediators and SIgA and correlate their levels with clinical data.

Methods

• Patients with COPD were recruited from Centro Hospitalar do Baixo Vouga and Agrupamento dos Centros de Saúde do Baixo Vouga. They were enrolled in a PR programme twice a week during 3 months. Sociodemographic, anthropometric, clinical data and saliva samples were collected from participants during 9 months as shown in Figure 1. Eleven age and sex matched healthy subjects were evaluated once.

• Inflammatory mediators and SIgA were measured in saliva samples from patients with COPD and healthy subjects by Sandwich ELISA method.

Fig. 1 — Chronology of saliva and data collection of patients with COPD.

Fig. 2 — Scheme of processing and analysis of saliva samples.

Results

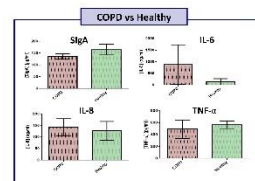• Twenty patients with COPD were enrolled in this study (%70 male; 72.8±6.9 years; FEV1pp 46.2±14.1).

Fig.3 — Levels of SIgA, IL-6, IL-8 and TNF-α in saliva of patients with COPD and healthy subjects. SIgA and TNF-α levels show a trend for a reduction in patients with COPD. IL-8 and IL-6 levels show the opposite.
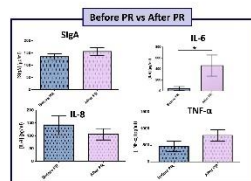
Fig.4 — Levels of SIgA, IL-6, IL-8 and TNF-α before and after PR. After PR, there is a trend for an increase of SIgA and TNF-α and a trend for a decrease of IL-8. There is a significant increase of IL-6, after PR [Wilcoxon test: p= 0.0313]. *p<0.05
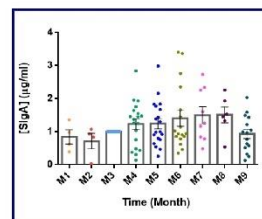

Fig.5 — SIgA levels during the 9 months of study. Each column represents the mean of values normalized with the level measured at month 3. During PR there is a trend for an increase of SIgA.
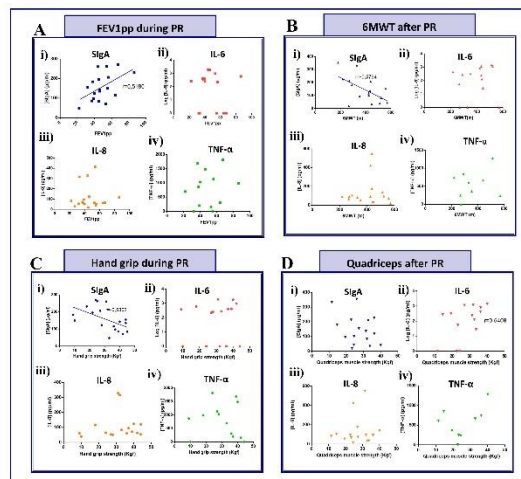

Fig.6 — A) Correlation between FEV1pp and inflammatory mediators and SIgA during PR. A- i) There is a significant positive correlation between FEV1pp and SIgA (Pearson test: r=0.5180; p=0.0277 (<0.05)). A- ii); iii); iv) There is no significant correlation between FEV1pp and log(IL-6), IL-8 or TNF-α respectively.

B) Correlation between 6MWT (6 minute walking test) and inflammatory mediators and SIgA after PR. B– i) There is a significant positive correlation between 6MWT and SIgA (Pearson test: r=0.5794; p=0.0187 (<0.05)). B- ii); iii); iv) There is no significant correlation between 6MWT and log(IL-6), IL-8 or TNF-α respectively.

C) Correlation between hand grip strength and inflammatory mediators and SIgA during PR. C- i) There is a significant negative correlation between handgrip strength and SIgA (Pearson test: r = -0.5302; p= 0.0236 (<0.05)) C- ii); iii); iv) There is no significant correlation between hand grip strength and log(IL-6), IL-8 or TNF-α respectively.

D) Correlation between quadriceps muscle strength and inflammatory mediators and SIgA after PR. D– ii) There is a significant positive correlation between log(IL-6) and quadriceps muscle strength (Spearman test: r=0.5408; p=0.049 (<0.05)). D- i); iii); iv) There is no significant correlation between quadriceps muscle strength and SIgA, IL-8 or TNF-α respectively.

Conclusions

• Patients with COPD tend to have lower levels of SIgA and TNF-α and higher levels of IL-6 and IL-8, than healthy subjects.

• After PR, there is a significant increase of IL-6. SIgA and TNF-α tend to increase and IL-8 tend to decrease, after PR.

• Clinical data are mostly correlated with the SIgA levels.

• Overall, this study suggests that PR influences the immune response of patients with COPD.