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Pathophysiologic pathway of obstructive airways diseases. A preliminary study on the underlying cellular and molecular mechanisms

Via patofisiológica das doenças obstrutivas da via aérea. Estudo preliminar dos mecanismos celulares e moleculares subsequentes

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Clínica, realizada sob a orientação científica do Professor Doutor Pedro Oliveira do Departamento de Química da Universidade de Aveiro e da Professora Doutora Beatriz Porto e Mestre Cláudia Oliveira do Laboratório de Citogenética do ICBAS/UP

“For from him and through him and for him are all things.”

O júri

Presidente

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Palavras-chave Doenças obstrutivas da via aérea; stress oxidativo; instabilidade cromossómica; danos mitocondriais; antioxidantes

Resumo

Doenças obstrutivas da via aérea, como asma e doença pulmonar obstrutiva crónica, são patologias multifatoriais que afetam uma grande parte da população e estão associadas a um elevado nível de stress oxidativo (SO). Este leva a consequências celulares como morte celular, disfunção mitocondrial e danos de DNA que, quando não reparados, dão origem a instabilidade cromossómica (IC) Assim, neste trabalho os objetivos foram: implementar, numa população controlo, um conjunto de técnicas de avaliação de SO e do estado redox da mitocôndria, de maneira a serem utilizadas num futuro estudo com pacientes com doenças obstrutivas da via aérea; confirmar, em células destes pacientes, os elevados níveis de IC previamente descritos em pacientes asmáticos, de maneira a alargar a população do estudo; explorar a eficácia de um tratamento *in-vitro* com antioxidantes para reduzir os níveis elevados de IC. Foi possível implementar, um conjunto de técnicas de avaliação de SO numa população controlo, para posterior utilização em estudos com pacientes. Também, foi possível confirmar um aumento dos níveis de IC, induzida com DEB, em linfócitos da população em estudo, comparativamente com a população controlo. Este resultado sugere a existência de uma hipersensibilidade dos pacientes a agentes indutores de SO. Para finalizar, o tratamento prévio com antioxidantes revelou-se eficaz na diminuição destes níveis elevados de IC.

Keywords Obstructive airway diseases; oxidative stress; chromosome instability; mitochondrial damage; antioxidants

Abstract Obstructive airway diseases (OAD), such as asthma and chronic obstructive pulmonary disease (COPD), are multifactorial pathologies that affect a large part of the population and are associated with high levels of oxidative stress (OS). This OS leads to cellular consequences, including cell death, mitochondrial dysfunction, and DNA damage that, when not repaired, gives rise to chromosome instability (CI). Thus, in this work, the objectives were: to implement, in a control population, a set of techniques to assess OS and the redox state of mitochondria, in order to be used in a future study with OAD patients; confirm, in cells from OAD patients, the high levels of CI previously described in cells from asthma patients, in order to enlarge the population size; to explore the effectiveness of an *in-vitro* antioxidant treatment in reducing high levels of CI. It was possible to implement, a set of OS assessment techniques, which can be used in future studies with OAD patients. Also, it was possible to confirm higher levels of CI, induced by DEB, in lymphocytes from OAD patients, comparatively to controls. This result suggests that OAD patients have an hypersensibility to OS-inducer agents. Finally, prior treatment with antioxidants proved to be effective in reducing these high levels of CI.

Index

Abbreviations:

ATP	Adenosine triphosphate
ALA	alpha lipoic acid
BER	Base excision repair
CI	Chromosome instability
COPD	Chronic obstructive pulmonary disease
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide trisphosphates
DSBs	Double-strand breaks
FA	Fanconi anemia
GFs	Growth factors
HR	Homologous recombination
ICLs	Interstrand crosslinks
MMP	metalloproteinases
MMR	Mismatch repair
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NHEJ	Nonhomologous end joining
NER	Nucleotide excision repair
OAD	Obstructive airway diseases
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCE	Sister chromatid exchange
SSBs	Single-strand breaks
TNF-α	Tumor Necrosis Factor

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1. Introduction

1.1. Obstructive airway diseases: asthma & chronic obstructive pulmonary disease (COPD)

Obstructive airways diseases (OAD) are a group of disorders composed by three different pathophysiological components: airway inflammation, airway obstruction, and airway hyperresponsiveness. Chronic inflammation plays a crucial role in all obstructive lung diseases being airway obstruction the result. Airway obstruction can have dynamic components (bronchospastic) and static components (structural). Different pathologies are included in OAD, such as cystic fibrosis, bronchiectasis, asthma, and chronical obstructive pulmonary disease (COPD). However, asthma and COPD are the most prevalent and with serious consequences[1].

1.1.1. Asthma characterization and worldwide prevalence

Asthma is a chronic airway inflammatory disease with obvious heterogeneity and variability, leading to multiple clinical phenotypes and inflammatory endotypes[2,3]. It is characterized by bronchial obstruction, mucus overproduction, airway remodeling, bronchial hyper-responsiveness, and over-production of reactive oxygen species, originated from inflammatory cells in the airways and intravascular compartment[4,5].

The complexity of asthma relies on several pathophysiologic mechanisms, which can interact and may not be present in all patients or at all time[6]. The reversibility of asthma is associated with mild disease. With disease progression, varying degrees of remodeling of the airways might lead to irreversibility. Pulmonary infection is present in patients with mild and moderate asthma, even though it stays relatively stable during lifetime. During exacerbations patients respond quickly to treatment and remain clinically stable during maintenance therapy. On the other hand, in cases of severe asthma, patients remain clinically symptomatic and present different airway behavior, in result of persistently low expiratory flow rates and failure to respond adequately to treatment[7]. Airborne allergens and viral respiratory

infections were identified as the two major environmental factors which contribute to persistence of asthma[5].

Asthma affects approximately 339 million individuals worldwide, being one of the main causes of death, and its incidence rises each year[8]. According to the World Health Organization, it is estimated that in 2025 the number of asthma patients might increase to 400 million[2].

1.1.2. COPD characterization and worldwide prevalence

COPD affects approximately 380 million people and is the fourth leading cause of death. It has been estimated that COPD will be the third leading cause of death in 2030[9].

COPD embraces three pathological conditions: emphysema, chronic bronchitis, and small airways disease[10]. COPD is an avoidable and treatable disease characterized by progressive airflow limitation that could be fully or partially irreversible. The disease is clinically characterized by excessive cough, sputum production, shortness of breath, chest tightness and by progressive decline in lung function. That can be exacerbated by exogenous factors such as cigarette smoke [10,11]. These factors are present in airway mucus secretion. Mucus is present in the apical epithelial surfaces of respiratory tract and works as a barrier against airborne pollutants and toxins. In cases of chronic airway inflammatory diseases, mucus hypersecretion is present and works as a characteristic of these diseases[12,13].

Occupational and environmental exposures to chemical smoke, dusts, and other lung are risk factors for this disease[14]. Only 15-20% of smokers develop COPD, indicating that factors beyond exposure to inhalational toxicants are important. Genetic factors are also involved, but the only established genetic risk factor for COPD is the deficiency of alpha-1 antitrypsin, affecting 3-10% of patients[15].

Unlike asthma, that appears early in life and its symptoms vary from day to day, COPD is a slowly progressing disease with continuous declining of lung function, with a long asymptomatic phase. COPD can manifest itself in four different stages, from “at risk” to

"very severe". The sooner the diagnosis is carried out the causes are identified, like exposure to smoke, the sooner the disease progression can be slowed[14].

1.1.3. Asthma-COPD overlap syndrome

As mentioned, both asthma and COPD are obstructive airway inflammatory diseases. More than 40% of patients with COPD report simultaneously a historic of asthma, and this dual diagnosis increase with age. In the presence of these two diseases a more rapid disease progression is reported. Also, asthma has been reported as a risk factor for COPD[16].

Asthma and COPD present different inflammation pathways, however they can overlap. Asthmatic patients are characterized by an elevated level of eosinophils and overstimulation of allergen-specific CD4+ T cells by antigen-presenting cells like lung macrophages and dendritic cells[1,9]. Otherwise, COPD presents high level of $CD8^+$ T cells and neutrophils that characterize the chronic inflammation associated with the disease[1,17]. Although the patterns of inflammation are heterogeneous, they can have a significant overlap. Asthmatics who smoke or exhibit severe fixed obstruction present an increased number of neutrophils, similar to COPD. Also, like in asthma, eosinophilic inflammation has been detected in COPD patients that present a greater reversibility of airway obstruction[1]. Interestingly, in both diseases, a neutrophilic inflammation is detected, leading to the increase steroid resistance[1,18].

Increasing age, smoking, airway hyperresponsiveness, and disease exacerbations in both asthma and COPD are factors for airway remodeling and accelerated loss of lung function[1,19]. The "Dutch hypothesis" (Figure 1) presents an explanation based in two major suppositions: that asthma and airway hyperresponsiveness predispose patients to develop COPD later in life; and that asthma, COPD, bronchitis, and emphysema are different expressions of a single airway disease. Patients that present chronic bronchitis, emphysema, or both, are not considered to have COPD, except if airflow obstruction is present. Asthmatic patients with airflow obstruction reversible are also not considered to have COPD. Asthma-

COPD overlap syndrome is present when asthmatic patients develop an airflow obstruction that is not completely reversible[1,20].

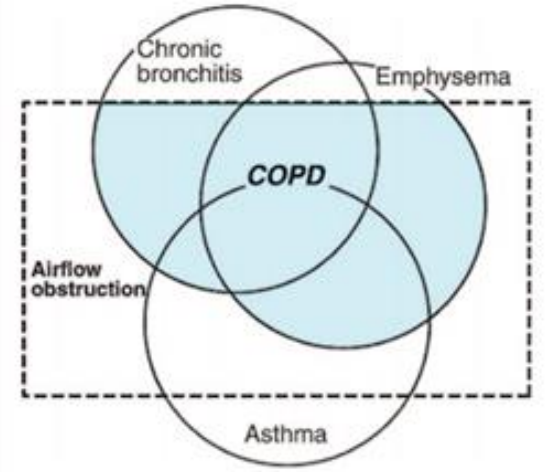


Figure 1 - Dutch Dutch hypothesis. The overlap of the pathological and clinical features of chronic bronchitis, emphysema, COPD and asthma are characterized[1]. The color blue represents the asthma-COPD overlap syndrome.

Concluding, patients with COPD and asthma demonstrate unique clinical features including an increase in respiratory exacerbations. Improved monitoring and prevention of this exacerbations may improve quality of life of these patients and potentially survival[1].

1.1.4. Inflammatory processes and cellular and molecular alterations

Inflammation is a natural defense mechanism of the human body, activated upon exogenous and endogenous stimuli**.** Inflammatory response in asthma and COPD involves a wide range of immune cells including mast cells, eosinophils, neutrophils, T-lymphocytes and also airway epithelial cells and smooth muscle cells [21].

Neutrophil mediated airway inflammation was found to be prevalent in various types of asthma. Although neutrophils play a pivotal role in primary defense against invading bacterial, fungal, and viral infections, these immune cells disperse a wide range of mediators that affect the airway lumen in asthma patients[21]. Cells involved in inflammation pathway of asthmatic patients when exposed to an allergen, generate reactive oxygen species (ROS),

such as hydroxyl radicals, superoxide, peroxydes, peroxynitrite and nitric oxide[4]. Due to uncontrolled generation of inflammatory cells, asthmatic patients present a higher production of ROS that leads to lipid peroxidation and increased oxidative stress (OS)[21]. In case of severe asthma with severe exacerbation, a significant elevation of ROS in their blood is present, and these species could have a different role depending on their producing cells. In epithelial and in bronchial smooth muscle cells, ROS likely induce local tissue damages and aggravates inflammation.

In peripheral blood mononuclear cells from asthma patients, the increased ROS were observed with any apparent mitochondrial dysfunction and could participate in the activation of circulating T lymphocytes, potentially playing a role in the "homing" of immune cells to the inflammatory upper airways. In fact, studies have shown that these ROS did not seem to be mainly produced by mitochondria and could have been produced by NADPH-oxidase and/or by xanthine oxidase[4]. Interestingly, the same study demonstrated that when comparing healthy subjects with severe asthma patients with severe exacerbation, a significant increase in their mitochondrial respiratory chain was observed together with increased ROS production[4]. However, more studies have to be performed in order to understand the connection between mitochondria and the immune response in asthma.

Significant increase in the concentration of IL-4 was also observed in asthmatic patients. IL-4 activates neutrophils on allergic inflammation and enhances the generation of IL-8 and tumor necrosis factor (TNF-α) while suppressing IL-1β. IL-4 is another inflammatory marker responsible for the induction of vascular cell adhesion molecule-1 on endothelial cells. It is responsible for the adhesion of inflammatory cells on the endothelial cells for migration (diapedesis) and support for allergic reactions. IL-4, IL-5 and IL-13 were implemented as potent inflammatory markers for the antiasthmatic activity and TNF-α is considered as the systemic inflammation marker in severe asthma by enhancing circulation of neutrophils[21]. Higher levels of TNF-α were evidenced in severe asthmatic patients compared with moderate asthmatic patients[22].

Alterations in metabolome are equally present in cases of asthma. Metabolome reflects genome-environmental interactions and metabolites play a crucial role in homeostasis and disease, contributing to processes such as redox balance, oxidative stress, signaling, apoptosis and inflammation. Several circulating metabolites in asthma differ from those in healthy individuals[3,6].

Concerning to patients with COPD, a different inflammation pathway is present, as shown in Figure 2. A harmful stimulus in lungs, mainly cigarette smoke, activate macrophages and epithelial cells to release cytokines that in turn recruit more immune cells from the circulation (mainly neutrophils and $CD8^+$ T cells). These cells activate fibroblasts leading to fibrosis and abnormal tissue repair. Neutrophils release proteases that are involved in alveolar destruction and release of mucus[23]. Proteases have an important role in cellular and tissue homeostasis. Metalloproteinases (MMP) are zinc-dependent endopeptidases and are critical components during the cell response to their microenvironment. MMPs can both activate and deactivate effector molecules like cytokines and growth factors (GFs). Likewise, cytokines can also activate or regulate the secretion of MMPs[9].

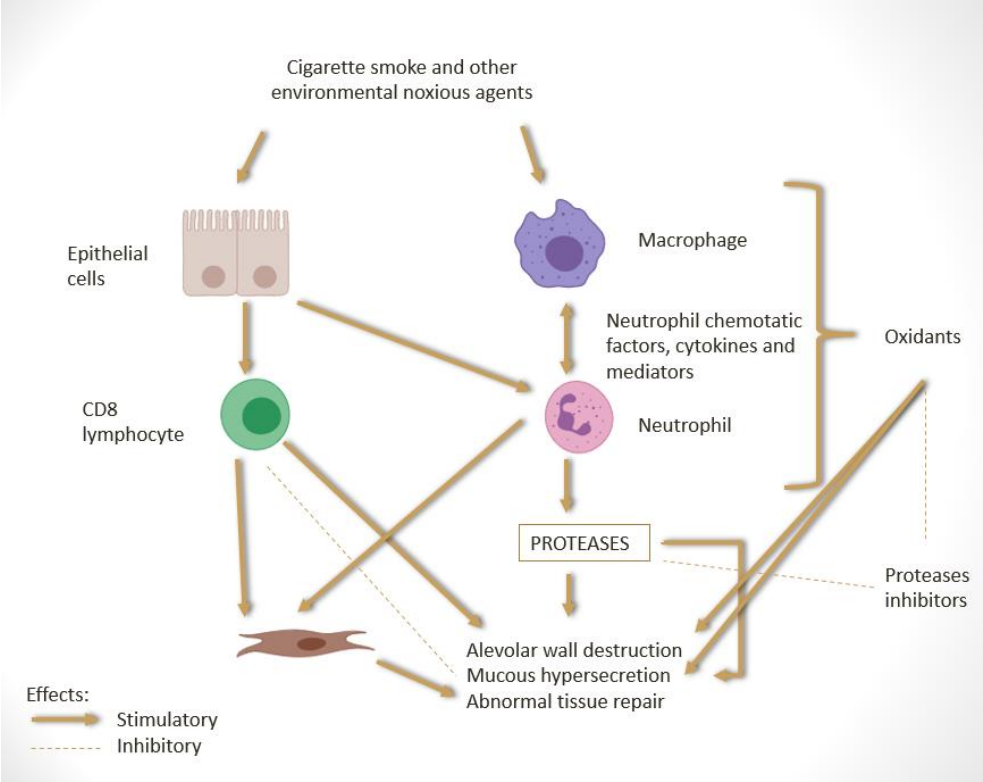


Figure 2 - Inflammation pathway in patients with COPD. Cigarette smoke act as a stimulus to the activation of macrophages that release cytokines. These latter recruit neutrophils and CD8 cells that have a crucial role in the tissue repair and progression of the disease[23].

Alveolar wall contains a variety of MMPs like MMP-12 (elastase), neutrophil elastase, and others that are also involved in mucus hypersecretion, a component of COPD disorder. The three MMPs directly involved in COPD, by modulating secretion leading to a disease progression and exacerbation, are MMP-9 (gelatinase), MMP-12 and MMP-8 (collagenase)[9,24]. Together they can destroy all extra cellular matrix and lung parenchyma, causing emphysema[24]. Cigarette smoking that is the principal cause of alveolar inflammation in patients with COPD, is also the main source of MMP-12, by cigarette smoke-exposed macrophages. They recruit more neutrophils and macrophages, aggravating the situation of neutrophil elastase to cause emphysema. Other proteins also exposed to smoking secrete prothrombin and plasminogen from the alveolar spaces and converts them to thrombin and plasmin (proteases). Proteinase-activated receptor 1 is activated and promotes the secretion of MMP-12 and other inflammatory proteins, and the cycle remains. In cases with high levels

of OS, to decrease the effect of these proteases, protease inhibitors and antioxidants can be used[9].

Concluding, own to the uncontrolled generation of inflammatory cells, both in asthmatic and COPD patients, which gives rise to increased production of ROS, it is of most importance to better characterize the cellular consequences of OS, and its harmful effect in OAD patients.

1.2. Oxidative stress (OS)

1.2.1. Reactive oxygen species

Free radicals are high reactive chemical species that interact and damage biomolecules such as proteins, lipids, DNA, and carbohydrates. They are molecules intrinsically instable and reactive. In biological systems, one of the most important types of free radicals are reactive oxygen species (ROS) [12]. Elevated levels of these species result in a state of OS. Irreversible damage to biomolecules is induced resulting in cell and tissue injury, and in onset of inflammatory diseases such as respiratory diseases[12,25].

In lungs, there are endogenous and exogenous sources of ROS. Endogenous sources refer to the production of free radicals by cells, such as epithelial, endothelial, neuroendocrine, smooth muscle, fibroblasts and immune cells (macrophages, neutrophils and T cells)[12]. The enzymes responsible for the production of ROS in lungs are cytochrome c oxidase, NADPH oxidase, myeloperoxidase and xanthine oxidase, being the mitochondria the principal source of excess of ROS[26]. The exogenous sources of ROS correspond to the inhalation of airborne toxicants such as cigarette smoke, biomass fuel smoke, gaseous emissions, ozone, chemical toxins, pesticides, and pollen grains, being the cigarette smoke the main contributor for free radicals in human lungs. In addition to possessing intrinsic oxidative potential, these airborne toxicants activate NADPH oxidase, stimulating the production of ROS, recruiting inflammatory cells (macrophages and neutrophils) and damaging mitochondria[12].

1.2.2. OS: mitochondrial and DNA damage

The presence of ROS in lungs results in oxidative damage responsible for the alteration of different mechanisms, which are associated to lung diseases. Lipid peroxidation refers to the oxidation of cellular membrane lipids, and when uncontrolled is the early biological process induced by oxidative stress. High levels of lipid peroxidation led to programmed cell death, i.e. apoptosis, inactivation of crucial proteins and activation of proinflammatory responses and transcriptions factors[12,27].

The mitochondria, responsible to produce ATP by oxidative phosphorylation, can be severely affected by the exposure to high levels of OS, leading to an increase in the mitochondrial respiratory chain or even mitochondria dysfunction[12,28].

DNA is a macromolecule that can be affected by the presence of high levels of OS. DNA is protected by histones and nuclear membranes, however, is also susceptible to attack from free radicals, especially at double-bonds[29]. DNA damage is represented by single- and double-strand DNA breaks. They result by the effect of ROS in a sequence of radical reactions within the DNA backbone, leading to the breakage of molecule[29,30]. This damage causes severe obstacle to cellular processes such as transcription and replication. In long term, the accumulation of ROS in DNA can lead to carcinogenesis[30].

1.2.3. Mechanisms of antioxidant defense

In order to combat the oxidative stress, the human body owns different systems of antioxidant mechanisms. The antioxidant response has the function of protecting lungs from inhaled environmental oxidants and for that, lungs are endowed with both non-enzymatic and enzymatic antioxidant defenses[12].

Respiratory tract epithelial lining fluid works as a protector of the airways and is rich in many non-enzymatic antioxidant scavengers. They interact directly with the inhaled oxidants and detoxify them, preventing the direct contact of these toxicants with the underlying epithelium[12]. The major's antioxidants molecules are glutathione (GSH), ascorbic

acid, uric acid, and vitamin E. GSH is the most important antioxidant present in respiratory tract epithelial lining fluid and with superior levels than in plasma. GSH acts on ROS products like hydroxyl, H_2O_2, hypochlorous acid and lipid peroxyl radicals generated during exposures to inhaled oxidants and allergens[31]. Being one of the main antioxidant molecules in the human body and having a crucial role in lungs, GSH levels are used as a hallmark feature of pulmonary diseases including asthma and COPD[12]. Ascorbic acid directly reduces the oxidative potential present in cigarette smoke or particulate matter and inhibits production of ROS by NADPH oxidase[32]. The ascorbic acid also has a role in the reduction of oxidized antioxidants in respiratory tract epithelial lining fluid, such as vitamin E, leading to the maintenance of the total antioxidant capacity of the lungs during oxidative insult. Vitamin E, on the other hand, neutralizes ROS and attenuates self-propagating lipid peroxidation reactions in the airways[12]. Uric acid is the main antioxidant in nasal secretion and helps in the removal of inhaled ozone, neutralizing the oxidative potential of particulate matter in humans. Abnormal levels of serum uric acid are associated with greater risk of COPD[33].

Enzymatic antioxidants systems are also present in lungs in order to minimize oxidative stress. The main pulmonary antioxidants enzymes are superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, hemeoxygenase-1, peroxidoxin-1, thioredoxin and thioredoxin reductase. In presence of environmental oxidants, these antioxidant proteins are activated, and work coordinated, helping in efficient detoxification of ROS and lipid peroxidation in lungs[12]. In Figure 3, it is represented a summary of the mechanisms, enzymatic and non-enzymatic, involved in antioxidant defenses in the lungs.

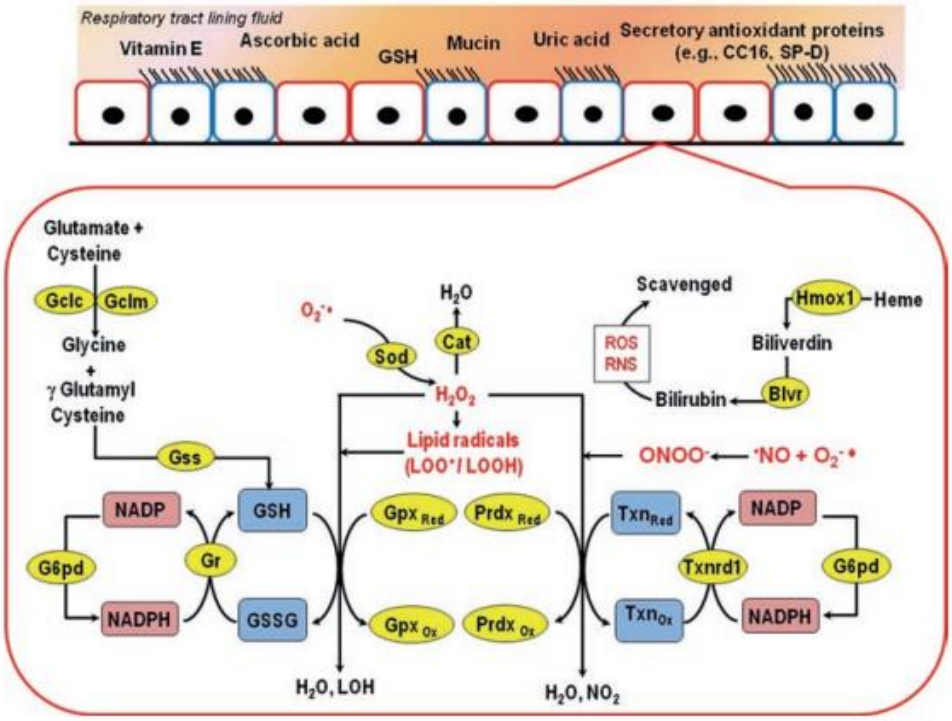


Figure 3 - Antioxidant defenses in lungs. Representation of respiratory tract lining fluid with the non-enzymatic antioxidant defense, and the enzymatic defense in epithelial cells. Gpx - glutathione peroxidase; Prdx – peroxiredoxins; Txnrd -Thioredoxin reductase; Txn – thioredoxin; Hmox1 - hemeoxygenase-1; Blvr – biliverdin [12].

Concluding, in case of exposure to airborne toxicants, a state of OS is induced in lungs, causing high levels of ROS. This leads to DNA damage and cell death. Even though the antioxidant defense mechanisms come into action, when there is an imbalance, i.e. when the presence of ROS is higher than antioxidant defense, other implications emerge, like mitochondrial dysfunction, epigenetic alterations, pro-fibrotic signals, lipid and protein oxidation, inflammation, mucus hypersecretion and endoplasmic reticulum stress. All these alterations contribute to a final state of lung disease, as illustrated in Figure 4 [12].

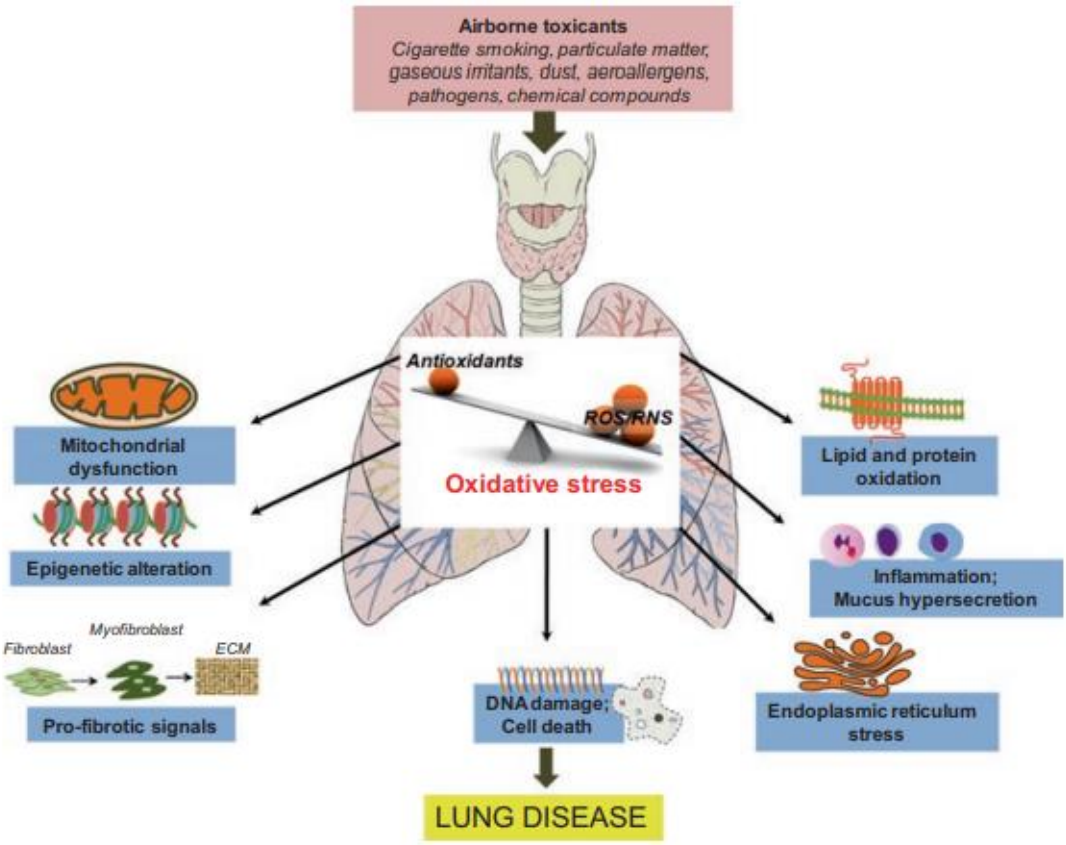


Figure 4 - Oxidative stress-mediated events in lung diseases. Alterations that occur in a case of imbalance between antioxidants and ROS/RNS[12].

1.3. Control mechanisms of cellular division and DNA repair

1.3.1. DNA replication and cellular division

DNA replication is the process whereby organisms duplicate their DNA before each cell division. To copy a DNA strand into a complementary DNA strand, is necessary the separation of the DNA helix in two template strands. Each one will originate a new DNA complementary strand. During replication, some errors can occur, for example, alterations in the nucleotides (complementary base-pairing), changes in helix geometry, tautomeric forms of the DNA bases, that when incorporated in the new DNA chain originate mutations[34]. DNA polymerase is the responsible for the first proofreading step just before a new nucleotide is covalently added to the DNA chain[35]. When errors escape from the replication machine, a strand-directed mismatch repair system come into action and removes these replication

errors[34]. In human cells, the genetic material is present in the form of chromosomes and the DNA replication takes place during only one part of the cell cycle, the DNA *synthesis phase* or S phase[35]. However, it is not only at this phase, or in DNA replication, that errors can occur. There can also be a lot of alterations during G1, G2 and M phases of cell division (Figure 5). One of the most dangerous types of DNA damage are the double-strand breaks (DSBs) that happen when both strands of the double helix are broken. The principal causes for these types of breaks are ionizing radiation, replication errors, oxidizing agents and other metabolites. If the reparation of these breaks does not occur, they lead to the breakdown of chromosomes into smaller fragments [34].

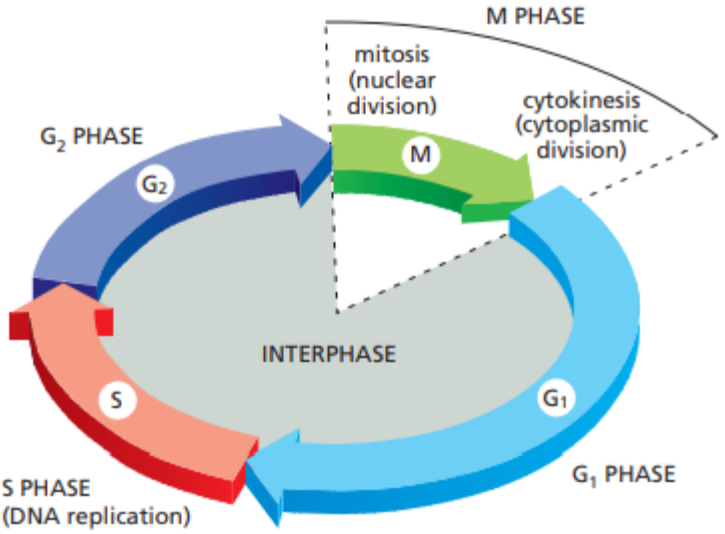


Figure 5 - The cell cycle and its different phases. The longest phase is Interphase where G1 phase, S and G2 phase are inserted, occurring DNA replication. The M phase, or mitosis, corresponds to nuclear division and consequently cytoplasmatic division, originating two cells[34].

1.3.2. Control and repair mechanisms

One of the control mechanisms is the cell-cycle control system that manages cell-cycle progression at three points. The first one is at late G1, where the cell enters the cell cycle and proceeds to S phase. This is the start of transition and where the evaluation of the environment occurs. The second point is the G2/M transition where the cell enters to mitosis. Here, not

only the environment is evaluated but also the DNA is replicated. The last checkpoint is the metaphase-to-anaphase transition where is analyzed if the chromosomes are attached to the spindle, stimulating sister-chromatid separation, and leading to the completion of mitosis and cytokinesis. If a problem is detected in any transition point, the cell is hold until that problem is resolved[36]. In these cases, breaks can be present in DNA, just in one strand, the single-strand breaks (SSBs) or in both strands, the double-strand breaks (DSBs). SSBs can be repaired by three different mechanisms: base excision repair (BER), nucleotide excision repair (NER) or mismatch repair (MMR)[37]. Being DSBs the most harmful for the cell, its repair mechanisms will be discussed in more detail.

As mentioned above some errors in DNA replication can be present in cells and homologous recombination (HR) is one of the mechanisms that allows DNA sequences to be maintained with very little change[34]. In HR occurs an exchange of DNA strands between a pair of homologous duplex DNA sequences, i.e. segments of double helix that are very similar or identical in nucleotide sequence. That way, one stretch of duplex DNA is allowed to act as a template to restore lost or damaged information on the second stretch of the duplex DNA. Thereby, HR can repair many types of damage because the template for repair is not limited to the strand complementary to that containing the damage, and one example is that HR is also possible in the repair of DSBs[38].

Another repair mechanism for DSBs is the nonhomologous end joining (NHEJ). In this pathway, the broken ends are brought together and rejoined by DNA ligation, but a loss of nucleotides occurs at the site of joining. This leads to the presence of mutations at the site of breakage. Besides that, another problem could be present in cases NHEJ. As there is no mechanism to ensure that the two ends to be joined were originally next to each other in the genome, NHEJ generate rearrangements in which one broken chromosome becomes covalently attached to another. The result is chromosomes with two centromeres (dicentric chromosomes) and fragments of chromosomes without centromere[34]. In Figure 6 is represented the functioning of these two repair processes.

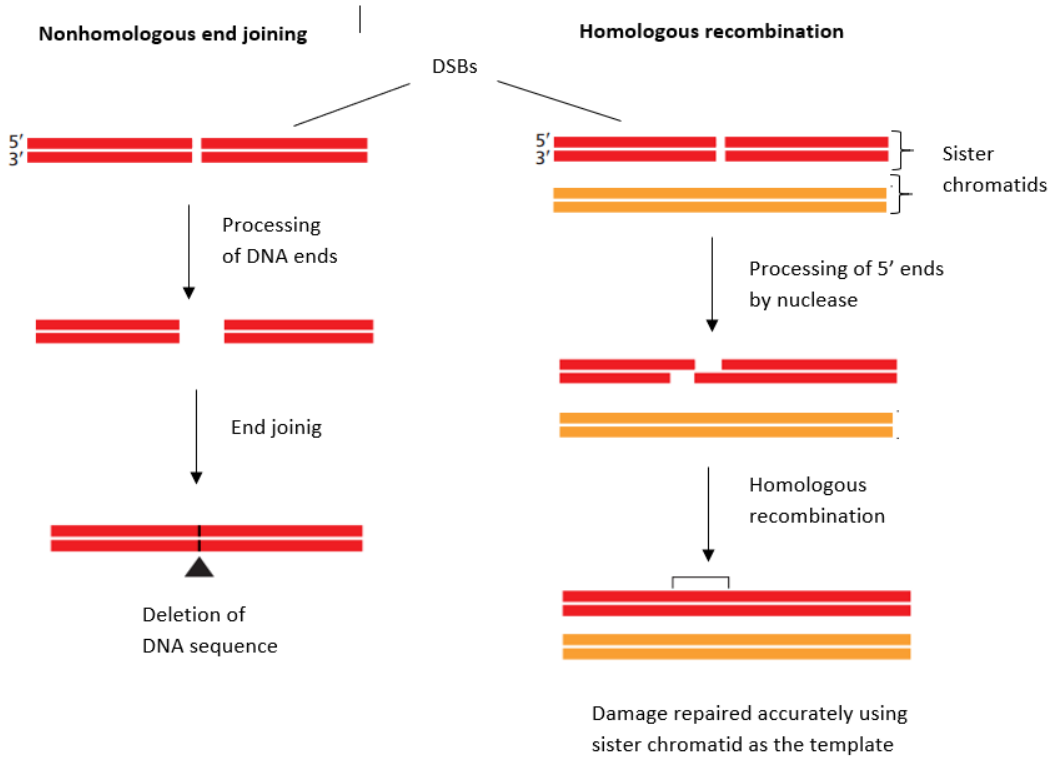


Figure 6 - The two processes of DSBs repair. On the left side the nonhomologous end joining repair where the original DNA sequence is altered when repairing a broken chromosome. On the right side is represented the HR where the original DNA sequence is restored by using sister chromatid of a DNA sequence as the template[34].

1.4. Chromosome instability (CI)

1.4.1. Failure in repair mechanisms and consequent effect on chromosomes

Replication of DNA containing interstrand crosslinks (ICLs) induces DSBs and is also a potential inducer of sister-chromatid exchange (SCE). SCE represents the reciprocal exchange of DNA strand between identical sister chromatid during replication, and that way, DSBs are mainly resolved through the production of SCE, involving the HR. DSBs in DNA that yield two broken ends, which can be directly recombined with one another, generally do not induce SCE. A replication fork collapse resulting in only one broken double strand-end[39]. In replication not also occurs the DNA synthesis but before that, DNA unwinding. DNA

unwinding is possible by the action of helicases that modify the structure of the DNA helix leading to the separation of the two DNA strands. However, to originate the breaks in the DNA strands, the replication fork needs to be paused, also denominated as stalled replication fork. Many impediments can stall the replication fork, but one of the main problems is the depletion of deoxynucleotide trisphosphates (dNTPs). Without dNTPs, the polymerase complex of replication fork cannot continue and slows down and arrests[40]. DNA lesions, such as ICLs, difficult-to-replicate regions, and collision with transcription machineries are also impediments that lead to stalled replication forks. When the replication stress is prolonged, stalled replication forks can undergo irreversible for breakage, resulting in genome instability and cell death. In Figure 7 it is represented the fork protection mechanism to minimize the genotoxic effects of replication stress by stabilizing, repairing, and restarting stalled forks[41].

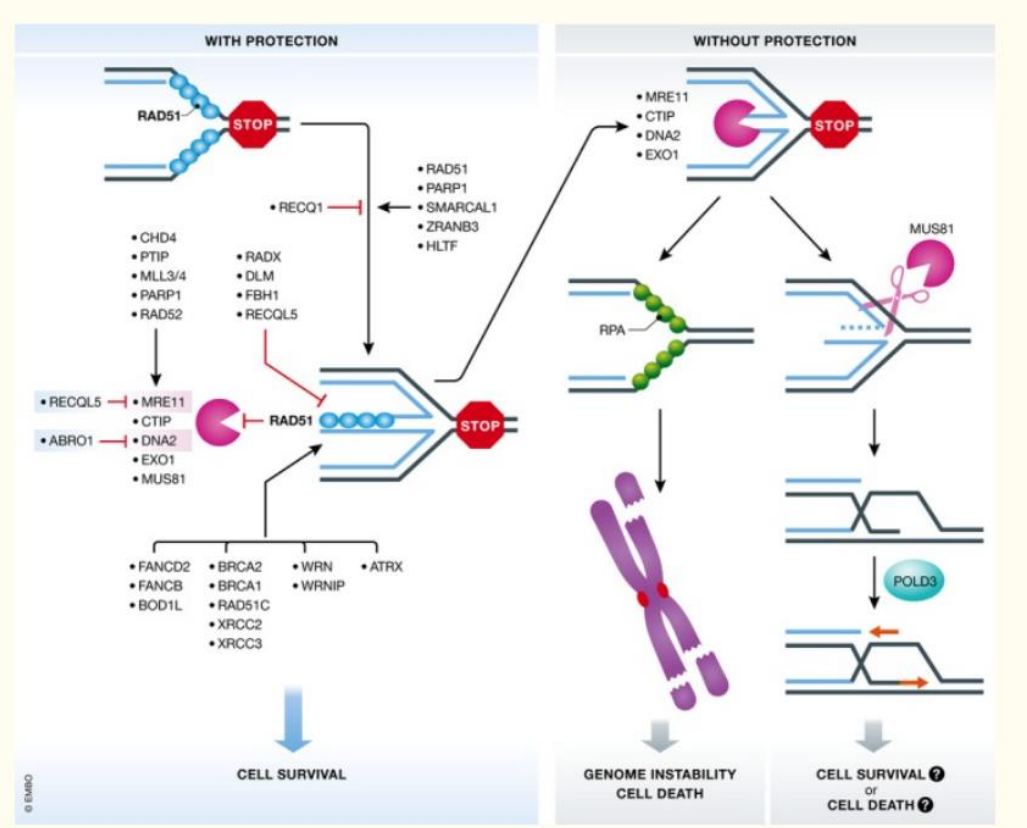


Figure 7 - Representation of stalled replication fork with and without protection. In cases without protection the stalled fork will lead to genome instability and cell death[41].

Increased DSBs and failure in their different repair mechanisms are at the origin of random chromosomal changes present in the cell, leading to chromosome instability (CI). CI is expressed at cellular level by the presence of chromatid and chromosome breaks, dicentric chromosomes and rings. In the most severe forms, it is also expressed by the formation of radial structures, in which broken ends with only small regions of sequence homology are joined. Radial structures are tandem arrays of chromosomes connected via recombined sister chromatids, created by chromatids erroneously fused to one another[39].

The canonical example of disease with increased CI, where the described radial figures are the hallmark, is Fanconi anemia (FA) a rare genetic disorder caused by pathogenic variants in any of the 22 genes that belong to the FANC/BRCA pathway, responsible for DNA repair and genomic stability. [39,42]. FA cells are characterized by oxidative DNA

damage, increased lipid peroxidation, free iron levels, ROS overproduction, mitochondrial dysfunction, and GSH depletion, being in a permanent pro-oxidant state. As a consequence, FA is linked with increased CI and cell death. The basis for the diagnosis of FA is the hypersensitivity of FA cells to OS-related genotoxic agents, such as diepoxybutane (DEB)[39].

In Figure 8 it is represented the pattern of chromosome breaks that can occur in FA cells as a consequence of CI. In DEB-induced lymphocyte cultures from FA patients, 30%-100% of the cells present multiple chromosomal breaks and the most particular feature is the presence of radial structures, due to increased frequency of illegitimate recombination[39].

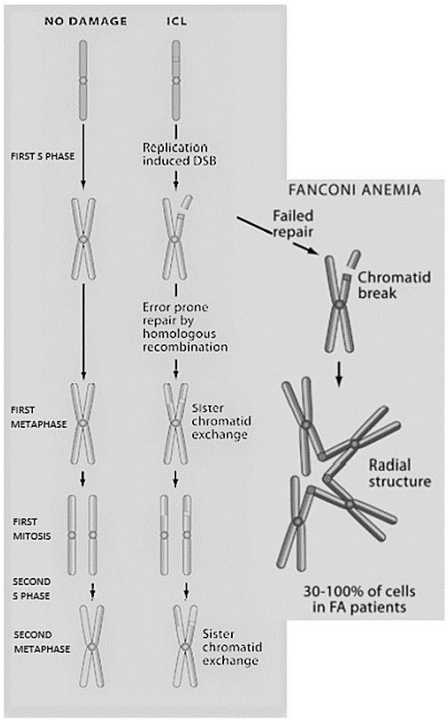


Figure 8 - Representation of chromosomal changes that can occur to DNA-ICL damage. In a cell with DNA ICL, replication is blocked, leading to the formation of DSBs. In normal cells, DSBs are repaired by HR, frequently resulting in SCE. The repair of ICL leads to the creation of a mutation at or near the site of the ICL. These DSBs can be removed by illegitimate recombination with nonhomologous sequences on other chromosomes, yielding radial structures[39].

1.4.2. OS-related CI cells from OAD patients

As already mentioned, a high and constant state of OS leads to DNA damage, in particular increased CI[29]. A recent study demonstrated that lymphocytes from severe asthmatic patients had increased levels of CI, both in spontaneous and DEB-induced lymphocyte cultures. When lymphocytes from these patients were treated with antioxidants, a decrease in CI was observed[43].

To better understand how CI correlates with OAD patients' clinical situation, further studies must be conducted, with an enlarged population, including not only asthma but also COPD patients. COPD, as mentioned above, is associated with an overproduction of ROS, circulating pro-inflammatory cytokines, and acute-phase proteins. OS with high levels of hydroxyl radicals, superoxide anions, and hydrogen peroxide go out of balance, leading to lung disease[44]. Consequently, OS may lead to DNA oxidation which, in turn, can lead to DSBs and CI. Thus, it can be hypothesized that in COPD patients there may also be present an increased level of CI, comparable to that detected in lymphocyte cultures from asthmatic patients.

1.4.3. Protective effect of antioxidants against CI

In conditions with high OS levels, the human body has systems for antioxidant defense. However, when there is an imbalance between OS and antioxidant defense, external help may be needed in order to balance this situation. One possibility is the use of external antioxidants.

Two external antioxidants, N-acetylcysteine (NAC) and alpha-lipoic acid (ALA), have been used in a previous study to counteract the effect of OS-induced CI in cells from FA patients[45]. NAC, derived from the amino acid L-cysteine and a thiol compound, has shown an excellent efficiency in increasing the levels of GSH, being fundamental for the regulation of the production of this antioxidant in cases of oxidative stress. NAC also promotes detoxification by acting directly on ROS, modulating the levels of pro-inflammatory

cytokines, being useful in preventing infections. ALA, in turn, is a natural compound, a mitochondrial nutrient that is predominant in its reduced form (DHLA)[42]. It acts as a potent antioxidant reducing vitamin E, vitamin C, and coenzyme Q10, and as a cofactor for many mitochondrial enzymes, mainly dehydrogenases. That way is directly involved in the ROS detoxification process. Like NAC, ALA also has the ability to increase and regulate GSH levels, maintaining the antioxidant status of cells and reducing pro-inflammatory conditions through interaction with NFkB[42,45].

In a recent study it was shown that NAC and ALA had an efficient action in reducing CI in lymphocyte cultures from severe asthmatic patients[43]. Further studies in a larger population, including both asthma and COPD patients, are needed, in order to confirm this protective effect.

2. Objectives

Considering the importance to proceed an ongoing study that focus on a more complete cellular characterization of OS and related CI in OAD patients, this work had two main goals: to implement a protocol for evaluation of OS and redox state in mitochondria; to better characterize the levels of CI in cells from OAD patients.

For the evaluation of OS and redox state of mitochondria the specific aim was:

- To optimize, in a control population, specific techniques that meet the needs of a future study directed to OAD patients, namely:
    - Evaluation of OS-related damage through the analysis of protein oxidation, protein nitration and lipid peroxidation by the Slot-blot technique;
    - Evaluation of total cellular ROS production by CM-H2DCFDA probe;
    - Evaluation of mitochondrial membrane potential by JC1 probe;
    - Evaluation of mitochondrial bioenergetics by Agilent Seahorse XF analyzer;
    - Evaluation of mitochondrial function through resazurin analysis

For evaluation of the levels of CI in cells from OAD patients, the specific aims were:

- To confirm the previously described increased levels of CI in primary lymphocytes from a population of asthma patients, enlarging the population size, including other OAD patients.
- To further explore the efficacy of an *in-vitro* antioxidant cocktail as a putative treatment.

3. Material and Methods

3.1. Population

3.1.1. Selection of patients

This study included 35 controls, for the establishment of internal reference values and techniques, and 12 OAD patients (11 asthmatics and 1 COPD patient) recruited by the Service of Medicine of CHUP (Centro Hospitalar Universitário do Porto).

In order to be selected as a control to this study, participants should be at least 18 years old, and complete and sign the free, prior, and informed consent for this study. In order to exclude any respiratory symptoms, participants filled out a specific questionnaire and performed a spirometry test. In the context of the Sars-CoV-2/COVID-19 pandemic and due to the high risk of transmission by aerosolization, a spirometry test cannot be performed without being sure that the volunteer is negative for the virus. Thus, COVID-19 antigen rapid tests were performed before the spirometry test.

As for the patients selected for this study, participants should be at least 18 years old, have already been diagnosed with OAD, completed and signed the informed consent for this study and filled all the specific inquiries.

3.1.2. Inquiries and Informed consents

To identify the sociodemographic variables and clinical scales that could be important to characterize the participants' lifestyle, health and clinical history, a literature review was done, in order to include the specific information in the inquiries. Informed consents were also designed for the purpose of comply with all the ethics committee guidelines. The final documents for this specific study are in the attachments (Attachment 2).

3.1.3. Sample collection

From each participant, 27 mL of venous blood were collected by antecubital venipuncture, into vacuum tubes with lithium heparin.

3.2. Techniques

3.2.1. List of reagents

Acetic acid (Fisher Chemicals, Thermo Fisher Scientific Inc., USA); ALA (Sigma-Aldrich, St. Louis, MO, USA); BSA (Sigma-Aldrich, St. Louis, MO, USA); Colcemid (GIBCO, Invitrogen Corporation, USA); CM-H_2DCFDA probe (Thermo Fisher Scientific Inc., USA); DEB (Sigma-Aldrich, St. Louis, MO, USA); DMSO (Sigma-Aldrich, St. Louis, MO, USA); EDTA (Sigma-Aldrich, St. Louis, MO, USA); Fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA); Fetal calf serum (Gibco, Invitrogen Corporation, USA); Giemsa solution (Merck, Darmstadt, Germany); HBSS (Biowest, MO, USA); HCl (Fisher Chemicals, Thermo Fisher Scientific Inc., USA); Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA); Hoechst (Thermo Fisher Scientific Inc., USA); JC1 probe (Tetrathylbenzimidazolylcarbocyanine iodide) (Sigma-Aldrich, St. Louis, MO, USA); KCl (Sigma-Aldrich, St. Louis, MO, USA); Lymphoprep™ (STEMCELL Technologies, Vancouver, Canada) L-glutamine (Sigma-Aldrich, St. Louis, MO, USA); Goat anti-4-hydroxynonenal antibody (AB5605, Merck Millipore, Temecula, CA, USA); Goat anti-rabbit IgG antibody (AP307P, Sigma-Aldrich,St. Louis, MO, USA); Methanol (Fisher Chemicals, Thermo Fisher Scientific Inc., USA); NAC (Sigma-Aldrich, St. Louis, MO, USA); PBS (Biowest, MO, USA); Penicillin (PEN-STEP, GIBCO, Invitrogen Corporation, USA); Phosphate buffer saline solution (Sigma-Aldrich, St. Louis, MO, USA); Phytohemagglutinin (PHA) (GIBCO, Invitrogen Corporation, USA); Ponceau S (MB19201, NZYTech, Lisboa,Portugal) Pluronic acid (Sigma-Aldrich, St. Louis, MO, USA); RPMI (Sigma-Aldrich, St. Louis, MO, USA); Rabbit anti-DNP antibody (D9656, Sigma-Aldrich, St. Louis, MO,USA); Rabbit anti-nitro-tyrosine antibody (9691S, Cell Signaling Technology, The Netherlands); Rabbit anti-goat IgG antibody (AP107P,Sigma-Aldrich, St. Louis, MO, USA) RPMI without phenol red (Gibco, Invitrogen Corporation, USA); SDS (Thermo Fisher Scientific Inc., USA); Streptomycin (PEN-STEP, GIBCO, Invitrogen Corporation, USA) Pierce™ BCA Protein assay Kit (Thermo Fisher Scientific Inc., USA).

3.2.2. Technical procedures

- **Cell cultures and CI analysis**

For cell cultures, the 0.5 mL of blood collected was cultured in a complete medium of RPMI with 15% of fetal bovine serum, penicillin (10,000 units/mL), streptomycin (10,000 µg/mL), and L-glutamine (29 mg/mL). Phytohemagglutinin (5 µg/mL) was added in order to stimulate the cultures and these were incubated at 37ºC with 5% CO_2 atmosphere, for 72h. [45]. In the appropriately selected cultures, DEB was added at the concentrations of 0.05 µg/mL and 0.1 µg/mL, 24h after culture initiation. DEB is a suspected carcinogen of unknown risk, so for precaution, all procedures were made in a flow chamber, using appropriate individual protection equipment. Colcemid (4 µg/mL) was added for 1h of incubation, after 3 days of culture. Cells were harvested after a hypotonic treatment with KCl (75 mM) and fixed 3 times in a 1:3 iced solution of acetic acid: methanol. Cell suspensions were maintained at 4^0C until cytogenetic analysis.

The cytogenetic analysis was performed by microscopic observation. For that, the suspensions were dropped onto microscope slides and stained for 5 min with Giemsa solution (4%) diluted in phosphate buffer saline solution. For each experiment 50 metaphases per sample were analysed, in a blinded fashion. To avoid bias in cell selection, consecutive metaphases that appeared intact, with sufficient well-defined chromosome morphology, were selected for study. Each cell was scored for chromosome number and the number and the types of structural abnormalities. The structural abnormalities considered were breaks (achromatic areas wider than a chromatid), fragments (also scored as breaks), dicentric chromosomes, ring chromosomes and chromatid exchange configurations (triradial and tetraradial figures), being the last three scored as rearrangements and considered as two breaks. Gaps, that are achromatic areas less than a chromatid in width, were excluded in the calculation of chromosome breakage frequencies. The CI parameters evaluated were percentage of aberrant cells and mean number of breaks per cell.

- **Peripheral blood mononuclear cells isolation**

The density gradient medium Lymphoprep™ was pipetted into a SepMate™ tube via the insert hole according to the manufacturer's instructions. Whole blood was diluted in a 1:1 ratio with cold PBS, supplemented with 2% FBS, and gently added through the walls of the inside of the tube. The SepMate™ tubes were then centrifuged at 1200×g for 10min (with brake on) at 4ºC. Plasma and peripheral blood mononuclear cells (PBMCs) were poured into a separate 50 mL tube and washed twice with the PBS (2% FBS). During all the process of lymphocyte isolation, the samples were maintained in ice.

- **Cell counting**

For cell counting a Countess^{tm} automatic cell counter was used according to manufacturer's instructions. Briefly, 10 µL of sample were mixed with 10 µL of Triptan Blue and loaded into the Countess for reading.

The automatic result presents several data such as total cell concentration, viable cell concentration, dead cells concentration, viability, average viable cell size, average dead cell, number of viable cells counted, and number of dead cells counted. For the present study, cell viability was selected.

- **Extraction and quantification of total proteins**

Total proteins from PBMCs were extracted using 50 µL of SDS. After incubation at room temperature for 15-20 min, a centrifugation at 14000xg for 20 min was made.

For protein quantification, diluted samples (1:10 in PBS) were prepared in a transparent 96 well-plate, and three replicates were carried out for each sample. As a protein standard curve, several dilutions of BSA (0, 50, 100, 200, 500, 1000 µg/mL) were used. Then, 190 µL of Working Reagent (prepared by mixing 300 µL of Reagent B in 15 mL of Reagent A, from Pierce™ BCA Protein Assay Kit) was added to each well and the plate was incubated for 30

min, at 37ºC protected from light. The absorbance (595 nm) was read on a Synergy™H1 multi-mode microplate reader.

- **Evaluation of OS-related damage by Slot-blot**

The OS-related damage was evaluated on PBMCs through the analysis of protein oxidation, lipid peroxidation, and protein nitration by Slot-blot technique and using specific antibodies.

For the analysis of protein nitration and lipid peroxidation evaluation, samples were diluted to a concentration of 50 ng/µL. For protein oxidation, the content of carbonyl groups was evaluated. Protein samples of 5 µg were derivatized using 2,4-dinitrophenylhydrazine (DNPH) to obtain 2,4-dinitrophenyl (DNP) and then diluted to a final concentration of 1 ng/µL. The slot blot technique was performed using a Hybri-slot manifold system. A staining with Ponceau S was performed for total protein normalization. The resulting PVDF membranes were incubated overnight at 4ºC with a rabbit anti-nitro-tyrosine antibody (1:1000), a goat anti-4-hydroxynonenal antibody (1:5000) and a rabbit anti-DNP antibody (1:5000), respectively. Membranes were then incubated with a rabbit anti-goat IgG antibody (1:5000) or goat anti-rabbit IgG antibody (1:5000). Blots were visualized with Clarity™ Western ECL Substrate and read using a Bio-RadChemiDoc XR system. Densities from each band were quantified using Image Lab Software.

- **Evaluation of total cellular ROS production**

Total cellular ROS production in PBMCs was evaluated using the fluorogenic dye CM-H_2DCFDA, according to the manufacturer's instructions.

For this technique, $2.5x10^5$ PBMCs were incubated with 750 µL of a 5 µM CM-H_2DCFDA and 0.1% (w/w) Hoechst solution, prepared with HBSS. Then, 100 µL of the sample solution was transferred into a black 96 well-plate and incubated at 37ªC for 30 min. For each sample, three replicates were carried out. PBMCs incubated in a 0.1% H_2O_2 solution

were used as a positive control. Fluorescence (495/529 nm; excitation/emission) was measured on a Synergy™ H1 multi-mode microplate reader.

- **Evaluation of mitochondrial potential**

The mitochondrial membrane potential of PBMCs was evaluated using the fluorogenic dye 5-5′,6-6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC1), according to the manufacturer ’s instructions.

In resume, $2.5x10^5$ PBMCs were incubated in a JC1 solution (0.65 µg/mL in HBSS) at 37 °C for 30 min. PBMCs incubated in a 20% DMSO solution were used as a positive control. Then, cells were centrifuged at 500xg for 5 min and washed twice with HBSS. After that, the PBMCs were transferred to a black 96-well plate so that monomers and JC1 aggregates (485/530 nm - excitation/emission; 535/590 nm - excitation/emission, respectively) could be read on a Synergy™ H1 multi-mode microplate reader. As an indicator of mitochondrial membrane polarization, the ratio of JC1/monomer aggregates (Red/Green ratio) was used, where an increased ratio indicates hyperpolarization, and a decreased ratio indicates depolarization.

- **Evaluation of mitochondrial bioenergetics by Agilent Seahorse XF analyzer**

According to the manufacturer’s instructions, the Oxygen Consumption Rate (OCR) and the Extracellular Acidification Rate (ECAR) were obtained using an Agilent Seahorse XF24 Extracellular Flux Analyzer. For this experiment, the Seahorse XF Cell Mito Stress Test kit was used. With this assay kit, multiple parameters including, basal respiration, ATP-linked respiration, maximal and reserve capacities, and non-mitochondrial respiration are obtained.

In brief, PMBCs were seeded in a 24-wells cell culture plate at a density of 1×10^6 cells per well in 2mL of RPMI for 24h, at 37ºC. Three technical replicates were carried out for each condition. After the incubation period, the plate was centrifuged for 10 min, and the cells were transferred into the Seahorse XF24 microplate with 500 µl of assay medium,

supplemented with sodium pyruvate, glutamine, and glucose. Prior to analysis, the plate was placed in a non-CO2 incubator for 1h at 37ºC, in order to avoid any temperature variances that might affect experimental results. During the analysis, 1.5 µM Oligomycin, 1 uM carbonyl cyanide-4-phenyl-hydrazone (FCCP), and 0.5 µM of Antimycin A and rotenone, were added to determine mitochondrial and non-mitochondrial function.

- **Evaluation of mitochondrial function through resazurin analysis**
    - Kinetic analysis

In order to evaluate metabolic activity of the mitochondria through a resazurin assay, PMBCs were seeded in a 96-wells plate at a density of 3×10^5 cells per well in RPMI medium for 24h, at 37ºC. Three replicates were carried out for each condition. After incubation, resazurin was added for a final concentration of 44 µM. After 2 hours of incubation with resazurin, specific drugs were added, according to the intended experiment. Fluorescence measurements (550/588 nm; excitation/emission) were performed using a Synergy™ H1 multi-mode microplate reader, each hour for 8 hours.

    - Single-point analysis

In order to assess cell viability in each of the conditions performed in cell cultures for IC evaluation, a single-point measurement with resazurin was performed.

After 68h of incubation, 198 µL of the culture was transferred into a 96-wells plate, and resazurin was added for a final concentration of 44 µM. Triplicates of each condition were carried out. After 4 hours of incubation with resazurin, a single fluorescence measurement (550/588 nm; excitation/emission) was performed using a Synergy™ H1 multi-mode microplate reader.

3.2.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9, version 9.0.0 software. Graphical and tabular results are expressed as mean ± standard error of mean (SEM). Statistical comparison between groups was done using a paired t-tests and a Two-factor mixed ANOVA followed by a multi-comparison Turkey's test. $p < 0.05$ was considered significantly different.

4. Results and Discussion

4.1. Optimization of techniques for evaluation of OS and redox state of mitochondria

In order to implement a protocol for evaluation of OS and redox state of mitochondria, to be used in a future study directed to OAD patients, the specific techniques that follow were optimized in a control population. For each experiment, peripheral blood from healthy blood donors were used.

- **Optimization of PBMCs isolation**

PBMCs isolation is the first step in blood samples processing, and it plays a major role in the success of the remaining techniques. Therefore, a set of experiments was performed in order to implement the best isolation technique, which requires: the minimum time consuming without inducing extra cell damage or influence subsequent techniques

Isolation using Histopaque-1077:

The protocol that is currently in use at the Laboratory of Cytogenetics is a density gradient centrifugation, using Histopaque-1077, according to the manufacturer's instructions. In brief, this protocol consisted in adding 15 mL of Histopaque-10777 to a Falcon tube and then carefully add 15 mL of a peripheral blood sample through the walls of the tube and centrifuge at 400xg for 30 minutes (with break on). After centrifugation, the opaque surface containing the PBMCs was carefully aspirated carefully with a Pasteur pipette and the plasma, gradient medium and erythrocytes were discarded. The PBMCs were transferred to a clean falcon tube and washed twice with PBS calcium and magnesium free (250xg for 10 minutes).

Even though this isolation technique enables to obtain PBMCs with good quality, the process of centrifugation is time-consuming. This problem is a particular concern in the case

of a study with a large number of samples. So, a new technique, to overcome this problem, was evaluated.

Isolation using erythrocyte lysis method

In order to solve the time-consuming problem of Histopaque-1077 isolation, an erythrocyte lysis protocol was proposed. This protocol is a quick and efficient method of for erythrocyte lysis.

In resume, 10 mL of a peripheral blood sample were added into a falcon tube with 40 mL of lysing solution containing ammonium chloride and incubated for 10 minutes at room temperature. After the incubation period, centrifugation at 300xg for 5 minutes at 4ºC (break on) was performed. The supernatant was discarded, and the cells were washed 2 times with PBS. After PBMCs isolation the pellet was stored in a cryopreservation solution (SBF + 4% DMSO) and stored at -80ºC.

Some studies have already shown that the method that uses ammonium chloride to lyse erythrocytes, can be quite aggressive to cells[46]. In order to confirm if the isolation protocol used in this study did not create any extra damage to PBMCs, a DNA damage analysis was performed using the comet assay or single-cell gel electrophoresis technique. Briefly, after embedding in agarose on a microscope slide, cells were lysed, keeping DNA organized in a form of nucleoid, which were then electrophoresed. With this assay it is expected that undamaged DNA does not move, and damaged DNA disrupts and consequently loses its compact structure and drift out the nucleus. This damage forms a comet-like image (tail) viewed by fluorescence microscopy. The extent of DNA damage is expressed by the quantity of fluorescent DNA in the tail[47].

For the DNA damage analysis, two different conditions were defined. A non-exposed control, where spontaneous damage of PBMCs was assessed, and a positive control condition, where cells were exposed to DEB (0.1 µg/mL), an inducer of DNA damage. Figure 9 illustrates the results obtained in this experiment.

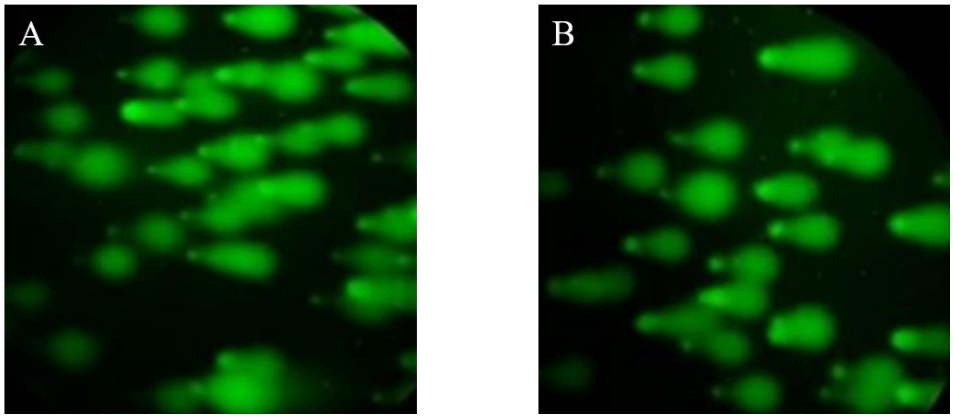


Figure 9 - Representative illustration of results from comet assay slide scoring. Comparison of DNA damage between (A) Spontaneous DNA damage of non-exposed PBMCs and (B) Induced DNA damage of DEB exposed PBMCs.

Analyzing the obtained results, both conditions showed cells with very high levels of DNA damage, namely "hedgehog" type comets. This type of damage is normally observed after exposure of cells to genotoxic agents and might also represent the very early stages of DNA breakdown in apoptosis[48].

Hereupon, it was hypothesized that, in the present study, the specific damage observed both spontaneously and when exposed to DEB, may be due to the method of PBMCs isolation.

Isolation using SepMate^{TM} Tubes

In order to eliminate the DNA damage variable caused by the erythrocyte lysis protocol and to optimize the protocol time of Histopaque-1077, it was decided to try a new form of PBMCs isolation using SepMate ^{TM} tubes.

The SepMate^{TM} tube enables PBMCs isolation in just 15 minutes since the insert of this tube allows to quickly separate blood compounds over the density gradient medium. After centrifugation, isolated PBMCs are simply poured off into a new falcon tube.

The final protocol for this technique is already described in materials and methods, page 24.

The use of SepMateTM tube for the PBMCs isolation reduced in 40 minutes the total time of the lymphocyte isolation protocol, without compromising the viability of the cells, as seen in Figure 10.

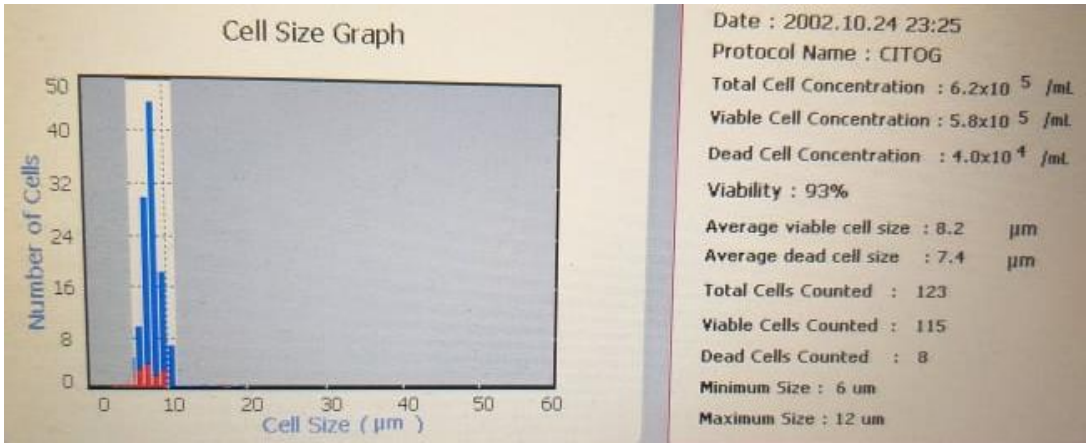


Figure 10 - Data obtained in Countess^{TM} for isolated PBMCs from a peripheral blood sample using the SepMate^{TM} tubes. It is possible to see that this technique provides accurate values of cell viability in a short period of time.

In conclusion, this optimized isolation method will be used in the future work.

- **Evaluation of OS-related damage by Slot-Blot**

For evaluation of OS-related damage, an *in vitro* model was created, using the alkylating agent DEB as an inducer of OS in normal cells. This model, when implemented, will be used for comparison with cells from OAD patients.

A model of in vitro increased OS

Increased cellular OS was evaluated through the analysis of carbonyl groups (protein oxidation), protein nitration and lipid peroxidation. For this, lymphocyte cultures were exposed to different DEB concentrations (0.005 µg/mL, 0.01 µg/mL, 0.05 µg/mL, 0.1 µg/mL, and 0.2 µg/mL), and the OS-related damage was evaluated at different time points (1h, 2h, 6h and 24h). Lymphocyte cultures without DEB exposure were used as a control.

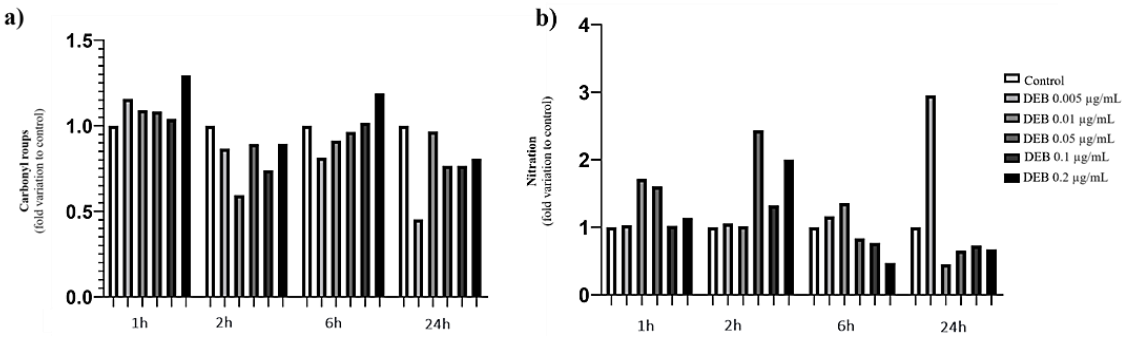


Figure 11 – Evaluation of oxidative stress-related damage in lymphocyte cultures exposed to different concentrations of DEB (0.005 µg/mL, 0.01 µg/mL, 0.05 µg/mL, 0.1 µg/mL and 0.2 µg/mL) at different exposure times (1h, 2h, 6h and 24h). Damage was evaluated by the effect of DEB on (a) carbonyl groups and (b) protein nitration. Results are presented as fold variation to control and expressed as mean. (n=3).

As shown in Figure 11, addition of DEB to lymphocyte cultures did not cause considerable variations, evaluating both protein oxidation and nitration, concerning either the parameters dose-effect or exposure time. Some sporadic expressive variations can be explained by technical artifacts.

Therefore, it was not possible to create a model for increased OS-related damage, evaluating either protein oxidation or nitration.

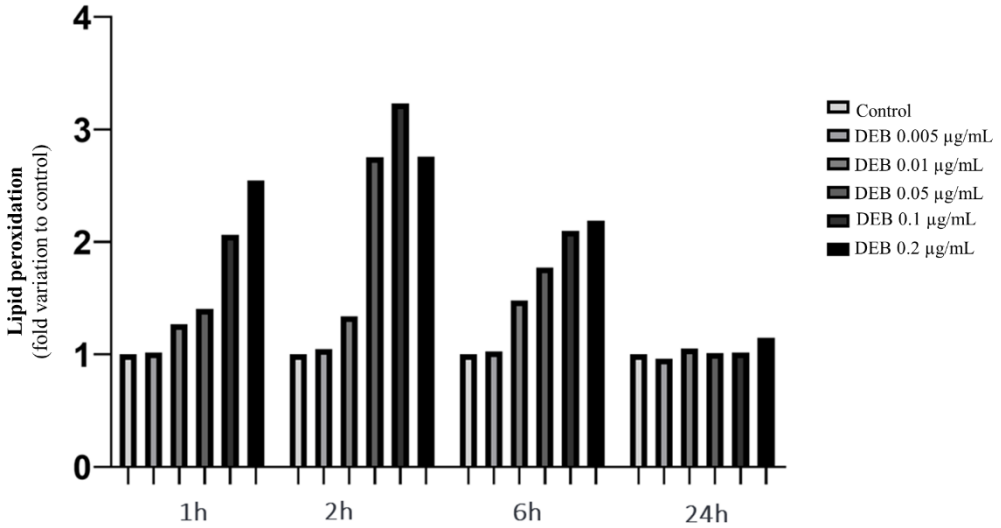


Figure 12 – Evaluation of oxidative stress-related damage in lymphocyte cultures exposed to different concentrations of DEB (0.005 µg/mL, 0.01 µg/mL, 0.05 µg/mL, 0.1 µg/mL and 0.2 µg/mL) at different

exposure times (1h, 2h, 6h and 24h). Damage was evaluated by the effect of DEB on protein peroxidation. Results are presented as fold variation to control and expressed as mean. (n=3).

Figure 12 shows that at the first two hours of DEB exposure, at high doses (0.05 µg/mL, 0.1 µg/mL, and 0.2 µg/mL), PBMCs presented increased levels of lipid peroxidation when compared to control. Since peroxidation corresponds to the formation of peroxides and peroxyl radicals in the presence of O_2, it is expected that with OS increase (represented by the increase in DEB concentration), oxygen radicals increase and consequently peroxidation[49]. Concerning exposure time, after 6h of exposure to DEB, the increased levels of lipid peroxidation start to decrease, reaching similar levels to the control at 24h of DEB exposure. This result could be suggested by the already described adaptive mechanisms of cells. This population consists of healthy controls, so it is expected that these cells can generate an adaptive response when exposed to an OS inducer agent.

In summary, it was concluded that in a future work with OAD patients, whenever it is intended to evaluate OS-related damage, only lipid peroxidation will be considered. As far as the test conditions are concerned, only the highest DEB concentrations (0.05 µg/mL, 0.1 µg/mL and 0.2 µg/mL) will be used, as they showed higher differences when compared to control. Also, DEB 0.05 µg/mL and 0.1 µg/mL are the concentrations used in CI analysis, which might be of great importance for comparative studies. Regarding the exposure time, it was concluded that the most interesting timepoint for this study would be 2 hours of DEB-exposure, since after this timepoint a possible adaptive response of the cells to OS may occur.

- **Evaluation of total cellular ROS production by CM – H_2DCFDA probe**

Evaluation of total cellular ROS production by CM-H_2DCFDA probe was also performed. CM-H_2DCFDA probe is a chloromethyl derivate of H_2DCFDA and is used as an indicator for cellular ROS. Through the passive diffusion of CM- H_2DCFDA into cells, its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation of H_2DCF by

ROS converts the molecule to 2', 7' dichlorodihydrofluorescein (DCF), which is highly fluorescent. Measuring the fluorescence allows the assessment, in real time, of ROS levels in the cells[50].

In order to validate this technique, PBMCs were incubated with an antioxidant treatment of NAC+ALA for different periods of time (2h, 3h, 4h and 5h). Once CM-H_2DCFDA allows the determination of total cellular ROS production, with an exposure to an antioxidant treatment, it was expected that the endogenous levels of ROS, presented at 0h, would decrease. This way, it was possible to validate the proper functioning of the probe.

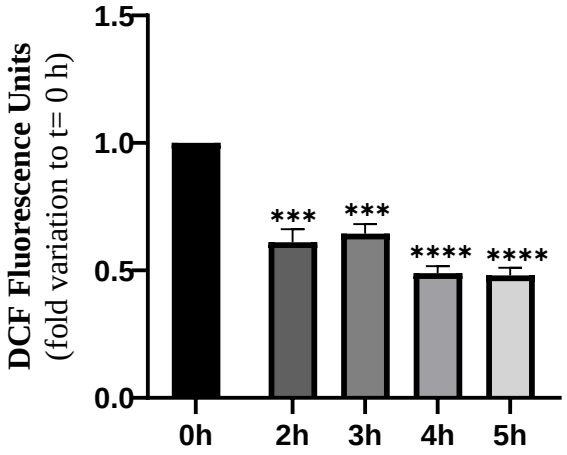


Figure 13 – Evaluation of endogenous ROS production in PBMCs at baseline (T=0 h) and with an antioxidant treatment for 2h, 3h, 4h and0 5h, using the fluorogenic dye CM-H2DCFDA. Results are expressed as mean ± SEM (n=3). ****p<0.0001; ***p<0.001.

As shown in Figure 13, endogenous ROS presented at 0h was significantly decreased with 2h, 3h, 4h and 5h ($p= 0.002$, $p= 0.003$, $p<0.001$ and $p< 0.001$, respectively) of the specific antioxidant treatment. In this way, it was possible to validate the CM-H_2DCFDA probe's function for PBMCs. When compared the incubation times between them, no significant differences were found. So, for future experiments, the 2h period of incubation was chosen, as it allows a better time management without compromising the obtained results.

- **Evaluation of mitochondrial potential by JC1 probe**

One of the objectives of the present work was to implement techniques to evaluate the mitochondrial potential in PBMCs. The first method tested was evaluation through JC1 probe. JC1 is a cationic dye that accumulates in energized mitochondria. At low concentrations (low mitochondrial membrane potential), JC1 remains in monomeric form in mitochondria and yields green fluorescence (530±15 nm), and at high concentrations (high mitochondrial membrane potential) JC1 aggregates, which shows an intense red fluorescence (590±17.5 nm). The ratio between the two wavelengths (Red/Green) is proportional to the potential and varies among different cell types[51].

In order to optimize this technique, a protocol that was already established for another type of cells was adapted[52].

JC1 probe concentration assessment

The first aim was to identify the ideal concentration of the JC1 probe, for PBMCs. For that, two different concentrations, 0.65 µM and 2 µM, were tested.

In brief, for this experimental design, $2.5x10^5$ of cells were added to 750 µL of JC1 solution (with 0,1% Hoechst), for each concentration, and incubated at 37ºC for 30 minutes. After incubation period, the PBMCs were washed with RPMI, resuspended in 750 µL of RPMI, and then, 200µL were transferred to a 96 well-plate.

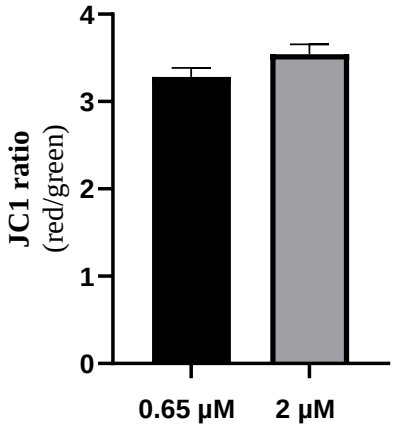


Figure 14 – Evaluation of JC1 ratio in PMBCs at different probe concentrations (0.65 µM and 2 µM). Results are expressed as mean ±SEM (n = 3).

Analyzing the ratios obtained in Figure 14, the results do not present differences between the use of 0.65 µM and 2.5 µM of JC1. Therefore, in subsequent protocols, in order to be as cost-effective as possible without compromising the results, it was decided that the ideal concentration of JC1 to use is 0.65 µM.

In this experiment, Hoechst 33342 dye was used for microscopic analysis of the nucleus. However, it was found out that Hoechst 33342 has the same wavelength as the JC1 aggregates. As seen in Figure 15, both spectra overlap and consequently, the fluorescence obtained should not be considered if Hoechst was used. It was concluded that, as Hoechst has the same wavelength as JC1 probe, it should only be used for observation of this specific probe through fluorescence microscopic imaging and not for fluorescence quantitative measurements.

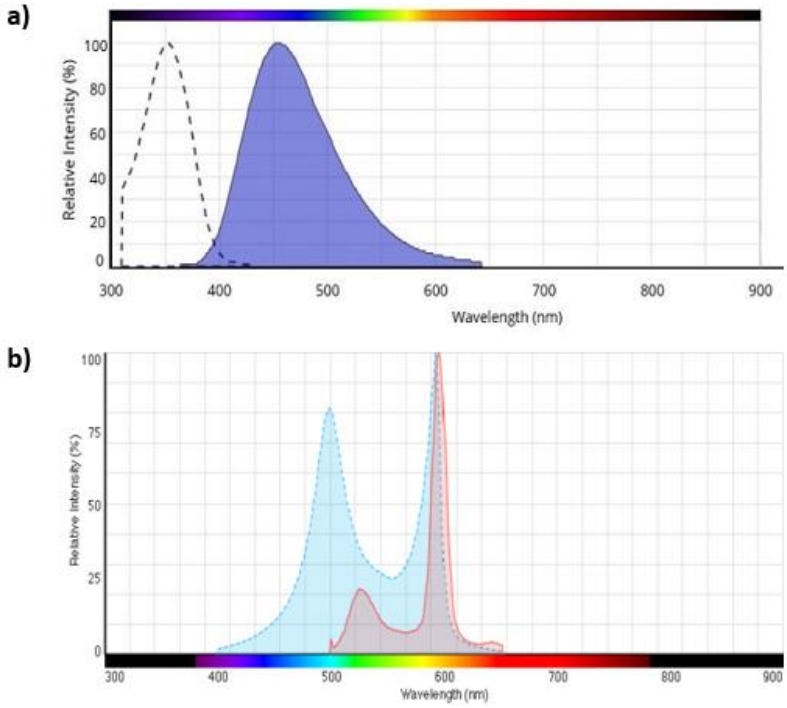


Figure 15 - a) Fluorescence spectra of Hoechst 33342. b) Fluorescence spectra of JC1 probe. It is possible to see that the spectra of both components have the same wavelength (≈400 nm to ≈650nm)[53].

Although the results of the experiment with Hoechst are not 100% viable, it does not invalidate the decision of the best concentration of JC1 to be used. The influence of Hoechst fluorescence interfered equally at both concentrations and therefore the comparison between the ratios is still valid. Nevertheless, a new experiment was done in order to confirm this data.

JC1 protocol validation (without Hoechst)

In order to validate the JC1 protocol for PBMCs, the protocol previously done was replicated, without the addition of Hoechst.

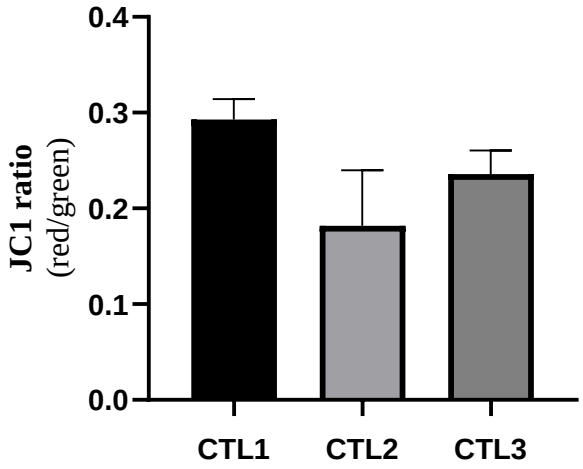


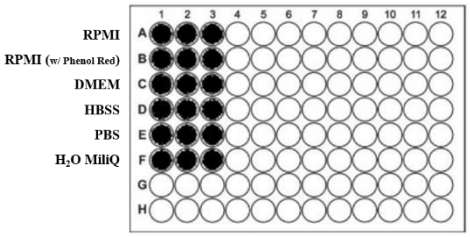
Figure 16 - Evaluation of JC1 ration in PMBCs from 3 controls. Results are expressed as mean ±SEM.

Considering the data in Figure 16, all the controls had very low ratios, contrary to what would be expected, since it was a control population. These results could be interpreted in two ways: either all controls had mitochondrial membrane depolarization or something in the technique was interfering with the fluorescence measurements, leading to an increase in the green light. It was hypothesized that the culture medium (RPMI) could have autofluorescence. In the first screening, this increase in the green fluorescence, probably caused by the medium, might have been masked by the presence of Hoechst, since it was interfering with the red signal.

Evaluation of medium autofluorescence

In order to rule out RPMI interference in fluorescence readings, and to find a substitute, an autofluorescence assessment of the following solutions was carried out: RPMI, RPMI (without Phenol Red), DMEM, HBSS, PBS 1x, and H2O MiliQ. The 96-wells plate design and respective results are present in Figure 17.

a)



b)

RED Ex.530 – Em. 590

	1	2	3	4	5	6	7	8	9	10	11	12
A	35	35	33	13	25	20	15	13	13	18	19	16
B	252	252	243	15	15	16	17	14	21	16	14	10
C	473	485	468	17	14	16	20	17	16	13	18	16
D	31	26	28	15	11	18	20	10	15	15	20	12
E	15	16	15	18	10	16	8	11	17	17	14	17
F	16	11	12	14	19	14	13	15	25	10	11	8
G	11	19	20	18	17	11	18	14	14	16	21	16
H	16	14	18	8	19	16	11	15	17	10	13	14

GREEN Ex.485 – Em. 528

	1	2	3	4	5	6	7	8	9	10	11	12
A	5824	5705	5814	83	72	70	81	68	65	61	70	64
B	7532	7833	8025	60	58	68	59	56	61	65	62	64
C	11476	11502	11684	61	71	69	59	65	47	71	65	49
D	77	89	75	67	72	73	60	67	68	55	75	69
E	59	76	67	51	72	59	65	60	68	72	56	60
F	72	70	65	60	70	67	65	45	57	64	67	63
G	58	57	45	70	57	53	59	69	71	62	64	60
H	74	61	73	63	67	54	55	69	73	60	62	56

Figure 17 – Evaluation of autofluorescence. a) 96-wells plate design for autofluorescence test; b) Plate reader output with the results of autofluorescence test. (Green 485/530 nm - excitation/emission and Red 535/590 nm - excitation/emission).

Analyzing the fluorescence values obtained at red wavelength, it was possible to conclude that both the RPMI with phenol red and DMEM (which also has phenol red in its composition) presented autofluorescence in this spectrum. Therefore, was concluded that these media are not suitable for this type of assay, as they increase the obtained JC1 aggregates values.

Regarding the green signal, all the culture media, despite having phenol red or not, presented autofluorescence in this spectrum. This could be explained by the presence of L-Glutamine in these media, which has a range of fluorescence from 480-560 nm, overlapping with the green signal of JC1 monomeres[54]. HBSS and PBS do not have red or green autofluorescence, being suitable to use in JC1 protocol. HBSS, unlike PBS, has glucose in its composition, which is essential to cells viability.

In conclusion, to the final protocol of JC1 assays with PBMCs it was established that HBSS would be used.

- **Evaluation of oxygen consumption rate by Agilent Seahorse XF analyzer**

To evaluate the oxygen consumption rate (OCR), the Seahorse XF Cell Mito Stress test was used, offering a real-time analysis of metabolism with limited numbers of viable cells[55]. The expected results of this test are represented in Figure 18.

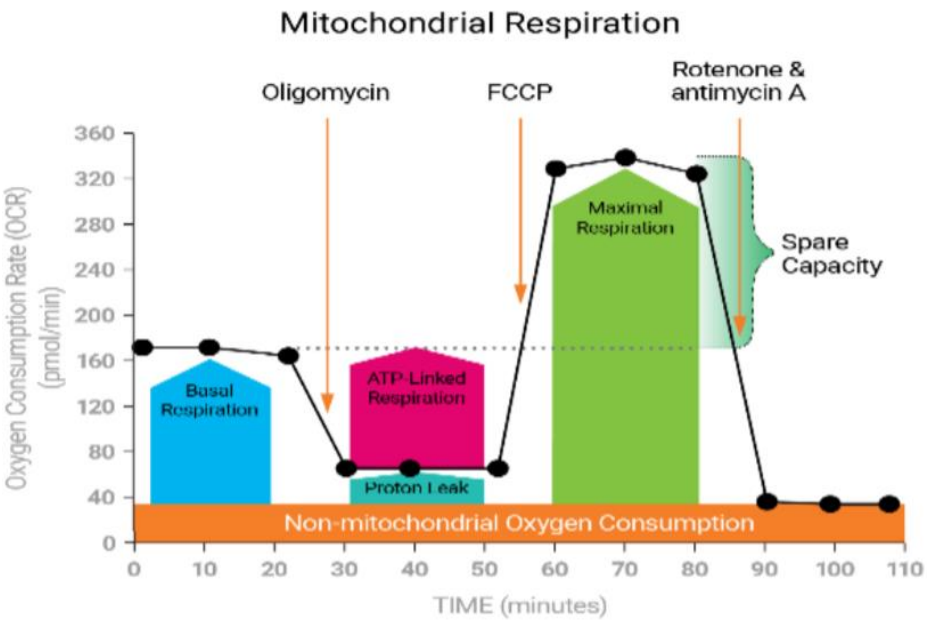


Figure 18 - Key parameters of mitochondrial function obtained in an Agilent Seahorse XF Cell Mito Stress analyze.[56]

In resume, this test starts with an evaluation of cells basal respiration, and it is followed by a serial injection of oligomycin, FCCP, and rotenone/antimycin A. Oligomycin is an ATP synthase (complex V) inhibitor. It decreases electron flow through the electron transport chain (ETC), resulting in a reduction in OCR, which is linked to cellular ATP production[55,57]. FCCP is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential[55]. As a result, electron flow through the ETC is uninhibited, and oxygen consumption by complex IV reaches the maximum. The FCCP-stimulated OCR can then be used to calculate spare respiratory capacity, which is a measure of the ability of the cell to respond to increased energy demand or under stress. Rotenone and antimycin A, are inhibitors of complex I and complex III, respectively. With this mix, the

mitochondrial respiration is completely blocked and is possible to evaluate non-mitochondrial respiration[55,58].

Seahorse XF Cell Mito Stress protocol adaptation for PBMCs

The objective of this first screening was to adapt, the Seahorse XF Cell Mito Stress protocol, that was already established for adherent cells, to non-adherent PBMCs. There were two main parameters to test in this first screening: the ideal plate coating for PBMCs and the number of PBMCs needed to have enough signal. Three different coating were used: collagen, cell-tack™ and polylysine, each tested for two concentrations of PBMCs $5x10^5$ and $1x10^6$. Since the plate space was limited, it was only tested polylysine with $5x10^5$ PBMCs.

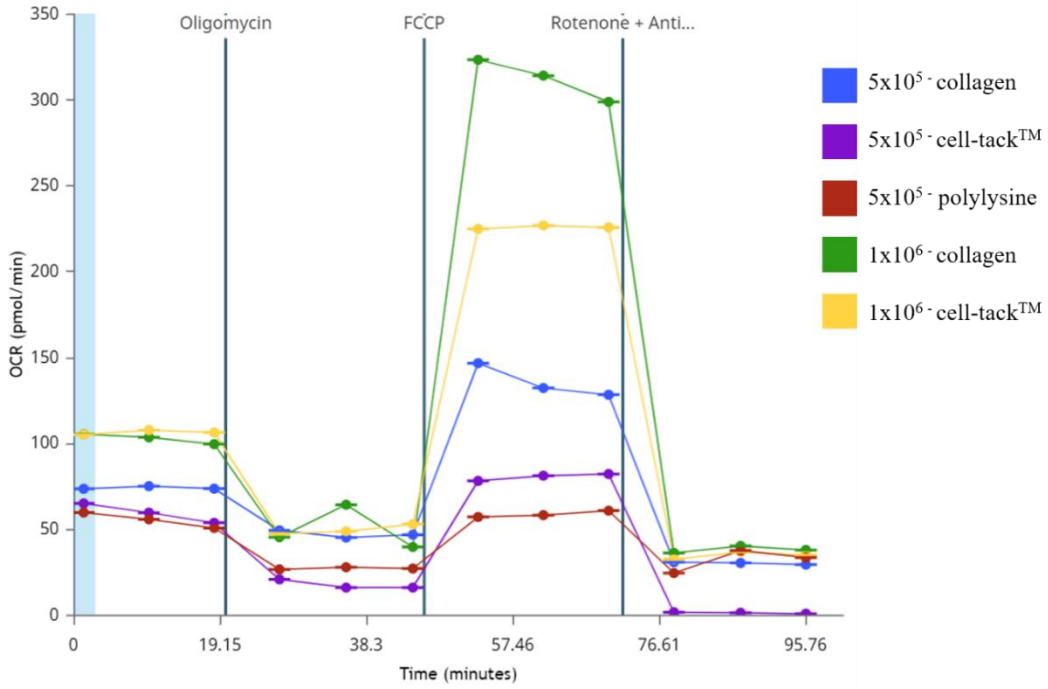


Figure 19 - Oxygen consumption rate of PBMCs. Evaluation of the ideal plate coating and PBMCs concentration.

As shown in Figure 19, the conditions with $1x10^6$ PBMCs had a better response when compared to the lower concentration. In fact, in the concentration of $5x10^5$ PMBCs, after FCCP injection, the maximal respiration presented was similar to their respective basal respiration. So, since this parameter is crucial to evaluate the ability of the cell to respond

under stress, it was decided that for future works, $1x10^6$ PBMCs was the suitable concertation.

As for the ideal plate coating, when comparing collagen with cell-tack™, it is possible to verify that cell-tack™ presented a more stable OCR, with less artefacts than collagen. Therefore, cell-tack™ will be used as the plate coating for future works.[59]

Assessment of an antioxidant treatment

In order to evaluate if an antioxidant treatment with NAC+ALA could improve the cells respiratory capacity, PMBCs from two controls were incubated with and without the AO cocktail, for 24h at 37ºC.

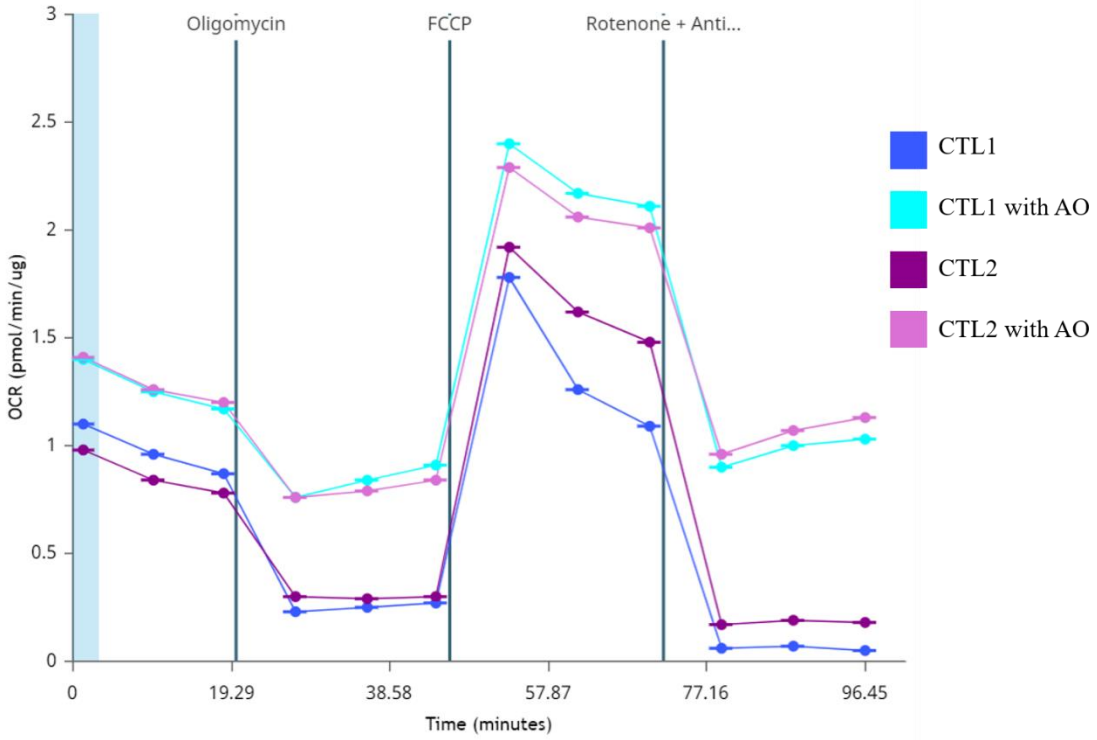


Figure 20 - Oxygen consumption rate of PBMCs. Evaluation of the effect of an antioxidant cocktail in PBMCs respiratory capacity.

In Figure 20, it is possible to see that the respiratory capacity of PBMCs was increased in both controls with the antioxidant treatment. However, even though visually there seems to be an increase in the maximum respiratory capacity with the antioxidant treatment, when analyzing the respiratory spare capacity, it is possible to see that there were no big differences between conditions, in both controls (0.92 to 1 and 1.14 to 1.02 pmol/min/µg protein, respectivly). These results are in line with what was expected, since these are two healthy controls, without any major source of endogenous mitochondrial stress.

In conclusion, the adaptation of the Seahorse XF Cell Mito Stress protocol for PBMCs was successfully achieved, being ready to apply in a future work with OAD patients.

- **Evaluation of mitochondrial function through resazurin analysis**

Resazurin is a blue, non-fluorescent dye used in viability assays and cytotoxic determination. It allows to measure metabolic activity and proliferation of living cells through the bioreduction of the dye. The oxidized form of the resazurin (blue) is reduced and simultaneously the fluorescent intermediate (red) increases.

The first approach to the resazurin protocol was a kinetic analysis of the fluorescence behavior for eight hours. Doing this type of study, it is possible to analyze in real time the mitochondria metabolic activity. In order to optimize this protocol for PBMCs, several experiments were performed.

Ideal cell concentration

The first step was to identify the ideal number of cells for this specific protocol. For that, a kinetic study was done with the following conditions: $1x10^5$, $2x10^5$, $3x10^5$, $4x10^5$, $5x10^5$ and $6x10^5$ cells. For all of the concentrations, spontaneous and DEB 0.1µg/mL induced conditions were compared. As an initial study, the fluorescence was read for 360 minutes, each 30 minutes. The results are present in Figure 21.

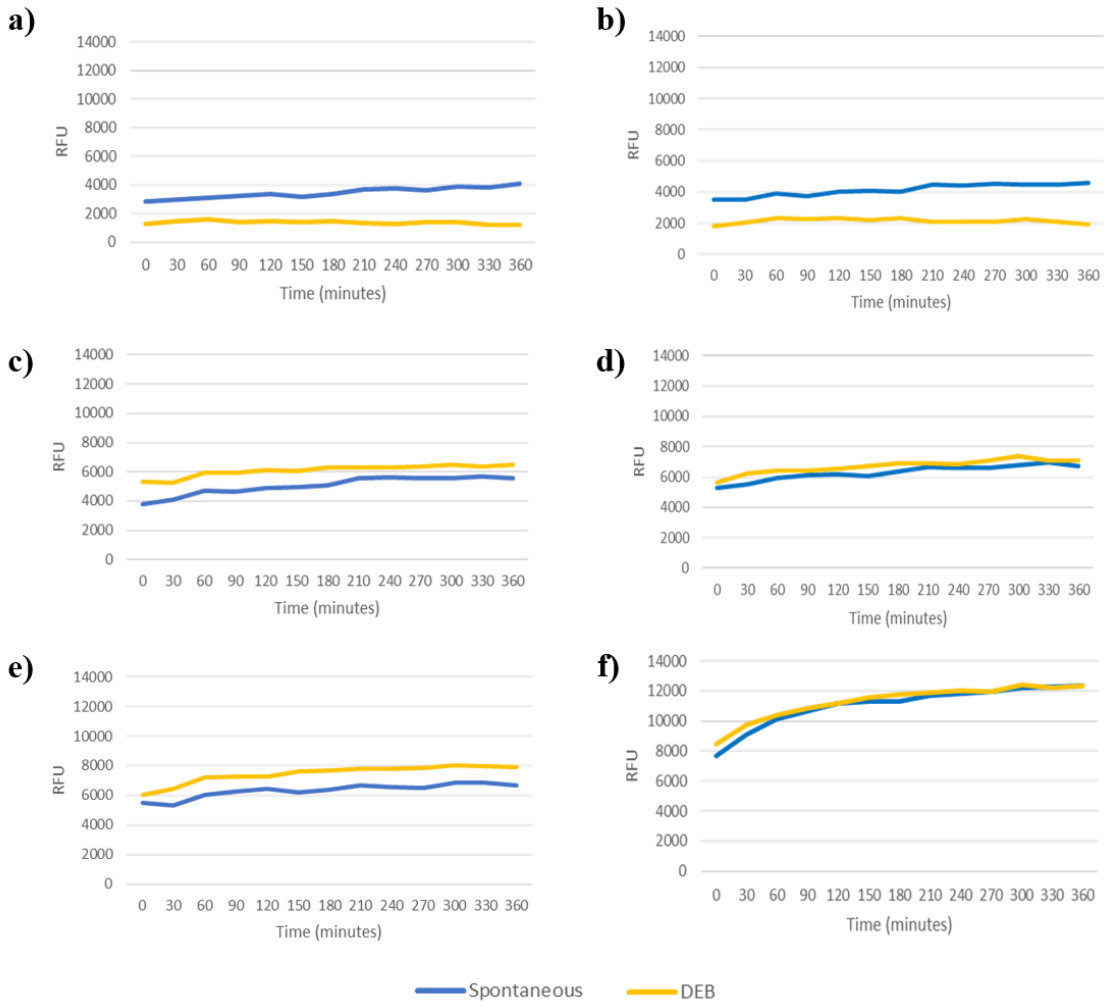


Figure 21 – Evaluation of ideal PBMCs concentration for resazurin assay. Comparative kinetic study of resazurin fluorescence in spontaneous and DEB 0.1µg/mL induced cultures, for 360 minutes, each 30 minutes. a-f) Graphic representation of $1x10^5$ to $6x10^5$ PBMCs, respectively (n=3).

The aim of this experiment was to find a concentration of cells that gave enough fluorescent signal to do an analysis. Since this was a normal control, it was pretended that not many differences were found between the spontaneous and DEB induced conditions. The $1x10^5$ and $2x10^5$ concentrations were excluded because the fluorescent signal was very weak, even after the six hours of incubation. In the other hand, in the $6x10^5$ concentration, the signal was so high that it was impossible to find any differences between the two conditions. That said, the best concentrations for future works were $3x10^5$, $4x10^5$or $5x10^5$ PBMCs per well.

Therefore, in order to minimize the number of need cells, and considering that there was already a protocol that used $3x10^5$ cells for a different variation of this resazurin protocol, this was the chosen concentration[60].

Evaluation of DEB effect

After the determination of the ideal number of cells, the next step was to determine the concentration of DEB at which cells should be exposed to. For that, another kinetic study was accessed. The following conditions were evaluated: Spontaneous, DEB 0.1 µg/mL, DEB 1 µg/mL, DEB 10 µg/mL and DEB 100 µg/mL. The fluorescence was read each hour for 8h. The results are presented in the Figure 22.

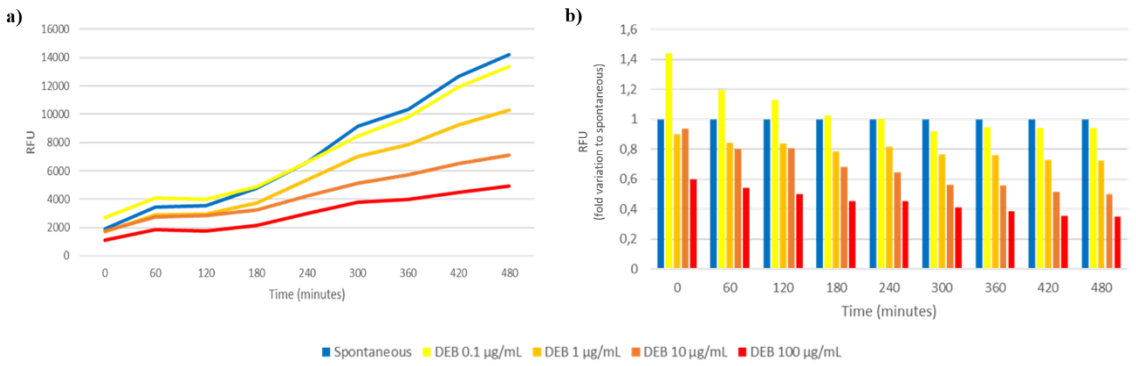


Figure 22 - Evaluation of DEB effect in PBMCs. a) Comparative kinetic study of resazurin fluorescence in spontaneous and DEB (0.1 µg/mL, 1 µg/mL, 10 µg/mL, and 100 µg/mL) induced cultures, for 480 minutes, each 60 minutes. b) Normalized results of a), fold variation to spontaneous condition (n=2).

As shown in Figure 22 there was a uniform correlation between concentration and fluorescence intensity, with the lowest concentration of DEB having almost the same pattern as the spontaneous conditions. Thus, for future experiments the 0.1 µg/mL and 10 µg/mL concentrations were chosen, in order to obtain different degrees of damage in cells.

Determination of the timepoint for the antioxidant treatment

This experiment aimed to explore the action of antioxidants in the decrease DEB-induced oxidative stress. After the determination of DEB concentration, the next parameter

was to determine the timepoint for the antioxidant treatment. Two possibilities were proposed: adding the antioxidants 1h30 before DEB exposure, or adding antioxidants ate the same time as DEB. For that, another kinetic study was done with the antioxidant cocktail NAC+ALA, added in DEB-induced (0.1 µg/mL and 10 µg/mL) cultures. The results are present in Figure 23.

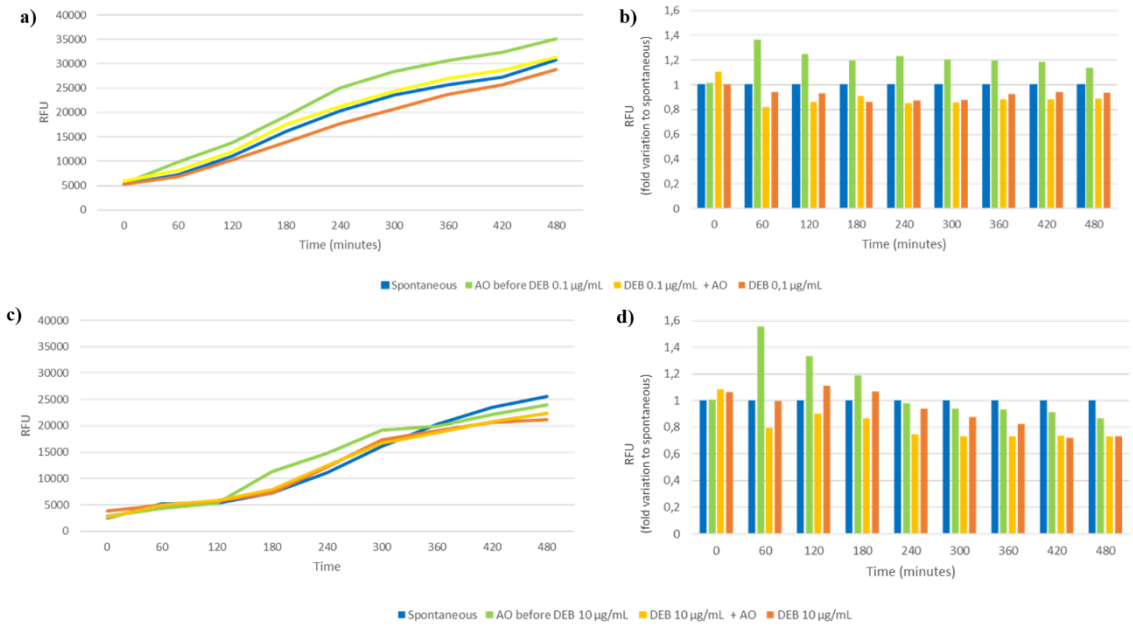


Figure 23 -Determination of the timepoint for the antioxidant treatment. a) and c) Comparative kinetic study of resazurin fluorescence in spontaneous and DEB (0.1 µg/mL and 10 µg/mL, respectively) induced cultures, and antioxidant effect at different timepoints, for 480 minutes, each 60 minutes. b) e d) Normalized results of a) and c), respectively, fold variation to spontaneous condition (n=2).

In Figure 23 it is possible to see that when cells were pre-treated to an antioxidant cocktail before DEB exposure, there was a better response of cells, in term of cell viability. The damaged caused by DEB exposure was compensated, surpassing even the cell viability levels of the spontaneous condition. In cells pre-treated with the antioxidant and exposed to the higher concentration of DEB (10 µg/mL), cell viability eventually decreased overtime, possibly due to the depletion of antioxidants in the media. Regarding the results obtained from the conditions where antioxidants were added ate the same time as DEB, no differences were found when compared to the conditions that only were exposed to DEB.

Determination of other OS inducers

In the previous experiments, DEB was the OS-inducer of choice, since it is already established as being gold-standard for IC studies, and thus, comparative studies could be carried out between the results of the resazurin assay and those obtained in IC. However, unlike the IC protocol, where cells were exposed to DEB for a long period of time (48h), in this resazurin assay, cells were exposed to DEB only for 480 minutes. Taking this to account, there was a necessity to study the action of OS-inducers with a rapid effect in cells. For that, mitomycin (MMC) (0.1µM and 1µM) and staurosporine (STS) (5 µM) were proposed.

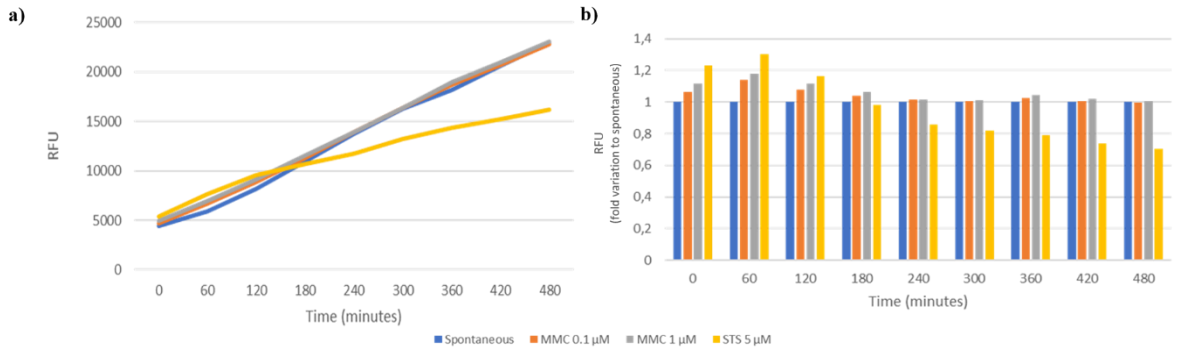


Figure 24 – Determination of other oxidative stress inducers. a) Comparative kinetic study of resazurin fluorescence in spontaneous and Mitomycin (MMC) (0.1µM and 1µM) and Staurosporine (STS) (5 µM), for 480 minutes, each 60 minutes. b) Normalized results of a), fold variation to spontaneous condition (n=2).

As shown in Figure 24, there were no differences between the concentrations of MMC used. On the other hand, STS presented an interesting decrease in cell viability when compared with the spontaneous condition. Thus, another experiment was proposed with more STS (1µM, 2.5 µM and 5 µM) concentrations.

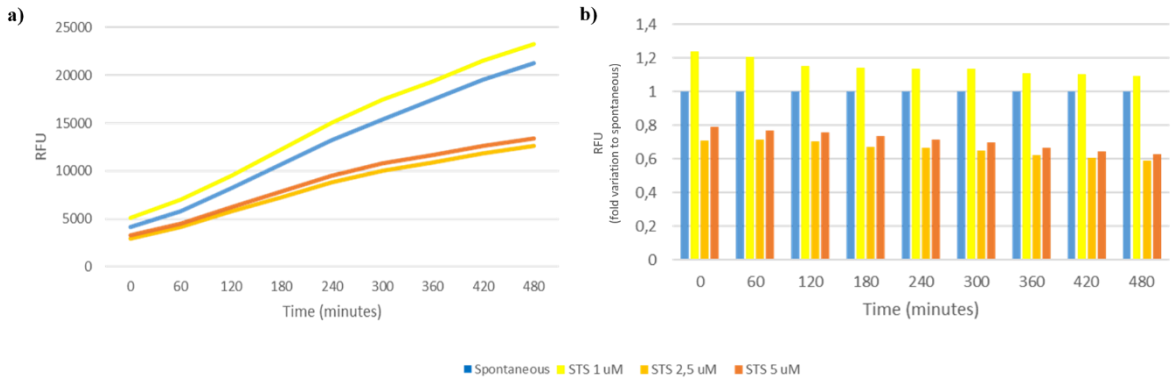


Figure 25 - Determination of STS concentration. a) Comparative kinetic study of resazurin fluorescence in spontaneous and Staurosporine (STS) (1µM, 2.5 µM and 5 Mm), for 480 minutes, each 60 minutes. b) Normalized results of a), fold variation to spontaneous condition (n=4).

In Figure 25 is possible to see that 1 µM STS concentration does not present differences in cell viability when compared to the spontaneous condition. However, when cells were exposed to the concentration of 2.5 µM or 5 Mm of STS, there was a similar decrease in both when compared to the spontaneous condition. Thus, 2.5 µM was the ideal concentration of STS to be used in future resazurin assays.

Single point resazurin analysis

In order to assess cell viability in each of the conditions performed in cell cultures for IC evaluation, a single-point measurement with resazurin was proposed. With this single-point measurement, a direct comparative study could be done between cell viability after 72h of culture and the IC results. The detailed protocol is in material and methods, page 26.

For this experiment, Spontaneous and DEB (0.05 µg/mL, 0.1 µg/mL, 0.25 µg/mL, 0.5 µg/mL and 1 µg/mL) induced cultures were evaluated.

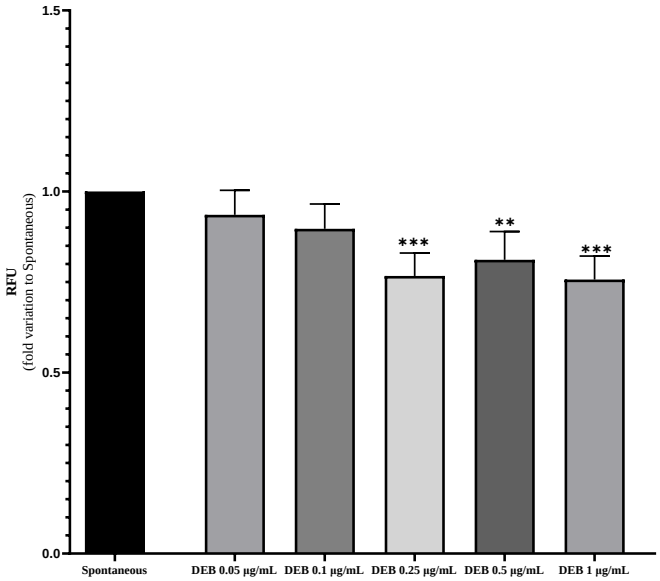


Figure 26 - Comparative single-point study of resazurin in spontaneous and different concentrations of DEB (0.05 µg/mL, 0.1 µg/mL, 0.25 µg/mL, 0.5 µg/mL and1 µg/mL) Results are expressed as mean ± SEM (n=5). ***p<0.001; **p<0.01.

As shown in Figure 26, as the concentration of DEB increases, cell viability decreases. Significant differences are observed in DEB concentrations of 0.25 µg/mL, 0.5 µg/mL and 1 µg/mL ($p=0.004$, $p=0.0048$ and $p=0.0003$, respectively). This decrease in cell viability is in line with what was expected, since with an increase in the concentration of OS-inducer agents, cells lose their defense capacity, thus leading to an increase in cell death[61].

In conclusion, the implemented techniques for evaluation of OS and redox state of mitochondria were optimized and protocols have been established for a future application in studies directed to OAD patients.

4.2. Evaluation of CI in cells from OAD patients

Several studies have already shown the cellular effects of OS, which can lead to DNA damage. Excess of non-repaired OS-damage can lead to SSBs or DSB, being the last ones the more harmful, which have as a direct manifestation the increment of CI[30]. Therefore, in OS-related clinical situations, such as OAD, it is of most importance to evaluate the levels of CI.

- **Comparative study between the CI levels in lymphocyte cultures from OAD patients and from controls**

In this study we aimed to confirm, in a larger population of OAD patients, the increased levels of CI previously described in a population of asthma patients[62]. The CI parameters analyzed in each sample were the percentage of aberrant cells and the mean number of breaks per cell (Attachment 1), either in spontaneous or DEB-induced lymphocyte cultures.

- *Spontaneous CI*

As shown in Figure 27, the difference between the levels of spontaneous CI in lymphocytes from the OAD patients and from controls is not significant, evaluated either by the percentage of aberrant cells (p=0.7642) and mean number of breaks per cell (p=0.9348).

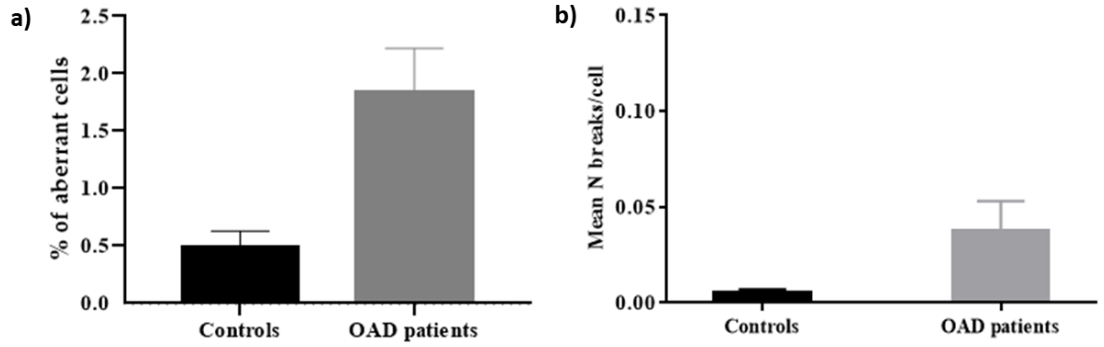


Figure 27 – Comparative study between control cultured lymphocytes (n=35) and OAD patients (n=11), in the levels of spontaneous chromosome instability. a) Evaluation of CI by percentage of aberrant cells; b) Evaluation of CI by number of breaks per cell. Data are expressed as Mean ± SEM. Differences between groups were evaluated by Two-factor mixed ANOVA followed by a multi-comparison Turkey's test (α= 0.05).

- *DEB-Induced CI*

DEB-induced lymphocytes from OAD patients showed an increased level of OS-related CI endpoints, when compared to controls, in cultures with high doses of DEB. As shown in Figure 28, significant differences in both the percentage of aberrant cells and the mean number of breaks per cell ($p<0.01$ and $p<0.1$, respectively) were observed in the cultures with the DEB concentration 0.1 μg/mL. The cultures with the DEB concentration 0.05 μg/mL did not show significant differences, neither in percentage of aberrant cells, nor in the mean number of breaks per cell ($p=0.9999$ and $p=0.9806$, respectively). Figure 29 illustrates the difference in CI from selected metaphases from a control and an OAD patient in DEB-induced (0.1 μg/mL) cultures.

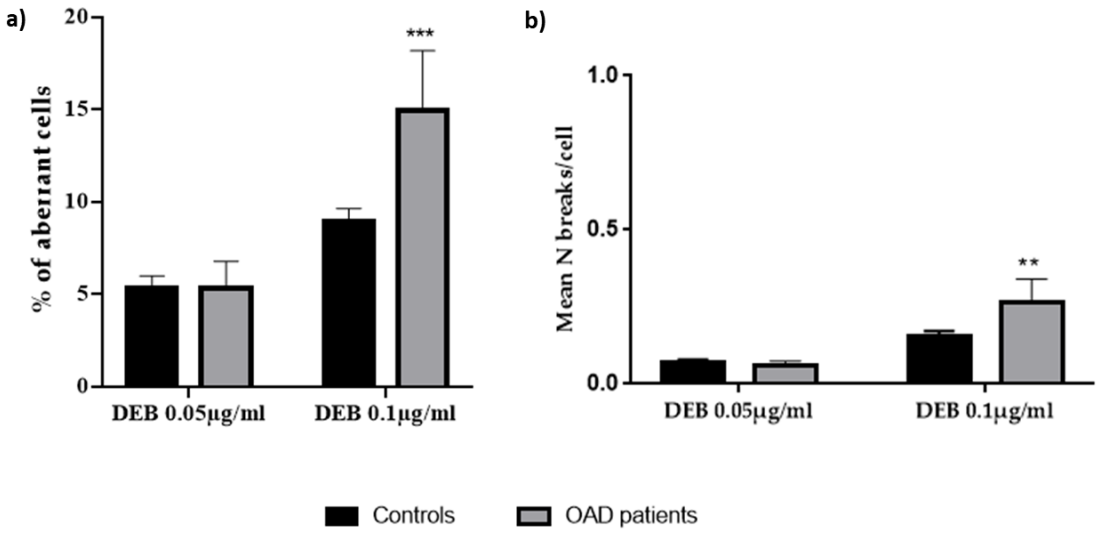


Figure 28 - Comparative study between control cultured lymphocytes (n=35) and OAD patients (n=11), in the levels of induced chromosome instability, by exposure to DEB at different concentrations. a) Evaluation of CI by the percentage of aberrant cells; b) Evaluation of CI by the mean number of breaks per cell. Data are expressed as Mean ± SEM. Differences between groups were evaluated by Two-factor mixed ANOVA followed by a multi-comparison Turkey's test ($\alpha= 0.05$). ***$p<0.001$; **$p<0.01$.

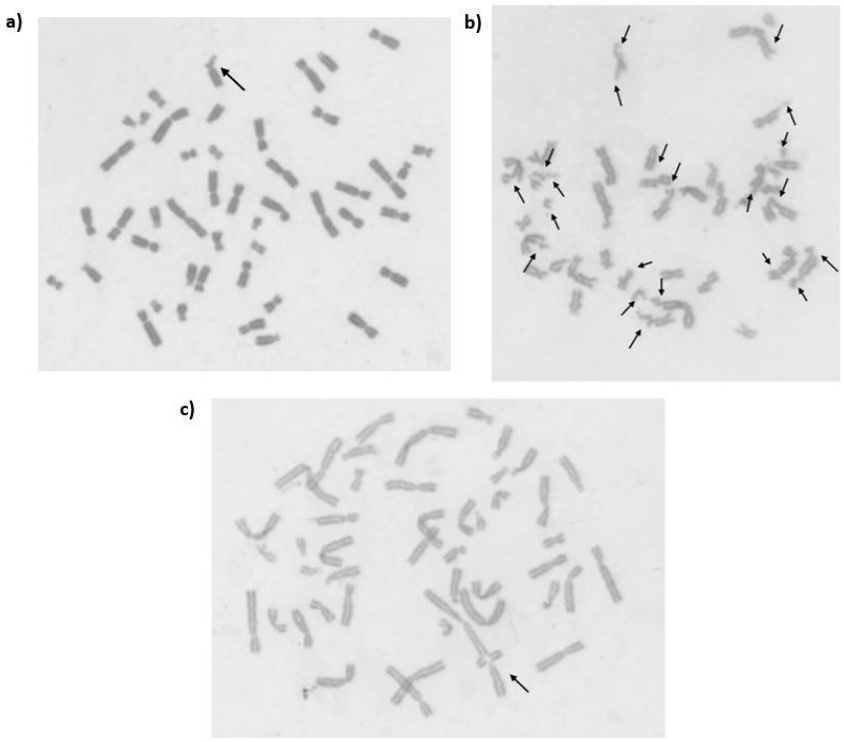


Figure 29 – Illustration of the difference in DEB-induced metaphases (DEB 0.1 µg/mL) in number of breaks per cell from lymphocyte cultures of controls and OAD patients. a) Selected metaphase from a control: one chromatid break; b) and c) Selected metaphase from an OAD patient: multiple breaks and complex rearrangement.

In the present study, and regarding spontaneous CI, when comparing the values between controls and OAD patients, despite an increase in the percentage of aberrant cells and number of breaks per cell in OAD lymphocytes, this increase was not statistically significant. This result is not in accordance with the previous study where a significant difference was observed[43]. This fact may be explained by the small sample size available for this study, with only 12 OAD patients (11 asthmatic patients and 1 COPD).

DEB-induced lymphocyte cultures were used in order to evaluate cell sensitivity to the clastogenic effect of exogenous OS. The results showed a significant increase of DEB-induced CI at the highest dose in lymphocytes from OAD patients, when compared to controls. This data is particularly relevant, because it suggests that OAD patients might have a higher sensitivity to the cumulative genotoxic effect of OS[63].

It is known that OAD patients are not only characterized by high levels of OS, but also by high levels of inflammation. Correlations between DNA damage in asthma patients and their inflammatory state have already been described[2,64]. In fact, asthmatic patients can be classified in different phenotypes, such as mild and severe asthma, according to the levels of inflammation[65]. Concerning the asthmatic population in this study, it was not available enough clinical information to distinguish between both phenotypes. This fact might have a major impact in the mean levels of CI from this population, since it has been shown previously that, mild asthma patients do not have a significant increase in CI levels when compared with the control population[62].

After confirming the presence of increased levels of DEB-induced CI in lymphocytes from OAD patients, we focused on reducing the harmful cumulative genotoxic effect, using antioxidant treatments.

- **Evaluation of the effect of an antioxidant treatment in CI levels in lymphocyte cultures from AOD patients**

It was intended to further explore if an *in-vitro* antioxidant with NAC and ALA, already described in the preliminary study with asthma patients, would be effective in the reduction of the levels of CI in cells from OAD patients.

The CI parameters analyzed in each sample were the percentage of aberrant cells and the mean number of breaks per cell (Attachment 1) in DEB-induced lymphocyte cultures.

As shown in Figure 30, lymphocyte cultures from OAD patients exposed to DEB 0.01 µg/mL and pre-treated with NAC + ALA present a significant decrease in the level of CI, considering the percentage of aberrant cells ($p<0.01$). Interestingly, the IC values obtained in OAD patients with the antioxidant treatment reached values similar to those observed in the control population (see Figure 29).

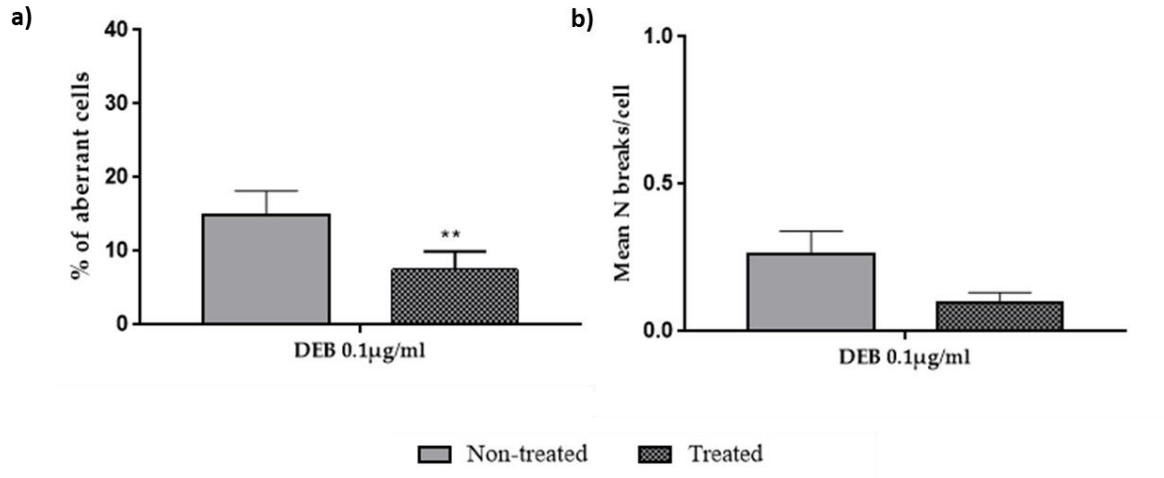


Figure 30 - Comparative study between OAD patients (n=11) treated with and without antioxidants (NAC+ALA), to evaluate levels DEB-induced (DEB 0.1 µg/mL) chromosome instability. a) Evaluation of CI by percentage of aberrant cells; b) Evaluation of CI by number of breaks per cell. Data are expressed as Mean ± SEM. Differences between groups were evaluated by paired t-test. **p<0.01.

In summary, lymphocytes from OAD patients, when exposed to high doses of DEB, presented a significant increase in the levels of CI, when compared to controls. This damage was reduced when lymphocytes were pre-treated with an antioxidant cocktail of NAC+ALA. Studies have already revealed that the NAC+ALA cocktail actually has an effect on decreasing OS-related CI[45]. This effect could be explained by the specific function of each antioxidant. NAC is a thiol compound that acts directly on ROS, promotes detoxification, and plays a role in the inflammation process as it modulates proinflammatory cytokines. It is also known as a precursor for GSH synthesis, increasing its intracellular levels[66]. ALA has the potential to induce a substantial increase in intracellular GSH, and being a mitochondrial agent with direct action on the ROS scavenger, with great antioxidant power, is able to maintain the cell's oxidative state[45].

In conclusion, it increased CI levels were observed in lymphocytes from the OAD patients, confirming the previously described CI increment in cells from an asthma population. Also, the previously described antioxidant treatment with NAC and ALA proved to be effective in reducing this damage.

5. Conclusion

This work is part of an ongoing larger study that focuses on a wider cellular characterization of OAD, including evaluation of OS and redox state of mitochondria and evaluation of OS-related CI levels.

In order to evaluate OS and redox state of mitochondria, the first objective was to optimize, in a control population, several techniques that meet the needs for a future implementation in a OAD patient's study. This was accomplished and specific techniques were implemented, namely: evaluation of OS-related damage through the analysis of protein peroxidation; evaluation of the total cellular ROS production by CM-H2DCFDA probe; evaluation of mitochondrial membrane potential by JC1 probe; evaluation of mitochondrial bioenergetics by Agilent Seahorse XF analyzer and evaluation of mitochondrial function through resazurin analysis.

This study was performed in a limited control population. As a next step, all these techniques should be consistently validated in an enlarged control population, before being used in future studies with OAD patients, in order to establish the range of normal values. However, in the present work it was not possible to achieve this control validation, own to time constrictions.

The second objective of this work was to confirm, in an enlarged population of OAD patients, the increased levels of CI that were previously characterized in lymphocytes from a population consisting of asthma patients. It was possible to confirm that, in cases of exposure to high doses of DEB, lymphocytes from OAD patients presented higher levels of CI, compared to controls. Additionally, an antioxidant treatment with NAC and ALA proved to be effective in reducing the high CI levels observed in those OAD patients.

Nevertheless, this work still has some limitations. As mentioned, one of the aims was to confirm the increased levels of CI in an enlarged population of OAD patients, including not only asthmatic but also COPD patients. However, due to the Covid-19 pandemic context,

it was very difficult to have access to blood samples from patients with pulmonary diseases. Consequently, a small number of samples was obtained during the period time of the experimental work. In fact, only one COPD blood sample was obtained. Therefore, as a perspective for a future work, it is intended to continue this study with a wider population of COPD patients.

6. References

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7. Annexes

7.1. Annex 1:

Table A – Evaluation of Chromosome Instability in spontaneous and DEB-induced conditions in controls and OAD patients, with and without antioxidants treatment. Evaluation in terms of percentage of aberrant cells and mean number of breaks per cell. Data is expressed as Mean ± SEM.

Group of individuals	***Spontaneous***		***DEB 0.05 µg/mL***		***DEB 0.1 µg/mL***	
	% aberrant cells	*N breaks/cell*	*% aberrant cells*	*N breaks/cell*	*% aberrant cells*	*N breaks/cell*
Controls	0.60 ± 0.27	0.006 ± 0.0027	8.25 ± 1.39	0.091 ± 0.015	12.4 ± 2.37	0.154 ± 0.027
OAD patients	2.00 ± 0.49	0.0375 ± 0.015	5.83 ± 1.69	0.059 ± 0.017	15.91 ± 3.54	0.245 ± 0.076
OAD patients + AO	1.7 ± 0.61	0.017 ± 0.006	4.7 ± 0.98	0.048 ± 0.010	10 ± 3.40	0.114 ± 0.0371

Table B - Evaluation of Chromosome Instability in spontaneous and DEB-induced conditions in OAD patients without antioxidants treatment (n=12). Evaluation in terms of percentage of aberrant cells and number of breaks per cell.

	SPONTANEOUS		***DEB 0.05 µG/ML***		***DEB 0.1 µG/ML***	
OAD	*% aberrant cells*	*N breaks/cell*	*% aberrant cells*	*N breaks/cell*	*% aberrant cells*	*N breaks/cell*
1	2	0,02	0	0	12	0,14
2	0	0	0	0	6	0,06
3	0	0	2	0,02	10	0,14
4	0	0	18	0,2	32	0,52
5	4	0,06	4	0,04	6	0,06
6	2	0,02	8	0,08	14	0,18
7	2	0,02	5	0,08	15	0,2
8	3	0,04	9	0,1	41	1,01
9	3	0,04	2	0,02	7	0,14
10	2	0,03	3	0,03	8	0,27
11	1	0,01	8	0,08	22	0,35
12	3	0,03	5	0,05	6	0,08

Table C - Evaluation of Chromosome Instability in spontaneous and DEB-induced conditions in Healthy control blood donors (n=35). Evaluation in terms of percentage of aberrant cells and number of breaks per cell.

	SPONTANEOUS		***DEB 0.05 µG/ML***		***DEB 0.1 µG/ML***	
CONTROLS	*% aberrant cells*	*N breaks/cell*	*% aberrant cells*	*N breaks/cell*	*% aberrant cells*	*N breaks/cell*
1	1	0,03	11	0,17	8	0,18
2	1	0,01	5	0,06	11	0,25
3	1	0,01	3	0,06	8	0,24
4	1	0,01	3	0,03	0	0
5	0	0	3	0,05	8	0,13
6	0	0	3	0,05	7	0,12
7	0	0	2	0,04	4	0,08
8	0	0	5	0,06	4	0,05
9	1	0,01	3	0,04	6	0,1
10	0	0	3	0,03	6	0,07
11	0	0	3	0,03	6	0,08
12	0	0	7	0,11	8	0,11
13	0	0	4	0,07	4	0,04
14	0	0	3	0,06	9	0,16
15	1	0,01	4	0,01	17	0,35
16	0	0	7	0,08	15	0,29
17	0	0	2	0,02	16	0,37
18	0	0	1	0,01	6	0,14
19	1	0,01	5	0,06	12	0,2
20	0	0	6	0,09	13	0,35
21	0	0	1	0,01	8	0,09
22	0	0	0	0	5	0,07
23	0	0	0	0	4	0,06
24	0	0	10	0,12	6	0,18
25	1	0,01	3	0,05	9	0,14
26	2	0,02	12	0,13	13	0,14
27	4	0,04	11	0,13	13	0,2
28	0	0	14	0,22	5	0,06
29	1	0,01	6	0,06	9	0,11
30	2	0,02	12	0,12	19	0,33
31	0	0	8	0,08	9	0,15
32	0	0	1	0,01	13	0,23
33	0	0	10	0,13	9	0,11
34	0	0	3	0,04	12	0,16
35	0	0	12	0,2	10	0,12

Table D - Evaluation of Chromosome Instability in spontaneous and DEB-induced conditions in OAD patients with antioxidants treatment (n=10). Evaluation in terms of percentage of aberrant cells and number of breaks per cell.

	SPONTANEOUS		***DEB 0.05 µG/ML***		***DEB 0.1 µG/ML***	
OAD TREATED	*% aberrant cells*	*Number breaks/cell*	*% aberrant cells*	*Number breaks/cell*	*% aberrant cells*	*Number breaks/cell*
1	0	0	5	0	14	0,04
2	3	0	10	0	21	0,04
3	0	0,02	4	0	4	0,02
4	0	0,02	2	0,08	4	0,26
5	0	0,08	0	0,06	6	0,08
6	2	0	2	0,06	2	0
7	4	0	10	0,05	38	0,22
8	6	0,03	6	0,11	10	0,26
9	2	0	6	0,04	0	0,05
10	0	0	2	0,02	1	0,02

7.2. Annex 2:

CONSENTIMENTO INFORMADO, LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO EM INVESTIGAÇÃO
de acordo com a Declaração de Helsínquia e a Convenção de Oviedo

Por favor, leia com atenção a seguinte informação. Se achar que algo está incorreto ou não está claro, não hesite em solicitar mais informações. Se concorda com a proposta que lhe foi feita, queira assinar este documento.

Título do estudo: Avaliação da fisiopatologia das doenças obstrutivas das vias aéreas e dos seus mecanismos celulares e moleculares.

Instituições: Instituto de Ciências Biomédicas Abel Salazar (ICBAS-UP) em parceria com o Centro Hospitalar Universitário do Porto (CHUP) e o Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA).

Explicação do estudo: Este estudo tem como principais objetivos: 1) avaliar os potenciais mecanismos celulares e moleculares subjacentes às doenças obstrutivas das vias aéreas; 2) analisar o efeito de stress oxidativo na fisiopatologia dessas doenças; 3) verificar *in vitro* o efeito de um cocktail de antioxidantes para a potencial prevenção de episódios. Esperamos que o resultado deste estudo possa contribuir para **melhorar a saúde e qualidade de vida** das pessoas que sofrem atualmente destas doenças.

Se concordar, pedimos que:

- Preencha e assine o consentimento informado, livre e esclarecido;
- Preencha um questionário de exclusão de sintomas respiratórios e realize uma avaliação de espirometria, teste não invasivo e indolor, realizado por profissionais de saúde (30 min);
- Permita a colheita de sangue para avaliar indicadores biológicos celulares e moleculares, sem punção adicional para o dador;
- Responda ao questionário geral com questões de saúde, nutrição e histórico de exposições ocupacional e ambiental (15 min) que um elemento da nossa equipa lhe irá fazer.

Saiba que:

- A participação é voluntária e poderá desistir do estudo em qualquer altura e sem qualquer tipo de consequência.
- Toda a informação recolhida será estritamente confidencial. Quer os questionários quer os tubos de colheita de sangue serão codificados e tornados anónimos no ato de colheita e deixarão de poder ser relacionados consigo. Apenas o Investigador Principal sujeito a sigilo profissional terá acesso à chave de codificação, armazenada em local protegido, de acesso restrito e diferente do material recolhido.
- Os dados recolhidos só poderão ser consultados pelos responsáveis científicos do estudo e ser objeto de publicação científica, mas apenas de forma anónima e agregada, isto é, em termos de percentagens ou de dados numéricos, nunca individualmente e não poderão ser relacionados consigo, isto é, com a sua identificação.
- [**para incluir após aprovação pela Comissão de Ética**] O estudo mereceu parecer favorável da Comissão de Ética do Departamento de Ensino e Formação e Investigação (DEFI) do Centro Hospitalar Universitário do Porto e do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Muito obrigado pela sua colaboração

DECLARAÇÃO DE CONSENTIMENTO INFORMADO E ESCLARECIDO [1]

Avaliação da correlação entre doenças do obstrutivas das vias aéreas e putativos mecanismos celulares e moleculares.

Eu, abaixo-assinado, _____ compreendi a explicação escrita e verbal que me foi dada acerca deste projeto de investigação, tomando conhecimento dos objetivos, métodos, benefícios previsíveis e riscos potenciais, bem como das garantias de confidencialidade previstas para os dados que disponibilizo. Foi-me dado tempo de reflexão e oportunidade de fazer as perguntas que julguei necessárias, obtendo respostas satisfatórias. Sei que tenho o direito de recusar a qualquer momento, sem qualquer prejuízo, a minha participação no projeto através do contacto com o investigador responsável abaixo identificado. Sei também que não serei ressarcido de quaisquer despesas decorrentes da participação.

Se terminado este projeto existir ainda alguma das amostras biológicas por mim facultadas, autorizo que sejam conservadas para utilização em estudos futuros devidamente aprovados pela Comissão de Ética do DEFI?

☐ **SIM** ☐ **NÃO**

Autorizo a utilização dos resultados obtidos, devidamente anonimizados, para publicações científicas?

☐ **SIM** ☐ **NÃO**

Estas são as condições em que decido livremente participar no estudo/investigação

Assinatura: … … … … … … … ..... … … … … … ... … … … … … … … … … … … … … … Data: …… /…… /……..

Pelo investigador responsável

_____

Investigador responsável
Cláudia Oliveira
Contacto institucional: +351 22 0428254 | csoliveira@icbas.up.pt

[1] Feito e assinado em duplicado sendo entregue um exemplar ao participante, juntamente com o documento informativo

DECLARAÇÃO DE CONSENTIMENTO INFORMADO E ESCLARECIDO [1]

Avaliação da correlação entre doenças do obstrutivas das vias aéreas e putativos mecanismos celulares e moleculares.

Eu, abaixo-assinado, _____ compreendi a explicação escrita e verbal que me foi dada acerca deste projeto de investigação, tomando conhecimento dos objetivos, métodos, benefícios previsíveis e riscos potenciais, bem como das garantias de confidencialidade previstas para os dados que disponibilizo. Foi-me dado tempo de reflexão e oportunidade de fazer as perguntas que julguei necessárias, obtendo respostas satisfatórias. Sei que tenho o direito de recusar a qualquer momento, sem qualquer prejuízo, a minha participação no projeto através do contacto com o investigador responsável abaixo identificado. Sei também que não serei ressarcido de quaisquer despesas decorrentes da participação.

Se terminado este projeto existir ainda alguma das amostras biológicas por mim facultadas, autorizo que sejam conservadas para utilização em estudos futuros devidamente aprovados pela Comissão de Ética do DEFI?

☐ **SIM** ☐ **NÃO**

Autorizo a utilização dos resultados obtidos, devidamente anonimizados, para publicações científicas?

☐ **SIM** ☐ **NÃO**

Estas são as condições em que decido livremente participar no estudo/investigação

Assinatura: … … … … … … … ..... … … … … … ... … … … … … … … … … … … … … … Data: …… /…… /……..

Pelo investigador responsável

_____

Investigador responsável
Cláudia Oliveira
Contacto institucional: +351 22 0428254 | csoliveira@icbas.up.pt

[1] Feito e assinado em duplicado sendo entregue um exemplar ao participante, juntamente com o documento informativo

CONSENTIMENTO INFORMADO, LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO EM INVESTIGAÇÃO
de acordo com a Declaração de Helsínquia e a Convenção de Oviedo

Por favor, leia com atenção a seguinte informação. Se achar que algo está incorreto ou não está claro, não hesite em solicitar mais informações. Se concorda com a proposta que lhe foi feita, queira assinar este documento.

Título do estudo: Avaliação da fisiopatologia das doenças obstrutivas das vias aéreas e dos seus mecanismos celulares e moleculares.

Instituições: Instituto de Ciências Biomédicas Abel Salazar (ICBAS-UP) em parceria com o Centro Hospitalar Universitário do Porto (CHUP) e o Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA).

Explicação do estudo: Este estudo tem como principais objetivos: 1) avaliar os potenciais mecanismos celulares e moleculares subjacentes às doenças obstrutivas das vias aéreas; 2) analisar o efeito de stress oxidativo na fisiopatologia dessas doenças; 3) verificar *in vitro* o efeito de um cocktail de antioxidantes para a potencial prevenção de episódios. Esperamos que o resultado deste estudo possa contribuir para **melhorar a saúde e qualidade de vida** das pessoas que sofrem atualmente destas doenças.

Se concordar, pedimos que:

- Preencha e assine o consentimento informado, livre e esclarecido.
- Preencha um questionário de avaliação de sintomas respiratórios e realize uma avaliação de espirometria, teste não invasivo e indolor, realizado por profissionais de saúde (30 min);
- Permita a colheita de sangue para avaliar indicadores biológicos celulares (5 min). A colheita será efetuada num momento programado com a equipa de saúde.
- Responda ao questionário geral com questões de saúde, nutrição e histórico de exposições ocupacional e ambiental (15 min) que um elemento da nossa equipa lhe irá fazer.

Saiba que:

- A participação é voluntária e poderá desistir do estudo em qualquer altura e sem qualquer tipo de consequência.
- Toda a informação recolhida será estritamente confidencial. Quer os questionários quer os tubos de colheita de sangue serão codificados e tornados anónimos no ato de colheita e deixarão de poder ser relacionados consigo. Apenas o Investigador Principal sujeito a sigilo profissional terá acesso à chave de codificação, armazenada em local protegido, de acesso restrito e diferente do material recolhido.
- Os dados recolhidos só poderão ser consultados pelos responsáveis científicos do estudo e ser objeto de publicação científica, mas apenas de forma anónima e agregada, isto é, em termos de percentagens ou de dados numéricos, nunca individualmente e não poderão ser relacionados consigo, isto é, com a sua identificação.
- [**para incluir após aprovação pela Comissão de Ética**] O estudo mereceu parecer favorável da Comissão de Ética do Departamento de Ensino e Formação e Investigação (DEFI) do Centro Hospitalar Universitário do Porto e do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Muito obrigado pela sua colaboração

DECLARAÇÃO DE CONSENTIMENTO INFORMADO E ESCLARECIDO [1]

Avaliação da fisiopatologia das doenças obstrutivas das vias aéreas e dos seus mecanismos celulares e moleculares.

Eu, abaixo-assinado, _____ compreendi a explicação escrita e verbal que me foi dada acerca deste projeto de investigação, tomando conhecimento dos objetivos, métodos, benefícios previsíveis e riscos potenciais, bem como das garantias de confidencialidade previstas para os dados que disponibilizo. Foi-me dado tempo de reflexão e oportunidade de fazer as perguntas que julguei necessárias, obtendo respostas satisfatórias. Sei que tenho o direito de recusar a qualquer momento, sem qualquer prejuízo, a minha participação no projeto através do contacto com o investigador responsável abaixo identificado. Sei também que não serei ressarcido de quaisquer despesas decorrentes da participação.

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☐ **SIM** ☐ **NÃO**

Autorizo a utilização dos resultados obtidos, devidamente anonimizados, para publicações científicas?

☐ **SIM** ☐ **NÃO**

Estas são as condições em que decido livremente participar no estudo/investigação

Assinatura: … … … … … … … ..... … … … … … ... … … … … … … … … … … … … … … Data: …… /…… /……..

Pelo investigador responsável

_____

Investigador responsável
Cláudia Oliveira
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DECLARAÇÃO DE CONSENTIMENTO INFORMADO E ESCLARECIDO [1]

Avaliação da fisiopatologia das doenças obstrutivas das vias aéreas e dos seus mecanismos celulares e moleculares.

Eu, abaixo-assinado, _____ compreendi a explicação escrita e verbal que me foi dada acerca deste projeto de investigação, tomando conhecimento dos objetivos, métodos, benefícios previsíveis e riscos potenciais, bem como das garantias de confidencialidade previstas para os dados que disponibilizo. Foi-me dado tempo de reflexão e oportunidade de fazer as perguntas que julguei necessárias, obtendo respostas satisfatórias. Sei que tenho o direito de recusar a qualquer momento, sem qualquer prejuízo, a minha participação no projeto através do contacto com o investigador responsável abaixo identificado. Sei também que não serei ressarcido de quaisquer despesas decorrentes da participação.

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Assinatura: … … … … … … … ….. … Data: …… /…… /……..

Pelo investigador responsável

_____

Investigador responsável
Cláudia Oliveira
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[1] Feito e assinado em duplicado sendo entregue um exemplar ao participante, juntamente com o documento informativo

Codificação do individuo

Data: _____

Hora de início: _____ : _____
Hora de fim: _____ : _____

QUESTIONÁRIO GERAL

A Dados Pessoais

1. Género

☐ Feminino ☐ Masculino

2. Qual a sua idade?

_____

3. Qual a sua data de nascimento? (dd/mm/aaaa)

_____-_____-_____

4. Qual o Código Postal da sua residência? (primeiros 4 digitos) _____

B Dados de Saúde

Abreviaturas: NS (não sei); NR (não respondo)

5. Teve/tem algum problema de saúde relevante ?

☐ SIM ☐ NÃO ☐ *NS/NR* Qual?_____

6. E nos últimos 2 meses?

☐ SIM ☐ NÃO ☐ *NS/NR* Qual?_____

7. No último ano fez algum tipo de exame radiológico (Raios X)?

☐ SIM ☐ NÃO ☐ *NS/NR* Qual?_____

8. Tomou alguma vacina nos últimos 12 meses?

☐ SIM ☐ NÃO ☐ *NS/NR* Qual?_____

9. No último ano foi submetido(a) a alguma intervenção cirúrgica?

☐ SIM ☐ NÃO ☐ *NS/NR* Qual?_____

10. No último ano recebeu alguma transfusão sanguinea?

☐ SIM ☐ NÃO ☐ *NS/NR* Qual?_____

11. Alguma vez um médico lhe diagnosticou uma das doenças indicadas que o(a) obrigue ou tenha obrigado a tratamento continuado?

☐ SIM ☐ NÃO ☐ *NS/NR*

11.1. Se sim, qual(is) e quando é que lhe foi(ram) diagnosticada(s) por algum médico?

	Idade de Diagnóstico	Realizou algum tratamento		
		Sim	Não	Não sei
☐ Depressão	____ anos	☐	☐	☐
☐ Epilepsia	____ anos	☐	☐	☐
☐ Dislipidemia (ex.colesterol elevado)	____ anos	☐	☐	☐
☐ Excesso de peso/obesidade	____ anos	☐	☐	☐
☐ Diabetes	____ anos	☐	☐	☐
☐ Hipertensão	____ anos	☐	☐	☐
☐ Rinite alérgica (pó, animais, etc.)	____ anos	☐	☐	☐
☐ Doença pulmonar obstrutiva crónica	____ anos	☐	☐	☐
☐ Bronquite crónica	____ anos	☐	☐	☐
☐ Lupus ou artrite	____ anos	☐	☐	☐
☐ Urticária/dermatite	____ anos	☐	☐	☐
☐ Conjuntivite alérgica	____ anos	☐	☐	☐
☐ Asma	____ anos	☐	☐	☐
☐ Alergias. Qual(is)? _____	____ anos	☐	☐	☐
☐ Doença neurodegenerativa. Qual? _____	____ anos	☐	☐	☐
☐ Doença do coração. Qual(is)? _____	____ anos	☐	☐	☐
Doença dos rins. Qual(is)? _____	____ anos	☐	☐	☐
Cancro. Qual(is)? _____	____ anos	☐	☐	☐
Outro. Qual? _____	____ anos	☐	☐	☐

12. Toma pílula contraceptiva ou faz terapêutica hormonal de substituição? ☐ SIM ☐ NÃO

12.1. Há quanto tempo? _____

13. Está a tomar algum medicamento neste momento? ☐ SIM ☐ NÃO ☐ *NS/NR*

13.1 Se sim, quais e para quê? (usar MAÍUSCULAS)

Doença/Situação	Nome	Posologia	Toma	Prescrito
			◯ Crónica ◯ Temporária	☐ SIM ☐ NÃO ☐ *NS/NR*
			◯ Crónica ◯ Temporária	☐ SIM ☐ NÃO ☐ *NS/NR*
			◯ Crónica ◯ Temporária	☐ SIM ☐ NÃO ☐ *NS/NR*
			◯ Crónica ◯ Temporária	☐ SIM ☐ NÃO ☐ *NS/NR*
			◯ Crónica ◯ Temporária	☐ SIM ☐ NÃO ☐ *NS/NR*
			◯ Crónica ◯ Temporária	☐ SIM ☐ NÃO ☐ *NS/NR*

14. Nos últimos 12 meses, alguma vez usou comprimidos anti-histamínicos (Atarax, Zyrtec, Claritine, Telfast, Xyzal, Aerius, Levrix, Rinialer, Ceterizina, Loratadina; Kestine) **para rinite/alergias?**

☐ SIM ☐ NÃO ☐ *NS/NR*

15. Nos últimos 12 meses, alguma vez usou inaladores (Pulmicort nasal, Flutaide, Nasomet, Eutidil, Rontilona, Aeromax, Budesonido nasal) **para rinite alérgica/alergias?**

☐ SIM ☐ NÃO ☐ *NS/NR*

16. Fez ou está a fazer vacinas antialérgicas ou injeções para as alergias?

☐ SIM ☐ NÃO ☐ *NS/NR*

17. Alguma vez lhe foi diagnosticado por um médico alergia a algum alimento?

☐ SIM ☐ NÃO ☐ *NS/NR*

17.1. Se sim, a que alimento? _____

17.2. Este diagnóstico foi efetuado com base numa prova de provocação oral com o alimento feito no hospital?

☐ SIM ☐ NÃO ☐ *NS/NR*

C Estilos de Vida

18. Consome habitualmente álcool? ☐ SIM ☐ NÃO

18.1. Que quantidade de álcool consome em média, por dia?_____

18.2. Que tipo de bebida alcoólica bebe habitualmente? ☐ Vinho ☐ Outra. Qual_____

19. É atualmente fumador? ☐ SIM ☐ NÃO

19.1. Com que idade começou a fumar?_____anos

19.2. Quantos cigarros/maços ou mL fuma por dia? _____ cigarros/dia ou _____ maços/dia ou _____mL/dia

20. Alguma vez fumou? ☐ SIM ☐ NÃO

20.1. Com que idade começou a fumar?_____anos

20.2. Com que idade deixou de fumar?_____anos

20.3. Razão? ☐ Saúde ☐ Outra _____

21. Tem contacto regular com fumadores:

Em casa	☐ SIM	☐ NÃO
No trabalho	☐ SIM	☐ NÃO
Nas suas atividades	☐ SIM	☐ NÃO

21.1. Esteve exposto ao fumo do tabaco em que locais e com que frequência?

Em casa	No Trabalho/Na Escola	Em lazer (café, restaurante, etc.)
☐ Nunca	☐ Nunca	☐ Nunca
☐ Esporadicamente	☐ Esporadicamente	☐ Esporadicamente
☐ Diariamente (menos de 1h)	☐ Diariamente (menos de 1h)	☐ Diariamente (menos de 1h)
☐ Diariamente (entre 1h e 3h)	☐ Diariamente (entre 1h e 3h)	☐ Diariamente (entre 1h e 3h)
☐ Diariamente (mais de 3h)	☐ Diariamente (mais de 3h)	☐ Diariamente (mais de 3h)

22. Habitos Alimentares:

22.1. Consumo de chá ☐ SIM ☐ NÃO Frequência _____ /semana

22.2. Consumo de café ☐ SIM ☐ NÃO Frequência _____ /semana

22.3. Consumo de fruta ☐ SIM ☐ NÃO Frequência _____ /semana

22.4. Consumo de vegetais ☐ SIM ☐ NÃO Frequência _____ /semana

22.5. Consumo de peixe e carne ☐ SIM ☐ NÃO Frequência **peixe** _____ /semana

carne _____ /semana

23. Tomou nos últimos 12 meses suplementos nutricionais (vitaminas/minerais)? ☐ SIM ☐ NÃO

23.1. Se SIM, qual(ais) o nome(s) e em que quantidade tomou nos últimos 12 meses?

Nome/marca comercial	Quantidade diaria	Quantidade de embalagens

D Exposição Ocupacional e Ambiental

24. No seu local de trabalho está exposto(a) a algum tipo de agente químico (por exemplo solventes)**, microbiológico ou físico** (por exemplo ruído)**?**

☐ SIM ☐ NÃO ☐ *NS/NR*

25. Teve no passado, alguma profissão em que estava exposto(a) a algum tipo de agente químico (por exemplo solventes)**, microbiológico ou físico** (por exemplo ruído)**?**

☐ SIM ☐ NÃO ☐ *NS/NR*

25.1. Se sim, entre _____ Idade início e _____ Idade fim

*Se respondeu **NÃO** às perguntas 24 e 25, passe para a pergunta 27*

26. No seu local de trabalho, a que compostos está/esteve exposto(a) e com que frequência? Utiliza(va) habitualmente algum tipo de equipamento de proteção individual?

	Frequência		Uso de equipamentos de proteção individual		
	Diária	Semanal	Não	Sim	Se sim* (assinale todos que se apliquem)
Solventes (ex: benzeno, metanol, xileno)	☐	☐	☐	☐	**M**☐**C**☐**O**☐**L**☐**S**☐**R**☐**A**☐
Partículas (ex: madeiras, farinhas)	☐	☐	☐	☐	**M**☐**C**☐**O**☐**L**☐**S**☐**R**☐**A**☐
Pesticidas	☐	☐	☐	☐	**M**☐**C**☐**O**☐**L**☐**S**☐**R**☐**A**☐
Outros compostos químicos Qual(is)? _____	☐	☐	☐	☐	**M**☐**C**☐**O**☐**L**☐**S**☐**R**☐**A**☐
Radiação (ex: raios-X, beta e gama)	☐	☐	☐	☐	**M**☐**C**☐**O**☐**L**☐**S**☐**R**☐**A**☐
Ruído	☐	☐	☐	☐	**M**☐**C**☐**O**☐**L**☐**S**☐**R**☐**A**☐
Vibrações	☐	☐	☐	☐	**M**☐**C**☐**O**☐**L**☐**S**☐**R**☐**A**☐
Outros agentes físicos Qual(is)? _____	☐	☐	☐	☐	**M**☐**C**☐**O**☐**L**☐**S**☐**R**☐**A**☐
Outro tipo de exposição relevante Qual(is)? _____	☐	☐	☐	☐	**M**☐**C**☐**O**☐**L**☐**S**☐**R**☐**A**☐

* M – máscara; C – capacete; O – óculos; L- luvas; S – sapatos de proteção; R – roupa de proteção/touca; A - auriculares.

27. Vive perto (cerca 500 m) de alguma fábrica, aterro/lixeira ou zona industrial?

☐ SIM ☐ NÃO ☐ *NS/NR*

27.1. Se sim, qual? _____

28. A sua casa encontra-se próxima (cerca de 200 m) **de uma área de cultivo** (horta, pomar, vinha…) **que seja pulverizada com pesticidas?**

☐ SIM ☐ NÃO ☐ *NS/NR*

29. Utiliza pesticidas em sua casa, no seu jardim/vasos ou nos seus animais?

☐ SIM ☐ NÃO ☐ *NS/NR*

29.1. Se sim,com que frequência? Diária ☐ Semanal ☐ Sazonal ☐

29.2. Utiliza equipamento de proteção individual (exemplo: luvas, máscara)? Não ☐ Sim ☐

Qual (is)? _____

FIM

MUITO OBRIGADA PELA SUA COLABORAÇÃO

Codificação do individuo

QUESTIONÁRIO DE ESTADO DE SAÚDE (SF-36V2)

As questões que se seguem pedem-lhe opinião sobre a sua saúde, a forma como se sente e sobre a sua capacidade de desempenhar as atividades habituais. Pedimos que leia com atenção cada pergunta e responda o mais honestamente possível.

Para cada um dos itens a seguir, assinale com um círculo o número que melhor descreve a sua saúde. Certifique-se que seleciona apenas uma resposta para cada alínea.

Agradecemos desde já a sua disponibilidade em responder às questões que se seguem. As suas respostas serão tratadas anonimamente.

1. Em geral, diria que a sua saúde é:

Ótima	Muito boa	Boa	Razoável	Fraca
1	2	3	4	5

2. Comparando com o que acontecia há um ano, como descreve o seu estado geral atual:

Muito melhor	Com algumas melhoras	Aproximadamente igual	Um pouco pior	Muito pior
1	2	3	4	5

3. As perguntas que se seguem são sobre atividades que executa no seu dia-a-dia. Será que a sua saúde o(a) limita nestas atividades? Se sim, quanto?

		Sim, muito limitado(a)	Sim, um pouco limitado(a)	Não, nada limitado(a)
a.	Atividades violentas, tais como correr, levantar pesos, participar em desportos extenuantes;	1	2	3
b.	Atividades moderadas, tais como deslocar uma mesa ou aspirar a casa;	1	2	3
c.	Levantar ou pegar nas compras da mercearia;	1	2	3
d.	Subir vários lanços de escadas;	1	2	3
e.	Subir um lanço de escadas;	1	2	3
f.	Inclinar-se, ajoelhar-se ou baixar-se;	1	2	3
g.	Andar mais de 1 Km;	1	2	3
h.	Andas várias centenas de metros;	1	2	3
i.	Andar uma centena de metros;	1	2	3
j.	Tomar banho ou vestir-se sozinho/a;	1	2	3

Codificação do individuo

4. Durante as últimas 4 semanas teve, no seu trabalho ou atividades diárias, algum dos problemas apresentados a seguir como consequência do seu estado de saúde físico?					
	Sempre	A maior parte do tempo	Algum tempo	Pouco tempo	Nunca
a. Diminuiu o tempo gasto a trabalhar ou outras atividades;	1	2	3	4	5
b. Fez menos do que queria?	1	2	3	4	5
c. Sentiu-se limitado/a no tipo de trabalho ou outras atividades;	1	2	3	4	5
d. Teve dificuldade em executar o seu trabalho ou outras atividades (por exemplo, foi preciso mais esforço);	1	2	3	4	5

5. Durante as últimas 4 semanas, teve com o seu trabalho ou com as suas atividades diárias, algum dos problemas apresentados a seguir devido a quaisquer problemas emocionais (tal como sentir-se deprimido(a) ou ansioso(a))?					
	Sempre	A maior parte do tempo	Algum tempo	Pouco tempo	Nunca
a. Diminuiu o tempo gasto a trabalhar ou outras atividades	1	2	3	4	5
b. Fez menos do que queria?	1	2	3	4	5
c. Executou o seu trabalho ou outras atividades menos cuidadosamente do que era costume	1	2	3	4	5

6. Durante as últimas 4 semanas, em que medida é que a sua saúde física ou problemas emocionais interferiram no seu relacionamento social normal com a família, amigos, vizinhos ou outras pessoas?

Absolutamente nada	Pouco	Moderadamente	Bastante	Imenso
1	2	3	4	5

7. Durante as últimas 4 semanas teve dores?

Nenhumas	Muito fracas	Ligeiras	Moderadas	Fortes	Muito fortes
1	2	3	4	5	6

8. Durante as últimas 4 semanas, de que forma é que a dor interferiu com o seu trabalho normal (tanto o trabalho fora de casa como o trabalho doméstico)?

Absolutamente nada	Pouco	Moderadamente	Bastante	Imenso
1	2	3	4	5

Codificação do individuo

9. As perguntas que se seguem pretendem avaliar a forma como se sentiu e como lhe correram as coisas nas últimas quatro semanas.					
	Sempre	A maior parte do tempo	Algum tempo	Pouco tempo	Nunca
a. Se sentiu cheio/a de vitalidade?	1	2	3	4	5
b. Se sentiu muito nervoso/a?	1	2	3	4	5
c. Se sentiu tão deprimido/a que nada o/a animava?	1	2	3	4	5
d. Se sentiu calmo/a e tranquilo/a?	1	2	3	4	5
e. Se sentiu com muita energia?	1	2	3	4	5
f. Se sentiu deprimido/a?	1	2	3	4	5
g. Se sentiu estafado/a?	1	2	3	4	5
h. Se sentiu feliz?	1	2	3	4	5
i. Se sentiu cansado/a?	1	2	3	4	5

10. Durante as últimas quatro semanas, até que ponto é que a sua saúde física ou problemas emocionais limitaram a sua actividade social (tal como visitar amigos ou familiares próximos)?

Sempre	A maior parte do tempo	Algum tempo	Pouco tempo	Nunca
1	2	3	4	5

11. Por favor, diga em que medida são verdadeiras ou falsas as seguintes afirmações.					
	Absolutamente verdade	Verdade	Não sei	Falso	Absolutamente falso
a. Parece que adoeço mais facilmente do que os outros;	1	2	3	4	5
b. Sou tão saudável como qualquer outra pessoa;	1	2	3	4	5
c. Estou convencido(a) que a minha saúde vai piorar;	1	2	3	4	5
d. A minha saúde é ótima;	1	2	3	4	5

MUITO OBRIGADA PELA SUA COLABORAÇÃO

Codificação do individuo

QUESTIONÁRIO DE EXCLUSÃO DE SINTOMAS RESPIRATÓRIOS

As doenças respiratórias produzem sintomas, tais como:

- TOSSE
- CANSAÇO / FALTA DE AR / DIFICULDADE EM RESPIRAR
- EXPECTORAÇÃO / ESCARRO
- PIEIRA / CHIADEIRA / GATINHOS.

Alguns destes sintomas podem surgir sem que haja doença respiratória. Quando são produzidos por uma doença respiratória são frequentemente ligeiros no início e aumentam muito lentamente a sua intensidade ao longo do tempo. Por estes motivos estes sintomas são frequentemente desvalorizados pelos doentes e até por médicos e enfermeiros, atrasando muitas vezes o diagnóstico das doenças respiratórias que os produzem.

Agradecemos desde já a sua disponibilidade em responder às questões que se seguem. As suas respostas serão tratadas anonimamente.

1. Lembra-se se tossiu nas últimas 8 semanas? ☐ SIM ☐ NÃO

1.1. Se respondeu **SIM** assinale com uma (X) na escala que se segue a intensidade da sua tosse:

TOSSE NORMAL **TOSSE INTENSA E CONSTANTE**

0	1	2	3	4	5	6	7	8	9	10

2. Sente cansaço ou falta de ar ou dificuldade a respirar fora do normal? ☐ SIM ☐ NÃO

3. Deita expetoração ou escarro pela boca todos os dias? ☐ SIM ☐ NÃO

4. A sua respiração produz pieira, chiadeira ou gatinhos? ☐ SIM ☐ NÃO

5. É fumador? ☐ SIM ☐ NÃO

6. Faz tratamentos para doença respiratória? ☐ SIM ☐ NÃO

7. Faz tratamentos para doença cardíaca? ☐ SIM ☐ NÃO

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Codificação do individuo

QUESTIONÁRIO ACT- Teste de controlo da Asma

Este questionário irá ajudar-nos a medir o impacto que a asma está a ter no seu bem-estar e no seu quotidiano.

Agradecemos desde já a sua disponibilidade em responder às questões que se seguem. As suas respostas serão tratadas anonimamente.

Para cada um dos itens a seguir, assinale com uma (X) o quadrado que melhor o descreve presentemente. Selecione apenas uma resposta para cada pergunta.

					Pontuação
1. Durante as últimas 4 semanas, quanto tempo é que a asma o/a impediu de fazer as suas tarefas habituais no trabalho, na escola/universidade ou em casa?					
1 Sempre	2 A maior parte do tempo	3 Algum tempo	4 Pouco tempo	5 Nunca	
2. Durante as ultimas 4 semanas, quantas vezes teve falta de ar?					
1 Mais do que 1 vez por dia	2 1 vez por dia	3 3 a 6 vezes por semana	4 1 ou 2 vezes por semana	5 Nunca	
3. Durante as ultimas 4 semanas, quantas vezes os sintomas da asma (pieira, tosse, falta de ar, aperto ou dor no peito) o/a fizeram acordar de noite ou mais cedo do que é costume de manhã?					
1 4 ou mais noites por semana	2 2 ou 3 noites por semana	3 1 vez por semana	4 1 ou 2 vezes	5 Nunca	
4. Durante as ultimas 4 semanas, quantas vezes usou os seus medicamentos para alivio rápido, em inalador ou nebulizador, como por exemplo Salbutamol?					
1 3 ou mais vezes por dia	2 1 ou 2 vezes por dia	3 2 ou 3 vezes por semana	4 1 vez por semana ou menos	5 Nunca	
5. Como avalia o seu controlo da asma nas ultimas 4 semanas?					
1 Não controlada	2 Mal controlada	3 Mais ou menos controlada	4 Bem controlada	5 Completamente controlada	
				PONTUAÇÃO GERAL	

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Codificação do individuo

QUESTIONÁRIO CAT- Teste de controlo da DPOC

Este questionário irá ajudar-nos a medir o impacto que a DPOC está a ter no seu bem-estar e no seu quotidiano.

Agradecemos desde já a sua disponibilidade em responder às questões que se seguem. As suas respostas serão tratadas anonimamente.

Para cada um dos itens a seguir, assinale com uma (X) a opção que melhor o descreve presentemente. Certifique-se que seleciona apenas uma resposta para cada pergunta.

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

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