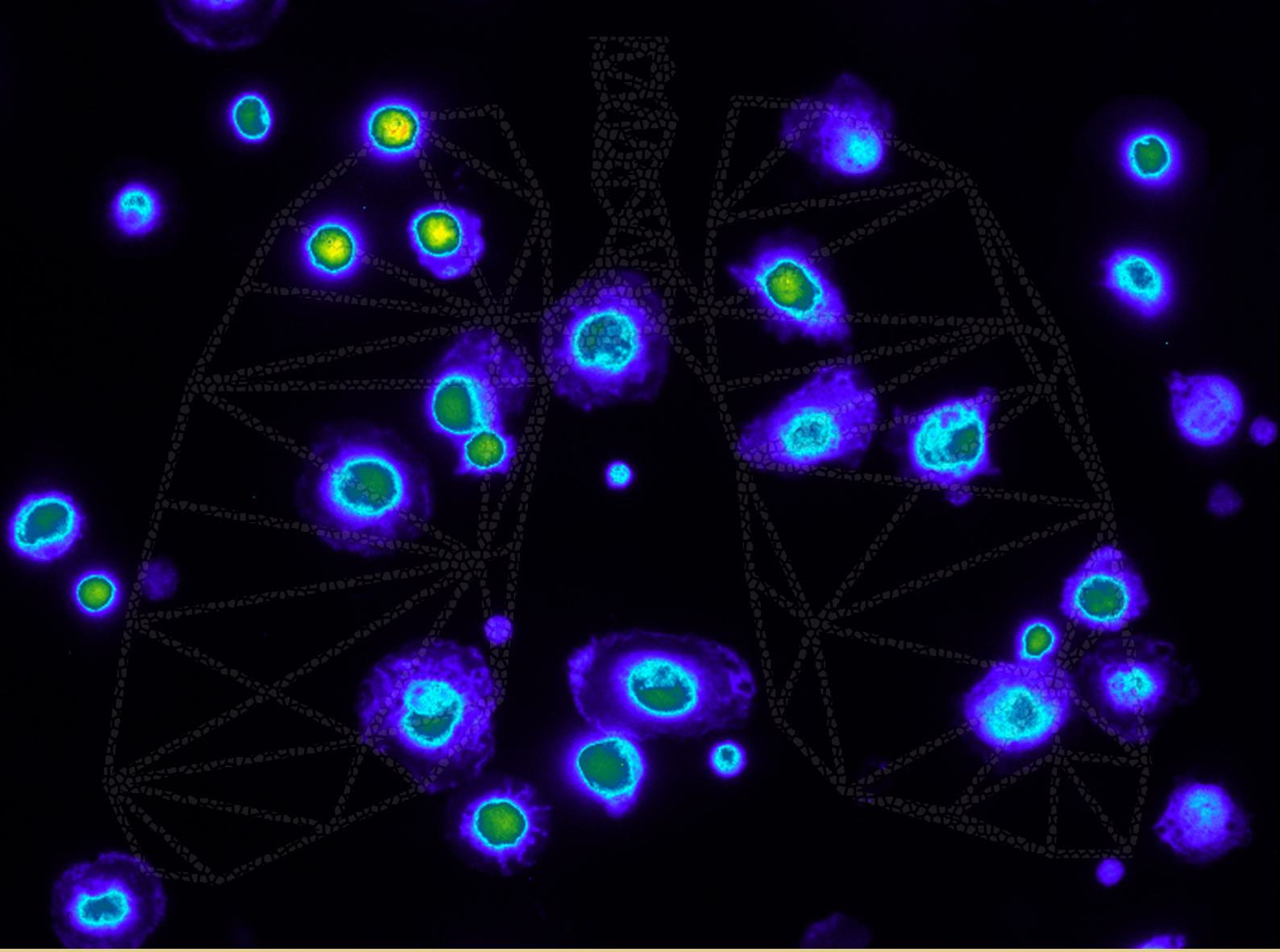

Tiago Manuel Pombo Alfaro

ADENOSINE RECEPTORS IN INFLAMMATORY LUNG DISEASES

Tese de Doutoramento em Ciências da Saúde - Ramo de Medicina, orientada pelo Prof. Doutor Carlos Robalo Cordeiro e pelo Prof. Doutor Rodrigo Cunha e apresentada à Faculdade de Medicina da Universidade de Coimbra

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“It takes a village to do research”

Mireles-Cabodevila E, Stoller JK. Research during fellowship: ten commandments. Chest. 2009

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Resumo / Abstract

Resumo

As doenças pulmonares inflamatórias crónicas, incluindo a asma, a doença pulmonar obstrutiva crónica e doenças pulmonares intersticiais são uma causa crescente e significativa de incapacidade e mortalidade precoce à escala global. Há uma necessidade urgente e reconhecida de novas terapêuticas, mas os alvos identificados nos estudos *in vitro* e modelos animais não têm sido aplicados com sucesso em humanos. A modulação dos recetores purinérgicos é uma abordagem particularmente promissora no controlo da inflamação e promoção da homeostasia. Um conjunto extenso e robusto de dados pré-clínicos demonstraram a presença e atividade destes recetores em células e tecidos envolvidos na inflamação pulmonar e a sua modulação foi eficaz em modelos animais de doença. No entanto, a aplicação destes resultados em ensaios clínicos tem sido dececionante. Alguns autores argumentam que essa ineficácia resulte de diferenças na distribuição e efeitos dos recetores purinérgicos entre as espécies.

O objectivo deste estudo foi a avaliação dos efeitos dos recetores da adenosina na doença pulmonar humana. Em última análise pretende-se obter uma prova de conceito na manipulação dos recetores da adenosina para o tratamento de doenças pulmonares humanas. Foi aplicada uma estratégia de três passos. Primeiro, estudaram-se os efeitos do consumo crónico de doses moderadas de cafeína, um antagonista não seletivo dos recetores A_1 e A_{2A} da adenosina, na saúde respiratória, possibilitando assim um estudo não intervencional do efeito destes recetores em humanos. De seguida, avaliaram-se os níveis pulmonares dum percursor da adenosina, permitindo a avaliação da ativação endógena dos recetores purinérgicos na doença pulmonar humana. Finalmente, desenvolveu-se um método laboratorial que avalia um marcador robusto de ativação celular em macrófagos alveolares humanos frescos e não modificados. Salienta-se que o macrófago alveolar apresenta especial importância na patogénese das doenças pulmonares inflamatórias.

Os estudos epidemiológicos mostraram efeitos globalmente benéficos da cafeína nas doenças respiratórias, incluindo na asma e na tosse pós-infeciosa, bem como na função respiratória e na mortalidade de causa respiratória. Os efeitos na DPOC e sarcoidose não foram claros. Não se observou correlação entre o consumo de cafeína e o risco ou gravidade da apneia do sono. Quando se mediram os níveis pulmonares de trifosfato de adenosina (ATP), encontraram-se níveis elevados em doentes com sarcoidose, comparativamente àqueles com pneumonite de hipersensibilidade, sugerindo que o ATP pode ser útil como biomarcador auxiliar de diagnóstico. Finalmente, em macrófagos alveolares humanos, os recetores A_{2A} da adenosina sofreram alterações adaptativas da sua densidade e a sua ativação reduziu os transientes de cálcio livre intracelular causados pela exposição a um estímulo inflamatório. Estes resultados

suportam a proposta de que os recetores A_{2A} da adenosina são um alvo promissor no tratamento das doenças respiratórias inflamatórias, desde que seja utilizada uma dose suficientemente eficaz.

Em conclusão, foram realizados dois estudos laboratoriais e quatro estudos epidemiológicos focados nos efeitos da adenosina em doença respiratória humana e obteve-se evidência que suporta um papel relevante das purinas na patogénese das doenças pulmonares inflamatórias humanas. Estes resultados sustentam a realização de ensaios clínicos com agonistas dos recetores A_{2A} da adenosina em doenças pulmonares inflamatórias.

Abstract

Chronic inflammatory lung diseases, such as asthma, chronic obstructive pulmonary disease and interstitial lung diseases are a major and rising global source of disability and early death. An urgent need for new therapies is widely recognized, but the identification of therapeutic targets in *in vitro* and animal studies has not been successfully translated into effective human treatments. The modulation of purinergic receptors is a particularly promising new strategy for the control of inflammation and promotion of homeostasis. A breadth of preclinical data has shown that adenosine receptors are active in cells and tissues with a role in pulmonary inflammation and their modulation leads to functional improvements in animal models. The application of these strategies in clinical trials was, however, disappointing. Some authors argue that this lack of effectiveness is related to inter-specific differences in distribution and effects of adenosine receptors.

This study aimed to assess the effects of adenosine receptors in human disease. The final objective was to obtain a proof-of-concept for the use of adenosine receptor modulation for the treatment of human disease. A three-step approach was used. First the effects of chronic consumption of caffeine, a non-selective antagonist of the A_1 and A_{2A} adenosine receptors, were studied, allowing for a non-interventional evaluation of their effects on human respiratory disease. The levels of an adenosine precursor in the lung were then assessed, allowing for an appraisal of the expected endogenous activation of purinergic receptors in human disease. Finally, a laboratory method was developed for the study of a robust marker of cell activation in fresh, unchanged human alveolar macrophages, which have a prominent role in the pathogenesis of inflammatory pulmonary disease.

The epidemiological studies showed that caffeine intake has positive effects in some lung diseases, such as asthma and post-infectious cough, as well as in lung function and respiratory mortality. The results on COPD and sarcoidosis were less clear. No correlation was found between caffeine consumption and the risk or severity of sleep apnoea. The measurement of the lung levels of adenosine triphosphate (ATP), revealed higher levels in patients with sarcoidosis compared to those with hypersensitivity pneumonitis, suggesting that ATP could useful as a diagnosis biomarker. Finally, adenosine A_{2A} receptors underwent adaptive changes of density and their activation reduced the intracellular free calcium transients resulting from exposure to an inflammatory stimulus in human alveolar macrophages. This supports the contention that adenosine A_{2A} receptors are a promising therapeutic target for treating inflammatory lung disorders in humans, provided that a sufficiently effective dose of agonist is used.

In conclusion, these two laboratory and four epidemiological studies focused on the effects of adenosine in human lung disease found evidence that supports a relevant role for purines in the pathogenesis of human inflammatory lung disease. These results support the further testing of adenosine A_{2A} receptor agonists for the treatment of inflammatory lung diseases.

General index

General index

Abbreviations and acronyms

Abbreviations and acronyms

A_1R	adenosine A_1 receptor
$A_{2A}R$	adenosine A_{2A} receptor
$A_{2B}R$	adenosine A_{2B} receptor
A_3R	adenosine A_3 receptor
ADA	adenosine deaminase
ADP	adenosine 5’-diphosphate
AHI	apnea-hypopnea index
AIP	acute interstitial pneumonia
AK	alkaline phosphatase
ALI	acute lung injury
AM	alveolar macrophage
AMP	adenosine 5’-monophosphate
ARDS	acute respiratory distress syndrome
ARIC	atherosclerosis risk in communities study
ATP	adenosine 5’-triphosphate
ATS	American Thoracic Society
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BALT	bronchus-associated lymphoid tissue
BHL	bilateral hilar lymphadenopathy
BMI	body mass index
cAMP	cyclic adenosine 3’,5’-monophosphate
CHD	coronary heart disease
COP	cryptogenic organizing pneumonia
COPD	chronic obstructive pulmonary disease

CPAP	continuous positive airway pressure
CT	computed tomography
CTD	connective tissue disease
CV	cardiovascular
DIP	desquamative interstitial pneumonia
DNA	deoxyribonucleic acid
E-NPP	ecto-nucleotide pyrophosphatase/phosphodiesterase
E-NTPDase	ecto-nucleoside triphosphate diphosphohydrolase
ER	emergency room
ERS	European Respiratory Society
ESS	Epworth sleepiness scale
FCT	Fundação para a Ciência e Tecnologia
FDA	United States Food and Drug Administration
FEV1	forced expiratory volume in 1 second
FMLP	formyl-methionyl-leucyl-phenylalanine
FVC	forced vital capacity
GOLD	global initiative for chronic obstructive lung disease
HBSS	Hank's buffered saline solution
HDAC	histone deacetylase
HP	hypersensitivity pneumonitis
HPFS	health professionals follow-up study
HR	hazard ratio
IIP	idiopathic interstitial pneumonia
IL	interleukin
ILD	interstitial lung disease
IPF	idiopathic pulmonary fibrosis

IQR	interquartile range
LAM	lymphangioleiomyomatosis
LIP	lymphocytic interstitial pneumonia
LPS	lipopolysaccharide
LTOT	long term oxygen therapy
MCP	monocyte chemotactic protein
mRNA	messenger ribonucleic acid
N/A	not available
nAChR	nicotinic acetylcholine receptor
NHANES	national health and nutrition examination survey
NHS	nurse health study
NIH	National Institutes of Health
NOS	Newcastle-Ottawa scale
NS	not significant
NSIP	non-specific interstitial pneumonia
OECD	Organization for Economic Co-operation and Development
OR	odds ratio
OSA	obstructive sleep apnea
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PI3K-δ	phosphoinositide-3-kinase-δ
PKA	protein kinase A
PLCH	pulmonary Langerhans cell histiocytosis
PLCO	prostate, lung, colorectal and ovarian
PPADS	pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid

PPFE	pleuro-parenchymal fibroelastosis
PUD	peptic ulcer disease
RASON	respirable antisense oligonucleotide
RB-ILD	respiratory bronchiolitis with interstitial lung disease
RCT	randomized control trial
RNA	ribonucleic acid
ROI	region of interest
SD	standard deviation
SE	standard error
SF-12	short form health survey 12
TH1	type 1 T helper cell
TH17	type 17 T helper cell
TH2	type 2 T helper cell
TReg	regulatory T cells
USA	United States of America
WASOG	World Association Of Sarcoidosis and Other Granulomatous Disorders
WHO	World Health Organization

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Institutions and Funding

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Publications

Publications included in this thesis

1. **Alfaro TM**, Monteiro RA, Cunha RA, Cordeiro CR. Chronic coffee consumption and respiratory disease: A systematic review. Clin Respir J. Forthcoming 2017.

2. Lopes PO, **Alfaro TM**, Lopes P, Cunha RA, Cordeiro CR. Caffeine consumption and exacerbations of chronic obstructive pulmonary disease: retrospective study. Rev Port Pneumol. 2015;21(5):271-5.

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5. Rosa BS, **Alfaro TM**, Queiroz F, Casalta J, Bártolo R, Tome AR, et al. Pulmonary ATP levels in hypersensitivity pneumonitis and sarcoidosis – preliminary study (submitted). Sarcoidosis Vasc Diffuse Lung Dis.

6. **Alfaro TM**, Rodrigues DI, Tomé ÂR, Cunha RA, Robalo Cordeiro C. Adenosine A2A receptors are up-regulated and control the activation of human alveolar macrophages. Pulmonary Pharmacology & Therapeutics. 2017;45:90-4.

Chapter 1
Introduction and objectives

Clinical problem and research needs

Epidemiology of respiratory diseases

Chronic respiratory diseases affect hundreds of millions worldwide and are associated with a remarkable disease burden. The global respiratory mortality is staggering, with about four million people dying every year from chronic respiratory disease.[1] The Global Burden of Disease Study 2015 revealed that the main chronic respiratory causes of morbidity in 2015 were chronic obstructive pulmonary disease (COPD), asthma and interstitial lung diseases (ILD).[2] Interestingly, interstitial lung diseases were the only ones showing an increase between 2005 and 2015. The data on age-adjusted mortality from 2013 were similar, with growing mortality from ILD between 2003 and 2013.[3]

In Portugal, the mortality resulting from respiratory diseases is increasing, and it is currently the third leading cause of death. In fact, Portugal has the third highest respiratory mortality in the European Union, and the second highest mortality from pneumonia in the Organisation for Economic Co-operation and Development (OECD).[4]

There are several contributing factors for this notable burden of respiratory disease. First, continued tobacco smoking is the main driver for the development of COPD and lung cancer.[1] The increasing life expectancy and population ageing are associated with higher prevalence of a multitude of chronic diseases, including those affecting the lungs.[5] Pollution and poor air quality also contribute to the development of airway diseases.[1] Finally, there is a significant gap in medical research leading to the development of new and effective therapies for the prevention and treatment of the most frequent inflammatory lung diseases.[6] A clear contrast can be made with cardiovascular diseases. Although cardiovascular diseases are still the main global cause of death, a steady reduction in mortality has been occurring in the last decades, which largely results from a significant effort and investment in research, leading to the development of effective preventive and treatment strategies, such as statins and angiotensin modulators.[7] Some specific characteristics of the respiratory system also have to be considered. The lungs are particularly vulnerable to infectious and other noxious hazards, as they are simultaneously exposed to the internal and external environment. This unique character led to the evolutionary development of complex local immune mechanisms for defence against external invaders in parallel to the development of precise mechanisms of self-tolerance. It is therefore unsurprising that, besides frequent infections, the lungs are affected by a number of diseases characterized by immune dysfunction and chronic inflammation.[8]

The inflammatory and interstitial lung diseases

The most prevalent and important chronic inflammatory lung diseases are asthma, COPD and interstitial lung diseases.[2] Asthma affects over 300 million people worldwide, but the majority of patients can be controlled by the use of inhaled steroids and bronchodilators.[9] About 5 to 10% of patients seem to be refractory to steroids, and are therefore at increased risk for morbidity and mortality.[10] Asthma is very heterogeneous and should probably be considered a syndrome rather than a single disease. One of the proposed strategies for improved control of severe asthma is the development of targeted therapies for specific phenotypes, which are termed endotypes when associated with a distinct pathophysiology.[11] Although significant variation is recognized, the pathophysiology of allergic asthma (the most frequent phenotype) is characterized by Type 2 helper T cells (T_H2) inflammation, with airway predominant inflammation. A number of cells are involved, but T_H2 lymphocytes and eosinophils are predominant, with mast cells, basophils, neutrophils, monocytes, macrophages and airway epithelial cells having a lesser role.[12] Some patients tend to exhibit a later presentation and worse response to steroids, associated with a neutrophil predominant inflammation, and a Type 1 and Type 17 T helper cell (T_H1 and T_H17) cytokine profile.[13] The net result of airway inflammation in asthmatic patients is the development of hyperreactivity leading to repeated periods of wheezing, shortness of breath, cough and chest tightness, which can lead to hospitalization, respiratory failure, and death.[9]

Chronic obstructive pulmonary disease (COPD) is also frequent, affecting over 380 million individuals worldwide. The disease affects only adults and is caused by significant exposure to noxious particles or gases, mainly cigarette smoking, biomass, and occupational exposures.[14] Some recent data however, suggests that a significant proportion of individuals develop the disease as a result of abnormal lung development and reduced growth.[15] Although asthma and COPD share a number of characteristics, both leading to airflow limitation, the latter is characterized by a different type of inflammation, with CD8 T-cells, neutrophils and macrophages having a predominant role. This inflammation involves mainly the peripheral airways and lung parenchyma, causing bronchiolitis and emphysema, both leading to increased airflow resistance. Patients with COPD tend to have accelerated loss of lung function, and persistent symptoms, mainly shortness of breath. Exacerbations are an important manifestation of the disease and are characterized by worsening symptoms, enhanced airway and systemic inflammation leading to loss of quality of life, hospitalization, respiratory failure and death.[14] The current pharmacological treatment of COPD is based on the use of bronchodilators, as these improve symptoms and quality of life, while reducing the risk for exacerbations. In the past, there was wide use of inhaled steroids, but several studies revealed that the disease is characterized by steroid-resistant inflammation and steroids actually increase the risk for pneumonia in this

population.[16] Interestingly, theophylline, a nonselective purinergic antagonist and phosphodiesterase (PDE) inhibitor seems to reverse this steroid resistance. The mechanism for this action is probably related to an enhanced histone deacetylase (HDAC) activity, resulting from selective inhibition of phosphoinositide-3-kinase-δ (PI3K-δ).[17]

The interstitial lung diseases (ILD) are an extensive and heterogeneous group of pulmonary disorders characterized by diffuse primary involvement of the space between alveoli and capillaries, termed pulmonary interstitium. The clinical and radiological manifestations are broadly shared within this group of disorders, but are mostly non-specific. A proportion of these diseases have an identifiable cause, which can be an inhaled antigen, a drug, a systemic auto-immune disease or other factors.[18] For most however, the cause remains unknown, and are thus named idiopathic ILDs. The pathophysiology is highly variable between ILDs, with some exhibiting inflammation leading to pulmonary fibrosis, some developing fibrosis with no previous identifiable inflammation, and some evolving mostly with formation of granulomas.[19, 20] The classification of ILDs has been a matter of discussion, and several proposals have been produced. A subgroup of idiopathic disorders, the idiopathic interstitial pneumonias (IIP) has been the subject of a recent international task force updated report.[21] A representation of the classification of ILDs including the IIP can be seen in figure 1.

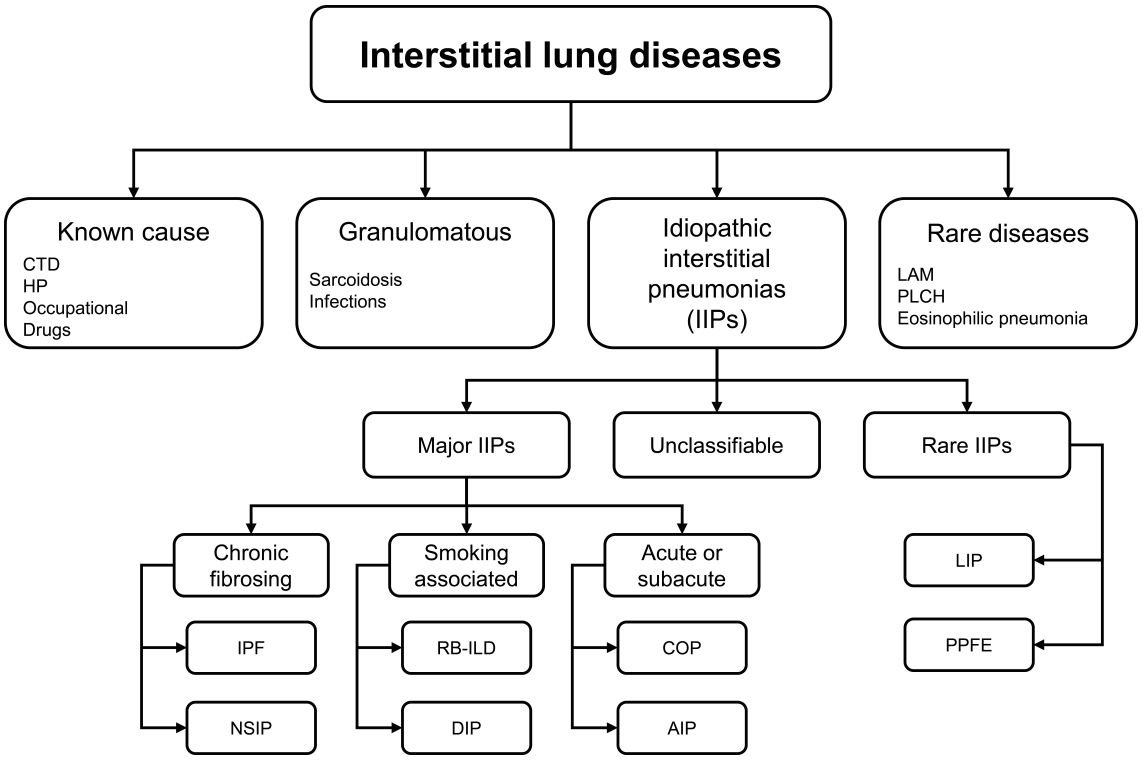

Figure 1: classification of interstitial lung diseases. The idiopathic interstitial pneumonias are now classified as major, unclassifiable or rare. IIP: idiopathic interstitial pneumonias; CTD: connective tissue

disease; HP: hypersensitivity pneumonitis; LAM: lymphangioleiomyomatosis; PLCH: pulmonary Langerhans cell histiocytosis; IPF: idiopathic pulmonary fibrosis; NSIP: non-specific interstitial pneumonia; RB-ILD: respiratory bronchiolitis with interstitial lung disease; DIP: desquamative interstitial pneumonia; COP: cryptogenic organizing pneumonia; AIP: acute interstitial pneumonia; LIP: lymphocytic interstitial pneumonia; PPFE: pleuro-parenchymal fibroelastosis.

The ILD group includes over 200 different medical conditions. Although most are rare when considered separately, the group is responsible for about 15% of all outpatients referred to a typical pulmonary medicine clinic.[22] Furthermore, some diseases, such as idiopathic pulmonary fibrosis (IPF) are associated with an especially poor prognosis, with a median survival upon diagnosis of three years, which is worse than most types of cancer.[23] Finally, several recent studies reported increasing prevalence and population mortality from IPF, although it is still unclear whether this is due to increasing life-expectancy, improved diagnosis or a real increase in population prevalence.[24] Concerning treatment, steroids and other immunomodulatory drugs are commonly used in order to reduce the inflammatory component and prevent disease progression. This strategy proved ineffective in IPF, and these patients are now managed with anti-fibrotics such as pirfenidone and nintedanib.[25] Importantly, these therapies only reduce the rate of disease progression, and IPF patients continue to have a poor prognosis. For young patients, lung transplant is the only strategy which can lead to long-term survival.[26, 27] For the non-IPF ILDs, the treatment of the specific cause is favoured when possible, but the response is quite variable, and frequently there is progression to respiratory failure and death.[26]

In conclusion, one of the main challenges for the reduction of the global respiratory disease burden is the fact that many of these chronic lung diseases are characterized by inflammation and or fibrosis that does not respond to currently available anti-inflammatory treatments, including steroid and non-steroid anti-inflammatory drugs. In the last 40 years, only a few novel classes of drugs were introduced for non-malignant respiratory diseases. These include anti-PDE-IV drugs, which reduce exacerbations in a sub-group of COPD patients and anti-fibrotic drugs, which reduce the otherwise inexorable progression of pulmonary fibrosis in IPF.[28] New and effective therapies are urgently needed.

Purine modulation as a new strategy for the treatment of inflammatory lung diseases

Purines and purinergic signalling

Purines are heterocyclic aromatic organic molecules, typically known for their role as building blocks for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Adenosine, a nucleoside, is formed by the purine adenine attached to a ribose moiety. Adenosine has a prominent role as the main mechanism for cellular energy transfer when attached to three phosphate molecules forming adenosine triphosphate (ATP). Cyclic adenosine monophosphate (cAMP) is an important intracellular second messenger, involved in many signal transduction pathways. Finally, these molecules have a key role as extracellular signalling molecules acting on cell membrane receptors and serving as neuromodulators or mediating cell to cell communication.[29] The main functions of purines are depicted in figure 2.

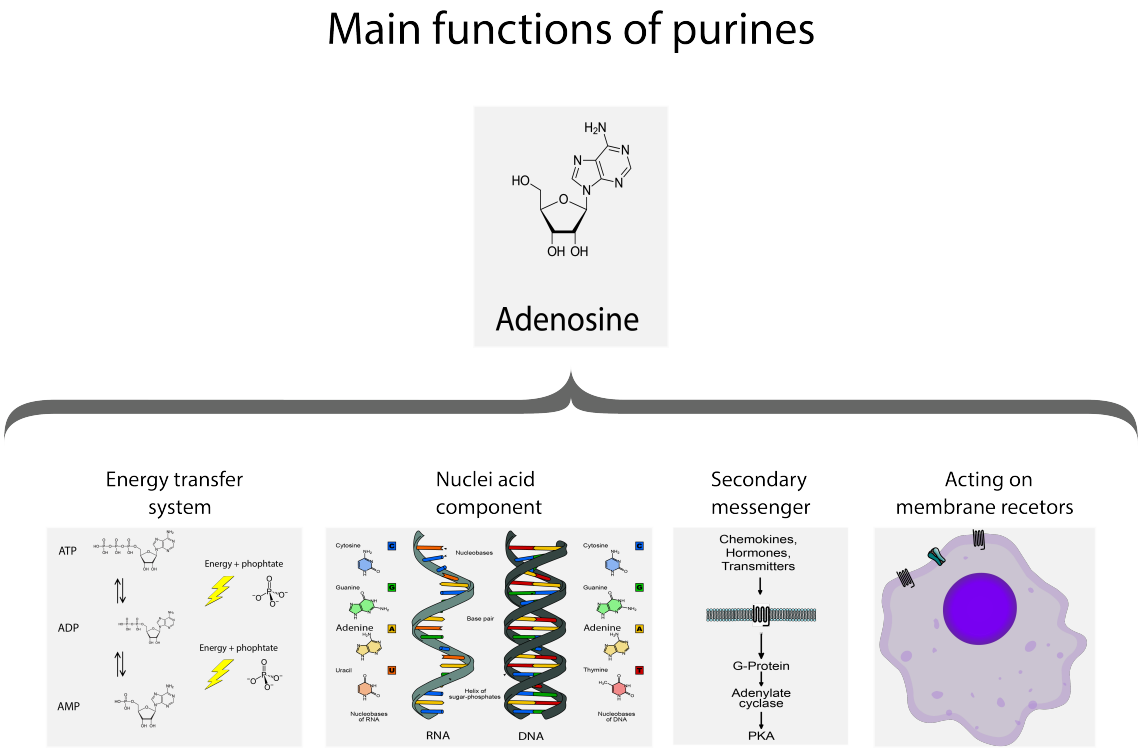

Figure 2: the main four functions of purines, part of ATP↔ ADP↔ AMP energy transfer system, one of the components of nucleic acids, second-messenger system and acting on cell membrane receptors. ATP: adenosine 5'-triphosphate; ADP: adenosine 5'-diphosphate; AMP: adenosine 5'-monophosphate; RNA: ribonucleic acid; DNA: deoxyribonucleic acid; PKA: protein kinase A.

The signalling function of purines (Figure 3) is mediated by a family of receptors that are classified under two groups: P1 receptors are preferentially activated by adenosine and are metabotropic G-protein-coupled receptors; P2 receptors are preferentially activated by ATP (and UTP, UDP and ADP) and can be either metabotropic G-protein coupled P2Y receptors or ionotropic (ion-gated) P2X receptors.[30, 31] For each group, a number of receptor types has been identified. Different receptors vary on distribution and affinity for agonists and antagonists, resulting in distinct and sometimes opposing effects. The net result of purinergic modulation is therefore dependent on the dynamic interaction between the release and metabolism of adenosine precursors, adenosine degradation and presence and density of each receptor on the target cells.[32]

Importantly, the extracellular levels of ATP and adenosine are especially increased following tissue stress or injury.[33, 34] Increased ATP levels result from passive release from necrotic cells and active release from cells during inflammation, hypoxia or apoptosis. The mechanisms for active release include exocytosis from intracellular vesicles and transport via membrane channels or transporters.[35, 36] ATP affects most inflammatory cells and when in high levels leads to wide pro-inflammatory effects, including chemotaxis, release of cytokines, production of oxygen free radicals and inflammatory cell proliferation.[35] Extracellular ATP is rapidly dephosphorylated to adenosine through the action of membrane-bound and soluble nucleotidases from four families: ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), ecto-5'-nucleotidase/CD73, and alkaline phosphatase (AK).[37] The resulting adenosine acts on extracellular receptors that have been identified in most inflammatory and structural cell types. Its effects are mostly anti-inflammatory including inhibition of endothelial adhesion, reduced release of pro-inflammatory cytokines, reduced production of superoxide anion and enhanced release of IL-10, an anti-inflammatory cytokine.[35] The net result is that adenosine acts as a mechanism for the resolution of inflammation and it thus considered a retaliatory metabolite, promoting homeostasis and healing.[38] Based on this, purinergic receptors are a promising target for new therapies for inflammatory diseases.[32, 39]

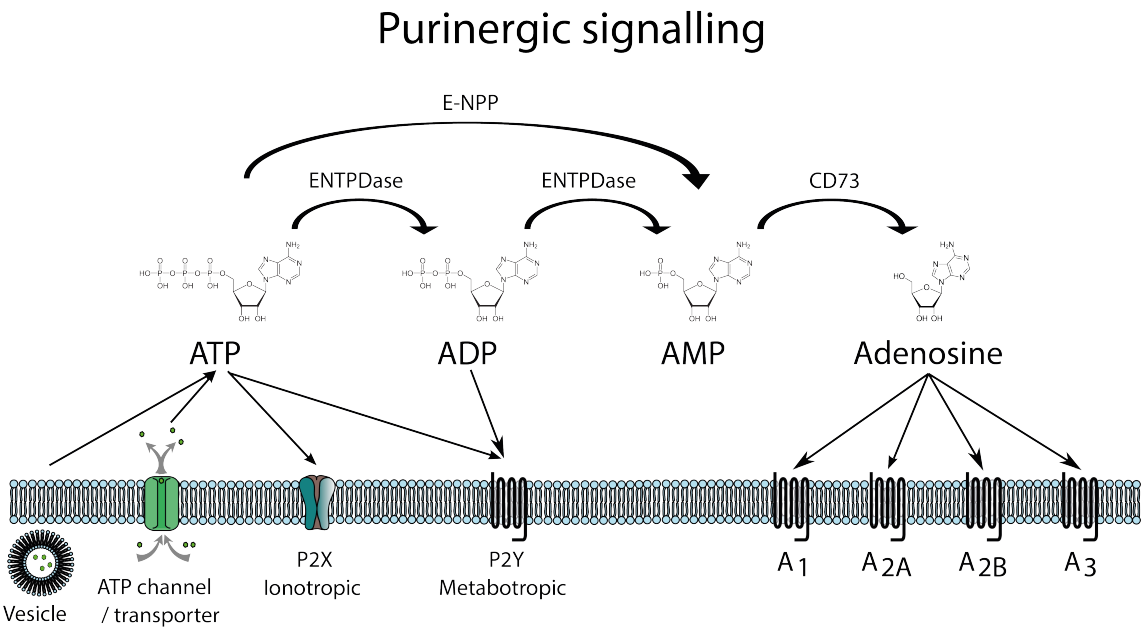

Figure 3: main forms of purinergic signalling. Adenosine triphosphate is released from cells through exocytosis or channels/transporters and acts directly on P2 receptors. The degradation of ATP by extracellular nucleotidases leads to the formation of adenosine diphosphate (which can act on P2Y receptors), adenosine monophosphate, and adenosine. Adenosine acts through four metabotropic receptors, A_1, A_{2A}, A_{2B} and A_3. E-NPP: ecto-nucleotide pyrophosphatase/phosphodiesterase; E-NTPDase: ecto-nucleoside triphosphate diphosphohydrolase; ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate.

Purinergic control of respiratory disease

A substantial amount of evidence on the purinergic control of respiratory diseases comes from preclinical studies, mostly performed on cell lines and animal models. This is in contrast to limited data coming from clinical studies, which may contribute to the current lack of approved purinergic-based therapies for inflammatory lung disease.

Preclinical studies

Some of the most promising preclinical data comes from studies on cells and tissues with a significant role on the pathogenesis of pulmonary disease. These include both immune cells, such as leukocytes and structural cells, such as alveolar epithelial cells. A brief review on the

purinergic control of the most important players is presented. The main studied effects of adenosine on lung cells is illustrated in figure 4.

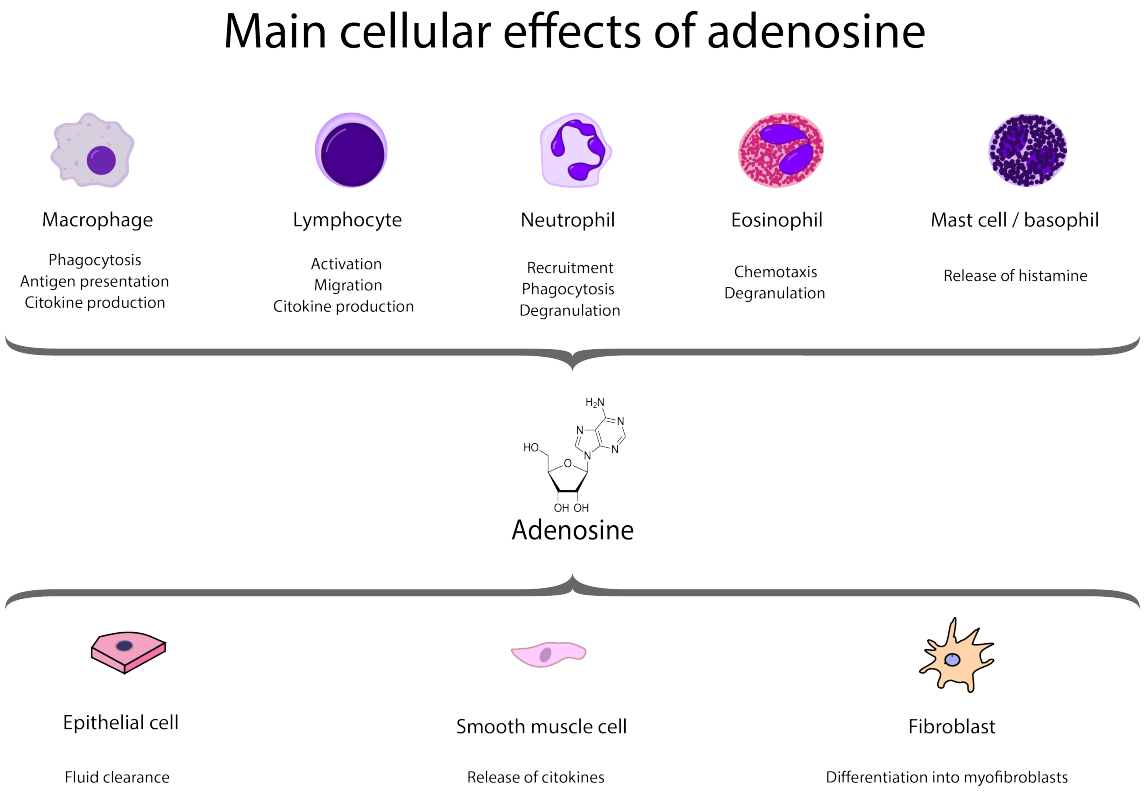

Figure 4: main effects of adenosine on different cell types with a role in pulmonary inflammatory diseases. The effects of adenosine are dependent on the receptor type, and can thus be positive or negative.

Alveolar macrophages

The alveolar macrophage (AM) is the chief lung immune cell in physiological conditions. AMs differentiate from early-life blood monocytes upon exposure to the lung environment and then reside in the alveoli, having a surprisingly prolonged lifespan.[40] These are the first line of defence against any hazardous foreign element prior to the migration of other leukocytes, such as neutrophils. Macrophages display numerous immune functions, including phagocytosis, antigen processing/presentation, lymphocyte priming and release of both pro and anti-inflammatory mediators.[41] Accordingly, AMs are critical orchestrators for the initiation, maintenance and resolution of a number of pulmonary inflammatory diseases, such as COPD,[42] interstitial lung disease[41] and acute lung injury (ALI).[43] Concerning adenosine, human alveolar macrophages express messenger RNA (mRNA) for all four P1 receptors, with a predominance of A_1R and

$A_{2A}R$ subtypes. Furthermore, exposure to lipopolysaccharide (LPS) led to a 400-fold increase in $A_{2A}R$ expression, a decrease in A_1R and $A_{2B}R$ (10 fold), and no changes in A_3R.[44] Adenosine has been shown to modulate macrophage behaviour, including differentiation, phagocytosis, oxidative burst, and mediator production and release.[45] Importantly most of these effects were shown in either animal or human monocyte-derived macrophages, and no inferences on the effects of adenosine in human alveolar macrophages can be made with certainty.

Lymphocytes

Lymphocytes are the second most frequent leukocytes in the blood, usually amounting to 25 to 35% of the white blood cell count. They are responsible for the adaptive immunity and express clonally distributed antigen receptors, so that each cell clone recognizes one specific antigen. Lymphocytes are usually subdivided in three populations: B-cells mature in the bone marrow and recognize antigens through surface receptors, evolving into plasma cells and secreting antibodies. T-cells mature in the thymus and also recognize antigens, but do not secrete antibodies. Instead, they secrete cytokines that activate or inhibit most of the components of the immune system, including both innate and adaptive response cells. Natural killer (NK) cells resemble T-cells, but they do not need to mature in the thymus and are capable of lysing virus-infected and tumour cells.[46] The normal lung has an estimated resident population of 10 billion T-cells, a number comparable to that of the blood.[47] These are distributed in different compartments: the epithelial surface, bronchus-associated lymphoid tissue (BALT), the interstitium and the vasculature. Their main functions are antibody production, cytotoxic activity, cytokine production and immune tolerance. T-cells have a central role in the pathogenesis of some of the most frequent lung disorders. In allergic asthma, CD4+ T-lymphocytes with a T_H2 cytokine profile are usually observed, whereas in COPD, CD8 cytotoxic lymphocytes are more frequent.[48] The interstitial lung diseases are more heterogeneous regarding CD4+ T-lymphocytes. Sarcoidosis and hypersensitivity pneumonitis are associated with a CD4 T_H1 profile, but a shift to a T_H2 profile is associated with evolution to fibrosis.[49, 50] Idiopathic pulmonary fibrosis, a strictly fibrotic disease. displays a T_H2 type response.[51]

Human T lymphocytes express adenosine $A_{2A}R$, $A_{2B}R$ and A_3R, but their presence and effects can vary according to the lymphocyte subtype.[52-54] The overall effect of adenosine is inhibitory, with reduction of T-cell activation, release of cytokines, mobility, adhesion to endothelium and migration. A subset of T cells, named regulatory T cells (T_{Reg}) and characterized by FoxP3 and CD25 expression express the ectonucleotidases CD39 and CD73, which degrade extracellular ATP and ADP, leading to increased presence and effects of extracellular adenosine. This is probably one of the mechanisms explaining the immunosuppressive effects of T_{Reg}

lymphocytes.[55] There are two clinical examples of the importance of the adenosine for lymphocyte function. One is the disease characterized by genetic deficiency in adenosine deaminase (ADA), leading to profound suppression of lymphocyte functions and severe infections. The second is cancer immunology, as it has been shown that one of the main mechanisms by which cancer evades immune surveillance is the enhanced release of adenosine in hypoxic cancer tissue.[56, 57]

Neutrophils

Neutrophils are the most abundant leukocytes in the peripheral blood and can be rapidly recruited to inflamed peripheral sites, such as the lung. Although their main known function is antimicrobial, neutrophils are involved in the pathogenesis of numerous non-infectious pulmonary diseases, including COPD, IPF, ischemia-reperfusion and acute lung injury. Neutrophils can act as both producers and target cells of adenosine, in an autocrine or paracrine fashion.[58] Adenosine, acting on cell-membrane receptors, regulates most neutrophilic inflammatory mechanisms, whether antimicrobial or proinflammatory. These include recruitment, adhesion and transmigration, release of inflammatory mediators, phagocytosis, degranulation, oxidative burst and cell death.[59, 60]

Eosinophils

Eosinophils are marrow derived granulocytes mostly known for their physiological role in the defence against parasites and their central pathological role in atopic diseases, including asthma.[61] Concerning adenosine, studies have shown that the A_3R inhibits degranulation and chemotaxis of human eosinophils.[62]

Mast cells and basophils

Mast cells and basophils are two different cell types that share the same precursor and have similar functional roles, mostly connected to T_H2 immune responses which is important for defence against helmints and in allergic diseases. While mast cells reside in peripheral tissues and have a typical lifespan of weeks to months, basophils remain in peripheral blood and have a typical lifespan of 60 hours. Both cell types are infrequent, with basophils comprising about 1% of blood leukocytes.[63] Adenosine, acting on A_2R, inhibited histamine release from human basophiles.[64] For human mast cells, low adenosine concentration enhance the release of

histamine, while higher concentrations inhibit it.[65] The specific P1 receptors responsible were identified in the mouse, but seem to be different in humans.[66]

Epithelial cells

Human alveolar type II cells express both $A_{2A}R$ and $A_{2B}R$.[67] Cultured human bronchial epithelial cells secrete adenosine in response to stretching, reducing endothelial permeability.[68] The net effect has not been studied in humans, but in mice, intra-tracheal instilled adenosine acts on $A_{2A}R$ and A_3R and enhances alveolar fluid clearance. Physiological concentrations act on A_1R and have the reverse effect.[69]

Smooth muscle cells

The bronchial smooth muscle is a major player in the pathogenesis of obstructive lung diseases. Besides bronchoconstriction, these cells are also active in the release of proinflammatory molecules, such as IL-2, 5, 6, 8 & 12, as well as monocyte chemotactic protein (MCP) 1, 2 & 3.[70] Human bronchial smooth muscle cells express $A_{2B}R$, which when activated leads to enhanced release of the pro-inflammatory cytokine IL-6 and the chemokine MCP-1.[71]

Fibroblasts

Fibroblasts are mesenchymal cells that can be found in the stroma of most tissues and organs. In the lung, they are usually found in the adventitia of vascular structures and airway. Fibroblasts are important for several physiological processes such as development, wound healing and tissue repair. Under pathological conditions, activated fibroblasts differentiate into myofibroblasts which are central to the pathogenesis of tissue fibrosis, the hallmark lesion of IPF, also seen in the small airways of patients with COPD.[72, 73] Human lung fibroblasts, like smooth muscle cell express mainly adenosine $A_{2B}R$. The activation of $A_{2B}R$ leads to release of IL-6 and differentiation into myofibroblasts. Interestingly, tissue hypoxia, which is usually caused by intense inflammatory stimuli, amplifies this response.[74]

Animal models

Animal models simulate the clinical and pathological characteristics of a specific disease in a laboratory animal. Their use allows for the study of complex interactions in the pathogenesis of a disease, as well as the development and testing of preventive and therapeutic strategies.[75] However, the need for inducing the disease in the animal coupled to interspecies molecular differences may mean that these models do not accurately predict the pathogenesis and response to treatment in human disease.[76] A particularly striking example of this limitation has been seen in cancer, where promising cures in mouse models have not been often translated into similar results in humans.[77]

Adenosine deaminase (ADA) is the enzyme responsible for the degradation of adenosine and desoxyadenosine to inosine. Knock-out mice for this enzyme are viable, but display greatly increased systemic levels of adenosine, creating an useful model for the study of its effects on organs and systems. On the respiratory system, severe inflammatory changes are observed that resemble obstructive airways diseases. These include increased airway neutrophils and macrophages, tissue eosinophilia, mast cell degranulation, mucous cell metaplasia and airway hyperreactivity, ultimately resulting in respiratory failure and death at three weeks of age.[78, 79] Replacement therapy with ADA leads to reduced adenosine levels and leads to improved health and survival. When temporary treatment is administered, animals develop pulmonary fibrosis, which was probably not observed otherwise because animals died before developing this stage of lung disease.[80] The use of ADA deficient mice allowed the exploration of the use adenosine receptor modulation, either by genetic removal or the use of pharmacological agonists and antagonists for adenosine receptors in these animals. Overall, the A_1R[81] and $A_{2A}R$[82] have a protective role, reducing inflammation, whereas the $A_{2B}R$[83] and A_3R[84] seem to have a negative effect enhancing inflammation.

Another useful approach is the pharmacological modulation of adenosine receptors in induced disease models. This has been done in animal models of allergy and asthma, smoking exposure, acute lung injury and pulmonary fibrosis. The availability of knock-outs for adenosine receptors also allows the development of these same models in an animal lacking one of these receptors. In the models of allergy, the A_1R[85], $A_{2B}R$[86] and A_3R[87] promote inflammation, while the $A_{2A}R$[88] reduces it. In models of acute lung injury, the A_1R[89, 90] promotes tissue injury, whereas the $A_{2A}R$ and $A_{2B}R$ are protective.[91] In a mouse model of cigarette smoke exposure, the $A_{2A}R$ reverses the decrease in bronchoalveolar lavage macrophage numbers and inhibits neutrophil activation.[88] The administration of bleomycin has been used as a model for pulmonary fibrosis. On this model, $A_{2B}R$ seems to have a negative role in the acute phase, and a protective role in the chronic phase.[92]

Human observations

Theophylline (methylxantine) has been used as a treatment for asthma since 1922. Although the initial hypothesis was that its favourable effects were mediated by the inhibition of phosphodiesterase, subsequent observations showed that this only occurs under much higher than clinical effective tissue concentrations.[17] In 1979, Fredholm suggested that therapeutic gains were probably related to adenosine antagonism.[93] Based on this, and the observation that adenosine leads to tracheal smooth muscle contraction in *in vitro* studies, Cushley and Holgate showed that the inhalation of adenosine results in bronchoconstriction on patients with asthma, but not in healthy controls.[94] Aminophylline, the ethylenediamine salt of theophylline prevents this effect.[95-97] A similar observation was performed in patients with COPD, by using cyclic AMP, a stable adenosine precursor.[98] In fact, the inhalation of cAMP is currently recommended as a clinical test for the investigation of non-specific airway hyper-responsiveness, which is invariably found in patients with asthma. Although the precise mechanisms for this response are not fully known, the most probable explanation is that adenosine acts on $A_{2B}R$ on the cell membrane of mast cells, leading to the release of mediators and ultimately causing bronchoconstriction.[99]

Another important source of evidence on the purinergic control of respiratory disease comes from studies evaluating changes in the levels and/or metabolic pathways of adenosine in patients with respiratory disease. In physiological conditions the tissue levels of extracellular adenosine are quite low, varying from 40 to 600 nM. Patients with asthma[100] and COPD[101] have increased pulmonary levels, and these are further elevated when asthmatics are subjected to an allergen challenge or exercise.[102-104] The observation that patients with idiopathic pulmonary fibrosis and severe COPD display increased pulmonary levels of CD73 and ADA, which promote increased adenosine levels, is in agreement with these results.[105]

Several studies have also shown changes in adenosine receptor levels in patients with respiratory disease. Bronchial biopsies from asthmatics have increased levels of A_1R[106] in the bronchial epithelium and smooth muscle. All four adenosine receptors were identified in the lung parenchyma of patients with COPD, and the A_1R, $A_{2A}R$ and $A_{2B}R$ display decreased affinity, which might be partially caused by chronically increased adenosine levels in the lungs of these patients.[107] Patients with asthma or COPD have higher expression of A_3R in lung eosinophils, also suggesting a role for this receptor in the pathophysiology of eosinophil-related disorders.[62] Finally, Selman *et al.* reported on the genetic changes in the accelerated variant of idiopathic pulmonary fibrosis and found increased expression of the $A_{2B}R$ gene.[108]

The development of human adenosine-based effective therapies for respiratory diseases has been disappointing so far. EPI-2010, an A_1R respirable antisense oligonucleotide (RASON) was ineffective in a phase II on moderate/severe persistent asthma.[109] Polosa and Blackburn argue

that some concerns over the use of antisense oligonucleotides in humans may have led to the use of sub-therapeutic doses.[110] The use of $A_{2A}R$ agonists has also been proposed as a treatment for asthma, but CGS21680 causes hypotension in animal models, precluding its systemic use in humans.[111] The use of inhaled $A_{2A}R$ agonists GW328267X, and UK432,097 partially resolved this, but they were nonetheless ineffective in phase II trials in asthma[112] and COPD.[113] Importantly, there is still the possibility that sub-therapeutic doses were used, as no cardiovascular effects were noted.[110] Further adenosine-based clinical studies are ongoing, including a phase III study on an oral A_1R antagonist PBF-680 for asthma[114] as well as phase I study on an $A_{2B}R$ antagonist for IPF.[115]

Bronchoalveolar lavage as a clinical and research tool

Bronchoalveolar lavage (BAL) is a low risk technique for the collection of both cellular and acellular components of the lower respiratory tract.[116] The procedure is usually performed through bronchoscopy under conscious sedation and consists on wedging the bronchoscope tip on a 5th or 6th order bronchus, followed by instillation and immediate aspiration of a small amount of warm saline. This allows for the sampling of about one million alveoli, with collection of cells and solutes. BAL is frequently performed for diagnosis of suspected interstitial lung disease. The differential cell count from the collected fluid is helpful for establishing a specific ILD diagnosis, when conjugated with clinical, imaging, and in some cases, biopsy data. The procedure is generally safe and the main concerns are the development of post-BAL transient fever and/or loss of lung function.[117]

Apart from clinical applications, BAL has been extensively used for research.[118] Being a safe and widely available way of evaluating the lower respiratory tract, BAL has been instrumental for the study of the immune mechanism of the lung during health and disease. An important advantage of the wide clinical use of the technique is that part of the collected sample can be used for cell or solute research without the need to perform specific procedures on the patient. Specifically concerning the development of new therapies for inflammatory lung diseases, the use of BAL may be useful for the identification of new therapeutic targets.[119] This results from the fact that live, unchanged cells can be collected and tested for the presence and effects of drug targets using both morphological and dynamic studies, such as the one that was applied in the current project.

Objectives

The inflammatory lung disorders constitute a major unmet health need, as currently available therapies are still limited, while their associated morbidity and mortality are increasing.

The modulation of adenosine receptors is a particularly promising strategy for the treatment of inflammatory lung disorders, but the implementation of this approach in human disease is very limited. One of the main reasons for this are the difficulties in the translation of the positive and promising results from preclinical studies to human applications, probably related to the inter-species differences in adenosine receptors distribution and effects.

The main objective was the study of the effects of the $A_{2A}R$ in human disease. This was achieved using a translational approach, coupling a clinical and epidemiological analysis with the pharmacological laboratory study of unchanged and uncultured human cells.

Three main strategies were followed. Caffeine, a widely consumed component of several beverages is a non-selective modulator of A_1R and $A_{2A}R$.[120] The aim was to study the effects of caffeine on respiratory disease allowing for a non-interventional assessment of the modulation of adenosine receptors on the risk and evolution of human respiratory disease. Next, the levels of an adenosine precursor in the lung were quantified, allowing to estimate the expected endogenous activation of adenosine receptors in human respiratory disease. Finally a laboratory method was developed to evaluate the anti-inflammatory effects of adenosine receptors in human cells with a role in the pathogenesis of inflammatory pulmonary disease. The use of a robust marker of cell activation in fresh cells, that are essentially free of manipulation allows for a simple translation of these results to human clinical trials.

Our ultimate objective is to provide a proof-of-concept for the use of adenosine receptor modulation in the treatment of human respiratory disease, by directly studying humans and human cells and thus overcoming the limitation of inter-species variation in the role of adenosine receptors.

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Chapter 2

Chronic coffee consumption and respiratory disease: a systematic review

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ORIGINAL ARTICLE

WILEY

Chronic coffee consumption and respiratory disease: A systematic review

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Abstract

Purpose: The widespread consumption of coffee means that any biological effects from its use can lead to significant public health consequences. Chronic pulmonary diseases are extremely prevalent and responsible for one of every six deaths on a global level.

Methods: Major medical databases for studies reporting on the effects of coffee or caffeine consumption on a wide range of non-malignant respiratory outcomes, including incidence, prevalence, evolution or severity of respiratory disease in adults were searched. Studies on lung function and respiratory mortality were also considered.

Results: Fifteen studies, including seven cohort, six cross-sectional, one case control and one randomized control trial were found. Coffee consumption was generally associated with a reduction in prevalence of asthma. The association of coffee with natural honey was an effective treatment for persistent post-infectious cough. One case-control study found higher risk of chronic obstructive pulmonary disease (COPD) with coffee consumption. No association was found with the evolution of COPD or sarcoidosis. Coffee was associated with a reduction in respiratory mortality, and one study found improved lung function in coffee consumers. Smoking was a significant confounder in most studies.

Conclusions: Coffee consumption was associated with some positive effects on the respiratory system. There was however limited available evidence, mostly from cross sectional and retrospective studies. The only prospective cohort studies were those reporting on respiratory mortality. These results suggest that coffee consumption may be a part of a healthy lifestyle leading to reduced respiratory morbidity.

KEYWORDS
asthma, caffeine, coffee, COPD, respiratory disease, systematic review

1 | INTRODUCTION

Chronic respiratory diseases cause a significant worldwide burden of disease. Most have an inflammatory component, serving as the main driver (eg, asthma), failing to contain (eg, lung cancer) or promoting damage (eg, infections) from the disease.[1] Several epidemiological studies have pointed to an effect of coffee consumption on the risk for development or progression of respiratory disorders.[2] Caffeine is the most widely consumed bioactive substance.[3] Despite being generally recognized as safe by the United States Food and Drug Administration (FDA), a number of studies have found associations between caffeine intake and the risk for disease.[4] However, preclinical studies found anti-inflammatory effects from caffeine consumption,[5] and an epidemiological analysis found a positive association between coffee consumption

and lung function.[6] This possibility is strengthened by findings of positive coffee-related health effects on other systems. Caffeinated coffee could prevent the neuroinflammation-induced acute and probably the long term neurodegenerative changes. In fact, several cross-sectional and longitudinal population-based studies suggested a protective effect of coffee, tea, and caffeine use against late-life cognitive impairment/decline, although the association was not found in all cognitive domains investigated.[7,8] The chronic consumption of coffee was also associated to a reduced risk of cardiovascular disease, including coronary heart disease, stroke and heart failure.[9]

The results from studies on the respiratory system tend to be scattered through different disciplines (respiratory physiology, nutrition, toxicology and respiratory medicine), hindering the development of clear evidence-based conclusions and recommendations regarding the effects of coffee and caffeine consumption on the risk for development and worsening of respiratory disease.

We performed a systematic review for the association between coffee and caffeine consumption and the risk for development and progression of non-malignant respiratory disease in adults. We intended to analyse the full range of effects of coffee and caffeine on the respiratory system, and so we included studies on a wide and heterogeneous range of respiratory diseases, as well as effects on lung function and mortality. The results from this analysis will improve our capacity for advising both patients and the general population on their coffee consumption habits.

2 | METHODS

2.1 | Data sources, search and study selection

A search was performed in Pubmed, Web of Science, SCOPUS and Embase for clinical studies evaluating the association between chronic coffee or caffeine consumption and any non-malignant respiratory outcome in adults. The complete search strategy can be consulted in the electronic Supporting Information. The Cochrane Central Registry of Controlled Trials, and the World Health Organization (WHO) International Clinical Trials Registry Platform were also searched using a similar strategy. We included published articles from any date (up to the end of august of 2016) and language that reported the association between caffeine or coffee consumption for more than seven days and any non-malignant respiratory outcome, such as incidence, prevalence, evolution or severity of respiratory disease, including death. After the initial search, the references were downloaded to a reference managing software (Endnote version X7, Thompson Reuters – The United States), and duplicate records were deleted. A selection of the relevant studies was performed by two independent authors (TMA and RM), based on the title and abstract. The full text of the selected articles was then downloaded and reviewed by the same authors. Any discordance was resolved by consensus. The references list of the selected articles and the author's personal files were also scanned. All the case reports, studies not reporting outcomes, animal studies, reviews and studies on paediatric population or on the acute effects of coffee or caffeine were excluded.

2.2 | Quality assessment and data extraction

Randomized controlled trials were assessed for risk of bias according to the recommendations from the Cochrane Handbook for Systematic Reviews of Interventions version 5.1.0, a domain based evaluation of seven domains.[10] For observational studies, The Newcastle–Ottawa Scale, a point based evaluation of eight components was used.[11] For cross-sectional studies the scale was adapted by removing the points for outcomes at the start of the study and follow-up. The data extraction was performed using an electronic database. The following data was recorded for each study: authors, year, journal, country, study design and population, including number, age, gender and follow-up. The specific exposure to coffee or caffeine and form of assessment were also recorded. The outcomes included incidence, prevalence, severity and mortality from respiratory disease. Lung function outcomes were also recorded.

2.3 | Data synthesis

In view of the major differences in exposures and outcomes between the included studies, the authors did not combine data from the studies.

3 | RESULTS

Our initial search strategy yielded a total of 5612 records after exclusion of duplicates. These included 5611 from databases (Pubmed, Web of knowledge, SCOPUS and Embase) and 1 from personal files scanning. Following the initial selection based on title and abstract, a total of 29 candidate studies were gathered. Upon evaluation of the full text, 14 of these were excluded. The reasons for exclusion are shown on Figure 1. One article was excluded as a it was subjected to a reanalysis because of methodological concerns for a fraction of the original data.[12,13] One of the trials on the effects of coffee and honey on persistent post-infectious cough,[14] included all the patients from two previous reports,[15,16] so these two were excluded. The final analysis included 15 studies. The main characteristics and results from the included studies can be consulted in Table 1. Most studies relied on the quantification of coffee consumption, and only four tried to quantify caffeine from various sources. One was a randomized controlled trial

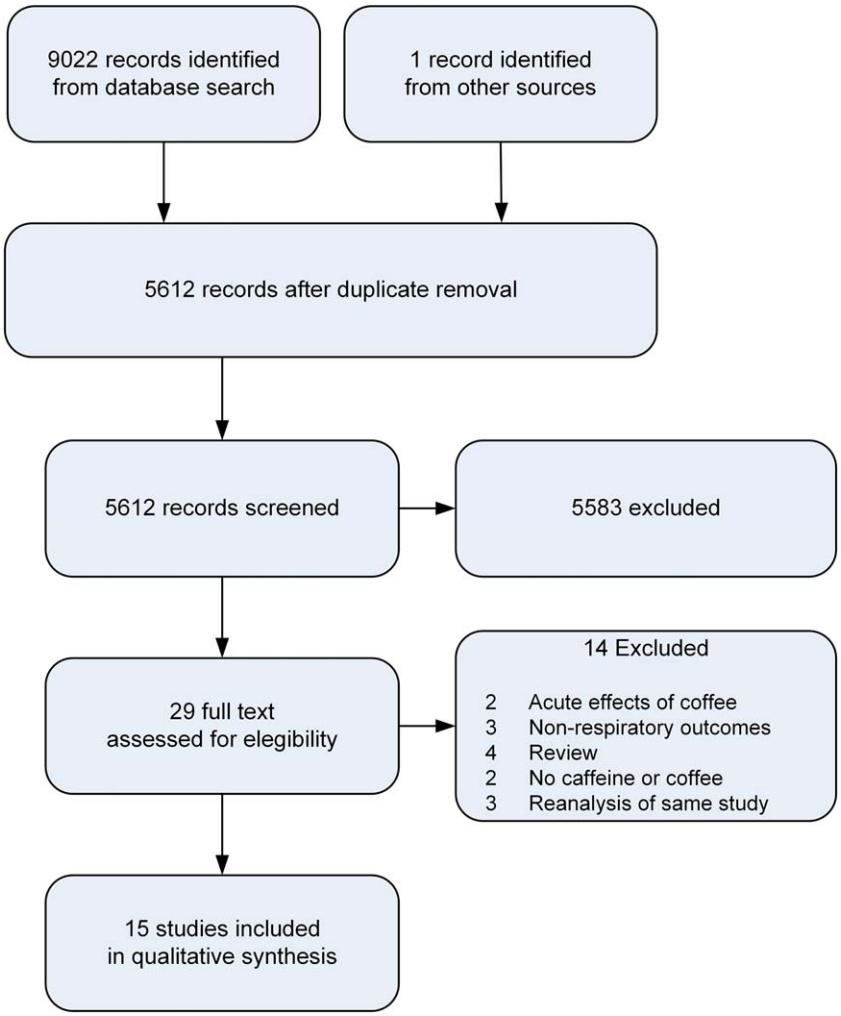

FIGURE 1 Literature search for the systematic review. The searched databases included Pubmed, Web of Science, SCOPUS and Embase. Other sources include personal files and references from other articles. The reasons for exclusion after full-text analysis are presented

on the use of caffeine and honey to treat persistent cough and fourteen were observational, including seven cohort, six cross-sectional and one case-control study. On the observational studies, the outcomes included single-point lung function, risk (asthma and COPD), evolution (sarcoidosis and COPD), severity (asthma) and mortality from respiratory disease.

3.1 | Asthma

We found three studies assessing the association between coffee consumption and asthma. Pagano et al. analysed a large representative sample of the general Italian population, and found a significant reduction in the prevalence of asthma in coffee consumers.[17] This effect showed some dose dependency up to three cups of coffee/day. The authors estimated a reduction of 5% prevalence for one cup and over 20% for two or more cups of coffee per day. Annesi et al. reported a similar study, in a sample of general French population.[18] They found no association, but the sample included less than 100 asthmatics (out of 16,220 subjects). In the same article, Annesi reports no association between coffee consumption and prevalence of bronchial hyperreactivity in a sample of 323 male French policemen from a 1985 study.[19] Finally, an American study by Schwartz et al. used the data from NHANES II, a random sample of American population.[20] They also found a significantly reduced risk of having asthma in coffee drinkers, with some signs of a dose-response pattern.

Two studies from a Californian group evaluated asthmatics for the use of caffeine as self-treatment. The first study found that 6% of a sample of 482 asthmatics under specialist care reported using coffee or black tea as self-treatment in the previous year.[12,13] This use was associated with an increased severity of disease and significantly higher probability of repeated emergency visits and hospitalization. The second study recruited patients with asthma or rhinitis from a population based random sample and found that 26% of the asthmatics had used caffeine (coffee or black tea) to treat breathing or nasal symptoms in the previous year.[21] The investigators reported a small, non-significant, negative effect on the health status of the patients with asthma or

TABLE 1 Main characteristics from the included studies

Nr	Author year	Country	Study type (follow-up)	Population Setting	Exposure	Outcome	Results		Adjustments	Quality
1	Pagano et al.[17] 1999	Italy	Cross-sectional	Gen. population N = 72,284	Dietary coffee	Asthma prevalence	Cups/day 0 1 2 ≥3	Relative risk 1 0.93 (0.83–1.04) 0.79 (0.69–0.91) 0.78 (0.69–0.88)	Age, gender, smoking, geographical area, education and alcohol consumption.	S: 3 C: 2 O/E: 0
2	Annesi et al.[18] 1990	France	Cross-sectional	Gen. population N = 16,220	Dietary coffee or tea	Asthma prevalence	Cups/day 0 1 2 ≥3	Odds ratio 1 1.03 0.86 0.96	N/A	S: 2 C: 0 O/E: 0
3	Schwartz et al.[20] 1992	USA	Cross-sectional	Gen. population N = 12,095	Dietary coffee	Asthma prevalence	Cups/day 0 1 2 ≥3	Odds ratio 1 0.84 (0.60–1.17) 0.66 (0.47–0.92) 0.70 (0.53–0.93)	Age, gender, smoking and race.	S: 3 C: 2 O/E: 0
4	Blanc et al.[12,13] 1997/2000	USA	Cross-sectional	Asthmatics Specialized care N = 482	Self-treatment with coffee or black tea	≥2 ER visits in 12 months Hospitalization in 12 months	Users Users	OR: 3.4 (1.4–8.5) OR: 2.9 (1.0–7.8)	Age, gender, marital status, education, income, severity of asthma, general health status and atopy.	S: 3 C: 1 O/E: 0
5	Blanc et al.[21] 2001	USA	Cross-sectional	Asthma or rhinitis pts Gen population N = 300	Self-treatment with caffeinated products	Differences in SF-12	SF-12 dimension Physical Mental	Mean difference 2.8 1.0	N/A	S: 3 C: 2 O/E: 0
6	Hirayma et al.[22] 2012	Japan	Case-control	COPD pts Specialized care Community controls N = P:277; C:340	Dietary coffee	Risk of COPD	mL/day < 30 30 to 140 > 140	Odds ratio 1 1.37 (0.82–2.28) 1.68 (1.02–2.75)	Age, gender, smoking, BMI, disease, education, and alcohol.	S: 3 C: 2 O/E: 2
7	Lopes et al.[23] 2015	Portugal	Retrospective cohort (3y)	COPD pts Specialist care N = 90	Continuous dietary caffeine	Self-reported exacerbations/ year	Correlation Caffeine (mg/day)/ exacerbations (n/year)	$P > .05$	Age, gender, smoking, FEV1, oxygen therapy, hypertension, stroke and peptic ulcer disease.	S: 3 C: 2 O/E: 1

(Continues)

TABLE 1 (Continued)

Nr	Author year	Country	Study type (follow-up)	Population Setting	Exposure	Outcome	Results		Adjustments	Quality
8	Costa et al.[24] 2011	Portugal	Retrospective cohort (5.1y)	Sarcoidosis patients Specialist care N = 46	Continuous dietary caffeine	Continuous mean loss of FVC	Correlation Caffeine (mg/day)/loss of FVC(%/year)	$P > .05$	Smoking and drug treatment.	S: 3 C: 2 O/E: 2
9	Raeessi et al.[14] 2014	Iran	RCT (1w)	Patients with persistent cough Specialist care N = 205 (28 + 24 + 97 + 30 + 26)	Honey vs Coffee vs coffee + honey vs steroid vs placebo	Cough frequency	Arm	Cough at end of study No (0), low (1), moderate (2), high (3) mean (SD)	N/A	–
							62.5 g of honey/day	1.4 (0.5)		
							8.75 g coffee/day	1.8 (1.3)		
							8.75 g coffee + 62.5 g honey/day	0.2 (0.5)		
							40 mg prednisolone/day	2.4 (0.6)		
							Placebo	2.7 (0.5)		
10	Nettleton et al.[6] 2009	USA	Cross-sectional	Gen. population N = 10,658	Dietary coffee	Lung function	Cups/day	FEV1 in L (SE)	Age, gender, smoking, race, activity, BMI and centre.	S:3 C: 2 O/E: 1
							0	2.96 (0.01)		
							<1	3.00 (0.01)		
							1	2.97 (0.01)		
							2 to 3	2.99 (0.01)		
							≥4	3.02 (0.01)		
11	Freedman et al.[25] 2012	USA	Prospective cohort (14y)	Gen. population N = 402,260	Dietary coffee	Risk of respiratory mortality	Cups/day	HR in males	Age, smoking, BMI, race, education, alcohol, health status, diabetes, marital status, physical activity, diet, vitamins and hormone therapy.	S: 3 C: 2 O/E: 2
							0	1		
							<1	1.05 (0.87–1.27)		
							1	0.93 (0.77–1.11)		
							2 to 3	0.83 (0.70–0.98)		
							4 to 5	0.83 (0.69–1.00)		
							≥6	0.81 (0.65–1.00)		
							Cups/day	HR in females		
							0	1		
							<1	1.09 (0.91–1.31)		
							1	0.84 (0.69–1.01)		
							2 to 3	0.79 (0.67–0.93)		
							4 to 5	0.65 (0.53–0.79)		
							≥6	0.77 (0.61–0.99)		

(Continues)

TABLE 1 (Continued)

Nr	Author year	Country	Study type (follow-up)	Population Setting	Exposure	Outcome	Results		Adjustments	Quality
12	Odegaard et al.[26] 2015	Singapore	Prospective cohort (15.1y)	Gen. population N = 52,584	Dietary coffee	Risk of respiratory mortality	Cups/day 0 <1 1 ≥2 Cups/day 0 <1 1 ≥2	HR in ever smokers 1 0.99 (0.74–1.32) 0.99 (0.8–1.11) 1.06 (0.86–1.3) HR in never smokers 1 0.75 (0.57–0.99) 0.76 (0.62–0.92) 0.66 (0.53–0.82)	Age, gender, dialect, education, year of interview, activity, sleep, BMI, hypertension, and diet.	S: 3 C: 2 O/E: 3
13	Saito et al.[27] 2015	Japan	Prospective cohort (18.7y)	Gen. population N = 90,914	Dietary coffee	Risk of respiratory mortality	Cups/day 0 <1 1 to 2 3 to 4 ≥5	HR 1 0.78 (0.64–0.94) 0.63 (0.50–0.79) 0.60 (0.41–0.88) 0.79 (0.45–1.40)	Age, gender, smoking, area, alcohol, BMI, hypertension, diabetes, exercise, diet and job status.	S: 3 C: 2 O/E: 3
14	Ding et al.[28] 2015	USA	Three prospective cohort studies, NHS, NHS2 and HPFS	Female nurses N = 167944 NHS and NHS2 Male health professionals N = 40557 HPFS	Dietary coffee	Risk of respiratory mortality	Cups/day 0 ≤1 1.1–3.0 3.1–5.0 >5 Cups/day 0 ≤1 1.1–3.0 3.1–5.0 >5	HR 1 0.9 (1.77–1.05) 0.84 (0.73–0.97) 1.04 (0.89–1.22) 1.32 (1.10–1.58) HR in never smokers 1 0.91 (0.67–1.23) 0.88 (0.66–1.19) 0.94 (0.65–1.36) 0.62 (0.3–1.31)	Age, hypertension, hypercolesterolemia, diabetes, BMI, physical activity, diet, smoking, alcohol, menopause, hormone use and caffeinated/ decaffeinated coffee	S: 2 C: 2 O/E: 3

(Continues)

TABLE 1 (Continued)

Nr	Author year	Country	Study type (follow-up)	Population Setting	Exposure	Outcome	Results		Adjustments	Quality
15	Loftfield et al.[29] 2015	USA	Prospective cohort (9y)	Gen. population N = 90,317	Dietary coffee	Risk of mortality from chronic respiratory diseases	Cups/day	HR	Age, sex, smoking, race, education, marital status, employment, diabetes, BMI, vitamin, hormone therapy, ibuprofen, aspirin, alcohol and diet	S: 3 C: 2 O/E: 3
							0	1		
							<1	0.93 (0.64–1.35)		
							1	0.73 (0.48–1.08)		
							2 to 3	0.78 (0.57–1.08)		
							≥4	0.68 (0.48–0.97)		
						Risk of mortality from influenza and pneumonia	Cups/day	HR		
							0	1		
							<1	1 (0.63–1.59)		
							1	1.11 (0.70–1.75)		
							2 to 3	0.82 (0.54–1.25)		
							≥4	0.55 (0.32–0.95)		

Values within parenthesis correspond to 95% confidence interval. Studies are ordered by endpoint. N/A: non-available; w: week; m: month; y: year; gen.: general; pts: patients; ctr: controls; OR: odds ratio; HR: hazard ratio; N/A: not available; ER: emergency room; SF-12: short form health survey 12, a simplified health related quality of life (HRQoL) score. Higher values indicate better HRQoL; USA: United States of America; COPD: chronic obstructive pulmonary disease; BMI: body mass index; CV: cardiovascular; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; RCT: randomized control trial; SD: standard deviation; SE: standard error; Quality column: S: selection; C: comparability; O/E: outcome/exposure.

rhinitis using caffeine for self-treatment, but no association between this practice and self-reported disease severity.

3.2 | COPD

We found two studies evaluating the effects of caffeine on COPD. A Japanese case control study including 277 patients and 340 controls found a positive association between caffeine/coffee consumption and the prevalence of COPD.[22] The authors adjusted the analysis for smoking status (current, former or never smoker), but not for duration or number of smoked cigarettes per day. A recent Portuguese study found no correlation between caffeine consumption and the risk for exacerbations on a cohort of 90 patients with COPD.[23] Unlike in most other studies, patients were asked for caffeine consumption in a long time frame (20 years).

3.3 | Sarcoidosis

We only found one study focusing on the effect of caffeine on sarcoidosis. Costa et al. enrolled 46 sarcoidosis patients and probed for correlations between chronic caffeine consumption and the evolution of lung function and radiological abnormalities.[24] Both analyses were negative, before and after adjustment for major possible confounders. The authors also compared caffeine consumption between the patients and a sample from the general population and found no differences. Finally, there was a tendency for reducing caffeine consumption upon diagnosis. The conclusions from this study are limited by its small sample size and retrospective nature.

3.4 | Persistent post-infectious cough

Raeessi et al. reported a randomized controlled trial using caffeine and honey for the treatment of persistent post-infectious cough.[14] This trial included all patients previously reported by the same group,[15,16] and compared the use of coffee associated with honey, coffee, honey, systemic steroid and placebo (guaifenesin) for the treatment of persistent post-infectious cough. A total of 205 patients were randomized between 2003 and 2011. A per-protocol analysis found higher effectiveness in the group assigned to coffee associated with honey. The mechanism for this effect is not clear, but the authors emphasized the low cost and availability of this intervention.

3.5 | Lung function

Nettleton et al. conducted a cross-sectional analysis of the Atherosclerosis Risk in Communities (ARIC) study, a large population based cohort study.[6] They found a positive association between coffee consumption and improved lung function, before and after adjustment for major confounders. Upon stratification by smoking status, this effect was only present in long term quitters and non-smokers. The authors hypothesized that smoking could interfere with the effects of caffeine in lung function through accelerated metabolism of caffeine or dampening of the anti-oxidant effects of coffee. Alternatively, smoking may overwhelm any beneficial effects of coffee on lung function.

3.6 | Respiratory mortality

The effects of coffee on respiratory mortality have been assessed by five recent studies. Freedman et al. analysed the data from the NIH-AARP Diet and Health Study, including over 400,000 subjects and more than 50,000 registered deaths.[25] They found a reduced risk of death because of respiratory causes in coffee drinkers. Furthermore, this decrease was observed in both caffeinated and decaffeinated coffee, and was not changed upon stratification by smoking status (P value for interaction = 0.14). The primary endpoint was all-cause mortality and this was also positive, with lower all-cause mortality for coffee drinkers. In another prospective cohort study, Odegaard et al. followed 52,584 subjects for a mean of 15.1 years, registering 10,029 deaths.[26] A reduced respiratory mortality upon consumption of coffee and black tea was only observed in never-smokers. Again, the results for all-cause mortality were similar, with reduction only in never-smoking coffee and black tea drinkers. A third study, reported by Saito et al, was performed in Japan and included 90,914 participants, with a mean follow-up of 18.7 years.[27] An inverse relationship between coffee consumption and respiratory mortality was found. Following stratification by gender and smoking status, this effect was confirmed in smoking men, but not in never-smoking men. Importantly, very few deaths were recorded in never-smoking males. Interestingly, the authors performed an analysis on adding sugar to coffee, and found no changes in mortality.

Ding et al. analysed the association between coffee consumption and overall and specific-cause mortality in three large prospective cohort studies, the Nurse Health Study (NHS) and Nurse Health study 2 (NHS2), which involved only female nurses, and the Health Professionals Follow-up Study (HPFS), which involved only male health professionals.[28] The study included a total pooled population of 208,501 subjects with 31,956 deaths. A positive association between coffee and mortality from respiratory disease was reported by the authors, although the statistical analysis showed non-linearity for this association. The authors also found a non-linear association between coffee consumption and all-cause mortality, with lower risk in consumers of one to five cups per day and higher risk on those consuming more than five cups per day, when compared with non-

consumers. The authors hypothesized that this non-linear association could be the result from residual confounding by smoking and repeated the full analysis restricting for never smokers. This revealed a linear negative association between coffee and all-cause mortality and no significant association with respiratory mortality. Importantly, only 385 deaths from respiratory disease were recorded in the never-smoking subjects, and the analysis for respiratory mortality was not adjusted for gender. For the overall mortality, the results were reported for the both the pooled population as well as for each study, which were gender restricted.

Loftfield et al. reported the association between coffee consumption and overall and cause-specific mortality on the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial, a large population-based US cohort study.[29] A total of 90,317 participants were analysed, corresponding to 8,718 deaths. The authors chose to report separately on the ten leading causes of death in the United States, including mortality from chronic lower respiratory disease and from influenza/pneumonia. They found a negative association between coffee and both of these causes. The analysis for overall mortality was also positive. A separate analysis for decaffeinated coffee showed similar results in magnitude and direction, but did not reach statistical significance, probably because of a small number of decaffeinated coffee consumers in the studied population.

3.7 | Quality assessment

The results of the quality assessment of the included studies are also shown on Table 1. Separate scores for patient selection, comparability and outcome/exposure are presented for cohort, case-control and cross-sectional studies. The randomized trial by Raeessi et al. was considered to have a low risk of bias for all domains except the risk of incomplete outcome data. A detailed assessment of quality for each study is shown in Tables S1 to S4 in the Supporting Information.

4 | DISCUSSION

Our main finding was that coffee seems to be associated with positive effects on the respiratory system. Coffee consumption was associated with lower prevalence of asthma and was an effective treatment for persistent cough, when combined with honey. Interestingly, coffee is also frequently used for self-treatment of asthma (even in patients with access to medical care). The data on COPD, a major cause of disability and death, are scarce and do not point to protection. The study on sarcoidosis was fairly small and the negative results should be interpreted with caution. Finally, studies found an association between coffee consumption, improved lung function and reduced mortality from respiratory disease.

An important caveat is that only a small number of studies on chronic caffeine and coffee consumption and respiratory health are available, most of which are cross sectional or retrospective cohorts. There are also some inconsistent findings, and no longitudinal data. The only prospective studies were those on mortality and there was only one randomized control trial reporting on persistent post-infectious cough.

Some results were partly discordant. The studies of Pagano et al. and Schwartz et al. showed reduced asthma prevalence in coffee drinkers, but Annesi et al. found no such association in a French cohort. One possible explanation for these negative results is the low prevalence of asthma and shorter follow-up in the French study. Considering the findings by Blanc et al, of worse asthma disease in patients using caffeine as self-treatment, we argue that this effect may be related to patients using caffeine instead of proven more effective therapies. However, these studies were retrospective, and causality was not proven.

Importantly, this systematic review used a functional classification of disease, considering asthma as an obstructive lung disease. Allergy, however, is a major component of the pathophysiology of asthma,[30] and several immune and non-immune cell types with a role in allergic inflammation display adenosine receptors.[31] It is therefore possible that caffeine, acting as an adenosine receptor antagonist, may modulate allergic inflammation and lead to changes in the risk or evolution of asthma. Interestingly, there have been reports of allergy to caffeine, and some authors even suggest that caffeine consumption may contribute to chronic idiopathic urticaria.[32] In summary, it is probable that the effects of caffeine consumption on asthma are quite complex, acting as a bronchodilator, a modulator of allergic inflammation and in some cases as an allergen.

Considering respiratory mortality, we found some heterogeneous results, but a global reduction in respiratory mortality in coffee drinkers was observed, especially in non-smokers. Residual confounding by smoking, even after extensive adjustment as was performed in the study by Ding et al. is an important issue for these studies.

This proposed overall benefits of coffee may come from several mechanisms. Caffeine, the major pharmacologically active component of coffee, is a non-selective adenosine receptor antagonist and has been associated with significant systemic anti-inflammatory effects.[33,34] These chronic effects may be mediated by changes in adenosine receptor density, as has been shown in pre-clinical studies.[35] Caffeine is also a mild bronchodilator, and this could explain the effects of coffee on the prevalence and treatment of asthma.[2] Other components of coffee and tea may be active, including polyphenols and other anti-oxidants.[36] In fact, some studies showed similar benefits from caffeinated and non-caffeinated coffee, suggesting that caffeine is not the only important factor.[37]

The major confounder for the studies on the effects of coffee is smoking. Cigarette smoking is a major worldwide driver of respiratory morbidity and mortality, and studies have shown increased coffee consumption in both current and former smokers.[38,39] Smoking can impact the effects of coffee consumption on respiratory disease in several ways. Active smokers have an increase in hepatic metabolism and reduced serum levels of caffeine for the same level of consumption, thus reducing the effects from caffeine in coffee.[40] Smoking leads to a robust oxidative stress and inflammatory environment,[41] which can overwhelm any possible anti-inflammatory and anti-oxidative properties of coffee. Finally, most studies perform adjustments for smoking based on self-reporting by subjects. It is possible that subjects underreport the socially undesirable habit of smoking while accurately reporting the more acceptable habit of coffee drinking, leading to bias. Also, some studies adjust for smoking based only on classification on current, former or never smoker, and do not take account of the duration and intensity of this habit.

This is the first systematic review of the effects of coffee and caffeine on respiratory disorders as a group. We used a wide search strategy in order to identify all available studies on the effects of coffee and caffeine on respiratory disorders. These results may be helpful in guiding clinicians when advising respiratory patients on health promoting behaviour.

A number of limitations should however be discussed. Since most of the included studies are non-interventional, the reported associations do not necessarily mean causation. It is possible that some reported positive effects of coffee are because of reverse causation, with healthy subjects drinking more coffee. In fact, the study by Costa et al. showed that patients with sarcoidosis seem to reduce caffeine consumption upon diagnosis, probably because of the widespread notion that caffeine is part of an unhealthy lifestyle.[24] The positive effects of decaffeinated coffee in some studies may mean that other components of coffee are causing the effects on health, while other sources of caffeine (tea, caffeinated soda, chocolate) may be less relevant. Most studies analyse only baseline consumption of caffeine and smoking, and do not adjust for changing habits, another possible source of bias. The possibility of publication bias has to be considered, as it is possible that negative results were not submitted or published. Although this review is centred on the effects of caffeine and coffee on a single organ system, it is possible that the reported results reflect both systemic (anti-inflammatory and anti-oxidative) and local (bronchodilator) effects of caffeine and coffee. Specifically, none of the included mortality studies focused only on respiratory disorders and most showed similar results for all-cause and respiratory mortality. Furthermore, only some causes of respiratory mortality were included, and only the main cause of death was considered. We used the Newcastle–Ottawa Scale (NOS) for assessing the quality of the selected observational studies, but this scale is still under evaluation and to our best knowledge there is no published report on its validation. We stress, however, that recent systematic reviews on respiratory disease have used the same strategy.[42,43]

In conclusion, this systematic review has shown significant positive effects of coffee consumption on the respiratory system, including better lung function, lower prevalence of asthma and lower respiratory mortality. One Japanese case-control study suggested a positive association between caffeine intake and the development of COPD. There was relatively limited evidence, mostly from cross-sectional and retrospective cohort studies. These were mainly non-interventional, and no causal inference can be made. Although there is a need for further studies, currently available data argue for a global benefit from coffee consumption in respiratory patients or those at risk for respiratory disease. Chronic respiratory disorders are extremely frequent and a major cause of disability and death. Our results suggest that coffee consumption may be a part of a healthy lifestyle leading to reduced respiratory morbidity.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

TMA wrote the protocol, performed the initial search, selected included studies, extracted data and wrote the first draft. RAM reviewed the protocol, selected included studies, and reviewed the first draft. RAC reviewed the protocol and corrected the manuscript. CRC supervised the whole project, reviewed the protocol and corrected the final manuscript.

ETHICS

The manuscript does not contain clinical studies or patient data.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

TABLE S1 Cohort studies
TABLE S2 Case-control studies
TABLE S3 Cross-sectional studies
TABLE S4 Randomized control trial

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Supplementary data

Title: Chronic coffee consumption and respiratory disease: a systematic review

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Search strategy for EMBASE

1. “Respiratory disease”/exp
2. “Pulmonary disease”/exp
3. Asthma/exp
4. COPD/exp
5. Pneumonia/exp
6. "Pulmonary fibrosis"/exp
7. Tuberculosis/exp
8. Emphysema/exp
9. “Lung function“/exp
10. “Pulmonary function“/exp
11. #1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10
12. Caffeine
13. Coffee
14. #12 OR #13
15. #11 AND #14

Detailed quality assessment

Table 1 - Cohort studies

Number	Author	Selection				Comparability	Outcome		
		Exposed	Non-exposed	Ascertainment	Outcome not at start		Assessment	Follow-up duration	Follow-up adequacy
7	Lopes *et al* 2015[1]	1	1	1	0	2	0	0	1
8	Costa *et al* 2011[2]	1	1	1	0	2	1	1	0
11	Freedman *et al* 2012[3]	1	1	0	1	2	1	1	0
12	Odegaard *et al* 2015[4]	1	1	1	0	2	1	1	1
13	Saito *et al* 2015[5]	1	1	0	1	2	1	1	1
14	Ding *et al* 2015[6]	0	1	0	1	2	1	1	1
15	Loftfield *et al* 2015[7]	1	1	0	1	2	1	1	1

Table 2 - Case-control studies

Number	Author	Selection				Comparability	Exposure		
		Case definition	Case representativeness	Control selection	Control definition		Ascertainment	Case/ control method	Non-response
6	Hirayma *et al* 2012[8]	0	1	1	1	2	0	1	1

Table 3 - Cross-sectional studies

Number	Author	Selection			Comparability	Outcome
		Exposed	Non-exposed	Ascertainment		
1	Pagano *et al* 1999[9]	1	1	1	2	0
2	Annesi *et al* 1990[10]	1	1	0	0	0
3	Schwartz *et al* 1992[11]	1	1	1	2	0
4	Blanc *et al* 1997/2000[12,13]	1	1	1	1	0
5	Blanc *et al* 2001[14]	1	1	1	2	0
10	Nettleton *et al* 2009[15]	1	1	1	2	1

Table 4 - Randomized control trial

Number	Author	Random sequence generation	Allocation concealment	Blinding of participants	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other bias
9	Raeessi *et al* 2014[16]	Low risk	Low risk	Low risk	Low risk	Unclear risk	Low risk	Low risk

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Chapter 3

Caffeine consumption and exacerbations of chronic obstructive pulmonary disease: retrospective study

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ORIGINAL ARTICLE

Caffeine consumption and exacerbations of chronic obstructive pulmonary disease: Retrospective study

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Coffee;
Respiratory Tract Diseases

Abstract
Background: The modulation of adenosine receptors has been proposed as new therapeutic target for chronic obstructive pulmonary disease, but studies in humans were negative. Caffeine is widely consumed and acts by non-selective modulation of these receptors, allowing for a non-interventional evaluation of the purinergic effects on COPD. We evaluated the effects of chronic caffeine consumption on the risk for COPD exacerbations.
Methods: Retrospective study including patients with COPD. The total number of exacerbations during a three-year period and the mean daily caffeine consumption in the last twenty years were evaluated. A univariate and multiple regression analysis were performed for evaluation of the significant predictors of exacerbations.
Results: A total of 90 patients were included. Most were males (82.2%) and had a mean forced expiratory volume in the first second (FEV1) of 57.0 ± 17.1% predicted. The mean daily caffeine consumption was 149.7 ± 140.9 mg. There was no correlation between the mean caffeine consumption and exacerbations ($p > 0.05$).
Discussion: Our results suggest that caffeine has no significant effect on the frequency of COPD exacerbations. These conclusions are limited by the sample size and the retrospective nature of the study.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive, non-reversible airflow obstruction, leading to disability and premature death. Exacerbations are frequent episodes of increasing symptoms and inflammation, besides a major driver of morbidity and

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mortality from the disease. Most exacerbations are caused by bacterial/viral infection or exposure to pollutants. Importantly, some patients seem to be especially prone to exacerbations (frequent exacerbator phenotype), and each episode increases the risk for a new one. COPD usually develops as a late complication of cigarette smoking, with biomass and occupational exposure having smaller roles.[1] The disease is highly prevalent, and the associated morbidity and mortality are growing. The World Health Organization estimates that COPD will be the third leading cause of worldwide death in 2030.[2] Current treatments have no significant effects on its progression, and so new therapies are urgently needed.[3]

One potential new therapeutic target is the modulation of purinergic receptors, which have been shown to have potent anti-inflammatory and immunomodulatory effects.[4] However, despite promising results in animal models, the clinical use of purinergic modulators in COPD has not shown efficacy, probably due to differences in receptor types and distribution among species.[5]

Caffeine, the most widely consumed psychoactive substance exerts its effects by non-selective antagonism of adenosine receptors.[6] This allows for an estimation of the potential of adenosine receptor modulation on COPD patients, by studying the consequences of caffeine intake. Our objective was to evaluate the effects of chronic caffeine consumption on the risk of exacerbations of COPD patients.

Materials and methods

We performed a retrospective study, including patients with COPD under follow-up at the outpatient consult of the Unit of Pneumology of an Academic Hospital in Portugal. The inclusion criteria were a diagnosis of COPD according to international guidelines[1] and performance of at least one post-bronchodilator spirometry in the three previous years. The exclusion criteria were a diagnosis of another pulmonary or systemic inflammatory disease, asthma, pregnancy and non-consent. Patients were selected from the outpatient clinics database and contacted by telephone. After oral consent was given, a standardized questionnaire was applied. The purpose of the interview was the estimation of mean daily caffeine consumption in the last twenty years, and the number of exacerbation in the last three years. Patients were questioned about the mean daily consumption of the main dietary caffeine sources: espresso = 100 mg, instant coffee (cup) = 60 mg, decaffeinated coffee = 3 mg, tea (leaves, herbs or berries) = 30 mg, instant tea (cup) = 20 mg, cola-drinks (300 ml can or bottle) = 18 mg.[7] The average daily consumption of caffeine was calculated by multiplying the amount of caffeine content in each source by its mean daily consumption. An exacerbation was defined as a period of worsening symptoms leading to urgent medical evaluation. The subjects were also asked about their demography, habits and clinical history, including comorbidities. The questions about comorbidities focused on hypertension, stroke and peptic ulcer disease as the study investigators considered that these diseases could impact caffeine consumption. Patient's clinical files were reviewed for data on COPD diagnosis and lung function tests.

The statistical analysis was performed using the STATA software package version 13.1 (StataCorp, USA). The continuous variables were characterized using measures of central tendency (mean) and distribution (standard deviation), and the categorical variables were characterized using proportions. The group differences in quantitative variables were tested with Student's t test for independent variables or Wilcoxon rank-sum test, according to normality, as tested by Shapiro–Wilk. For more than two groups, ANOVA or Kruskal–Wallis was used. The correlations were tested using Pearson or Spearman coefficient, depending on normality. The relationships between categorical variables were tested using chi-square. For the analysis of the effect of caffeine consumption on the frequency of exacerbations, a multiple regression model was built using a step-down procedure. A p-value < 0.05 was considered statistically significant. The sample size was also calculated using STATA. For a power of 0.8 and α-value of 0.05, a sample of 85 individuals was necessary to test for a correlation of 0.3 between caffeine consumption and exacerbation rate.

Results

An initial sample of 100 patients was contacted but 10 were excluded due to an alternative diagnosis (8) or concomitant systemic inflammatory disease (2). Our final sample included 90 patients. The demographical and clinical characteristics of the study population are described in Table 1. Patients were predominantly male, with a mean age of 73.0 ± 10.6 years. The majority were smokers (15.6%) or former smokers (46.7%), with a mean of 56.0 ± 33.2 pack years of tobacco lifetime exposure. Former smokers had quit 17.5 ± 12.0 years before this study. Concerning COPD severity, most patients had GOLD spirometric stage 2 disease, with a mean forced expiratory volume in the first second (FEV1) of $57.8 \pm 17.1\%$ predicted. A significant proportion (36.7%) were on long term oxygen therapy (LTOT). Comorbidities were common, including hypertension in 72.2% and stroke in 11.1% of patients. When comparing genders, we found differences in smoking history (31.3% of smokers or former smokers in females vs 68.9% in males, $p < 0.01$, chi square), and oxygen therapy (68.8% in females vs 29.7% in males, $p < 0.01$, chi-square). There were no other significant differences between genders in demographical or clinical features.

The patient's frequency of exacerbations can be seen in Table 2. Patients reported a mean of 2.33 ± 3.70 exacerbations during the three years of the study, with 0.74 ± 1.57 leading to admission. There was no significant correlation between age and the number of exacerbations ($p = 0.72$, Spearman). There was also no difference regarding gender ($p = 0.90$, Mann–Whitney), history of smoking ($p = 0.20$, Mann–Whitney) hypertension ($p = 0.59$, Mann–Whitney) and stroke ($p = 0.31$, Mann–Whitney). There was a trend for higher number of exacerbations for patients under LTOT ($p = 0.077$, Mann–Whitney), and an unexpected lower number of exacerbations in patients reporting a diagnosis of peptic ulcer disease (PUD) (mean of 0.95 ± 1.67 vs 2.73 ± 4.03, $p < 0.05$, Mann–Whitney). A

Table 1 Demographical and clinical characteristics of the study population. Values are expressed as mean ± standard deviation. FEV1: forced expiratory volume in the first second (% predicted); GOLD: global initiative for chronic obstructive lung disease (GOLD 1: FEV ≥ 80%, GOLD 2: 50 ≤ FEV1 < 80; GOLD 3: 30% ≤ FEV1 < 50%; GOLD 4: FEV1 < 30%).

	Total
Patients	90
Male n (%)	74 (82.2)
Age (years)	73.0 ± 10.6
Smoking status n (%)	
Current smoker	14 (15.6)
Former smoker	42 (46.7)
Non-smoker	34 (37.8)
Lifetime tobacco exposure (pack-years)	56.0 ± 33.2
FEV1 (% predicted)	57.8 ± 17.1
GOLD spirometric stage n (%)	
1	8 (8.9)
2	52 (57.8)
3	25 (27.8)
4	5 (5.6)
Comorbidities and complications n (%)	
Chronic respiratory failure under LTOTc	33 (36.7)
Hypertension	65 (72.2)
Stroke	10 (11.1)
Peptic ulcer disease	20 (22.2)

negative correlation was found between lung function and number of exacerbations, (r_S −0.37, $p < 0.001$, Spearman for % predicted FEV1). The analysis for exacerbations requiring admission led to similar results, except for a trend for less incidents in patients reporting history of peptic ulcer disease ($p = 0.054$, Mann–Whitney).

We then analyzed the consumption of caffeine in our population. The detailed data on caffeine consumption from our sample can be seen in Table 2. Patients reported a mean daily consumption of 149.7 ± 140.9 mg. There was no difference between genders ($p = 0.644$, Mann–Whitney). Subjects with a history of smoking reported higher caffeine consumption (177.1 ± 150.3 vs 104.5 ± 150.3 mg, $p = 0.014$, Mann–Whitney). There was no correlation between the number of pack-years smoked and caffeine consumption ($p = 0.35$, Spearman). Younger patients reported higher consumption (232.4 ± 188.7 for those younger than 65 years, 121.7 ± 107.9 for those between 65 and 80 years, and 129.7 ± 124.0 for those over 80 years, $p = 0.042$, Kruskal–Wallis). There were no differences in consumption between those under LTOT ($p = 0.345$, Mann–Whitney), with a history of stroke ($p = 0.292$, Mann–Whitney), hypertension ($p = 0.149$, Mann–Whitney) or PUD ($p = 0.525$ Mann–Whitney).

Finally, we examined the effects of caffeine consumption on the number of reported exacerbations in the last three years. On the univariate analysis, there was no correlation between caffeine consumption and either the total number of exacerbations ($p = 0.678$, Spearman) or the exacerbations requiring admission ($p = 0.822$, Spearman). On the multiple linear regression analysis, ulcer ($\beta = -1.53$, $p < 0.01$) and FEV1 ($\beta = 0.058$, $p < 0.05$) were found to explain 11% of the variability on exacerbations ($R^2 = 0.13$, $F(2,87) = 5.75$, $p < 0.01$). Caffeine consumption was not a significant predictor ($p > 0.05$).

Discussion

We report the first study evaluating the effects of caffeine consumption on the frequency of exacerbations in COPD patients. Our main finding was the lack of a significant effect of caffeine on this frequency, both before and after adjusting for major confounders. This is surprising, given that caffeine has been associated with an improved lung function in previous studies,[8] and lung function is a major predictor for the risk of COPD exacerbations.[9] Caffeine exerts its effects by the antagonism of adenosine receptors, potent modulators of inflammation in the lung and other organs.[4,6] There is evidence that the levels of adenosine are increased in patients with COPD,[10] and a number of preclinical studies suggest that adenosine receptor modulation may be an effective treatment for COPD.[5]

One possible explanation for these negative results is that caffeine consumption is only beneficial in non-smokers and long term former smokers. In fact, caffeine was only associated with better lung function in non-current smokers, and even former smokers who quit in the last 15 years seem to gain less from caffeine consumption than those who abandoned smoking earlier.[8] Some of the suggested mechanisms for this interaction include changes in caffeine metabolism that are induced by smoking, and an enhanced inflammation in smokers overwhelming the effects of caffeine. Another explanation for our results is confounding by smoking, a common problem in health studies of caffeine and disease.[11] The authors tried to overcome this with a multivariate regression analysis. COPD exacerbations may be characterized by a heightened or different form of inflammation that is resistant to the anti-inflammatory effects of caffeine, unlike stable disease. The found association between reported peptic ulcer disease and lower exacerbation rate was unexpected. In fact, previous studies have shown a positive association between PUD, worsening COPD[12] and mortality.[13] The 2015 edition of GOLD report recognizes that reflux is associated with an increased risk or exacerbations and poorer health status.[14,15] The mechanism for this association is not fully known. Our results may be caused by the self-report nature of these data and the use of anti-reflux therapies in patients who reported PUD, whereas other subjects may have non-identified, non-treated PUD or reflux.

This study has some limitations. The retrospective nature of the study and the self-reported nature of the clinical data may have induced bias. We evaluated caffeine consumption over a period of 20 years. Although some recall bias is to be expected from this questionnaire, subjects tend to maintain stable caffeine consumption throughout the years.

Table 2 Mean number of exacerbations in the last three years and mean daily caffeine consumption in the last 20 years according patient's epidemiological and clinical characteristics. LTOT: long term oxygen therapy; GOLD: global initiative for chronic obstructive lung disease (GOLD 1: FEV $\geq$ 80%, GOLD 2: 50 $\leq$ FEV1 < 80; GOLD 3: 30% $\leq$ FEV1 < 50%; GOLD 4: FEV1 < 30%).

	Exacerbations	*p*	Caffeine	*p*
Total	2.33 ± 3.70		149.7 ± 140.9	–
Gender		NS		NS
Male	2.27 ± 3.66		153.3 ± 140.8	
Female	2.63 ± 4.03		132.7 ± 144.6	
Age groups		NS		0.042
≤65 years	2.09 ± 2.19		232.4 ± 188.7	
>65 and ≤80 years	1.82 ± 3.19		121.7 ± 107.9	
>80 years	3.50 ± 5.26		129.7 ± 124.0	
Smoking status		NS		0.014
Never smoker	3.24 ± 4.95		104.5 ± 111.9	
Current/former smoker	1.79 ± 2.60		177.1 ± 150.3	
GOLD spirometric stage n (%)		0.002		NS
1	1.50 ± 2.62		202.8 ± 252.8	
2	1.58 ± 2.84		141.0 ± 125.1	
3	4.36 ± 5.02		151.8 ± 135.2	
4	1.40 ± 1.95		144.0 ± 117.8	
LTOT		NS		NS
Present	3.45 ± 5.03		121.2 ± 102.3	
Absent	1.68 ± 2.49		166.1 ± 157.5	
Hypertension		NS		NS
Present	2.23 ± 3.84		142.5 ± 144.9	
Absent	2.60 ± 3.38		168.2 ± 130.8	
Stroke		NS		NS
Present	1.20 ± 1.99		128.0 ± 175.4	
Absent	2.48 ± 2.85		152.4 ± 137.1	
Peptic ulcer disease		0.042		NS
Present	0.95 ± 1.67		128.0 ± 110.3	
Absent	2.73 ± 4.03		155.9 ± 148.6	

Other studies have successfully used a similar approach to estimate caffeine consumption in patients with Alzheimer's disease, as well as matched controls. [16] This was also a single-center study. The sample size was powered to test for a 0.3 correlation, and weaker associations may have been missed. Finally, we did not collect data on other variables that may have an influence on the risk for exacerbations, including patient's current treatment for COPD and coexistence of bronchiectasis. Some of the strengths of the study include a comprehensive assessment of the dietary sources of caffeine and a multivariate analysis for exacerbations, controlling for smoking and comorbidities.

In conclusion these results suggest that caffeine has no significant effects on the risk for exacerbations in COPD patients. These conclusions are limited by the sample size and the retrospective nature of the study. We propose that further studies of the use caffeine for COPD should focus on different clinical endpoints such as loss of lung function or quality of life.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.rppnen.2015.05.001.

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CENTRO DE PNEUMOLOGIA
Faculdade de Medicina da Universidade de Coimbra

CAFEÍNA E FREQUÊNCIA DAS EXACERBAÇÕES NA DOENÇA PULMONAR OBSTRUTIVA CRÓNICA

Questionário de Entrevista telefónica

Código do doente: _____

Data: ____/____/_____

Bom Dia/Boa Tarde.

O meu nome é Pedro Lopes estou a desenvolver um trabalho de investigação com o Dr. Tiago Alfaro no Serviço de Pneumologia dos HUC-Centro Hospitalar e Universitário de Coimbra sobre o consumo de cafeína e as doenças respiratórias. Gostaria de lhe pedir que respondesse a um questionário de 5 minutos sobre o seu consumo de cafeína e as crises da sua doença.

Todos os dados só servirão para estudo, pelo que garantimos o seu anonimato. Por favor tente lembrar-se e responda de um modo preciso.

Confirma que é seguido/a na consulta deste Serviço por DPOC/bronquite crónica/doença pulmonar?

Critério de inclusão:

- Doentes com DPOC que tenham realizado espirometria há menos de 3 anos.

Critérios de exclusão:

- Asma brônquica
- Doença inflamatória,
- Gravidez
- Não consentimento

Identificação e hábitos

Sexo:

- ☐ Masculino
- ☐ Feminino

Idade: ___ anos

Profissão: _____

É fumador?

- ☐ Sim
- ☐ Não

(se sim): **Há quanto tempo? _____**
Quantos maços em média por dia: ___

(se não):

Já alguma vez fumou?

- ☐ Sim — **Durante quantos anos fumou? ____**
  Quantos maços em média por dia? ____
- ☐ Não

Tem hipertensão arterial?

- ☐ Sim
- ☐ Não

Já teve algum AVC?

- ☐ Sim
- ☐ Não

Tem úlcera gástrica?

- ☐ Sim
- ☐ Não

Exacerbação: Evento de carácter agudo no curso natural da doença, caracterizado por uma mudança/agravamento nos sintomas habituais do doente, para além das variações normais do dia-a-dia, que pode resultar em necessidade de alteração na medicação habitual.

Pergunta?

- Quantas vezes se sentiu pior da sua doença respiratória e teve que consultar o médico (consulta ou urgência?)

Quantas vezes em 2010? _____exacerbações

Quantas obrigaram ao internamento? _____

Quantas vezes em 2011? _____exacerbações

Quantas obrigaram ao internamento? _____

Quantas vezes em 2012? _____exacerbações

Quantas obrigaram ao internamento? _____

Total dos últimos 3 anos? _____exacerbações

Quantas obrigaram ao internamento? _____

1- Tanto quanto se lembra, qual foi o seu consumo de bebidas com **cafeína,** nos respectivos anos:

Bebida	**Quantidade**	**1992**	**1993**	**1994**	**1995**	**1996**	**1997**	**1998**	**1999**	**2000**	**2001**
Café expresso	chávenas de café / dia										
Café instantâneo (inclui o "nescafé" misturado no leite)	chávenas de café / dia										
Descafeinado	chávenas de café / dia										
Chá (folhas, ervas, bagas)	chávenas de chá / dia										
Chá instantâneo	chávenas de chá / dia										
Bebida tipo "coca-cola" (Pepsicola, Spur-cola, etc)	lata ou garrafa 300ml/ dia										

Bebida	**Quantidade**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**
Café expresso	chávenas de café / dia										
Café instantâneo (inclui o "nescafé" misturado no leite)	chávenas de café / dia										
Descafeinado	chávenas de café / dia										
Chá (folhas, ervas, bagas)	chávenas de chá / dia										
Chá instantâneo	chávenas de chá / dia										
Bebida tipo "coca-cola" (Pepsicola, Spur-cola, etc)	lata ou garrafa 300ml/ dia										

Chapter 4

A preliminary study on the effect of caffeine consumption on the evolution of sarcoidosis

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A Preliminary Study on the Effect of Caffeine Consumption on the Evolution of Sarcoidosis

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Background: Sarcoidosis is a systemic granulomatous disease of unknown etiology that primarily affects the lungs. One commonly used therapy is methotrexate, an immunomodulating drug, acting through adenosine-mediated mechanisms. This led us to gauge the impact of caffeine, an antagonist of adenosine receptors, on the evolution of sarcoidosis. ***Methods:*** In a retrospective cohort study involving 46 patients diagnosed with sarcoidosis and followed up at the University Hospital of Coimbra, we ranked the evolution of sarcoidosis based on lung function (evaluated as the forced vital capacity) and computed tomography scan staging and applied a questionnaire to evaluate their estimated caffeine consumption over the past 20 years. ***Results:*** We found that the consumption of caffeine failed to modify the evolution of the disease, and this was not hindered either by smoking or drug therapy. Further, there was no difference in caffeine consumption between healthy individual and the patient before diagnosis of the disease. ***Conclusion:*** Overall, these preliminary findings suggest that caffeine consumption fails to affect the initiation or evolution of sarcoidosis, a conclusion that casts doubts on the interest of considering adenosine-based immunomodulatory strategies to manage sarcoidosis.

Introduction

Sarcoidosis is a systemic granulomatous disease that preferably involves the lungs; it can affect both genders and all ages, but is more frequent between the age of 20 and 39 years.[1] The cause of the disorder is still unknown, but environmental, genetic and immunologic factors are potentially responsible; accordingly, sarcoidosis is characterized by a Th1-like immune-inflammatory response involving activated macrophages and $CD4^+$ T lymphocytes.[2] Sarcoidosis is frequently asymptomatic, and in many cases diagnosed by routine chest X-ray; in other cases, dyspnea, dry cough, mucus, chest pain, or nonspecific constitutional symptoms occur.[1] Therapy for sarcoidosis, when required, is based on the use of oral corticosteroids, but their frequent long-term side-effects often lead to the prescription of immunomodulating drugs such as methotrexate.[3] Methotrexate is the most frequently used immunomodulating agent and its main mechanism of action is thought to involve indirect modulation of adenosine receptors.[4]

Adenosine is a purinergic nucleoside acting as a major STOP signal of the immune-inflammatory system through the activation of adenosine A_{2A} receptors.[5] The importance of this immunomodulation system is best illustrated by the current targeting of A_{2A} receptors to manage conditions as diverse as asthma, arthritis, organ transplant, cancer, or sepsis.[5] Notably, this adenosine modulation system is also targeted by the most widely consumed stimulating substance in the world, caffeine.[6]

Epidemiological studies have shown that chronic consumption of moderate doses of caffeine is associated with an improvement in lung function[7] and a symptomatic improvement in lung diseases such as asthma[8] or pulmonary emphysema.[9] The observation that caffeine can influence immune-inflammatory responses,[5] particularly in the lungs,[10] raises the possibility that caffeine consumption might have an impact on the evolution of sarcoidosis.

The purpose of this study is to gather preliminary information to gauge the impact of caffeine consumption on the clinical evolution of sarcoidosis. This was investigated in a retrospective cohort study including 46 patients with sarcoidosis and 49 controls, where their average consumption of caffeine during the 20 years before the study was evaluated and correlated to patient's clinical evolution, gauged using respiratory function and radiological data.

Methods

Enrolled cases and controls

This study enrolled 46 individuals of both genders, over 18 years old, smokers and nonsmokers, under different therapies, who attended the pulmonology outpatient consult at

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Table 1. Baseline Characteristics of Study Participants

		Patients	*Controls*
N		46	49
Male gender *n* (%)		26 (56.5)	21 (42.9)
Age	mean	48.2	41.71
	range	26–80	18–82
CT stage	I	6 (13.6)	—
at diagnosis*n* (%)	II	28 (60.9)	—
	III	6 (13.0)	—
	IV	4 (8.7)	—
Duration of disease Average years (range)		5.1 (1–34)	—

CT, computed tomography.

the University Hospital of Coimbra, and have been diagnosed with sarcoidosis according to 1999 ATS/ERS/WASOG recommendations. We also evaluated 49 healthy individuals of both genders, over 18 years old, with a similar average age to that of the patients' cohort. The average age, gender, staging, and follow-up of all individuals are summarized in Table 1. The individual characteristics of each of the patients can be accessed in the Supplementary Table S1 (Supplementary Data are available online at www.liebertonline.com/caf).

Estimation of caffeine consumption

The questionnaire used in this study was previously validated for the estimation of caffeine consumption in a Portuguese population[11]; here, it was applied to estimate the consumption of beverages containing caffeine (*Espresso* coffee, instant coffee, decaffeinated coffee, tea infusion, instantaneous tea, and cola-drinks) in cups or bottles per day in the last 20 years (1990–2009) while also registering sociodemographic characteristics such as gender, age, job, ethnic background, marital status, educational level, and dates of first symptoms and diagnosis. This questionnaire was completed through an interview, which was conducted face-to-face, or when this was not possible, by telephone, by two distinct researchers (I.C.C. and T.M.A.). All interviews to controls were performed face-to-face by one researcher (I.C.C.).

Caffeine intake was calculated by adding the estimated caffeine contents for the different consumed beverages. According to Barone and Roberts,[12] the following standardized values were used for caffeine contained in a cup or bottle of the sources–*espresso* coffee: 100 mg, instantaneous coffee: 60 mg, decaffeinated coffee: 3 mg; tea infusion: 30 mg, instantaneous tea: 20 mg, cola-drinks: 18 mg. The average daily intake of caffeine (mg/day) was estimated by calculating the cumulative intake of caffeine from all sources. The time interval used to estimate this average depended on the age of the individuals, since we only considered data from ages over 18 years, based on the social standard habits of coffee consumption in the Portuguese population.

Clinical evaluation

The clinical data were gathered through the analysis of the clinical files stored at the pulmonology outpatient consult of the University Hospital of Coimbra. This consisted of the staging of patients using either the chest X-ray (stage 0: normal; stage I: bilateral hilar lymphadenopathy [BHL]; stage II: BHL plus pulmonary infiltrates; stage III: pulmonary infiltrates [without BHL]; stage IV: pulmonary fibrosis) and/or the chest computed tomography (CT) using the Scadding criteria as well,[13] an analysis carried out by a qualified radiologist. Additionally, we evaluated the evolution of lung function for each patient by repeated measures of percent predicted forced vital capacity (FVC), which is routinely performed at our department using *Masterscreen PFT* or *Masterlab body*, from Jaëger (calibrated daily). All tests were performed by certified respiratory technicians, and predicted values were calculated using published referential equations.[14] The variation of patient's FVC values and CT staging were determined for each interval between consultations that varied between 5 and 332 months. These data were collected by two distinct researchers for all appointments between the time of diagnosis and the last observation.

Statistical analysis

The statistical analysis was performed using PASW Statistics software, version 18. The sample characterization was done by calculating measures of location (arithmetic mean and median) and measures of spread (standard deviation or interquartil range) for quantitative variables and by determining absolute and relative frequencies for qualitative variables.

Age and sex prevalence comparison between the study group and the controls was done using the Student's t test for independent samples and chi-square test, respectively. Caffeine consumption between the two groups was compared using Mann–Whitney test. Comparison of caffeine consumption pre- and postdiagnosis was performed using the Wilcoxon test. For nonsmokers and smokers (previous or actual), correlation of caffeine consumption with mean FVC variation per year and with CT stage variation was performed by calculating the Pearson and Spearman correlation coefficient, respectively. The same procedure was done considering the groups composed of individuals submitted to pharmacologic therapeutics and those who were not. All analyses were carried out establishing a significance at a 95% confidence level, unless otherwise explicitly defined.

Results

Impact of caffeine on the clinical evolution of sarcoidosis

We first assessed the impact of caffeine consumption on the variation of pulmonary function over time, gauged by the variation of FVC. This variation could only be determined for 29 patients since several patients had only one FVC determination in their clinical records. In this set of the cohort, we found a mean variation of FVC of $0.017\% \pm 1.278\%$ predicted per year. As shown in Figure 1A, there was no significant correlation between caffeine consumption and FVC variation (Pearson coefficient, $R = 0.091$, $p = 0.639$).

We next investigated the impact of caffeine consumption on the staging of the disease based on CT scan analysis. This analysis was carried out using 36 patients, who were subjected to more than one CT scan. In this set of the cohort, the median variation of CT stage was 0 with an interquartile range of 1. As shown in Figure 1B, there was no significant

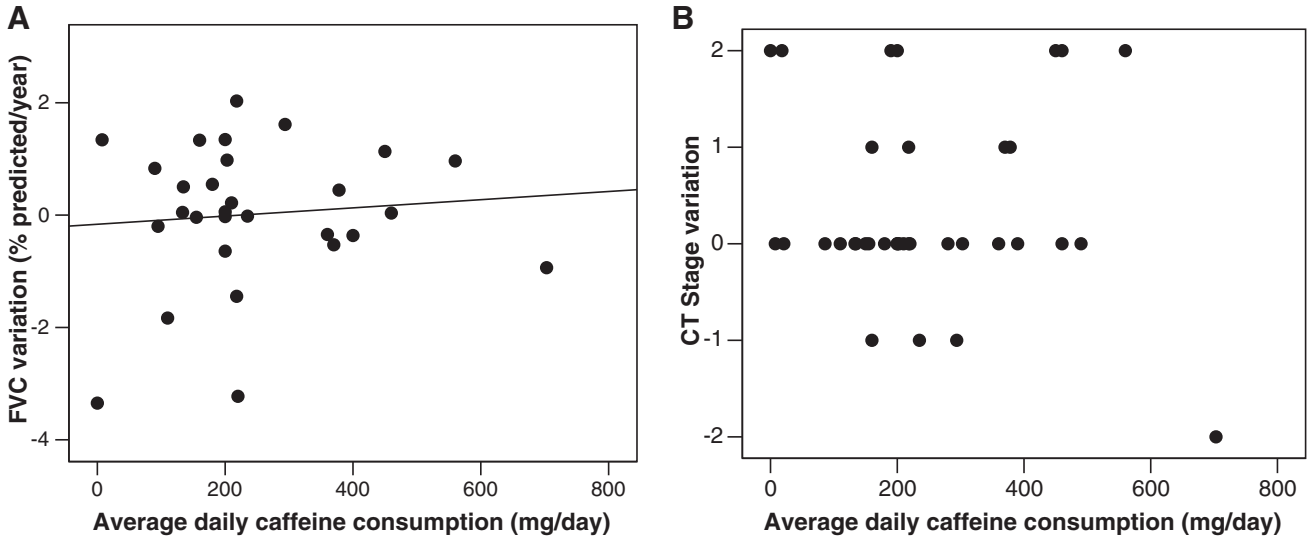

FIG. 1. Lack of effect of caffeine consumption on the clinical evolution of sarcoidosis, evaluated either through the yearly evolution of the pulmonary function (gauged by the variation of FVC) or the variation of CT scan staging between visits. All the individual data points for the 29 patients with more than one FVC determination **(A)** and for the 36 patients with more than one CT scan **(B)** are represented. FVC, forced vital capacity; CT, computed tomography.

correlation between caffeine consumption and the evolution of CT staging (Spearman coefficient, $R=-0.015$, $p=0.931$).

Possible influence of drug treatment or smoking habits on the impact of caffeine on disease evolution

We next tested if two potentially major interfering variables could hinder the effect of caffeine consumption on the evolution of sarcoidosis. We first investigated whether the introduction of a pharmacological management could be responsible for this lack of effect of caffeine: for this purpose, we segmented the initial cohort into two subgroups, namely treated and nontreated patients and investigated the impact of caffeine consumption in each of these groups in terms of variation of FVC and CT staging.

The mean variation of FVC for treated patients ($n=15$) was $0.203\% \pm 1.139\%$ predicted/year, and for nontreated patients ($n=14$) was $0.060\% \pm 1.361\%$ predicted/year. As shown in Figure 2A and B, caffeine consumption failed to significantly modify FVC variation in either treated ($R=-0.110$, $p=0.697$) or nontreated patients ($R=0.274$, $p=0.343$). The median variation of CT stage for treated ($n=20$) and nontreated ($n=16$) patients was 0 with an interquartile range of 1. Again, as shown in Figure 2C and D, caffeine consumption also failed to significantly modify the evolution of CT staging in either treated patients ($R=0.072$, $p=0.763$) or nontreated patients ($R=-0.083$, $p=0.759$).

We next investigated if smoking habits might interfere with the ability of caffeine to affect the evolution of sarcoidosis. To allow sufficient statistical power to apply a chi-square analysis, we grouped current and former smokers. The mean variation of FVC for nonsmokers ($n=22$) was $-0.137\% \pm 1.186\%$ predicted/year, and it was $0.845\% \pm 0.985\%$ predicted/year for smokers ($n=7$). As shown in Figure 3A, caffeine consumption did not affect FVC evolution in nonsmoking patients ($R=0.231$, $p=0.300$); however, as shown in Figure 3B, caffeine seemed to increase the loss of FVC in smoking patients ($R=-0.752$, $p=0.051$). However, this apparent effect of caffeine on the evolution of sarcoidosis in the subgroup of smoking patients was not confirmed when evaluating the CT staging. The median variation of CT stage for nonsmokers ($n=27$) is 0 with an interquartile range of 1 and it was 0 with an interquartile range of 2 for smokers ($n=9$). As shown in Figure 3C and D, caffeine was devoid of effects on the evolution of CT stating in either nonsmoking patients ($R=-0.180$, $p=0.369$) or smoking patients ($R=0.075$, $p=0.849$)

Comparison of caffeine consumption in patients and healthy individuals

We next evaluated if the consumption of caffeine in the previous 20 years was different in the cohort of patients analyzed compared with a group of healthy individuals. The group of healthy individuals, all Caucasians as the patients' group, had an average age (41.7 ± 14.2 years, $n=49$) slightly lower ($p=0.03$; t test for independent samples) than that of patients (48.2 ± 14.2 years, $n=46$), whereas gender distribution was not significantly different ($p=0.183$; chi-square test) between the two groups (42.9% and 56.5% males in healthy individuals and patients, respectively). We found that the average consumption of caffeine was not significantly different ($p=0.44$; Mann–Whitney test) in patients (236.3 ± 156.3 mg) compared with healthy individuals (203.9 ± 127.5 mg).

The last question we addressed was whether patients modified their habits of caffeine consumption upon diagnosis of sarcoidosis. The comparison between the average consumption of caffeine before and after diagnostic shows that there was a tendency for a decrease of caffeine consumption (-17.8 ± 132.9 mg, $p=0.638$, $n=46$) after diagnosis.

Discussion and Conclusions

The main conclusion of this preliminary study is that the consumption of caffeine fails to affect the evolution of

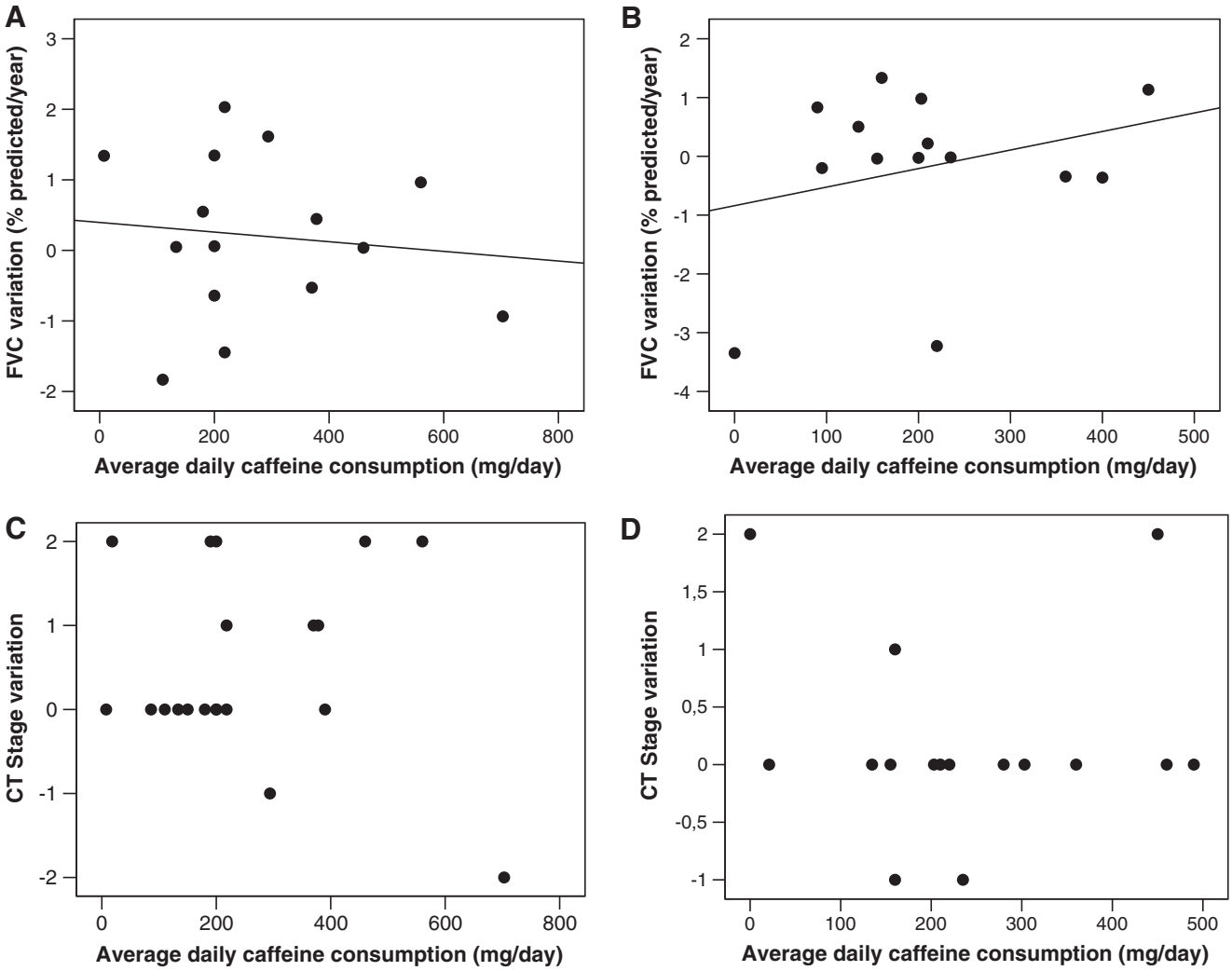

FIG. 2. The introduction of a drug therapy does not change the effects of caffeine consumption on the clinical evolution of sarcoidosis. In fact, the consumption of caffeine failed to modify the yearly evolution of the pulmonary function (gauged by the variation of FVC, between visits) either in treated ($n = 15$, **A**) or in nontreated patients ($n = 14$, **B**); likewise, the consumption of caffeine also failed to modify the variation of CT scan staging between visits either in treated ($n = 20$, **C**) or in nontreated patients ($n = 16$, **D**).

sarcoidosis. We further confirmed that neither the patients' smoking habits nor the introduction of pharmacotherapy were responsible for this lack of effect of caffeine. Further, the individuals who latter developed sarcoidosis consumed similar amounts of caffeine as control healthy individuals. This further attests to the lack of effect of caffeine in both the initiation and evolution of sarcoidosis.

This global conclusion is rather surprising in view of the proposed primary involvement of immune-inflammatory processes in the progression of sarcoidosis. As a matter of fact, in several other conditions where the abnormal functioning of the immune-inflammatory system plays a role, it has been shown that the consumption of caffeine affects the evolution of these diseases, such as arthritis[15] or diabetes.[16] In particular, the consumption of caffeine is associated with a modification of inflammatory parameters in healthy individuals, and individuals suffering from endothelial pathologies such as diabetes, cardiovascular dysfunction, or alcoholic liver injury.[17,18] Caffeine consumption also affects the incidence or evolution of different carcinomas, especially the ones displaying a stronger immunologic component.[19] This is generally in agreement with the ability of adenosine A_{2A} receptors, the likely target of nontoxic doses of caffeine,[6] to control immune and inflammatory responses.[5] Further, caffeine has also been reported to affect lung function and lung pathology[10,20,21] and to modulate inflammatory responses in the lung.[7] Thus, the present lack of evident effects of caffeine consumption on the evolution of sarcoidosis clearly favors the view that the evolution of this pathology is associated with specific imbalances in the response of particular lymphocytic populations,[22] rather than an overt hyperreactivity of the main components of the immune-inflammatory system.[1,23]

When attempting to detail the possible influence of two major factors that could interfere with the impact of caffeine consumption on the evolution of sarcoidosis, namely smoking and drug therapy, we further confirmed the lack of effect of caffeine in any of these subgroups of patients. Regarding

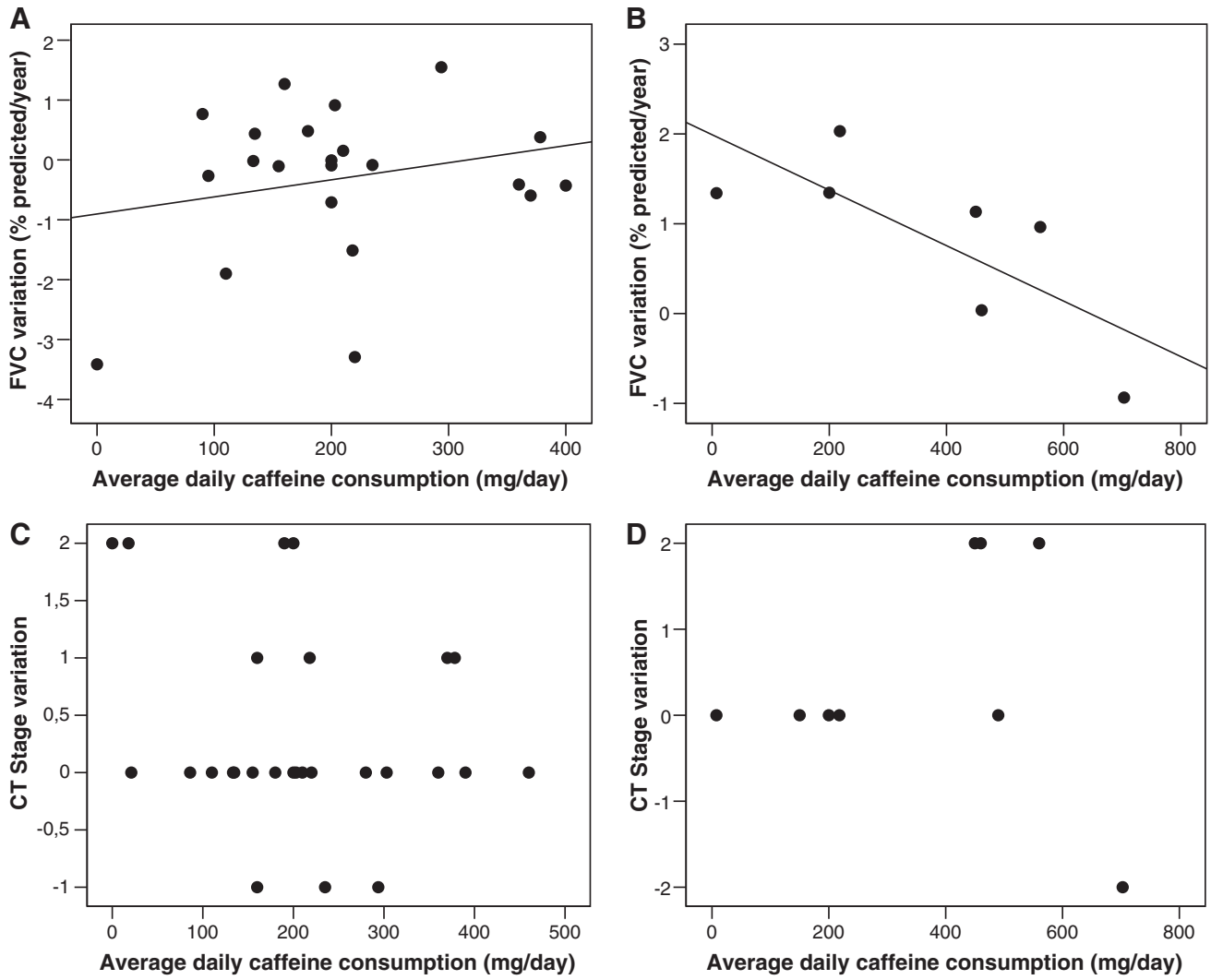

FIG. 3. Smoking habits do not seem to change the effects of caffeine consumption on the clinical evolution of sarcoidosis. The consumption of caffeine failed to modify the yearly evolution of the pulmonary function (gauged by measuring the variation of FVC, between visits) in nonsmoking ($n=22$, **A**) but seemed to enhance the deterioration of pulmonary function in smoking patients ($n=7$, **B**); however, the consumption of caffeine failed to modify the variation of CT scan staging between visits either in nonsmoking ($n=27$, **C**) or in smoking patients ($n=9$, **D**).

the smoking factor as a confounding parameter, it was observed that FVC but not CT scan staging was aggravated by caffeine in smoking patients, but the reduced number of cases fitting these criteria ($n=7$) precludes any conclusive statement at present. Interestingly, it has been documented that the effects of caffeine are dependent on nicotine consumption,[10] in accordance with the molecular interaction between adenosine and nicotinic receptors. Thus, this attempted segmentation further reinforces our main conclusion that caffeine consumption fails to affect the evolution of sarcoidosis.

With regard to the potential impact of the main pharmacotherapeutic options used to manage sarcoidosis, they are all known to affect the adenosine modulation system and hence potentially affect the actions of caffeine: corticosteroids interfere with the adenosine modulation system[24,25] and methotrexate is thought to indirectly act through adenosine receptors,[4] as initially proposed by Cronstein's group.[26] Thus, methothrexate was found to trigger the release of adenosine nucleotides upon its metabolism into methotrexate polyglutamate, which inhibits 5-aminoimidazole-4-carboxamide ribonucleotide transformylase and leads to an adaptive inhibition of AMP deaminase; these released adenosine nucleotides are then extracellularly converted into adenosine through the action of ecto-nucleotidases, prompting the activation of the anti-inflammatory adenosine A_{2A} receptors.[27]

The lack of effect of caffeine, an adenosine A_{2A} and A_1 receptor antagonist, on sarcoidosis, may be arguing against the importance of adenosine-based mechanisms on the therapeutic effects of methotrexate. Accordingly, it has been proposed that the anti-inflammatory effects of methotrexate may also involve non-adenosine-based mechanisms such as folate antagonism, inhibition of polyamines' production or control of cellular redox state, albeit none of these mechanisms is as likely as the indirect modulation of adenosine receptors.[28] Therefore, one should expect an interaction between chronic

caffeine consumption and the effect of methotrexate. This assumption should be carefully considered because caffeine has complex time-dependent actions over adenosine receptors that should preclude defining any index of activity of caffeine on adenosine receptors. Thus, on one hand caffeine can transiently block adenosine receptors (only when present in accordance with its short pharmacokinetic profile), but on the other hand the chronic consumption of caffeine can lead to an increase in the affinity and density of A_1 and A_{2A} receptors.[29,30] Therefore, caffeine is potentially expected to acutely blunt but chronically enhance the anti-inflammatory action of methotrexate. In accordance with this complex interaction between caffeine and methotrexate, initial animal and human studies in rheumatoid arthritis suggested that the chronic consumption of caffeine could prevent the therapeutic effects of methotrexate,[31,32] whereas other studies in rheumatoid arthritis or psoriasis showed no difference,[33,34] clearly showing that this issue is still unresolved.[35] Further, the rationale to use methotrexate in both sarcoidosis and rheumatoid arthritis is different: its use in rheumatoid arthritis is well established as a first-line therapy,[28] whereas in sarcoidosis it is generally used as a steroid-sparing agent.[3] Accordingly, the inflammatory modifications in both disorders are different. Thus, rheumatoid arthritis is an auto-immune and inflammatory disease that primarily affects synovial joints[36] and involves TNF-alpha- and IL-1β-mediated inflammation[37] and the recruitment of fibroblast-like and macrophage-like synoviocytes, macrophages, and B and T lymphocytes. In contrast, sarcoidosis involves the development of noncaseating granulomas associated with an evident $CD4^+$-T cell helper 1 response together with fibroblasts, B cells, and CD8 T lymphocytes.[1,38]

In conclusion, the present study suggests that caffeine consumption might not affect the onset or the evolution of sarcoidosis. It should be noted that the strength of these conclusions is limited by the size of the cohort of patients analyzed and by the design of the study as a retrospective collection of information. It is hoped that future prospective studies carried out in a larger population of patients suffering from this pathology may confirm the presently proposed conclusions. This tentative conclusion raises doubts concerning the development of adenosine-based therapeutic strategies to manage the purported inflammatory dysfunction associated with sarcoidosis, as has been successfully attempted in other diseases with an evident inflammatory component.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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Supplementary Data

Supplementary Table S1. List of all Sarcoidosis Patients, Including Information on Gender, Age, Year of Diagnosis, Therapy, Average Caffeine Consumption in the Last 20 Years (mg/Year), Yearly Variation of Forced Vital Capacity, and Chest Computed Tomography Scan Stage Modification

Case	*Gender*	*Age at diagnosis*	*Year of diagnosis*	*Therapy*	*Average daily caffeine consumption*	*Average yearly FVC change (%)*	*Chest CT stage variation*
C1	Male	36	2009	Steroids + azathioprine	293.8	1.61	−1
C2	Female	33	2009	0	303.0		0
C3	Female	66	2009	Steroids	150.0		0
C4	Female	64	2008	0	220.0	−3.23	0
C5	Female	58	2005	0	90.0	0.83	
C6	Male	53	2007	Steroids	560.0	0.96	2
C7	Female	35	2000	Steroids	133.3	0.05	0
C8	Male	27	2008	Steroids	200.0	1.35	0
C9	Female	26	2008	Steroids	241.0		
C10	Female	59	2007	Steroids	7.5	1.34	0
C11	Male	37	2007	0	21.0		0
C12	Female	64	1993	Steroids	180.0	0.55	0
C13	Female	72	2005	0	155.0	−0.04	0
C14	Male	52	2000	0	210.0	0.22	0
C15	Male	61	2008	Steroids	460.0	0.04	2
C16	Female	38	2003	0	390.0		
C17	Female	49	2005	0	235.0	−0.02	−1
C18	Male	42	2009	Steroids	218.0	2.03	0
C19	Male	35	2008	0	360.0	−0.34	0
C20	Male	50	2007	Steroids	218.0	−1.45	1
C21	Male	46	2003	Steroids	200.0	−0.64	2
C22	Female	52	2007	Steroids	703.0	−0.94	−2
C23	Male	43	2009	Steroids	18.0		2
C24	Male	54	1993	Steroids	390.0		0
C25	Female	27	2008	0	90.0		
C26	Male	47	2008	Steroids	378.2	0.45	1
C27	Male	27	2005	Steroids	86.0		0
C28	Female	35	2009	0	298.5		
C29	Male	45	2007	Steroids	200.0	0.06	0
C30	Male	34	2007	0	134.6	0.50	0
C31	Female	50	2005	Steroids	370.0	−0.53	1
C32	Male	35	2008	0	0.0		2
C33	Female	26	2008	0	157.5		
C34	Female	36	2007	0	0.0	−3.35	
C35	Male	57	2006	0	200.0	−0.03	
C36	Female	71	2006	Steroids	110.0	−1.83	0
C37	Male	77	2007	Azathioprine + infliximab	190.0		2
C38	Male	50	2005	0	450.0	1.13	2
C39	Male	48	2008	0	203.0	0.98	0
C40	Male	45	2010	0	460.0		0
C41	Male	64	2009	0	280.0		0
C42	Male	70	2010	0	490.0		0
C43	Male	45	2009	0	400.0	−0.36	
C44	Female	41	2009	0	160.0	1.33	−1
C45	Male	80	2002	0	95.0	−0.20	
C46	Female	53	2008	0	160.0		1

Follow-up begins with diagnosis and continues up to today. The therapeutics includes three kinds of drugs: steroids (prednisolone or deflazacort), azathioprine, and infliximab. For FVC variation the negative values represent loss of FVC, while the negative values on Chest TC variation represents a downstaging.

FVC, forced vital capacity; CT, computed tomography.

Chapter 5

Caffeine consumption in patients with obstructive sleep apnoea: retrospective study

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Original Research Articles

Caffeine Consumption in Patients with Obstructive Sleep Apnea: Retrospective Study

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Obstructive sleep apnea (OSA) is a frequent syndrome characterized by repeated pharyngeal obstruction during sleep. Some of its consequences include excessive daily somnolence, impaired cognition, and cardiovascular and metabolic dysfunction. Caffeine, the world's most widely consumed psychoactive drug, affects both the respiratory function and the sleep/waking cycle, but the pattern and effects of caffeine consumption on patients with OSA are mostly unknown. We performed a retrospective study evaluating caffeine consumption in the last 20 years of 48 patients with OSA and 49 healthy controls, and found no differences in caffeine consumption between the two groups (203.9 ± 147.8 mg/day vs. 203.9 ± 127.5 mg/day). Although there was no correlation between caffeine consumption and either risk factors or consequences of OSA, there was a positive correlation between caffeine consumption and severity of OSA upon adjustment for somnolence. A negative correlation between sleepiness and caffeine consumption was also found. In conclusion, the present study suggests that caffeine consumption is not associated with the development of OSA, but may have a correlation with the associated sleepiness and severity of the syndrome.

Introduction

Obstructive sleep apnea (OSA) is a syndrome characterized by periods of total or partial pharyngeal obstruction during sleep, with a prevalence in adults of 3–7% in men and 2–5% in women. The disease is strongly related to excess body weight (the main risk factor), alcohol and tobacco use, and craniofacial and upper airway changes. Other risk factors include male gender, menopause, and advanced age. This syndrome has been associated with a global cognitive disorder, an increased risk of traffic accidents, and a greater incidence of metabolic and cardiovascular disease.[1] Caffeine is the most widely consumed psychoactive substance in the Western world, increasing energy levels and reducing drowsiness.[2] The main mechanism of action is nonselective blockage of adenosine receptors, which are well known to control sleep and arousal,[3] sleep deprivation-associated attention and cognition, and to affect respiratory function.[2,4] Caffeine consumption is also associated with an improved cardiovascular and metabolic risk profile, and displays anti-inflammatory, lipolytic, antioxidant, and antidiabetic effects.[2] Since all these conditions are frequently observed in OSA patients, there are reasonable grounds to consider a hypothetical relationship between caffeine and OSA, which may result from two concurring mechanisms: first, caffeine consumption might decrease the risk of developing the disease through lipolytic effects; second, caffeine may act as self-medication for drowsiness and neurocognitive deficits. However, since the relationship between caffeine consumption and the development and evolution of OSA in adults is fundamentally unknown, this study was designed to evaluate caffeine consumption in a population of OSA patients, and its possible association with the development, evolution, and severity of the disease.

Methods

A retrospective study was performed, including patients attending the respiratory sleep diseases outpatient consult clinic of Coimbra University Hospital, between 2009 and 2011. The inclusion criteria were the performance of a simplified polygraphic sleep study leading to treatment with continuous positive airway pressure (CPAP), and the exclusion criteria were underage or nonconsent. A parallel group

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of healthy individuals, already reported in a previous study from our group, was also included.[5] The assessment of caffeine consumption was conducted through a standard validated questionnaire performed by telephone.[6] Caffeine consumption was calculated as the sum of its contents in various beverages consumed by the individual. We considered the main caffeine sources that were taken into account in previous studies[2,5,7]: espresso = 100 mg; instant coffee (cup) = 60 mg; decaffeinated coffee = 3 mg; tea (leaves, herbs, or berries) = 30 mg, instant tea (cup) = 20 mg, cola drinks (300 mL can or bottle) = 18 mg. Each participant was asked about the daily intake of each source of caffeine for the past 20 years. The average daily intake of caffeine was calculated by multiplying the amount of caffeine content in each source by the average daily consumption of each one. For the patient group, the average daily consumption of caffeine was calculated for two periods: before and after diagnosis of OSA, totaling 20 years. For the control group, the previous 20 years were considered. The relevant clinical data, including body mass index (BMI), associated diseases, smoking history, medication, Epworth Sleepiness Scale (ESS) score at the time of the OSA diagnosis, and apnea–hypopnea index, were obtained from the patients' clinical records. The data on medication, associated diseases, and smoking history were confirmed during the telephone interview, and reported at the time of OSA diagnosis.

Statistical analysis was performed using SPSS v20 (IBM Corp., Armonk, NY). The group differences in quantitative variables were evaluated with the Student's *t*-test for independent samples. The relationships between categorical variables were tested using the chi-square test. For the changes on caffeine consumption before and after OSA diagnosis, the Student's *t*-test for paired samples was used. Correlations were analyzed using Pearson's or Spearman's coefficient, depending on the normality of each variable, tested by the Kolmogorov–Smirnov test. A *p*-value of <0.05 was considered statistically significant.

Results

A total of 48 patients (42 males) were included, with an average age of 58.6 ± 10.7 years. The control group consisted of 49 individuals (21 males), with an average age of 41.7 ± 14.2 years (Table 1). The differences in both the gender and age distributions between the two groups were statistically significant ($p < 0.001$ for both comparisons). Although the average age was higher in the patients' compared to the control group, there was no correlation between age and OSA severity in this group, as evaluated by the apnea–hypopnea index. Similarly, there was no significant correlation between age or BMI and the ESS score. Regarding average daily caffeine consumption, we found no differences in caffeine consumption between

Table 1. Group Characteristics

	Patients	*Controls*	*Significance*
n	48	49	N/A
Gender	42M/6F	21M/28F	$p < 0.001$
Average age (years)	58.6 ± 10.7	41.7 ± 14.2	$p < 0.001$
Age range (years)	37–80	18–82	
BMI (kg/m^2)	32.0 ± 4.4	N/A	
Epworth Sleepiness Scale score	10.6 ± 5.93	N/A	
AHI (events/hour)	33.4 ± 22.7	N/A	
Caffeine consumption (mg/day)	203.9 ± 147.8	203.9 ± 127.5	NS
Smoking			
Smokers/former smokers, *n* (%)	26 (54.2)	N/A	
Caffeine (mg/day)	204.5 ± 136.0	N/A	NS (vs. nonsmokers)
Medication			
Hypnotics, *n* (%)	15 (31.3)	N/A	
Caffeine (mg/day)	178.8 ± 110.5	N/A	NS (vs. no hypnotics)
Antidepressants, *n* (%)	9 (18.8)	N/A	
Caffeine (mg/day)	250.7 ± 158.9	N/A	NS (vs. no antidepressants)
OSA complications			
CHD, *n* (%)	13 (27.1)	N/A	
Caffeine (mg/day)	193.9 ± 116.0	N/A	NS (vs. no CHD)
Stroke, *n* (%)	3 (6.25)	N/A	
Caffeine (mg/day)	282.4 ± 280.0	N/A	NS (vs. no stroke)
Cognitive complaints, *n* (%)	9 (18.8)	N/A	
Caffeine (mg/day)	198.6 ± 193.5	N/A	NS (vs. no complaints)
Psychiatric complaints, *n* (%)	11 (22.9)	N/A	
Caffeine (mg/day)	210.6 ± 168.4	N/A	NS (vs. no complaints)
Traffic accidents, *n* (%)	1 (2.1)	N/A	
Caffeine (mg/day)	160.0	N/A	NS (vs. no accidents)

Values are expressed as mean ± standard deviation. There were significant differences in age and gender between patients and controls, but not in caffeine consumption. The comparison of average caffeine consumption between patients with smoking, medication habits, or complications from OSA showed no significant differences from those without these characteristics.

N/A, not available; NS, not significant; BMI, body mass index; AHI, apnea–hypopnea index; OSA, obstructive sleep apnea; CHD, coronary heart disease.

OSA patients (203.9 ± 147.8 mg caffeine per day prior to the diagnosis) and healthy controls (203.9 ± 127.5 mg of caffeine per day). In the group of OSA patients, the average daily caffeine intake was 204.0 ± 147.8 mg for 17–19 years prior to diagnosis and 172.4 ± 106.5 mg for 1–3 years after diagnosis; this decrease in caffeine consumption ($M = 31.57$ mg) was not statistically significant ($p = 0.102$), with 16 patients reducing their caffeine intake after diagnosis, 13 increasing it, and 19 maintaining their habits. Regarding associated conditions in the OSA group, the average daily consumption of caffeine before diagnosis of OSA was similar between nonsmokers ($n = 22$; 203.3 ± 164.0 mg/day) and current or former smokers ($n = 26$; 204.5 ± 136.0 mg/day). Patients under treatment with hypnotics ($n = 15$) or antidepressants ($n = 9$) consumed a similar daily dose of caffeine as those that were not under any of these treatments. Regarding OSA consequences, we found a weak negative correlation between daily somnolence as evaluated by the ESS and caffeine consumption before diagnosis ($p = 0.03$; $R^2 = 0.099$). This relation was not significant for average daily caffeine consumption after the diagnosis ($p = 0.158$; $R^2 = 0.043$). The correlation between the severity of OSA and caffeine consumption before and after the diagnosis was not significant. However, when we adjusted caffeine consumption for somnolence, we found a weak positive correlation between OSA severity and average daily caffeine consumption ($p = 0.041$). Finally, with respect to the development of OSA complications, we found no significant differences in caffeine consumption between patients with or without history of coronary heart disease, stroke, traffic accidents, and cognitive or psychiatric complaints.

Discussion

The main findings of our study were the lack of an association between caffeine consumption and the development or severity of OSA, and a negative correlation between daytime somnolence and caffeine consumption in patients with OSA. As far as we are aware, this is the first detailed evaluation of caffeine consumption in patients with OSA. Despite the likely relationship between OSA and caffeine—excessive somnolence being the most frequent complaint and caffeine the most widely used self-strategy to deal with this complaint—there is a remarkable lack of information on this subject in the literature. A recent large U.S. community-based study by Aurora *et al.*[8] reported an association between caffeinated soda consumption and the presence and severity of sleep disordered breathing, but the measuring of caffeine consumption was based on the number of cups of various beverages consumed on an average day. Our study tried to quantify the typical daily consumption of caffeine from various sources in the last 20 years using a detailed questionnaire. Our findings indicate that caffeine does not influence the development of OSA, as patients and controls consumed the same amount of caffeine in the last 20 years. Notably, after adjustment for somnolence, we found a positive correlation between caffeine consumption and OSA severity. This finding is in line with the conclusions of the study from Aurora *et al.*

In conclusion, the present study suggests that caffeine consumption does not change the development of OSA, but may show a correlation with both the associated sleepiness and severity of the syndrome. These conclusions are limited by the small size of our cohort, the retrospective nature of the study, and the significant differences between the patient and control groups, and ought to be confirmed in a larger cohort.

Author Disclosure Statement

No competing financial interests exist.

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Chapter 6

Pulmonary ATP levels in hypersensitivity pneumonitis and sarcoidosis – preliminary study

Title: Pulmonary ATP levels in hypersensitivity pneumonitis and sarcoidosis – preliminary study

Running title: ATP levels in hypersensitivity pneumonitis and sarcoidosis

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Abstract

The interstitial lung diseases are a vast and heterogeneous group of conditions that share clinical and radiological features, generating frequent difficulties in their differential diagnosis. Extracellular adenosine 5'-triphosphate (ATP) is a multifunctional signaling molecule acting through the activation of purinergic P2 receptors, which are proposed to regulate lung inflammation and fibrosis. In order to evaluate the involvement of ATP in the pathophysiology of interstitial lung diseases and its role in their differential diagnosis, we quantified the ATP concentrations in the bronchoalveolar lavage fluid (BALF) of 5 patients diagnosed with hypersensitivity pneumonitis, 8 patients with sarcoidosis and 10 with other lung diseases, using the luciferin-luciferase luminescence method. The median level of ATP in the BALF supernatant of patients with sarcoidosis (1.65 ± 5.78 nM) was significantly higher than those of patients with HP (0.04 ± 0.38 nM). Furthermore, these ATP levels were negatively correlated with the BALF total cell count and positively correlated with the forced vital capacity. This raises the possibility for a role of the evaluation of pulmonary extracellular ATP in the differential diagnosis of ILD and suggests a different role of purinergic signaling on these diseases.

Main text:

Introduction

The interstitial lung diseases (ILD) comprise a heterogeneous group of conditions that share clinical and radiological features, creating difficulties in the differential diagnosis (1). Some of the most frequent ILDs include hypersensitivity pneumonitis (HP), a disease caused by intense and/or repeated inhalation of organic dusts and occupational antigens with further sensitization (2), and sarcoidosis, a multisystem granulomatous disorder of unknown cause (3). Extracellular adenosine 5'-triphosphate (ATP) is a multifunctional molecule controlling metabolism, signaling, and cell growth, through the activation of purinergic P2 receptors (4). Previous studies supported the notion that ATP has an important role on the regulation in lung inflammation and fibrosis (5). We now quantified the ATP concentration in the supernatant of bronchoalveolar lavage fluid (BALF) of patients with HP and sarcoidosis to gauge the usefulness of this method for the differential diagnosis between these disorders.

Methods:

A prospective study was performed, including patients referred for bronchoalveolar lavage at the University Hospital Center of Coimbra, as clinically indicated by the patient's physician. The exclusion criteria included clinical or laboratory signs of infection and a non-representative BALF. The study was aproved by the local ethics committee and informed consent was obtained. All the information was maintained anonymous throughout the study. Bronchoscopy was performed under local anesthesia with lidocaine. The bronchoscope was wedged in the middle lobe and a total of 150 mL of warm sterile saline was instilled and immediately recovered through manual aspiration. This BALF was filtered through sterile gauze and centrifuged at 380 g (10 minutes at 4°C). The supernatant was stored at -20ºC and then moved to -80ºC, until ATP analysis. Total cell count was performed using a particle counter (Coulter LH780). The differential cell count was performed on cytospin slides with May-Grünwald-Giemsa stain and lymphocyte subsets were evaluated by flow cytometry. ATP was quantified using Sigma ATP Bioluminescent Assay Kit (Sigma®), according to the manufacturer's instructions, using an automated plate reader (Wallac 1420, Perkin Elmer®, Sweden). Samples were measured in triplicate to define their mean luminescence. Standard curves were performed in 0.9% sterile saline solution. The samples under the detection limit of 0.016 nmol/L ($10^{-10.5}$M) of ATP were assigned this value. Preliminary experiments established kinetic stability, ATP dependency, as the signal was abolished by the use of apyrase and reproducibility as the same sample gave similar results when analyzed in different days. After ATP quantification, only patients with an established diagnosis were considered. Sarcoidosis was diagnosed according to WASOG criteria (3) and HP according to Richerson (6). Patients with other diseases were also included

based on the attending physician's final diagnosis. Three groups were considered: sarcoidosis, hypersensitivity pneumonitis, and other diagnosis. Clinical and functional data were gathered from the patient's clinical files.

The statistical analysis was performed using STATA 13.1 (StataCorp, USA). Values were expressed using median ± interquartile range (IQR). The differences between two groups were tested using mann-Whitney test. For more than two groups, we used Kruskal-Wallis followed by Dunn's test for pairwise analysis. The correlations between ATP concentrations and BALF cell numbers or lung function were tested using Spearman's correlation. All analyses were carried out at a two tailed 95% confidence.

Results

Twenty three patients were included (17F/6M), with a median age of 52.0 ± 20 years, similar between genders (F: 49.1±13.3 vs M: 51.5±19.5, p=0.62, Mann-Whitney). The clinical and laboratory characterization of each group can be seen in table 1. There were eight patients in the sarcoidosis group, five patients in the HP group and ten patients in the other diseases group (2 - lung cancer, 2 - pneumoconiosis, 2 - granulomatosis with polyangiitis, 1 - polymyositis, 1 - eosinophilic pneumonia, and 2 - leukemia). Concerning sarcoidosis, two subjects had stage I, five had stage II and one stage III thoracic disease, as evaluated by HRCT. All HP patients had chronic bird fancier's disease. No subject was a current smoker or under therapy with steroids or thienopyridines. Four patients with sarcoidosis and one patient in the other diseases' group were former smokers, none having COPD. Considering sarcoidosis and HP, the latter group had higher age, and worse lung function. On the BAL analysis, patients with HP had higher lymphocyte and neutrophil percentage, but lower CD4/CD8 ratio, when compared to sarcoidosis patients. ATP levels in BALF were significantly different between the three groups (p=0.027, Kruskal-Wallis). HP patients had a lower median ATP concentration in BALF than sarcoidosis patients (0.04 ± 0.38 vs 1.65 ± 5.78 nM/L, p=0.02, Dunn's post-hoc test).

Since smoking has been associated with an increase in BALF ATP levels, we compared the median BALF ATP levels in patients with and without a smoking history. There were no significant differences (1.46 ± 2.01 vs 1.74 ± 3.97 nmol/L, p=0.11 Mann-Whitney). Furthermore, when excluding patients with a smoking history, there is still a difference between the median BALF ATP levels of the three groups (p=0.028, Kruskal-Wallis). The difference between BALF ATP patients PH vs sarcoidosis is no longer significant, but there was a low number of sarcoidosis

patients in this analysis (0.04 ± 0.38 vs 0.94 ± 6.98 nmol/L, p=0.20, Dunn's post-hoc test).

When we probed for correlations between lung function and BAL ATP levels, we found a positive correlation for forced vital capacity (Spearman's rho = 0.54, p=0.024), but not for diffusion capacity. When we performed the same analysis for BALF cellular composition, we found a negative correlation between ATP concentration and the total number of cells (Spearman's rho =-0.63, p=0.001), as well as the number of neutrophils (Spearman's rho = -0.45, p=0.029) and lymphocytes (Spearman's rho = -0.72, p=0.0001).

Discussion

Our main finding was that patients with HP display significantly lower BALF ATP levels than sarcoidosis patients. We also found a positive correlation between ATP and patient's lung function, as well as a negative correlation between ATP and BALF cellular components. Importantly, HP patients had ATP levels close to the detection limit, which strengthens our conclusions as the group differences may be even higher. We ruled out possible interactions with BALF constituents by kinetic assay analysis, and confirmed the presence of ATP in BALF by the use of apyrase. Daily reproducibility was also shown.

The quantification of ATP in the BALF of sarcoidosis patients has been previously described (7) but there were significant differences in methodology.

Our method may overestimate purine concentration as the mechanical and osmotic forces generated during lavage may trigger ATP release by airway cells; there might also be an underestimation due to rapid ATP degradation in biological fluids and alveolar lining fluid dilution during the procedure. ATP degradation may also occur during sample processing, despite our care to prevent this, as all samples were equally manipulated, and had a similar storage period. Additional limitations of our study include the small cohort, the retrospective collection of clinical information and the absence of a formal control group, as BALF is not usually collected from healthy subjects. To overcome this, we included a group of patients with diverse lung diseases, thus serving as control.

The differences in the ATP levels between sarcoidosis and HP patients suggest that purines may serve different roles in the pathogenesis of these diseases. This warrants further study, especially to distinguish whether these differences stem from changes of the release or the catabolism of ATP.

In conclusion, this study suggests that HP patients have lower pulmonary extracellular ATP concentrations than patients with sarcoidosis and this could be related with the BALF cellular composition. If confirmed in a larger cohort, this prompts using pulmonary extracellular ATP levels in the differential diagnosis of ILD, particularly foreseeing the use of exhaled breath condensate, a non-invasive method that does not introduce osmotic or mechanical forces in the airways (8).

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Competing interests:

The authors declare that they have no competing interests.

Ethics approval and consent to participate:

The protocol was approved by the local ethics committee and patients provided informed consent.

		Sarcoidosis	HP	Others	Total	p value
Number		8	5	10	23	-
Age (years)		38 ± 9.5	57 ± 20	55.5 ± 18	52 ± 20	**0.016 #**
Males (%)		25.0	0.0	40.0	26.1	0.250 *
Former smokers (%)		50.0	0.0	10.0	21.7	0.062 *
Lung function	FVC (%)	97.3 ± 17.7	84.3 ± 28.3	111.69 ± 8.56	98.1 ± 19.3	**0.016 #**
	DLCO (%)	93.6 ± 29.1	42.5 ± 13.8	91.5 ± 26.3	86.5 ± 13.2	**0.031 #**
Bronchoalveolar lavage	Total cells (cels./µL)	100 ± 55	320 ± 190	80 ± 110	110 ± 110	0.160 #
	Macrophages (%)	84.5 ± 34.5	33 ± 22	71.5 ± 22	69 ± 42	**0.014 #**
	Lymphocytes (%)	9 ± 33	65 ± 40	13 ± 14	18 ± 32	**0.043 #**
	Neutrophils (%)	1 ± 3.5	18 ± 16	4 ± 6	4 ± 7	**0.018 #**
	Eosinophils (%)	0 ± 0	0 ± 0	0 ± 10	0 ± 2	0.327 #
	CD4/CD8 ratio	3.17 ± 11.83	0.95 ± 1.37	2.32 ± 3.05	2.21 ± 3.05	0.136 #
ATP (nmol/L)		1.65 ± 5.78	0.04 ± 0.37	1.72 ± 1.02	1.60 ± 2.27	**0.027 #**

Table 1: clinical and laboratory characteristics of the three groups of patients included.

The values for age, lung function and ATP quantification are expressed as median ± interquartile range. All p values are for differences between the three groups.* - chi-square test; # - Kruskal-Wallis test.

Chapter 7

Adenosine A_{2A} receptors are up-regulated and control the activation of human alveolar macrophages

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Adenosine A_{2A} receptors are up-regulated and control the activation of human alveolar macrophages

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ABSTRACT

Chronic inflammatory lung diseases remain a health concern and new anti-inflammatory treatments are needed. Targeting adenosine A_{2A} receptors ($A_{2A}R$) affords robust anti-inflammatory effects in animal models, but the translation of this promising strategy to humans has been challenging, possibly due to interspecies differences in receptor distribution and effects. Thus, we now assessed the efficiency of a selective $A_{2A}R$ agonist to control the activation of fresh human alveolar inflammatory cells. We collected bronchoalveolar lavage fluid from patients with interstitial lung disease and loaded alveolar cells with the intracellular free calcium probe FURA-2/AM. Calcium transients were then recorded in response to superfusion with a proinflammatory peptide (N-formylmethionyl-leucyl-phenylalanine - FMLP), in the absence or presence of the selective $A_{2A}R$ agonist CGS21680. In a second experiment, cells were continuously exposed to FMLP and $A_{2A}R$ density was assessed by immunocytochemistry. Sixteen patients were included, nine for analysis of calcium transients, and seven for immunocytochemistry. When alveolar macrophages were exposed to 100 nM FMLP for 120 s, a peak elevation of intracellular free calcium levels (97.0% over baseline) was recorded; CGS21680 (100 and 300 mM) significantly reduced this peak to 89.5% and 81.5%, respectively. The immunofluorescence analysis revealed a time-dependent increase of $A_{2A}R$ density in alveolar macrophage upon exposure to 1 μM FMLP, up to 148% of control at 6 h. These results show that pro-inflammatory stimuli up-regulate $A_{2A}R$ and their activation dampens the impact of pro-inflammatory stimuli. This supports that targeting $A_{2A}R$ is a promising therapy for human lung inflammatory diseases, especially for diseases with a strong inflammatory component.

1. Background

Pulmonary inflammatory diseases are a major global cause of disability and early death [1]. Airway inflammatory diseases alone, such as asthma and chronic obstructive pulmonary disease (COPD), affect over 200 million people each, with the latter being currently the third leading cause of death [2]. Interstitial lung diseases are an extensive and heterogeneous group of inflammatory and/or fibrotic pulmonary diseases that can be idiopathic or associated to specific exposures or systemic diseases. Although interstitial lung diseases have a relatively lower prevalence, a significant proportion of these patients progress to pulmonary fibrosis and respiratory failure, despite treatment [3].

The presently available anti-inflammatory strategies for pulmonary inflammatory disorders are largely based on the use of steroids, which are ineffective in delaying the evolution of COPD and have substantial side effects, including osteoporosis, hyperglycaemia and cataracts, when given systemically to manage interstitial lung diseases [3–5]. New anti-inflammatory therapies for pulmonary disease are urgently needed.

The modulation of the purinergic adenosine receptors is a promising strategy for the treatment of pulmonary inflammatory disorders [6]. Adenosine, a purine nucleoside, is kept at low

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concentrations under physiological conditions, but its extracellular levels rise several fold upon cellular dysfunction as a result of increased ATP catabolism and adenosine release [5]. This increased extracellular concentration of adenosine acts as a retaliatory metabolite, reducing inflammation and promoting tissue repair [5]. Its effects are exerted through four G-protein coupled membrane receptors, A_1, A_{2A}, A_{2B} and A_3 receptors, and adenosine A_{2A} receptors ($A_{2A}R$) play a major role in dampening the activation of neutrophils, macrophages and lymphocytes [5]. Animal models revealed that $A_{2A}R$ are dynamically engaged to control inflammation, since they can be up-regulated under inflammatory conditions to adapt their efficiency to a heightened cellular activity [6,7]. Accordingly, a robust protective role of $A_{2A}R$ agonists was shown in several preclinical models of asthma, COPD and acute lung injury, whereas genetic deletion or antagonism of $A_{2A}R$ led to disease worsening in the same models [7].

Unfortunately, the translation of these results to human studies has been challenging. Initial clinical studies using selective $A_{2A}R$ agonists for rhinitis, asthma and COPD were negative [8–10]. Some possibilities were considered to explain these results: one hypothesis proposed that the lower efficiency of $A_{2A}R$ agonists in humans could result from a significant inter-species variability in the presence and distribution of $A_{2A}R$ in the various tissues and cell types [11] or to an inability of human $A_{2A}R$ to alter their efficiency under inflammatory conditions. In fact, most of the studies on the anti-inflammatory effects of $A_{2A}R$ were performed in animal models or in human cell-cultures, which may not be representative of the human disease conditions [11,12].

We now aimed to assess the dynamics of $A_{2A}R$ and the efficiency of a selective $A_{2A}R$ agonist on fresh human alveolar inflammatory cells, which are representative of human disease conditions.

2. Methods

2.1. Patient and sample collection

We performed a prospective study including patients referred for bronchoscopy with bronchoalveolar lavage (BAL) for diagnosis of interstitial lung disease at the University Hospital of Coimbra in Portugal. The inclusion criteria were the presence or suspicion of an interstitial lung disease at diagnosis stage, informed consent and age over 18 years. The exclusion criteria were clinical or laboratory signs of infection, underage, pregnancy, non-consent and chronic treatment with steroids. All patients had a clinical indication for the exam, and no clinical exams were performed solely for this study. The protocol was approved by the local ethics committee and patients provided informed consent. The bronchoscopy and bronchoalveolar lavage were performed according to the department's general practice. The bronchoscope was introduced by the nares or mouth with the patient on dorsal decubitus, under local anaesthesia with lidocaine. After passing through the upper airway, the bronchoscope tip was wedged in one of the middle lobe segments and three 50 mL syringes of warmed saline were gently injected in the work channel and immediately aspirated. For this study, 10 mL of the pooled fluid was collected. The remaining fluid was sent for the central hospital laboratory for total and differential cell counts. For total cell count a Coulter cell counter was used. For the differential cell count, 50 mL of lavage fluid was spun at 500 × *g* for 10 min at 4 °C. The supernatant was discarded, and the cells were re-suspended in 1 mL of Hank's buffered saline solution (HBSS). A slide was prepared on a cytospin 3 centrifuge using a 650 rpm speed with a low acceleration for 10 min. Staining was performed using May-Grünwald-Giemsa procedure [13]. A total of 500 cells were counted and each cell type recorded as a proportion (%) of the total number of cells.

2.2. Reagents

All reagents were purchased from Sigma unless otherwise specified. The composition of the Hank's buffered saline solution (HBSS) was (mM): 136.9 NaCl, 4.2 $NaHCO_3$, 0.34 NaH_2PO_4, 0.44 KH_2PO_4, 5.4 KCl, 1.3 $CaCl_2$, 1 $MgSO_4$, 5.6 glucose. The composition of the phosphate buffered saline (PBS) was (mM): 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.47 KH_2PO_4. For the immunocytochemistry analysis, the following antibodies and dilutions were used: goat polyclonal anti-$A_{2A}R$ (SC-7504, dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-CD68 (Novocastra NCL-L-CD68 no dilution; Menarini Diagnostics, Paço de Arcos, Portugal), donkey anti-goat IgG labelled with Alexa Fluor® 488 (ab150129, dilution 1:100; Abcam, Cambridge, UK), donkey anti-mouse IgG labelled with Alexa Fluor® 594 (ab150116, dilution 1:100; Abcam).

2.3. Intracellular free calcium analysis

The collected BAL was kept at 4 °C and transported to the laboratory within 4 h of collection. The remaining protocol was performed at controlled room temperature (20 °C). The cell viability was tested using a 0.2% solution of trypan blue in a Neubauer chamber. Samples with less than 50 cells/μL or less than 90% viability were discarded. The BAL fluid was diluted (1:1) in HBSS with 1% penicillin/streptomycin (P/S), and the cells were plated in photoetched gridded coverslips with 12 mm diameter (Bellco glass, NJ, USA) during 60 min. The cells were then washed with HBSS and loaded with 5 μM FURA-2/AM with 1% bovine serum albumin and 0.4% pluronic F-127 in HBSS for 90 min. The coverslips were washed with HBSS and kept in the same medium until analysis. The intracellular free calcium analysis was performed using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Jena, Germany). The coverslips were mounted in a RC-25 chamber (Warner Instruments, Harvard, UK) and placed under a continuous superfusion of 1 mL/min of HBSS. A Lambda DG4 xenon light (Sutter Instruments, Novato, CA, USA) was used to alternatively excite the cells with UV light centred at 340 and 380 nm with an exposure time of 1 s. The fluorescence was captured through a 40× oil objective and a 510 nm band-pass filter (Carl Zeiss) connected to a digital camera (Cool SNAP; Roper Scientific, Trenton, NJ, USA). For each coverslip, the superfusion protocol consisted of 2 min of basal medium, 10 min of medium or $A_{2A}R$ agonist, followed by 2 min of formyl-methionyl-leucyl-phenylalanine (FMLP, final concentration of 100 nM), also without or with the $A_{2A}R$ agonist, CGS21680 (10–300 nM). At the end of the experiment, the cell coordinates were recorded. For cell type identification, the cells in the coverslips were fixed with ice-cold methanol for 10 min and stained with May-Grünwald-Giemsa. The analysed cells were located using the gridded coverslip coordinates and each cell type was identified by its staining pattern.

The offline image analysis was performed using the software ImageJ version 1.48v (NIH, USA). A region of interest (ROI) was manually selected for each cell and the average fluorescence intensity was measured for both series (340 and 380 nm image stacks). Previous experiments in cell-free coverslips established the background intensity (112 arbitrary units). A graph of the corrected ratio, R, the average fluorescence light intensity emitted upon alternated excitation at 340 and 380 nm (R = F340/F380) for each cell was created. Changes in R correspond to the changes in the levels of cytosolic free calcium [14,15]. For each cell, a baseline

value, peak value, and percentage change were calculated using Microsoft Excel (Microsoft, Redmond, USA).

2.4. Immunocytochemistry analysis

The same procedure of collection and viability analysis were used. Briefly, the cells were diluted (1:1) in HBSS with 1% P/S and plated in glass coverslips for 60 min. After washing in HBSS, the coverslips were exposed to HBSS with 1% P/S and FMLP 1 μM at 37 °C for 1.5, 3 and 6 h in a 95% O_2 and 5% CO_2 atmosphere. A higher concentration of FMLP was chosen for this protocol in order to achieve a supra-maximal stimulation of cells. Appropriate controls without FMLP were also prepared. The coverslips were fixed in 4% paraformaldehyde for 10 min, and kept in phosphate buffer with saline (PBS) at 4 °C until further analysis.

For $A_{2A}R$ immunocytochemical analysis, the cells were first permeabilized with 0.25% Triton X-100 in PBS for 5 min. After washing, the cells were blocked with 5% normal horse serum (NHS) for 60 min. The primary antibodies (goat polyclonal anti-$A_{2A}R$ and mouse monoclonal anti-CD68) were incubated in PBS with 5% NHS overnight at 4 °C. After washing, the secondary antibodies (Alexafluor 488-labelled donkey anti-mouse IgG and Alexafluor 594-labelled donkey anti-goat IgG) were incubated in PBS with 3% NHS for 2 h. The nuclei were then counter-stained with DAPI (1:5000 for 5 min), washed and mounted with DAKO S3023 Fluorescence Mounting Medium (DAKO, Glostrup, Denmark). Controls for non-specific binding were performed by omitting the primary antibodies in separate coverslips. The coverslips were imaged using a Zeiss Axio imager Z2 fluorescent microscope (Carl Zeiss, Jena, Germany), photographing six fields per coverslip in different areas covering all quadrants. The offline analysis was also performed using the ImageJ software. A region of interest for the macrophages was manually selected using a manual threshold command in the Alexafluor 488 channel (CD68 staining). The mean fluorescence intensity for each ROI was then measured in the Alexafluor 594 channel ($A_{2A}R$ staining). The mean absolute fluorescence in this last channel was then calculated and averaged for each time point in samples from each patient.

2.5. Statistical analysis

The statistical analysis was performed using the software STATA 13.1 (StataCorp, College Station, USA). The numerical variables were analysed using measures of central tendency (median) and distribution (interquartile range - IQR), and the categorical variables were expressed using proportions (percentage). The differences between two groups were tested using the Mann-Whitney *U* test, and the differences between more than two groups were tested using the Kruskall-Wallis test. A post-hoc test was used to compare specific pairs. A 95% two-tailed confidence was considered for all tests.

3. Results

3.1. Patient population

A total of sixteen patients were included, nine for the calcium transients, and seven for the immunocytochemistry analysis. The patient's clinical and BAL characteristics are reported in Table 1. A slight female predominance and a median age of 52.0 (IQR 14.5) were observed. Most had a final diagnosis of interstitial lung disease. The BAL differential cell count found a predominance of macrophages in 11 out of 16 patients (Table 1).

3.2. Intracellular free-calcium analysis

We analysed a total of 706 macrophages from 9 patients. When the alveolar macrophages were exposed to 100 nM of FMLP for 120 s, we recorded a 97.0% median (IQR 68.1) peak elevation above baseline of intracellular free calcium levels, as assessed by FURA-2 fluorescence imaging. The median basal FURA-2 ratio (F340/F380) was 0.384 ± 0.076. When the experiment was repeated under superfusion with the selective adenosine A_{2A} receptor ($A_{2A}R$) agonist CGS21680, a concentration-dependent reduction in this peak to 89.5± 61.3% at a concentration of 100 nM and to 81.5± 71.4% at a concentration of 300 nM was recorded ($p < 0.05$, Kruskall-Wallis; $p < 0.05$ control *vs*. 300 nM CGS21680, Dunn's test). Importantly, superfusion with CGS21680 at various concentrations did not cause significant changes or transients in intracellular free calcium. Representative recordings of intracellular free calcium in alveolar macrophages are displayed in Fig. 1b and the average FMLP-induced calcium transients in the absence or presence of CGS21680 are shown in Fig. 1a.

3.3. Immunocytochemistry

When the cells were exposed to 1 μM FMLP, we observed a time-dependent increase of $A_{2A}R$ staining in CD68-stained macrophages (Fig. 2a). As shown in Fig. 2b, after 6 h of exposure to 1 μM FMLP, the cells displayed a median increase of $A_{2A}R$/CD68 fluorescence of 147.6% (SD 28.4) when compared to the control ($p < 0.01$, Kruskall-Wallis; $P < 0.05$ 1.5 vs 6 h, Dunn's test).

4. Discussion

Our main finding was that adenosine A_{2A} receptors ($A_{2A}R$) can undergo adaptive changes of density and that their activation reduces the intracellular free calcium transients resulting from exposure to an inflammatory stimulus in human alveolar macrophages.

Anti-inflammatory effects resulting from $A_{2A}R$ activation have been shown in several animal and cell culture models. However, subsequent clinical studies in pulmonary diseases were negative [8–10]. Some authors argued that this lack of efficacy stemmed from the use of infra-therapeutic doses for fear of cardiovascular toxicity [11]. Others pointed to major inter-species variability in purinergic receptor presence and distribution to explain the lack of effect [11]. Our study showed that $A_{2A}R$ reduces the FMLP-induced cell activation of freshly collected human alveolar macrophages. Furthermore, we also showed that the exposure of human alveolar macrophages to a strong pro-inflammatory stimulus increased $A_{2A}R$ density. This suggests that $A_{2A}R$ agonists will be optimally effective in human disorders characterized by particularly robust inflammation such a sepsis or ischemia-reperfusion syndrome. Accordingly, previous studies have found striking effects of $A_{2A}R$ agonists in animal models of these conditions [16,17]. It can be expected that these effects are also present in lung neutrophils, eosinophils and possibly lymphocytes. However these cells are normally present in much smaller population in normal lungs as well as in most chronic pulmonary diseases [18], precluding the direct analysis of the effects of $A_{2A}R$ in these cells in our pulmonary samples. Importantly, radioligand studies in lymphocytes, neutrophils and platelets from the peripheral blood of healthy human volunteers have shown that these cells express $A_{2A}R$ with similar sensitivities, and the activation of this receptor leads to elevated intracellular cAMP levels [19]. A study by Anderson et al., showed that in neutrophils, the anti-inflammatory effects of $A_{2A}R$ were mediated by cAMP-dependent re-sequestration of FMLP-induced transients of cytosolic calcium [20].

Table 1
Clinical and BAL characteristics of the patient population. All values are expressed as n, % or median ± IQR. No patient was under medication with systemic steroids. The patients with asthma (two in the calcium study) were both on a medium dose of an inhaled steroid. BAL: bronchoalveolar lavage; IQR: interquartile range; TCC: total cell count.

	Calcium study	Immunocytochemistry	Total
Patients (n)	9	7	16
Male n (%)	4 (44.4%)	3 (42.9%)	7 (43.8%)
Median age (years)	56.0 ± 8.0	45.0 ± 14.0	52.0 ± 14.5
Diagnosis (n)	Sarcoidosis – 4	Sarcoidosis – 4	
	Asthma – 2	Rheumatoid lung – 1	
	Lupus lung – 2	Emphysema - 1	
	Hypersensitivity pneumonitis - 1	Hypersensitivity pneumonitis - 1	
Smoking habits	Never smokers: 8	Never smokers: 6	
	Smokers: 1	Former smokers: 1	
BAL			
TCC (cells/μL)	150.0 ± 70.0	140.0 ± 177.0	145.0 ± 85.0
Macrophages (%)	72.0 ± 37.0	63.0 ± 27.0	66.5 ± 31.5
Lymphocytes (%)	18.0 ± 20.0	27.0 ± 24.0	21.5 ± 28.5
Neutrophils (%)	8.0 ± 3.0	1.0 ± 6.0	5.5 ± 7.0
Eosinophils (%)	0.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.5

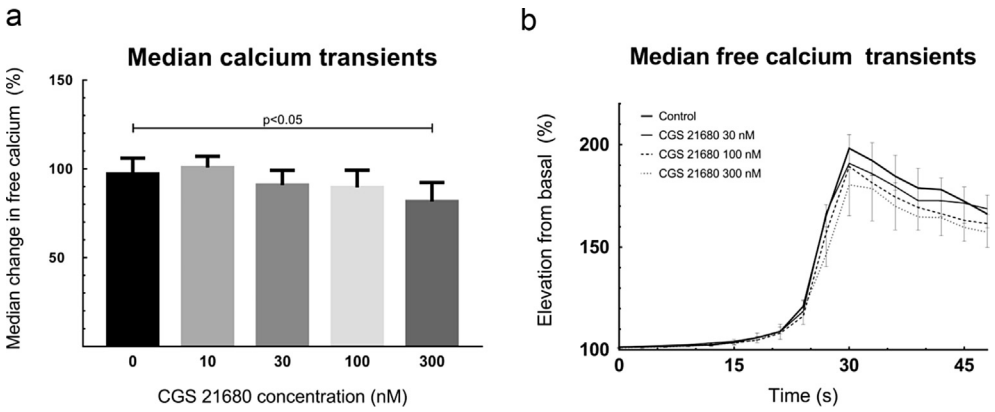

Fig. 1. (a) Median elevation of intracellular free calcium as assessed by FURA-2 ratio in human alveolar macrophages upon stimulation with the pro-inflammatory polypeptide FMLP (100 nM, formyl-methionyl-leucyl-phenylalanine), in the absence or presence of the adenosine A_{2A} receptor ($A_{2A}R$) selective agonist CGS21680 (10–300 nM). The error bars display the 95% confidence interval of the median. (b) Time course of the variation of the FMLP (100 nM)-induced calcium transients in the absence or presence of CGS21680: only the concentrations of 30, 100 and 300 nM of CGS21680 are depicted for clarity. The values are medians and the error bars are the 95% confidence interval of the median.

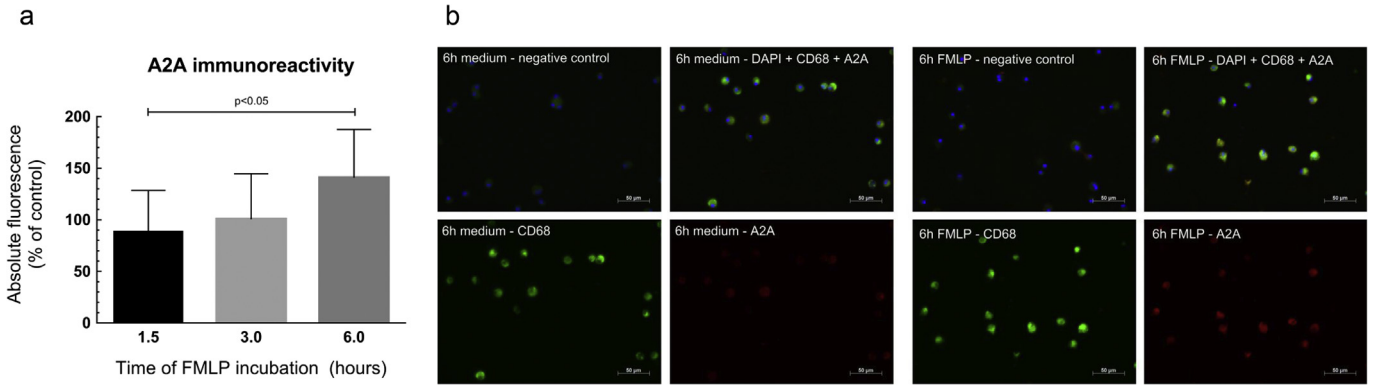

Fig. 2. (a) Time course of the relative increase of median adenosine A_{2A} receptor ($A_{2A}R$) immunodensity in human alveolar macrophages upon exposure to 1 μM of the pro-inflammatory polypeptide FMLP (formyl-methionyl-leucyl-phenylalanine). The error bars display the 95% confidence interval of the median. (b) Representative image of human alveolar macrophages without or with exposure to 1 μM FMLP. Blue: nuclear staining; Green: CD68 staining; Red: $A_{2A}R$ staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This study has several limitations. First, we evaluated a small number of patients with different diseases, and we did not use a control group. However, the use of a healthy control group would imply exposing these individuals to the risks of a bronchoscopy procedure. Also, since $A_{2A}R$ seem to be less abundant in the absence of inflammation [5], the addition of a control group would probably not change our conclusions. The inclusion of a homogenous population of patients with a defined condition could facilitate the translation of these results to new treatment for such condition. However, we studied calcium transients, an intracellular second messenger that mediates activation of macrophages by a variety of stimuli. We argue that by reducing intracellular free calcium

transients, a wide spectrum effect is expected in diverse pulmonary inflammatory conditions, such as asthma, COPD and some interstitial lung diseases. Importantly, our immunocytochemistry results suggest that these effects should be more pronounced in the presence of strong inflammatory environment.

The study also has significant strengths. The use of fresh alveolar macrophages allows for an improved generalization of these results to the pathophysiology of human disease. This prevented modifications on the cell behaviour that would occur after cell culture. Similarly, some differences are to be expected between cells collected from a patient *versus* those from commercial cell culture line. The use of human cells precludes any bias by inter-species dissimilarities in distribution and effects that are known to exist in purinergic receptors. Finally, the use of calcium transients, a robust marker of cell activation allows for a very wide application of these results in human pulmonary diseases.

In conclusion, we tested the efficiency of an $A_{2A}R$ agonists in fresh human alveolar macrophages and found an ability of $A_{2A}R$ to undergo up-regulation and to attenuate FMLP-induced intracellular free calcium transients. This supports the contention that $A_{2A}R$ is a promising therapeutic target for treating inflammatory lung disorders in humans, provided that a sufficiently effective dose of $A_{2A}R$ agonists is used.

5. Declarations

Ethics approval and consent to participate

The protocol was approved by the local ethics committee and patients provided informed consent. Ethics committee protocol number: 053-CE-10.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TMA performed the experiments, analysed the data and wrote the first manuscript draft. DIR performed part of the immunocytochemistry experiments. ART set up the calcium analysis experiment and participated in the data analysis. RAC set up and wrote the project, supervised all experiments and reviewed the manuscript. CRC supervised the collection of human samples and reviewed the manuscript.

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Chapter 8
Further studies

Effects of an A_{2A} adenosine receptor antagonist in human alveolar macrophages

Introduction and methods

Further studies on the effects of $A_{2A}R$ in human alveolar macrophages were made using the selective antagonist SCH58261.[1] The use of antagonists has several advantages, for example in radioligand studies, obviating the effects of high and low receptor affinity states.[2] However, in functional studies, the use of an antagonist is not so much linked to studying the function of a receptor, but to demonstrate its involvement in the effects of an agonist and to probe for the tonic activation of the receptor by its endogenous ligand.[3]

The effects of SCH58261 on the intracellular calcium transients of human alveolar macrophages were tested using the same protocol as for the agonist CGS21680 (chapter 7).[4] Briefly, consecutive patients referred for bronchoscopy with bronchoalveolar lavage (BAL) for diagnosis of interstitial lung disease at the University Hospital of Coimbra in Portugal were included. Those with signs of infection or under systemic steroids were excluded. After collection of the informed consent, bronchoscopy and BAL collection were performed according to the Department's general practice, which entails the use of 150 mL of warmed saline. For this study, 10 mL of the pooled fluid was collected. The total and differential cell counts were performed in the central hospital laboratory. For the intracellular free calcium analysis, fresh human BAL cells were plated in gridded coverslips, loaded with the calcium probe FURA-2/AM and exposed to a two minute superfusion of the proinflammatory peptide formyl-methionyl-leucyl-phenylalanine (FMLP) 100 nM in the absence and presence of SCH58261 in different concentrations (30–300 nM). The calcium probe allows evaluating the changes of intracellular free calcium in response to the FMLP stimuli and its modification upon exposure to the SCH58261 compound.[5] After the experiment, the macrophages were identified through the May-Grünwald-Giemsa staining characteristics and the coverslip coordinates. The offline analysis was performed using the software ImageJ version 1.48v (NIH, USA) and Microsoft Excel (Microsoft, Redmond, USA).

Results

A total of three patients were studied, including a 61 years-old male with asthma, a 31 year-old female with sarcoidosis and a 58 year-old female with sarcoidosis. All three had a lymphocyte-predominant BAL cell count. A total of 216 cells were assessed. When alveolar macrophages were exposed to FLMP 100 nM, a median increase in FURA-2/AM ratio of 107.8% (IQR: 54.8) was observed. The median basal FURA-2 ratio (F340/F380) was 0.299 ± 0.042. When the same experiment was repeated under superfusion with SCH58261, a selective $A_{2A}R$ antagonist, calcium transients were unchanged for SCH58261 at 30 and 100 nM, but increased to 170.2% (IQR: 58.6) upon exposure to SCH58261 300 nM ($p<0.001$, Kruskall-Wallis; $p<0.001$ control *vs*. 300 nM SCH58261, Dunn's test). The median FMLP-induced calcium transients in the absence or presence of SCH58261 are shown in Figure 1. Importantly, when cells were exposed to SCH58261 without FMLP, no changes in intracellular free calcium were observed.

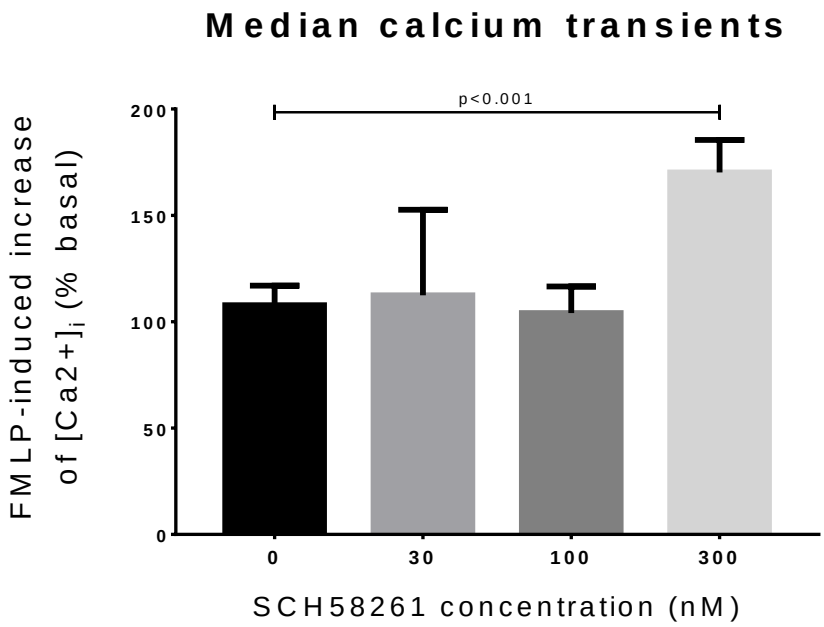

Figure 1: Median elevation of intracellular free calcium as assessed by FURA-2 ratio in human alveolar macrophages upon stimulation with the pro-inflammatory polypeptide FMLP (100 nM, formyl-methionyl-leucyl-phenylalanine), in the absence or presence of the adenosine A_{2A} receptor ($A_{2A}R$) selective antagonist SCH58261 (10-300 nM). The error bars display the 95% confidence interval of the median.

Discussion and conclusions

These results are in agreement with those reported in the paper “Adenosine A_{2A} receptors are up-regulated and control the activation of human alveolar macrophages” - Chapter 7.[4] The amplification of calcium transients that was observed for the highest dose of SCH58261 may mean that even under superfusion there may be some basal effects of adenosine acting on $A_{2A}R$, which will be reduced by the antagonist. It is however surprising that the effects of SCH58261 were observed only with 300 nM, but not with 100 nM. In fact, the affinity of SCH58261 for human $A_{2A}R$ in the low nanomolar range[6] would predict that 100 nM should be a supra-maximal concentration, causing effects similar to these expected for 300 nM. This prompts the alternative possibility that SCH58261 may be acting through non-$A_{2A}R$-mediated effects, a possibility that cannot be discarded since the selectivity of SCH58261 for A2AR has only been experimentally defined in the mouse brain.[7] In view of the limited number of patients so far studied, further studies are warranted to disentangle these different possibilities.

Interaction between purinergic and cholinergic receptors in human alveolar macrophages

Introduction and methods

This study is based on the hypothesis that the purinergic and cholinergic systems exhibit significant functional interactions in the human lung. This is supported by previous research showing that AMP-induced bronchoconstriction is prevented by anti-cholinergic agents[8] and adenosine triphosphate (ATP), a source of adenosine, is co-released with acetylcholine from parasympathetic lung terminals.[9] In fact, this interaction has been shown in animal models: the activation of nicotinic receptors in rat alveolar macrophages led to significant reduction in ATP-induced intracellular free calcium transients.[10]

This experiment was designed to test whether cholinergic receptor modulation led to changes in ATP or FMLP-induced intracellular calcium transients in human alveolar macrophages. Importantly, although ATP can lead to robust intracellular free calcium transients, FMLP was chosen instead as a trigger of intracellular calcium recruitment for two reasons: first, there was previous experience in the characterization of the effects of FMLP; secondly, this would limit the possibility that ATP would be extracellularly metabolized into adenosine to control this calcium response in macrophages, as previously shown in chapter 7. It was also tested whether the antagonism of P2X receptors changes FMLP responses as both ATP and FMLP caused a robust intracellular free calcium elevation in previous studies.[11, 12]

The methods for patient selection, bronchoalveolar lavage collection, and laboratory specimen handling were the same as for the experiment on the effects of $A_{2A}R$ in human alveolar macrophages (chapter 7).[4] For this experiment however, the calcium changes were induced by either ATP 100 µM or FMLP 100 nM. Three experiments were undertaken: 1 - macrophage stimulation with ATP in the absence and presence of the potent nicotinic agonist epibatidine;[13] 2 - macrophage stimulation with FMLP in the absence and presence of epibatidine; 3 - activation of macrophages with FMLP in the absence and presence of the generic ATP P2X receptor antagonist pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS).[14]

Results

A total of five patients were studied, two for the ATP/epibatidine, three for the FMLP/epibatidine, and four for the FMLP/PPADS experiment. The patients' clinical characteristics are detailed in table 1. All had a macrophage predominant BAL cell count, except for the patient with simple pulmonary eosinophilia, who had an eosinophilic BAL fluid.

Table 1: clinical and BAL characteristics of the included patients. All values are expressed as n, % or median ± interquartile range. No patient was under medication with systemic steroids. BAL: bronchoalveolar lavage.

Patients (n)		5
Male sex n (%)		4 (80%)
Median age (years)		66 ± 29
Diagnosis (n)	Sarcoidosis	1
	Idiopathic nonspecific interstitial pneumonia	1
	Asthma	1
	Polymyalgia rheumatica	1
	Simple pulmonary eosinophilia	1
Smoking habits (n)	Ever smokers	0
	Never smokers	5
Bronchoalveolar lavage (median ± IQR)	Total cell count (cells/µL)	90 ± 20
	Macrophages (%)	82 ± 5
	Lymphocytes (%)	12 ± 3
	Neutrophils (%)	2 ± 3
	Eosinophils (%)	0 ± 1

ATP and epibatidine

A total of 75 macrophages from two patients were studied. When the cells were exposed to 100 µM of ATP, a 128.7% median (IQR 48.0) peak elevation in intracellular free calcium was observed. The median basal FURA-2 ratio (F340/F380) was 0.326 ± 0.045. When the macrophages were exposed to ATP in the presence of epibatidine 100 nM, there were no significant changes in this peak elevation of intracellular free calcium, 128.73 +- 48.08 vs. 129.44 +- 53.85%, p>0.05, Kruskall-Wallis.

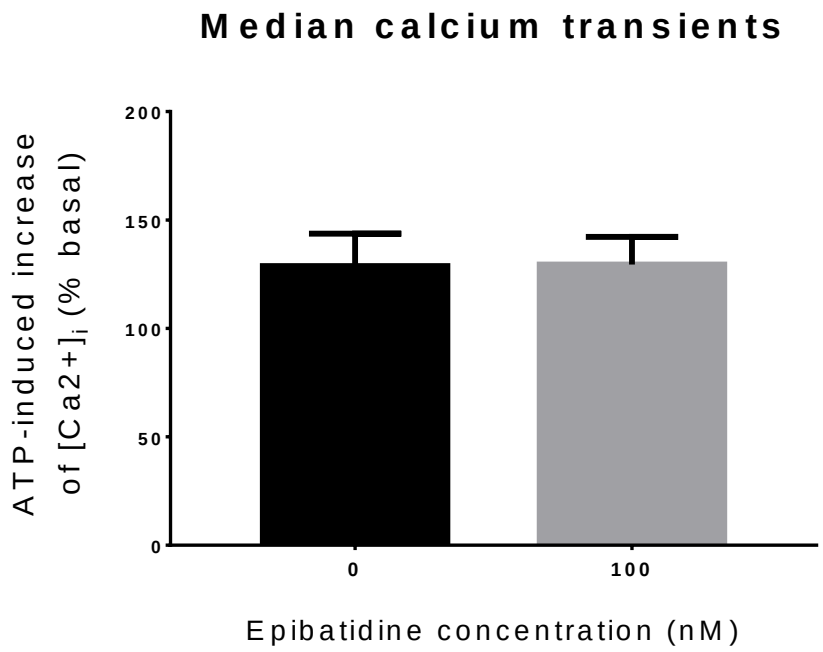

Figure 2: Median elevation of intracellular free calcium ($[Ca^{2+}]_i$) as assessed by FURA-2 ratio in human alveolar macrophages upon stimulation with adenosine triphosphate (ATP) 100 µM, in the absence or presence of the cholinergic β2-containing receptor agonist epibatidine 100 nM. The error bars display the 95% confidence interval of the median.

FMLP and epibatidine

A total of 133 macrophages from three patients were studied. Exposure to 100 nM FMLP led to a 92.9% median (IQR: 47.9) peak elevation above baseline of intracellular free calcium levels. The median basal FURA-2 ratio (F340/F380) was 0.326 ± 0.042. When the cells were exposed to FMLP in the presence of the epibatidine 100 nM there were no significant changes in peak elevation of intracellular free calcium, 92.96 +- 47.9 % vs. 106.13 +- 58.43 %, p>0.05, Kruskall-Wallis.

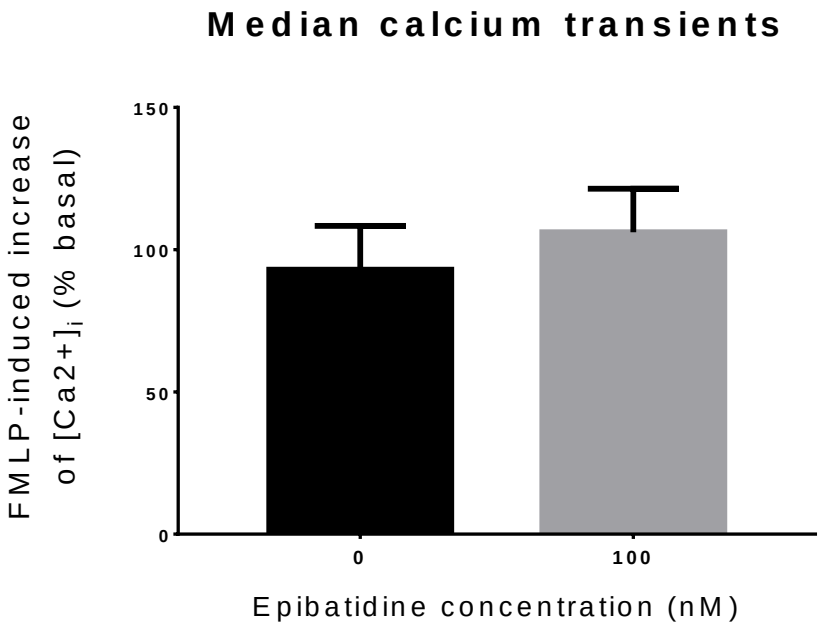

Figure 3: Median elevation of intracellular free calcium as assessed by FURA-2 ratio in human alveolar macrophages upon stimulation with the pro-inflammatory polypeptide formyl-methionyl-leucyl-phenylalanine (FMLP) 100 nM, in the absence or presence of the potent cholinergic agonist epibatidine 100 nM. The error bars display the 95% confidence interval of the median.

FMLP and PPADS

The effects of PPADS in FMLP-induced intracellular free calcium transients were studied in two patients for a total of 70 macrophages. Again, there were no differences between experiments performed in the absence and presence of PPADS, peak calcium elevation of 95.24 +- 40.30 vs. 97.20 ± 52.50%, p>0.05, Kruskall-Wallis. The median basal FURA-2 ratio (F340/F380) was 0.325 ± 0.074.

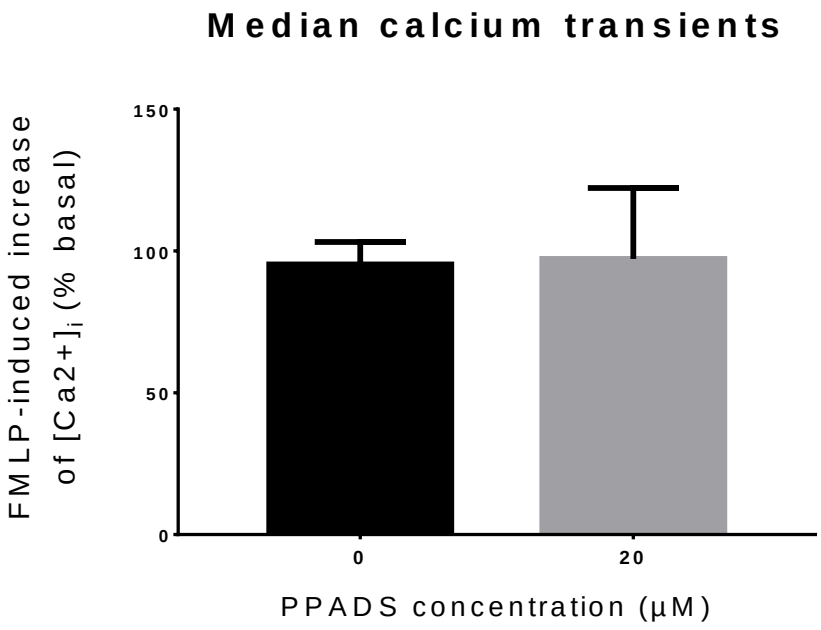

Figure 4: Median elevation of intracellular free calcium as assessed by FURA-2 ratio in human alveolar macrophages upon stimulation with the pro-inflammatory polypeptide formyl-methionyl-leucyl-phenylalanine (FMLP) 100 nM, in the absence or presence of the ATP P2X receptor antagonist pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) 20 µM. The error bars display the 95% confidence interval of the median.

Discussion and conclusions

These preliminary results indicate that the activation of P2 receptors (i.e. exposure to ATP) can trigger an increase of intracellular free calcium with an amplitude similar to that of FMLP. However, when FMLP is used as a trigger of calcium responses in human alveolar macrophages, then there is no participation of P2X receptors, since the generic antagonist of these receptors, PPADS, was devoid of effects. The present preliminary results also seem to exclude a relevant participation of β2-containing acetylcholine receptors in the control of calcium responses in human alveolar macrophages since epibatidine failed to significantly modify the responses triggered by either ATP or FMPL. Therefore, the present results do not support the hypothesis of an interaction between the purinergic and cholinergic receptor systems in the control of human alveolar macrophages.

Previous studies showed expression of multiple P2 receptors in mouse and rat macrophages, with P2Y1, P2Y2, P2Y4, P2X4, and P2X7 evoking ATP-mediated increases of cytosolic calcium levels.[15] Human alveolar macrophages also express mRNA for most P2 receptor types (P2X1, P2X4, P2X5 and P2X7 as well as $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{13}$ and $P2Y_{14}$).[11] Regarding functional studies, only P2X7, P2Y1, P2Y2, and P2Y11 lead to elevations in $[Ca^{2+}]_i$.[11]

Concerning nicotinic acetylcholine receptors (nAchR), an RT-PCR study showed that rat alveolar macrophages express subunits α3, α5, α9, α10, β1, and β2 by RT-PCR. In these cells, nicotine reduced ATP-induced elevations in $[Ca^{2+}]_i$ independently of extracellular calcium, showing a modulatory (atypical) effect of nicotinic receptors.[10] Interestingly, although the nicotinic anti-inflammatory effects on extra-pulmonary macrophages are dependent on α7-subunit nAChR, this does not occur in alveolar macrophages, where an α9/α10-subunit composition appears to be the most likely explanation for this effect.[16] In human alveolar macrophages, an RT-PCR analysis showed that the expression of α3, α4, α6, α7 and β2 subunits is significantly increased in smokers, compared to non-smokers.[17]

Some other possible explanations are that cholinergic receptors are either not present in the cell membrane, or present in low density, precluding any effects in our experimental model. Importantly, none of the studied patients was a smoker or former smoker. A detailed immunocytochemical characterization of cholinergic receptors in human alveolar macrophages, and their changes in response to an inflammatory stimulus and smoking would be useful for a better understanding of these results.

Immunohistochemical characterization of purinergic and cholinergic receptors in the human lung

Introduction and methods

This study was also derived from the hypothesis that the purinergic and cholinergic systems might interact in the control of pulmonary disease. It was hypothesized that this interaction might occur in non-inflammatory cells, such as parasympathetic lung terminals.

The human lung has a dense nervous system, particularly on the airways.[18] An important function of this neural network is the maintenance of the bronchial smooth muscle tonus. In fact, parasympathetic tonus is the main reversible component of bronchoconstriction in patients with chronic obstructive pulmonary disease and one of the main targets for treatment in this disorder.[19]

This task sought to characterize the presence of purinergic and cholinergic receptors in airway parasympathetic terminals. A two-step design was followed: the immunohistochemical identification of cholinergic lung terminals in lung slides, followed by concomitant identification of purinergic receptors in the same terminals, using immunofluorescence.

A protocol was established with the Department of Pathology of the Faculty of Medicine of the University of Coimbra, which provided archived redundant lung tissue from operated lung cancer patients. These samples were part of a cancer biobank and previous ethical board approval and informed consent were provided for this biobank. The lung tissue was fixed in formaldehyde and embedded in paraffin according to the Pathology Department's standard practice.

For the immunohistochemistry protocol, lung tissue blocks were cut into 4 µm sections and lifted onto poly-L-lysine coated glass slides. Deparaffinization and rehydration were performed using xylene and decreasing ethanol concentrations. This was followed by antigen retrieval using a 10 mM citric acid solution in phosphate-buffered saline (PBS) with a pH of 6.0 at 65ºC for 20 minutes. Non-specific blocking and permeabilization was performed using PBS with 5% horse serum and 0.25% triton X-100 for 20 minutes at room temperature. The primary antibodies were diluted in the same blocking solution and incubated overnight at 4ºC. This was followed by washing in PBS with 2% horse serum and incubation with the secondary antibody, diluted in the same solution for two hours at room temperature. Finally, the nuclei were counter-stained with DAPI (1:5000 for 15 minutes), and the slide washed and mounted with DAKO S3023 Fluorescence Mounting Medium (DAKO, Glostrup, Denmark). Appropriate controls for non-specific binding were performed by omitting the primary antibodies in parallel experiments.

Results

A rabbit polyclonal antibody was used for the human vesicular acetylcholine transporter in human lung slides, but the staining pattern was not consistent with the previous reports on parasympathetic lung terminals.[20] Further attempts were made using rabbit polyclonal anti-choline acetyltransferase antibody, but the staining pattern was also inconsistent with cholinergic terminals. In parallel, the lung slides were stained for adenosine receptors (A_1 and A_{2A}), ionotropic ATP receptors ($P2X_2$, $P2X_3$, $P2X_5$ and $P2X_6$), and for the metabotropic ATP receptor $P2Y_1$. The antibody specifications and dilution for each stain are described in table 3.

Table 3: specifications and usage conditions of the primary antibodies.

Receptor	Antibody	Type	Origin	Dilution
A_1	Abcam – ab82477	Polyclonal	Rabbit	1:100
A_{2A}	Santa Cruz – sc7504	Polyclonal	Goat	1:50
$P2X_2$	Alomone – apr003	Polyclonal	Rabbit	1:100
$P2X_3$	Alomone – apr026	Polyclonal	Rabbit	1:200
$P2X_5$	Santa Cruz – sc15191	Polyclonal	Goat	1:200
$P2X_6$	Santa Cruz – sc15197	Polyclonal	Goat	1:200
$P2Y_1$	Alomone – apr009	Polyclonal	Rabbit	1:100

A representative picture and staining interpretation for each tested receptor is presented on figures 5 to 11.

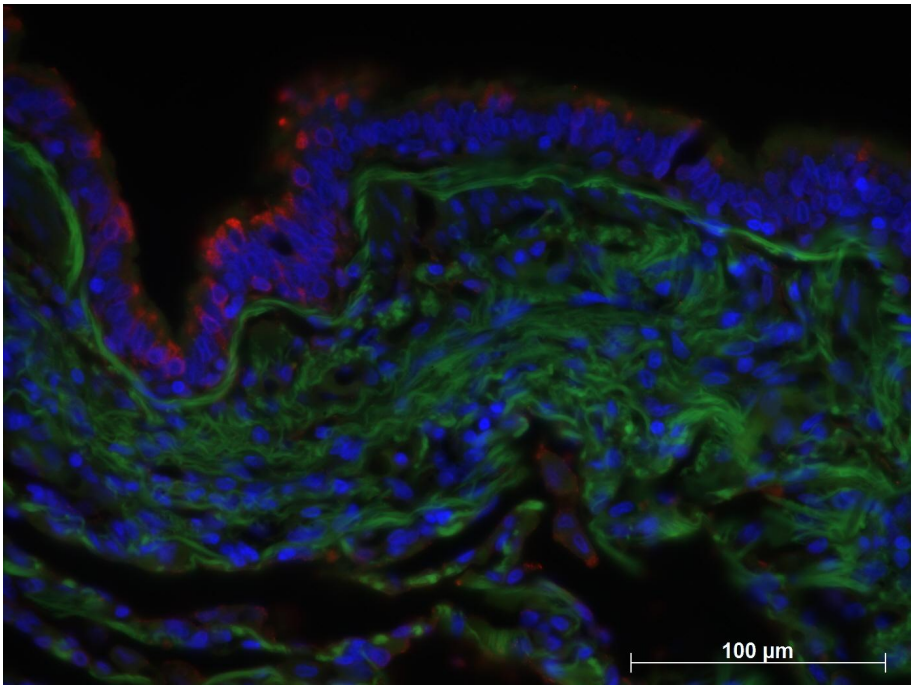

Figure 5: Immunohistochemistry for adenosine A_1 receptor (A_1R): A mostly apical cytoplasmic immunostaining on bronchiolar epithelial cells was observed, suggesting that these cells have significant A_1R cytoplasmic expression. Blue: DAPI; Green: auto-fluorescence; Red: A_1R.

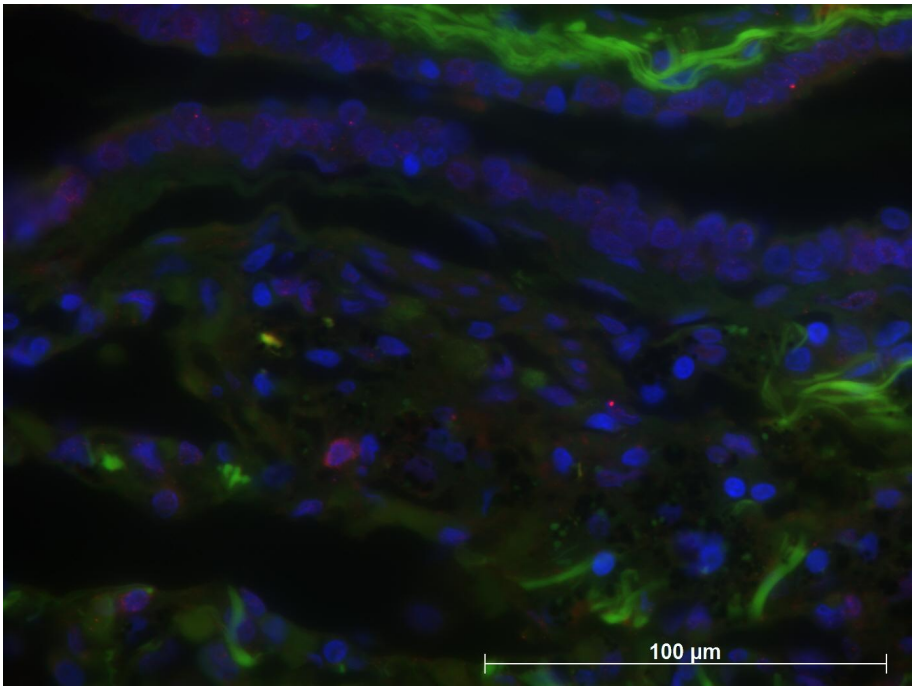

Figure 6: Immunohistochemistry for adenosine A_{2A} receptor ($A_{2A}R$): low intensity immunostaining on bronchial epithelial and numerous sub-epithelial cells was observed. Blue: DAPI; Green: auto-fluorescence; Red: $A_{2A}R$.

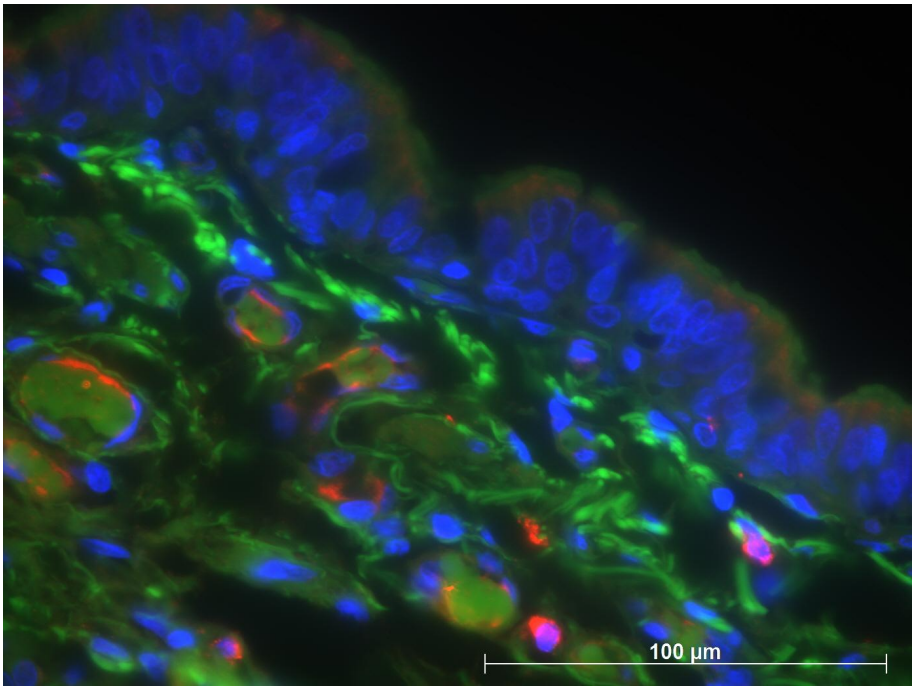

Figure 7: Immunohistochemistry for $P2X_2$ receptor. $P2X_2$ immunostaining concentrated in sub-epithelial vessels, possibly endothelial cells. There was also cytoplasmic staining in several non-identified sub-epithelial cells. Blue: DAPI; Green: auto-fluorescence; Red: $P2X_2$ receptor.

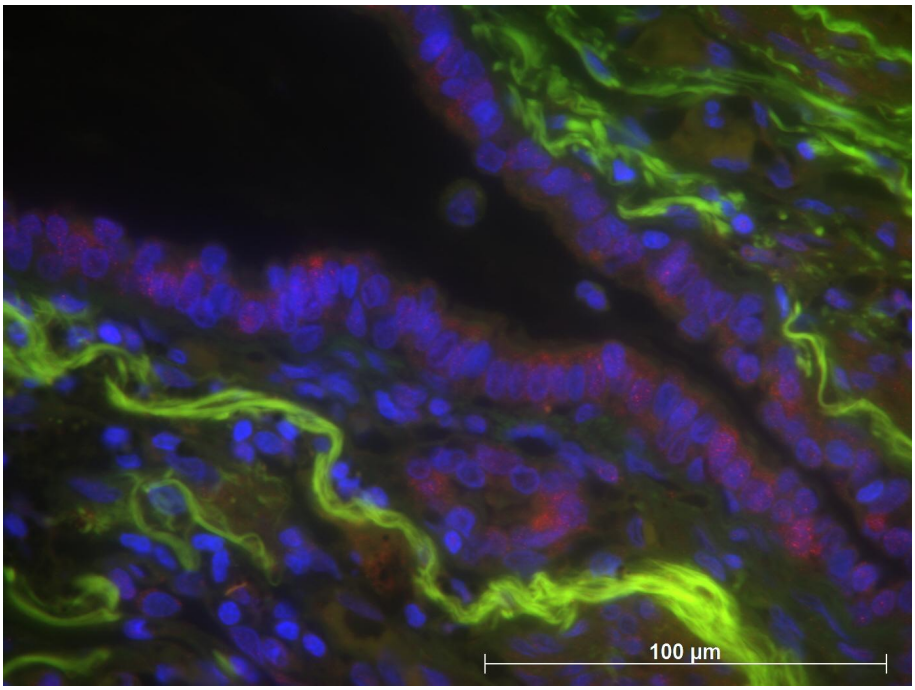

Figure 8: Immunohistochemistry for $P2X_4$ receptor: there was mild cytoplasmic immunostaining in bronchiolar epithelial cells. Unlike the A_1 receptor, there was no apicobasal staining gradient. Blue: DAPI; Green: auto-fluorescence; Red: $P2X_4$ receptor.

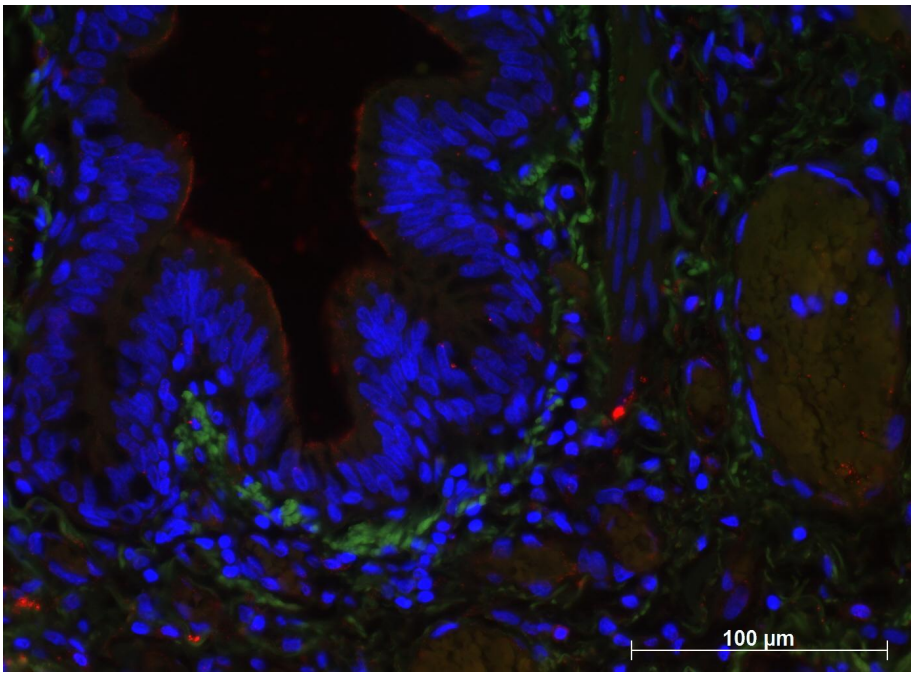

Figure 9: Immunohistochemistry for $P2X_5$ receptor: there was strong immunostaining on the apical border of bronchial epithelial cells. Blue: DAPI; Green: auto-fluorescence; Red: $P2X_5$ receptor.

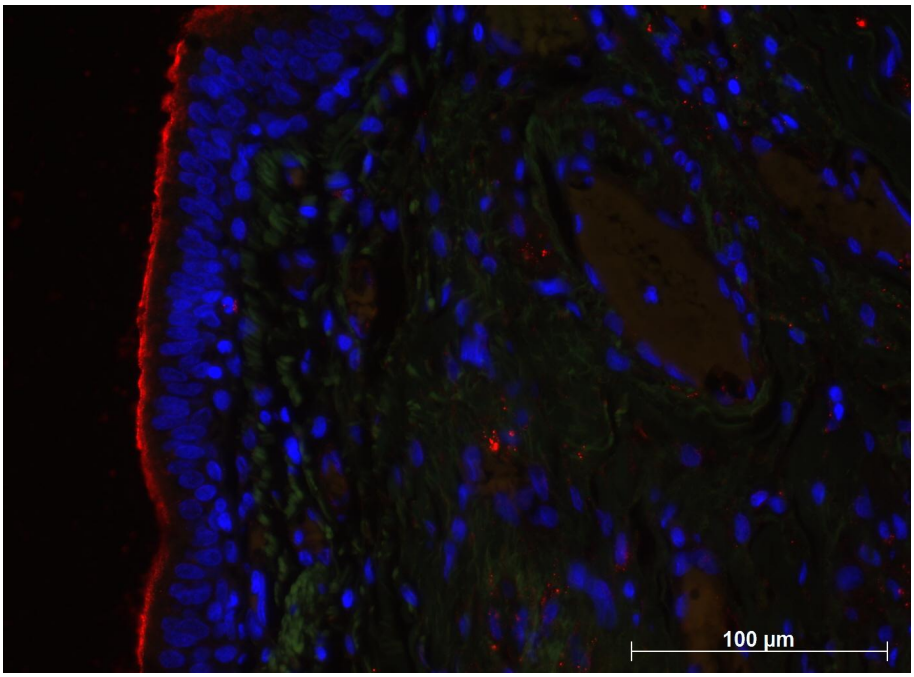

Figure 10: Immunohistochemistry for $P2X_6$ receptor: a similar pattern to $P2X_5$ receptor was found. Blue: DAPI; Green: auto-fluorescence; Red: $P2X_6$ receptor.

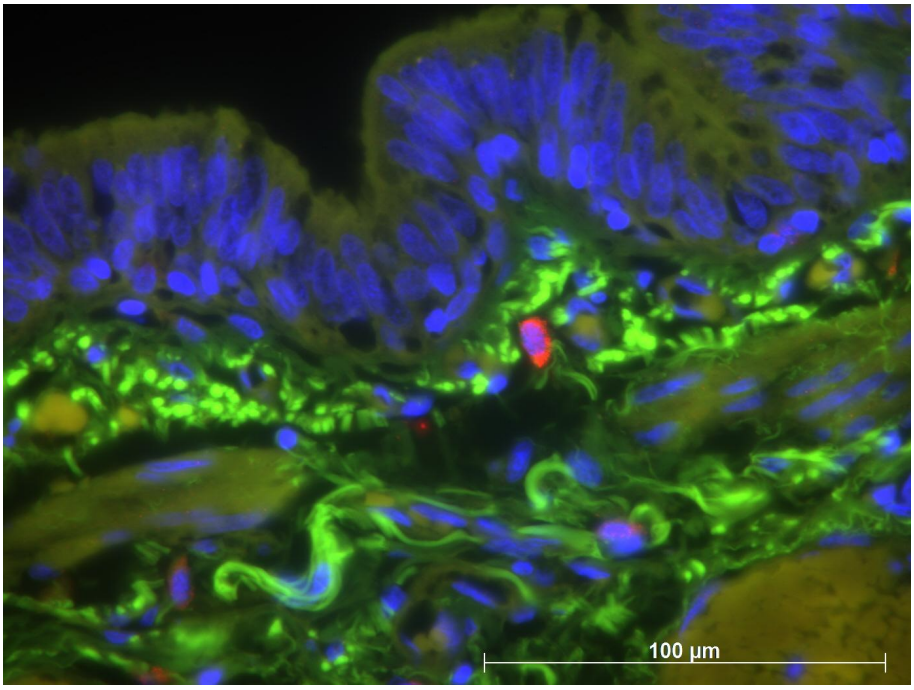

Figure 11: Immunohistochemistry for $P2Y_1$ receptor: there was cytoplasmic immunostaining for occasional sub-epithelial unidentified cells. Blue: DAPI; Green: auto-fluorescence; Red: $P2Y_1$ receptor.

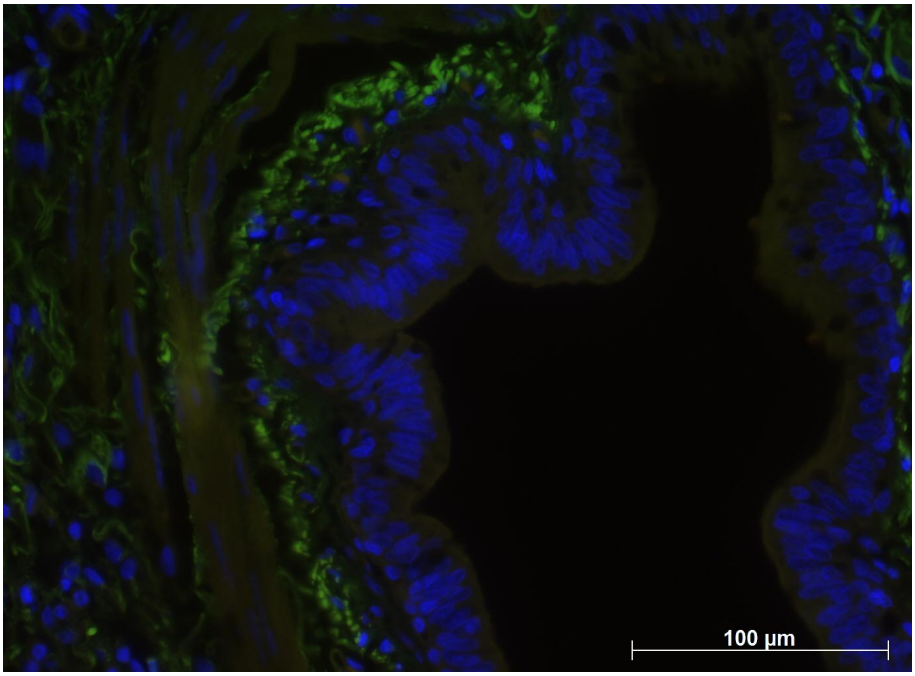

Figure 12: negative control. This was performed by omitting the primary antibodies. Blue: DAPI; Green: auto-fluorescence.

Discussion and conclusions

This task was severely hindered by the difficulties encountered in the identification of airway parasympathetic terminals. The immunostaining for A_1R and $A_{2A}R$ revealed that they are mostly located in the cytoplasm of epithelial bronchial cells. There was increased variability in distribution in P2 receptor distribution, with $P2X_2$ receptors being probably present in endothelial cells, $P2X_4$ and $P2Y_1$ in the cytoplasm of epithelial cells, and $P2X_5$ and $P2X_6$ in the apical border of epithelial bronchial cells.

The expression and function of purinergic receptors in the human lung have been previously studied. Brown detected weak expression of A_1R in the epithelium of healthy subjects using immunohistochemistry,[21] but these results were not confirmed by Varani[22] and by Versluis,[23] both using immunohistochemistry in smokers and former smokers. In the latter studies, the A1R was only expressed in macrophages. Rollins performed laser capture of ciliated human bronchial epithelium from healthy organ transplant donors and found no expression of A_1R using real-time PCR.[24] The $A_{2A}R$ has been identified in epithelial cells, smooth muscle cells, endothelial cells and infiltrating cells by several groups, similar to current findings. $A_{2B}R$ are expressed in mast cells and macrophages and A_3R have the same distribution as $A_{2A}R$.[23]

Concerning P2 receptors, All P2X receptors except $P2X_6$ been identified in the human pulmonary vasculature.[25] $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ have been identified in human bronchial epithelial cells.[26, 27] The metabotropic receptors $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{14}$ are expressed in human bronchial epithelial cells,[25, 28, 29] whereas pulmonary endothelial cells express $P2Y_1$ and $P2Y_2$.[30] Importantly, P2X and P2Y receptors are also expressed by several immune cell types that are found in the lungs, including macrophages, neutrophils and mast cells.[11, 15]

The current findings are similar to previous reports except for A_1R and $P2Y_1$ receptors. A possible explanation for the different findings of A_1R expression is variability due to the antibodies as discordant findings have already been presented by other groups. Regarding the $P2Y_1$ receptor, the current findings are based on immunohistochemistry, whereas the previous studies are based on RT-PCR.[28, 29]

In conclusion, a number of purinergic receptors (A_1, A_{2A}, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2Y_1$) were identified in the human lung by immunohistochemistry, but the difficulties identifying the airway cholinergic terminals prevent any conclusion regarding their presence in parasympathetic airway terminals.

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Chapter 9
Discussion, conclusions and further research

Discussion

This multimodal approach to study the effects of purinergic receptors in human inflammatory lung disease found general anti-inflammatory effects for the A_{2A} adenosine receptor ($A_{2A}R$) in human lung macrophages. When measuring the lung levels of adenosine triphosphate (ATP), higher levels were found in patients with sarcoidosis compared to patients with hypersensitivity pneumonitis. The studies on caffeine intake showed mostly beneficial effects for lung diseases, including asthma, and post-infectious cough as well as for lung function and respiratory mortality. The studies in COPD and sarcoidosis were less clear. Finally, no correlation was found between caffeine consumption and the risk and severity of sleep apnoea. The additional data gathered do not support the hypothesis that the cholinergic and purinergic systems interact in the control of human pulmonary inflammatory disorders, but this task was severely limited by difficulties in the identification of cholinergic lung terminals using immunohistochemistry.

The main strength of this project was that it was fully based on human samples. Most studies on adenosine receptors were performed in animal models, and the translation of these results to humans was likely hampered by inter-species differences in receptor distribution and effects.[1] In this study, freshly collected macrophages were used for the calcium experiment. These are probably free of the cellular changes that are induced by cell culture and other manipulations. In fact, most studies on macrophages are performed in monocyte-derived macrophages or in cancer-derived cell cultures. Another strength was the use of a translational method, where *in vitro* pharmacological results are correlated with epidemiological studies on the effects of a modulator of adenosine receptors.

The present studies of calcium transients support the notion that adenosine $A_{2A}R$ agonists may be useful for the treatment of human inflammatory lung diseases in which macrophages are involved. Changes of calcium level are a robust marker of cell activation and can therefore mirror a wide spectrum of inflammatory pathways.[2] Thus, the testing of adenosine $A_{2A}R$ agonists in inflammatory lung conditions is warranted, especially in those conditions associated with a robust inflammatory component, since it was confirmed that there is an increased expression of $A_{2A}R$ in macrophages upon exposure to a potent inflammatory stimulus. It is worth noting that animal studies were particularly promising for the effects of adenosine receptor modulation in acute lung injury and ischemia-reperfusion injury.[3, 4]

The quantification of ATP levels in bronchoalveolar lavage may be useful as a novel biomarker for the differential diagnosis of interstitial lung diseases. The different levels of this adenosine precursor may mirror different pathogenic mechanisms and possibly help selecting the diseases

where adenosine may have a more important role, supporting the testing of adenosine based therapies.

Caffeine is a widely consumed non-selective adenosine receptor antagonist.[5] The present studies on the effects of caffeine on the risk and evolution of respiratory diseases are thus an indirect way of studying the health effects of adenosine receptor modulation in humans. The effects of caffeine may however be related to several mechanisms. Caffeine at the levels which are found in regular coffee consumers leads to blockade of A_1R and $A_{2A}R$, with a higher affinity for A_1R.[6] The positive effects that were observed are most likely related to the antagonism of this receptor. It is unknown if the effects on $A_{2A}R$ are also important. It is worth to note that most caffeine is consumed in the form of coffee, which is known to have components with anti-oxidant properties such as polyphenols.[7] The best way to study the effects of the modulation of each adenosine receptor is the use of selective agonists for that receptor. Their use is, however, only currently acceptable after *in vitro* studies have found positive effects, such as the one now carried out in alveolar macrophages. It is argued that the results from this study support the notion that selective adenosine agonists should be tested for the treatment of strong inflammatory lung conditions such as acute lung injury.

Some limitations of the present project should be discussed. Most of these limitations have already been mentioned in the individual chapters; they are related to the inclusion of a heterogeneous population of patients in the study of calcium transients and the sample sizes of the studies in bronchoalveolar ATP levels and caffeine consumption. Furthermore, the results from the epidemiological studies, including the systematic review, may be partly dependent of non-caffeine components of coffee.[8]

Considering the group of studies, the conclusion that adenosine acting on $A_{2A}R$ has positive effects in the pathogenesis of inflammatory lung diseases is limited by the fact that only macrophages were studied, and other cells, such as lymphocytes may eventually display an inverse effect when activated by the same receptor. The correlation between laboratory studies and epidemiological studies is limited by the non-selective nature of caffeine as an adenosine receptor antagonist. However, the completely non-interventional nature of these studies justifies their performance as the first step in a comprehensive evaluation of the effects of adenosine receptors in human respiratory inflammatory disease.

Conclusions and further research

In conclusion, this project encompasses two laboratory studies and four epidemiological studies directed at the effects of adenosine in human lung disease and found evidence that supports a relevant role for purines in the pathogenesis of human inflammatory lung disease. These results support further testing of $A_{2A}R$ agonists for the treatment of inflammatory lung diseases.

This project fostered the development of a close collaboration between a hospital-based clinical unit and a basic science laboratory and provided advanced laboratory research training for a Pulmonologist, leading to the development of truly translational research.

Based on the results from this project, some further research can be proposed. One possibility is the extension of the analysis of calcium transients to different cell types, and possibly well-defined single-disease patient cohorts. The main limitation is that this protocol is very time and labour-intensive. One way to overcome this is the use of cell cytometry for calcium analysis of a cell population, instead of a single cell analysis.[9] The analysis of one receptor in single disease patient cohort will facilitate testing the same receptor in a clinical trial for the same population. Based on the present results, patients with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) are a promising target.

Another proposal is to further test possible interactions between purinergic and other cell communication systems. The initial results obtained on cholinergic nicotinic receptors were negative and this was not further pursued. However, the use of a cytometry based analysis would make further testing possible, allowing for the evaluation of a higher number of receptors, which was not feasible using the method used in this project.

A final proposal is to consider a detailed analysis of the use of regadenoson in patients with inflammatory lung diseases. Regadenoson is an $A_{2A}R$ agonist that is regularly used in clinical practice for cardiac pharmacological stress testing. The introduction of regadenoson in clinical practice was accompanied by concerns that it could have negative consequences in patients with asthma or COPD, but this was not the case.[10] Since these studies were performed to look at negative consequences for this drug, a detailed study looking at any positive effects of the typical use of regadenoson in patients with respiratory inflammatory disease could strengthen the present suggestion to further test this same drug in respiratory patients.

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