DEPARTMENT OF LIFE SCIENCES

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Pollen Proteases and Allergic Disorders

Dissertation submitted to the University of Coimbra to meet the necessary requirements to obtain a Master degree in Biochemistry, conducted under the scientific orientation of Professor Paula Verissímo, PhD (University of Coimbra)

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I dedicate this thesis to my brother, David Lourenço Gaspar, the main reason I finished my work. I miss you, I love you… I believe you would be proud of me…

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List of Abbreviations

ADAM - A Disintegrin and Metalloproteinase

AEBSF – 4-(2-Aminoethyl) benzenesulfonyl fluoride

AIC - Air liquid interface

AJ – Adherent junctions

AMC – 7-Arnino-4methylcoumarin

ANP – Atrial Natriuretic Peptide

APC – Antigen-presenting cell

APRIL – a proliferation-inducing ligand

ASL - Airway surface liquid

BAFF – B cell activating factor

BSA – Bovine albumin serum

CAPS – *N*-cyclohexyl-3-aminopropanesulfonic acid

CBA – Cytometric Bead Array

CGRP - Calcitonin-gene-related peptide

CHAPS – 3[(3-Cholamidopropyl) dimethylammonio]-propanesulfonic acid

CLAP – Protease inhibitor mixture consisting of chymostatin, leupeptin, antipain and pepstatin

CS – Cigarette smoke

DAG - diacylglycerol

DAKO – DakoCytomation Fluorescent Mounting Medium

DC – Dendritic cell

DMEM – Dulbecco's Modified Eagle Medium

DTT – Dithiothreitol

E-64 – *N*- [N- (L-3-trans-carboxyirane-2-carbonyl) -L-leucyl] -agmatine

EDTA - Ethylenediaminetetraacetic acid

EGF – Epidermal growth factor or EGF

EMEM – Eagle's minimal essential medium

FBS – Fetal bovine serum

FcεR – Fc receptors for IgE

GM-CSF - Granulocyte-macrophage colony-stimulating factor

HEPES – *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*

ICAM - Intercellular adhesion molecules

IFN – Interferon

IgE – Immunoglobulin E

IL – Interleukin

IP3 - Inositol trisphosphate

JAM - Junctional adhesion molecule

LDH – Lactate dehydrogenase

LT – Lymphocyte T

MHC – Major histocompatibility complex

MMP – Matrix metalloproteinase

MS/MS – Mass spectrometry

NADPH - Nicotinamide adenine dinucleotide phosphate

NO - Nitric oxide

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

PAF – Platelet-activating factor

PAGE – Polyacrylamide gel

PAR – Protease-Activated Receptor

AEBSF – Commercial name for AEBSF

PGE_2 - Prostaglandin E2

pI – Isoelectric point

PKC – Protein kinase C

PLC – Phospholipase C

Pl3K – Phosphoinositide 3-kinase

PMSF - Phenylmethanesulfonyl fluoride

PVDF - Difluoreto de polivinilideno

RITC-Dex 70S – Rhodamine B Isothiocyanate-dextran

RhoGEF – Rho guanine nucleotide exchange factor

SDS – Sodium dodecyl sulfate

SLPI – Secretory leukocyte peptidase inhibitor

SNP – Single-nucleotide polymorphism

TEMED – Tetramethylethylenediamine

TGF - Transforming growth factor

Th – T helper cells

TJ – Tight junctions

TLCK – N-α-p-tosyl-l-lysine chloromethyl ketone

TLR3 - Toll-like receptor 3

TNF – Tumor necrosis factor

TPCK - L-1-tosylamido-2-phenylethyl chloromethyl ketone

Tris – 2-Amino-2-hydroxymethyl-propane-1,3-diol

TSLP - Thymic stromal lymphopoietin

VIP – Vasoactive intestinal peptide

ZO – *Zonula Occluden*

α1-PI - α1-antitrypsin

Abstract

Allergic disorders, namely asthma, are increasingly prevalent in the development world, and for that reason taken as a serious public health issue. Allergic disorders are recognized as inflammatory disorders of the respiratory airways, and are considered to be triggered in part by pollen. Pollen allergies, where respiratory allergic reactions are induced by pollens, have been showed to be increasing in Europe (Burney *et al.,* 1997; D´Amato *et al.,* 2000). Pollen grains when inhaled and contact the respiratory mucosa are hydrated, releasing allergenic material and proteases. Proteases have been shown to cause epithelium barrier breakdown allowing allergens to contact cells of the immune system, initiating allergic response and perpetuate inflammation (Cortes *et al.,* 2006; Runswick *et al.,* 2007; Takai *et al.,* 2011; Vinhas *et al.,* 2011).

This study focused on three pollen species with different allergenic potential that are widespread over the Mediterranean area: *Chenopodium sp.* (moderate allergenic), *Eucalyptus sp.* (low allergenic) and *Plantago sp.* (moderate allergenic).

Identification of proteolytic activity present in the pollen extracts occurred using zymographys and enzymatic assays, which allowed the perception of high molecular weight proteases with acidic pI. Also, it was observed a majority presence of serine and metalloproteinases in all pollen extracts, with some interesting cysteine activity in *Chenopodium sp.* and *Eucalyptus sp.* pollen extracts.

In order to comprehend the mechanism subjacent to lung epithelium breakdown caused by the exposure to pollen, we resorted to a Human epithelial cell line, Calu-3. The proteolytic activity present in all three pollen extracts were capable of inducing an increase of epithelial permeability in a time-dependent manner and *in vitro* cell detachment by disruption of intercellular protein complexes formed between epithelial cells, namely E-cadherin, Occludin, ZO-1 and Claudin-1. This is obviously due to a direct digestive action of proteases on the respiratory airway epithelium. The effect of *Chenopodium sp.* was tremendous compared to the other two pollen extracts possibly correlated also to the significant presence of both serine and cysteine activity.

The loss of epithelial effectiveness can also occur indirectly through activation of PAR-2 receptors widely expressed on the apical surface of lung epithelial cells. Serine proteases have the ability in activating proteolytically PAR-2 receptors, which

coupled to G-signaling cascades increase phospholipase C levels which in turn lead to increased intracellular Ca^{2+} levels (Berger *et al.*, 2001; Schechter *et al.*, 1998; Ubl JJ *et al.*, 2002). This induces a suggested release of chemokines, cytokines and neurotransmitters through signaling cascades. The proinflammatory role of PAR-2 has been supported by many studies (Sun *et al.*, 2001; Asokananthan *et al.*, 2002; Adam *et al.*, 2006).

Increased intracellular Ca^{2+} levels were induced when exposed to the pollen extracts from *Chenopodium sp.* and *Eucalyptus sp.* After exposure to pollen extract from *Plantago sp.* no visual increase of intracellular calcium was observed, possibly due to a low protease concentration present in the extract. The effect observed was again tremendous for *Chenopodium sp.*, concurrent to what was obtain for induced increased transepithelial permeability and disruption of protein junctions. All pollen extracts were also able to induce the production and release of pro-inflammatory cytokines, namely IL-6 and IL-8.

In the natural environment the respiratory system is exposed to multitude of pollens with overlapping pollination seasons: time and geographic dependent, as verifiable through epidemiologic studies. For that reason, we studied combinations of pollen extracts. The purpose was to observe possible alterations of proteolytic activity when combined. Two combinations of pollen extracts were studied: *Chenopodium sp.* with *Eucalyptus sp.* and *Chenopodium sp.* with *Plantago sp*. Basically, we observed that the presence of *Chenopodium sp.* was able to potentiate and increase the effect of the other pollen extracts when combined via directly and indirectly in lung epithelium disruption.

These studies conducted in this research work are of great importance in obtaining a deeper knowledge of the mechanisms involved in the development of allergic disorders to pollens. Only by these studies will it be possible to correlate proteolytic activity with loss of respiratory airway epithelium integrity and the potentiating of allergic responses. Important also, is the fact that our work suggests that even less allergenic pollens are likely to participate in allergic sensitization and airway inflammation, possibly through access facilitation to a variety of allergens and exposing them to cells of the immune system. These results can be of great interest in the attempt of developing future therapies for allergic disorders.

Resumo

As doenças alérgicas, nomeadamente a asma, têm vindo a aumentar no mundo desenvolvido. As doenças alérgicas são reconhecidas como patologias inflamatórias das vias respiratórias, despoletadas por pólen. Alergias polínicas, induzidas pela exposição a pólen, têm vindo a crescer na Europa (Burney *et al.,* 1997; D´Amato *et al.,* 2000). Grãos de pólen, após inalados, chegam à mucosa das vias aéreas onde são hidratados, libertando o seu conteúdo: material alergénico e proteases. Tem sido demonstrado a capacidade das proteases causarem fragilização do epitélio respiratório permitido alérgenos contactar com as células do sistema imunitário, iniciando a responsa alérgica e perpetuando a inflamação (Cortes *et al.,* 2006; Runswick *et al.,* 2007; Takai *et al.,* 2011; Vinhas *et al.,* 2011).

Neste trabalho foram estudados três pólenes distintos, bem disseminadas pela zona Mediterrânea, com distintas capacidades alergénicas: *Chenopodium sp.* (alergenicidade moderada), *Eucalyptus sp.* (alergenicidade baixa) e *Plantago sp.* (alergenicidade moderada).

A identificação da atividade proteolítica presente nos extratos polínicos foi possível através de zimografias e ensaios enzimáticos específicos, que permitiram demonstrar a presença de proteases com alto peso molecular e pI acídico. Verificou-se uma maioritária atividade serínica e metalloproteinase, como também uma atividade cisteínica adicional nos extratos polínicos do *Chenopodium sp.* e *Eucalyptus sp.*

De forma a compreender o mecanismo subjacente à perda de integridade do epitélio após exposição ao pólen, recorremos a uma linha de células epiteliais Humanas, Calu-3. A atividade proteolítica presente nos extratos polínicos foi responsável pelo destacamento celular *in vitro*, verificando-se um aumento da permeabilidade transepitelial, como resultado da disrupção dos complexos proteicos intercelulares formados entre as células epiteliais, nomeadamente a E-caderina, Ocludina, ZO-1 e Claudina-1. Este efeito sugere uma acção directa de degradação das proteases sobre o epitélio respiratório. O efeito do *Chenopodium sp.* foi muito superior, possivelmente correlacionado com a presença bastante significativa de atividades serínica e cisteínica.

A perda de integridade do epitélio também pode ser, indiretamente, resultado de uma ativação dos receptores PAR-2, largamente expressos na superfície apical das

células epiteliais. Proteases serínicas possuem a capacidade de ativar proteoliticamente os receptores PAR-2, que acoplados com proteínas G sinalizadoras, aumentam os níveis de fosfolipase C que por sua vez aumentam os níveis intercelulares de Ca^{2+} (Berger *et al.*, 2001; Schechter *et al.*, 1998; Ubl JJ *et al.*, 2002). É sugerido uma indução de libertação de citoquinas, quimiocinas e neurotransmissores através de cascatas sinalizadoras. Este papel pró-inflamatório dos PAR-2 é referenciado em vários estudos científicos (Sun *et al.*, 2001; Asokananthan *et al.*, 2002; Adam *et al.*, 2006).

A exposição dos extratos polínicos do *Chenopodium sp.* e *Eucalyptus sp.* induziram um aumento dos níveis intercelulares de Ca^{2+}. Em relação ao extrato polínico do *Plantago sp.* não se verificaram alterações visíveis nos níveis intercelulares de cálcio, provavelmente devido a uma baixa concentração de proteases no extrato. Mais uma vez este efeito foi muito mais significativo para o *Chenopodium sp.*, concordante com o aumento elevado induzido da permeabilidade transepitelial e na degradação das juncões proteicas Todos os extratos polínicos induziram a libertação de citoquinas proinflamatórias, nomeadamente IL-6 e IL-8.

No ambiente natural o sistema respiratório é exposto a uma multiplicidade de pólenes distintos que tenham períodos de polinização sobrepostos, dependente a nível geográfico e temporal verificável por estudos epidemiológicos. Por esta razão, foram estudados duas combinações de pólen, *Chenopodium sp.* com *Eucalyptus sp.* e *Chenopodium sp.* com *Plantago sp*., de forma a observar possíveis alterações a nível da atividade proteolítica quando combinados. Basicamente, observou-se que a presença do *Chenopodium sp.* era capaz de potenciar o efeito do outro pólen quando combinado, quer diretamente ou indiretamente na perda de integridade do epitélio pulmonar.

Estes estudos conduzidos neste trabalho são de grande valor no aprofundar do conhecimento nos mecanismos envolvidos no desenvolvimento das doenças alérgicas induzidas pelo pólen. Só com estes estudos será possível correlacionar a atividade proteolítica com a perda de integridade epitelial e a potenciação da resposta alérgica. Importante, o nosso trabalho sugere que mesmo pólenes menos alergénicos provavelmente participam na sensibilização alérgica e inflamação das vias aéreas, possivelmente facilitando o acesso a uma variedade de alérgenos com uma consequente exposição às células do sistema imunitário. Estes resultados podem ser de grande interesse na tentativa de desenvolvimento de futuras terapias para as doenças alérgicas.

Chapter 1

Introduction

1- Introduction

1.1- Allergic Disorders

Allergic disorders are immediately associated to a set of common symptoms such as runny nose, irritations, eczema, hives, hay fever and asthma. These general and uncomfortable manifestations result as consequences of the immune system. Allergic disorders, such as asthma, are increasingly prevalent in the developed world, and for that reason considered to be a big issue in global public health (Galli *et al.*, 2008). Allergic disorders affect about 40% of the European population (D'Amato *et al.*, 2007).

1.1.1- Type 1 Hypersensitivity

The term "allergy" was first introduced by von Piquet in 1906. The term when introduced was referent to any situation that induced changes in immune reactivity. This was, naturally, associated to both, protective immunity as well as hypersensitive reactions. Nowadays, the term is used almost synonymously to describe excessive responses of the immune system, where exposure to an allergen provokes a series of adverse physiological events (Kay *et al.*, 2001). In particular, when the response is specifically IgE-mediated, which reports to a hereditary predisposition to produce IgE antibodies against environmental allergens or allergens with cross-reactivity, this is referred to as type 1 hypersensitivity reactions or atopy.

These substances referred to as allergens, present in food and the environment, are identified as foreign by antibodies circulating in blood vessels, as well as, in other organic liquids. These antibodies (IgE) are produced accordingly to the exposure of allergens (derived for example, from plant pollen, house dust mites, molds and animal dander). IgE associated to mast cells are found in the cell coating present in the respiratory and digestive system. When IgE is in contact with a specific allergen, an immediate and explosive release of chemical substances from mast cells occurs, originating an intense inflammation, yielding allergy symptoms.

1.1.2- Immune System

The immune system is composed by a network of organs, cells and molecules, which objectively intend to keep the homeostasis of the organism, acting upon general aggressions. The immune system is divided into classes, action dependent, in innate immunity and adaptive immunity. The innate immunity, or non-specific, is our first line

of defense. This type of immunity is based on a set of non-specific cellular and humoral mechanisms that trigger a fast response. It provides physical, chemical and biological barriers, specialized cells and soluble molecules, present in every individual. The principal effector cells of this type of immunity are: mast cells, neutrophils, dendritic cells and natural killer cells, which provide a response that include inflammatory and phagocytosis mechanisms.

Oppositely, the adaptive immune system depends on the activation of specialized cells, lymphocytes, as well as, soluble molecules produced by these cells. The most important features of this type of immunity are: specific and diverse recognition, memory and response specialization, auto limitative and tolerant to components of the proper organism (Cruvinel *et al.*, 2010). In adaptive immunity, as implied by the name, an adaptation occurs, at a molecular level to the allergen´s structural conformation.

Allergens are recognized by small functional regions of the molecule, called epitopes. Epitopes are minimal peptide units of allergens that are obviously recognized by the immune system, and for that reason is a requirement for all immune responses, including allergic sensitization. But, the presence of appropriate epitopes is not sufficient to confer allergic potential to a protein. It´s significant to consider other factors like proteolysis resistance, glycosylation and enzymatic activity. The intrinsic potential of an allergen is expressed in susceptible individuals, if exposure occurs in sufficient amount. The differences between individual susceptibility for allergic disorders are complex and naturally dependent on age, environmental and hereditary factors (Holgate *et al.*, 1999). Nevermore, these epitopes allow discrimination between different allergens, and most importantly the identification of those who are foreign and not part of the organism. Although lymphocytes are the principal cells involved in this immunity response, it´s antigen presenting cells (APCs) that play an essential role in their activation and presenting allergens associated to molecules of the major histocompatibility complex to lymphocytes.

The mucosal surfaces are continuously exposed to a vast array of foreign proteins and pathogens. For this recognition antigen presenting cells (APCs) are fundamental, which have been identified in lungs to be majority dendritic cells, and not mast cells, as initially thought. These cells form a tight network throughout the airway

epithelium, ideally situated to monitor the external environment and sample inhaled allergens (Upham & Stumbles, 2003). B Lymphocytes and epithelial cells are also APCs, and are found to be strategically localized in points of entrance in the organism, so capture and incorporation of proteins from the circulate medium is possible (Holt *et al.*, 1999).

At the crossroads of innate and adaptive immunity, and as the majority type of APCs alongside epithelial cells in the airways, dendritic cells (DCs) are an important part of determining how allergic responses are initiated and perpetuated (Hamida Hammad, 2008). But not only are DC´s responsible for the initiation and amplification of the immune response, they also regulate the qualitative nature of these events and are also viewed as key mediators of immune tolerance (Upham & Stumbles, 2003). Dendritic cells, specialized in the capture and presentation of allergens to lymphocytes, are considered a bridge between innate and adaptive immunity, since elements of innate immunity are determinate in activation and attraction, while these cells are responsible for sensitization of T Lymphocytes of the adaptive immunity.

Allergens are captured, processed and partially digested within the cell and presented at the surface incorporated in major histocompatibility complexes class II molecules. After, APCs suffer a maturation process, migrating to lymphatic nodules, while the complex MHC class II/ peptide are transported to the cell´s surface. The complexes MHC class II/peptide can be specifically recognized by receptors of antigens existent on T Lymphocytes. The recognition is responsible for intracellular stimulating signals that are capable of activating T helper cells (Th). Afterwards, leading to a cell differentiation into T helper cells type 1 (Th1) or type 2 (Th2) (Cory *et al.*, 1999).

Mast cells can also act as APCs, where the recognition of epitopes of allergens is possible through receptors expressed on their surface, potentiating B Lymphocytes to produce specific antibodies. The antibodies produced by these cells have the same specificity as the receptor, binding to the same epitopes (Cory *et al.*, 1999; Holgate, 1999). An effective production of antibodies depends on cooperation between B Lymphocytes and T helper cells. This cooperation can be mediated by a cell-cell contact or the release of cytokines by T helper cells that will activate specific molecules on the surface of B Lymphocytes (Platts-Mills, 2002). The identities of released cytokines that

stimulate B Lymphocytes affect the intensity and quality of antibody production (Fig. 1).

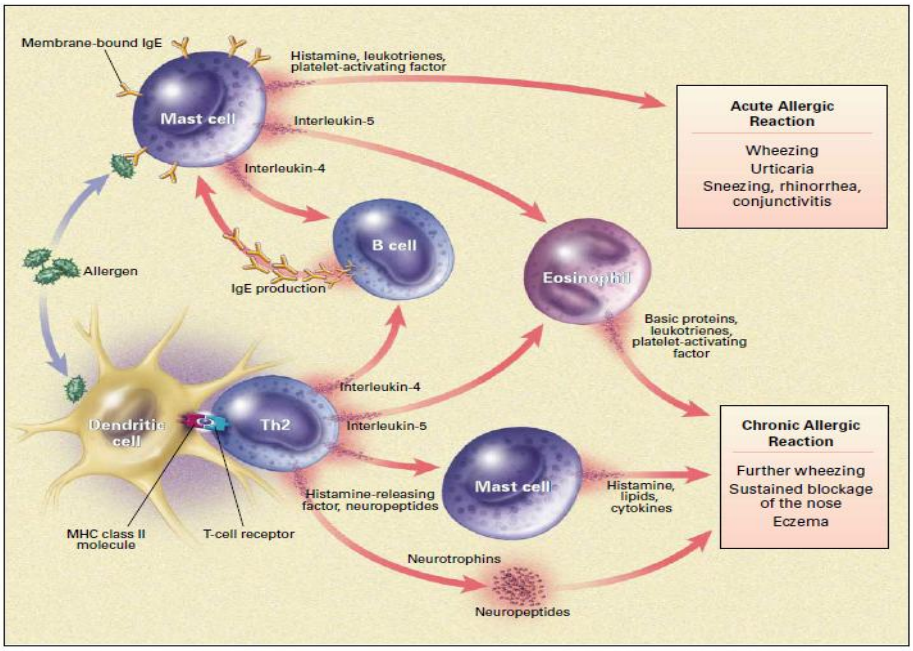


Figure 1 – Pathways that lead to acute and chronic allergic reactions. IgE produced by B Lymphocytes bind to receptors FcƐRI existent on the surface of mast cells and eosionphiles inducing the immediate response, also referred to as the acute allergic reaction. The binding of the allergen to dendritic cells stimulates T Lymphocytes to release diverse molecules that are responsible and develop the allergic response and inflammation (Adapted from Kay, 2001).

Since T helper cells can suffer two distinct maturation processes, this will also cause different characteristics in the responses. Typically, T helper cells type 2 produce essentially cytokines like interleukins (IL)-4, IL-5, IL-6, IL-10 and IL-13, which stimulate B Lymphocytes to produce antibodies, as well as, promotes the differentiation of mast cells and eosionphiles (Holt *et al.*, 1999). On the other hand, T helper cells type 1, typically produce IL-12, interferon (IFN)-Ɣ and tumor necrosis factor (TNF) type α/β, that regulate negatively IgE synthesis. Once established the type of Th response, it tends to maintain because of the suppressive effects of the cytokines produced by Th1 cells on the development of Th2 cells, and vice-versa (Cory *et al.*, 1999) (Fig. 2).

Until recently, the potential to induce a type Th1 or Th2 cell response was not fully clear whether it depended on intrinsic properties of the epitopes, or conferred by other characteristics of the allergen or the immune system (Holgate *et al.*, 1999). Recent studies revealed that the response nature of T helper cells to epitopes depend on the

affinity for the MHC complex class II, the quantity of allergen as well as its biochemical properties (Holt *et al.*, 1999).

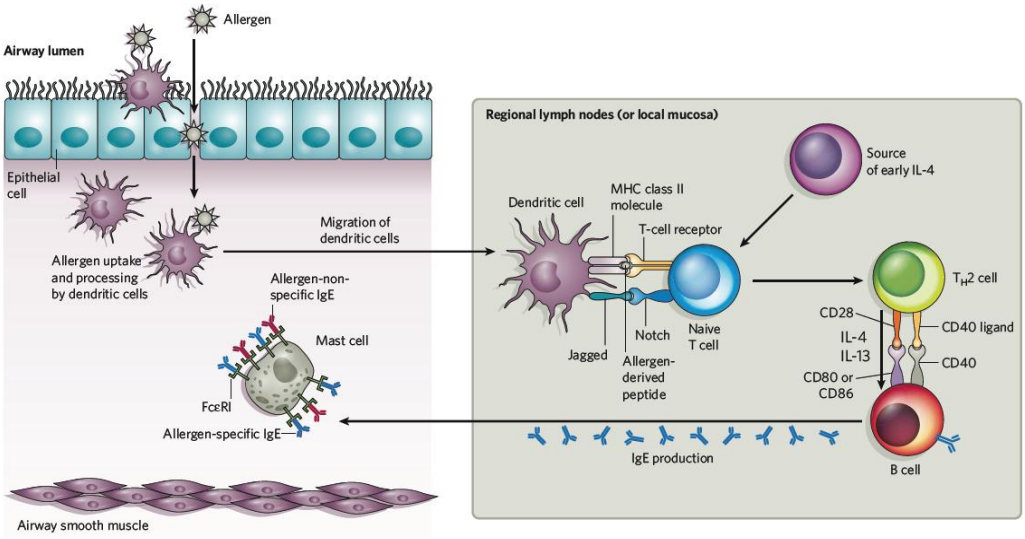


Figure 2 – Sensitization to allergens in the airway. Allergens can be presented to dendritic cells in the airway lumen. But can also enter either through disrupted epithelium (not shown) or by cleaving tight junctions between epithelial cells due to proteolytic properties of the allergen, gaining access to submucosal dendritic cells. Activated dendritic cells mature and migrate to regional lymph nodes, presenting the peptides of the processed allergen linked to the complex MHC class II to naïve T Lymphocytes. The native T Lymphocytes can acquire characteristics of T helper 2 cells. T-helper 2 cells produce IL-4 and IL-13, and B cells are stimulated to produce IgE. IgE diffuses locally and is then distributed systematically. IgE binds to a high affinity receptor FcƐRI, on the mast cells, thereby sensitizing them to respond to a later re-exposure (Adapted from Galli, 2008).

Molecules of IgE bind specifically to allergen epitopes through variable domains (Fab) and to specific receptors on the surface of leucocytes (FcƐR) through constant regions (Fc) (Huby *et al.*, 2000). In the presence of a specific allergen, the cross-link between two or more molecules of IgE at the surface of leucocytes leads to the aggregation of FcƐR receptors. This phenomenon conducts to an influx of calcium in the cell, which is responsible for exocytose of existing interior granules, namely pre-formed pro-inflammatory mediators (Platts-Mills, 2002; Goldsby *et al.*, 2000). These mediators are responsible for symptoms of immediate allergic reaction, such as increase of vascular permeability, vasodilatation and edema (Pearlman *et al.*, 1999; Oettgen *et al.*, 1999). The aggregation of FcƐR receptors leads equally to synthesis induction and also subsequent release of neo-formed mediators, like products of arachidonic acid

(prostaglandins and leukotrienes) and cytokines (Williams *et al.*, 2000) (Fig. 3 & Table 1).

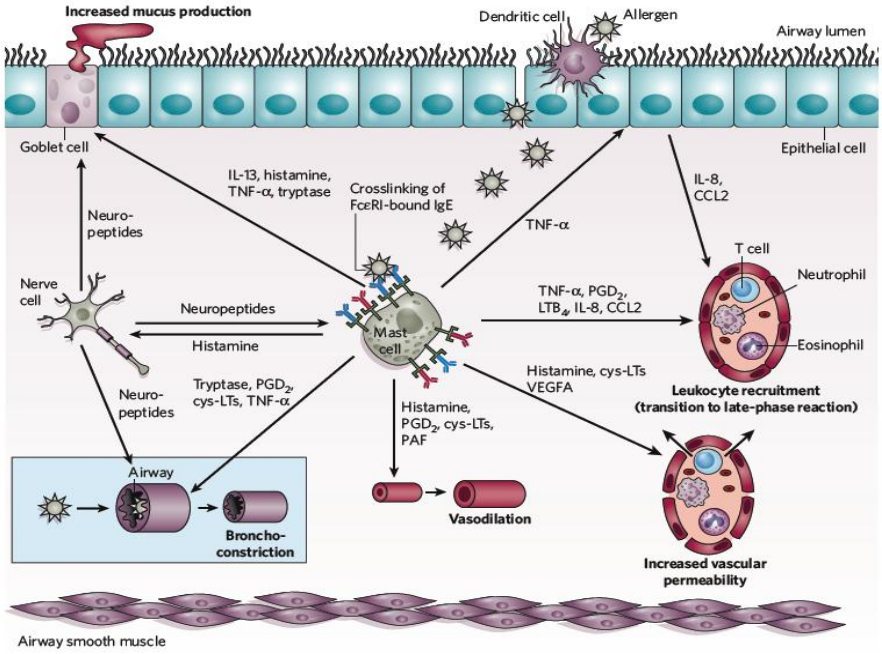


Figure 3 – Early phase of airway inflammation induced by allergens. The IgE molecules that are bound to FcƐRI receptors on the mast cells can be specific for different allergens. The recognition of an allergen, and specific binding of an IgE, induces FcƐRI aggregation, which allows mast cells to secrete mediators, increase the synthesis of cytokines, chemokines and growth factors. This results in vasodilatation, increased vascular permeability and increased mucus production. Also important for contextualization, is the fact the mast cells contribute to the transition to late-phase response (Adapted from Galli, 2008).

Table 1- List of inflammatory mediators and role in allergies. These are the principal mediators released by mast cells after cross-linking of the receptors FcƐRI and their contribution to early and late-phase allergy reactions (Adapted from Goldsby *et al.*, 2000).

Neo-formed Mediators (Derived from arachidonic acid)	**Role in Allergies**
Prostaglandins	√ Vascular dilation and permeability √ Broncoconstriction √ Increase of neutrophils chemotaxis
Leukotrienes	√ Edema √ Increase of vascular permeability √ Mucus production

Neo-formed Mediators (Cytokines)	
TNF-α	√ Increase of chemotaxis and fibroblast growth √ Phagocytosis √ Increase of histamine and tryptase release by mast cells √ Increase of eosinophils cytotoxicity
IL-4	√ Positive regulation of Th2 Lymphocytes promoting a continuous cytokine production (IL-6) and IgE by T Lymphocytes
IL-5	√ Eosinophil recruitment
IL-6	√ Increase production of IgE √ Growth and differentiation of T cells √ Mucus production
IL-8	√ Basophil recruitment
Pre-formed Mediators	
Histamine	√ Increase of vascular permeability √ Broncoconstriction √ Mucus production √ Stimulation of suppressive T Lymphocytes
Heparin	√ Increase of endothelial cell migration √ Regulation of the activity of tryptase, chymase and neutrophils elastase
Chemotactic substances	√ Recruitment of eosinophils and neutrophils
Tryptase	√ Activation of components from the complement system, responsible for degranulation of mast cells √ Activation of PAR-2 receptors *(Protease Activated Receptors*) at a endothelial and epithelial level, increasing the vascular permeability
Chymase	√ Decrease in fibroblast growth √ Degradation of substance P, a broncoconstrictor released by sensorial neurons that nerve the respiratory epithelium

	√ Conversion of angiotensin I in II, a potent vasoconstrictor
PAF	√ Increase of vascular permeability √ Smooth muscle contraction √ Increase of monocytes chemotaxis √ Platelet aggregation

However for a late response, it is the contribution of neo-formed mediators like, for example, cytokines IL-8 and IL-5 that recruit and activate, respectively, basophiles and eosinophils leading to the amplification of the inflammatory reaction, or still, IL-4 that regulates positively Th2 cells promoting the synthesis of cytokines and IgE by B Lymphocytes (Goldsby *et al.*, 2000; Pearlman *et al.*, 1999; Oettgen *et al.*, 1999) (Fig. 4). It is with this situation that allergy reaches a chronic state and possible associated to asthma (Chang, 2000).

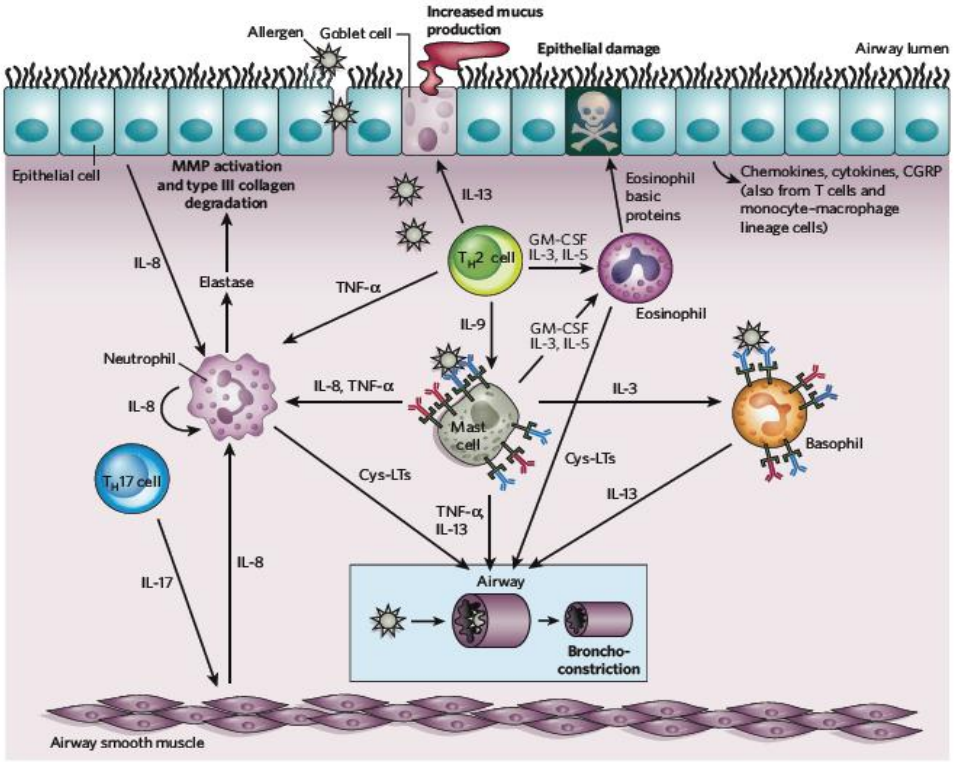


Figure 4 – Late-phase of airway inflammation induced by allergen exposure. The late-phase reactions have many features in common with the early phase (Fig. 3), except the fact that in late-phase the reactions typically occur hours after the allergen exposure. Late-phase reactions include actions from innate and adaptive immune system, as

well as the active participation of cells from the resident tissue. In the late-phase, it's convenient to mention the activation of matrix metalloproteinases (MMPs) and also the secretion of several other mediators CGRP, calcitonin-gene-related peptide; Gm-CSF, granulocyte-macrophage colony-stimulating factor; Th 17 cell, IL-17-producing Th cell. If allergen exposure is continuous or repetitive, inflammation persists, and may cause the transition to a chronic allergic inflammation (Adapted from Galli, 2008).

1.1.3- Allergic Asthma

Asthma is characterized by the increase of hypersensibility in the respiratory airways (AHR, *Airways HyperResponsiveness*) as a response to chemical, pharmacological and/or immunological stimuli. The most common symptoms, whether isolated or simultaneous episodes, include: wheezing, coughing, chest tightness and broncoconstriction (Kay, 2001; McFadden & Gilbert, 1992).

Allergic asthma requires an initial sensitization of respiratory mast cells by IgE antibodies specific to exposed allergens. Which leads to an, already described, early-phase where subsequent inhalation of allergens cause the cross-link of IgE receptors resulting in activation of secretory pathways that lead to release of histamine, leukotrienes, prostaglandins, PAF, and a vast number of cytokines and chemokines. This causes specific alterations of the immediate response, vasodilatation, vascular permeability, broncoconstriction, as well as, the transition to the late-phase response. The late-phase, also previously described, involves a set of cells, Th2 lymphocytes, eosinophils, monocytes and basophils. In nonallergic asthma this scenario is distinct, because an inflammatory early-phase is not visible, being the mechanism involved virtually identical to a late-phase (Kaplan, 2005).

It´s common that patients with initially only one allergic disorder eventually tend to develop other allergic disorders. This is often referred to as the allergic march. This process may result of a vicious cycle in which the function of epithelial barrier is diminished as an inflammatory consequence. This obviously increases exposure of the immune system to original allergens as well as additional allergens, with subsequent sensitization to new allergens through specific IgE production (Galli *et al.*, 2008; Porter *et al.*, 2010). This concept is strong, because nonallergic asthma (intrinsic asthma) refers to a population of asthmatics in whom there is no evidence of IgE-mediated hypersensitivity, accounting for approximately only 40% of adult and 10% of child

asthmatics. Also, nonallergic asthma demonstrates histological and biochemical features strongly similar to asthma associated with allergies. For this reason, they have been considered to possess the same immunopathologic entity, even thought obvious differences in initiating mechanisms (Kaplan, 2005; Humbert *et al.*, 1999).

Allergies for that reason have been considered to be the principal risk factor for asthma. Already studies have proved and established a correlation between asthma and IgE levels in blood serum (Williams *et al.*, 2000). The repeated cycles of T helper cell activation and eosinophils activation/recruitment, which occur during allergic reactions, result in structural and functional modifications on the airway tissue, which are characteristically of an asthmatic state (Platts-Mills, 2002 & Barnes *et al.*, 1998). These modifications result from an exaggerated deposition of collagen in subepithelial layers, which cause a decrease in the diameter of the respiratory airways, as well as, hypertrophy and hyperplasia of smooth airway muscle link to alterations in impulses of the autonomic nervous system (Holgate, 2002; Holt *et al.*, 1999).

Even though allergens are present on daily bases in the natural environment and the majority of asthmatics are allergic to at least one or more inhaled allergen, only a small portion of allergic individuals manifest asthma symptoms. For that reason, a common question researchers have, is why isn't the progression of allergic sensitization to asthma expression an automatic and obligatory process, when all the necessary elements seem to be present in allergic individuals (Platts-Mills, 2002).

Even so, asthma and respiratory airway inflammation depend on additional factors that may directly or indirectly be associated to allergies. For example, viral infections (Johnston *et al.*, 1996), the threshold of sensitization to the allergen in atopic individuals (Peat *et al.*, 1996), tobacco smoke (Michel *et al.*, 1997) and environmental pollution (D'Amato, 2000). Genetic factors also seem to be greatly involved, for example in the recognition of allergens by the MHC complex class II (Holgate, 2002; Platts-Mills, 2002).

Recent findings and observations indicate that the origin of asthma may have an inflammatory component, which naturally means that the products of inflammatory cells play a critical role in this pathology (Ying *et al.*, 2006). These conclusions focused on studies that concentrated on lymphocytes and interactions between T cells and eosionphiles. For example, malformed T Lymphocytes with expressed marker CD25

(IL-2 receptor) and EG_2^+ eosinophils (cleaved form of an eosinophil cationic protein) where found in biopsies of asthmatic bronquial mucosa (Azzawi *et al.*, 1990; Jeffery *et al.*, 1980). Also, an increase of the number of activated Th-lymphocytes $CD4^+$ and eosinophils $EG2^+$ was verified in nasal tissues after late-phase reaction induced by an allergen (Durham *et al.*, 1992; Kay, 1991), as well as , a predominance of memory T lymphocytes ($CD4^+$/$CD45RO^+$) (Frew & Kay, 1991; Hellquist & Karlsson, 1992).

However, it´s becoming clear that by itself inflammation is not able to explain many features characteristic of chronic asthma. The bronchial epithelium in asthmatics is structurally disturbed. Differently from other airway pathologies, airway epithelial shedding seems characteristic of asthma even thought similarities in infiltration of inflammatory components. Thus, either asthmatic epithelium is more susceptible for damage or has an altered response for injury (Holgate *et al.*, 2000).

1.2- Respiratory System

1.2.1- Morphology

Epithelium was once attributed a passive role in serving as a physical barrier preventing inhaled allergens, pathogenic and toxic agents access to the submucosal tissue. Recent studies recognize that airway epithelial cells are more than just a complex physical barrier, responding actively to inhaled antigens, initiating and augmenting airway host defensive mechanisms. Well positioned epithelial cells, throughout the airway can regulate both innate and adaptive immunity through production of functional molecules, as well as, via physical interactions with cells of the immune system (Kato & Schleimer, 2007).

The respiratory system has a conducting parte and a respiratory portion. The conducting parte includes the nasal cavity, nasopharynx, larynx, trachea, bronchi and bronchioles (terminal). The respiratory portion consists of alveolar ducts, alveolar sacs, alveoli and bronchioles. The mature airway is a complex structure lined by a continuous layer of epithelial cells, over a layer of connective tissue that contains a network of capillary tubes, elastic tissue, fibroblasts, and cells that can participate in the inflammatory response like lymphocytes, neutrophils, eosinophils and mast cells (Thompson *et al.*, 1995).

Many types of cells have been discovered in the airway. On the surface of epithelium of the proximal lower respiratory tract, ciliated cells predominate. The proximal region forms a pseudoestratified epithelium, constituted majority by ciliated cells visible in the luminal surface, and minority by basal cells and goblet cells. Basal cells are in contact with much of the basement surface, anchoring their neighbor epithelial cells. The epithelial surface extends into the ducts of mucosal glands, where it is characterized by a variable proportion of ciliated and mucous cells (De Poitiers *et al.*, 1980). In the distal airway, ciliated cells are not present, Clara cells and basal cells predominant, giving a more columnar appearance, and not ciliated, to the epithelium (Thompson *et al.*, 1995; Plopper *et al.*, 1983).

Each of the principal cell components of the pulmonary epithelium, which were mentioned until this point, has very different and unique histological features. Goblet cells have large granules that secret continuously mucous (Mariassay *et al.*, 1984). Basal cells on the other hand are plan and have a pyramidal form (Evans *et al.*, 1989). Ciliated cells are characterized by prominent cilia. Lastly, Clara cells are columnar shaped cells, with apical surfaces which bulge into the airway lumen. Clara cells and histochemical studies suggest that the cell is active in secretion of protein, In particular, CC10, a low-molecular protein that appears involved in the inhibition of elastase (Singh *et al.*, 1988).

Further along the airway, at the alveolar level, the epithelium is very thin and predominantly constituted by type 1 pneumocytes interspersed with type 2 pneumocytes (Crapo *et al.*, 1982). Type 1 pneumocytes cover 95% of alveolar area, and are small and thin cells, allowing because of their form a relative small distance to the endothelial surface with capillary tubes permitting efficient gas exchanges (Crapo *et al.*, 1982). Type 2 pneumocytes are cuboidal in appearance, and have apical surface covered with micovilli and their cytoplasm has numerous of lamellated inclusions. These lamellated inclusions are composed of lipids (predominantly phospholipids) and proteins. They are secreted to the apical surface of alveolar epithelia as pulmonary surfactant. This reduces the superficial tension at the surface of alveolus and also has immuno-modulatory activity (Hamm *et al.*, 1992; Hohlfeld, 2002) (Fig. 5).

At the alveolar level, type 1 cells, being most exposed in the alveolus are the most susceptible in injuries. These cells also lack the ability of cellular repair and

mitosis. Type 2 cells are more resistant to injury. The normal physiological response to injury is the destruction and exfoliation of type 1 cells, followed by proliferation of type 2 cells and finally differentiation into type 1 phenotype. The control of the proliferative response of type 2 cells depends on several factors, that include stimulation with growth factors (EGF and TGF-α) (Kumar *et al.*, 1988). Migration, proliferation and differentiation of epithelial cells are necessary features for repairing the injury accompanying airway inflammation (Thompson *et al.*, 1995).

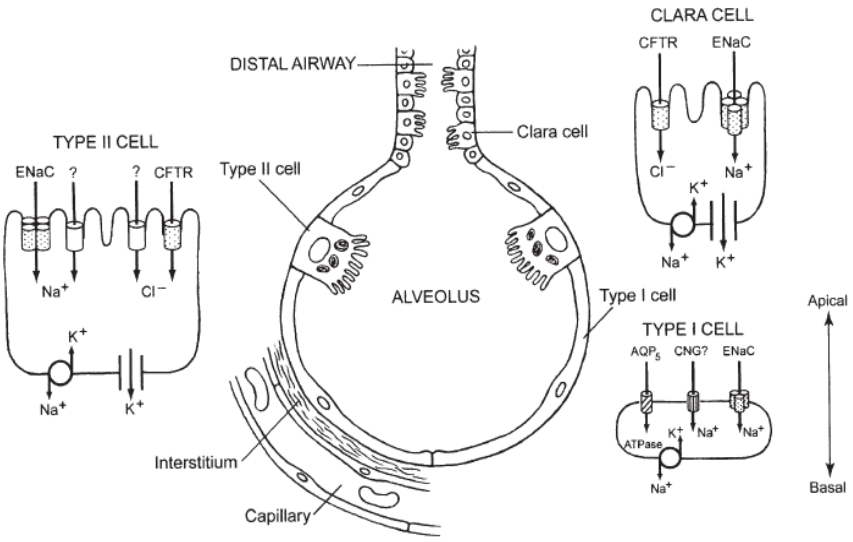


Figure 5 – Morphology of the distal pulmonary epithelium, as well as the main cell components of this airway region, such as type 1 and 2 pneumocytes and Clara cells (Adapted from Matthay *et al.*, 2005).

On a daily basis a person inhales approximately 10,000 liters of air. The nose and upper airway act as a filter to large particulates, and warm and humidify the inhaled air. Even so, the lower airway is exposed daily to a vast variety of agents, whether particles, gas, smoke or biological material. For this reason the pulmonary epithelium has several functions to preserve the normal respiratory activity: coordinated interactions between mucous secretion and ciliary movement, modulation of cell activity in inflammatory response, and to function as a protective barrier (Thompson *et al.*, 1995).

1.2.2- Mucociliary Clearance

After deposition of particles in the lower airways on the mucous surface, these become trapped in mucous. For that reason the efficient clearance of particles inhaled,

depends upon trapping in mucous and clearance of the mucous by cough and ciliary activity. The apical membrane of the airway epithelium is covered by a layer of fluid (*ASL, Airway Surface Liquid*) which consists of two phases. One phase is more liquid mucous, surrounding ciliated cells, while gel-phase mucous rich in mucins, have stickiness properties in order to trap particles. Mucins are a group of complex high-molecular glycoproteins. The physiochemical properties of mucins make them sticky, and for that reason ideal for nonspecific binding to trap particles. In addition to nonspecific binding, mucins also have many carbohydrate receptors that can establish specific interactions with a number of bacterial species: *Haemophilus influenza, Streptococcus pneumonia and Staphilococcus aureus* (Plotkowski *et al.*, 1993). However, it seems clear that immune effector cells and their products also play a role in regulating and altering mucociliary function (Thompson *et al.*, 1995) (table 2).

Factor	Ciliary motility	Mucus velocity	Source
β-agonists	↑	↑	Hormonal
Bradykinin	↑↑	↑	Airway neurones
Histamine	±	↑	Eosinophils, mast cells
Interleukin-1β	↑	?	Macrophages
Leukotrienes	±	?	Eosinophils
Major basophilic protein	↓	?	Eosinophils
Nitric oxide	↑	?	Airway epithelium
Oxidants	↓	↑	PMNs Macrophages
Substance P	↑	↑	Airway neurones
Tumour necrosis factor-α	↑	?	Macrophages

PMN: polymorphonuclear cells.

Table 2- Factors of effector cells and products that can alter mucociliary function (Adapted from Thompson *et al.*, 1995).

In the distal region, where cilia cells are not present, the particle clearance is managed by macrophages and cough. Clara cells and pneumocytes also assist in clearance by producing surfactant which causes changes of charge on the surface of the particles.

1.2.3- Modulation of Inflammatory Cell Function

There is much experimental evidence to suggest that airway epithelial cells interact with inflammatory cells via a number of pathways. Airway epithelial cells not only mediate and activate innate immune responses but also regulate adaptive responses

through interactions with DC, T and B cells (Kato & Schleimer, 2007). Epithelial cells have the capacity to recruit inflammatory cells by releasing chemotactic substances, to direct migration across the epithelium by the expression of cell surface molecules, and to modulate the activity of inflammatory cells via production of cytokines. These pathways all lead to amplification in the inflammatory response (Thompson *et al.*, 1995). However, some cytokines may demonstrate anti- and pro-inflammatory effects, and epithelial cells may release cytokines with predominantly anti-inflammatory effects. These opposite effects induced by cytokines control inflammatory processes, as well as, consequentially chronic inflammation of the airway epithelium (Denburg, 1990). Many chemotactic factors released by epithelial cells may be involved in the activation of neutrophils, macrophages, eosinophils and lymphocytes.

Epithelial airway cells are capable of inducing migration of DCs into epithelium via CCL20 (Chemokine (C-C motif) ligand 20) production (Pichavant *et al.*, 2005; Reibman *et al.*, 2003). Recruitment of DCs to the airway epithelial is essential for adaptive immune B, T and NK cell-mediated response that are important in inflammatory processes. Recent studies believe that cytokines produced by epithelial cells can induce attraction and differentiations of monocytes from the blood stream, given arise to an expansion of DCs in the lung. This seems possible, because epithelial DCs are recruited from the blood, but the number of DCs circulating is too low to correlate with the expansion observed. The mechanisms behind DC differentiation are incompletely understood (Kato & Schleimer, 2007).

Nitric oxide (NO) is a well known vasodilating agent with also evidence of regulation on chemotactic of inflammatory cells. In an inflammation context of the inferior portion of the respiratory system, neutrophils, macrophages and bronchus epithelial cells release NO when stimulated. Obviously, this may suggest a complex interaction between epithelial and inflammatory cells through NO production (Moncada & Higgs, 1993).

Besides the stimulation and recruitment of inflammatory cells, recent studies suggest the participation of epithelial cells in negative regulation of inflammatory cell activity, through synthesis of Transforming Growth Factor type β (TGF-β). TGF-β, present in the interstitial fluid of the epithelial cells, has anti-inflammatory properties,

and is responsible for the inhibition of cytokines produced by macrophages and the proliferation of T cells IL-12 dependent (Kehrl *et al.*, 1986).

Epithelial cells can produce other mediators with inflammatory properties like prostaglandin E_2 (PGE_2) and IL-6. PGE_2 amongst other functions can reduce the production of chemotactic factors by macrophages (Christman *et al.*, 1991). IL-6 is bifunctional, with pro- and anti-inflammatory effects.

1.2.4- Anti-Protease/Protease Balance

Maintaining a balance between proteases and anti-proteases in the epithelium is critical for protection of lung tissue. Otherwise, disturbance of this balance can result in pathologies like asthma. The set of pulmonary inflammatory cells, which include, neutrophils, macrophages, basophils and mast cells are all major protease sources. The activation of these inflammatory cells with subsequent release of proteases to the extracellular region, cause a high increase concentration of proteases. Cleavage and degradation of pulmonary parenchymal proteins and direct effects on the airway have been postulated as a result of the high concentration of proteases (Malech & Gallin, 1987). In normal conditions, the airway epithelium and lung parenchyma is protected from the effect of these proteases because of the presence of an excess of anti-proteases (Thompson *et al.*, 1995).

For example, α_1-protease inhibitor and secretory leucoprotease inhibitor (SLPI) are inhibitors for serine proteases. Differently, α_1-macroglobulin is not class specific, and inhibits proteases from four different classes: serine, metalloprotease, cysteine and aspartic (Thompson *et al.*, 1995).

In the inferior respiratory system, anti-proteases derive from diverse sources, including pulmonary macrophages. SLPI derives entirely from pulmonary epithelium and is presumably the principal inhibitor of elastase from neutrophils, but as well as, of chymase, cathepsin G, trypsin and chymotrypsin (Abe *et al.*, 1991; Franken *et al.*, 1989).

Imbalance of lung proteases and anti-proteases has been associated to inflammatory lung disease. The increase in proteases contributes, either through morphological or functional mechanisms, to a hypersecretory state, which characterizes chronic airway inflammations (Snider, 1981).

1.2.5- Epithelial Barrier

Multicellular organisms have specialized cells, epithelial and endothelial, that form barriers between tissues and different compartments. These cells are polarized, having an apical and basolateral domain and adhere to each other through complexes that form junctions (Matter & Balda, 2003). Histological studies demonstrated the presence of these cell junctions in the respiratory epithelium (Breeze & Wheeldon, 1977; Schneeberger *et al.*, 1978). Cell junctions are specializations of the plasmatic membrane that have a role in maintaining the interaction and link between cells or between cells and the extracellular matrix, but also contain components that are crucial of signaling pathways that regulate epithelial proliferation and differentiation. There are fundamentally three types of junctions: Adherens Junctions (AJ), Desmosome and Tight Junctions (TJ). Under normal circumstances, the healthy bronchial epithelium is an impermeable barrier offering resistance to the paracellular flow of macromolecules and infectious agents, as wells as, limiting significantly ion diffusion (Winton *et al.*, 1998).

Intercalated to the intracellular complex junctions, are gap junctions, composed of conexin proteins forming intercellular pores allowing exchanges of small hydrophilic molecules between cells (Cascio *et al.*, 2005) (Fig. 6).

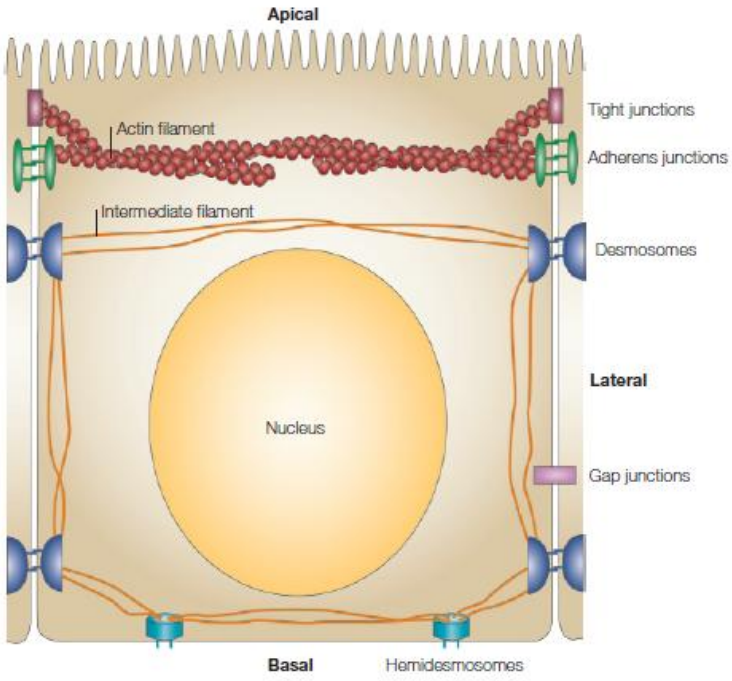


Figure 6 – Representation of epithelial intracellular junctions on a polarized epithelial cell. Tight junctions and adherent junctions are linked to the actin cytoskeleton while desmosomes and

hemidesmosomes are linked to the intermediate filaments (Matter & Balda, 2003).

Tight junction permeability varies widely accordingly to different epithelia, or as a response to a variety of physiological, pharmacological and pathological conditions (Grumbiner, 1987; Balda, 1992). All the classical second messengers including Ca^{2+}, adenosine 3´, 5´-cyclic monophosphate (cAMP), G proteins, protein kinase C (PKC) have been reported to influence the properties of barriers tight junctions (Balda, 1992; Anderson & van Italie, 1995).

Desmosomes are not restricted locally to a site and can be distributed alongside the entire lateral membrane. These proteins are important for the epithelial integrity since they link to intermediate filaments. Adherens junctions form a continuous belt and function to hold neighboring cells together through Ca^{2+} dependent cell-cell adhesion molecules that are linked to actin filaments, called cadherins (Grumbiner, 1987). Both of these junction types are associated to the catenin family of proteins that regulate cell-cell interactions and junction structure (Gallicano *et al.*, 1998).

Tight junctions are the most apical component of the junctional complex and function as a barrier separating the apical from basolateral domains, controlling lateral diffusion of lipid and membrane protein. TJ also prevent the exposure of lymphocytes and APCs to inhaled allergens, and promote physical separation between ligand and receptor which can be activated with binding resulting from the lost of integrity of the epithelium. TJs are composed of a branching network of continuous sealing strands form by various transmembrane proteins and cytosolic complexes (Anderson & Van Itallie, 1995) (Fig. 7).

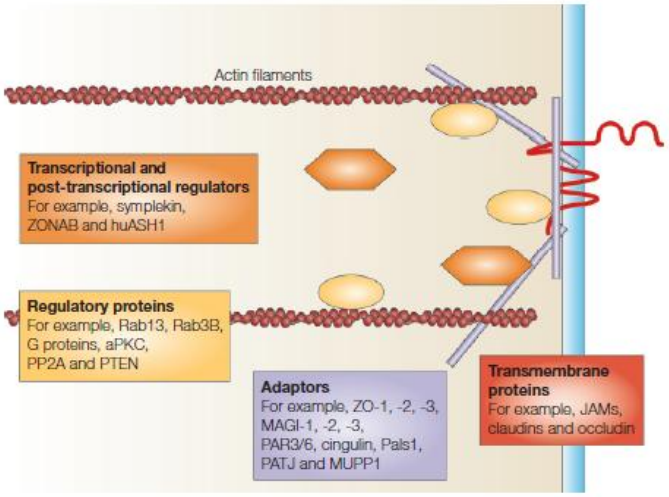


Figure 7 – The biochemical composition of epithelial tight junctions. Junctional components, including cytosolic complexes and transmembrane proteins are visible in the representational figure (Matter & Balda, 2003).

The physical barrier formed by the tight junctions is apparently dependent on the structural organization of actin. The correct arrangement of transmembrane proteins is crucial for TJ functionality, and at this level the cytosolic complexes are vital (Denker & Nigam, 1998; Fanning *et al.*, 1999).

1.2.5.1- TJ Transmembrane Proteins

Identified in 1993 as a tight junction component, occludin is a 65-kDa phosphoprotein that originally was thought to be the main sealing protein (Tsukita & Furuse, 1999). This Ca^{2+} independent intercellular adhesion molecule has four transmembrane domains, and it´s cell distribution affects the occludin arrangement in neighbor cells (Balda *et al.*, 1998). At the moment, occludin physiologic functions are unclear, but possibly at a TJ level, involved in the formation of aqueous pores, electrical barrier and signaling events (Fanning, 1999; Tsukina *et al.*, 2001; Saitou *et al.*, 1998).

Claudins (Claudin-1 and Claudin-2) identified as integral components of TJ and obviously possess several functional characteristics consistent with the role in barrier formation, have a molecular mass in the range of 23-kDa. Recent data suggest that Claudin-1 and Claudin-2 belong to a protein family with at least 20 members. Claudins possess also four transmembrane domains, but do not have any sequence homology with occludin (Fanning, 1999; Tsukina *et al.*, 2001; Furuse *et al.*, 1998). Claudins display a varied differential expression, which is postulated to explain the differences observed in permeability among different tissues along the epithelium (Mitic *et al.*, 2000; Coyne *et al.*, 2003).

The junctional adhesion molecule (JAM) was also identified as an integral transmembrane protein of TJ. JAM is a glycosylated 43-kDa protein, which belongs to the immunoglobulin superfamily (González-Mariscal *et al.*, 2003). JAM seem to be directly or by interaction with occludin involved in the formation of TJ, but also linked to the regulation of immune cell transport through the paracellular barrier (Martin-Padura *et al.*, 1998).

1.2.5.2- TJ Cytosolic Complexes

The most important cytosolic proteins associated to the cytoplasmatic surface of TJ are ZO-1 (*Zonula Occludens*-1), ZO-2, ZO-3, Cingulin and 7H6 (Tsukina *et al.*, 2001; Denker *et al.*, 1998). Also, various signaling molecules were localized in these binding complexes, such as, G proteins, protein kinase C and protein tyrosine kinases (Denker *et al.*, 1996; Dodane *et al.*, 1996). Internal signaling pathways may contribute in the regulation of TJ properties (Anderson *et al.*, 1995).

ZO-1, as well as, homologous ZO-2 and ZO-3 have PDZ domains. These PDZ domains existent in ZO-1, ZO-2 and ZO-3 are totally available to recruit proteins. Recent data suggest that these PDZ domains bind to the carboxyl end of Claudins (Itoh *et al.*, 1999) and JAM (Ebnet *et al.*, 2000). The ZO interaction with occludin is not mediated by PDZ domains, but does occur, and evidence suggests that the interaction is responsible for recruiting and organizing occludin at a TJ level (Fanning, 1999). ZO-1 binds to the actin cytoskeleton, important since structural organization of actin has been linked to the ability of paracellular barrier formation by TJ (Denker *et al.*, 1998; Fanning *et al.*, 1998).

1.3- Airborne Allergens

An allergen is considered *major* if in a specific allergic group it reacts with IgE in more than 50% of the patients (Larsen & Lowestein, 1996). With the increase in hypersensitivity type 1 in the world population, the identification, isolation and characterization of proteins that cause allergies IgE-mediated has become the main objective of investigations in this field. The efficiency of diagnostic and treatment depend on the usage of well characterized allergens or extracts. Unfortunately, the sources contain a variety and diversity of substances majority yet to be known (van Ree, 1997).

Allergens can be divided into two groups: those originated from the natural environment and those from a chemically contaminated environment. In the last group, these allergens are usually low-weight chemical compounds and elements of the nature of heptanes. Some examples are metals, drugs, latex and additives to food products. From the natural environment group, the most frequent representatives are the airborne allergens, which include for example pollen of different plants and mould fungi spores,

and are usually proteins with molecular mass superior to 10-kDa (Puc, 2003). Table 3, exemplifies some characterized *major* airborne allergens from different sources.

Table 3 – Examples of some of the most popular *major* airborne allergens, their source and biochemical characterization (Chapman *et al.*, 2009; Donnelly *et al.*, 2006; Helbling & Reimers, 2003; Horner *et a*l., 1995; Vijay & Kurup, 2004; RCSB Protein Data Base (www.rcsb.org); IUIS Allergen Nomenclature (www.allergen.org)).

Source		Allergen	Molecule Mass (kDa)	Biochemical Characterization
Domestic Animals	*Felis domesticus*	Fel d 1	36	Unknown
	Canis familiaris	Can f 1	25	Lipocalin
Fungi	*Aspergillus fumigatus*	*Asp f 5*	40	Metalloproteinase
		Asp f 10	34	Aspartic protease
		Asp f 13	34	Serine protease
		Asp f 18	34	Serine protease
	Alternaria alternata	*Alt a 2*	25	Aldehyde dehydrogenase
	Cladorporieum herbarium	*Cla h 10*	53	Aldehyde dehydrogenase
	Penicillium notatum	*Pen ch 13*	32	Serine protease
Mites	*Dermatophagoides pteronyssinus*	*Der p 1*	25	Cysteine protease
		Der p 3	30	Serine protease
		Der p 9	24	Serine protease
Pollen	*Parietaria judaica*	*Par j 1*	10	Lipid transport protein
	Parietaria officinalis	*Par o 1*	15	Lipid transport protein
	Lolium perenne	*Lol p 1*	27	Expansin

	Mbrosia elatior	*Amb a 1*	38	Pectate lyase
	Artemisia vulgaris	*Art v 1*	47	Defensin

Allergens from mites and pollen are the principal airborne allergens to promote allergic disorders (Tovey *et al.*, 1981; D´Amato *et al.*, 1984).

The pollen grain is the part of the flowering plant life cycle, and is a specialized structure that harbors the flowering plant male gametes. Pollen has a variety of shapes and sizes. The external wall is important for the physical and chemical strength of pollen, for that reason the majority are resistant to a large pH range, as well as, high temperatures. In dry atmosphere pollen may remain stable for centuries (Taketomi *et al.*, 2006). The allergic ability of pollen is not related to the abundance of protein, since structural and content differences confer different allergic responses (King, 1979).

Beside the pollen grain, allergens can be present in other plant structures, like the roots, stamens, leafs, seeds and fruit, which can be observed for example in *Ambrosia spp*., *Plantago spp*. and in multiple types of pasture grass. Allergy can be triggered by excreted substances by the plant, like in corn, cotton plant and herbaceous (Grote *et al.*, 1988; van Ree *et al.*, 1992).

Another important mechanism of allergy induction is cross-reactivity. This mechanism occurs since there are similarities in the amino-acid sequences between distinct antigenic proteins, which lead to cross reactions amongst different allergens and the same specific antibody. The principal pollen allergens from plants that coexist in temperate climatic zones are structurally and immunologically similar, and amongst species from the same genus, the levels of similarity can reach 90% (Matthiesen et al., 1991).

Pollen allergens are water-soluble proteins or glycoproteins, being capable of inducing an IgE-mediated inflammatory response in seconds. Many of these protein allergens have been biochemically characterized in defensins, lipid transport proteins, expansins, lyases, amongst others that are important for plant development and protection.

Pollen allergy has had a tremendous clinical impact everywhere in Europe, and series of evidence suggest that a prevalence of allergic reactions pollen induced on the Europe continent has increased a trend clearly evident in the Mediterranean area (D´Amato *et al.*, 1998).

1.4- Pollen

1.4.1- *Chenopodium sp.*

Chenopodium sp. is a perennial plant that belongs to the goosefoot family, Chenopodiaceae (Pauli G, 2000). This weed is found in all parts of the world and grows on all types of soils (Valenta R, 2007). Pollinosis and allergic sensitization to this pollen has been reported in European countries and North America, among other countries (Barderas *et al*, 2004). This pollen is characterized by having pollen grains of small to medium dimensions (10-28 μm), moderate allergic potential and with a period of pollination comprehended between April and October (Portuguese Society Aerobiology – www.rpaerobiologia.com). The crude extract of C. album pollen is a mixture of both allergic and non-allergic compounds (Vahedi, *et al*, 2010) (Fig. 8).

Previously reported, three *Chenopodium sp.* allergens have been identified and characterized: Che a 1, Che a 2 and Che a 3. Che a 1, is considered to be the principal allergen, and is a glycoprotein of molecular mass about 17 kDa and composed of 143 amino acids residues, recognized in more than 77% of *C. album* allergic patients. The sequence of this allergen exhibits 27-45% homology with other well known allergens of the Ole e 1-like protein family (Barderas *et al.*, 2002). However limited cross-reactivity was detected between Che a 1 and Ole a 1 allergens (Gadermaier *et al.*, 2004).

Besides Che a 1, two other allergens, Che a 2 (profillin) an about 14.4 kDa protein and Che a 3 (polcalcin) an about 9.5 kDa protein, were cloned, purified and characterized. C. album allergic patients showed 55 and 46% reactivity to Che a 2 and Che a 3, respectively (Nouri *et al*, 2011). These two allergens are relevant in *Chenopodium* pollen, since they are candidates involved in IgE cross-reactivity with other allergens, explaining the highly frequent polysensitization of patients allergic to chenopod.

Che a 2, profillin, represents a ubiquitous actin-binding protein involved in signal transduction and, can be found as cross-reactive pan-allergen in almost all plants

species. Che a 3 is also highly cross-reactive pollen, belonging to a family of calcium-binding proteins, termed polcalcins. These proteins have also been characterized in pollens of various plants (trees, bushes, grasses, weeds and flowering plants), and are extremely potent allergens. Both Che a 2 and Che a 3, have been showed to display high cross-reactivity with the corresponding profillin and polcalcin from the olive tree, due to their conservative amino acid sequence (Rodriguez *et al.*, 2007).

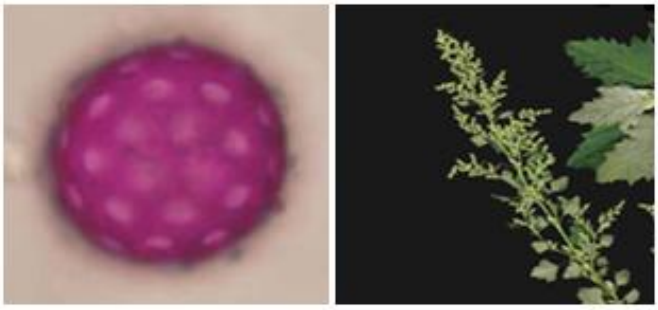


Figure 8 – Images of the pollen grain and herbaceous flowering plant from the goosefoot family, *Chenopodium sp.*, respectively (Adapted from Portuguese Society Aerobiology – www.rpaerobiologia.com).

1.4.2- *Plantago spp.*

The Plantago genus of the Plantaginaceae family comprises approximately 250 species. This pollen is an important cause of Pollinosis in the temperate regions of North America, Europe and Australia (Spieksma *et al.*, 1980). In terms of flowering, this tends to occur from May to October, and the pollen grains are usually in terms of dimension, 19 to 39 µm (Portuguese Society Aerobiology – www.rpaerobiologia.com). Plantain pollen is generally considered as one of the most important dicotyledons that cause allergic diseases in Europe (Merrett *et al.*, 1980; Mehta & Wheeler, 1991) (Fig 9).

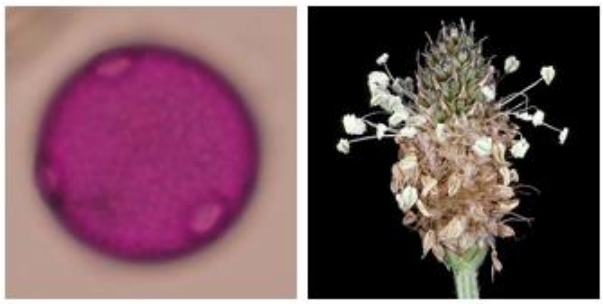


Figure 9 – Images of the pollen grain and the herbaceous ruderal plant, *Plantago sp.* respectively (Adapted from Portuguese Society Aerobiology – www.rpaerobiologia.com).

The role of Plantago in pollinosis has usually been overlooked, because of the fact that plantain pollen is in the same season as other relevant allergenic plants, such as grasses, and because the monosensitization to plantain pollen is not frequently found among allergic patients (Gadermaier *et al.*, 2004; Calabozo *et al.*, 2003). The plantain allergen as been reported to have cross-reactivity with the grass group 5 allergens (Asero *et al.*, 2000).

The *major* allergen of plantain pollen, designated Pla l 1, has been purified and characterized. This allergen has been found with about 17 and 20 kDa, which correspond to the unglycosylated and glycosylated forms, respectively, of the protein Pla l 1 (Calabozo *et al.*, 2002). Also, a 32-36 kDa protein has been found, and is suggested to be a dimeric form of the same Pla l 1 allergen (Calabozo *et al.*, 2001). These allergens display, also, approximately 40% sequence identity to Ole e 1 (Gadermaier *et al*, 2004).

1.4.3- *Eucalyptus spp.*

Eucalyptus belongs to the Myrtaceae family. In general terms, are trees but in some rare cases can be shrubs. The pollen from these trees has a triangular structure, with a medium dimension, with size ranging 13 to 35 µm. Allergens existent in the pollen are considered to have low allergenic potential. The pollination period occurs from November to July (Portuguese Society Aerobiology – www.rpaerobiologia.com). In terms of identification and characterization of allergens in this pollen, still no references have been made in the literature (Fig 10).

Figure 10 – Images of the pollen and tree, *Eucalyptus sp.* respectively (Adapted from Portuguese Society Aerobiology – www.rpaerobiologia.com).

1.5- Proteolytic Activity in Airborne Pollen Allergens

After inhaled, contact with mucous from the airway epithelium promotes hydration of the pollen grains. Hydration causes the grain of pollen to release its contents, a variety of solutes, which include proteins. The pollen grains, due to their large size tend to concentrate on the upper airways, however the contents released are able to access the distal airway, and for that matter, the epithelial cells of the lung epithelium (Hassim *et al.*, 1998; Widmer *et al.*, 2000). The epithelial surface can be targeted with high levels of concentration of solutes (Baraniuk *et al.*, 1988).

The pollen grains contain a variety of enzymes that compose an important portion of the quantity of existent protein, situated on exterior layers being, for that reason, released in the first minutes of hydration (Widmer *et al.*, 2000; Blanchard *et al.*, 1976). An example of these enzymes is NADPH oxidase which is present in the pollen grains of many trees and herbaceous plants, which have important physiologic functions like protection against pathogenic agents, growth and plant development (Boldogh *et al.*, 2005; Ritsick *et al.*, 2005). It is known that after pollen inhalation, at a pulmonary epithelial level, NADPH oxidase is responsible for the increase in reactive oxygen species and glutathione oxidase. This leads to oxidative stress, which is known to function as a signal for neutrophils recruitment and consequentially the development of an inflammatory response, previous to any antigen contact with APCs (Boldogh *et al.*, 2005).

It has already been demonstrated pollen proteolytic activity in extracts (Knox *et al.*, 1970) and since then, investigations concentrate on the characterization of these enzymes. Biologically, these proteases are in general related to plant germination, through specific cleavage of protein precursors and mobilization of stored proteins. However, these proteases have been pointed to be involved in mechanisms related with the development of allergic disease (Widmer *et al.*, 2000 & Hassim *et al.*, 1998), either as an enhancer or as an inductor.

In terms of classification, these proteases seem to be from principally four classes: serine, cysteine, aspartic and metalloproteinase. Among all the known proteases, one third are classified as being serine proteases (Hedstrom, 2002), ubiquitous in prokaryotes and eukaryotes (Déry *et al.*, 1998).

1.5.1- Pulmonary Homeostasis Disruption

The significance of protease activity was first highlighted more than a decade ago when it was discovered that the *major* allergen from the house dust mite, *Dermatophagoides pteronyssinus* (Der p 1), was a cysteine protease (Donnelly *et al.*, 2006). Considerable advances have been made in elucidating the mechanisms by which proteolytic enzymes may either enhance or function as allergens. The proteolytic activity found in pollen is suggested to cause allergic disorders through alteration of pulmonary homeostasis (Bagarozzi *et al.*, 1996; Bagarozzi *et al.*, 1998; Cortes *et al.*, 2006; Vinhas *et al.*, 2011).

Hassim and collaborators showed that proteases released by various allergenic pollens provoke epithelial cell detachment in mouse models, and also are not inhibited by endogenous proteases, like SLPI and α1-PI (Hassim *et al.*, 1998). On the other hand, pollen from *Ambrosia artemisiifolia*, principal cause of pollinosis in North America, was characterized as a non-allergic serine protease type chymotrypsin and trypsin. This protease inactivates the specific elastase inhibitor of neutrophils, α1-PI, which can leave to an uncontrolled degradation of inflamed tissue and potentiate respiratory complications associated to allergies (Bagarozzi *et al.*, 1996).

Kalsheker and colleagues (1996), suggest the cleavage of α1-PI as an important mechanism in the development of allergic disorders like asthma. This serine protease type chymotrypsin, is also capable of hydrolyze of two neuropeptides, substance P (SP) and vasoactive intestinal peptide (VIP), which are neurotransmitters involved in the modulation of the diameter of the respiratory airways, mucous secretion and vascular permeability (Barnes, 1991; Stanisz *et al.*, 1988). Substance P is a potent broncoconstrictor, released by sensorial neurons that nerve the respiratory epithelium, blood vessels and smooth muscle (Barnes, 1991). VIP is released by efferent autonomous neurons, with effect on relaxation of human bronchial and vascular smooth muscle (Stanisz *et al.*, 1988), as well as, anti-inflammatory properties by inhibiting the typical neutrophils recruitment of the inflammatory response (Sergejeva *et al.*, 2004). Naturally, the degradation of VIP and substance P lead to a disturbance in the equilibrium of contraction and relaxation effects of the lungs, that favors asthma development allergy related (Bagarozzi *et al.*, 1998; Widmer *et al.*, 2000).

The serine protease type trypsin, found in the *Ambrosia artemisiifolia* pollen induces alterations in the balance of the rennin-angiotensin II system of the airways, maintained stable in normal conditions by endogenous proteases (Thompson *et al.*, 1995; Bagarozzi *et al.*, 1998). This system is composed of neuropeptide mediators highly susceptible to proteolytic inactivation, like atrial natriuretic peptide (ANP), inhibitor of vascular contraction, and angiotensin II, potent vasoconstrictor (Thompson *et al.*, 1995). The hydrolyze of these mediators result in an increase of vascular permeability, inflammation and edema which may lead to asthmatic complications often present after pollen exposure (Bagarozzi *et al.*, 1998).

Recently, Der p 1 was shown to cleave and inactivate several anti-proteases, including α_1-antitrypsin, elafin and SLPI (Brown *et al.*, 2003). The inactivation of these leads to a shift in the immune environment to a pro-inflammatory allergic response (Sakata *et al.*, 2004).

Pollen from *Parietaria judaica*, Urticaceae family, is the largest cause of pollinosis in the Mediterranean area, where this type of allergy is associated to 50% of asthmatic cases (Colombo *et al.*, 1998; D´Amato *et al.*, 1992). In our laboratory, studies confirmed the presence of proteolytic activity in extracts of *Parietaria judaica* (Teixeira Dias, 1997). Later, a 98-kDa aminopeptidase from *Parietaria* was isolated, purified and characterized, with implication of proteolytic activity on various neuropeptides, such as, substance P, VIP, angiotensin I and II (Cortes *et al.*, 2006). Also, from our laboratory, proteolytic activity of pollen diffusates with distinct allergenicity from *Olea europea*, *Dactylis glomerata*, *Cupressus sempervirens* and *Pinus sylvestris* was confirmed and showed to be responsible in compromising the airway epithelial barrier (Vinhas *et al.*, 2011).

1.5.2- Epithelial Barrier Disruption

The primary risk factor for development of allergic sensitization is the delivery of allergens across the mucosal epithelium. Paracellular channels of the epithelial layer are normally sealed by tight junctions. Tight junctions, macromolecular assemblies that form continuous rings at the apices of epithelial cells, form a physical barrier between inhaled allergens and the immune system (Donnelly *et al.*, 2006).

Despite the epithelial barrier, some allergens gain access to the subepithelial layer and contact APCs, leading to an allergic reaction. Experimental evidences suggest a rupture of the barrier, in concrete a disruption of tight and adherent junctions (Holt, 1993), which causes pulmonary epithelial damage and leads to an amplification of the inflammatory components of the allergic response (Fig. 11). The intrinsic proteolytic activity of some allergens or other components released after pollen hydration can contribute to rupture of the epithelial barrier (Hassim *et al.*, 1998). Clinical studies point to abnormal morphologies of tight junctions to be an important characteristic of the asthmatic condition (Thompson, 1998).

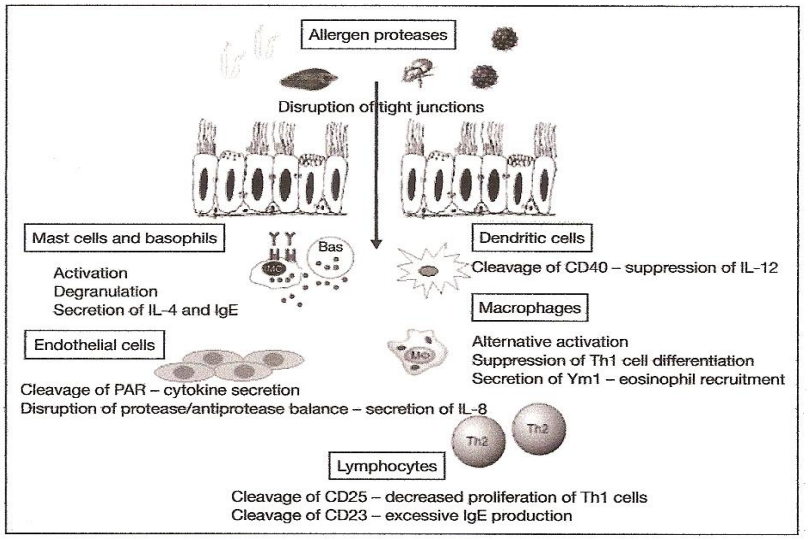


Figure 11 – Disruption of the integrity of the epithelial barrier by hydrolyze of tight junctions as a result of the Proteolytic activity of allergens leading to respiratory airway inflammation (Adapted from Donnelly *et al.*, 2006).

Wan and collaborators, in studies with house dust mites allergens, suggested that penetration of these high molecular mass proteins where via tight junctions (Wan *et al.*, 1999; 2000; 2001). The principal allergens of *Dermatophagoides pteronyssinus* are biochemically active, being that the allergens from group 1 have cysteine activity while from groups 3, 6 and 9 have serine activity (Robinson *et al.*, 1997). They all induce pro-inflammatory mediator release, and can be blocked in the presence of cysteine and serine inhibitors (Adam *et al.*, 2006; Asokananthan *et al.*, 2002). Der p 1 has already been implicated in cell detachment *in vitro* on trachea epithelial cells (Herbert *et al.*, 1995; Thompson, 1998). Also like Der p 1, Pen ch 13, an allergen from *Penicillium notatum*, has serine activity which action is responsible for hydrolyze of occludin (Tay *et al*, 2006). Der p 1 is also responsible for cleavage of other protein sites, like claudin-1

and ZO-1 (Wan *et al.*, 1999; 2000; 2001). Even thought ZO-1 is an intracellular protein, its hydrolysis can occur as a secondary phenomenon to tight junction degradation. However, after exposure of Der p 1, epithelial cells are able to synthesize *de novo* occludin and recuperate cell viability (Wan *et al.*, 2001). Recent studies also point out the fact that this allergen induces cell apoptosis, phenomenon believed to occur independently to tight junction protein hydrolyze (Baker *et al.*, 2003).

Evidences of fungi allergens, *Alternaria* and *Aspergillus*, biochemically active as well, have the ability to interact with epithelial cells and induce detachment and the release of cytokines IL-6 and IL-8 (Kauffman *et al.*, 2000).

Runswick and others, showed the ability of four allergic pollen species, *Ambrosia trífia*, *Poa pratensis*, *Betula pendula* and *Lilium longifolium*, to hydrolyze tight junctions on epithelial cells from MDCK (Madin-Darby Canine Kidney). Obviously, the cleavage of tight junctions increases epithelial permeability, facilitating the allergen passage and contact cells from the immune system. Notably, the four pollen extracts were inhibited by serine and cysteine protease inhibitors (Runswick *et al.*, 2007).

Pollen extracts from *Lolium perenne* and *Acacia longifolia*, also were shown to be responsible for cell detachment when incubated will trachea epithelial cells. Markers of cell damage and necrosis, such as high levels of LDH (Lactate dehydrogenase) or Tripan Blue staining were absent in recuperated cells after exposure *in vitro* with pollen extract of *Acacia longifolia*, confirming the persistence of cell viability (Hassim *et al.*, 1998).

Finally, as another example, studies using a cell line of human alveolar epithelial with pneumocytes type II characteristics (A549), observed that the pollen extract from *Parietaria judaica* was also associated to cell detachment. In this case, it was observed a complete inhibition with the addition of 10% of serum, which is known to have various inhibitors, and by PMSF, a specific serine protease inhibitor. After pollen extract exposure of *Parietaria judaica* the adherent cells maintain their ability to proliferate, confirming cell viability. Pollen from *Parietaria judaica* has already been implicated in hydrolyze of *in vitro* tight junction proteins such as claudin and occludin, as consequence of the presence of a serine aminopeptidase that has not been yet identified as an allergen (Resende, 2001; Cortes *et al.*, 2006).

1.5.3- Activation of Protease-Activated Receptors

Protease-activated receptors (PARs) are intriguing G protein-coupled receptors that are rapidly grasping a lot of attention of researchers. These receptors are unconventional, in the fact that they do not bind to free moving soluble ligands but instead require specific site-directed serine proteolysis cleavage which allows posteriorly a self-activation by their new amino-terminal, called tethered ligands. For that reason, PARs are considered to be sensors of extracellular proteolytic environments.

Four PARs have been identified, PAR-1, PAR-2, PAR-3 and PAR-4. PARs are 7-transmembrane proteins coupled to G proteins. Serine proteases cleave the amino acids at a specific site of the extracellular N-terminus of the molecule exposing a new N-terminal ligand domain that binds to another site on the same molecule, thereby activating the receptor. This amino acid cleavage site is specific for each particular PAR. The proteolytic activation is irreversible, and once cleaved, the receptors are degraded in lysosomes (Reed & Kita, 2004). PAR activation contributes to a variety of physiological and pathophysiological roles in various tissues and cells, that include circulatory, gastrointestinal, respiratory and central nervous system (Hollenberg & Compton *et al.*, 2002; Macfarlane *et al.*, 2001; Ossovskaya & Bunnett, 2004; Kida *et al.*, 2006).

All four PARs have been detected in the normal human lung, however with distinct distribution patterns in airway structures. PAR-2 is widely expressed on the apical surfaces of the lungs, which include epithelial cells, airway smooth muscle, fibroblasts, vascular smooth muscle and endothelial cells. PAR-2 is selectively activated by trypsin and trypsin-like enzymes. PAR-2 on cell airway epithelium causes the release of prostaglandin and matrix metalloproteinase (MNP) (Cocks & Moffatt, 2001).

Although much is known about the potential functions of PARs, there remain some substantial obstacles for the understanding of proteases and PARs in physiology and disease (Ossovskaya & Bunnett, 2004). Activated PARs couple to G-signaling cascades increasing phospholipase C levels which in turn lead to increased intracellular Ca^{2+} levels (Berger *et al.*, 2001; Schechter *et al.* 1998; Ubl JJ *et al.*, 2002). Increased levels of Ca^{2+} involve effects that include secretion, degranulation and smooth muscle contraction. The coupling to G proteins possibly varies among different PARs and

different cells, and the response almost certainly depends on the interaction with other signaling cascades stimulated by agents like chemokines, cytokines and neurotransmitters (Fig. 12). The activation of G proteins also generates transcriptional responses. This gene transcription produces integrins, chemokines, cytokines as well as cyclooxigenase 2. PARs cause edema, promotes angiogenesis and fibrosis, enhance IgE production, leukocyte infiltration and airway hyperresponsiveness (Reed & Kita, 2004).

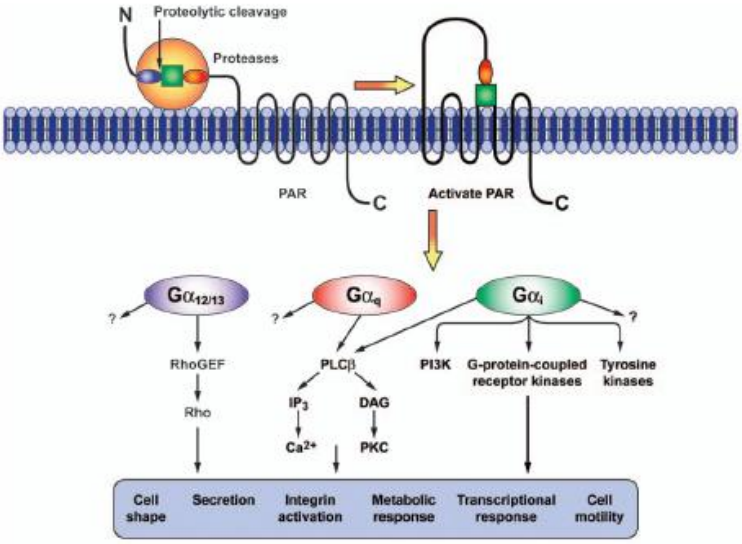


Figure 12 – Coupling to G proteins with PAR activation. Signal transduction pathways of the PAR response. RhoGEF, Rho guanine nucleotide exchange factor, PLCβ, phospholipase Cβ ; PI3K, Phosphoinositide 3-kinase; IP3, Inositol triphosphate; DAG, diacylglycerol; PKC, protein kinase C (Adapted from Reed & Kita, 2004).

The proinflammatory role of PAR-2 has been supported by guinea pig, mice and human studies. Patients with asthma express increased levels of PAR-2 on respiratory epithelial cells but not macrophages nor smooth airway muscle (Knight *et al.*, 2001; Roche *et al.*, 2003), suggesting a disequilibrium between pro- and anti-inflammatory activities that will favor the proinflammatory actions. Also, in the absence of PAR-2, lower levels of bronchial reactivity and IgE are observed (Barrios *et al.*, 2003; Chambers *et al.*, 2001; Ebeling *et al.*, 2005; Schmidlin *et al.*, 2002; Heijink & Kauffman, 2009).

Recent data indicates that the acute allergen challenge increases the permeability of both bronchial microvascular and epithelium barriers (Winter *et al.*, 2006). PAR-2 is

suggested to indirectly compromise the integrity of the epithelium barrier, through the disruption of tight junctions (Fig. 13). Exogenous allergens, including fungi, house dust mites and cockroaches, proteolytically active, are known to cause breakdown of epithelial barriers through PAR-2 mediated mechanisms.

PAR-2 also induces a transepithelial current through CFTR (cystic fibrosis transmembrane conductance regulator) in Calu-3 cells (Sato *et al.*, 2005). These findings suggest that PARs can regulate anion and fluid secretion in airway epithelia (Cho *et al.*, 2010).

Mites such as *Dermatophagoides pteronyssinus*, possess endogenous proteases such as cysteine (Der p 1) and serine proteases (Der p 3, 6 and 9), and can activate PARs, leading to inflammation through epithelia cell detachment and IgE production. Mite allergens can also induce cytokine production (Sun *et al.*, 2001; Asokananthan *et al.*, 2002; Adam *et al.*, 2006).

Recent studies have shown that functional PAR-2 when activated can induce the release of matrix metalloproteinase-9 (MMP-9), granulocyte monocytes-colony stimulating factor, eotaxin, PGE_2, IL-6 and IL-8. These findings suggest that PAR-2 participates in lung inflammation (Vliagoftis *et al.*, 2001; Vliagoftis 2000; Sun *et al.*, 2001; Asokananthan *et al.*, 2002).

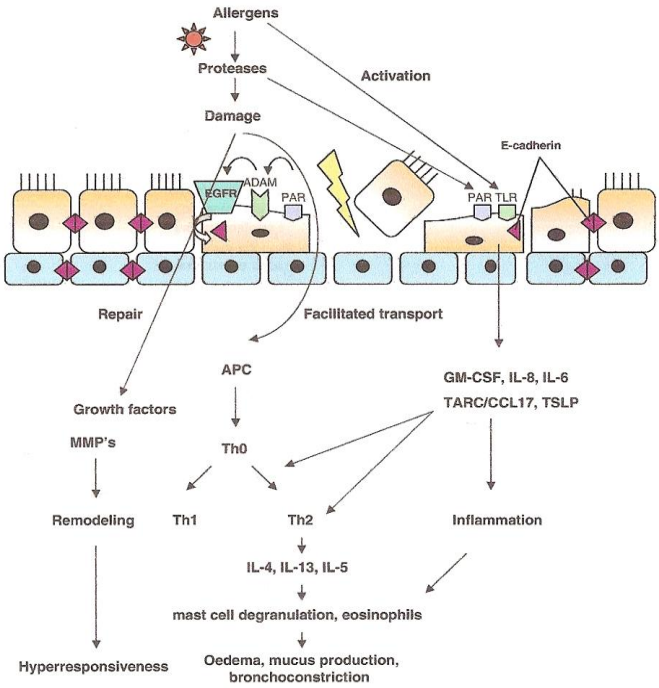


Figure 13 – Representation of intracellular cascades of inflammatory response by immune cells that result with the activation of PAR-2 receptors located on the airway epithelium when exposed to pollen proteases (Adapted from Heijink & Kauffman, 2009).

1.6- Aim of the Research Work

Allergic disorders have become a public epidemic health issue at a global level. Pollens are an important cause for allergic disorders. Airborne allergens, specifically pollen, when hydrated release their contents, which include proteases, allergens, NADPH oxidase and other proteins. Allergic responses result from an allergen interaction with immune cells, but for this to occur allergens must gain access through the epithelial barrier. Recent studies suggest an integrity loss of the respiratory epithelium as a consequence of proteolytic activity of proteases that compose pollen grains. Epithelium disruption allows allergens to gain access and potentiate an immune response as well as a sensitization to the allergen.

The proteolytic property is suggested to be the central biochemical characteristic that attributes these molecules an intrinsic allergic potential, as well as, ability to persist in the organism. We therefore intend to associate protease activity with disruption of the epithelial barrier.

In this project we aim, firstly, to identify and characterize proteolytic activity of three pollens with distinct allergic potential: *Chenopodium sp.*, *Eucalyptus sp.* and *Plantago sp*. This step will include the determination of protein and proteolytic profiles of pollen extracts and characterization of their proteolytic activity using a specific enzymatic assay.

Since the most effective alternative treatment of allergies and asthma has been to avoid exposure to allergic proteins, it is vital to understand interaction mechanisms of proteases on epithelial cells. Clinical studies confirm that asthma is associated to an increase in the epithelial permeability and disruption of intercellular protein complexes, for example, tight junctions. For that reason, we then aim to focus on the evaluation and correlation between the enzymatic activities of the pollen extracts with epithelial permeability, cell detachment and the identification of the disruption targets on a Human airway epithelial cell line, Calu-3.

We intend to evaluate the effect of existent proteases in the pollen extracts on the inflammatory component. Primarily we intend to confirm activation of PAR-2, which are expressed similar to lung epithelium, on the Calu-3 cell line. Lastly, establish a correlation with the quantity of cytokines released as a consequence of exposure to pollen extracts of *Chenopodium sp.*, *Eucalyptus sp.* and *Plantago sp*.

It´s our interest to try and underlie this proteolytic activity and connect it with the development of allergic disorders, in order to contribute to a better understanding of the disorder and help in new treatment approaches.

Chapter 2

Material and Methods

2- Material and Methods

2.1- Pollen Extracts from *Chenopodium sp.*, *Eucalyptus sp*. and *Plantago sp.*

The protein extraction from *Chenopodium sp.*, *Eucalyptus sp*. and *Plantago sp.* pollen (Allergon) was obtained by hydration of 20 mg/ml with 50mM Tris-HCl buffer pH 7.4, at room temperature for 2 hours with slow agitation. The homogenized was centrifuged at 12000 rpm during 10 minutes at 4ºC, and supernatant was recovered. These obtained extracts were all subjected to filtration using a 0,45µm porous filter. These fractions are referred to as the pollen extracts.

2.2- Concentration of Pollen Extracts

The pollen extracts when experimentally required were concentrated. For that reason, the pollen extracts where placed in centricon filter devices with 30kDa membranes and centrifuged during 10 minutes at 3500g, 4º C. The fractions resultant from this concentrating method where termed concentrated extracts.

2.3- Protein Quantification of the Pollen Extracts

The total protein concentration of the pollen extracts was determined by using the BioRad method accordingly to the protocol provided by BioRad, and using bovine albumin serum (BSA) as standard.

2.4- Protein Profile of Pollen Extracts of *Chenopodium sp.*, *Eucalyptus sp*. and *Plantago sp.*

2.4.1- SDS-PAGE Electrophoresis of Proteins

The different pollen extract samples were electrophoretically separated in polyacrylamide (PAGE) gels under denatured conditions, in the presence of 0,2% SDS, using BioRad´s *Mini-Protean II* system (Laemmli, 1970). Before applying the samples to the gels, they were denatured during 10 minutes at 80ºC with denaturing solution concentrated 2x (100mM Tris/Bicine, 4% SDS (m/v), urea 6 M, 4% β-mercaptoethanol (v/v) and Brilliant Blue. Besides the samples, a protein pre-stained standard with known molecular masses between 10 and 250 kDa (Precision Protein Standards from BioRad) was applied to every gel. Protein separation occurred in 10% and 12% polyacrylamide gels in electrophoresis buffer 100mM Tris/Bicine, 0,1% SDS.

2.4.2- Staining with Coomassie Brilliant Blue R250

In order to fix and stain the proteins separated by electrophoresis, the gels were placed in a solution with 0.25% Coomassie Brilliant Blue R-250 (m/v), 50% methanol (v/v) and 10% of acetic acid (v/v), during 30 minutes. The discoloration was managed by successive washes with a 25% (v/v) methanol and 5% (v/V) acid acetic solution.

2.4.3- Staining with Silver Nitrate

In some situations, it was difficult to visualize the protein bands of the samples with Coomassie Brilliant Blue staining, and for that reason we proceeded with Silver Nitrate staining of the gels.

In first place, protein fixation was accomplished by dipping the gel 30 minutes in a 50% methanol (v/v) and 10% acetic acid (v/v) solution, followed by another 30 minutes in a second solution of 5% methanol (v/v) and 7,5% acetic acid (v/v). After fixation, two 10 minute washes with 50% ethanol and 30% ethanol solutions were carried out. Gels were sensitized by incubation during 1 minute in 0,02% (m/v) of sodium thiosulfate, then proceeded with 2 washes, 20 minutes each, with distilled water. For staining, gels were left in 0,2% Silver Nitrate for 20 minutes. Revelation was managed by a solution of 0,25% (v/v) formaldehyde, 3% (m/v) sodium carbonate anhydrous and 0,001% (m/v) sodium thiosulfate. Once the pretended coloration was achieved the revelation was stopped with a solution of 0,4M Tris and 2,5% (v/v) acetic acid. The gels are subsequently stored in distilled water at room temperature.

2.5- Zymography

2.5.1- One-dimensional

The Zymography was realized co-polymerizing 1mg/ml of gelatin in a 10% or 12% polyacrylamide gel in the presence of 0,2% SDS (SDS-PAGE). Initially, the gelatin was solubilized in 1,5M Tris-HCl buffer pH 8.8, by heating in a microwave, and only after was the rest of the components added. The samples, neither denatured by heat nor reduced, were applied to the gel, diluted only in a 1:1 solution of 125mM Tris-HCl pH 6.8, 4% (m/v) SDS and 20% (v/v) glycerol. The proteins were separated electrophoretically in electrophoresis buffer, accordingly to what was described in 2.4, at 20 mA/gel and 4ºC, using a *Mini protean II* system from BioRad. After the

electrophoresis, SDS was removed from the gel washing with 50 mM Tris-HCl pH 7.4 with 0,25% (v/v) Triton X-100, during 30 minutes accompanied with a moderate agitation. After a quick passage through distilled water, the gel is incubated in 50mM Tris-HCl pH 7.4, overnight at 37ºC. Finally, two washes with distilled water are carried out, proceeding to staining with Coomassie Brilliant Blue, accordingly to 2.4.2, in order to reveal proteolytic activity.

2.5.2- Two-dimensional

The first dimension for protein separation accordingly to their isoelectric point (pI), Immobiline Dry Strips pH 3-10, 7 cm (Amersham) were used. Samples of pollen extracts, with approximately similar protein concentrations, were added to a rehydration buffer with no reducing agents (3% (m/v) CHAPS; 0,2% (v/v) anfolinas; 0,001% (m/v) bromophenol blue) in a 1:1 proportion. The strips were placed in an isoelectric focusing tray with the samples and covered with the rehydration solution. The rehydration occurred at 50 V and 20ºC during 12 hours in a Protean IEF Cell from BioRad. After this step, electrode wicks, previously humidified, were inserted between the strips border and the respective electrode. This step is important to remove the excess of water, salt and proteins that don't have pI values comprehended between the pH range of the strip. The strips were immediately covered with mineral oil in order to avoid dehydration. The isoelectric focusing occurred at 20ºC in a linear gradient voltage under the following conditions: until 250V during 30 minutes; until 4000V during 2 hours; and finally at 4000V during 3 hours.

After isoelectric focusing, the strips were dipped in an equilibration SDS buffer with, 50 mM Tris-HCl pH 8.8, 30% (m/v) glycerol, 2% (m/v) SDS and 0,002% bromophenol blue, during 20 minutes with light agitation. For protein molecular mass separation, each strip was washed in electrophoresis buffer (described in 2.4) and immobilized with 0,5% agarose also prepared in electrophoresis buffer on the top of the zymography, composed of 1mg/ml gelatin and 10% acrylamide. The second dimension and the detection of proteolytic spots occurred as described in 2.5.1.

2.6- Native-PAGE

The different pollen extract samples were electrophoretically separated in polyacrylamide (PAGE) gels, just like an SDS-PAGE described in 2.4.1, except not

under denatured conditions, in absence of SDS, using BioRad´s *Mini-Protean II* system (Laemmli, 1970). Before applying the samples to the gels, they were loaded with a solution concentrated 2x (200mM Tris-HCl pH 6.8, 30% glycerol and 0,01% bromophenol blue). Besides the samples, a protein pre-stained standard with known molecular masses between 10 and 250 kDa (Precision Protein Standards from BioRad) was applied to every gel. Protein separation occurred in 10% polyacrylamide gels in electrophoresis buffer 100mM Tris/Glicine pH 8.3. The gels where then incubated in 50mM Tris-HCl pH 7.4 buffer with 100µM Phe-AMC during 30 minutes at 37ºC. After incubation period washed rapidly with sample buffer and revealed with Versadoc detector.

2.7- Characterization of Proteolytic Activity

2.7.1- Enzymatic Assays to Determine Preferential Substrates

The proteolytic activity of the pollen extracts and combinations were determined by using peptide substrates coupled to a fluorescent molecule, 7-amino-4-methylcoumarin (AMC). For this enzymatic assay, 200µl of the pollen extracts were incubated with 2µl of fluorescent substrates accordingly to the concentrations represented on Table 4. Hydrolyze of the fluorescent substrates causes the release of the AMC group, which is visible by the increase of fluorescence. The fluorescence (Ex_{380nm}, Em_{460nm}), was monitored in intervals of 15 seconds, during 20 minutes at 37ºC with a 96 well plaque, using SpectreMAX-GeminiEM. It´s also necessary the construction of a standard AMC curve, to allow quantification of AMC released along the time.

Table 4 – Fluorescent AMC substrates and their effective concentrations used in the enzymatic assays.

Substrate (AMC)	Effective Concentration (mM)
Ala-AMC	0.1
Met-AMC	0.1
Leu-AMC	0.1
Arg-AMC	0.1
Bz-Arg-AMC	0.1
Lys-AMC	0.1

Phe-AMC	0.1
Ala-Pro-Arg-AMC	0.1

2.7.2- Identification of the Proteolytic Class through Inhibition of Enzymatic Activity

The identification of protease classes present in the pollen extracts was possible through profile analyze obtained by inhibiting the proteolytic activity using specific inhibitors, accordingly to Table 5. Obviously, the inhibitors used were specific to all the proteolytic classes known, in concentrations in accordance to values found in the literature.

Enzymatic activity was assayed, in 96 well plaques, where 180µl of pollen extract and 20µl of specific inhibitors were added and incubated for 20 minutes at 37ºC. After, 2µl of the preferred AMC substrate of each pollen extract, determined with the enzymatic assay described above was added, the florescence was monitored for 20 minutes using SpectreMAX-GeminiEM. The result is presented in percentage of residual activity in comparison to control condition without inhibition, for this, 180µl of pollen extract was incubated with 20µl of buffer Tris-HCl pH 7,4 and subjected to the same exact conditions.

Table 5 – Specific inhibitors and metallic ions used in the enzymatic assay for inhibition of proteolytic activity, and respective effective concentration.

Inhibitor	**Protease Classes**	**Effective Concentration (mM)**
Pepstatin	Aspartic	0.01
E-64	Cysteine	0.1
AEBSF	Serine	100
TPCK	Serine type Chymotrypsin	1
TLCK	Serine type Trypsin	1

Bestatin	Aminopeptidases	0.1
Amastatin	Aminopeptidases	0.01
EDTA	Metalloproteases	100
Divalent ions		
Ca^{2+}		10
Mg^{2+}		10
Mn^{2+}		10
Zn^{2+}		10

2.8 - Cell Culture

For an experimental model Calu-3 cells were used, which is a Human bronchial epithelial cell line (American Type Culture Collection). Cells were cultured in Dulbecco's modified Eagle´s medium (DMEM - Sigma) supplemented with 10% heat-inactivated (56ºC for 30 minutes) fetal Bovine Serum (FBS - Gibco, Barcelona, Spain), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). The cell culture was maintained in 75 cm^2 flasks using 15 ml of medium and in a humidified atmosphere with, 5% CO_2: 95% air at 37ºC. When confluence was reached, the cells were diluted 1:2 or 1:3, depending on the experiment. But to accomplish this, cells had to be trypsinized at 37ºC during 10-15 minutes using a solution composed by 0,25% (m/v) trypsin (Gibco), 1 mM EDTA in a saline medium with 130 mM NaCl, 3mM KCl, 1 mM Na_2PO_4, 30 mM Hepes, 10 mM glucose at pH 7.3. DMEM medium was renewed twice per week, and cells were only used in scientific experiments between passages 28 and 50.

2.9- Transepithelial Permeability Measurement

Calu-3 cell line was seeded on transwell filter inserts with 0,4µm pore size placed into 12-well tissue culture plates (Corning, Bath, UK), using a air-interfaced culture, at a density of 1.5×10^5 cells/cm^2, following cell culture described in 2.8. Cells were examined daily microscopically, for evaluation of features like confluence, integrity and uniformity of the epithelial cell monolayer. Also, on a daily basis, the chambers, termed inserts, were first washed with PBS buffer (10mM Na_2PO_4; 1,8mM KH_2PO_4; 137mM NaCl; 2,7mM KCl at pH 7.4) and then culture medium was renewed. In these conditions, cells were maintained in culture for approximately 10 days, in order

to reach confluence. After reaching confluence, cells were washed twice with PBS buffer, incubated 2 hours in culture serum free medium and for 6 hours incubated with the stimuli. The stimulus, naturally, pollen extracts and pollen extracts treated with 1mM AEBSF inhibitor, previously incubated during 20 minutes. As control, cells were incubated with culture serum free medium at 37ºC and with 5% CO_2. In this technique, for calculation purposes, it´s necessary another control, termed "control cell-free", treated like the control but differs because of the absence of cells in the inserts.

In order to obtain a time course, after 1, 3, 6 and 12 hours of stimuli, 50 µl of 10µM Rhodamine Isothiocyanate (RITC)-dextran (70-kDa) (Sigma), a fluorescent molecule prepared in PBS, was added to the inserts of the transwells, upon the confluent epithelial cell monolayer. Samples were subsequently collected from the basolateral parte of the chamber, 30 in 30 minutes, during 4 hours, and only one sample was collected from the apical part of the chamber at the last time point. Also, in order to see a possible inhibition of the protease effect, cells forming the monolayer in the inserts of transwells were subjected to a stimulus of 12 hours with pollen extracts treated with AEBSF 1mM, adding RITC-dextran and proceeding the same way with the collection of samples, described above. These samples were placed on a 96-well opaque plate, in order to read fluorescent values using a SpectraMax Gemini EM fluorescent plate reader (Molecular Devices) with an excitation wavelength of 530±25nm and emission wavelength of 590±35nm (SoftMax Pro v5 software).

The obtain fluorescence for each point is normalized relatively to the florescence of the respective apical chamber and this ratio is later adjusted to the volume of the basolateral chamber, which decreases 50 µl, 30 in 30 minutes. The corrected ratio is placed in order of the time, being that curve slopes correspond to the diffusion rate of RITC-DEX. The slope is converted from minutes to seconds and divided by the area of the filter (in this case, 1.1 cm^2), giving the transepithelial permeability in cm/s. These transformations are repeated for the "control cell-free" and the result, corresponding to the permeability of the filter membrane, is then subtracted to the value of the permeability determined for each stimuli.

2.10- Cell Viability

Calu-3 cell line is cultured in 12 well plates at a density of 5×10^4 cells/cm^2. Once the cells reached confluence, they were washed with culture serum free medium

and incubated with pollen extracts during 6 hours, at 37ºC and 5% CO_2. After, the contents of each well were collected and transferred into eppendorf tubes, these were centrifuged at 2000 rpm during 5 minutes, at 4ºC, in order to collect the detached cells. These were washed twice with culture medium and reseeded in 12 well plates. After 24 and 48 hours, the plates were observed microscopically to evaluate proliferation status of the subculture of detached cells.

2.11- Cell Extracts

In order to evaluate the integrity of integral proteins of tight junctions, cell extracts of Calu3 were prepared after 6 hours stimuli with different pollen extracts.

Cells were seeded at 5×10^5 cells/cm^2 density in 6 well culture plates (Corning). After 7 days in culture, cells were washed with PBS and culture serum free medium, and then incubated for 6 hours with different pollen extracts and combinations, at 37ºC and 5% CO_2. Cells were also exposed to pollen extracts and combinations treated with 1 mM AEBSF and denatured by heat (95ºC during 15 minutes). The control for this experience was cells incubated with culture serum free medium.

After stimuli period, adherent cells were washed twice with PBS containing a mix of protease inhibitors (1 μg/ml CLAP – Chymostatin, leupeptin, antipain and Pepstatin; 1 mM DTT; 0.1 mM PMSF). Next, 150μl of PBS with inhibitors was added to the culture plate, and then preceded to scrap the adherent cells into eppendorf tubes. The cell extracts were then centrifuged at 14 000 rpm for 5 minutes at 4°C. The pellet was then resuspended in 150 μl of heated denaturing solution 2x (100 mM Tris-Bicine, 6M urea, 4% SDS, 4% β-mercaptoethanol and bromophenol blue). After 10 minutes in ice, the denatured extracts were sonicated 3 times during 5 second pulses and centrifuged again at 14 000 rpm during 5 minutes. Finally, the supernatant was collected and denaturated at 80ºC during 10 minutes.

2.12- Western Blot

For Western blot analyze of proteins in cell extracts, prepared in 2.11, an electrophoretically separation was proceeded using a 10% SDS-PAGE gel (described in 2.4.1). After the electrophoresis, the proteins were transferred from the gel to a PVDF membrane (Millipore). The electrotransference occurred in 10mM CAPS with 10%

methanol, in a TransBlot Cell system (BioRad), overnight, at 4ºC and under a constant voltage of 40V.

Immunoblotting was performed using a SNAP system (BioRad). The PVDF membranes were blocked, washing 3x with 100µl of 2,5% milk in TBS-T (20 mM Tris-HCl; 137 mM NaCl; 0,1% Tween 20). The membranes were then incubated with the primary antibody diluted in 0,5% (m/v) milk in TBS-T: mouse monoclonal anti–E cadherin (1:500), mouse polyclonal antioccludin (1:500), rabbit monoclonal anticlaudin-1 (1:250) and mouse polyclonal anti-ZO-1 (Zymed Laboratories, Barcelona, Spain). After, the membranes were washed 3x with 100µl of 0,5% milk in TBS-T, followed by an hour of incubation with the respective alkaline phosphatase-conjugated secondary antibody: anti-rabbit (1:5000) or anti-mouse (1:20000) (GE Healthcare), diluted in 0,5% milk in TBS-T. Next, the membranes were washed again with 3x 100µl of TBS-T, and proceeded to incubate these in ECF reagent (GE Healthcare) for 5 minutes. When the membranes were dry, detection of the bands was possible using the VersaDoc detector. All the procedures were done at room temperature.

These membranes can be re-incubated several times with different primary and secondary antibodies, and for this it was necessary a stripping procedure to remove previous bands labeled. The membranes were placed in distilled water during 5 minutes, then 5 minutes in 0,2M NaOH and finally in water again for another 5 minutes.

As a internal control for quantity and levels of degradation of protein in each sample, immunoblots were normalized by labelling the membranes to β-actin mouse (Sigma) diluted in 0,5% milk in TBS-T (1:5000), which is an ubiquitous protein in cells.

The quantification of the density of labelled protein bands resultant through Western blot analyzes was managed by ImageJ software.

2.13- Immunocytochemistry

Calu-3 cells were seeded at 1.5×10^5 cells/cm^2 density in 12-transwell plates (Corning, Bath, UK), at 37ºC and 5% CO_2. These cells were maintained in culture approximately 10 days until reaching a confluent state, and for that reason the formation of a cell monolayer. Then, these cells were washed with PBS and culture serum free

medium, and incubated for 6 hours with different pollen extracts and combinations. As control, cells were incubated in culture serum free medium.

After stimuli incubation period, the monolayer of cells, were washed twice with PBS and then fixed in -20ºC methanol during 10 minutes. After newly washing with PBS, cells were permeabilized with 0.3% (v/v) Triton X-100 in PBS, at 4ºC during 10 minutes. The epithelial cells were then incubated with 10% (m/v) BSA in PBS-T (PBS with 0,1% (v/v) Tween 20), for 1 hour at room temperature in order to block nonspecific labeling. At this point, incubations with primary antibodies diluted in 10% (m/v) BSA in PBS-T occurred, overnight at 4°C: mouse monoclonal anti-occludin (1:300), rabbit polyclonal anti-claudin-1 (1:100), mouse monoclonal anti-ZO1 (1:100) (Zymed Laboratories, Barcelona, Spain) or mouse monoclonal anti-E-cadherin (1:100) (BD Biosciences, Franklin Lakes, USA). The cells were then washed 5x with PBS solution with 0.1% Tween and 0.1% gelatin, in order to incubate with the appropriate secondary antibodies diluted in 10% (m/v) BSA in PBS-T, for 1 hour at room temperature: Alexa Flour 594 rabbit (1:1000) and Alexa Flour 488 mouse (1:1000) (Invitrogen). After, the fluorescent dye Hoechst 33342 (Sigma, 0.5 μg/ml) was used to stain nuclei.

Subsequent to another set of washes the transwell membrane inserts were cut and mounted on glass lamellae, using a fluorescent mounting medium, termed DAKO (DakoCytomation Fluorescent Mounting Medium). The cell imaging was performed on a Zeiss LSM 510 confocal microscope, using a 63× oil objective.

It was also important the observation of subsequent inhibition of protein tight junction degradation by proteases. For that reason, the cells forming the monolayer were exposed to pollen extracts denatured 30 minutes at 95ºC. After the 6 hour stimuli incubation period on the confluent monolayer of cells, the pollen extracts were removed. Then these transwells proceeded to incubation periods with primary and secondary antibodies, exactly like described above.

2.14- Single-Cell Imaging

In order to evaluate the functionality feature of PAR-2 receptors in allergies, we monitored the variations of intracellular calcium-free levels [Ca^{2+}] in single cells following stimulation with different pollen and specific combinations.

Cells were loaded with 5 µM Fura-2/AM (Molecular Probes), 0.1% fatty acid-free BSA and 0.02% pluronic acid F-127 (Molecular Probes) in Krebs solution with calcium (132mM NaCl, 4mM KCl, 1,4mM MgCl2, 1mM CaCl2, 6mM glucose, 10mM HEPES, pH 7.4), for 60 minutes in an incubator with 5% CO_2: 95% air at 37ºC. After, cells were exposed to a 10 minute incubation period at room temperature to a blocking solution, (solution of 0,1% fatty acid-free BSA) in order to obtain a complete hydrolysis of the probe. The glass coverslip was mounted on an RC-20 chamber in a PH3 platform (Warner Instruments, Hamden, CT, http://www.warneronline.com) on the stage of an inverted fluorescence microscope (Axiovert 200; Carl Zeiss).

Cells were washed with Krebs solution and stimulated by pollen extracts. The variations of [Ca^{2+}] were evaluated by quantifying the ratio of the fluorescence of Fura-2, measured at 340 and 380 nm (excitation) and 510 nm (emitted). These measurements were possible using a Lambda DG4 apparatus (Sutter Instrument, Novato, CA, http://www.sutter.com) and a 510 nm band-pass filter (Carl Zeiss) before the fluorescence acquisition occurred with a 40x objective and a CoolSNAP digital camera (Roper Scientific, Trenton, NJ, http://www.roperscientific.com). Acquired values were processed using the MetaFluor software (Universal Imaging Corp., West Chester, PA, http:// www.moleculardevices.com).

2.15- Flow Cytometry

Calu-3 cells were seeded at 5×10^4 cells/cm^2 density in 48 well plates at 37ºC and 5% CO_2. When confluence was reached, these cells were washed with PBS and culture serum free medium, and subsequently incubated with pollen extracts and combinations between them. The pollen extracts were diluted in a proportion 1:1 with culture serum free medium. For this experiment, diverse controls were prepared, control with cells incubated only with culture serum free medium, another control with cells incubated only with buffer 50 mM Tris-HCl pH 7.4 and finally a control with cells incubated with culture serum free medium and 1mM AEBSF.

Samples were collected after 6 hours of incubation with the pollen extracts and with the intended pollen extract combinations. For this reason, after each period of incubation, 150 µl of cell supernatant was collected and frozen immediately at -80ºC. The inhibition effect was also intended for study, and for that reason pollen extracts and combinations were denatured at 95ºC during 30 minutes, and then incubated with the

cells during 6 hours.

The quantification of the released cytokines by Calu-3 cells, in the samples collected after the incubation periods was possible using the Cytometric Bead Array (CBA) method. Cytokine levels were measured using the Human Basic FlowCytomix Multiplex kit (Bender MedSystem GmbH, Vienna, Austria) according to the manufacturer's instructions. Briefly, the assay is based on a mixture of beads of different size, coated with capture antibodies specific for each cytokine to be analyzed, and a biotin- conjugated second antibody mixture to detect the cytokine of interest. After washing to remove unbound antibody, a streptavidin-phycoerythrin reagent was added. On removal of unbound material by washing, the bead suspension was analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Bead size and bead fluorescence were the means of identifying the type of cytokine, and phycoerythrin fluorescence was used as a measure of cytokine concentration as determined with standards of known concentrations. Results were analyzed with FlowCytomix Pro 2.4 Software (Bender MedSystem GMbH) and expressed as picogram per milliliter for each cytokine.

2.16- Statistical Analyze

The results of this research project are represented in terms of average ± standard error bar of the number of experiments realized. For statistical treatment, the program selected was Graphpad, using 2way and One-way ANOVAs followed by Bonferroni´s post test. The differences were considered significant for values of $P<0,05$.

Chapter 3

Results

3- Results

3.1- Epidemiological Studies of Allergic Pollen

The atmosphere content in pollen allergens depends and varies according to climate, geography and vegetation. In Europe, the main pollinosis period extends to about half of the year, from spring to autumn. The Mediterranean area, with its characteristic climate conditions of mild winters and sunny days with dry summers, differ in their vegetation from that of central and northern Europe. The typical allergenic pollen plants of Mediterranean climate zone are trees and grasses, namely cypress and Parietaria (D´Amato & Liccardi, 1994; D´Amato *et al.*, 1998, 1991).

With data obtained through aerobiological and allergological studies on the presence and prevalence of allergic airborne pollens, it´s possible to design calendars with the approximated flowering periods of plants from the sampled area. Three pollen seasons have been identified for the Mediterranean area:

- A winter season from December to March characterized by the presence of pollens of some trees like Cupressaceae (*Cupressus* and *Juniperus*), Corilaceae, Acaciae (Mimosa) and some Betulaceae.

- A season from April to June dominated by the pollination of grasses, like *Parietaria* and *Olea* trees. Overlapping with this season, March to May, *Platanus* pollen, which have some allergenic importance in some Mediterranean areas as France, Spain and other parts.

- Finally, a summer-autumn season, from August to October, marked by *Parietaria*, Gramineae, pollens of herbaceous plant, such as mugwort and Chenopodiaceae (D´Amato *et al.*, 1998).

Grass pollen is by far the most important cause of pollinosis throughout the Europe continent and also the Mediterranean area. It´s interesting to note in European cities, while the atmospheric concentration of grass pollen is decreasing the prevalence of allergic disorders are increasing. The decrease of atmospheric grass pollen is obviously related to a substantial decrease in grassland over large regions of the

continent. However, the increase in allergic disorders is obviously related and caused by other factors, including increased air pollution levels.

Plant pan-allergens share highly conserved sequence regions, structure and function. They are responsible for extensive IgE cross-reactivity among a variety of allergenic sources, even between unrelated sources. The impact of pan-allergenicity is lower in patients living in geographical regions such as central and northern Europe, where grass and Betulaceae have clear distinct pollen periods, and are the almost only source of pollen allergens. However, this situation is very different in regions like southern Europe. Here, besides grasses being the most relevant cause of allergies, other pollen species play an important role, and have near and even overlapping pollen seasons, such as olive pellitory, cypress, Russian thistle among others (Barber *et al.*, 2008).

In Portugal, other pollen species are frequent such *as Pinus sylvestris*, *Plantago sp.*, *Chenopodium sp.* and others that also contribute to the prevalence of allergic disorders. Accordingly to The Portuguese Aerobiology Network, which undertakes continuous airborne pollen monitoring at five different stations, they reported the following results in terms of percentage of pollen counts:

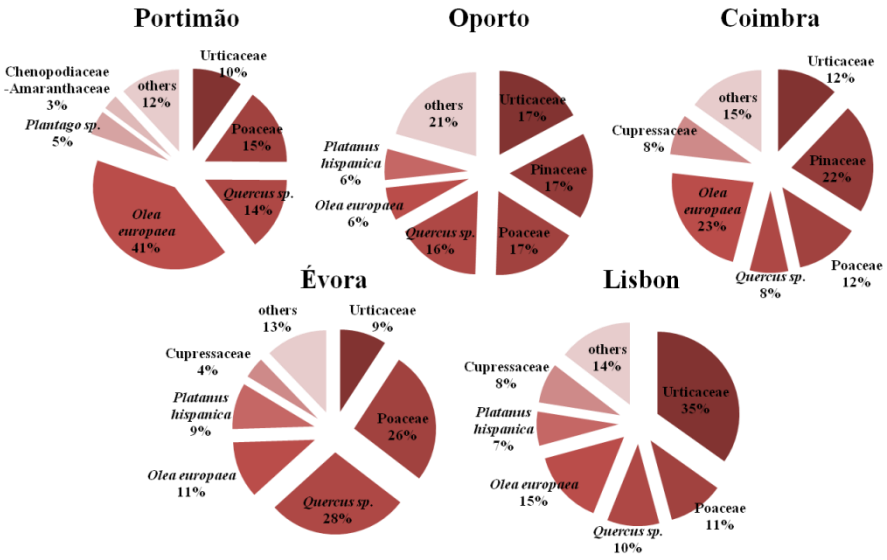


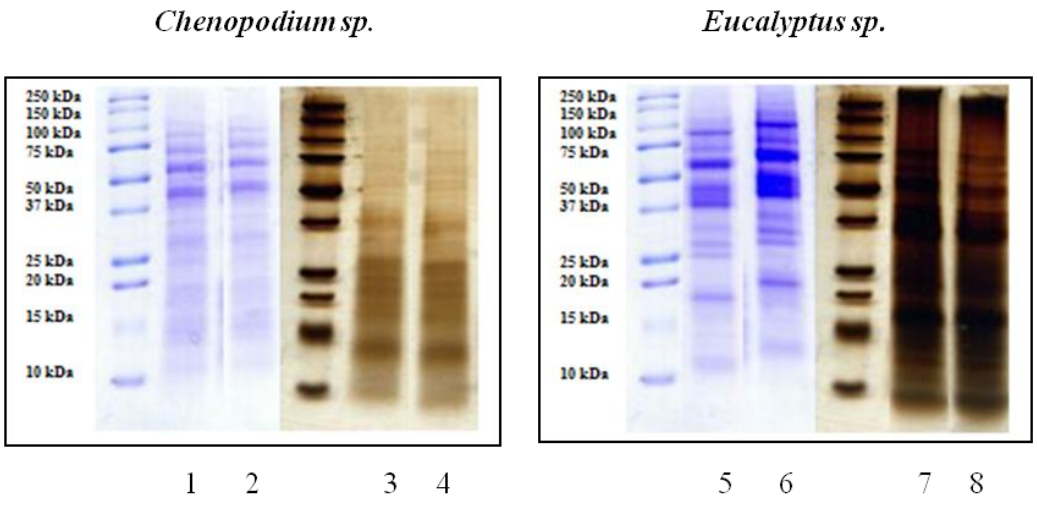
Table 6 – Circular tables with the results in terms of pollen counts from five different stations (Adapted from The Portuguese Aerobiology Network: Airborne pollen results 2002-2006).

Importantly, the atmospheric pollen concentration varied year to year and between the 5 regions explainable do to various factors such as, landscape issues, weather conditions before and during the pollination period, floral phenology of the species releasing the pollen, and others (The Portuguese Aerobiology Network: Airborne pollen results 2002-2006). The overlapping pollination periods and cross reactivity make it vital to study not only pollen from different plant species isolated but also combined, to mimic natural conditions found in the atmosphere.

3.2- Protein Profile of Pollen Extracts of *Chenopodium sp.*, *Eucalyptus sp.* and *Plantago sp.*

Pollen extracts of each plant species was extracted by hydration in 50 mM Tris-HCl pH 7.4 (~20mg of pollen/ml), during 2 hours at room temperature with slow agitation in order to mimic the same process of hydration that occurs in the respiratory airway after pollen inhalation.

The pollen extracts of *Chenopodium sp.*, *Eucalyptus sp.* and *Plantago sp.* were analyzed by SDS-PAGE in order to obtain a protein profile in terms of molecular mass. The quantity of total protein released was superior in pollen extracts with low allergenicity and larger pollen grain dimensions, approximately 0,8mg/ml for *Eucalyptus sp*. While, for *Chenopodium sp.* and *Plantago sp.* with moderate allergenic potential and small dimensions of their pollen grains, had an average of total protein released of 0,35mg/ml and 0,2mg/ml, respectively. In order to enhance the sensitivity of visual identification of the protein profile, it was necessary to resort to staining with Silver Nitrate, staining method more sensitive then Coomassie Blue (Fig. 14).



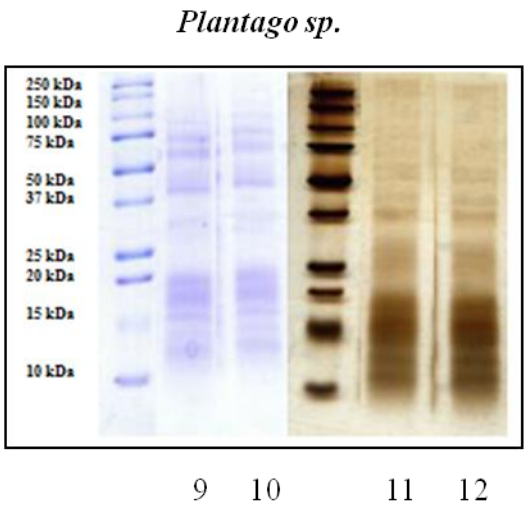


Figure 14 – Protein profile of the pollen extracts in 12,5% SDS-PAGE gels. Staining was preformed with Coomassie Blue, first two profiles, and with silver nitrate, last two profiles, for each pollen extract. 1 and 3) 3,5µg of pollen extract from *Chenopodium sp.* 2 and 4) 5µg of pollen extract from *Chenopodium sp.* 5 and 7) 8µg of pollen extract from *Eucalyptus sp*. 6 and 8) 12µg of pollen extract from *Eucalyptus sp*. 9 and 11) 2µg of extract from *Plantago sp*. 10 and 12) 3µg of pollen extract from *Plantago sp*.

Chenopodium sp. has been targeted in previous studies and research on the identification of protein and allergens of this pollen extract. From the obtained protein profile, the SDS-PAGE allows the visual perception that the total quantity of protein released on hydration is much inferior to *Eucalyptus sp.* In existent literature, proteins have already been identified from this pollen extract by SDS-PAGE, and can also be observed in the protein profile obtained, corresponding to estimated molecular masses of 97, 85, 66, 45, 39, 18, 15, 10 kDa (Fig. 14 & Tehrani *et al.*, 2010). The majority of the protein content of this pollen extract has high molecular mass. From this protein profile, bands corresponding to already identified allergens che a 1 (glycoprotein), che a 2 (profillin) and che a 3 (polcalcin), with approximate molecular weights of 17, 14.4 and 9.5 respectively are easily identified on the SDS-PAGE (Gadermaier *et al.*, 2004). In a study conducted by Tehrani *et al.*, in spite of antigenic differences among pollens from some species of Amaranthaceous/Chenopodiaceous family, there are significant IgE cross reactivity, especially those proteins of high molecular weight. Moreover, the proteins with apparent molecular mass of 45, 39 and 66 KDa are suggested to be the common allergic components among pollens from these species. It was also proven that the protein with estimated molecular mass of 66KDa is the most imunoreactive protein

of *Chenopodium sp.*, since the frequency of IgE-binding to this protein was higher in comparison to the others (Tehrani *et al.*, 2010).

Accordingly to Figure 14, observing the protein profile of the pollen extract from *Eucalyptus sp.* we can easily prove the high quantity of total protein released after hydration. Also, observable is the presence of a larger abundance of proteins with high molecular weight compared to a few number of low molecular weight proteins. These low molecular weight proteins often are identified as allergens, interesting when correlating with *Eucalyptus sp*. low allergenic potential. Since no allergens have been identified in the extracts of this pollen species no corresponding protein band and molecular weights can be made. Even so, it is vital to list the approximate molecular masses of at least 12 protein bands easily observed and perceptive in the SDS-PAGE: ~100, 90, 70, 55, 44, 40, 38, 30, 27, 25, 14 and 12 kDa.

Observing the protein profile correspondent to pollen extract of *Plantago sp*.it is perceptive a larger amount of proteins of low molecular weight than those of high molecular weight. In this pollen extract there has not been much characterization of allergenic proteins besides the already identified *major* allergen, Pla l 1 (glycoprotein). As already described this allergen can be found with about 17, 20 and also 32-36 kDa, corresponding, respectively, to the unglycosylated, glycosylated and dimeric form. Besides these, it becomes important to mention other well defined and perceptive protein bands present in the SDS-PAGE with estimated molecular masses of 40, 65, 70 and 90 kDa (Fig. 14).

3.3- Proteolytic Profile of Pollen Extracts of *Chenopodium sp., Eucalyptus sp.* and *Plantago sp.*

The presence of proteolytic activity in pollen extracts has been highlighted in response of the ability to cause alterations in the pulmonary homeostasis. This proteolytic activity of pollen extracts may contribute in the initiation of an allergic process through an epithelium disruption, allowing allergens to gain access to subepithelial layers and contact with cells of the immune system, namely APC (Cortes *et al.*, 2006; Runswick *et al.*, 2007). For this reason, we aim to investigate the existence of proteases in the different pollen extracts.

3.3.1- One-Dimensional Zymography

The presence of proteases in these pollens diffusates was assessed by gelatin zymography. Zymography is an enzymatic assay which uses gelatin as a substrate co-polymerized in an SDS-PAGE gel. Gelatin is used, since it is derived from collagen and for that reason efficient in the detection of a range of proteases. For the identification of proteolytic activity in the pollen extracts, these were prepared in non denaturing conditions in order to preserve the enzymatic activity, and subjected to an electrophoresis in a polyacrylamide gel containing 1mg/ml gelatin. After the electrophoretical separation, SDS was removed using Triton X-100, incubated overnight at 37ºC and in buffer pH 7,4. Finally staining with Coomassie Blue allowed the detection of enzymatic digestion spots that corresponded to non-colored bands (Fig. 15).

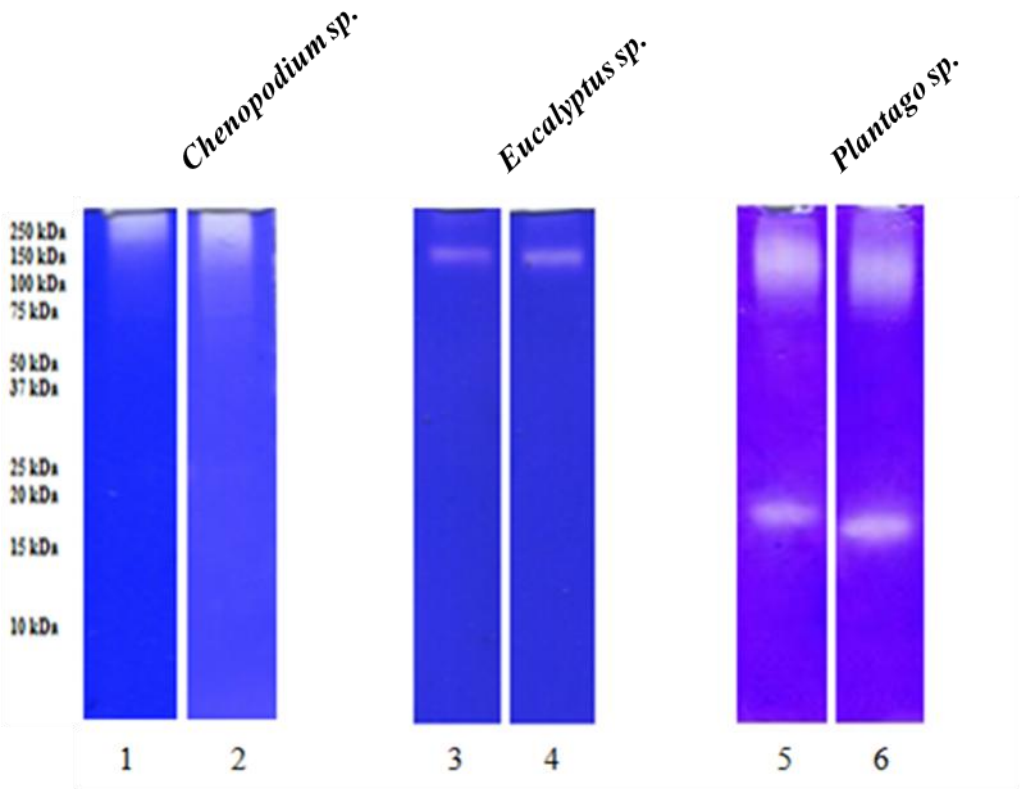


Figure 15 – Proteolytic profiles of pollen extracts obtain by zymography 12% polyacrylamide co-polymerized with 1mg/ml gelatin. 1) 3,5µg of the pollen extract from *Chenopodium sp.* 2) 5µg of the pollen extract from *Chenopodium sp.* 3) 8µg of the pollen extract from *Eucalyptus sp*. 4) 12µg of the pollen extract from *Eucalyptus sp*. 5) 2µg of the pollen extract from *Plantago sp*. 6) 3µg of the pollen extract from *Plantago sp*.

This enzymatic assay showed that all pollen diffusates contained proteases able to digest the gelatin substrate. The prevalence of high molecular weight proteases is perceptive, with the exception of a low molecular protease present in the pollen extract of *Plantago sp*. Specifically in terms of *Chenopodium sp.*, this activity is situated between 250-100 kDa. Proteases from *Eucalyptus sp* are found located between 150-100 kDa. The proteases from *Plantago sp.* on the other hand are located between 250-75 kDa and 20-15 kDa (Fig. 15).

It is extremely important to underline the fact that even though *Plantago sp.* and *Chenopodium sp.* release low amounts of protein content, they produced an intense degradation band. This result implies that *Plantago sp.* and *Chenopodium sp.* have a large concentration of proteases with intense proteolytic activity. *Eucalyptus sp.* in terms of total protein quantity in the zymography is present 4x to 6x more when compared to the *Chenopodium sp.* and *Plantago sp* pollen extracts, respectively. (Fig. 15).

3.3.2- Two-dimensional Zymography

Additionally, pollen proteolytic activity was rapidly characterized through two-dimensional zymography. For first dimension, concentrated pollen extracts samples were submitted to an isoelectric focusing, using pH 3-10 strips, were separation occurred accordingly to their pI. The second dimension, separation occurs accordingly to their molecular mass, on a gelatin zymography. Revelation of enzymatic digested spots was possible through Coomassie Blue staining (Fig. 16).

Pollen extracts samples were concentrated using centricon filter devices of 30kDa, in order to increase the total quantity of protein. This method intends to remove proteins of low molecular weight and concentrate the samples of pollen extracts in high molecular weight proteins, region normally comprised of the majority of proteases.

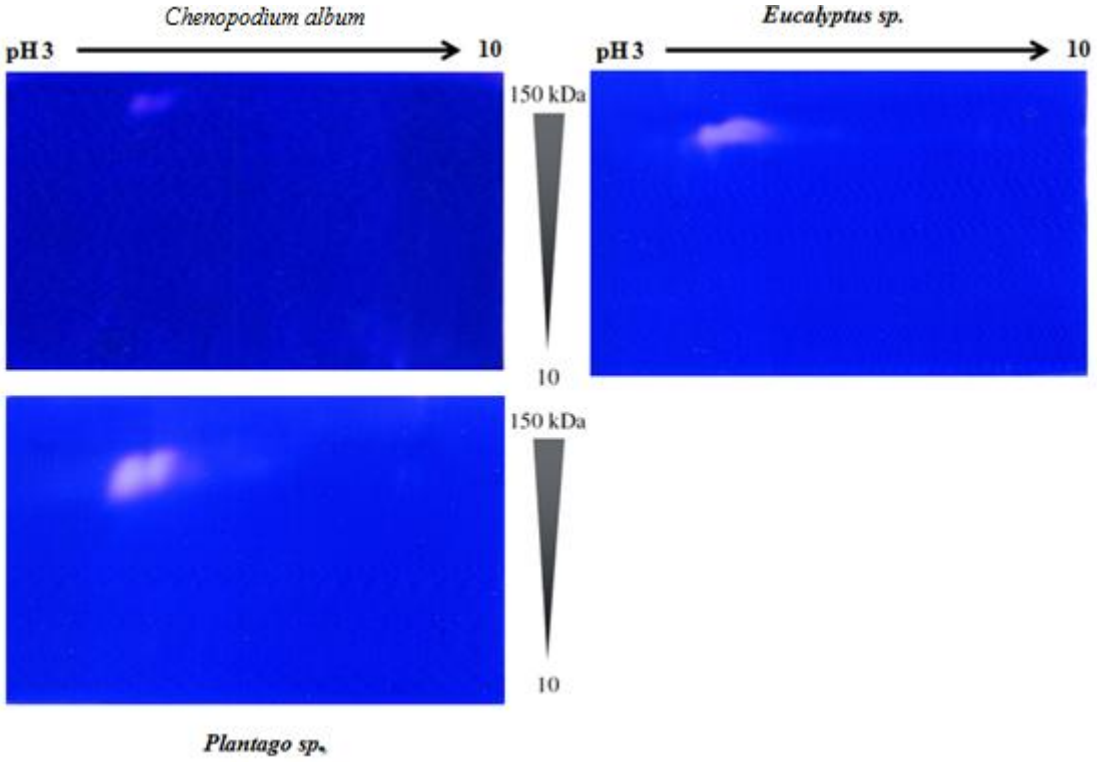


Figure 16 – Proteolytic 2D-PAGE profile of the concentrated pollen extracts of *Chenopodium sp.*, *Eucalyptus sp.* and *Plantago sp.* The total quantity of each sample of concentrated pollen extract was ~100µg.

The proteolytic activity of the pollen extracts of *Chenopodium sp.*, *Eucalyptus sp.* and *Plantago sp.* are a result of proteins located in a region of molecular mass superior to 55 kDa and an acidic pI. These spots of enzymatic digestion obtained in this 2D analysis point to the probability of existence of similar proteases between the three species of pollen. Therefore, it is acknowledgeable the presence of high molecular weight spots with low pI, within all three pollen diffusates, suggesting that pollen grains with distinct allergenic capacities might release identical proteases.

3.3.2- Native-PAGE

In order to conduct a complete protein profile, a native-PAGE was then realized to draw conclusions relative to their biological activity which remains intact during this technique (Fig. 17).

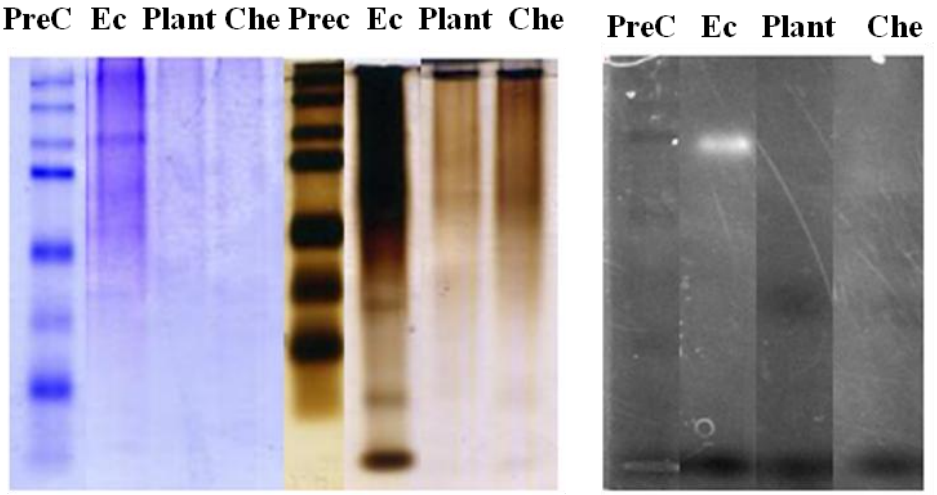


Figure 17 – Protein profile of the pollen extracts in 10% PAGE gels. Staining was preformed with Coomassie Blue and with Silver Nitrate, for each pollen extract, image on the left. For *Eucalyptus sp.*, *Plantago sp*. and *Chenopodium sp.*, the quantity of total protein used was 12µg, 3µg and 5µg respectively. After, the gel was incubated in the preferential AMC substrate, Phe-AMC, image on the right.

Analyzing the gel on the left, the most important aspect to highlight is the presence of high molecular mass proteins in all three pollen extracts and the disappearance of the low molecular mass proteins that appeared on the SDS-PAGE, shown previously. This is naturally a result of the proteins acquiring and remaining in their native structure, with more aggregation and negative density of charge. After, to see whether these proteins had protease activity, we incubated the gel in the preferential AMC substrate of the pollen extracts, Phe-AMC (data showed next). For *Eucalyptus sp.* it´s well observable, again, the protease activity of the protein with molecular mass estimated around 100kDa. For *Plantago sp.* there is no visible protease band, which might be due to the fact of this pollen extract having the least amount of total protein quantity. Finally, for *Chenopodium sp.*, no intense band is visible. Although, a slight smear can be deciphered at a molecular mass approximate to 70kDa, which may correspond to the already identified allergen at 66kDa.

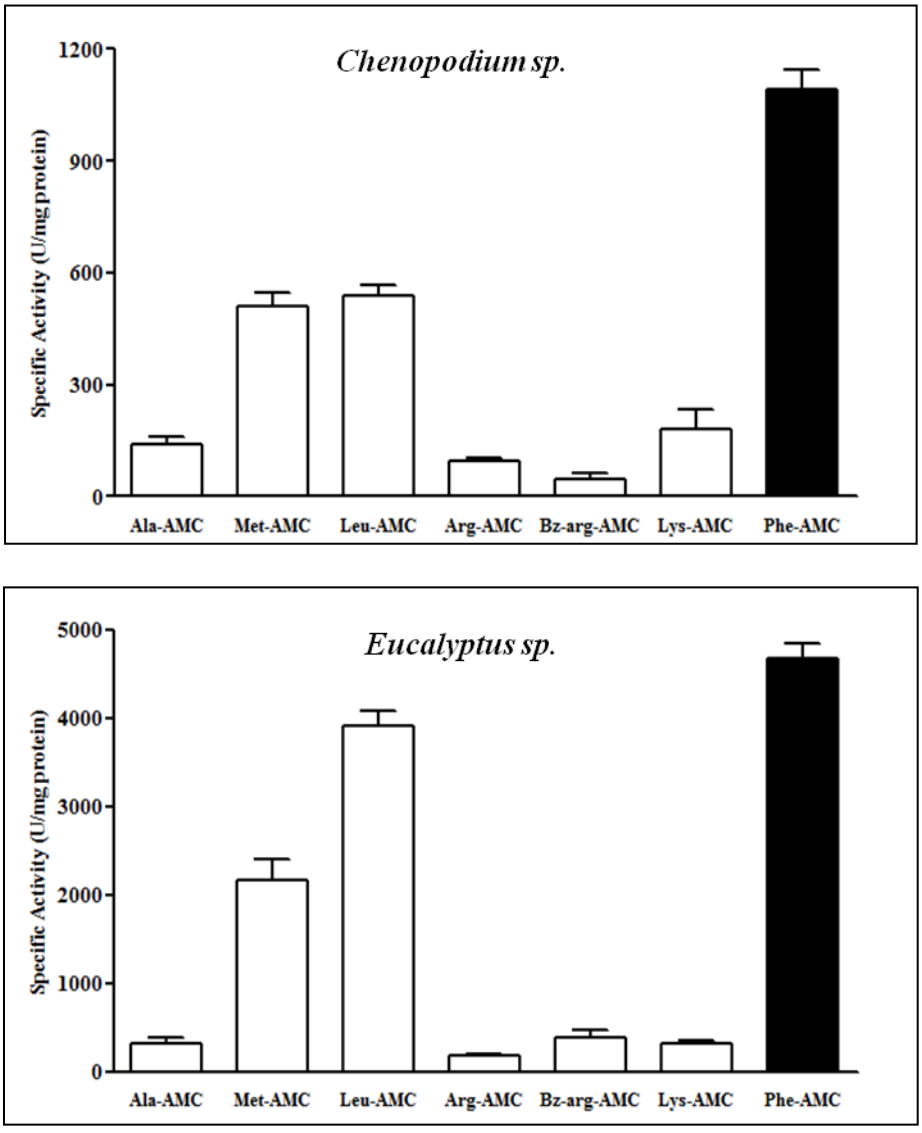
3.4- Characterization of the Proteolytic Activity of *Chenopodium sp., Eucalyptus sp. and Plantago sp.*

The characterization of proteases in pollen extracts of the three different species responsible for enzymatic digested spots evident in the one and two dimensional zymography, was possible using a well define enzymatic assay, which intended to

determine the preferential substrate of the pollen extracts, observing the highest enzymatic activity of the proteases. After, with the preferential substrate determined, another enzymatic assay using diverse inhibitors was used allowing the identification of the specific classes of proteases present in the extracts.

3.4.1- Preferential Substrate of the Pollen Extracts Proteases

The proteolytic activity of the pollen extracts was determined by using peptide substrates coupled to a fluorescent molecule, 7-amino-4-methylcoumarin (AMC). Hydrolyze of the fluorescent substrates causes the release of the AMC group, which is visible by the increase of fluorescence. The fluorescence (Ex_{380nm}, Em_{460nm}), was monitored in intervals of 15 seconds, during 20 minutes at 37ºC. The results were converted into bar graphs, were the specific activity of each pollen extract can be observed towards each different AMC coupled substrate (Fig. 18).



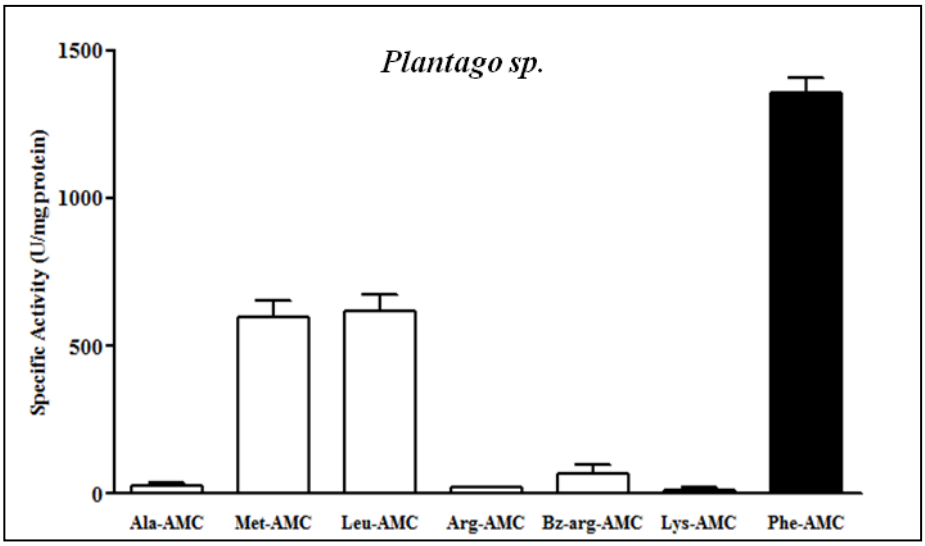


Figure 18- Substrate specificity of pollen proteolytic activity from *Chenopodium sp.*, *Eucalyptus sp.* and *Plantago sp.* The black bars point out the preferred substrate for each pollen extract, which will also be the substrate used for the inhibition assays. One unit of activity = pmol AMC released/min.

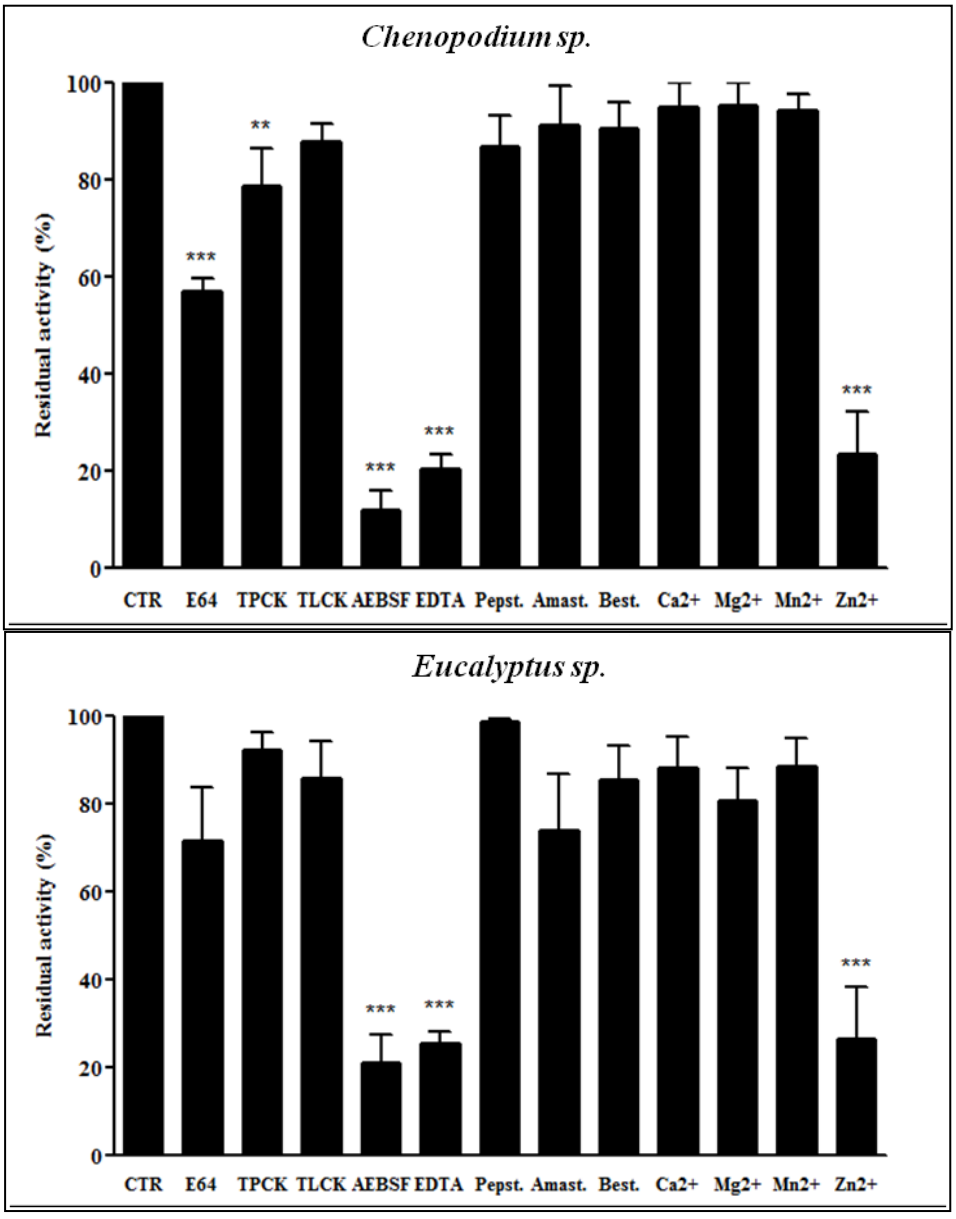
Taking into account Figure 18, it is easily verifiable that all pollen diffusates have proteolytic activity against a variety of AMC coupled substrates. For the pollen extracts of *Chenopodium sp.*, *Eucalyptus sp.* and *Plantago sp.* there are three AMC coupled substrates with respective and high activity, which are Met-AMC, Leu-AMC and Phe-AMC. Also, coincident is the fact that for all three pollen extracts the preferred substrate is Phe-AMC. This substrate was for that reason used in the evaluation of pollen protease class specificity. Visual from the results presented, *Eucalyptus sp.* appears to have greater specific activity when compared to *Chenopodium sp.* and *Plantago sp.* which are quite similar to each other.

Complementary studies were made for this enzymatic assay, in order to try other AMC conjugated substrates. The substrates used were Suc-LLVT-AMC, Suc-AADP-AMC, Suc-APA-AMC, Gly-Pro-AMC, Gly-Pro-Arg-AMC, AAP-AMC, Phe-Arg-AMC and VPA-AMC. The enzymatic assay was carried out in the exact same conditions as described above. The data of these results were not shown because they were considered irrelevant, since the enzymatic activity resultant from the incubation of these AMC conjugated substrates with the pollen extracts was extremely low when compared to the AMC substrates presented above.

Important highlight is the fact that these enzymatic assays were preformed with the initial pollen extracts, without any purification process. For that matter, the proteolytic activity detected is the result of the contribution of the totality of proteases existent in the pollen extract.

3.4.2- Inhibition Profile

The identification of protease classes present in pollen extracts was determined by using specific inhibitors, and analyzing the enzymatic profile obtained after 20 minutes of incubation of the inhibitors with the pollen extracts and then with the addition of the preferred substrate (Phe-AMC). The results were also converted into bar graphs in terms of percentage of residual activity when compared to control condition without inhibition (Fig. 19).



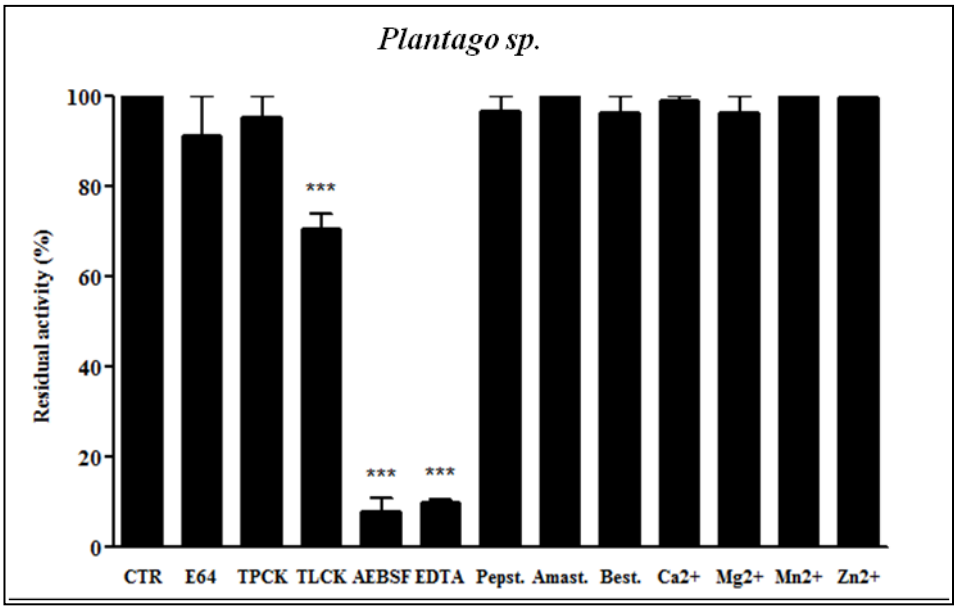


Figure 19- Effect of class-specific inhibitors on pollen diffusates proteolytic activity. Results are represented in terms of residual activity percentage when compared to control condition without inhibition. The results were analyzed statistically using Dunnett´s post-test: ***P < 0.001; **P < 0,01; *P < 0.05 (n=3).

In literature, the majority of identified protease allergens belong principally to four enzymatic classes: serine, cysteine, aspartic and metalloproteinases. For this reason, the inhibitors used in this enzymatic assay were class-specific for those four types of proteases.

In general, the results from this enzymatic assay highlighted the existence of a preferential serine and metalloproteinase activity in the three species of pollen, consistent with the high inhibition of AEBSF and EDTA, respectively. In terms of serine proteases, this presence was even more specific for *Chenopodium sp.*, with statistically significant inhibition of TPCK, class-specific for chymotrypsin-like serine proteases, as well as for *Plantago sp.* with statistically significant inhibition of TLCK, class-specific for trypsin-like serine proteases.

Importantly, *Chenopodium sp.* pollen appears to have cysteine activity present and statistically significant, corresponding to the inhibition with E-64 (Fig. 19). The presence of this specific protease is also verifiable, because TPCK and TLCK although specific for serine protease, are known to inhibit irreversibly cysteine proteases. This cysteine activity seems to be present also in *Eucalyptus sp.* pollen although not statistically significant.

In particular for *Chenopodium sp.* and *Eucalyptus sp.* knowing that divalent ions, such as Zn^{2+}, with high inhibition percentage for these pollen extracts, are involved in a inhibition mechanisms of aminopeptidases N, and the fact that AEBSF also inhibits partially aminopeptidases N, its plausible to conclude that aminopeptidases may exist in these two pollen extracts (Chandu *et al.*, 2003; Cortes, 2006). Importantly, this seems to occur since Amastatin, Bestatin and Pepstatin, possess a reversible inhibition mechanism while AEBSF inhibits irreversibly. This leads to low and not statistically significant inhibition of aminopeptidase and aspartic peptidases, respectively.

Aspartic activity appears to not exist in the pollen extracts, column evidenced by inhibition with Pepstatin (Fig. 19). This may be due to the fact that the substrates used in the enzymatic assay, namely, Phe-AMC, are not specific for aspartic proteases.

Complementary studies were performed in order to confirm this inhibition profile and try to obtain a better characterization of the proteases that exist in the pollen extracts. In the exact same conditions, this enzymatic assay was performed using Leu-AMC instead of Phe-AMC, and was observed that the overall profile did not suffer alterations.

3.5- Action of Pollen Extracts from *Chenopodium sp.*, *Eucalyptus sp.* and *Plantago sp.* on the Integrity of Human Epithelial Cells (Calu-3)

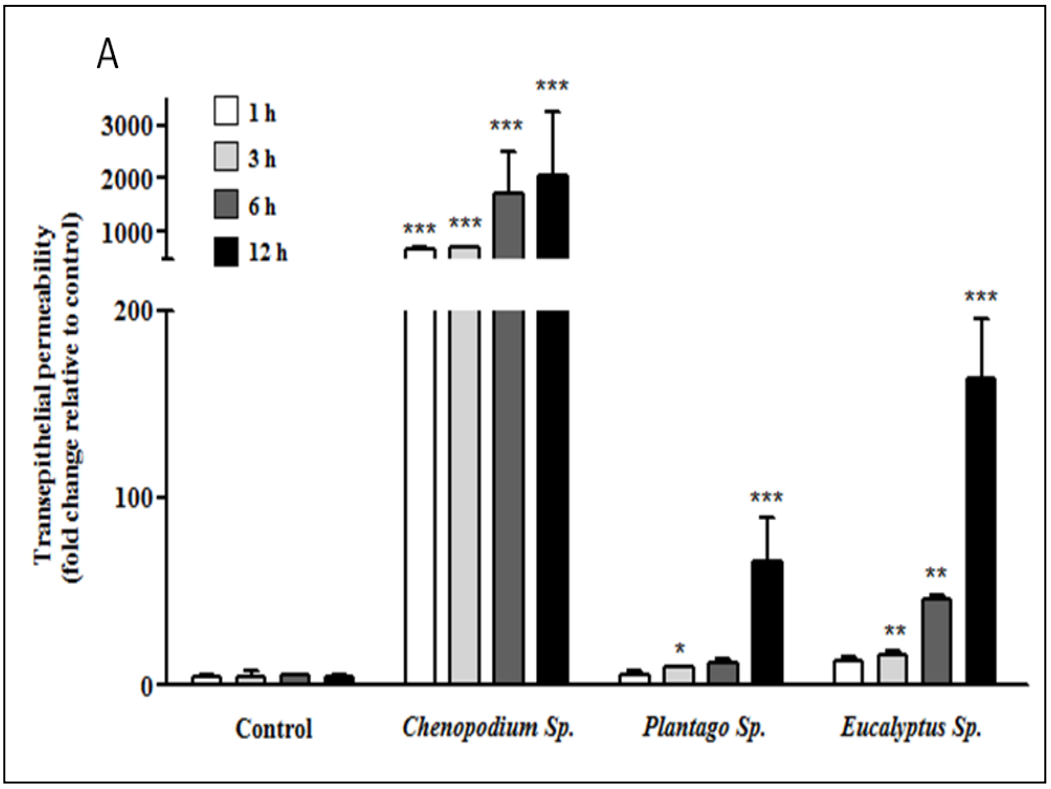
The initiation process of allergic sensitization implies the transposition of the epithelial barrier by allergens. The precise mechanism of how the allergens overcome the epithelial barrier is yet to be fully understood, and for that reason has been the target of many research studies. From existent literature, it has been suggested that proteases from pollen extracts cleave intercellular protein junctions, increasing transepithelial permeability and promoting contact with APC cells in subepithelial layers which leads to the development of allergic responses (Cortes *et al.*, 2006; Runswick *et al.*, 2007; Hassim *et al.*, 1998).

Studies on the action of pollen extracts on cell integrity will be possible using an adequate cell line, Calu-3. Calu-3 cells are resultant from adenocarcinoma of the Human bronchial submucosal glands. The Calu-3 cell line is one of the few respiratory cell lines that form functional tight junctions, *in vitro,* as well as, high transepithelial resistance,

allowing a functional model for airway epithelial barrier studies. This cell line has become the principal cell line for transepithelial permeability studies because it produces functional and differentiated epithelial Human cells. The culture conditions employed is obviously critical for the characteristics of the resultant monolayer. For this reason, Calu-3 cells were grown by using an air-liquid interface culture (AIC), which mimics and resembles the *in vivo* environment and polarized epithelium cells (Grainger *et al.*, 2006).

3.5.1- Transepithelial permeability

The transepithelial permeability is a parameter that allows an evaluation of the epithelial integrity and cell-cell contacts. For this reason, the flux of RITC-dextran 70S through the Calu-3 monolayer formed in inserts of transwells cultured in AIC conditions, was measured after 5 different conditions. These conditions were: 1 hour stimuli, 3 hour stimuli, 6 hour stimuli, 12 hour stimuli and 12 hour treated with AEBSF 1mM inhibitor stimuli, with pollen extracts from *Chenopodium sp.*, *Eucalyptus sp.* and *Plantago sp.* The results were converted into two separate graphs, the first representing a time-course and the second focusing on the inhibitor effect verified on the 12 hours stimuli (Fig 20 A&B).



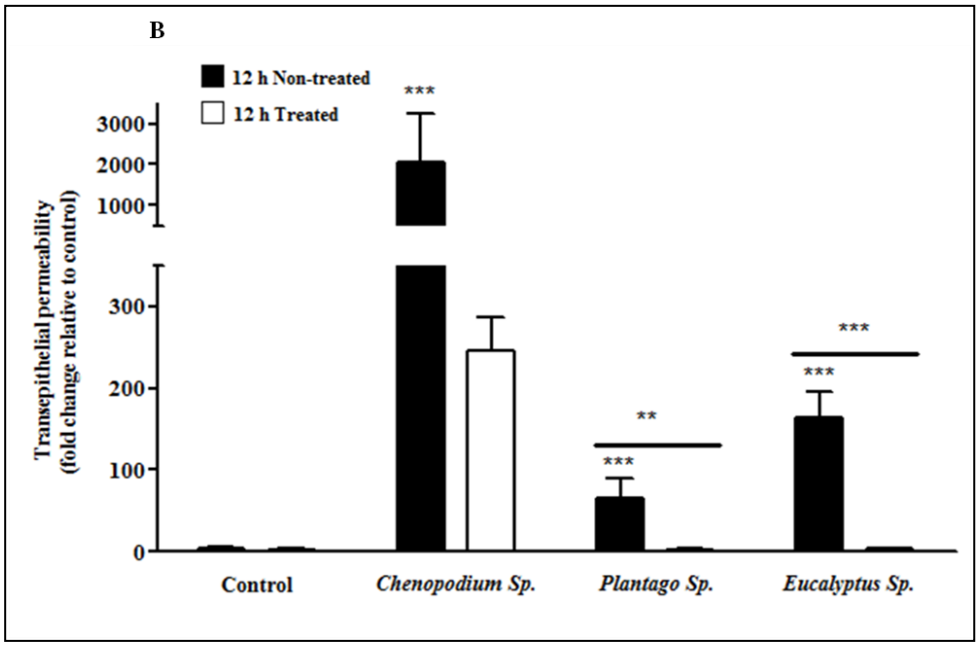


Figure 20- Effect of pollen proteases from *Chenopodium sp.*, *Eucalyptus sp.* and *Plantago sp.* on the paracellular permeability of the epithelial cell monolayer of Calu-3. A) Calu-3 cells for this matter were exposed to different time-points of pollen extracts stimulus. B) Calu-3 cells were also incubated with pollen diffusates treated with 1mM AEBSF and incubated for 12 hours. The results were analyzed statistically using Bonferroni´s post-test: ***P < 0.001; **P < 0,01; *P < 0.05 (n=3).

All pollen extracts induced an increase in transepithelial permeability in a time-dependent manner. When measured the transepithelial flux of RITC-dextran the effect of *Chenopodium sp.* on Calu-3 cells is perceptively more when compared to *Plantago sp.* and *Eucalyptus sp*, being the increment statistically significant on all four time points. Even so, the effect of *Plantago sp.* and *Eucalyptus sp.* are well evident and statistically significant in some time points studied (Fig. 20 A)).

In order to determine if this effect is related to proteases, the pollen extracts where pre incubated with AEBSF 1mM and Calu-3 cells for a 12 hour period stimuli, period corresponding to the largest induced transepithelial permeability and highly statistically significant in all three pollen extracts. The inhibitor AEBSF, is a serine protease inhibitor, and was already proven by the enzymatic AMC assays, shown above, able to inhibit all three pollen diffusates. For *Plantago sp.* and *Eucalyptus sp.* we observe a statistically significant reversal of the pollen extracts by the AEBSF inhibitor. For *Chenopodium sp.* diffusates AEBSF inhibitor reversed only partially the effect.

Obviously, as shown in the enzymatic AMC assays shown above, the AEBSF inhibitor does not block completely the protease activity of the pollen diffusates which may explain the protease activity that maintained functional (Fig. 20 B)).

Also, importantly, is the fact that higher variations of epithelial permeability are not linked directly to total quantity of protein released upon hydration, but dependent on the amount or specificity of proteases released. This can be verified by comparing the ability of *Chenopodium sp.* to induce higher variations of epithelial permeability compared to *Eucalyptus sp.* despite the fact that *Eucalyptus sp.* releases the largest amount of protein content. Oppositely, *Plantago sp.* is the pollen that releases the lowest amount of protein content when compared to *Eucalyptus sp.* and *Chenopodium sp.*, and also induces the lowest variation of epithelial permeability.

Also from these results, the experiments that were carried out from this point forward were always using the highest protein concentration obtained for each pollen extract and 6 hours incubation periods for stimulus. This incubation period of 6 hours was chosen, because besides the fact it being the lowest period with significant results, it was necessary to have a compromise between quantifiable degradation effects and cell detachment. This means, periods lower than 6 hours, pollen extracts difficulty will induce quantifiable degradation for the exception of *Chenopodium sp.,* but on the other hand incubation periods higher than 6 hours will induce cell detachment making it impossible to quantify degradation effects of pollen extracts.

3.5.2- Degradation of Protein Intercellular Junctions

It became important to evaluate cell viability of confluent Calu-3 cells after the exposure to pollen extracts. For this, the pollen extracts and detached cells were jointly removed after the 6 hour incubation period. These cells were washed several times and re-seeded on multiwell plates, incubated with culture medium favorable for proliferation.

Microscopic observation of these Calu-3 subcultures exposed previously to different pollen extracts allowed confirmation of induced morphological changes. The detached cells appeared to lose their ability to proliferate never reaching a confluent status. Therefore, the action of pollen extracts on Calu-3 cells appears to cause loss of cell viability.

As reported above, it was shown that exposure of pollen extracts induced an increase in transepithelial permeability, as well as, cell detachment on Calu-3 cells. In an attempt to understand the precise mechanism through witch this occurs the degradative action on protein junctions was evaluated. Accordingly to what has been suggested in literature, pollen proteases have the ability to cause degradation of these protein intercellular junctions via directly or indirectly, resulting in a loss of lung epithelium integrity (Vinhas *et al*., 2011).

In this study, Calu-3 cells were resorted to again because of the fact of being one of the few respiratory cell lines that form functional protein intercellular junctions, *in vitro*, and also have high transepithelial resistance. Cell extracts of Calu-3 were prepared after exposure to the pollen extracts during a 6 hour incubation period. With these samples, the degradation status of E-cadherin, Claudin-1, ZO-1 and Occludin intercellular junction protein were studied. Also, cells were treated with 1mM of AEBSF inhibitor, in order to evaluate the decrease or total inhibition of degradation of protein intercellular junctions by proteases (Fig. 21, 22 & 23).

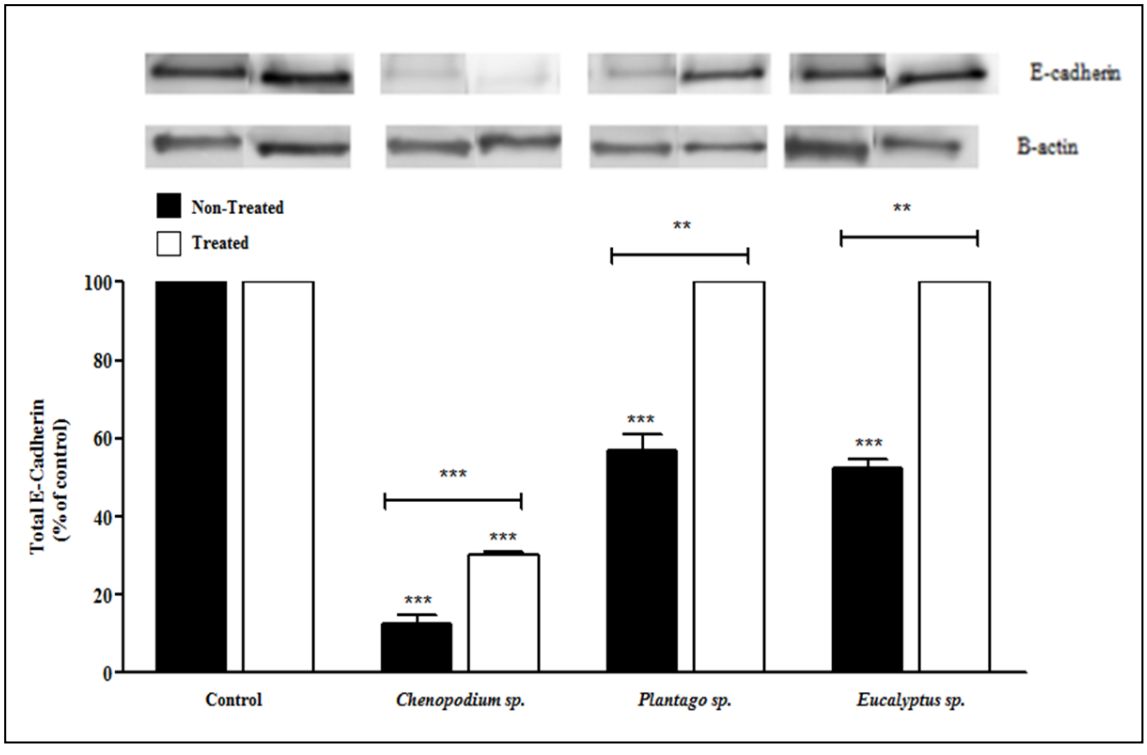
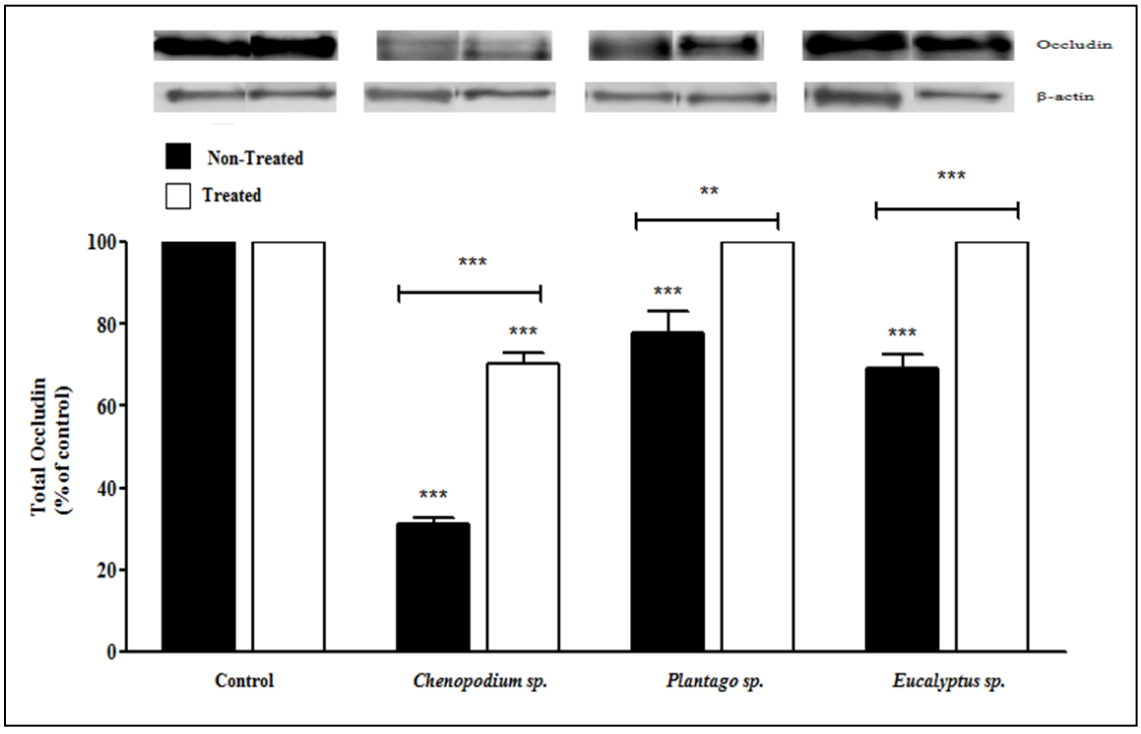


Figure 21- Effect of pollen proteases from *Chenopodium sp.* (0,7 mg), *Eucalyptus sp* (1,6 mg) and *Plantago sp.* (0,4 mg) on the degradation of an adherent junction, E-cadherin formed between epithelial cells of the Calu-3 monolayer analyzed by Western blot. Black bars, are

referent to the exposure of Calu-3 cells to different pollen extracts stimulus during 6 hours of incubation period. White bars, refer to a condition where the pollen extracts were pre-treated with 1mM of AEBSF, in order to evaluate the inhibition of the degradation of E-cadherin. The results were analyzed statistically using Bonferroni´s post-test: ***P < 0.001; **P < 0, 01; *P < 0.05 (n=3).

First, the effect of pollen extracts was analyzed on an adherent junction, E-cadherin (120kDa). All pollen extracts caused extensive and statistically significant degradation to this intercellular junction, responsible for decreases inferior to 50% of the quantity of this protein. *Chenopodium sp.* has a more drastic effect when compared to *Plantago sp.* and *Eucalyptus sp.* Also very important, was the statistically significant inhibition of the degradation ability of the proteases when treated with 1mM AEBSF. This inhibition was total for *Plantago sp.* and *Eucalyptus sp.* which confirm the large amount of serine proteases. Even though *Chenopodium sp.* was inhibited, the high ability of degradation potential reconfirms the presence of other proteases besides serine proteases, capable of inducing degradation actions on intercellular proteins.



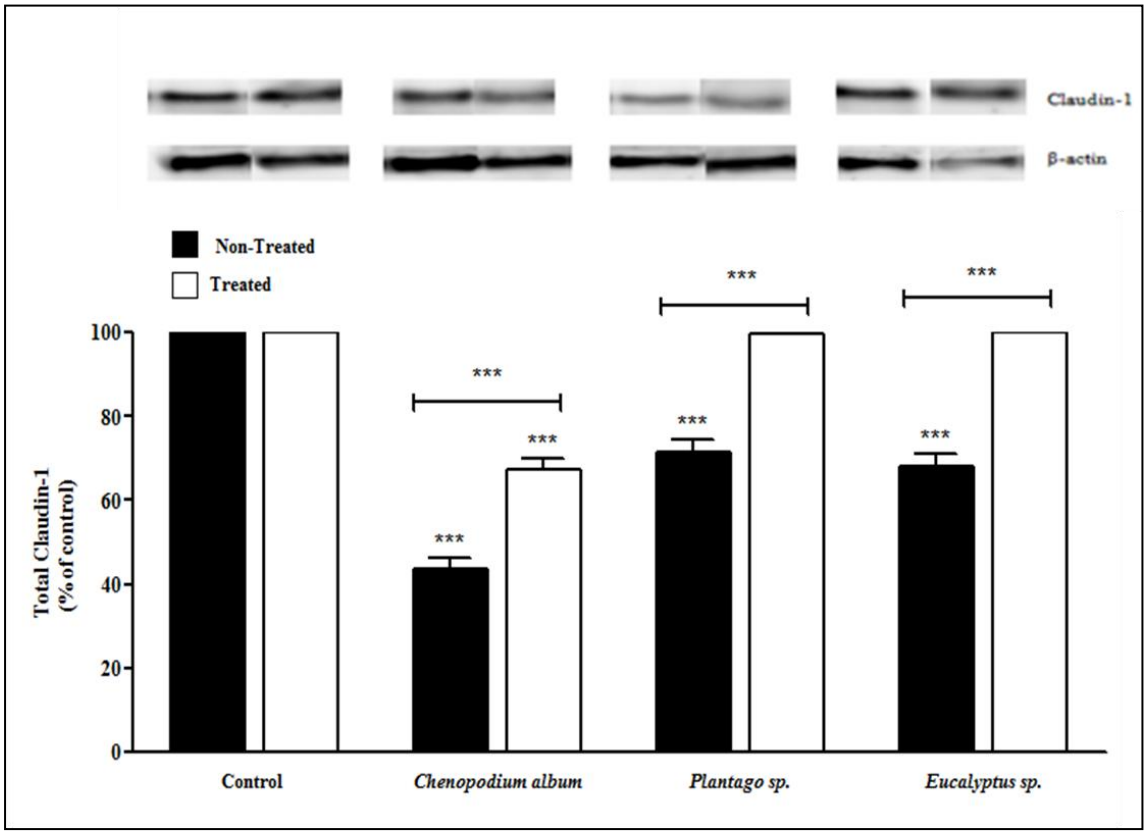


Figure 22- Effect of pollen proteases from *Chenopodium sp.* (0,7 mg), *Eucalyptus sp.*(1,6 mg) and *Plantago sp.*(0,4 mg) on the degradation of the tight junctions, Occludin and Claudin-1 formed between epithelial cells of the Calu-3 monolayer analyzed by Western blot. Black bars, are referent to the exposure of Calu-3 cells to different pollen extracts stimulus during 6 hours of incubation period. White bars, refer to a condition where the pollen extracts were pre-treated with 1mM of AEBSF, in order to evaluate the inhibition of the degradation of Occludin and Claundin-1. The results were analyzed statistically using Bonferroni´s post-test: ***P < 0.001; **P < 0, 01; *P < 0.05 (n=3).

After an adherent junction, the effect of pollen extracts was analyzed on tight junctions, Occludin (59kDa) and Claudin-1 (23kDa). All pollen extracts caused statistically significant degradation to these intercellular junctions, responsible for decreases of the total quantity of these proteins. *Chenopodium sp.´*s effect was again the most drastic when compared to *Plantago sp.* and *Eucalyptus sp.* Also very important, was again the statistically significant inhibition of the degradation ability of the proteases when treated with 1mM AEBSF. This inhibition was again total for *Plantago sp.* and *Eucalyptus sp.* As previously described *Chenopodium sp.* was also inhibited, but again not totally inhibited.

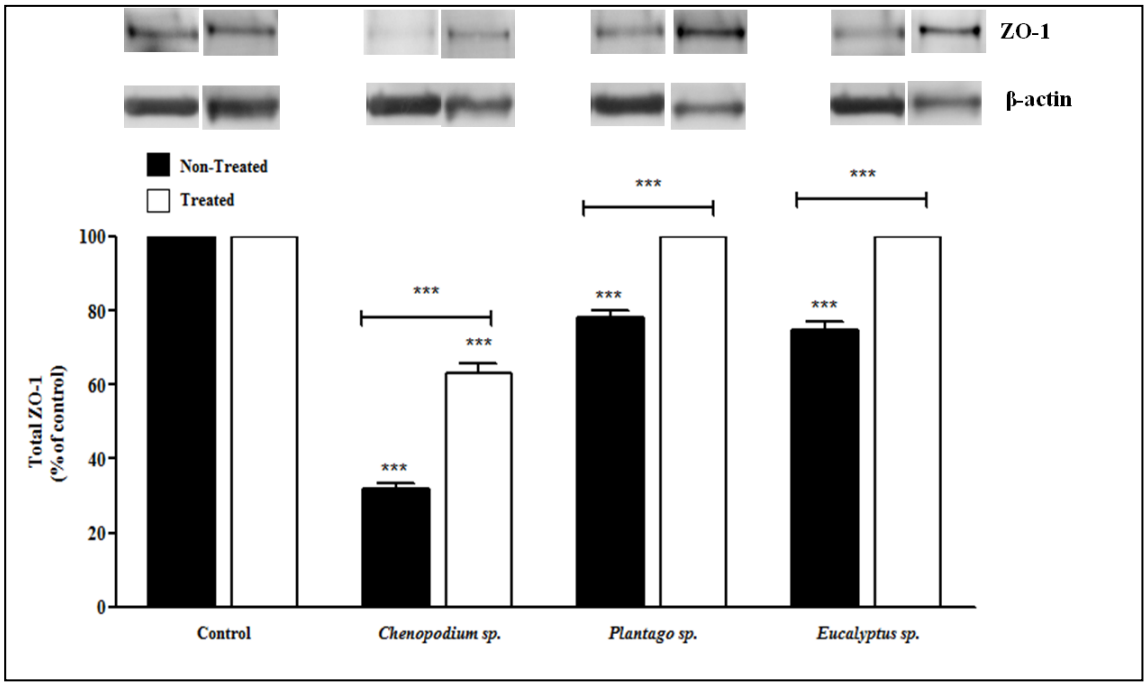


Figure 23- Effect of pollen proteases from *Chenopodium sp.* (0,7 mg), *Eucalyptus sp.* (1,6 mg) and *Plantago sp.* (0,4 mg) on the degradation of a citosolic protein complex of tight junctions, ZO-1, formed between epithelial cells of the Calu-3 monolayer analyzed by Western blot. Black bars, are referent to the exposure of Calu-3 cells to different pollen extracts stimulus during 6 hours of incubation period. White bars, refer to a condition where the pollen extracts were pre-treated with 1mM of AEBSF, in order to evaluate the inhibition of the degradation of ZO-1. The results were analyzed statistically using Bonferroni´s post-test: ***P < 0.001; **P < 0, 01; *P < 0.05 (n=3).

Finally the effects of pollen extracts were analyzed on a citosolic complex protein of the tight junctions, ZO-1. Again, all pollen extracts caused statistically significant degradation to these intercellular junctions, responsible for decreases of the total quantity of these proteins. *Chenopodium sp.´*s effect was again the most drastic when compared to *Plantago sp.* and *Eucalyptus sp.* Again, the same statistically significant inhibition profile of the pollen extracts was identical to the previous results already described.

In this study, the specific antibody labeling of E-cadherin, Occludin and ZO-1, allowed the visualization of other bands with lower molecular mass, which corresponded to products of degradation of these proteins. This was not visible when labeling specifically for Claudin-1.

These results obviously indicate a direct digestive action on the protein intercellular complexes, whether tight or adherent junctions, after exposure to pollen extracts, possible through their extracellular domains. This degradation is responsible for the increase in transepithelial permeability and posteriorly to cell detachment already verified.

The action of these pollen extracts upon the protein intercellular complexes was then further studied by imunoflourescence. This allowed, besides the re-confirmation of what was previously reported, a visual understanding of the degradation corresponding to interruptions in the continuous rings at the apices of epithelial cells caused by the assembly of these macromolecular proteins (Fig. 24).

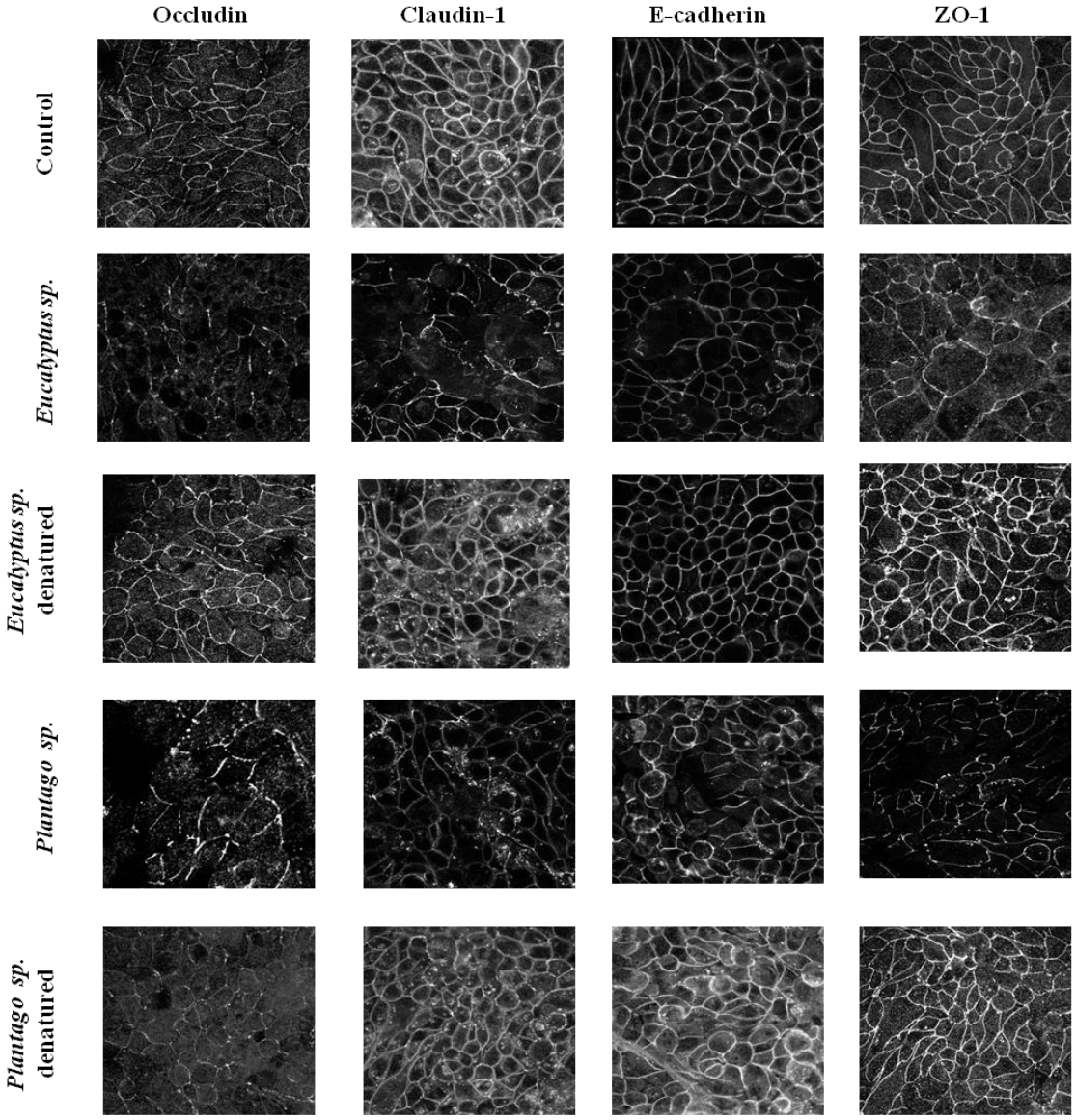


Figure 24- Effect of incubation of Calu-3 with pollen proteases from *Eucalyptus sp.* (0,4 mg) and *Plantago sp*. (0,1 mg) on the integrity of adherent junctions, E-cadherin; tight junctions, Occludin and Claudin-1 and a citosolic protein complex of tight junctions, ZO-1. Images were obtained using a confocal microscope, z-stacking diverse planes. Images were posteriorly treated with the selection of the best z plane obtained. Images are 150μm in dimension.

Observing figure 24, treatment of Calu-3 cells with the different pollen extracts alters the distribution pattern of the proteins that form the macromolecular assembly of intercellular protein complexes between epithelial cells, obviously as a result of a direct digestive action or a re-arrangement. Pollen extracts were denatured by heat, 95ºC during 30 minutes, in order to denature all classes of proteases and not only serine as realized in the Western blot experiment. This is due to the fact that for image quality all proteases have to be denatured, which in terms of Western blot does not have to occur, being a partial inhibition sufficient for the intended. Also, in denatured pollen extracts the degradation was reversed.

In particular for, *Chenopodium sp.*, images were impossible to obtain since this pollen extract induced high percentage of cell detachment. We intend to proceed in the future to dilutions in buffer Tris-HCl 50mM pH 7,4, until visual images are possible to be analyzed. Even so, this ability to induce high percentage of cell detachment is supported and in accordance to what was shown previously, with the high percentage of degradation of these intercellular protein complexes obtained by Western blot analysis.

Eucalyptus sp. had a very visible and similar digestive action on all proteins analyzed, which again is concurrent with results obtained in the Western blot experiment. The denatured pollen extract of *Eucalyptus sp.* also allowed a return of fluorescence intensity similar to control conditions. In this case, even in denatured conditions this pollen extract was capable of inducing a minimal digestive effect, barely visible by the imunoflourescence images. This effect was expected by this denatured pollen extract, since even after 30 minutes at 95ºC, there were still proteases with enzymatic activity. This residual activity was visible through enzymatic assays using the AMC coupled substrate, Phe-AMC (data not shown). The residual activity and the resistance of proteases to this denaturing process was suggested to be as a result of a

shielding protection made by the large amount of protein and pigments existent in this pollen extract.

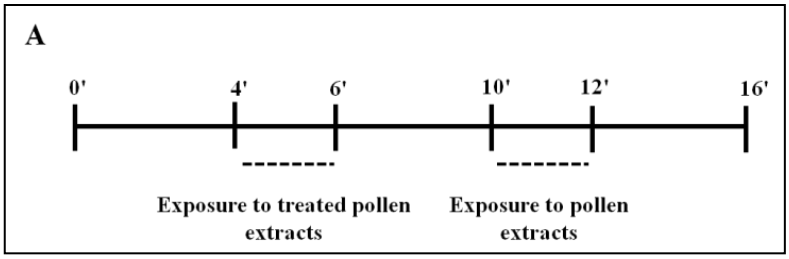
Finally, *Plantago sp.* was shown to also have the ability of causing interruptions in the continuous rings as a result of degradation of the proteins of adherent and tight junctions between epithelial cells. As shown by Western blot, the pollen extract has the ability to digest all proteins analyzed, but is the least aggressive. The denatured pollen extracts of *Plantago sp.* lost the ability to induce degradation, and therefore is observed a reversal, with more fluorescence intensity and well visible continuous rings.

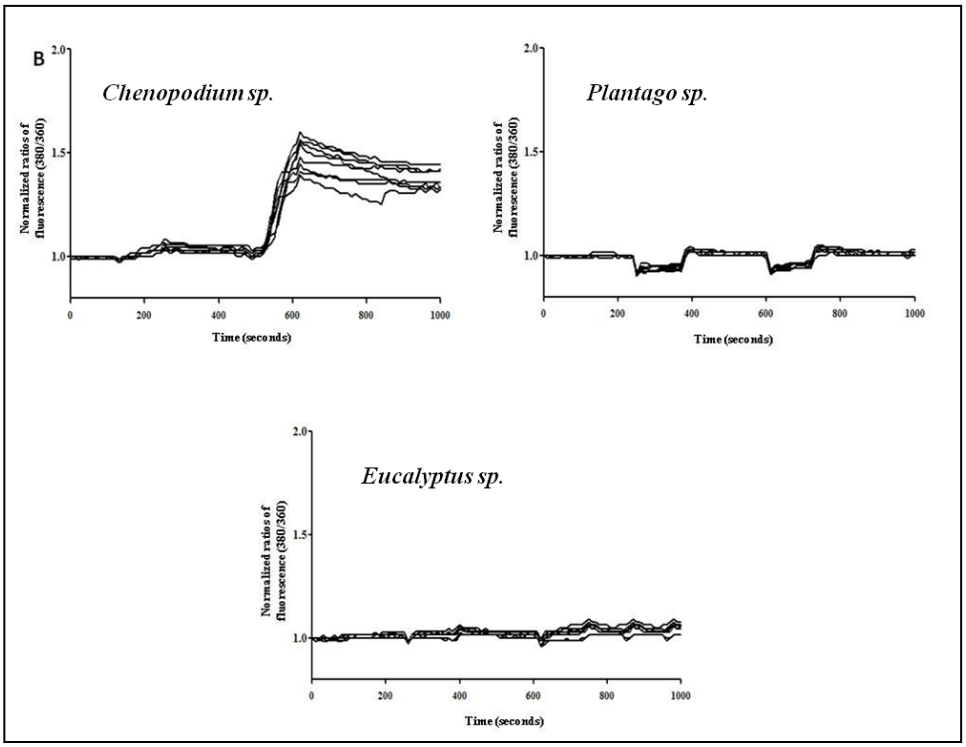
3.6- Inflammatory Component Induced by Pollen Extracts of *Chenopodium sp.*, *Plantago sp.* and *Eucalyptus sp.*

As previously mention, the research conducted intends to understand the mechanism through which proteases present in pollen extracts cause breakdown of the epithelial barrier via directly and indirectly, but never as separated or isolated pathways. PAR-2 is widely expressed on the apical surfaces of the epithelial cells of Calu-3, resorting again to this cell line for this analysis. For this reason, the next set of experiments focused on the activation of this receptor and the resulting events that followed prior activation.

3.6.1- PAR-2 Activation

Proteases seem to serve as ligands for PAR-2 receptors, proteolytically activated G-coupled receptors, inducing rapid increases of airway intracellular [Ca^{2+}] levels, which can be monitored by Single-Cell Calcium Imaging microscopic technique. For this, the florescence of Fura-2, linked to calcium, was monitored during programmed periods of time, when exposed to pollen extracts and denatured pollen extracts (30 minutes at 95ªC) of *Chenopodium sp.*, *Plantago sp.* and *Eucalyptus sp.* (Fig. 25).





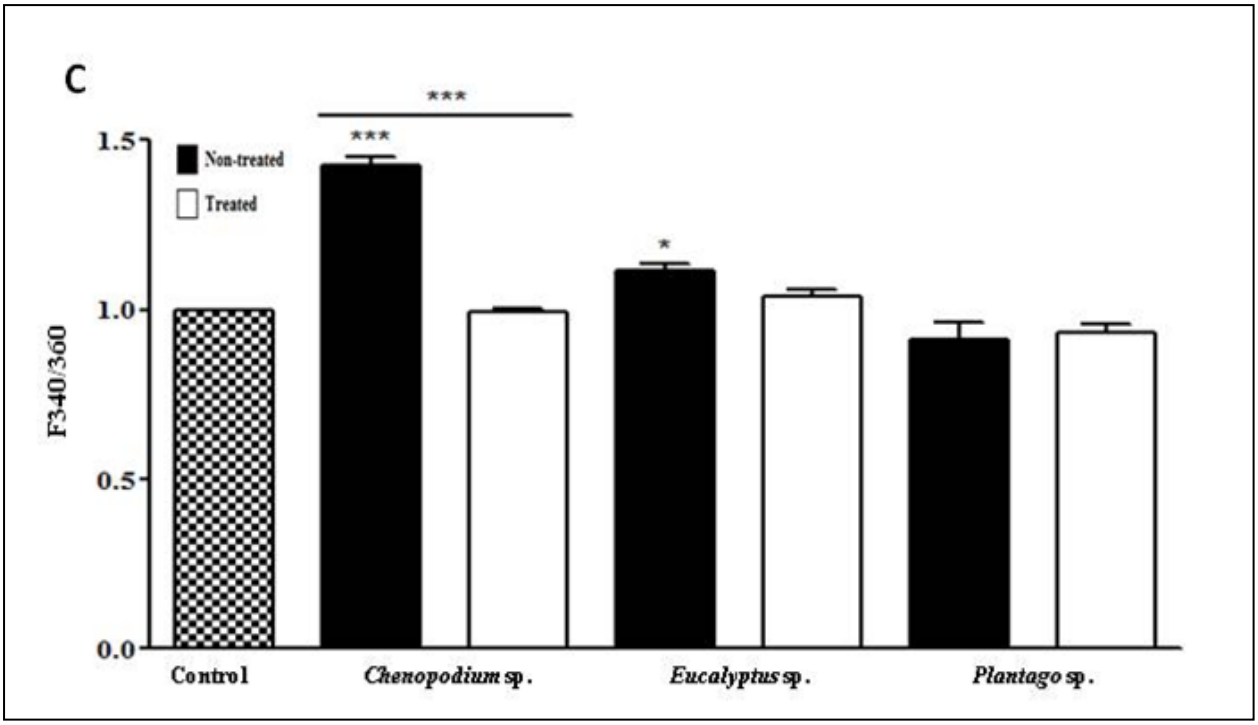


Figure 25- Effect of incubation of Calu-3 with pollen proteases from *Chenopodium sp.* (0,1 mg), *Eucalyptus sp.*(0,24 mg) and *Plantago sp*. (0,06 mg) on PAR-2 activation. A) Schematic representation of the time periods used for this experiment protocol. Dashed lines indicate Calu-3 exposure periods to denatured pollen extracts and after pollen extracts untreated. B) Profiles of representative cells of each experimental condition. Graphs are represented in terms of normalized ratio of fluorescence (340/380). C) Quantification of the variations of

intracellular [Ca^{2+}] mobilization after exposure to the stimuli. Black bars correspond to non-treated pollen extracts and white bars to denatured pollen extracts. These results were analyzed statistically using Bonferroni's post-test: ***P < 0.001; **P < 0,01; *P < 0.05 (n=3).

As already mention this experiment has specific time intervals, which were maintained in every following assay. First, cells were left at basal conditions for 4 minutes, after every stimuli was induced for 2 minutes and preceded by a wash with normal Krebs solution and a final period of 4 minutes to verify if cells were able to return to basal conditions (Fig. 25 A)).

Since PAR-2 is selectively activated by Trypsin and Trypsin-like enzymes, we first monitored the effect of Trypsin 0,25%. The effect observed was statistically significant, and for this reason was considered our positive control (data not shown). Also, this test was important to confirm the veracity of the technique showing that in fact PAR-2 exists on the surface of Calu-3 and that all cells respond in the same manner. Another control condition was tested, using 50mM Tris-HCl pH 7.4, buffer in which pollen is hydrated, and no significant alteration was observed in comparison to basal conditions. Discarding possible interferences of this buffer on activation of PAR-2 receptors (data not shown).

Observing the diverse images of obtained results, the first immediate conclusion is the fact that proteases existent in the pollen extracts are able to cause activation of PAR-2. The effect of *Chenopodium sp.* and *Eucalyptus sp.* are statistically significant, in terms of $[Ca^{2+}]_I$ variations, which is a result of activation of signal cascades due to PAR-2 receptor activation. *Plantago sp.* seemed incapable of activating PAR-2, which can be a consequence of low amount of proteases present in the pollen extract (Fig. 25 B) and C)).

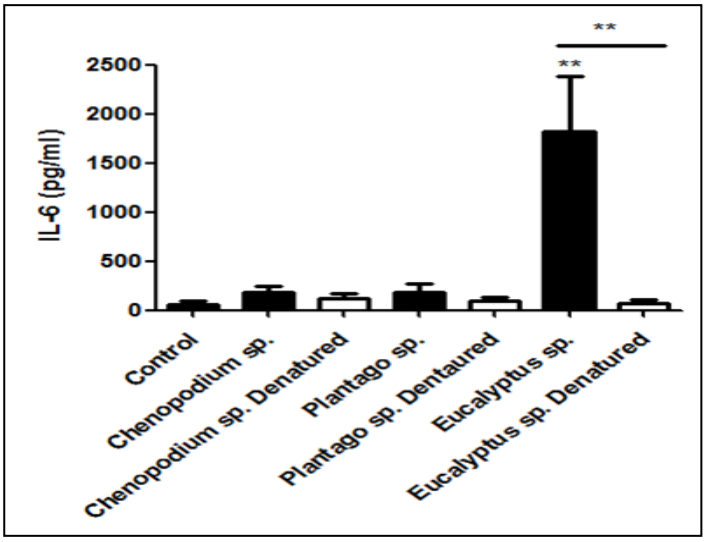
In order to backup the conclusions above, denatured pollen extracts were tested. The effect of these stimulus were similar to basal conditions, not inducing variation in $[Ca^{2+}]_I$ levels. This again indicates that the activation of PAR-2 is in fact a result of proteases present in the pollen extracts. This reversal situation was statistically significant for *Chenopodium sp.* and *Eucalyptus sp.* pollen extracts (Fig. 25 B) and C))

Important to highlight, *Eucalyptus sp.* denatured pollen extract caused slight increase visible in the curve, which is due to the fact that even after 30 minutes at 95ºC,

there were still proteases active, confirmed by the presence of a residual enzymatic activity present in the enzymatic assay. Also peculiar, was the effect of *Chenopodium sp.*, which was so significant, that cells were never able to return to basal conditions after exposure to this pollen extract (Fig. 25 B) and C)).

3.6.2- Induced Cytokine Release

Functional PAR-2 receptors, participate in lung inflammation and when activated induce the release of cytokines, such as, IL-6 and IL-8. For this reason, we resorted to flow cytometry to quantify these cytokines in samples collected from inserts of transwells plates, where Calu-3 cells were grown until a confluent status and then exposed to pollen extracts during a 6 hour incubation period (Fig. 26).



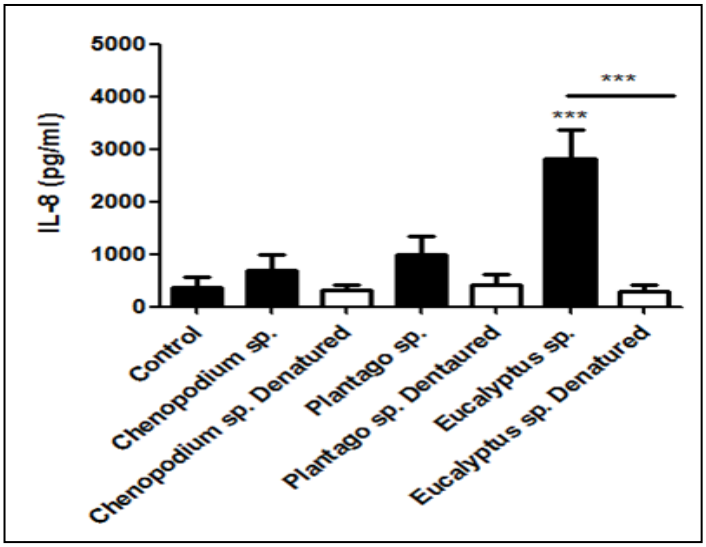


Figure 26- Effect of pollen proteases from *Chenopodium sp.* (0,01 mg), *Eucalyptus sp.*(0,4 mg) and *Plantago sp.*(0,1 mg) on the release of IL-6 and IL-8. Black bars correspond to the stimuli of each pollen extracts (except the control bar), while the white bars are referent to denatured pollen extracts (30 minutes at 95ºC). The results were analyzed statistically using Bonferroni´s post-test: ***P < 0.001; **P < 0, 01; *P < 0.05 (n=3).

All pollen extracts induced the release of IL-6 and IL-8. As expected, *Eucalyptus sp.* induced the release of IL-6 and IL-8 in a statistically significant manner, as for *Plantago sp.*, the amount of IL-6 and IL-8 after exposure to this pollen extract was not statistically significant. Interesting is the fact that *Chenopodium sp.*, even diluted 1:20 in buffer Tris-HCl pH 7,4 was able to induce a visible level of cytokine production.

In all pollen extracts, when denatured 30 minutes at 95ºC, the effect was reversed and the production of cytokines by the Calu-3 epithelial cells did not occur. This effect was statistically significant for *Eucalyptus sp.* In the case of denatured pollen extracts the values obtained of released cytokines are similar to the values found in control conditions (Calu-3 epithelial cells not exposed to any stimuli). Again, this establishes the connection to proteases existent in pollen extracts.

3.7- Combined Effect of Pollen Extracts

As already defined in the aims of this research work, we intend to investigate pollen extracts as isolated elements but also combined together to mimic natural conditions existent in the environment. The respiratory system is exposed to a multitude of airborne particles, namely pollen with overlapping pollination seasons. The underlining objective is to observe alterations in the activity of proteases when combined. For this purpose, we chose two interesting combinations: *Chenopodium sp.* with *Eucalyptus sp.* and *Chenopodium sp.* with *Plantago sp*. The first combination was intended to join moderate allergenic pollen with low allergenic pollen. Interestingly also in this combination is the fact that *Eucalyptus sp.* despite being low allergenic has a much higher enzymatic activity confirmed by the assay showed in comparison to *Chenopodium sp.* This is also a very promising characteristic of the mix, where there is one pollen specie with higher enzymatic activity than the other. The second combination is a mix of two moderate allergenic pollens, with identical enzymatic activities. Both of the combinations studied have *Chenopodium sp.* as common pollen,

this is purposely, since this pollen is by far the most aggressive to cells, as proven in results obtained in Western Blot analysis and transepithelial permeability (3.5).

3.7.1- Preferential Substrates of Combined Pollen Extracts of *Chenopodium sp.* with *Eucalyptus sp.* and *Chenopodium sp. with Plantago sp.*

The proteolytic activity of the combined pollen extracts was determined using the same conditions described above and the same peptide substrates coupled to AMC. In this case, we added 100µl of each pollen extract to the well, in order to obtain a final 200µl volume. The results were also converted into bar graphs, were the specific activity of each pollen extract can be observed towards each different AMC coupled substrate (Fig. 27).

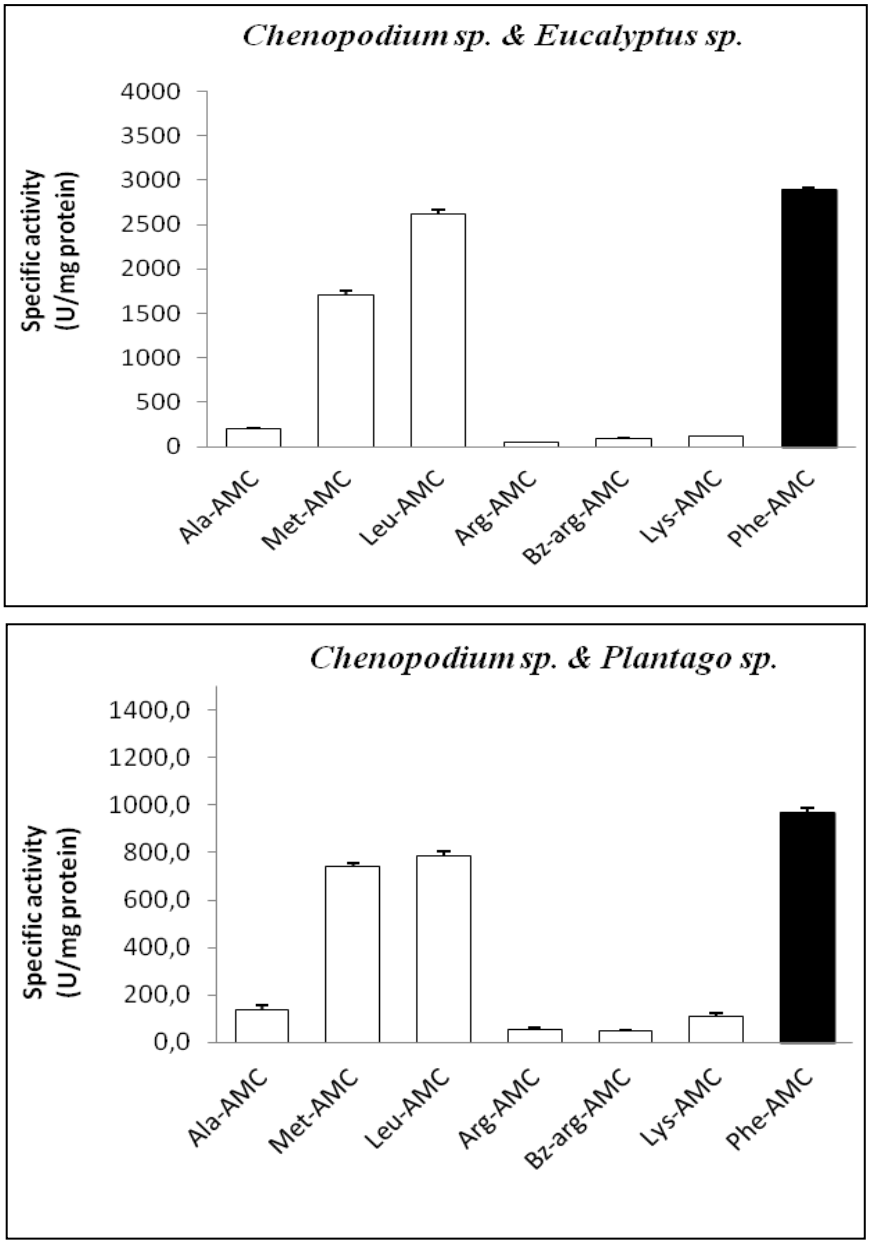
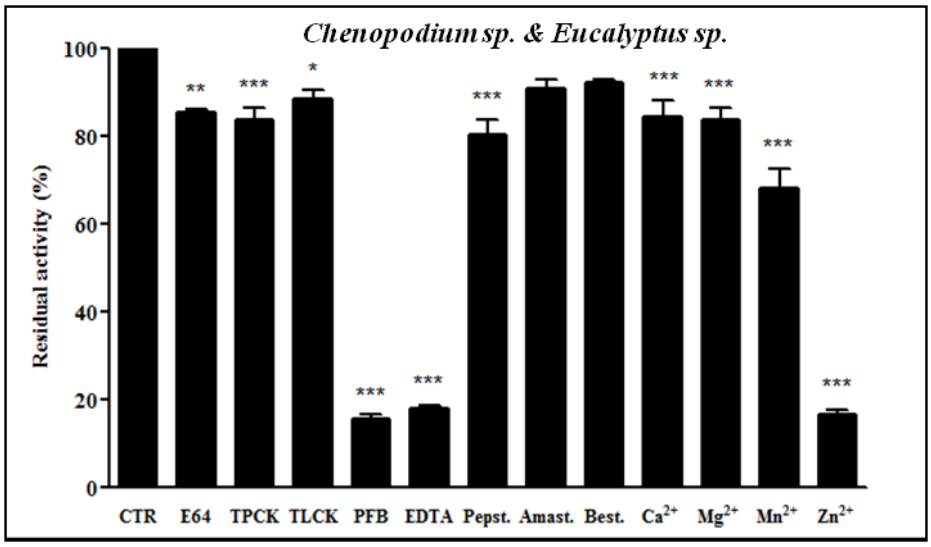


Figure 27- Substrate specificity of pollen proteolytic activity from *Chenopodium sp.* with *Eucalyptus sp.* and *Chenopodium sp.* with *Plantago sp*. The black bars point out the preferred substrate for each combined pollen diffusates, which will also be the substrate use for the inhibition assays. One unit of activity = pmol AMC released/min

Observing Figure 27, the combined pollen diffusates continue to have proteolytic activity against a variety of AMC coupled substrates, namely Met-AMC, Leu-AMC and Phe-AMC. Also, pollen extracts of *Chenopodium sp. with Eucalyptus sp.* and *Chenopodium sp.* with *Plantago sp.* continue to have Phe-AMC as their preferred substrate. This substrate was for that reason used in the evaluation of pollen protease class specificity. From this enzymatic assay it was well perceptive that the overall profile did not suffer alterations when pollen extracts were combined.

3.7.2- Inhibition Profile of Combined Pollen Extracts

The identification of specific protease classes present in combined pollen extracts was determined using the same conditions and same specific inhibitors. The enzymatic profile was obtained after 20 minutes of incubation of the inhibitors with the combined pollen extracts and then with the addition of the preferred substrate (Phe-AMC). The results were converted into bar graphs in terms of percentage of residual activity when compared to control conditions without inhibitor (Fig. 28).



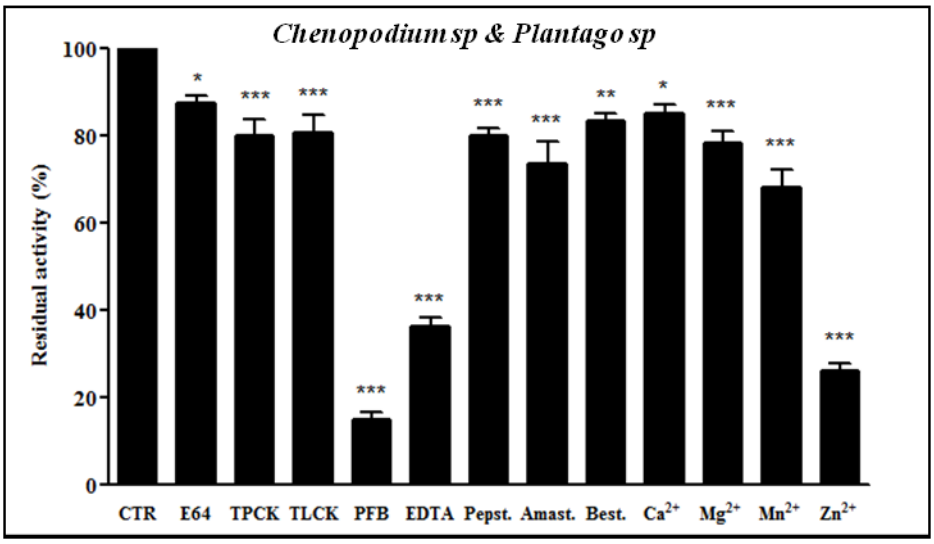


Figure 28- Effect of class-specific inhibitors on the proteolytic activity of combined pollen diffusates. Results are represented in terms of residual activity percentage when compared to control condition without inhibition. The results were analyzed statistically using Dunnett´s post-test: ***P < 0.001; **P < 0,01; *P < 0.05 (n=3).

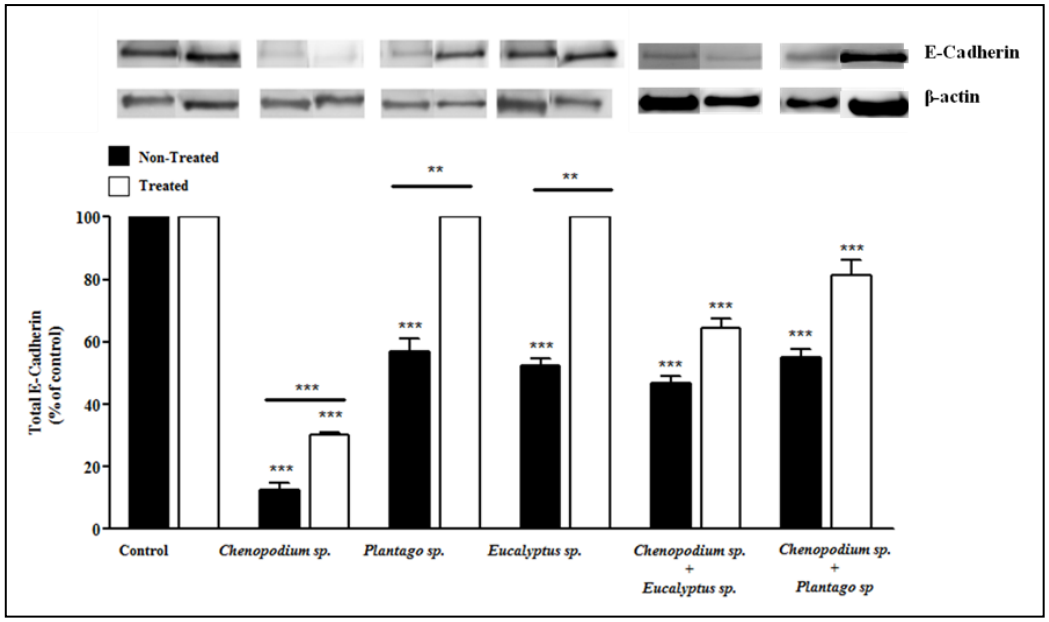
In terms of the combination *Chenopodium sp.* with *Eucalyptus sp.* it is still well perceptive and statistically significant the presence of serine, metalloproteinases and aminopeptidases N consistent with the inhibition of AEBSF, EDTA and Zn^{2+}, respectively. The only observable and significant difference of the combined profile compared to the isolated profile of the pollen extracts is the disappearance of cysteine activity confirmed by the poor inhibition of E-64. A possible explanation is the degradative effect of proteases on proteases resultant from the combination of pollen extracts.

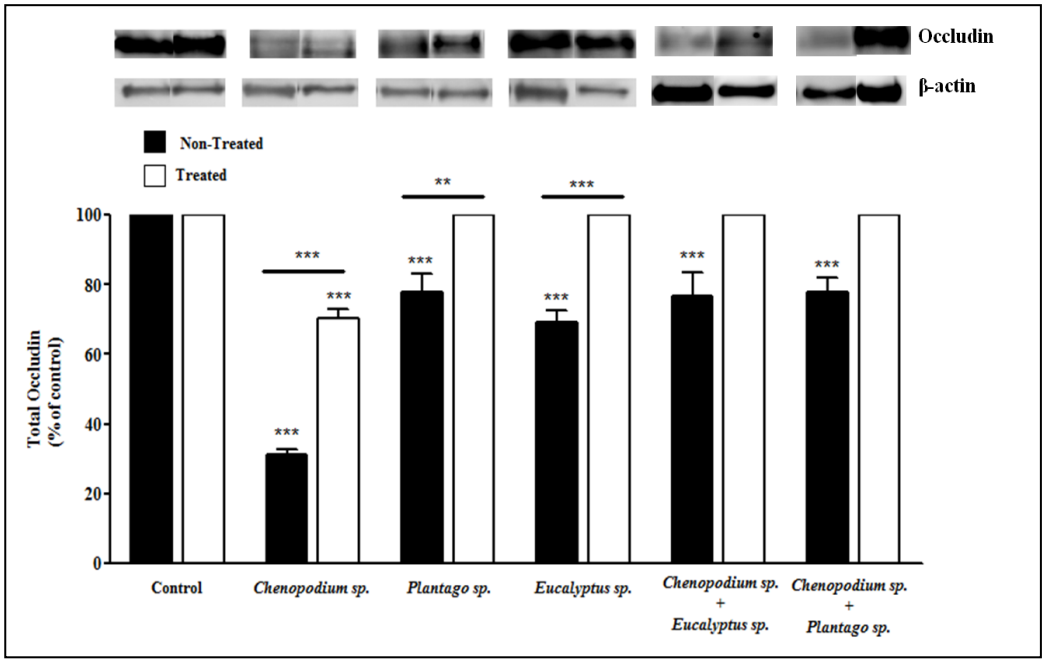
The profile of the combination between *Chenopodium sp.* with *Plantago sp.* has some visual differences. The serine, metalloproteinases and aminopeptidases N are still very perceptive and statistically significant, observable through the inhibition of AEBSF and TPCK, EDTA and Zn^{2+}, respectively. The two major differences, when compared to the isolated profiles, are the decrease of cysteine activity, with a lower inhibition of E-64. The second is a statistically significant inhibition of Amastatin, Bestatin and Pepstatin specific for aminopeptidases and aspartic proteases, respectively.

3.7.3- Degradation of Protein Intercellular Junctions by Combined Pollen Extracts

The same combined pollen extracts were analyzed to evaluate their action on the Calu-3 cell line.

In the same manner, cell extracts of Calu-3 were prepared after exposure to the combined pollen extracts during a 6 hour incubation period. With these samples, the degradation status of E-cadherin, Claudin-1, ZO-1 and Occludin intercellular junction proteins were studied. Also, cells were treated with 1mM of AEBSF inhibitor, in order to evaluate the decrease or total inhibition of degradation of the protein intercellular junctions by proteases (Fig. 29).





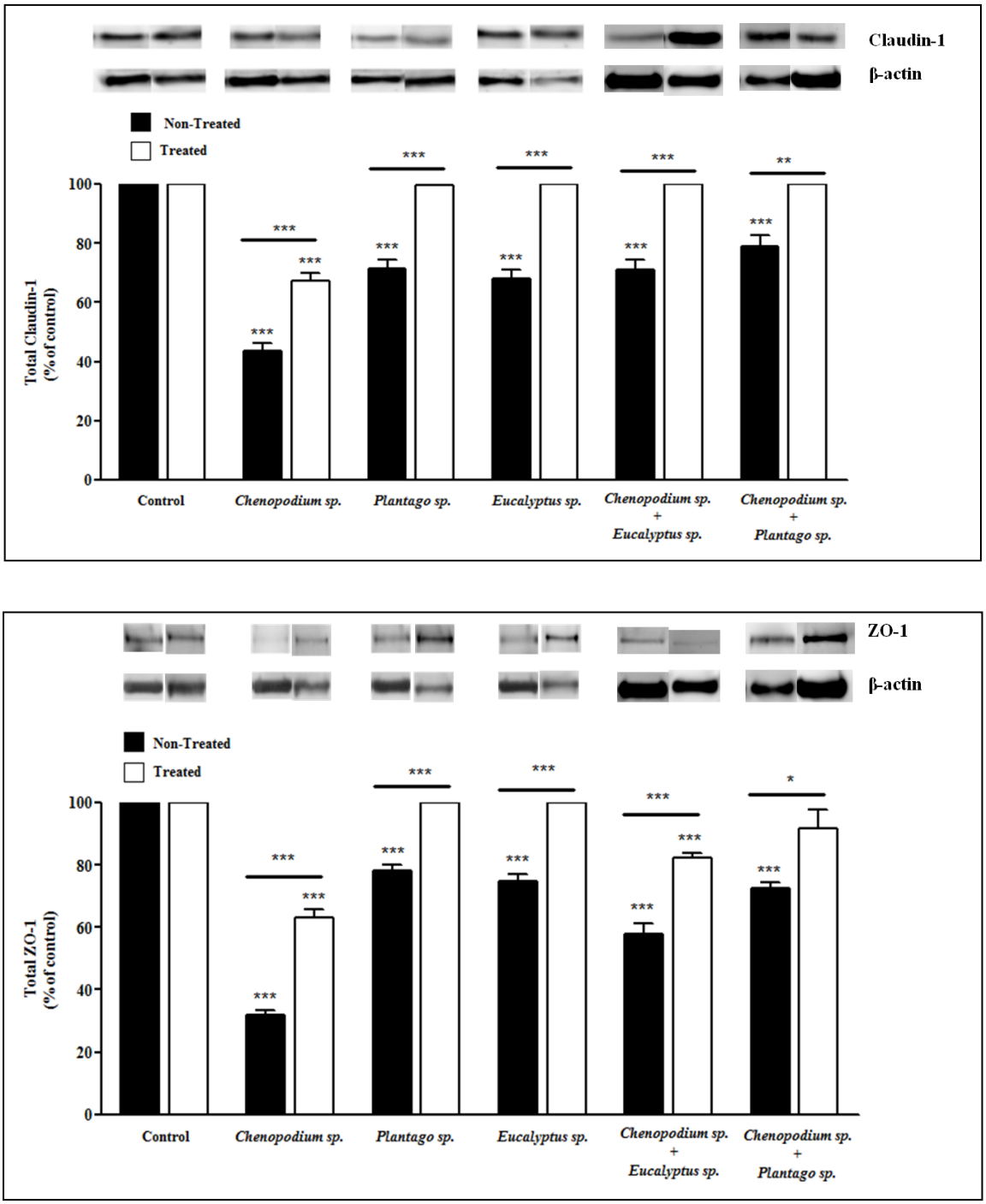


Figure 29- Effect of combined pollen proteases from *Chenopodium sp.* and *Eucalyptus sp.* (1,18 mg) and *Chenopodium sp.* and *Plantago sp.*(0,56 mg) on the degradation of E-cadherin, Occludin, Claudin-1 and ZO-1 intercellular protein complexes formed between epithelial cells of the Calu-3 monolayer analyzed by Western blot. Black bars, are referent to the exposure of Calu-3 cells to different pollen extracts stimulus during 6 hours incubation period. White bars, refer to a condition where the pollen extracts were pre-treated with 1mM of AEBSF, in order to evaluate the inhibition of the degradation of intercellular protein complexes. The results were analyzed statistically

using Bonferroni´s post-test: ***P < 0.001; **P < 0, 01; *P < 0.05 (n=3).

The action of these combined pollen extracts upon the protein intercellular complexes, namely E-cadherin was also further studied by imunoflourescence. This allowed, again the re-confirmation of what was previously reported by the Western blot, throw a visual understanding of the degradation of the continuous rings formed between the epithelial cells (Fig. 30).

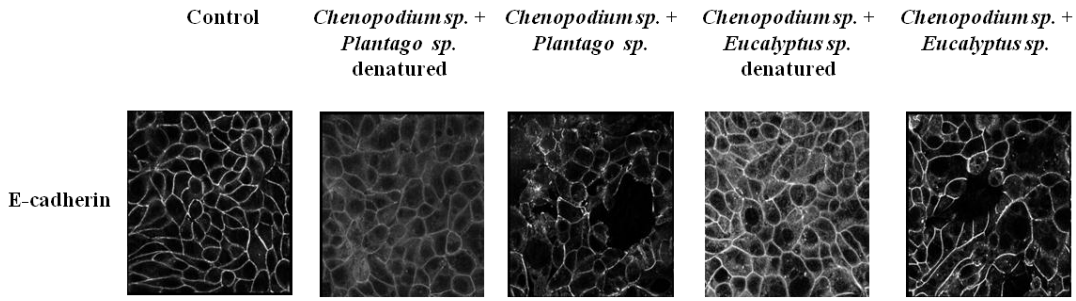


Figure 30- Effect of incubation of Calu-3 with combined pollen proteases from *Chenopodium sp.* and *Eucalyptus sp.* (0,3 mg) and *Chenopodium sp.* and *Plantago sp*. (0,14 mg) on the integrity of an adherent junction, E-cadherin. Images were obtained using a confocal microscope, z-stacking diverse planes. Images were posteriorly treated were the selection of the best z plane occurred. Images are 150μm in dimension.

Summarizing the conclusions taken by analyzing the results shown previously, combined pollen extracts also induce direct digestive action upon the proteins of the intercellular complexes formed between epithelial cells. Observable by the presence of interruptions on the continuous rings, lower fluorescence intensity compared to control conditions, morphologic alterations of the Calu-3 cells and regions of cell detachment. Also, as verified with isolated pollen extracts, denatured combined pollen extracts or combined pollen extracts pre-treated with 1mM of AEBSF inhibitor loss the ability to induce cell detachment and their degradative effect. These statements are in accordance and concurrent to the Western blot experiment and to the imunoflourescence images.

Although, all these results were in a sort of manner expected assuming what was already obtained for the isolated pollen extracts. What was truly important and intended to underline, is a suggested potentiation of the degradation effect when combined. This means, when combined with *Chenopodium sp.* it appears that the pollen extracts have more ability to cause disruption of the protein intercellular complexes, due to a probable

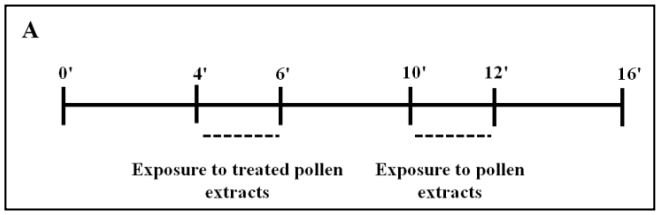
increase of the amount or specificity of proteases. In the enzymatic assays using AMC substrates, the specific activity of the combined pollen extracts with *Chenopodium sp.* despite visibly a little higher than expected, does not justify this assumption. But, combined with Western blot and imunoflourescence where it is also decipherable an increase of digestive effect, this assumption gains more veracity.

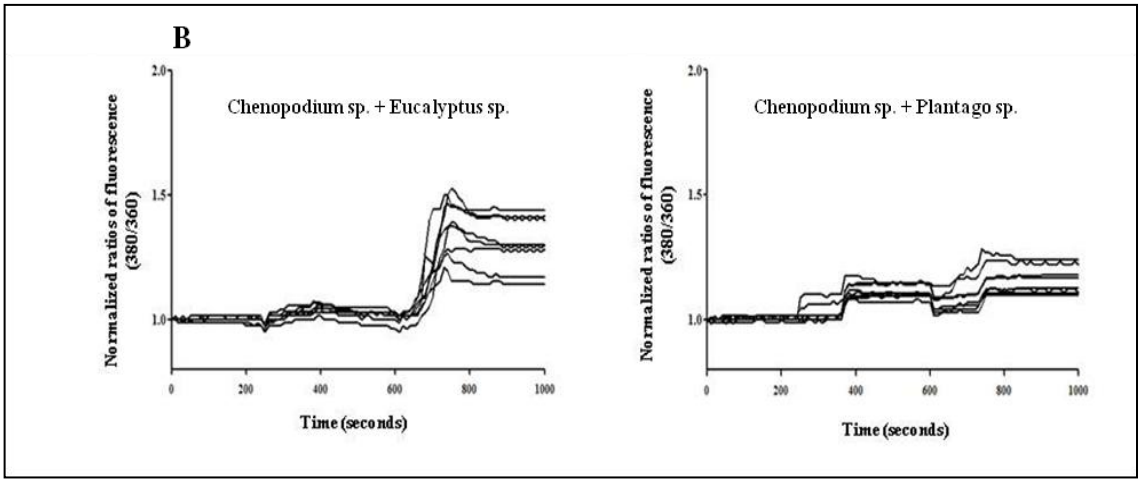
3.7.3- Combined Pollen Extracts Effect in the Inflammatory Component

The effect of combined pollen extracts on the inflammatory component was also studied, although the results represent preliminary results. For these to become scientifically reliable, it is fundamental to perform more repetitions of these experiments.

3.7.3.1- Combined Pollen Extracts Effect in PAR-2 Activation

The same experiment under the same conditions reported above in 3.6.1 was performed for combined pollen extracts (Fig. 31).





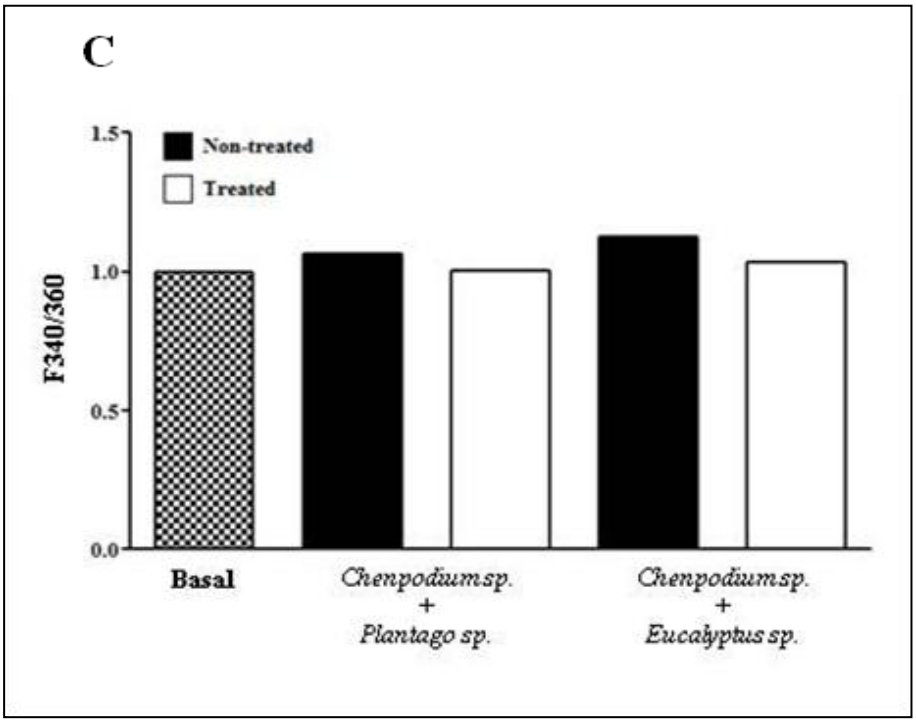


Figure 31- Effect of incubation of Calu-3 with combined pollen proteases from *Chenopodium sp.* and *Eucalyptus sp.* (0,2 mg) and *Chenopodium sp.* and *Plantago sp*. (0,08 mg) on PAR-2 activation. A) Schematic representation of the time periods used for this experiment protocol. Dashed lines indicate Calu-3 exposure periods to denatured combined pollen extracts and after combined pollen extracts untreated. B) Profiles of representative cells of each experimental condition. Graphs are represented in terms of normalized ratio of fluorescence (340/380). C) Quantification of the variations of intracellular [Ca^{2+}] mobilization after exposure to the stimuli. Black bars correspond to non-treated pollen extracts and white bars to denatured pollen extracts (n=1).

Observing the diverse images of obtained results, the first immediate conclusion is the fact that proteases existent in the combined pollen extracts are able to cause activation of PAR-2. This is obviously do to the effect of *Chenopodium sp.* combined with either *Eucalyptus sp.* or *Plantago sp.*, which in terms of $[Ca^{2+}]_I$ variations, is still very perceptive. *Plantago sp.* seemed incapable and *Eucalyptus sp.* had very low ability of activating PAR-2, but when combined with *Chenopodium sp.*, this situation was altered (Fig. 31 B & C)). *Chenopodium sp.* seems to potentiate the activation of these PAR-2 receptors, when combined with other pollen extracts. Also, very interesting was the fact that Calu-3 epithelial cells where incapable of returning to basal conditions after combined pollen extract exposure, resembling again to the profile obtained for *Chenopodium sp.*

In order to backup the conclusions above, denatured combined pollen extracts were tested. The effect of these stimulus were similar to basal conditions, not inducing variation in $[Ca^{2+}]_I$ levels. This again indicates that the activation of PAR-2 is in fact due to proteases (Fig. 31 B & C)).

3.7.3.2- Combined Pollen Extracts Induce Cytokine Release

The same experiment under the same conditions reported above in 3.6.2 was performed for combined pollen extracts (Fig. 32).

The result presented is a preliminary study. These results only allow to conclude that combined pollen extracts also induce the release of cytokines, namely IL-6 and IL-8. Also, that the denaturing process of combined pollen extracts inhibits and reverses the ability to induce cytokines, which once again establishes the connection with proteases.

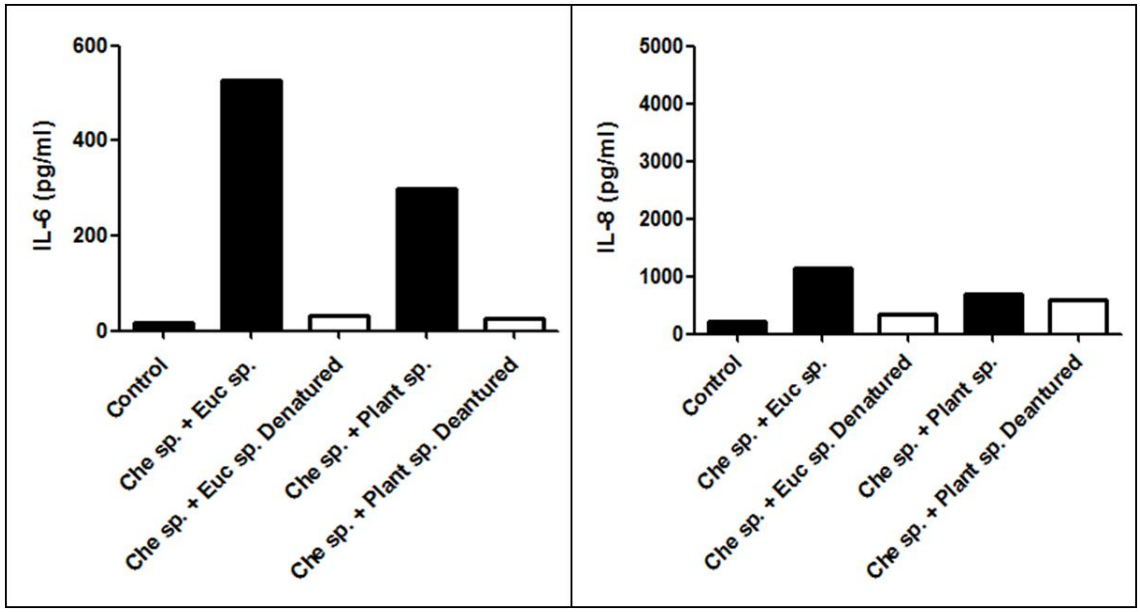


Figure 32- Effect of combined pollen proteases of *Chenopodium sp.* and *Eucalyptus sp.*(0,4 mg) and *Chenopodium sp.* and *Plantago sp.*(0,1 mg) on the release of IL-6 (image on the left) and IL-8 (image on the right). Black bars correspond to each stimuli with the pollen extracts (except the control bar), while the white bars are referent to denatured pollen extracts (30 minutes at 95ºC).

Chapter 4

Discussion

4- Discussion

Airborne pollen exposure results in many allergic disorders, namely asthma, which is an increasing cause of morbidity worldwide. For this reason and considerations for public health, the research interest on mechanisms behind the development of allergic disorders have gained interest.

Recently it has been attributed an increasing important role in the identification and characterization of proteolytic activity in allergic material, as found in dust house mites, fungus and pollens. This enzymatic activity has been suggested to be responsible in triggering allergic responses, as a result of an alteration in the integrity of lung epithelium, allowing allergens to gain access and contact APCs (Antigen Presenting Cells) (Hassim *et al*., 1998).

This research work allowed the identification of proteolytic activity of three pollen species with different allergic potentials: *Chenopodium sp.* (moderate allergenic), *Plantago sp.* (moderate allergenic) and *Eucalyptus sp.* (low allergenic). Experiments *in vitro* resorting to a Human airway epithelial cell line, Calu-3, permitted studies on the involvement of pollinic proteases on the disruption of the respiratory epithelium.

4.1- Proteolytic Profile of the Pollen Extracts

Pollen after inhaled is subjected to a hydration process at the surface of the respiratory epithelium, which leads to the release of large quantities of contend in a short period of time. Pollen grains carry proteins, namely allergens, proteases, NADH oxidase, lipoproteins; polysaccharides; lipids and phenolics. Very high quantities of pollen solutes can be concentrated in the epithelium mucosa dependent on the intensity of exposure of the individual to the pollen, geographic localization and time of year, reaching values of 50 mg/ml (Hassim *et al.*, 1998; Vinhas *et al*., 2011).

In this work, pollen hydration was performed in order to mimic the process of hydration of inhaled pollen grains that naturally occur in the respiratory airways when an individual is exposed. Each pollen type was hydrated with buffer Tris-HCl pH 7,4 in a 20 mg/ml concentration. Even though the initial proportion of pollen and buffer was the same for all three diffusates, the protein contends released vary amongst the pollen species. The quantity of total protein released was superior in pollen extracts with low allergenicity and larger pollen grain dimensions, approximately 0,8mg/ml for

Eucalyptus sp. While, for *Chenopodium sp.* and *Plantago sp.* with moderate allergenic potentials and small dimensions of their pollen grains, had an average of total protein released of 0,35mg/ml and 0,2mg/ml, respectively. This demonstrates distinct pollen ability to release protein content. Characterization of the protein profile of the three diffusates through SDS-PAGE revealed the presence of high molecular weight proteins in all pollen extracts.

Identification of proteolytic activity present in the different pollen extracts was possible resorting to a zymography. This enzymatic assay uses gelatin as substrate, and for that reason was initially conceived to identify enzymes that are capable of its degradation, such as collagenases and gelatinases. However, as a result of the heterogeneous structure of the polypeptides that form gelatin, hydrolyze of this substrate is possible for a large variety of proteases.

In all three pollen extracts, proteolytic activity was effectively detected, as a result of proteases of high molecular weight. In terms of *Chenopodium sp.* this activity is situated between 250-100 kDa, for *Eucalyptus sp.* proteases are found located between 150-100 kDa and finally for *Plantago sp.* proteases are located between 250-75 kDa and 20-15 kDa.

Two major conclusions can be taken from these results. First, the prevalence of high molecular weight proteases in all three pollen extracts. This is important and can be correlated to the fact that high molecular weight proteases have been identified and suggested responsible in the loss of lung epithelium integrity. This knowledge allows a step forward in the comprehension of the mechanism that permit allergens to gain access through the epithelial barrier (Runswick *et al*., 2007; Cortes *et al*., 2006; Vinhas *et al*., 2011).

Secondly, extremely important to underline the fact that even though *Plantago sp.* and *Chenopodium sp.* release low amounts of total protein content, they produced an intense degradation profile. This result implies that *Plantago sp.* and *Chenopodium sp.* have a large concentration of proteases with intense proteolytic activity. Visible is the fact that all three pollen diffusates release proteases in different proportions: pollens that are considered more allergenic release lower total protein contend but larger quantities of proteases, on the other hand, pollens considered less allergenic release larger amounts of total protein contends but have lower proteolytic activity.

Through a two-dimensional zymography, a deeper knowledge was obtained on the proteases released by pollen grains. Again, the results obtained are concurrent, revealing spots of proteolytic digestion at regions corresponding to high molecular weight. Additionally to high molecular weight features, this technique revealed an acidic isoelectric point of the proteases released. In a study conducted by Vinhas *et al*., proteases with high molecular weight and acidic pI were also encountered in four distinct pollen extracts. These results point to similar proteases existent in pollen extracts from different species and with different allergenic potential.

4.2- Characterization of the Proteolytic Activity

To evaluate and characterize specifically the proteolytic activity, namely in terms of substrate preference and protease class, present in pollen extracts we resorted to enzymatic assays using flourogenic substrates (AMC) and specific inhibitors. This technique allows a rapid and general search for proteases in pollen extracts. In literature, the majority of identified protease allergens belong principally to four enzymatic classes: serine, cysteine, aspartic and metalloproteinases. However, serine and cysteine proteases are the most commonly found in pollen extracts (Raftery *et al.*, 2003; Runswick *et al.*, 2007; Widmer *et al*., 2000).

In all pollen extracts, the substrate profile obtained was identical, being Phe-AMC the preferred but also high enzymatic activities shown for Met-AMC and Leu-AMC. This may indicate similarities in the proteases existent in the pollen extracts.

In general, the results from the inhibitor enzymatic assay highlighted the existence of a preferential serine and metalloproteinase activity in the three species of pollen, consistent with the high inhibition of AEBSF and EDTA, respectively. In terms of serine proteases, this presence was even more specific for *Chenopodium sp.*, with the presence of chymotrypsin-like serine proteases, as well as for *Plantago sp.* with trypsin-like serine proteases.

In particular for *Chenopodium sp.* and *Eucalyptus sp.* a presence of aminopeptidases N is suggested, as a result of high inhibition percentage of Zn^{2+} and AEBSF. Although, AEBSF is specific for serine proteases, it is known to inhibit partially aminopeptidases N, as well as, divalent ions such as Zn^{2+} (Chandu *et al.*, 2003; Cortes, 2006).

Lastly, *Chenopodium sp.* and *Eucalyptus sp.* pollen appear to have cysteine activity present in the pollen extracts. This activity corresponds to the inhibition with E-64. Also confirmed TPCK and TLCK, although specific for serine protease, are known to inhibit irreversibly cysteine proteases.

Complementary studies using Leu-AMC, another flourogenic substrate with high enzymatic activity for all three pollen extracts was used in order to try and obtain more information on the proteolytic characterization of the pollen extracts. However the overall profile did not suffer significant alterations.

These enzymatic assays were realized only for pH 7,4, however in order to detect a larger range of proteases it is convenient to vary pH conditions, as well as, increase the number of flourogenic substrates and specific inhibitors used in this enzymatic assay. Resorting to diverse techniques of identification and characterization of proteolytic activity it is also beneficial when working with pollen extracts, with potential for high and various numbers of proteases. Purification of the proteases present in the pollen extracts and identification through mass spectroscopy would allow a more specific and exact confirmation.

4.3- Action of Proteases on the Integrity of Respiratory Epithelium

The disruption of respiratory airway epithelium has been suggested to be the main cause for the development of allergic disorders. A disrupted lung epithelium loses the ability of paracellular barrier, allowing allergens to gain access to cells of the immune system originating an allergic response. A damaged epithelium interrupts the release of relaxant substances, which favors broncoconstriction symptoms.

For this reason, it becomes essential to determine the involvement of pollinic proteases in the disruption of lung epithelium and alteration of pulmonary homeostasis, given arise to the development of allergic disorders. For these studies, our laboratory resorted to Calu-3 epithelial cell line, one of the few cell lines that form differentiated epithelial Human cells with functional tight junctions, *in vitro*, as well as, high transepithelial resistance, allowing a functional model of airway epithelial barrier.

4.3.1- Increase of Transepithelial Permeability

All three pollen extracts cause an increase in transepithelial permeability in a time-dependent manner. This indicates that lung epithelium allows a larger flux of macromolecules through the monolayer barrier of cells when exposed to these pollen extracts.

The effect of *Chenopodium sp.* is tremendous, followed by *Eucalyptus sp.* and finally *Plantago sp.* As described previously in all three pollen extracts, serine and metalloproteinases were identified as the major protease components. In particular, *Chenopodium sp* and *Eucalyptus sp* showed to have also cysteine and aminopeptidase activity. This is interesting, because previous studies using Der p 1, allergen from house dust mite, with identified cysteine activity, was shown to induce epithelial disruption and induce an increase of transepithelial permeability (King *et al*., 1995; Wan *et al*., 1998). Obviously, serine activity is linked to this alteration of permeability of the barrier, because when pollen extracts are treated with AEBSF, specific serine inhibitor, a reversal of permeability is observed. But curiously, in *Chenopodium sp*. this reversal is far from complete, and in this pollen extract is where cysteine activity is more present and easily observable. Even treated with AEBSF, pollen extract from *Chenopodium sp.* has a larger effect on transepithelial permeability than untreated pollen extracts of *Eucalyptus sp.* and *Plantago sp.* Indicating that besides the already identified effect of serine activity in diverse studies present in literature, cysteine activity may also have a role in disruption of lung epithelium.

The loss of effectiveness of the epithelium barrier proven with these results, allows contends released after pollen hydration to gain access to submucosal layers of the epithelium and contact with immune cells, causing allergic responses. If correlations between different pollen extracts and common proteases activities with increased transepithelial permeability can be managed, as already described in many research studies (Cortes *et al*., 2006; Taverna *et al*., 2008; Vinhas *et al*., 2011), beneficial progress can be achieved, in a crucial step previous to allergen sensitization, highly opportune for eventual allergic treatments.

Epithelial cells reveal functions that overcome simple barrier functions, already described in studies (Thompson *et al*., 1995). The production of various mediators by epithelial cells contributes not only in a cell-cell interaction but also between cell-matrix

interactions, which is quite important for repairing the epithelium. After airway epithelial damage, epithelial cells at the edge of the injury appear to flatten and migrate across the matrix, to close the defect (Juhasz *et al.*, 1993).

Complementary studies using endogenous inhibitors would be fundamental, in order to evaluate the potential to reverse transepithelial permeability. For example, α_1-protease inhibitor and secretory leucoprotease inhibitor (SLPI) which are inhibitors of serine proteases. Differently, α_1-macroglobulin is not class specific, and inhibits proteases from four different classes: serine, metalloprotease, cysteine and aspartic (Thompson *et al.*, 1995). Interesting, Hassim and collaborators showed that proteases released by various allergenic pollens provoke epithelial cell detachment in mouse models, and also are not inhibited by endogenous proteases, like SLPI and α1-PI (Hassim *et al.*, 1998). On the other hand, Kalsheker and colleagues (1996), suggest the cleavage of α1-PI as an important mechanism in the development of allergic disorders like asthma.

4.3.2- Disruption of Protein Intercellular Complexes

Under normal circumstances, the healthy bronchial epithelium is an impermeable barrier offering resistance to the paracellular flow of macromolecules and infectious agents, as wells as, limiting significantly ion diffusion (Winton *et al.*, 1998) as a result of specialized cell junctions: Adherens Junctions (AJ), Desmosome and Tight Junctions (TJ) (Matter & Balda, 2003).

The increased in transepithelial permeability, cell detachment, as well as, the loss of cell viability incapable of proliferation can be a result of the disruption of these cell junctions when exposed to proteases present in the pollen extracts. Proliferation and differentiation of epithelial cells is also necessary in the repair of injured epithelium. These assumptions are suggested, taken into consideration various studies that point in this direction using pollinic proteases and house dust mites (Baker *et al.*, 2003; Cortes *et al.*, 2006; Runswick *et al.*, 2007; Wan *et al.*, 2001; Vinhas *et al.*, 2011).

Incubation of confluent Calu-3 cells with different pollen extracts during a 6 hour incubation period caused statistically significant degradation of intercellular proteins: E-cadherin, Claudin-1, Occludin and ZO-1. Western blot analysis allows confirmation of a preferential degradation of E-cadherin, by the pollen extracts. This

had already been verified by Cortes and collaborators in studies relatively to proteolytic action of a aminopeptidase of pollen from *Parietaria judaica* (Cortes *et al.,* 2006). This transmembrane protein linked to adherent junctions, has long been associated to contribute in the assembly of other specialized cell-cell junctions and epithelial integrity maintenance (Vinhas *et al.,* 2011; Jacob *et al.,* 2005; Cenac *et al.,* 2004). Even so, tight junctions namely Claudin-1 and Occludin, as well as, cytosolic complexes like ZO-1, were extensively and statistically significant disrupted after the exposure to the pollen extracts.

These results indicate a possible direct mechanism of degradative action on the extracellular domains of the transmembrane proteins. Inhibition of this degradative action was accomplished using a serine specific inhibitor AEBSF, which reversed the effect in all pollen diffusates, establishing the connection to proteases.

This intercellular protein complex degradation was further studied by imunoflourescence. The images obtained visually are totally concurrent with the results obtained and described previously with Western blot analysis. After 6 hour stimulus, with the different pollen extracts, interruptions in the continuous rings formed by the assembly between these protein complexes are visible, as well as, slight morphologic cell alterations. This effect was inverted, using denatured pollen (30 minutes at 95ºC), linking again the effect to the presence of proteases in pollen extracts.

Existent literature describes a regeneration of occludin after exposure to pollen extracts, as verified in studies by Runswick and Wan (Runswick *et al.,* 2007; Wan *et al.,* 1999). In order to complement our studies, visual identification and confirmation of a possible regeneration of airway epithelium after exposure to proteases present in pollen extracts is fundamental.

One of the important candidates that seem to play a role in both epithelial integrity as well as airway remodeling is a family of "a desintegrin and metalloproteinase" (ADAMs). This family of molecules seems to regulate formation of cell-cell and cell-matrix contacts and also regulate cell proliferation, cell survival, cell migration and airway remodeling (Heijink & Kauffman, 2009). Also, Metalloproteinases (MMPs) play an important role in epithelial integrity, repair and invasiveness through the extracellular matrix remodeling, the induction of growth factors (TGF-β and EGF) have been also implied in E-cadherin shedding (Heijink &

Kauffman, 2009). Many studies with single-nucleotide polymorphisms (SNPs) have been performed with these families of molecules and lead also to associations with asthma and allergic disorders (Foley *et al.*, 2007; Holgate *et al.*, 2006; Gosman *et al.*, 2006; Van Eerdewegh *et al.*, 2002; van Dieman *et al.*, 2005; Jongepier *et al.*, 2004; King *et al.*, 2004; Maretzky *et al.*, 2005; Hirao *et al.*, 2006).

4.3.3- PAR-2 Activation

The loss of effectiveness of the epithelium barrier can also occur by activation of PAR-2, a 7-transmembrane protein coupled to G proteins, widely expressed in the apical surface of epithelial cells. These receptors are unconventional, in the fact that they do not bind to free moving soluble ligands but instead require specific site-directed serine proteolysis cleavage which allows posteriorly a self-activation by their new amino-terminal, called tethered ligands. For that reason, PARs are considered to be sensors of extracellular proteolytic environments.

Serine proteases, for example trypsin and trypsin-like enzymes, cleave the amino acids at a specific site of the extracellular N-terminus of the molecule exposing a new N-terminal ligand domain that binds to another site on the same molecule, thereby activating the receptor (Reed & Kita, 2004). Activated PARs couple to G-signaling cascades increasing phospholipase C levels which in turn lead to increased intracellular Ca^{2+} levels (Berger *et al.*, 2001; Schechter *et al.* 1998; Ubl JJ *et al.*, 2002). Single-Cell Calcium Imaging, allowed the confirmation of induced increased intracellular Ca^{2+} levels when exposed to 2 minute stimuli periods with pollen extracts from *Chenopodium sp.* and *Eucalyptus sp.* After exposure to pollen extract from *Plantago sp.* no visual increase of intracellular calcium was observed, possibly due to a low serine protease concentration present in the extract. The inhibition of this effect was again possible with denatured pollen extracts (30 minutes at 95ºC), unequivocally pointing the effect to the presence of active proteases present in these extracts.

The effect observed was again tremendous and statistically significant for *Chenopodium sp.*, similar to what was obtain for increase transepithelial permeability and disruption of protein junctions. These findings are concurrent with studies that suggest PAR-2 indirectly compromises the integrity of the epithelium barrier, through the disruption of tight junctions. For example exogenous allergens, including fungi, house dust mites and cockroaches, proteolytically active, are known to cause breakdown

of epithelial barriers through PAR-2 mediated mechanisms. The effect of *Chenopodium sp.* was so disturbing that cells, even in a 2 minute period exposure, were unable to return to basal conditions, indicating possible cell detachment, altered cell morphology and even eventual initiation of apoptosis/necrosis processes.

The coupling to G proteins, successively increasing Ca^{2+} levels, is suggested to induce through signaling cascades the release of chemokines, cytokines and neurotransmitters.

The effect of pollen extracts on cytokine release by Calu-3 epithelial cell was studied using a technique named Cytometric Bead Array. The results obtained suggest that all pollen extracts induce the production and release of pro-inflammatory cytokines, namely IL-6 and IL-8, when compared to a control condition. This increase of cytokine production was again related to proteolytic activity present in the pollen extracts, since denatured pollen extracts (30 minutes at 95ºC) did not cause this increase in concentration of cytokines.

The proinflammatory role of PAR-2 has been supported by guinea pig, mice and Human studies. Mites such as *Dermatophagoides pteronyssinus*, possess endogenous proteases such as cysteine (Der p 1) and serine proteases (Der p 3, 6 and 9), and can activate PARs, leading to inflammation through epithelial cell detachment, IgE production and can also induce cytokine production (Sun *et al.*, 2001; Asokananthan *et al.*, 2002; Adam *et al.*, 2006). Recent studies have shown that functional PAR-2 when activated can induce the release of, amongst others, IL-6 and IL-8 (Vliagoftis *et al.*, 2001; Vliagoftis 2000; Sun *et al.*, 2001; Asokananthan *et al.*, 2002).

The production of inflammatory mediators by epithelial cells by interaction with allergens via mechanisms dependent on the proteolytic activity can help clarify and allow one step closer to treatment development. A time-course of production and successive release of cytokines would complement these results obtained.

4.4- Combined Pollen Extracts

As discussed previously pollen extracts were investigated as isolated elements, however in the natural environment the respiratory system is exposed to multitude of airborne particles, namely pollens, with overlapping pollination seasons, time and geographic dependent, as concluded through epidemiologic studies shown in 3.1. For

that reason, as a way to mimic natural conditions that we are exposed to in everyday situations, we studied combinations of pollen extracts. The purpose was to observe possible alterations of proteolytic activity when combined. Two combinations of pollen extracts were studied: *Chenopodium sp.* with *Eucalyptus sp.* and *Chenopodium sp.* with *Plantago sp*. The first combination was intended to join moderate allergenic pollen with low allergenic pollen. Secondly, a combination of two moderate allergenic pollens with identical enzymatic activities. Both of the combinations studied have *Chenopodium sp.* as common pollen, this is purposely, since this pollen is by far the most aggressive to cells, as proven in results obtained in Western Blot analysis and transepithelial permeability (3.5).

The combined pollen extracts continued to have activity for the same primordial three substrates Met-AMC, Leu-AMC and Phe-AMC. Also, pollen extracts of *Chenopodium sp. with Eucalyptus sp.* and *Chenopodium sp.* with *Plantago sp.* continued to have Phe-AMC as their preferred substrate.

In terms of the combination *Chenopodium sp.* with *Eucalyptus sp.* it´s still well perceptive and statistically significant the presence of serine, metalloproteinases and aminopeptidases N. The only observable and significant difference is the disappearance of cysteine activity. The profile of the combination between *Chenopodium sp.* with *Plantago sp.* has some visual differences. The serine, metalloproteinases and aminopeptidases N were still very perceptive and statistically significant. The major difference is again the decrease of cysteine activity. A possible explanation is the degradative effect of proteases on proteases resultant from the combination of pollen extracts.

Combined pollen extracts also induce direct digestive action upon the proteins of the intercellular complexes formed between epithelial cells. Observable by the presence of interruptions on the continuous rings, lower fluorescence intensity compared to control conditions, morphologic alterations of the Calu-3 cells and regions of cell detachment. Also, as verified with isolated pollen extracts, denatured combined pollen extracts or combined pollen extracts pre-treated with 1mM of AEBSF inhibitor loss the ability to induce cell detachment and their degradative effect. These statements are in accordance and concurrent to the Western blot experiment and to the imunoflourescence images.

What was truly important and intended to underline, is a suggested potentiation of the effect of degradation when combined. This means, when combined with *Chenopodium sp.* it appears that the pollen extracts have more ability to cause disruption of the protein intercellular complexes, due to a probable increase in the amount or specificity of proteases. This is proven by slight increases visible in enzymatic assays using AMC substrates and further confirmation by Western blot and imunoflourescence.

Interestingly also, is the fact that our research group previously described the same effect. Vinhas *et al.* described that *Pinus sylvestris* pollen potentiated the degradation activity of *Dactylis sp.* and *Olea sp*. Even more newsworthy is correlating this same effect of potentiation with the different characteristics between *Pinus sylvestris* and *Chenopodium sp.*, mainly in the enzymatic activity observed. Altogether, depending on the combination of pollens present in the environment, different reactions are induced and difficult to predict, as well as, cross reactivity episodes. This obviously increases even more the complexity of allergic disorders.

Combined pollen seems able to induce, indirectly, epithelium disruption through PAR-2 activation. Even though only preliminary studies were conducted, PAR-2 activation occurs and is obviously due to the effect of *Chenopodium sp.* combined with either *Eucalyptus sp.* or *Plantago sp.*, which in terms of $[Ca^{2+}]_I$ variations, is still very perceptive. *Plantago sp.* seemed incapable and *Eucalyptus sp.* had very low ability of activating PAR-2, but when combined with *Chenopodium sp.*, this situation was altered. *Chenopodium sp.* seems to potentiate the activation of these PAR-2 receptors, when combined with other pollen extracts. Also, very interesting was the fact that Calu-3 epithelial cells where incapable of returning to basal conditions after combined pollen extract exposure, resembling again to the profile obtained for *Chenopodium sp.* This effect was again inhibited when exposed to denatured pollen extracts, indicating PAR-2 activation is due to active proteases present in pollen extracts.

Combined pollen extracts were also able to induce the release of cytokines, namely IL-6 and IL-8. Also, the denaturing process of combined pollen extracts inhibits and reverses the ability to induce cytokines, which once again establishes the connection with proteases.

These studies conducted in this research work are of great importance in deeper knowledge of the mechanisms involved in the development of allergic disorders to pollens. Only by these studies will it be possible in correlating proteolytic activity with loss of respiratory airway epithelium integrity and the potentiation of allergic responses. Important also, is the fact that our work suggests that even less allergenic pollens are likely to participate in allergic sensitization and airway inflammation. These results can be of great interest in the attempt of developing future therapies for allergic disorders.

Chapter 5

Final Conclusion and Future Perspectives

Final Conclusions

This research work confirmed the identification of a majority of serine and metalloproteinase activity in the three pollen extracts, which are widespread over the Mediterranean area and have distinct allergenic potentials. Also identified, and somewhat relevant, was the presence of cysteine activity in pollen extracts from *Chenopodium sp.* and *Eucalyptus sp*. This proteolytic activity was a resultant of high molecular proteases with an acidic pI, released after a hydration process of pollen grains.

The respiratory epithelium, is part of the first line of defensive against contact with the external environment, and is daily exposed to a large variety of aggressors. These epithelial cells are exposed to high concentrations of pollen solutes, released upon hydration, being these contends composed by allergens and proteases, amongst others. In this way, this study suggests that the epithelial barrier is targeted *in vivo* by the proteolytic activity present in the pollen extracts. Thus, direct disruption of intercellular protein complexes occur to TJs, AJs and cytosolic complexes, resulting in the increase of transepithelial permeability, allowing interactions between allergens and cells from the immune system, APC´s, thereby amplifying the inflammatory response (Fig. 33). Also, the loss of effectiveness can be a result of an indirect digestive action of proteases due to the proteolytic activation of PAR-2 receptors widely expressed on the apical surface of epithelial cells, given also arises to inflammatory responses (Fig. 33).

In this research work, the targeted pollen species studied revealed some similarities in proteolytic activity but in different quantities. All pollen extracts, even *Eucalyptus sp.*, considered to be low allergenic, when incubated with Calu-3 epithelial cells caused a direct disruption of intercellular protein complexes, E-cadherin, Occludin, Caludin-1 and ZO-1 which led to increasing transepithelial permeability. Indirectly, we were able to demonstrate that *Chenopodium sp.* and *Eucalyptus sp.* were able to activate PAR-2 receptors and all pollen extracts were shown to release cytokines IL-6 and IL-8 after exposure to Calu-3 cells. In this manner, it is easily concluded that the proteolytic activity present in the pollen extracts, *in vivo*, have a role in the potentiation of allergic responses, either through direct or indirect pathways of intercellular protein complex disruption facilitating the passage of allergens to sub-layers of lung epithelium accessing and contacting cells of the immune system.

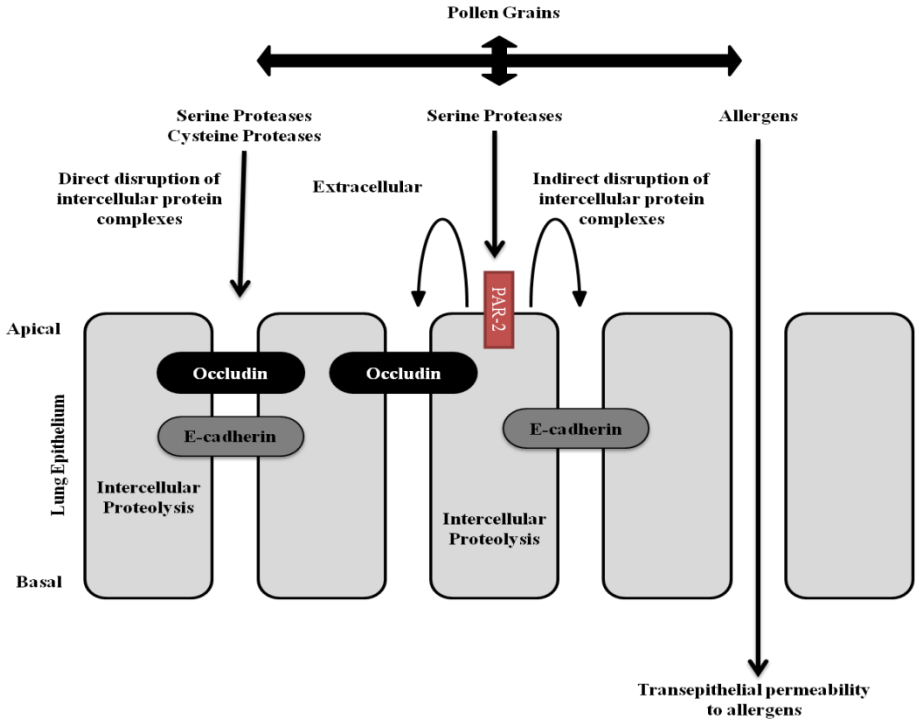


Figure 33- Proposed mechanisms, via directly or indirectly, for disruption of intercellular protein complexes formed between lung epithelium cells which increase transepithelial permeability to allergens, initiating allergic responses due to contact with immune cells (Image adapted from Wan *et al.*, 1999).

We concluded that not only pollen species considered to be highly allergenic, but also low allergenic pollen species, are involved in the disruption of intercellular protein complexes and consequentially airway inflammation. Since the respiratory airway is targeted by a multitude of airborne particles, these can either release large amounts of allergens and/or large amounts of proteases, which can disturb the respiratory epithelium and lead to sensitization to diverse allergens. What is very interesting and has to be necessarily highlighted is the fact that atopic patients develop allergic reactions to allergens with no proteolytic activity. This ability of proteases to promote allergy to allergens without functional activity is clearly critical. Also, increasingly frequent, epidemiologic studies worldwide confirm an increase of allergic reactions to pollen species traditionally considered low allergenic, like *Eucalyptus sp*.

Since allergic disorders are a serious worldwide issue, the identification of the proteases responsible for loss of lung epithelium barrier integrity are vital for full comprehension of the mechanisms involved in allergic responses , and this way in the development of inhibitors capable of being used for therapies against these health disorders.

Future Perspectives

- The results of this work suggest an obvious association between the proteolytic activity of different pollen extracts and lung epithelium disruption. For that reason isolation of these identified proteases in the pollen extracts would allow a better approach and knowledge to the specific mechanisms behind allergic responses and inflammation. The isolation of the identified proteases could also allow the specific characterization of these, for example through Mass Spectroscopy. Obviously all this together, could lead to development of specific inhibitors for the proteases, ideally for future therapies.

- Important is also studies to verify if the action of the pollen extracts can be inhibited by endogenous inhibitors such as SLPI and α1-PI, also observation of possible cleavage of neuropeptides important in pulmonary homeostasis (Substance P, VIP and angiotensin I).

- As allergic reactions have been described after the exposure to airborne pollen of species with different allergenic potentials, immune-reactivity tests on serum from allergic patients and quantification of IgE specific antibodies would allow deeper knowledge of the relationship between proteolytic activity and allergies.

- As already described in many studies, lung epithelium has the ability to repair and reassume a full integrity state. For example, regeneration of Occludin observed by Vinhas *et al.* after removal of the pollen stimuli. For this reason, first the attempt in comprehending the repair mechanism of the lung epithelium, by ADAM´s, metalloproteinases or other molecules would be knowledge fulfilling. Also, studies of cytotoxicity to evaluate eventual processes of apoptosis/necrosis and try establishing correlations with cell detachment and respiratory epithelium disruption.

- A recent issue involving environment pollution and cigarette smoke in allergic disease could be very promising. Studies revealed that urbanization and high levels of pollution are directly correlated to an increase of the frequency of pollen-induced respiratory allergy. Also, people from urban areas seem to be more affected by pollen-induced allergies than those from rural regions. The

pollutant content of the air interacts with pollen allergens inhaled, and may this way increase the atopic sensitization risk or exacerbate the symptoms of allergic individuals. Evidence suggest that pollutants facilitate the access of allergens inhaled to cells of the immune system, because of damage to epithelial cells which gives origin to an increase of epithelial permeability, inflammation and increased oxidative stress (D'Amato, 2000; 2002; Devalia *et al.*, 1998; Knox & Heslop-Harrison, 1970). Some studies have been performed with the purpose of evaluating the effect of cigarette smoke (CS) on the lung epithelium. CS exposure is known to increase epithelial barrier permeability in lungs, allowing allergens to gain access to the submucosal layer resulting in contact with immune cells. The mechanisms by which CS disrupts the epithelia are not fully clear, but it's suggested to involve alterations in the functions of tight junctions (Oliveira *et al.*, 2009). Recent data also suggest that CS inhibits the ability of epithelial cells to participate in airway repair (Wang *et al.*, 2001).

- These studies confirm that PAR-2 receptors widely expressed on the apical surface of epithelial cells were activated when exposed to pollen extracts. This experiment can be coupled to a follow up experiment, using inverted specific cleavage sites of amino acids which leave PAR-2 receptors inactive. If no variation of intracellular calcium concentration is observed, then undoubtedly PAR-2 receptors are activated when exposed to pollen extracts composed with proteases.

- Extremely significant for this research would be the use of tissue obtained from biopsies of healthy patients. This would allow greater approach to *in vivo* mechanism of allergic responses, were the effect of pollen would be studied via directly and indirectly on the disruption of lung epithelium. This could be the source of vital information on the development of future therapies.

- The surface of the respiratory epithelial mucosa is exposed to a multitude of airborne particles, namely different pollen species with coincident geographic and pollination season. As already introduced in this work, some combinations of pollen were studied. To understand if the presence of a large variety of pollen species contacting all at once the epithelium induces alterations in any way of

the allergic response, many other combinations of pollen and other elements must be studied.

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