Dissertation

THE SEASONAL NON-ALLERGIC CONJUNCTIVITIS SYNDROME

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used.

Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

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All co-authors have explicitly agreed to the use of their data in this thesis. Permission to reproduce figures and/or tables from the respective copyright holders have been obtained.

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Table of Contents

1. Abbreviations and Definitions

AKC: Atopic keratoconjunctivitis

ATD: Aqueous tear deficiency

BP: Blank with pollen extract

C: Control

CI: Cell index

CO_2: Carbon dioxide

Conc.: Concentration

CV%: Coefficients of variation

D: Distilled water

DC: Dendritic cells

DMEM: Dulbecco's Modified Eagle Medium

DPBS: Dulbecco's Phosphate Buffered Saline

Dur.: Duration

EDE: Evaporative dry eye

ELISA: Enzyme-linked immunosorbent assay

GLM: General Linear Models

GM-CSF: Granulocyte-macrophage colony-stimulating factor

IgE: Immunoglobulin E

IL-6: Interleukin-6

IL-8: Interleukin-8

kDa: Kilo-Dalton

LDH: Lactate Dehydrogenase

LMM: Linear mixed effects model

MGD: Meibomian gland dysfunction

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

NO_2: Nitrogen dioxide

O_3: Ozone

P: Probe

PALM: Pollen-associated lipid mediator

PM: Particulate matter

RAST: Radio-Allergo-Sorbent-Test

RTCA: impedance based xCELLigence real time cell analysis system

SD: Standard deviation

SNAC: Seasonal Non-Allergic Conjunctivitis

SNAR: Seasonal Non-Allergic Rhinitis

SS: Sjogren Syndrome

VKC: Vernal keratoconjunctivitis

WST-1: water-soluble tetrazolium, the sodium salt of 4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

2. List of Figures

Figure 10. Pipetting scheme for the WST-1 assay. Blank (B), control (C), probe 5mg/ml pollen extract (P1), probe 25mg/ml pollen extract (P2).
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 11. Zymography pattern of Birch pollen (lane 1, 2), Orchard grass (lane 3, 4).
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Figure 12. Zymography pattern of Corkscrew hazel pollen in different concentrations (lane 1, 2).
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 13. Zymography pattern of common Timothy (lane 1), Golden foxtail grass (lane 2) pollen.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 14. Zymography pattern of Korean fir (lane 1), Scots pine (lane 2), European silver fir (lane 3) pollen.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 15. PAGE of Common hazel (1), European alder (2), White willow (3), European ash (4) and Protein Standard (S) to estimate the molecular weight of the proteins by comparison of their visible bands.
Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom.

Figure 16. PAGE of Protein Standard (S), Korean fir (1), Scots pine (2) and European silver fir (3).
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 17. PAGE of Protein Standard (S).and Greater plantain (1), Orchard grass (2), Common timothy (3) and Golden foxtail grass (4).
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 18. PAGE of Protein Standard (S) and Elder (1).
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 19. Electrophoresis spectrum of Hazelnut pollen extract and tear fluid. (Lane 1: Hazelnut pollen. Lane 2: tear fluid. Lane 3: tears incubated with Hazelnut pollen extract.)
Reproduced from [6] with permission of publisher Kager, Basel, Switzerland.

Figure 20. PAGE of Corkscrew hazel. 1 = Tears (Non-Allergic subject), 2 = Tears (Non-Allergic subject) + Pollen, 3 = Pollen, 4 = Tears (Allergic subject) + Pollen, 5 = Tears (Allergic subject). In comparison to the lanes 1 and 5 single bands of the electrophoresis-spectrum of tear fluid disappeared and a few new bands appeared (arrows) within the lanes 2 and 4. This indicates that pollen proteins exert proteolytic activity destroying tear fluid proteins in allergic as well as non-allergic subjects.
Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom.

Figure 21. PAGE of European alder. 1 = Tears (Non-Allergic subject), 2 = Tears (Non-Allergic subject) + Pollen, 3 = Pollen, 4 = Tears (Allergic subject) + Pollen, 5 = Tears (Allergic subject). In comparison to the lanes 1 and 5 single bands of the electrophoresis-spectrum of tear fluid disappeared and new bands appeared (arrows) within the lanes 2 and 4. This indicates that pollen proteins exert proteolytic activity destroying tear fluid proteins in allergic as well as non-allergic subjects.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 22. PAGE of Common timothy. 1 = Tears (Non-Allergic subject), 2 = Tears (Non-Allergic subject) + Pollen, 3 = Pollen, 4 = Tears (Allergic subject) + Pollen, 5 = Tears (Allergic subject). In comparison to the lanes 1 and 5 single bands of the electrophoresis-spectrum of tear fluid disappeared and a few new bands appeared (arrows) within the lanes 2 and 4. This indicates that pollen proteins exert proteolytic activity destroying tear fluid proteins in allergic as well as non-allergic subjects.
Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom.

Figure 23. CHANG-cells incubated with Elder pollen extract at a concentration of 25 mg/ml after 5 hours.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 24. CHANG-cells incubated with European ash pollen extract at a concentration of 5 mg/ml after 5 hours.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 25. CHANG-cells incubated with European ash pollen extract at a concentration of 25 mg/ml after 5 hours.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 26. CHANG-cells incubated with Greater plantain pollen extract at a concentration of 25 mg/ml after 5 hours.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 27. CHANG-cells incubated with Korean fir pollen extract at a concentration of 25 mg/ml after 5 hours.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 28. CHANG-cells without pollen addition (control).
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 29. 96-well-microtiterplate of the WST-1 assay.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 30. Cell viability after incubation with 5 mg/mL pollen extracts for 5 hours. Statistically significant changes compared to the viability of the control are marked with an asterisk.
Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom.

Figure 31. Cell viability after incubation with 5 mg/mL pollen extracts for 24 hours. Statistically significant changes compared to the viability of the control are marked with an asterisk.
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Figure 32. Cell viability after incubation with 25 mg/mL pollen extracts for 5 hours. Statistically significant changes compared to the viability of the control are marked with an asterisk.
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Figure 33. Cell viability after incubation with 25 mg/mL pollen extracts for 24 hours. Statistically significant changes compared to the viability of the control are marked with an asterisk.
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Figure 34. Control cells after 48 hours in a cell culture E-plate with interdigitated micro-electrodes integrated into the bottom.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 35. Cells incubated with 5mg/ml Birch pollen extract after 48 hours in a cell culture E-plate with interdigitated micro-electrodes integrated into the bottom.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 36. Cells incubated with 25mg/ml European alder pollen extract after 48 hours in a cell culture E-plate with interdigitated micro-electrodes integrated into the bottom.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 37. Cells incubated with 25mg/ml Walnut pollen extract after 48 hours in a cell culture E-plate with interdigitated micro-electrodes integrated into the bottom.
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 38. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - Birch 25mg/ml, blue - Birch 5mg/ml, pink - Common hazel 25mg/ml, turquoise - Common hazel 5mg/ml, purple - European alder 25mg/ml, brown - European alder 5mg/ml, dark green - European hornbeam 25mg/ml).
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Figure 39. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - European hornbeam 5mg/ml, blue - Korean fir 25mg/ml, pink - Korean fir 5mg/ml, turquoise - Scots pine 25mg/ml, purple - Scots pine 5mg/ml, brown - European silver fir 25mg/ml, dark green - European silver fir 5mg/ml).
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 40. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - Walnut 25mg/ml, blue - Walnut 5mg/ml, pink - Maple 25mg/ml, turquoise - Maple 5mg/ml, purple - Lime tree 25mg/ml, brown - Lime tree 5mg/ml).
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 41. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - Horse chestnut 25mg/ml, blue - Horse chestnut 5mg/ml, pink - European ash 25mg/ml, turquoise - European ash 5mg/ml, purple - European chestnut 25mg/ml, brown - European chestnut 5mg/ml, dark green - Elder 25mg/ml).
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Figure 42. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - Elder 5mg/ml, blue - Common timothy 25mg/ml, pink - Common timothy 5mg/ml, turquoise - Orchard grass 25mg/ml, purple - Orchard grass 5mg/ml, brown - Golden foxtail grass 25mg/ml, dark green - Golden foxtail grass 5mg/ml).
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 43. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - White willow 25mg/ml, blue - White willow 5mg/ml, pink - Greater plantain 25mg/ml, turquoise - Greater plantain 5mg/ml, purple - Corkscrew hazel 25mg/ml, brown - Corkscrew hazel 5mg/ml).
Provided by Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

Figure 44. Pores of pollen.
Provided by Ass.-Prof. Dr. Karl Ettinger, Department of Earth Sciences, University of Graz, Austria.

Figure 45. Ruptured pollen, releasing starch granules (Coomassie blue).
Provided by a.o. Univ.-Prof. Mag. Dr. Otto Schmut and Dr. Dieter Rabensteiner, Department of Ophthalmology, Medical University of Graz, Austria.

3. List of Tables

4. Abstract in German

Hintergrund

Immer häufiger kann auch bei Nicht-Allergikern das Auftreten allergieähnlicher Symptome während der Pollenflugsaison beobachtet werden. Diese Erkrankung wurde als saisonales nicht-allergisches Konjunktivitis (SNAK) - Syndrom beschrieben. Haselnuss- und Birkenpollen sind bekannt dafür, menschliche Tränenflüssigkeit und Bindehautzellen zu schädigen. Wir haben den Einfluss weiterer Pollenspezies von unterschiedlichen Pflanzenfamilien auf menschliche Tränenflüssigkeit und Bindehautzellen untersucht.

Material und Methoden

Es wurden Pollen von unterschiedlichen regionalen Pflanzenfamilien (Adoxaceae, Betulaceae, Fagaceae, Juglandaceae, Malvaceae, Oleaceae, Pinaceae, Plantaginaceae, Poaceae, Salicacea, Sapindaceae) gesammelt. Um die proteolytische Aktivität der Pollen nachzuweisen, wurde eine Zymographie durchgeführt. Anschließend erfolgte die Inkubation menschlicher Tränenflüssigkeit mit Pollenextrakten und eine Untersuchung mittels Polyacrylamid Gel Elektrophorese (PAGE). Darüber hinaus wurden auch kultivierte menschliche Bindehautzellen (CHANG Zellen) mit Pollenextrakten inkubiert. Zytomorphologische Veränderungen wurden mikroskopisch und die Zellvitalität und Zelltod mittels Proliferations (MTS), Wasser löslichem Tetrazolium Salz (WST-1) und Laktat Dehydrogenase (LDH) Test, sowie dem Impedanz-basierten xCELLigence real time analysis system (RTCA) quantifiziert.

Ergebnisse

In der Zymographie zeigten verschiedene Pollenspezies Proteaseaktivität unterschiedlicher Muster. Die PAGE ergab, dass die Enzyme verschiedener Pollenspezies die Proteine der menschlichen Tränenflüssigkeit zerstören. Die Behandlung von Bindehautepithelzellen mit Pollenextrakten führte zu zytomorphologischen Veränderungen und einer meist deutlichen Verminderung der Zellvitalität, abhängig von der Konzentration des Pollenextrakts, sowie der Inkubationszeit.

Schlussfolgerungen

Pollen sind weit verbreitet in der Umwelt. Trotz anti-allergischer Therapie, leidet eine wachsende Anzahl von Menschen unter erheblichen Beschwerden während der Pollensaison. Pollenspezies unterschiedlicher Pflanzenfamilien zeigen eine deutliche proteolytische Aktivität, zersetzen menschliche Tränenflüssigkeit und schädigen Bindehautzellen, wodurch diese eine entscheidende Rolle bei der Pathogenese des SNAK-Syndroms spielen könnten.

5. Abstract in English

Purpose

Symptoms of ocular allergy can also be observed among proven non-allergy sufferers during pollen seasons. This disease was described as seasonal non-allergic conjunctivitis (SNAC) - syndrome occurring due to the influence of pollen enzymes. Hazelnut und birch pollen are known to degrade human tear fluid and conjunctival cells. We investigated the effect of further pollen species from different plant families on human tear fluid and conjunctival cells.

Material und Methods

Regional pollen species from different plant families (Adoxaceae, Betulaceae, Fagaceae, Juglandaceae, Malvaceae, Oleaceae, Pinaceae, Plantaginaceae, Poaceae, Salicacea, Sapindaceae) were collected. The proteolytic activity of the pollen was evaluated by Zymography. Subsequently, polyacrylamide gel electrophoresis (PAGE) of human tear fluid incubated with different pollen extracts was performed. Furthermore, human conjunctival cells (CHANG cells) were incubated with pollen extracts. Changes in cytomorphology were analysed by microscope and cell viability and cell death were assessed by a proliferation (MTS), a water-soluble tetrazolium (WST-1) and a lactate dehydrogenase (LDH) assay, as well as by the impedance based xCELLigence real time analysis system (RTCA), respectively.

Results

Zymography revealed protease activity of different pollen species, presenting diverse zymographic patterns. PAGE revealed the degradation of tear fluid proteins by pollen species from different plant families. Conjunctival cells incubated with pollen extracts typically showed cytomorphological changes and a decrease in cell viability. The degree of cell damage was dose- and time-dependent.

Conclusion

Pollen are widely present in the environment and an increasing number of people suffers from substantial complaints during flowering seasons, despite receiving common anti-allergic treatment. Pollen species from different plant families exert proteolytic activity and degrade human tear fluid as well as conjunctival cells, which may play a crucial role in the pathogenesis of SNAC.

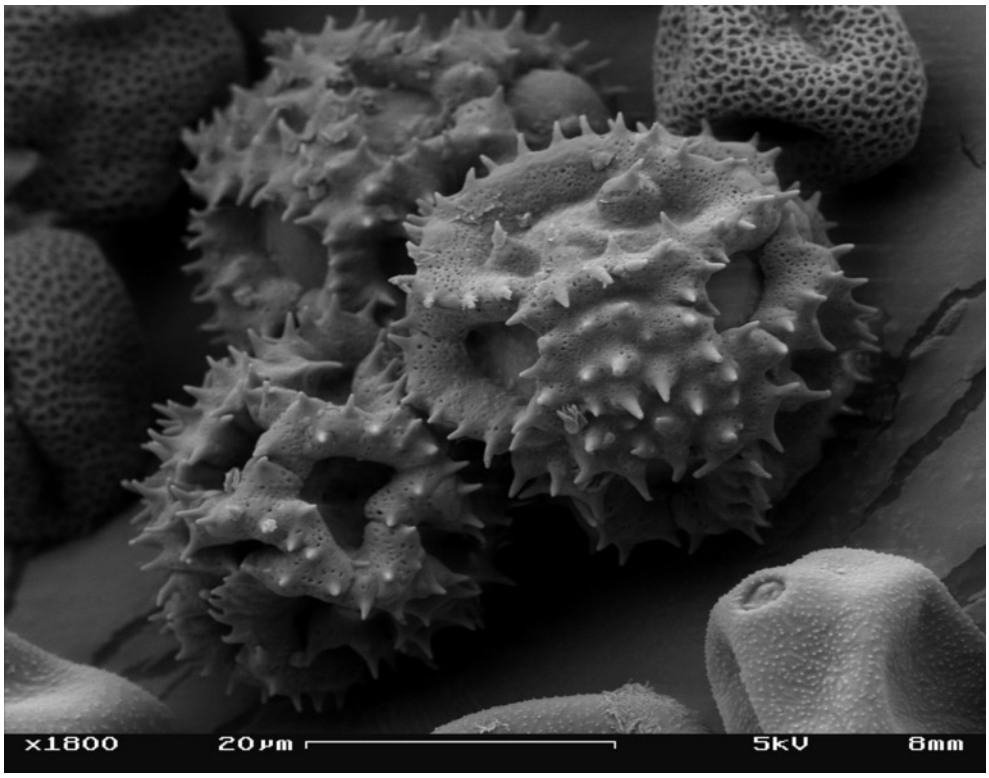
6. Introduction

Many people complain about itchy, reddened, sticky and watery eyes during the pollen-seasons. These symptoms frequently affect proven non-allergy sufferers and patients suffering from ocular surface disease as well. Lately, the existence of a so-called seasonal non-allergic conjunctivitis (SNAC) syndrome was proposed [1,6-11].

6.1 Pollen

During the last decades, increasing data is implying that pollen-induced allergic reactions become more prevalent in Europe, affecting approximately 40% of the people and thus having a substantial impact on the health care system [7,8]. Currently, there is no data on the European prevalence of pollen reactions affecting the eye available.

Figure 1. Electron microscopic images of different pollen species.



In Europe, the primary flowering season has a duration of about 6 months, beginning in spring very often lasting until late autumn [8].

From an allergological point of view, according the dissemination of airborne pollen species, there are five zones of vegetation throughout Europe. In the Arctic zone Birch pollen are present almost exclusively. In the Central zone, there are pollen of Birch, Deciduous forest and grasses. The Eastern zone comprises grasses, Mugwort and Ragweed pollen. The Mountains zone has mostly grass pollen, showing a delay in their season of pollination by 3-4 weeks, compared to grasses as at the level of sea. In the Mediterranean zone pollen of grasses, Cypress, Olive and Parietaria are present [8].

6.1.1 Nomenclature of allergen

The history of allergen nomenclature began with the use of "classical" biochemical techniques to fractionate allergens. Usually the researcher decided on the name of the fraction, which presented the highest allergenic potential.

During the 1940s to the 1950s the purification of allergens of house-dust and pollen grains was attempted by the use of electrophoretic techniques, salt precipitation and phenol extraction. Ten years later the "antigen E" of Ambrosia (ragweed) was the first allergen, which could be purified by the aid of ion exchange and usage of gel filtration media. Due to the reaction in one of five precipitin lines (labelled A–E) with polyclonal rabbit antibodies to Ambrosia in an Ouchterlony immunodiffusion test King and Norman decided on the name it that way. They revealed that antigen E was a strong allergen [9,10].

Allergens within the same genus or closely related genera that relate to each other according to their structure, but originate from different species are termed "Group". 40% up to approximately 90% of the sequence of amino acids can be identical. Possible functions on biology, as well as the tertiary structure are considered once defining a group of allergens. Group 2 mite allergens are a good example (Der f 2, Der p 2 and Lep d 2, Gly d 2 and Tyr p 2) show 40% to 88% of homology, and the group 5 Ambrosia allergens (Amb a 5, Amb t 5, and Amb p 5) show approximately 45% of homology [10-13].

6.1.2 Grass (Gramineae)

Although the prevalence of grass pollinosis varies in different areas, allergies to Gramineae pollen are known to be the most common pollen allergies in Europe and in many parts of the world [8].

Group 1 and group 5 allergens comprise the primary allergens of grass pollen. In 1992 Valenta et al could show that up to 95% of the subjects had specific IgE for group 1 and 80% of them for allergens belonging to group 5 [8,14].

Grass pollen antigens, as well as antigens from other allergenic grains of pollen can be released through pollen pores or fissures very quick, as soon as pollen are hydrated by body fluids on the mucosae of the eye, mouth or nose for example. Thus they are able to induce symptoms of hay-fever in patients who have been sensitized before [8].

The amount of Gramineae pollen in the ambient air is known to have an influence on the severity of symptoms in patients suffering from hay-fever. In the United Kingdom 10–50 grains/m^3 grass pollen in the atmosphere was already able to induce allergic symptoms [8,15]. Ten percent of the subjects sensitized to the pollen of Gramineae developed symptoms, when 10 grass-pollen grains/m^3were in the air, whereas more than 50 grains/m^3 were shown to induce symptoms in all of them [8,16,17]. A Spanish study of Antépara et al in 1995 found, that a pollen concentration over 37 grass-pollen grains/m^3 induced symptoms in all of the patients known to suffer from pollinosis [8,18]. Also, a Finish group of researchers was able to show, that at the begin of the pollen season, nasal symptoms were significantly associated with a concentration of less than 30 grass-pollen grains/m^3 [8,19].

The flowering period of the grasses (including at least 600 types and more than 10.000 species) in central, eastern and northern Europe begins in May and lasts till late July. Normally, one month earlier the Mediterranean pollination season starts.

In summary, the flowering season at the sea level starts two to three week earlier. The flowering period varies every year, due to the variability of the climate. The concentration of Gramineae pollen typically peaks one to two months after the start of the main season of pollination. In Europe grass pollination usually has its peak in June [8].

There is a decrease in the annual count of grass pollen. This might be due to alterations of the land use, as well as practices in agriculture, which reduced the amount of grasslands throughout Europe during the last decades. Nevertheless, there cannot be seen a decrease in the frequency of allergic grass-pollen sensitization in people [8,20].

Recent evidence suggests that, additionally to conjunctival and nasal symptoms, an exacerbation of asthma by the induction of an inflammatory mast- and T-cell, as well as eosinophil response can be triggered through the natural grass pollen exposure [8,21].

6.1.3 Trees

The most important allergenic tree-pollen of central, eastern and northern Europe are Birch (Betula) pollen. In Mediterranean regions, pollen of the Olive (Olea europaea) and Cypress (Cupressus) trees are known to be highly allergenic [8].

Fagales

The Fagales comprise three different families: the Betulaceae, the Corylaceae and the Fagaceae. Typically, they show high levels of cross reactivity according their allergenic potential, similar to the Gramineae [8].

In the north of Europe Betula is one of the trees producing a huge amount of allergenic pollen [8,22]. In the western European regions, the pollination season normally starts in late March, whereas in central and eastern Europe, it starts in early to the middle of April. In the north pollination begins in late April to the end of May, depending on the degree of latitude [8]. The pollen concentration peaks after 1-3 weeks. The length of the season depends on the temperature and therefore, can differ about 2-8 weeks annually. Depending on the region, there have been observed alternating periods of low and high pollen release, as well as differences in the duration of the flowering period every other year [8,23].

In Europe Corylaceae trees, Hazel and Alder (December-April), followed by Betula, Horn- and Hop hornbeam are the first to release their pollen grains. Alder and Hazel, showing an early flowering period and cross reactivity, are thought to be able to prime the allergic

sensitization to allergens of the Betulaceae family. Consequently, during the season of pollination of the Birch, clinical symptoms may get worse [8].

In central, eastern and western European areas the flowering period of the Birch can be extended by the pollination of the Oak. The season of the Oak is normally rather benign and starts before the flowering of the Beech. The pollination of the Sweet-chestnut happens in June and July in central and western Europe, as well as in the mountainous areas of southern Europe. In Alpine regions, the highest concentrations of Alnus viridis pollen can be found at the end of May and in early June [8].

Birch trees, Alder trees as well as Hazel trees are the most likely to induce different kinds of allergic disorders. A skin prick test positive to birch allergens could be found in 5% of The Netherlands' population and in up to 54% of the Swiss people. Due to the current developments in architecture and design, Birch has recently become popular as an ornamental plant. Especially, in northern Italy this seems to have led to an increased sensitization to Birch allergens[8]. Eriksson and Holmen performed a study on allergenic cross-sensitization. The study population comprised asthma and rhinitis patients, being older than 18 years of age. They could show, that there is often an association between Betula pollen allergens with allergens from other plants, who are known to primarily induce allergic symptoms affecting the nose. Furthermore, they observed, that respiratory symptoms mostly appeared at the beginning of the third decade of life [8]. An article by Bjorksten et al. on a possible association of being born in the flowering season of the Birch and the appearance of an allergy to these kinds of pollen, especially affecting the respiratory system, could not be confirmed until today [8,24].

Oleaceae.

Olea pollen often cause pollinosis in southern countries, like Spain, Greece, Italy, and Turkey as well [8,25-28]. The main flowering period lasts from the beginning of April to late June. Due to an improvement in diagnostics by altered agricultural practices the rate of allergy to Olea pollen seems to be on the rise [8,29]. Interestingly, changes of the environment, as well as the management of crops of the olive tree and its various cultivars are inducing changes concerning the allergenic potential of the plants [8,30].

In Olea europaea pollinosis the conjunctiva, as well as the nose are usually affected more often than the bronchia [8,29].

Interestingly, the frequency of a positive skin prick test to pollen of the Olive tree in adults and children having some sort of positive skin prick test in southern Italy is 13.49% and 8.33%, respectively [8]. In most of the patients there could be observed an association with other pollen grain derived allergens [8,29].

Patients who are sensitized to Olea pollen only, often show symptoms during the whole year. Interestingly, in Italy symptoms normally are not increasing in the flowering period of the olive. Florido et al. and Kirmaz and colleagues reported the same for Spain and Turkey [8,25,28]

The Oleaceae family also comprises other pollen species, like Fraxinus excelsior or Ligustrum vulgare. On the contrary, these hardly induce symptoms in sensitized subjects [8,31,32].

Cupressaceae

Cupressus arizonica, lusitanica, macrocarpa and sempervirens are the common species of the genus of the Cupressus. Every cypress is able to produce a considerable amount of anemophilous pollen. A significant part of the annual airborne pollen in Mediterranean regions is released by cypresses.

In the winter Cupressaceae pollen denotes for nearly one third of the pollen counted in Spain. Annually 20-40% of the total pollen count in Albania and Italy are released by cypresses [8,33-35].

During the last decades the prevalence of cypress pollinosis increased in southern countries [8,36-39]. In winter, where no other plant is shedding pollen, it is able to induce a so-called winter pollinosis [8].

The pollination of the cypress shows micro-sporophyllous maturation. The pollen mature from the plant's base to the top of it. Therefore, the flowering season can take more than a month. Furthermore, the pollen season is depending on meteorological factors and thus

shows a high annual inconsistency. The beginning and the length of the flowering of the Cupressus is hard to detect. Therefore, an Cypress allergy is very often problematic to treat [8].

Within the family of the Cupressacace (Cupressus, Cryptomeria and Juniperus) and among Taxacee a high cross-reactivity exists [8,40-42]. Since the flowering period of the Taxacee can overlap as well as precede the pollination of the Cypress, it can be difficult to distinguish which plant is causing allergic symptoms [8].

There is a high variability in the rates of sensitization to cypress pollen, ranging from 2.4% to 9.6% in the general population. In 2005 Charpin et al. reported sensitization rates of over 30% in pollinosis patients [8,43]. Allergies to cypress pollen are gaining importance, due to the use of Cupressus arizonica extracts and an increased awareness to cypress pollinosis, which otherwise could easily be misdiagnosed as viral infection because of its occurrence at the end of winter [8].

Compared to grass pollen allergy, in Cupressaceae pollinosis the prevalence of conjunctivitis is rather low compared to the prevalence of dry cough [8,43]. Nevertheless, there is a low prevalence of asthma in these subjects [8,44]. There have been reported positive results concerning the subcutaneous as well as the sublingual immunotherapy in patients suffering from allergies to cypress pollen [8,43].

6.1.4 Weed

Urticaceae

Concerning their allergenic potential, the genus Parietaria with the species Parietaria judaica and officinalis represent the most important species of the nettle family. Although they have been discovered in the United Kingdom as well, they can mostly be found in the coastal regions of the Mediterranean areas. [8,45].

Allergy to Parietaria pollen is rare in subjects younger than ten years of age [8,46]. Parietaria pollinosis is more common between the second and third decade of life, especially in towns located at the coast compared to the rural regions. Recently, there has

been reported an increased reactivity to Parietaria pollen in Europe and the United States [8,47,48].

The pollen of Parietaria is responsible for a multiseasonal symptomatology in Mediterranean regions, due to its long atmospheric persistence [8,49].

In their retrospective cohort study, Polosa et al reported, a significant increase in the risk to develop asthma, if subjects were sensitized to Parietaria judaica. They did not find any association for other pollen or house dust mite sensitizations [8,50].

In central and southern Italy about half of the subjects, monosensitized to Parietaria pollen, suffer from asthma or cough associated with rhinoconjunctivitis [8,51].

Although not reaching statistical significance, around Naples a lot of the patients monosensitized to Parietaria pollen were born during the respective pollen season. Therefore, they might have been exposed to this pollen species early [8]. During the last 15 years pollinosis symptoms in atopic subjects and particularly the number of positive skin prick tests to Parietaria spp rose [8,51]. There have been reported two severe cases of allergic reactions to orally consumed pistachio nuts due to cross-reactivity, in subjects mono sensitized to Parietaria [8,52].

Parietaria pollen is known to persist for a long time in the air. Therefore, it is often difficult to treat this type of pollinosis. There is evidence supporting the specific immunotherapy by the sublingual route [8,53,54].

Compositae

Comprising almost 20.000 species, the Compositae (Asteraceae) represent the largest plant family. Ragweed (Ambrosia) and Mugwort (Artemisia) are most likely to induce pollinosis.

Concerning Artemisia, well-known species are Artemisia annua and verlotorum, growing typically in the south of Europe and the species Artemisia vulgaris, which is growing in every part of Europe, in suburban as well as urban regions. The flowering period is from the end of July to the late August [8].

The genus Ambrosia, includes both Ambrosia artemisiifolia (short or common ragweed) and Ambrosia trifida (giant ragweed). It has been recognized as a major cause of allergic rhinitis for long. A sensitization to ragweed could be found in up to ten percent of the people in a random skin test study of a large population in the United States [8,55]. A recent study was able to correlate symptoms of asthma and rhinitis, as well as peak expiratory flow rates with the amount of Ambrosia pollen in the air [8,56].

The seasonal flowering periods of Ragweed and Mugwort are nearly the same. They also show increased cross-reactivity.

IgE reactivity for Ambrosia and Artemisia on Radio-Allergo-Sorbent-Test (RAST) and/or skin prick tests essentially identifies a co-sensitized subject, which is important for performing immunotherapy [8,57].

Short and Giant ragweed are known to show extensive cross-reactivity. Nevertheless, it has recently been suggested, that they are not equal in an allergological point of view. Therefore, diagnostic procedures and potential immunotherapy are ideally adapted to the Ragweed species growing in the corresponding surroundings [8,58].

6.1.5 Decorative plants

The dissemination of decorative plants has steadily increased during the last century. They can be found nearly everywhere, in the public, in offices and in homes. These plants are thought to provide aeroallergens right in our proximity, especially indoor plants. Recent data suggests, that the standard skin prick test should include these plants [8].

In 1987 Axelsson et al reported the first case of an allergic reaction to Weeping fig (Ficus benjamina) in gardeners [8,59]. There have been published numerous cases in the general population and even in non-atopic people [8,60,61]. Two-point-five percent of 2,662 patients with a skin test positivity to any aeroallergens, showed a reaction to Weeping fig [8,62]. The allergen was found to be in the juice of the plant [8,63]. The latex of Weeping fig can also trigger a Ficus-fruit syndrome by cross-reaction [8,64].

A recent study of 59 patients suffering from chronic rhinitis, who got in contact with indoor ornamental plants in a home environment, demonstrated a 78% sensitization to a decorative plant (e.g. Geranium, Ivy, Palm tree, Weeping fig and Yucca) [8,65].

6.1.6 Mediterranean allergenic pollen

The Mediterranean vegetation differs from other European regions, due to its dry summers and mild winters. Typical plants, who are producing allergenic pollen, are Cupressaceae, Oleaceae and Parietaria [8].

During the last decades in most parts of Europe, there has been established an increasing number of pollen-counting stations to form an observational network for further aerobiological and allergological studies [8]. The atmospheric pollen content was sampled at different Mediterranean regions. Therefore, three flowering periods were discovered [8,66]:

1. A mild winter pollen season (December-March) evident by Acaciae (mimosa), some Betulaceae, Corilaceae (hazel) and Cupressaceae (cypress and junipers) pollen [8].

2. An aggressive spring-summer pollination period (April-July) led by the flowering of Gramineae, Oleaceae and Parietaria. Some flowers in the areas of southern France and Spain, especially Platanus flowers, possess allergenic potential. Their pollination periods overlap from March to May [8].

3. A less noticeable summer–autumn season (August-October) with a small peak of Gramineae, Parietaria as well as herbaceous plants, like Artemisia and Goosefoot [8].

Throughout Europe, including the Mediterranean area, Gramineae pollinosis is the most important allergy to pollen. Depending on the population, IgE serum antibodies to Gramineae pollen allergens could be identified in 8% to 35% of young adults in Europe [8,67]. Interestingly, some studies could show that in several European cities on the one hand the concentration of the pollen of Gramineae in the atmosphere is falling, whereas on the other hand the number of people suffering from allergic rhinitis and asthma is on the rise [8,68]. The shrinkage in grassland over large areas of the continent has been accounted

responsible for the decrease in grass pollen concentrations. During the last three decades a significant reduction in areas covered by grass of approximately 40% could be observed [8,68]. An increased number of patients suffers from asthma and allergic rhinitis due to Gramineae pollen, which might triggered by environmental influences, like an increased air pollution [8,29,68,69].

The most allergenic pollen of the Oleaceae family in Mediterranean regions is produced by Olea europaea. It represents one of the most important reasons for seasonal respiratory allergy [8,40]. The flowering period starts in April and last till late June, occasionally causing severe pollinosis, affecting the eye, nose and bronchi as well. Oleaceae, like Betulaceae alternatingly produce a high and low amount of pollen every other year. These rhythm may be altered or even concealed by environmental influences, like the weather [8].

Since subjects with Olea pollinosis frequently show other atopic sensitizations (especially to Gramineae), it is often hard to find out, if the sensitization to pollen of Oleaceae or Gramineae predominates, [8].

Interestingly, the clinical symptoms of patients sensitized are often not restricted to the flowering period (May - June). They strangely occur during the whole year [8].

Betula, being the most powerful of the trees producing allergenic pollen in the north of Europe, is disseminating into the Mediterranean regions. Betula has also been used more often in modern urban gardens during the last years, especially in the north of Italy. The pollination of northern Betula normally peaks in May. In the south of Europe it can be measured in April. Spring-pollinating plants pollinate earlier in a warmer climate, whereas autumn-pollinating types show a reversed pattern [8,66].

The flowering of the cypress varies from one region to another. Increased concentrations could be observed in Mediterranean coastal regions. The Cypress pollinosis often affects the eyes and nose as well. In the cities of the Mediterranean regions, cypress pollen represent the most important allergen of the winter transported by air [8].

In the same way as the spreading of the birch, due to the increased popularity of Cupressaceae plants as an ornamental plant and for replanting purposes, this species has a growing impact on the epidemiology of pollinosis [8,70].

Although herb derived pollen like those from Mugwort and Plantain are of reduced importance, they may at least have some clinical relevance in the future. Mugwort for example has a marked sensitizing capacity. Ambrosia, which is almost spreading across all European regions, belongs to the same Compositae family [8].

6.2 Ocular Surface

The ocular surface comprises distinct highly specialised tissues, acting as an immune-competent barrier against environmental influences and infectious agents [71-73].

Pollen reactions primarily affect the tear film and the ocular surface. The surface of the cornea, the surface of the conjunctiva and the lid-margin, predominantly the areas where pollen attach around the eye (Figures 2-4).

Figure 2. Eye following exposure to airborne pollen.

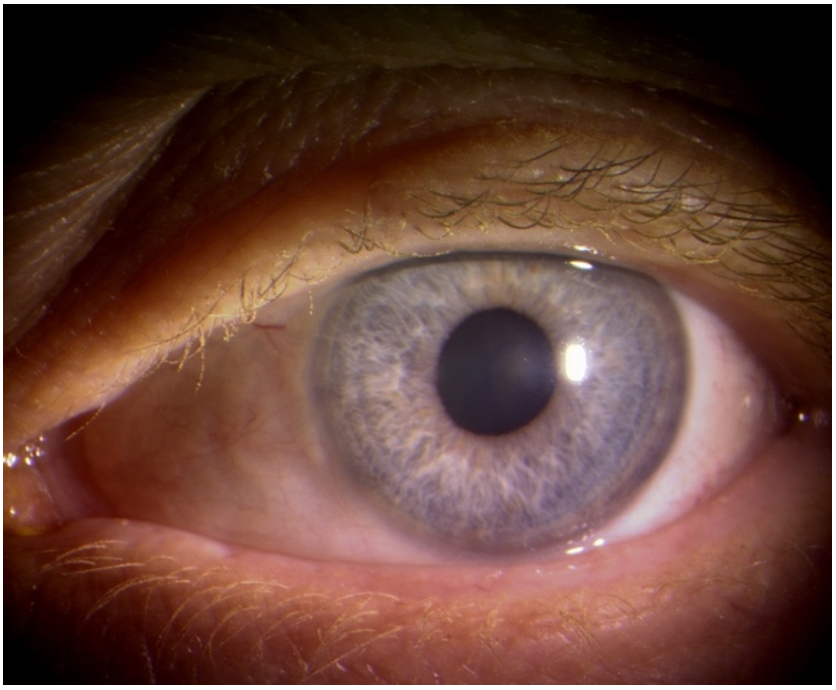


Figure 3. Pollen on the lashes.

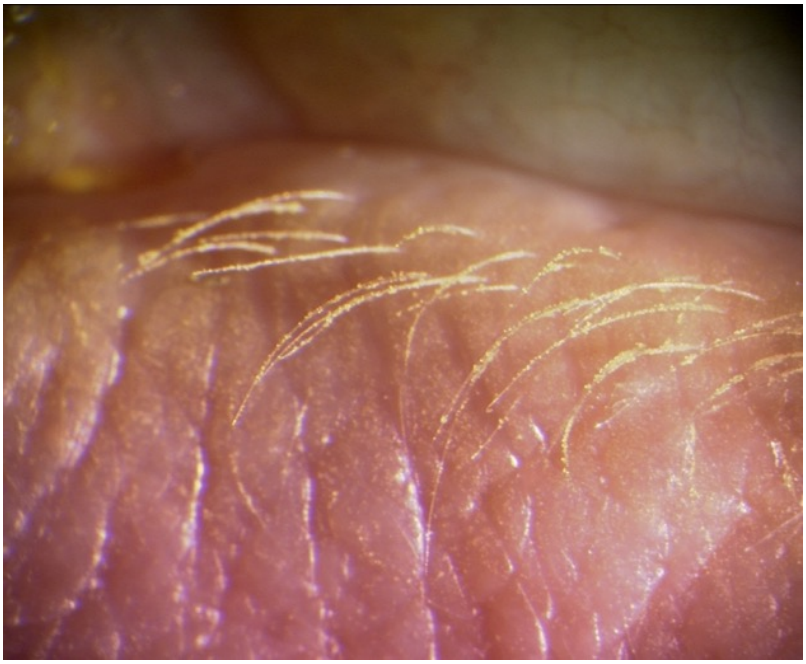
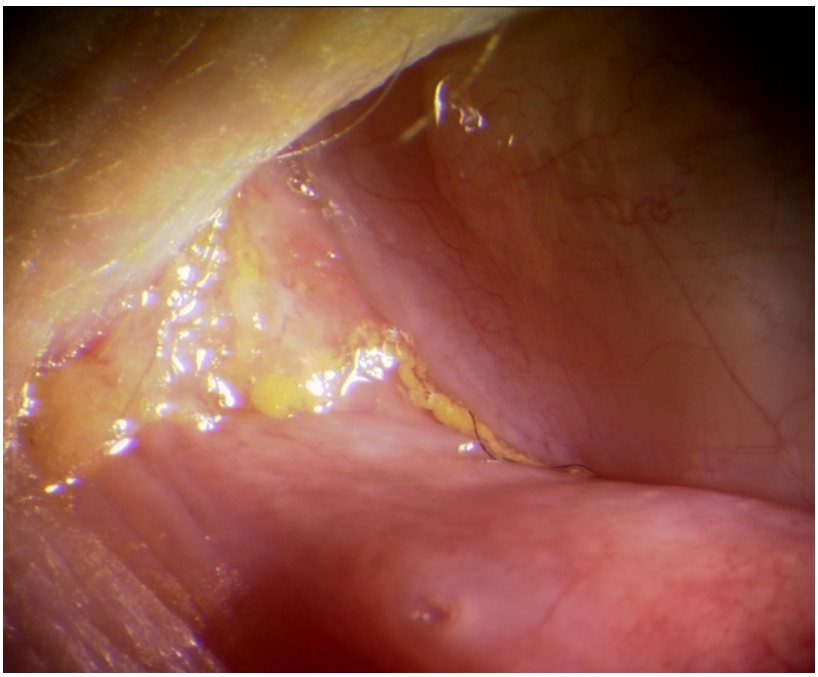


Figure 4. Pollen in the tear film.



6.2.1 Cornea

The cornea consists of multiple different layers and has to fulfil several physiological functions [71-73].

The corneal epithelium consists of non-keratinizing squamous epithelial cells, which are arranged in five to seven layers, resting on a 40-60µm thick basement membrane, called the Bowman's layer. Due to the constant interaction with the tear film, the superficial corneal epithelial cells are expressing mucin. Mucin not only plays an important role in providing a smooth optical surface, but also represents a fundamental component of the tear film itself. At the corneoscleral limbus the corneal epithelial cells blend with the conjunctival epithelial cells. Usually, the stem cells located at the corneoscleral limbus provide a lifetime source to replace and renew the corneal epithelium, whenever necessary [73,74].

The thickest layer, the corneal stroma is composed of distinctive collagen fibres and proteoglycans. They are produced, preserved, and renovated by scattered keratocytes. The collagen fibres are arranged with respect to one another and create a strong extracellular matrix by interacting with the proteoglycans. Preferably, the corneal stroma should not scatter light and thus allow the transmittance of more than 99% of the visible light [73].

The basement membrane for the corneal endothelium is called Descemet's membrane. In adults, it has a thickness of 10 to 15µm. It consists of an anterior banded zone, already deposited in-utero, and a posterior none-banded zone, laid down by the corneal endothelium. The Descemet is composed of a delicate latticework of collagen fibrils and corneal endothelial secretes. It is known to increase in thickness throughout life [71,73].

The corneal endothelium represents the innermost part of the cornea and is formed by a monolayer of non-replicating endothelial cells. These cells are essential to the maintenance of corneal transparency, while acting as a permeable barrier between aqueous humour and the corneal stroma, enabling the entry of nutrients into the avascular corneal. By metabolic pumps, ion transporters, and channels osmotically removing water from the cornea they maintain a level of stromal hydration compatible with the corneal transparency [73].

The avascular cornea is serving as a transparent optical tissue and environmental barrier as well [73].

6.2.2. Conjunctiva

The conjunctiva consists of a surface epithelium and a substantia propria [73].

The epithelium is just two to three cell layers thick and packed less regular than the corneal epithelial cells. Cuboidal stratified cells rest on a thin basement membrane, harbouring numerous organelles, particularly mitochondria. Their cell membranes show plenty microvilli and infoldings [73].

The substantia propria, the conjunctival connective tissue, serves as a reservoir of immune cells and contains numerous vessels. Mast cells, neutrophils and plasma cells, representing the cellular immune response, can be found [73].

It is also possible to divide the conjunctiva anatomically by different attachment zones:

The inner surface of the eyelids is covered by the palpebral conjunctiva, whereas the bulbar conjunctiva lies on the eye globe itself. Both parts are connected with each other and build the conjunctival fornix.

A distinctive portion of the palpebral conjunctiva adheres tightly to the underlying tarsus and therefore is referred to as tarsal conjunctiva. It cannot be separated, even not by sharp dissection. On the superior edge of the upper tarsus, as well as the inferior edge of the lower tarsus the palpebral conjunctiva adheres to the Müller's muscle and the lower eyelid retractors, respectively [73].

The globe is covered by the bulbar conjunctiva, which lightly adheres to the sclera. Parallel connective tissue fibres of Tenon's capsule obliquely insert into the bulbar conjunctiva, providing the anatomic arrangement, which is necessary for motility of the globe and a permanent coverage of the ocular surface by a smooth continuous mucosal surface [73].

A healthy conjunctiva protects the ocular surface from pathogens as a physical barrier and a source for immune cells. Moreover it plays a crucial role in tear film physiology by providing the mucus phase, as the inner layer of the tear film [71-73].

6.2.3. Stem Cells of the Ocular surface

The ocular surface epithelium is in a constant process of renewing. Approximately, every seven to ten days the corneal epithelium is completely renewed by stem cells. They are present in all self-renewing tissues. Usually they show a striking potential for clonic cell division and a long life. The proliferation should be essentially error-free, which is achieved by low mitotic activity and asymmetric deoxyribonucleic acid (DNA) segregation. This enables stem cells to keep their original DNA, while a new copy is passed along to the daughter cell. Normally, the poorly differentiated stem cells reside under special anatomic protection. In case of the corneal epithelium, stem cells can be found at the corneoscleral limbus. The limbal epithelial histology is characterized by approximately ten cell layers, in contrast to two to three cell layers of the conjunctival epithelium and seven to eight cell layers of the corneal epithelium. According to the keratin-like protein contents, limbal epithelial cells represent an intermediate between the corneal and conjunctival epithelium. The undetermined limbus stem cells produce corneal and conjunctival cell lines as well [73].

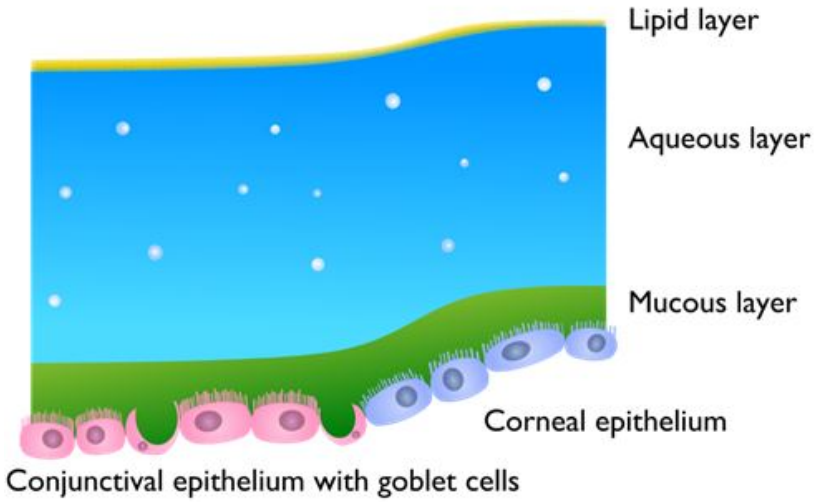
Earlier, there was the theory of conjunctival trans-differentiation as the source of corneal epithelial cells, but nowadays this has been replaced by the concept of corneal epithelial repopulation by stem cells. Normally, conjunctival cells do not repopulate the corneal epithelium, since these are not able to trans-differentiate into the phenotype of corneal epithelial cells. Nevertheless, in different disease states characterized by limbal stem cell damage or deficiency, phenotypical conjunctival cells start to repopulate the cornea, which is usually associated with corneal neovascularisation [73].

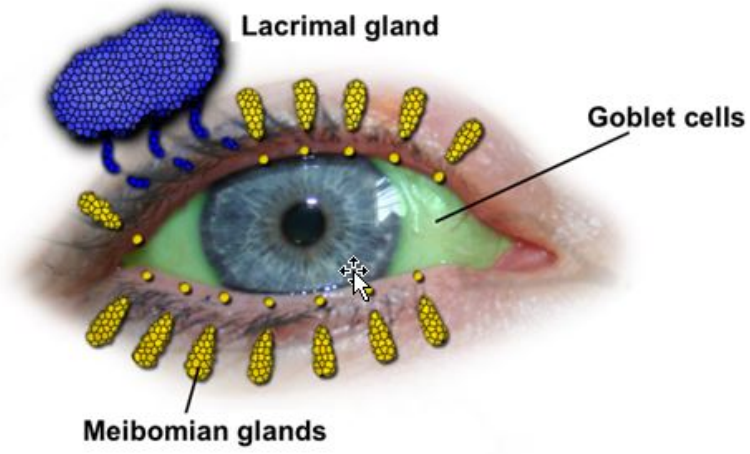
Concerning the conjunctiva, there are two independent cell kinetic systems, evolving from two separate stem cell regions on the ocular surface. The bulbar system resides at the limbus, whereas the palpebral system begins at the mucocutaneous junction of the lid-margin. Both systems are characterized by two compartments, a progenitor zone, where cells proliferate and a compartment of non-proliferating cells. Transient amplifying cells are generated by slow-cycling stem cells. The transient cells undergo cell-division before becoming mature conjunctival cells. Differentiation into conjunctival keratinocytes and goblet cells derives from a common bi-potent progenitor cell. Single conjunctival cell clones can change into goblet cells late in their life. The cells of the palpebral system stream toward the conjunctival fornices, where non-proliferating mature cells die [73].

6.3 Tear Film Physiology

The maintenance of the ocular surface integrity depends on a functioning tear film, which can be separated into three distinct layers (Figure 5). A sufficient volume and a perfectly balanced composition of all three layers of the tear film is essential to fulfil its optical and manifold physiological functions [71-73].

Figure 5. The three layers of the human tear film and their source of production.
Reproduced from [2] with permission of publisher Verlagshaus der Ärzte, Vienna, Austria





Mucous layer

The mucous layer adheres to the corneal and conjunctival epithelium. It mostly consists of glycosaminoglycans secreted by conjunctival goblet cells. Especially, the apical cytoplasm of the goblet cells contains membrane-bound mucin granules manufactured in the Golgi complex, for example MUC5AC, a large gel-forming mucin. Furthermore, corneal and conjunctival epithelial cells express membrane-bound mucin MUC1, 4 and 16, whereas some lacrimal gland cells express the small soluble mucin MUC7 [75].

By apocrine secretion goblet cells release mucin into their surrounding area. Depending on the location, the distribution of goblet cells within the conjunctiva varies. Most of them are located in the inferonasal fornix, particularly near the canalicular system. Bulbar conjunctival goblet cells are common at the temporal bulbar region. The mucus binds to the corneal and conjunctival epithelial microvilli and significantly improves the wettability of the ocular surface by its distinctive composition. Furthermore, the mucous layer is of utmost importance for the phenomenon of human tear fluid shear thinning. Shear thinning enables a variation in viscosity, resulting in a low tear viscosity at high shear rates, but very high viscosity at low shear rates. Therefore, drag during blinking is minimized and the tear film is more resistant to gravitational drainage in open eyes. Additionally, tear film mucus provides a protection against possible ocular surface micro traumas, which occur during blinking [5,73].

Aqueous layer

The main lacrimal gland and the accessory glands of Krause and Wolfring produce the aqueous layer of the tear film. The prevalence of the accessory lacrimal glands varies depending on the different regions of the conjunctiva. Krause glands are mostly found in the fornices, whereas Wolfring glands are more common in the area of the tarsal conjunctiva. Due to their difficult isolation, research on these accessory glands is still limited. Accessory secrete proteins have been compared to the proteins secreted by the main lacrimal gland and did not differ significantly. The main lacrimal gland is responsible for almost all electrolytes, proteins and water within the tear film [5,73].

Interestingly, the electrolyte concentration of the lacrimal fluid varies with flow rate. The fluid is hypertonic at a low and isotonic at an increased flow rate. Biochemical analysis detected sodium- (Na+), potassium- (K+), chloride- (Cl-), bicarbonate- (HCO3-), calcium- (Ca2+), and small amounts of other ions in lacrimal gland fluid. Except for K^+ and Cl^-, which are present in higher concentrations, the other electrolyte concentrations did not differ significantly from plasma concentrations. Different proteins have been identified in lacrimal gland fluid, like albumin, lactoferrin, lipocalin, lysozyme, epidermal growth factor, interleukins, secretory immunoglobulin A, several types of transforming growth factors and other tear specific proteins [5,73].

Also, the conjunctiva itself contributes to the aqueous layer of the tear film. The conjunctival epithelium can secrete Na^+, Cl^-, and water. Due to its large surface, it might be a significant source of electrolytes within tear fluid. Although they are able to secrete small amounts of electrolytes and water as well, the corneal epithelial cells might just play a minor role in the production of the aqueous layer of the tear film [5,73].

Lipid layer

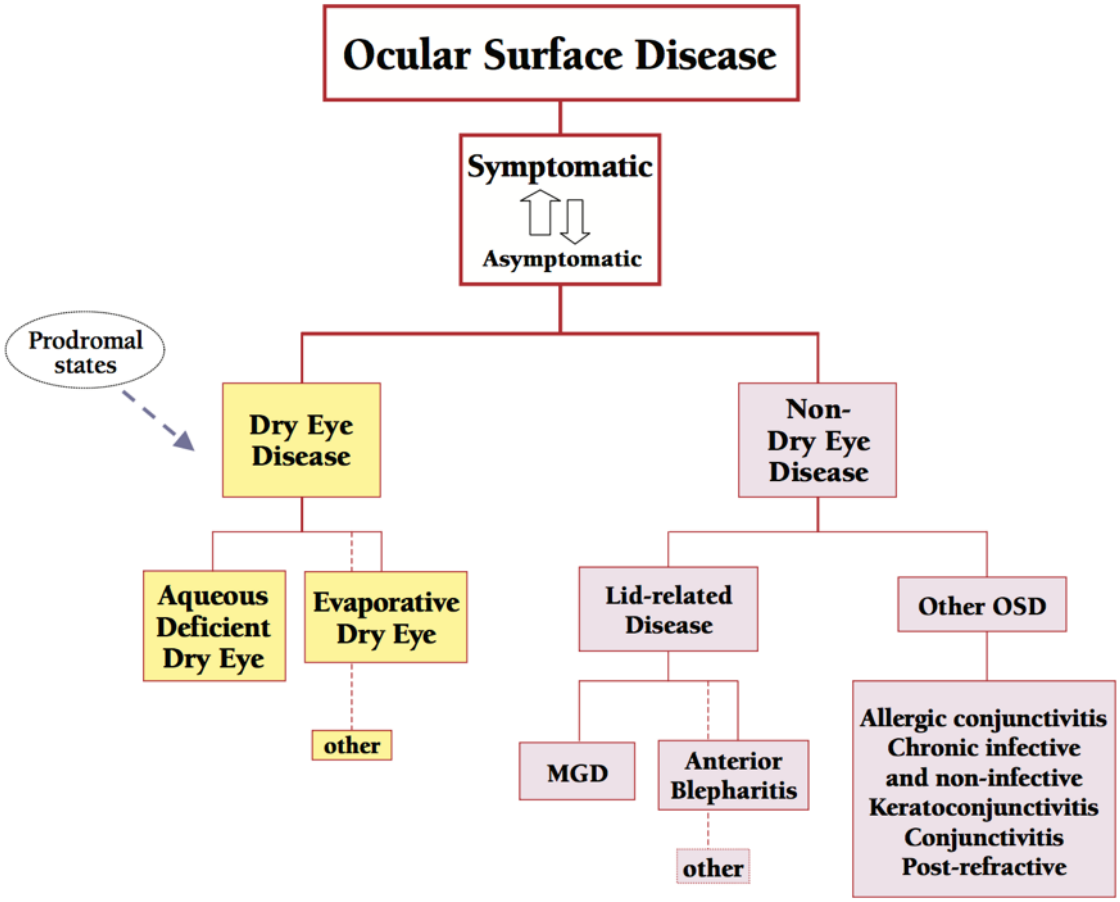
The outer lipid layer of the tear film is formed by the sebaceous secretion of the meibomian glands located along the lid margin and the glands of Zeis. The mechanical blinking action promotes the release of the secrete into the tear film [5,73,76]. The secrete is a complex mixture of different types of lipids, like wax monoesters, cholesteryl esters, (O-acyl)-ω-hydroxy fatty acids (OAHFA) and their esters, acylglycerols, diacylated diols, free fatty acids, cholesterol, and other polar and nonpolar lipids [77]. A functioning lipid layer stabilises the tear film, decreases evaporation and upholds a physiological tear break up time [5,73,76].

6.4 Ocular surface disorders

There are different kinds of ocular surface disorders (Figure 6), very often showing an overlap of symptoms. Thus, very often it is difficult to differentiate them from each other

by the clinical picture only. The incidence of some ocular surface disorders is thought to be connected to the presence of pollen [5].

Figure 6. Relationship between ocular surface disorders. Reproduced from [3] with permission of publisher Elsevier, Amsterdam, The Netherlands.



6.4.1 Allergic conjunctivitis

Allergic conjunctivitis is a common disease. Studies show that about 36% of the US population is affected and up to 64% of patients with allergic rhinitis suffer from associated ocular symptoms [78]. Williams et al suggest that allergic conjunctivitis occurs in almost 95% of the cases of allergic rhinitis, when patients are explicitly asked about ocular symptoms [79].

Hay fever (seasonal) and perennial allergic conjunctivitis are mostly IgE mediated immediate hypersensitivity reactions. Typically, the allergen is airborne, enters the tear

film and comes into contact with conjunctival mast cells that are equipped with allergen specific IgE antibodies. Consequently, degranulation of mast cells occurs and histamine and a variety of other inflammatory mediators are released. These promote vasodilation, edema, and recruitment of other inflammatory cells like eosinophils. The activation and degranulation of mast cells in a presensitized individual can be triggered very quickly, usually within minutes of the allergen exposure [80].

Very often patients with hay fever conjunctivitis also suffer from other atopic conditions, like allergic rhinitis or asthma. Usually, symptoms develop rapidly after exposure to the allergen and comprise itching, eyelid swelling, conjunctival hyperemia, chemosis, and mucoid discharge. For the clinician, intense itching is a hallmark symptom leading to the correct diagnosis. The symptoms are usually transitory and episodic [80].

Contact lens wear and a history of dry eye disease are possible contributing factors, as they can play a significant role in facilitating allergen contact with the ocular surface [5,80].

The final diagnosis of hay fever conjunctivitis is generally established clinically. Nevertheless, it is possible to perform conjunctival scraping in order to observe the characteristic eosinophils, which are not normally present on the ocular surface. If required, a challenge testing with a panel of allergens can be done as well [80].

6.4.2 Vernal Keratoconjunctivits (VKC)

Vernal, respectively springtime, keratoconjunctivitis predominately occurs in male children, who frequently, but not always, have a personal or family history of atopy. The disease may persist during the whole year, especially in tropical climates. The immunopathogenesis appears to comprise type I and type IV hypersensitivity reactions. Studies have identified the conjunctival inflammatory infiltrate in VKC as eosinophils, lymphocytes, plasma cells, and monocytes [80].

The symptoms consist of itching, blepharospasm, photophobia, blurred vision, and sometimes copious amounts of mucoid discharge [80].

The clinician differentiates two forms of VKC: palpebral and limbal.

In palpebral VKC the inflammation is located mostly on the palpebral conjunctiva, where a diffuse papillary hypertrophy, usually more prominently on the upper rather than the lower region, can be observed. Some degree of bulbar conjunctival hyperemia and chemosis may also be present. In severe cases, giant papillae resembling cobblestones may develop on the upper tarsus [80].

The development of limbal VKC can be alone or in association with palpebral VKC. It is more prevalent in hotter climates and occurs predominantly in patients of African or Asian descent. Generally, the limbus has a thickened, gelatinous appearance, with scattered opalescent mounds and vascular injection. Whitish so-called Horner-Trantas dots, representing macro-aggregates of degenerated eosinophils and epithelial cells, may be observed in the hypertrophied limbus of patients suffering from limbal VKC [80].

In VKC numerous types of corneal changes, especially associated with upper tarsal lesions, may develop. Most commonly punctate epithelial erosions in the superior and central cornea can be found. The formation of a pannus often occurs in the superior cornea and occasionally a 360° corneal vascularization may develop. The appearance of non-infectious epithelial ulcers with an oval or shield-like shape (the so-called shield ulcer) with underlying stromal opacification is possible. There has also been reported an association between VKC and keratoconus [80].

6.4.3 Atopic Keratoconjunctivits (AKC)

Differentiating VKC from AKC can be quite challenging. AKC is rarely diagnosed before puberty. It is thought to affect predominantly adults. Younger patients, presenting with AKC-like symptoms and atopic dermatitis, might usually be diagnosed with VKC [81,82].

The most prevalent clinical features of AKC are conjunctival hyperaemia and eczema, reported in up to 96% of the patients. Other clinical features include follicles, keratitis, and thickened dry skin, conjunctival papillae and Dennie-Morgan folds of the lower lid, as well as blepharitis. There is no single isolated clinical feature that can accurately differentiate between AKC and VKC [81].

Normally, VKC generally limits itself at puberty. It was recently suggested, that VKC may "evolve" into AKC in adulthood [83]. In the absence of typical clinical signs of VKC, e.g. Trantas dots, pseudoptosis, thick mucus discharge and/or shield ulcers a child with atopic dermatitis may be diagnosed with AKC and not VKC [82,83]. Children diagnosed with VKC may present with atopic dermatitis, not being a prerequisite for the diagnosis. On the contrary, evidence of atopic dermatitis has to be present for the diagnosis of AKC [82].

AKC and VKC require a specific treatment. The dermatologic manifestations of AKC need to be treated dermatologically. Although immunomodulators being effective treatments for severe AKC and VKC, lower concentrations may be required in patients with AKC [84]. Therefore, the early and accurate diagnosis of AKC and VKC is crucial for providing effective treatment strategies and improving patient outcomes [81].

6.4.4 Seasonal Non-Allergic Rhinitis / Conjunctivitis (SNAR / SNAC)

Many people complain about itchy, reddened, sticky and watery eyes during the pollen-seasons. These symptoms frequently affect proven non-allergy sufferers and patients suffering from ocular surface disease as well.

Already in 1991 Eriksson et al described several patients at the Allergy Clinic in Halmstad, Sweden suffering from rhino-conjunctival symptoms during pollen seasons. Despite of showing no signs of pollen allergy according to the common allergy tests, their periods of symptoms coincided with the pollen seasons. The disease was referred to as Seasonal Non-Allergic Rhinitis (SNAR) [85].

Wedback and colleagues compared patients with SNAR to patients with seasonal allergic rhinitis and persistent non-allergic rhinitis. They concluded, that the immunological mechanism of SNAR differed from the established allergic pathomechanism and is unknown so far [86].

In 2008 Wolf investigated the destruction of nasal mucus proteins by pollen proteases, a possible explanation for the development of SNAR [87].

Schmut et al. were first to describe the Seasonal Non-Allergic Conjunctivitis (SNAC) syndrome, which might occur due to the influence of pollen enzymes, especially proteases, on the tear film and the ocular surface. It is thought, that SNAC might induce a non-classical allergic inflammatory reaction [6,88-91].

The start of an allergic as well as inflammatory response after exposure to pollen grains is not quite understood yet. Following the hydration with body fluids, pollen release various allergens, NADPH oxidase, LTB-4- and PGE 2-like substances, proteins and enzymes. Proteases may ease the degradation of the body fluids and breakdown of epithelial barriers, acting as non-allergic inflammatory mediators [1,150-154].

There are various studies describing numerous pollen proteases. They are thought to be contributing to the pathogenesis of allergies. For example, the pollen of Ambrosia, Mesquite and Parietaria judaica are known to release characteristic proteases. Lung regulatory neuropeptides, like the vasoactive intestinal peptide and substance P can disable regulatory neuropeptides in the lung. It is speculated, that disabled bioactive peptides alter the equilibrium between contractile and relaxant properties in the respiration tract [92].

Inhaled allergens crossing the epithelial barrier are starting the sensitization process by at subepithelial dendritic cells (DCs). These are presenting allergenic peptides to naive T-lymphocytes, causing the production of specific IgE. Sampling of allergens occurs by interepithelial pseudopods of the DCs through proteins from junctional complexes of the epithelium [92,93].

It was found, that a 98-kDa aminopeptidase from P. judaica is able to degrade the intercellular adhesion proteins from tight junctions and adherence junctions. This leads to a detachment of epithelial cells [92,94]. The incubation with most allergenic pollen extracts of Ambrosia trifida, Betula, Lilium longifolium and Poa pratensis resulted in a loss of immunofluorescence labelling of tight junction proteins on epithelial cell lines. Runswick and colleagues were able to inhibit the effect by the addition of protease inhibitors [92,95].

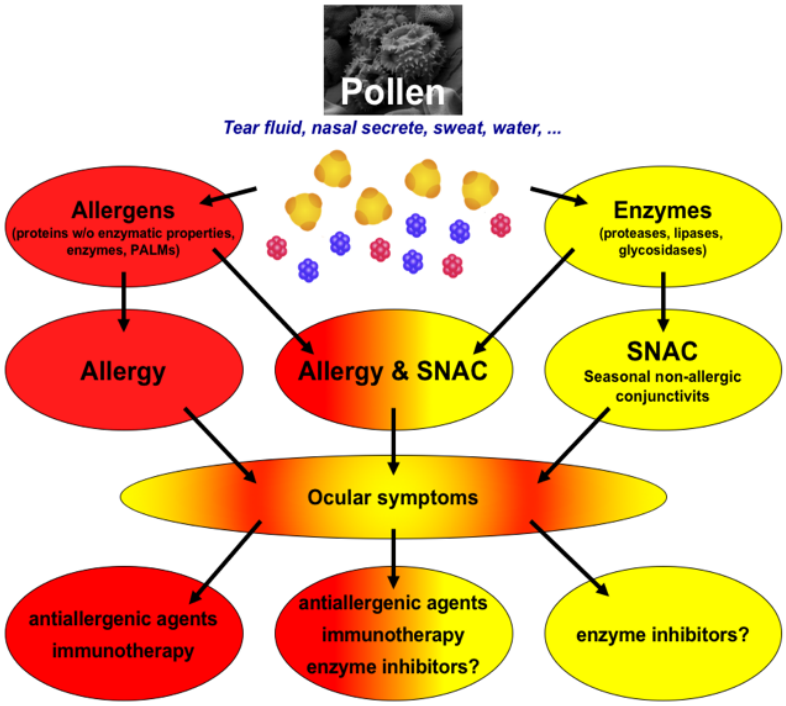
In a recent study Vinhas and colleagues analysed 4 widespread Mediterranean pollen types with different allergenic potential: Cupressus sempervirens, Dactylis glomerata, Olea europaea and Pinus sylvestris [92]. These species feature somewhat overlapping seasons of pollination in the European south. The flowering period of the Gramineae and the

Oleaceae from April to July is one of the most significant causes of pollinosis in Mediterranean regions [8,92,96]. The pollination of Cypress happens mostly December to March. It leads to high concentrations at the coasts of the Mediterranean regions, often inducing rhinoconjunctivitis [8,96].

Pine plants have been thought to have no allergenic potential, although they release large amounts of pollen throughout the whole year, peaking from March to May [92,97,98]. Nevertheless, Vinhas et al were able to show extensive proteolytic activity in these pollens. Although showing only distinct allergenic potential, these pollen grains were found to release proteases significantly. These might facilitate the sensitization to different atmospheric allergens by enabling the transportation of the allergen through the epithelial barrier and also by acting inflammatory directly [92].

Whether the enzymatic pollen reaction, affecting body fluids and mucosal surfaces, represents a separate entity or a subtype of an allergic disease has still to be determined. Mixed forms of SNAC and allergic reactions might occur as well. Figure 7 tries to illustrate the possible relationship between these two pathomechanisms.

Figure 7. Allergy and SNAC. Reproduced from [4] with permission of publisher Springer, Vienna, Austria.



Wolf et al found, that pollen proteases can destroy nasal mucus proteins. This degradation process of the nasal mucus was also suspected to trigger allergies, because allergens first have to pass the nasal mucus to get to the inflammatory cells in the nasal mucosa [87].

Recent studies of Tomazic and colleagues have shown, that pollen enzymes, especially proteases are able to trigger nasal mucus proteomic changes, reflecting an altered immune responses and epithelial permeability in patients with allergic rhinitis [99,100].

6.4.5 Dry Eye

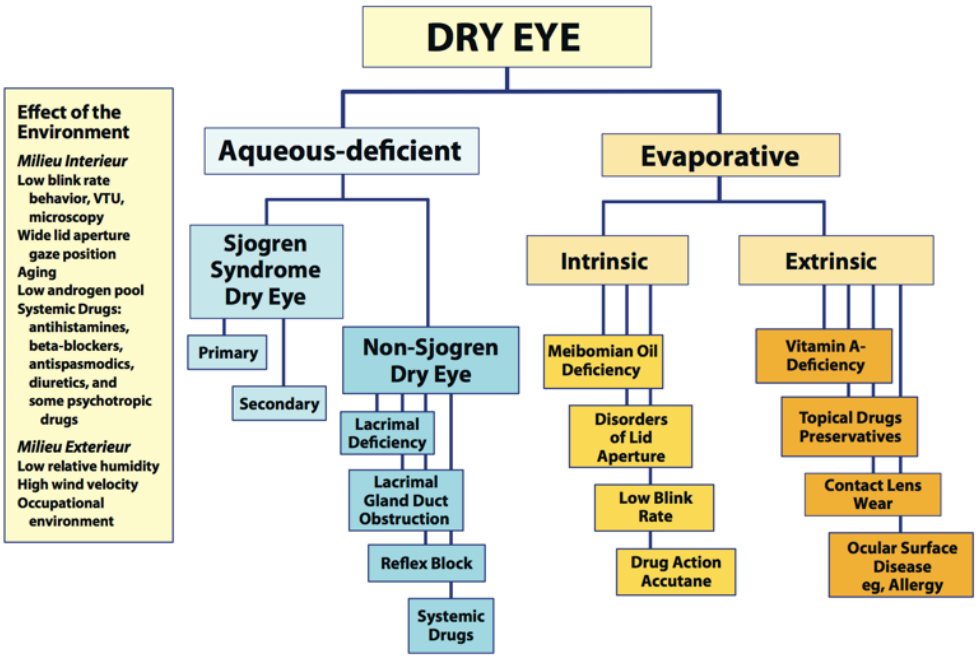
It is well-known that pollen contain different kinds of proteins. Some of them already have been identified as highly active enzymes, like proteases, lipases and glycosidases. These enzymes are known to be able to destroy important tear film components, like proteins, lipids and mucus representing chief components of a healthy tear film. Consequently, the

impaired tear film will show a reduced tear film break-up time and is thought to promote the development of a dry eye [89-91].

Dry eye disorders are a group of diseases resulting of different pathophysiological pathways, e.g. reduced tear volume and/or function, hyperosmolarity and inflammation. Unfortunately, most of them lead practically to the same clinical symptoms [5,71,101-103]. Dry eye triggered ocular symptoms provide a broad clinical spectrum ranging from mild transient to persistent irritation such as burning, itching, redness, pain, ocular fatigue, and visual disturbances [71,101-103].

Dry eye may be divided into two major pathogenetic categories (Figure 8). Either caused by a lack of tears (aqueous tear deficient dry eye, ATD) or by excessive evaporation (evaporative dry eye, EDE) [5]. It is important to note that these groups are not strictly separated from each other and mixed forms occur [103,104]. Ocular surface disease has a significant impact on the quality of life. Both, the intensity of symptoms and the clinical picture may vary during the day and those affected often feel impaired in their everyday life [3].

Figure 8. Major etiological causes of dry eye. Reproduced from [5] with permission of publisher Elsevier, Amsterdam, The Netherlands.



Even though ophthalmologists try to differentiate patients according to the leading causative factors of dry eye by these classifications, most of the patients suffering from dry eye present with a mixture of disease types [103].

Today ophthalmologists in industrialised countries face an increasing number of patients suffering from dry eye disorders. Multifactorial internal and environmental processes involving almost any of the components of the ocular surface and tear film are discussed to be causative for development of dry eye. Some studies even propose that a possible reason could the increased spreading of air conditioning systems and the increased work time in front of computer or other kinds of displays may play an important role. Here the question arises whether we face a relatively simple problem like a decrease in blinking rate or if there are still other unknown additional reasons for the development of dry eye [101,102,105,106].

Dry eye disease can occur alone or together with other ocular surface diseases, for example facilitating the contact of pollen allergens and enzymes with the ocular surface, when there is little or only non-functioning tear film left [5].

6.4.5.1 Aqueous tear deficiency

ATD is usually defined by a reduced production of tears. The amount of tear production can be assessed by a Schirmer test. Without prior application of a local anaesthetic filter paper stripes are bent at a notch and hooked into the lateral lower conjunctival sac for 5 minutes with the patient instructed to keep the eyes closed gently. The humidification of the filter paper stripe can be read and documented in millimetres. Schirmer values ≤ 5 mm / 5 min in at least one eye are referred to as aqueous tear deficiency [3].

Tear deficient dry eye can be further divided into Sjögren syndrome (SS) dry eye and non-Sjögren syndrome (non-SS) dry eye. The term of non-SS dry eye covers all other causes of tear deficiency than SS (Figure 8) [5].

6.4.5.2 Meibomian Gland dysfunction (MGD)

One of the most common causes of EDE is thought to be MGD. Very often a hyposecretory, obstructive condition of the meibomian glands can be observed. This is thought to be due to an increased keratinization of the glands' orifices and / or thickened meibum [76]. The literature also indicates a variant of hypersecretory MGD. There are controversial views regarding the definition, distribution and prevalence of MGD [107]. According to Nien and colleagues the hypersecretory MGD can primarily be found in younger patients [108]. Another common cause of EDE, frequently under diagnosed, is non-obvious lagophthalmus or incomplete blinking [109].

In different studies the prevalence of MGD in the general population is reported very variable with about 3.5% to 70%. It was found to be higher in the Asian than in the Caucasian population [110-112]. Frequencies of 20% to 90% were reported in clinical populations. Most of the investigations were performed on selected populations such as contact lens wearers or patients with dry eye [113-115]. A possible reason for the divergent results regarding the prevalence of MGD in the previously published literature is the use of different definitions for the existence of MGD [116]. The MGD report defines the presence of telangiectasia, erythema and irregularity of the lid margins, a shifting of the meibomian glands' orifices, as well as changes in the expressibility and quality of the meibum as signs of MGD [117,118].

6.5 Aim

The purpose of this study was to contribute to the understanding of the enzymatic reactions in pollen-exposed eyes and their connection to ocular surface diseases, like allergic, vernal, atopic conjunctivitis and dry eye. It has been shown, that Hazelnut und Birch pollen are able to degrade tear film components and damage ocular surface cells in a similar fashion as nasal mucus and mucosa [6].

We examined the effect of other pollen species from different plant families on human tear fluid and conjunctival cells[1].

Especially, the following questions were investigated:

1.) Do regional pollen species from different plant families show proteolytic activity?

2.) Are there differences in the Polyacrylamide gel-electrophoresis (PAGE) spectra of pollen species from different plant families?

3.) Which pollen species degrade human tear fluid and conjunctival cells?

4.) Does the incubation of conjunctival cells with pollen extracts from different pollen species induce cytomorphological changes?

5.) Does the assessment of cell viability by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and a Water-soluble tetrazolium, the sodium salt of 4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay, as well as the impedance based xCELLigence real time analysis system (RTCA) show a change in cell viability of conjunctival cells after the incubation with pollen extracts from different pollen species?

6.) Is it possible to further quantify cell death and cell lysis of conjunctival cells after the incubation with pollen extracts from different pollen species by a lactate dehydrogenase (LDH) release assay?

7. Materials and Methods

7.1 Pollen collection

During the last couple of years, from 2010 to 2016, we established a large collection of regional pollen species from different plant families (Adoxaceae, Betulaceae, Fagaceae, Juglandaceae, Malvaceae, Oleaceae, Pinaceae, Plantaginaceae, Poaceae, Salicacea, Sapindaceae) at the Department of Ophthalmology of the Medical University of Graz, Austria. Male inflorescences, which contain pollen, were collected in the surroundings of the city of Graz. The inflorescences were dried, sieved and stored in paper bags at room temperature [1].

An overview of the pollen, that have been collected and stored for further analysis is given in table 1.

Table 1. Pollen species collected. Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom and modified with permission of Dr. Dieter Rabensteiner.

Species	**Clade**	**Order**	**Family**
Birch	Rosids	Fagales	Betulaceae
Common hazel	Rosids	Fagales	Betulaceae
Common timothy	Commelinids	Poales	Poaceae
Corkscrew hazel	Rosids	Fagales	Betulaceae
Elder	Asterids	Dipsacales	Adoxaceae
European alder	Rosids	Fagales	Betulaceae
European ash	Asterids	Lamiales	Oleaceae
European chestnut	Rosids	Fagales	Fagaceae
European hornbeam	Rosids	Fagales	Betulaceae
European silver fir	Pinopsida	Pinales	Pinaceae
Golden foxtail grass	Commelinids	Poales	Poaceae
Greater plantain	Asterids	Lamiales	Plantaginaceae
Horse chestnut	Rosids	Sapindales	Sapindaceae
Korean fir	Pinopsida	Pinales	Pinaceae
Lime tree	Rosids	Malvales	Malvaceae
Maple	Rosids	Sapindales	Sapindaceae
Orchard grass	Commelinids	Poales	Poaceae
Scots pine	Pinopsida	Pinales	Pinaceae
Walnut	Rosids	Fagales	Juglandaceae
White willow	Rosids	Malpighiales	Salicaceae

For further analysis of the different pollen species we applied the established methods we have already published in our study on hazelnut and birch pollen in 2010 [6] and additionally performed a WST-1 [1]and LDH assay, as well as a RTCA [1].

7.2 Zymography

Zymography was performed to investigate the proteolytic activity of pollen with a 10% zymogram gelatine gel by Invitrogen Life Technologies Corporation, Carlsbad, USA. Staining was done with a colloidal blue staining kit acquired from Invitrogen Life Technologies Corporation, Carlsbad, USA [1,6].

Ten milligrams of pollen were incubated with 100 µl physiological saline for 1 hour at room temperature. The next day the pollen extract was centrifuged at 10.000 rpm for 5 minutes. Fifteen microliters of the supernatant were diluted with sample buffer at a ratio of 1:1. The remaining extract was filtered sterilely and again 15 µl were diluted with the sample buffer at a ratio of 1:1. Twenty microliters of the samples were applied to each well and the gel was placed in an electrophoresis chamber filled with running buffer. Zymography was performed for 60 minutes at 125 Volt [6].

Afterwards the zymogram gel was fixed with renaturing buffer for 30 minutes at room temperature and developed in developing buffer overnight at 37°C [6].

The gel was stained with a colloidal blue and destained with distilled water. The zymographic spectra were documented by a Minolta, RD 175 digital-camera [1,6].

7.3 Collection of tear fluid

After consultation with the local Institutional Review Board (IRB) of the Medical University of Graz, Austria, tear fluid was collected from five healthy volunteers with normal tear function (median age 57 years, range 25-69 years, 4 females, 1 male), as well as three subjects with known hay fever (median age 41 years, range 31-57 years, 2 females, 1 male). All subjects gave informed consent. The study has been conducted according to the principles expressed in the Declaration of Helsinki [1].

Fifty microliters of tear fluid were collected from the lower lateral tear meniscus with a capillary tube after stimulation with China mint oil applied to the skin around the zygomatic bone. The samples were transferred into plastic microtubes (Brand GmbH, Wertheim, Germany) and used for the experiment [1,6]. To perform the in-vitro

experiments repeatedly, tears were collected from the same subjects recurrently with an interval of at least one week in between the collections.

7.4 Polyacrylamide gel-electrophoresis (PAGE)

As previously described [6], 100 µl of physiological saline and 100 µl tear fluid, respectively, were laced with 10 mg of pollen and incubated at 37°C for 1 hour, afterwards the mixture was centrifuged at 10000 rpm for 5 minutes. Subsequently, 30 µl of pollen extract, 5 µl of the pollen-tear fluid mixture and 5 µl of tear fluid, respectively, were diluted with NuPAGE LDS 4 x sample-buffer in a ratio of 3:1. Fifteen microliters of pollen extract, 4 µl of pollen-tear fluid mixture and 4 µl of pure tear fluid, respectively, were pipetted into the wells of the pre-cast NuPAGE 4-12% Bis-tris gel, 1.0 mm x 10 well (Invitrogen Life Technologies Corporation, Carlsbad, USA). Electrophoresis was performed at 200 V, 78 mA, for 35 minutes, afterwards the gels were fixed in water-methanol-acetic acid (5:6:1) for 10 minutes, stained overnight with a colloidal blue stain kit (Invitrogen Life Technologies Corporation, Carlsbad, USA) and destained with distilled water [1,6].

To estimate the molecular weight of the proteins, a protein standard (Novex® Sharp Unstained Protein Standard, Life Technologies, Carlsbad, California, USA), consisting of 12 protein bands in the range of 3.5 - 260 kDa, was used[1].

The electrophoresis patterns were documented by a Minolta, RD 175 digital-camera [6]. All experiments were performed in duplicate and repeated twice[1].

7.5 Pollen extracts for experiments with CHANG cells

Twelve ml DMEM (Dulbecco's Modified Eagle Medium, Invitrogen, Carlsbad, California, USA) and 1% P/S (Penicillin/Streptomycin, Biochrom, Berlin, Germany) were added to 60 mg and 300 mg of pollen, respectively, which equals a concentration of 5 mg and 25 mg pollen per ml medium. These concentrations were chosen according to our prior experiments. The pollen suspensions were incubated for 24 hours at 4°C. Finally, the

suspensions were centrifuged at 4500 rpm for 5 minutes and the supernatant was filtered sterilely to receive the required pollen extracts [1,6].

7.6 Human conjunctival cells

Human conjunctival cells (CHANG cells, CCL-20.2, clone 1-5c-4m, Wong-Kilbourne derivates of CHANG conjunctiva) acquired from The American Type Culture Collection, ATCC Co., Manassas, VA, USA were used. The cell line is thought to be derived from normal epithelial conjunctiva, and is immortalised with HeLa marker chromosomes. The cells are positive for keratin and they grow adherently [1,6].

The cells were frozen at -196°C in fluid nitrogen. Before use they were defrosted and resuspended in 10 ml culture medium. To remove DMSO (dimethylsulfoxide) they were centrifuged at 800 rpm for 5 minutes, resuspended in 1000 µl culture medium and transferred into a 25 cm^2 culture flask purchased from Sarstedt GmbH, Wiener Neudorf, Austria. Finally, 4 ml DMEM containing 1% P/S and 10% fetal bovine serum (Fetal Bovine Serum, PAA, Pasching, Austria) were added to the flask and incubated in a CO_2 incubator (Heracell 240, Kendro Heraeus, Berlin, Germany) at 37°C. Every two to three days the medium was changed [6].

The assessment of cell growth, degree of confluence and photo documentation was performed by an inverse microscope (Axio Observer Z.1, Zeiss, Germany). As soon as the cells were at least 90% confluent, they were used for the experiments [6].

7.7 Cell suspension

First, cell culture medium was removed and the cells were rinsed with DPBS rinsing solution (Dulbecco's Phosphate Buffered Saline, Invitrogen, Carlsbad, California, USA). One millilitre trypsin was added to the cell monolayer and the flask was returned to the incubator for 2 minutes. The detachment of the cells was assessed under the microscope. To inactivate the effect of trypsin, the cells were resuspended in 3 to 5 ml of medium. Afterwards the cell suspension was transferred into a centrifuge tube and centrifuged at

800 rpm for 5 minutes. Finally, the medium was decanted and the cells were resuspended in 2 ml of medium [6].

To receive a cell suspension with the desired cell density cell count was determined by the CASY®1 Cell Counter + Analyser System - Model TT from Schärfe System GmbH, Reutlingen, Germany [1,6].

The CASY1 Cell Counter is used to control the quality of cell cultures. The cells are suspended in isotonic measuring solution (CASYton) and aspirated through a precision capillary with a defined size. While passing through the measuring capillary they are scanned with a frequency of 106 measurements per second in a low voltage field between two platinum electrodes. The resulting electrical signals give information about amount, concentration, diameter, volume and size distribution of the cells [6].

Nine point nine millilitres CASYton and 100 µl of cell suspension were pipetted into a CASY-cup and placed into the counter. After CASY1 cell amount determination the cell volume with desired cell density was calculated. Cell suspensions with an empiric cell density of 30.000 cells/ml for the MTS [6], WST-1 and LDH assay, as well as 100.000 cells/ml for the RTCA system were created.

7.8 Determination of cell viability by MTS assay

Evaluation of cell viability was performed by the Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (MTS) from Promega, Madison, WI, USA. The MTS assay is a colorimetric method for determination of the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. Yellow tetrazolium salt, MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, Germany), is bioreduced to purple formazan in the mitochondria of metabolically active cells. The reduction only takes place when mitochondrial reductase enzymes are active, and therefore conversion can directly be related to the number of viable cells [6].

Experiments were performed in 96-well-microtiterplates (Becton Dickenson, Franklin Lakes, NJ, USA) by applying 200 µl of cell suspension with a cell-density of 30.000 cells per ml into each well using a multi-channel pipette (Eppendorf Research®, Hamburg, Deutschland). According to the pipetting scheme (Figure 9) The outer wells of the plate were filled with distilled water, in order to avoid evaporation [6].

Figure 9. Pipetting scheme for the MTS assay. Distilled water (D), Blank (B), Blank with pollen extract (BP), control (C), probe (P).

	1	2	3	4	5	6	7	8	9	10	11	12
A	D	D	D	D	D	D	D	D	D	D	D	D
B	D	B	B	B	B	B	BP	BP	BP	BP	BP	D
C	D	C	C	C	C	C	P	P	P	P	P	D
D	D	C	C	C	C	C	P	P	P	P	P	D
E	D	P	P	P	P	P	C	C	C	C	C	D
F	D	P	P	P	P	P	C	C	C	C	C	D
G	D	D	D	D	D	D	D	D	D	D	D	D
H	D	D	D	D	D	D	D	D	D	D	D	D

After an incubation time of 24 hours at 37°C in a CO2 incubator cell growth was assessed and pollen extract was applied. First, medium was removed and each well was rinsed twice with 30 µl DMEM plus 1% P/S. 100 µl DMEM plus 1% P/S were filled into the blank wells and 100 µl pollen extract were applied to blank plus pollen-wells and the sample wells. 100 µl DMEM plus 1% P/S were filled into the control wells. Finally, the plates were incubated for 5 and 24 hours, respectively, at 37°C in a CO2 incubator [6].

After 5 and 24 hours, respectively, the 96-well plates were taken out of the incubator and cytomorphological changes were assessed by an inverse microscope and photo documented. Cell medium was removed, each well was rinsed with DMEM plus 1% P/S and 100 µl fresh DMEM plus 1% P/S were applied. Ten microliters of MTS-reagent were added and the plates were incubated for 2 hours at 37°C [6].

After incubation time the plates were evaluated. The absorbance of the formazan dye was measured at a wavelength of 492 and 620 nm using an ELISA-reader (Anthos 2010, ADAP-Software from Anthos Labtec Instruments GmbH, Krefeld, Germany) [6]. All experiments were performed at least in triplicate and repeated twice [1].

7.9 Determination of cell viability by WST-1-assay

Cell viability was determined by the WST-1 Cell Proliferation Assay from Roche Applied Science, Roche Diagnostics GmbH, Mannheim, Germany. A water-soluble tetrazolium, the sodium salt of 4-[3-(4iodophenyl)-2-(4- nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) is cleaved to formazan by cellular enzymes. The amount of formazan dye directly correlates to the number of active cells and was quantified at a wavelength of 450 versus 650 nm reference by a scanning multi-well spectrophotometer [119] (Anthos 2010, ADAP-Software from Anthos Labtec Instruments GmbH, Krefeld, Germany) [1].

The experiments were performed in 96-well-microtiterplates (Becton Dickenson, Franklin Lakes, NJ, USA) according to the manual. All experiments were performed at least in triplicate and repeated twice[1].

Figure 10. Pipetting scheme for the WST-1 assay. Blank (B), control (C), probe 5mg/ml pollen extract (P1), probe 25mg/ml pollen extract (P2).

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	B	B	B	B	B	B	B	B	B	B
B	C	P1	P1	P1	P1	P1	P1	P1	P1	P1	P1	P1
C	C	P1	P1	P1	P1	P1	P1	P1	P1	P1	P1	P1
D	C	P1	P1	P1	P1	P1	P1	P1	P1	P1	P1	P1
E	C	P2	P2	P2	P2	P2	P2	P2	P2	P2	P2	P2
F	C	P2	P2	P2	P2	P2	P2	P2	P2	P2	P2	P2
G	C	P2	P2	P2	P2	P2	P2	P2	P2	P2	P2	P2
H	C	P2	P2	P2	P2	P2	P2	P2	P2	P2	P2	P2

7.10 Measurement of cell proliferation by RTCA system

To dynamically monitor the phenotypic changes of cells in real time using electrical impedance data, we used the label-free xCELLigence RTCA system from ACEA Biosciences, San Diego, CA, USA [1].

Electrical impedance is measured by interdigitated micro-electrodes incorporated into the bottoms of cell culture E-plates (Omni Life Sciences, Bremen, Germany). The impedance data are displayed as a cell index (CI) value, providing quantitative information on the cells' biological status, including the cell number, viability and morphology. Cell proliferation and viability are monitored in real time by electrical impedance measurements. RTCA normally correlates well with other cell number and viability assays (e.g. WST-1, MTT) [120] [1].

For correction of the background absorbance 100 µl of cell culture medium were pipetted into the 16 wells of the E-Plate. One-hundred microliter of the cell suspension with a concentration of 100.000 cells/ml, equalling 10.000 cells/well, were added to each well [1].

For 24-hours the cells were incubated on the xCELLigence station inside the incubator. Every 20 minutes, electrical impedance was measured to assess cell proliferation and adhesion. On the next day, cell medium was removed, and the wells were washed with DPBS once. After that, 100 µl of the pollen extracts with concentrations of 5 mg and 25 mg pollen per ml medium, prepared as described above, were added accordingly [1].

The incubation of the control cells was performed with DMEM diluted with physiological saline at a 1:1 ratio. For three consecutive days, the electrical impedances were measured at 20 minute intervals [1].

Measurement data were displayed as impedance change over time, referred to as "Cell Index". The curves were normalized to the point of time before addition of the pollen extracts. All experiments were performed in duplicate and repeated twice [1].

7.11 Quantification of cell death and cell lysis by LDH assay

Cell death and cell lysis was quantified by a LDH release assay (Cytotoxicity Detection Kit Plus, Roche Diagnostics GmbH, Mannheim, Germany). The Cytotoxicity Detection Kit Plus is a colorimetric assay for the quantification of cytotoxicity and cytolysis.

Usually, cell death is assessed by quantifying plasma membrane damage. LDH is present in all cells and known as a stable cytoplasmatic enzyme. Damage of the plasma membrane leads to a rapid release of LDH into the cell culture supernatant.

The test principle involves the conversion of lactate to pyruvate by a LDH-catalysed reaction. Consequently, the yellow tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) is reduced to a formazan dye, which can be measured at a wavelength of about 500nm.

The experiments were performed in 96-well-microtiterplates (Becton Dickenson, Franklin Lakes, NJ, USA) in a similar fashion as the MTS assay according to the manual. All experiments were performed at least in triplicate and repeated twice.

7.12 Statistical analysis

We calculated the cell viability in percent on basis of the respective assay's extinctions. The extinctions of the control cells of each microtiterplate were defined as 100% cell viability for the particular microtiterplate [6].

Cell viability is given as mean with standard deviation (SD). Additionally, results for 20 pollen species were pooled into 11 plant families as a biological classification [1].

The effect of the added pollen of the 11 plant families on the viability of the Chang cells was assessed by a 4 General Linear Models (GLM) for each of the two exposure time conditions (5 h, 24 h) in the two pollen concentration conditions (5 mg, 25 mg). Dunnett's tests comparing each of the 11 families with the Control condition (no additive) were used as post-hoc tests to detect significant effects of the added pollen [1].

To test for differences in MTS between the 11 families and the 4 conditions we used a linear mixed effects model (LMM) with family, exposure time (5 h, 24 h) and concentration (5 mg/mL, 25 mg/mL) as fixed factors [1].

A p-Value of less than 0.05 was considered as statistically significant, all post-hoc tests of GLMs and LMM were corrected for multiple comparison with Dunnett's or Bonferoni correction, respectively [1].

To compare the means of the MTS and WST-1 assays for the 20 species, the SDs and Coefficients of variation (CV%) were juxtaposed [1].

8. Results

8.1 Zymography

It could be verified that the pollen used for the experiments contain active proteases, which possess enzymatic activity. The different pollen species showed distinctive zymographic spectra. Figures 11 - 14 show the zymography of some of the pollen, we investigated.

Figure 11. Zymography pattern of Birch pollen (lane 1, 2), Orchard grass (lane 3, 4). Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom.

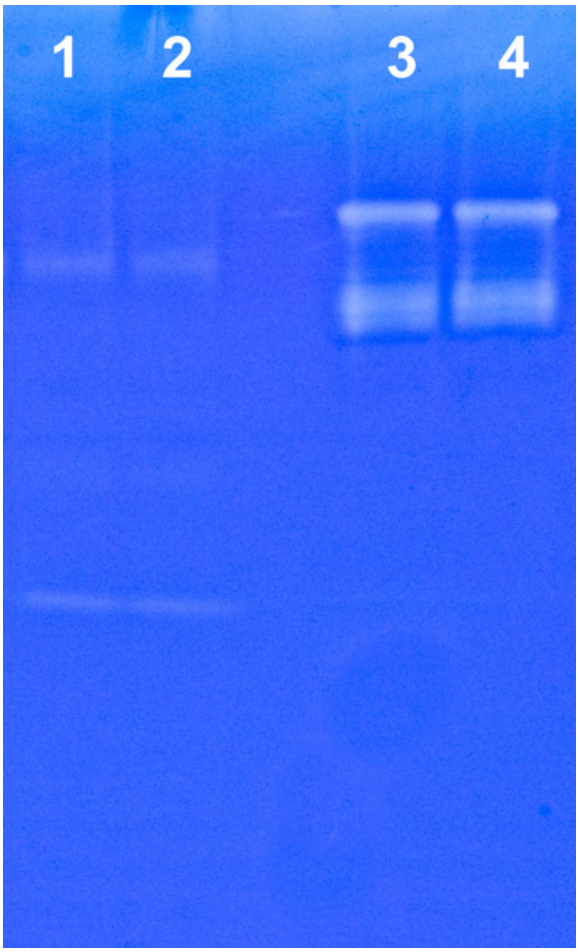


Figure 12. Zymography pattern of Corkscrew hazel pollen in different concentrations (lane 1, 2).

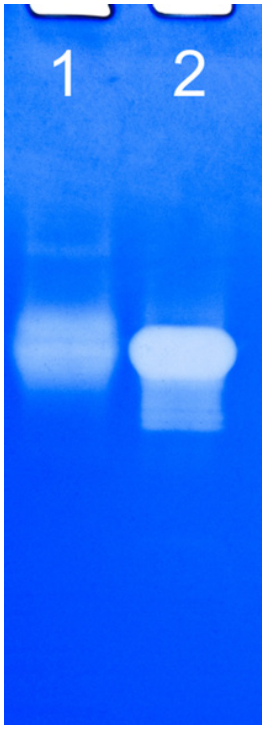


Figure 13. Zymography pattern of Common timothy (lane 1), Golden foxtail grass (lane 2) pollen.

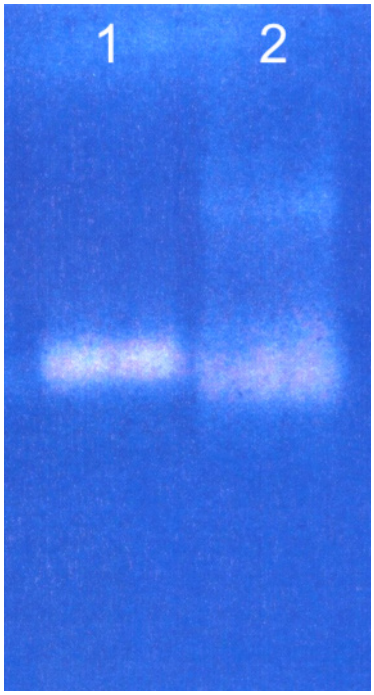
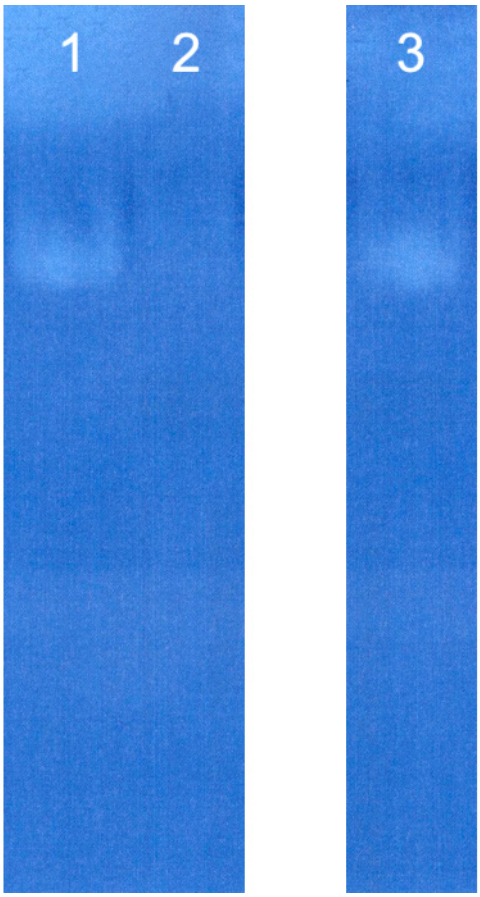


Figure 14. Zymography pattern of Korean fir (lane 1), Scots pine (lane 2), European silver fir (lane 3) pollen.



8.2 Polyacrylamide gel-electrophoresis (PAGE)

PAGE was performed in order to analyse proteins of pollen (Figure 15-18) and tear fluid (Figure 19-22).

Figure 15. PAGE of Common hazel (1), European alder (2), White willow (3), European ash (4) and Protein Standard (S) to estimate the molecular weight of the proteins by comparison of their visible bands.

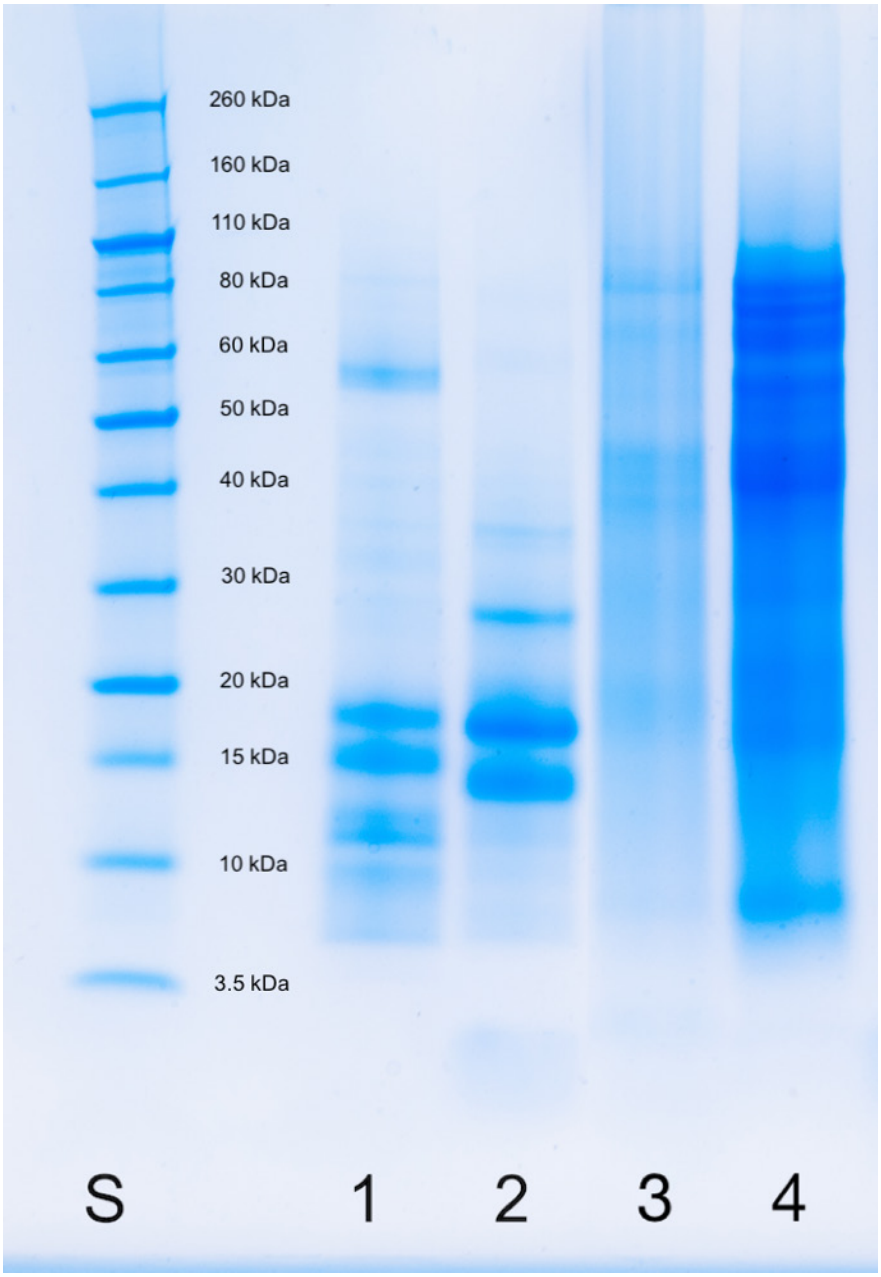


Figure 16. PAGE of Protein Standard (S), Korean fir (1), Scots pine (2) and European silver fir (3).

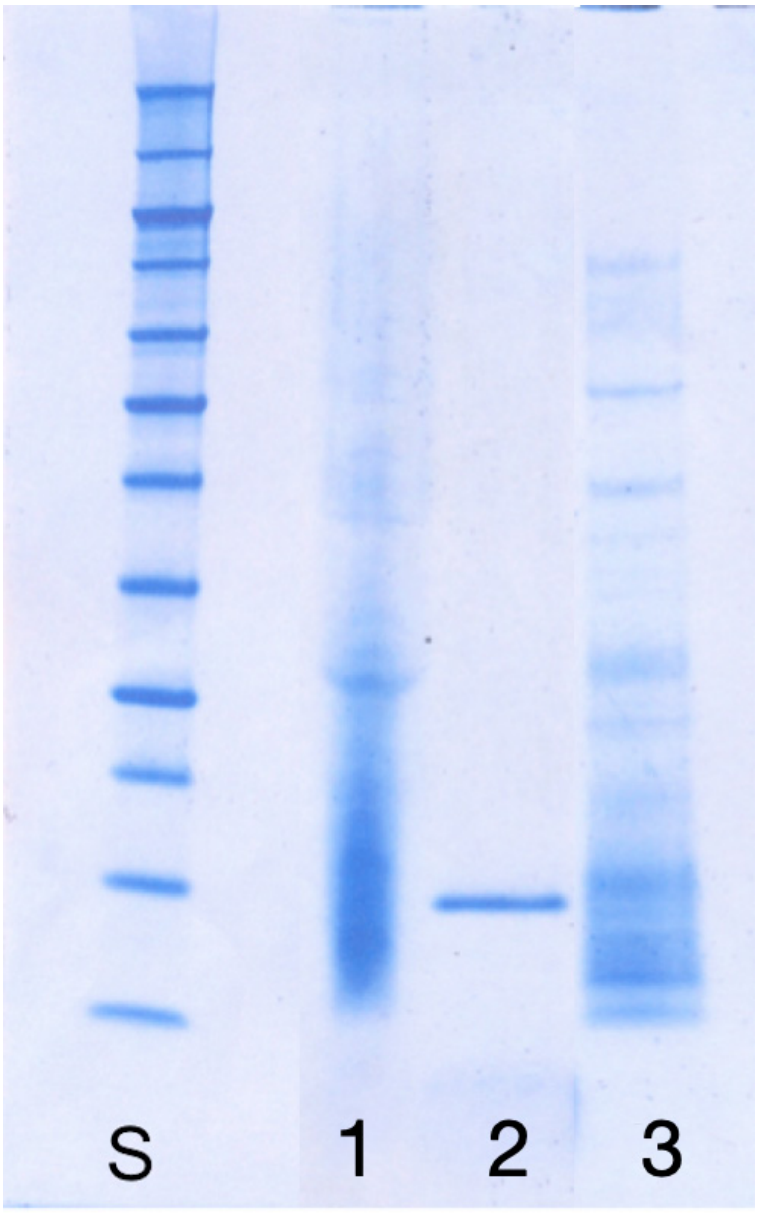


Figure 17. PAGE of Protein Standard (S).and Greater plantain (1), Orchard grass (2), Common timothy (3) and Golden foxtail grass (4).

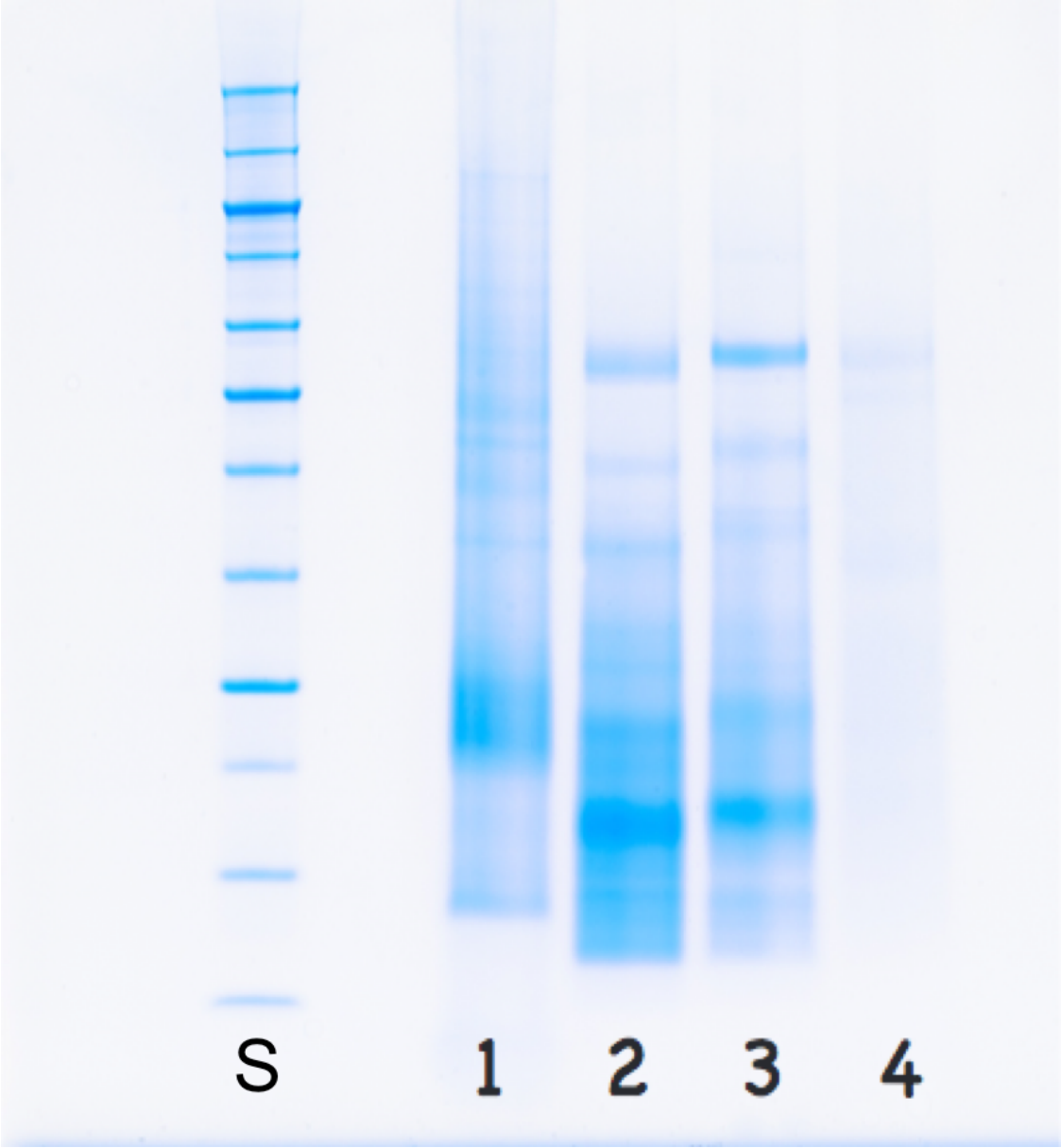
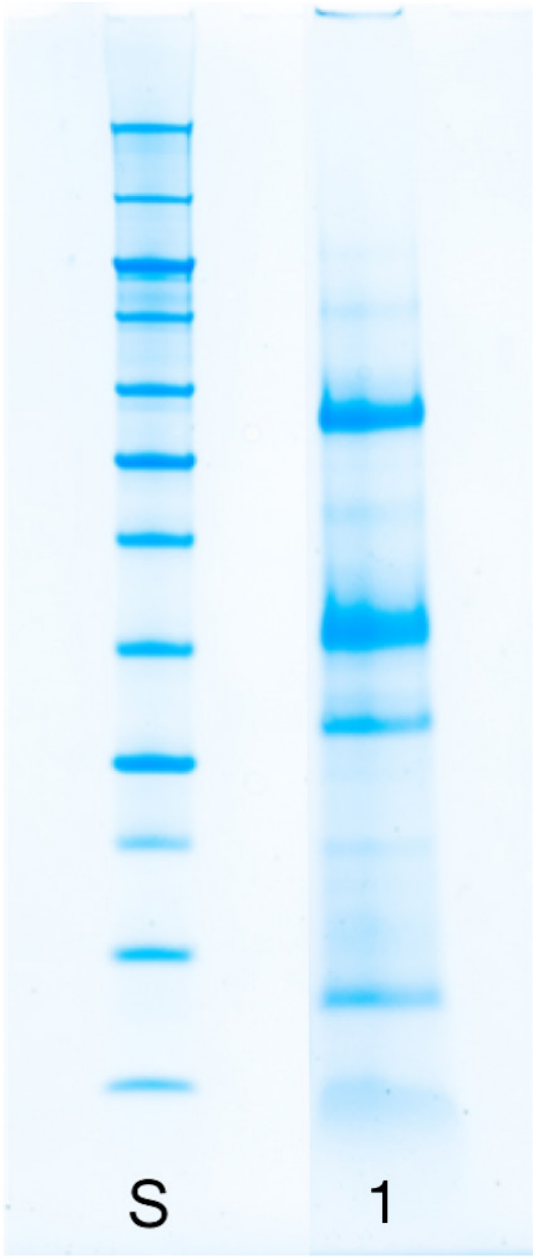


Figure 18. PAGE of Protein Standard (S) and Elder (1).



The electrophoresis-spectra of Hazelnut pollen extract, tear fluid and tear fluid incubated with pollen extract are shown in figure 19. Interestingly, after incubation of tear fluid with pollen extract, single bands of the electrophoresis-spectrum of tear fluid disappeared (arrows). This indicates that some pollen proteins exert proteolytic activity destroying tear fluid proteins. These can be obtained using other pollen extracts as well (Figures 20-22).

Figure 19. Electrophoresis spectrum of Hazelnut pollen extract and tear fluid. (Lane 1: hazelnut pollen. Lane 2: tear fluid. Lane 3: tears incubated with Hazelnut pollen extract.) Reproduced from [6] with permission of publisher Kager, Basel, Switzerland.

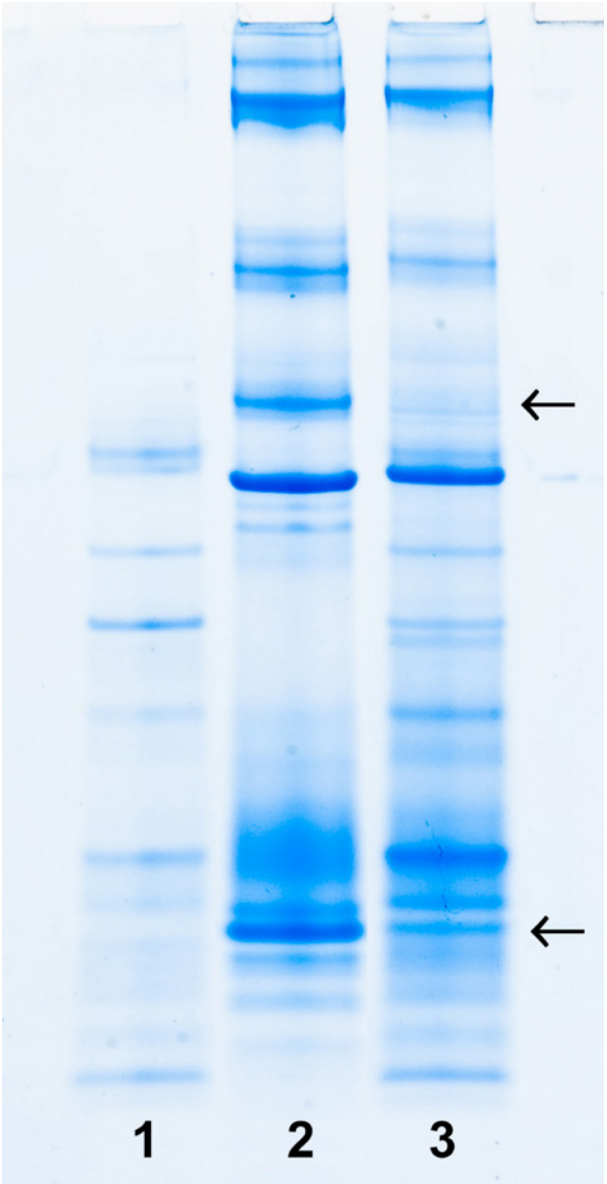


Figure 20. PAGE of Corkscrew hazel. 1 = Tears (Non-Allergic subject), 2 = Tears (Non-Allergic subject) + Pollen, 3 = Pollen, 4 = Tears (Allergic subject) + Pollen, 5 = Tears (Allergic subject). In comparison to the lanes 1 and 5 single bands of the electrophoresis-spectrum of tear fluid disappeared and a few new bands appeared (arrows) within the lanes 2 and 4. This indicates that pollen proteins exert proteolytic activity destroying tear fluid proteins in allergic as well as non-allergic subjects.

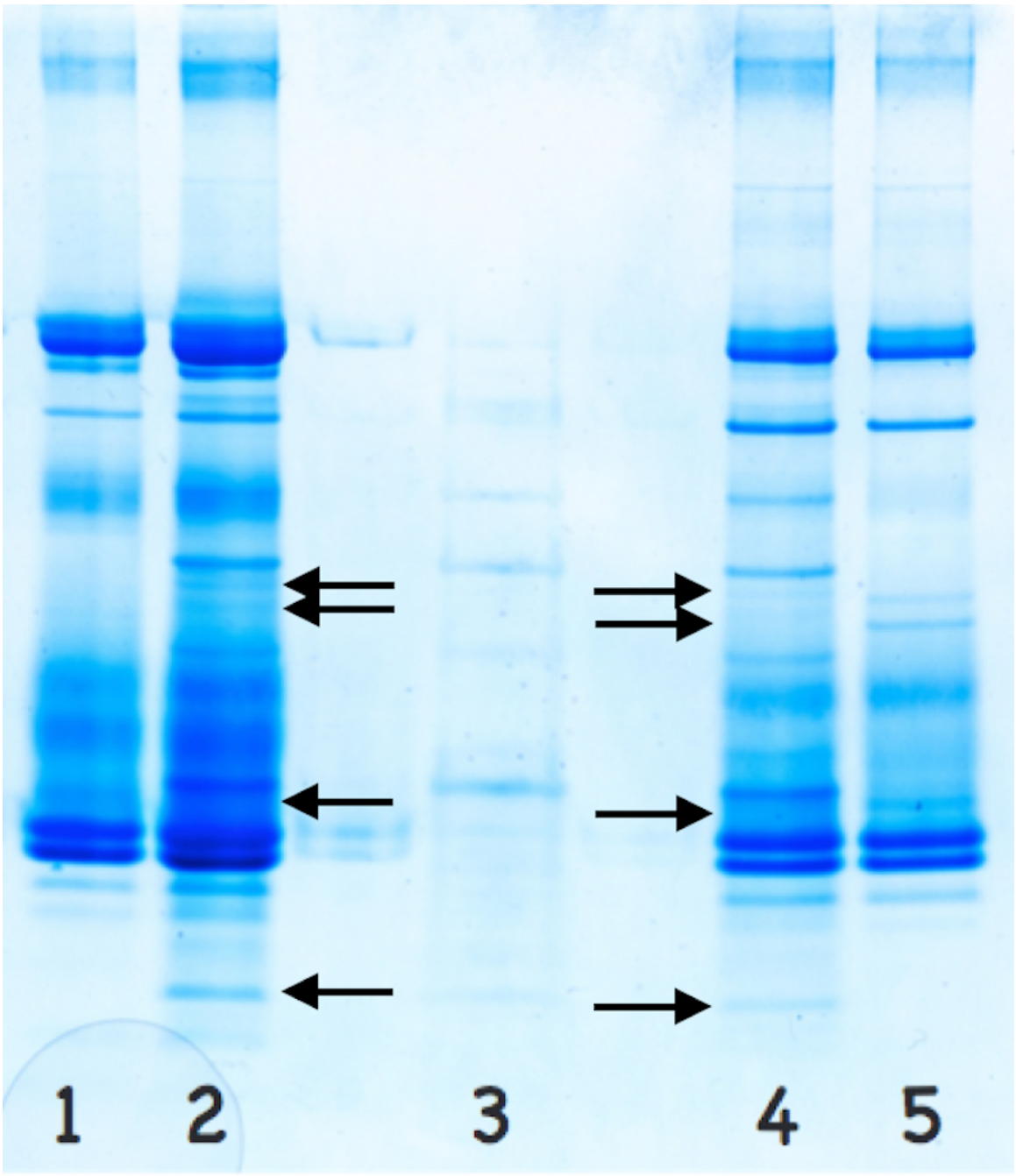


Figure 21. PAGE of European alder. 1 = Tears (Non-Allergic subject), 2 = Tears (Non-Allergic subject) + Pollen, 3 = Pollen, 4 = Tears (Allergic subject) + Pollen, 5 = Tears (Allergic subject). In comparison to the lanes 1 and 5 single bands of the electrophoresis-spectrum of tear fluid disappeared and new bands appeared (arrows) within the lanes 2 and 4. This indicates that pollen proteins exert proteolytic activity destroying tear fluid proteins in allergic as well as non-allergic subjects.

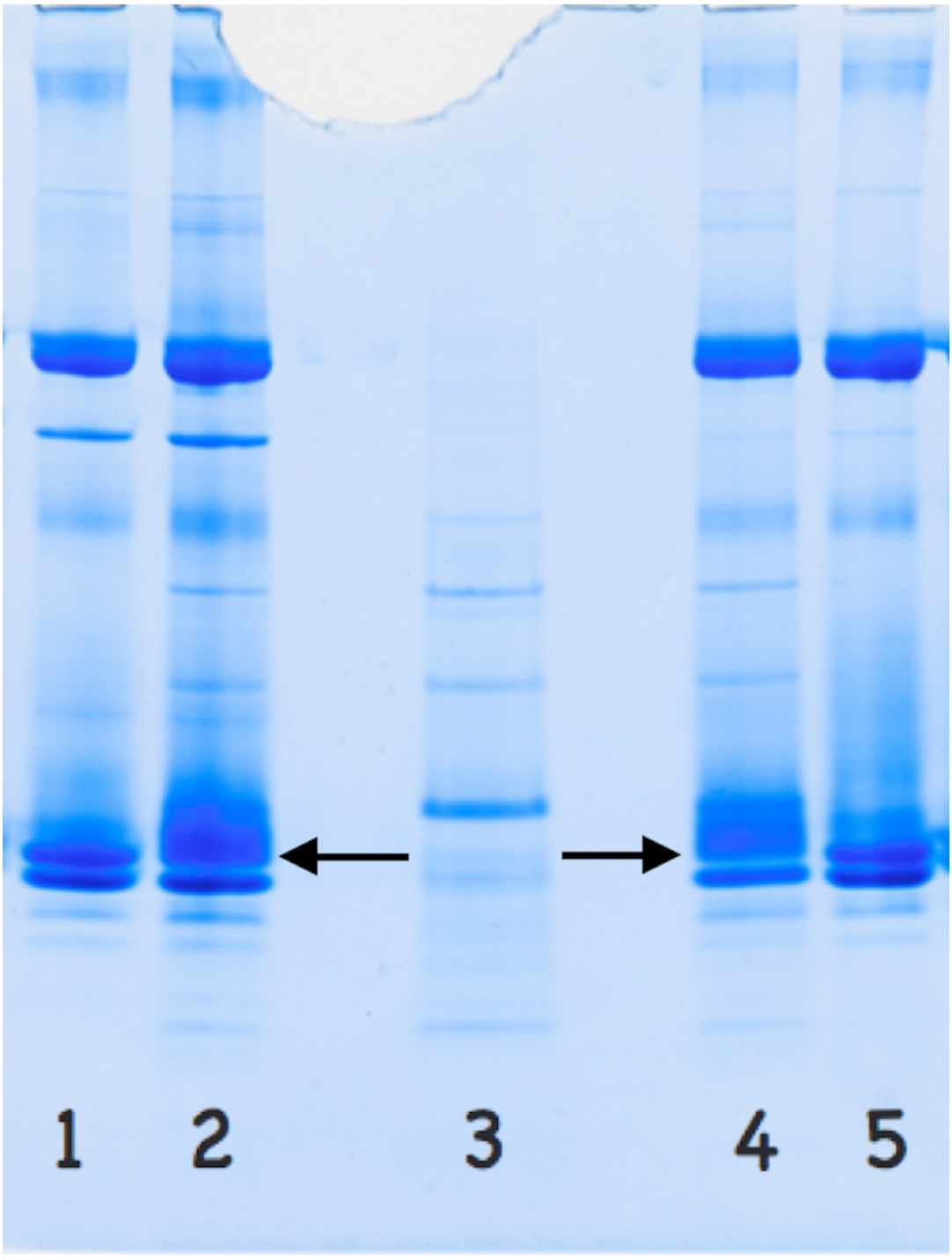
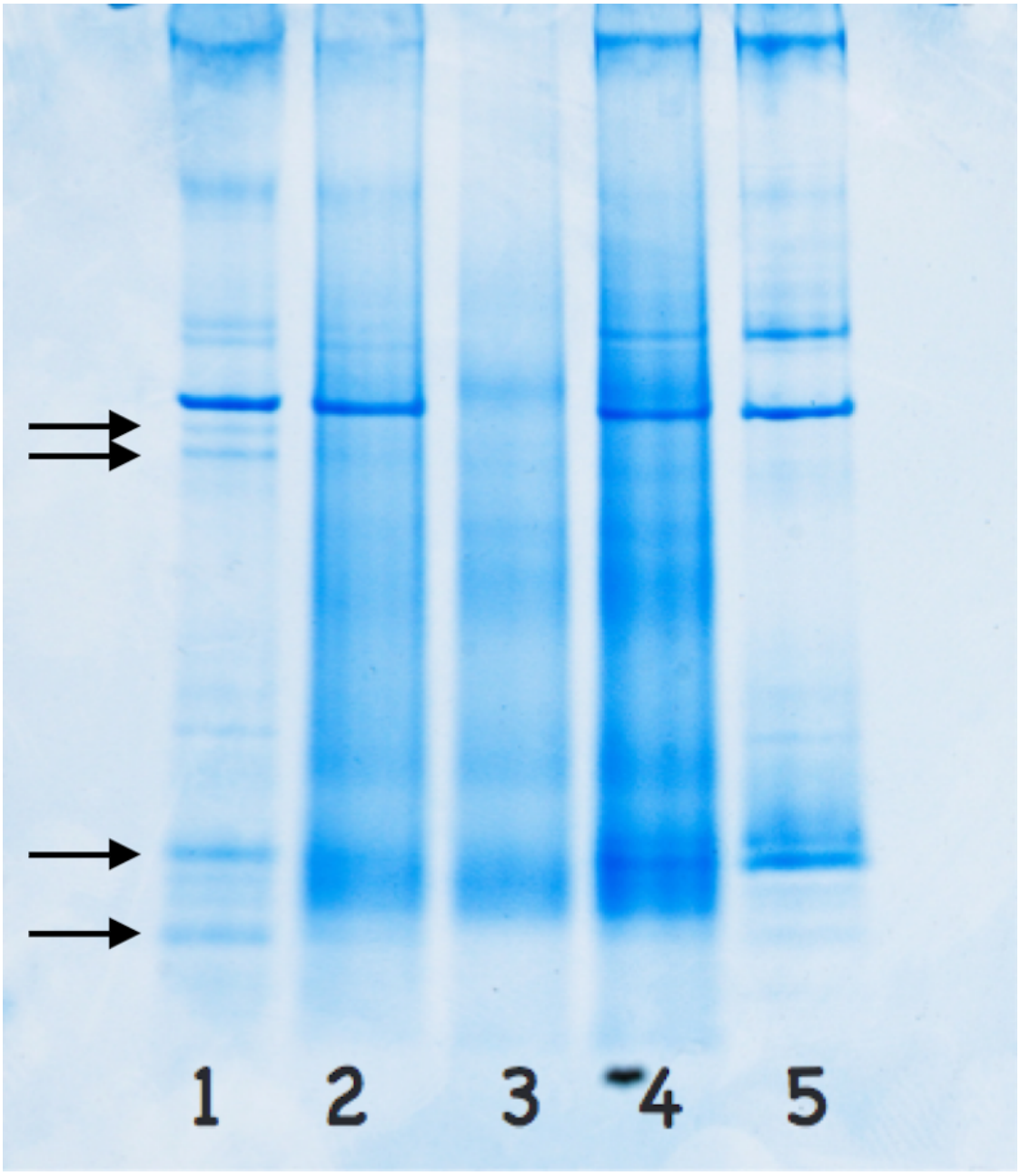


Figure 22. PAGE of Common timothy. 1 = Tears (Non-Allergic subject), 2 = Tears (Non-Allergic subject) + Pollen, 3 = Pollen, 4 = Tears (Allergic subject) + Pollen, 5 = Tears (Allergic subject). In comparison to the lanes 1 and 5 single bands of the electrophoresis-spectrum of tear fluid disappeared and a few new bands appeared (arrows) within the lanes 2 and 4. This indicates that pollen proteins exert proteolytic activity destroying tear fluid proteins in allergic as well as non-allergic subjects.
Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom.



Zymography revealed significant protease activity of most of the pollen, presenting different zymographic patterns. PAGE revealed the degradation of tear fluid proteins by most of the pollen species from different plant families. Zymography and PAGE results of the different pollen species are summarised in table 2.

Table 2. Zymography and PAGE of different pollen species. Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom and modified with permission of Dr. Dieter Rabensteiner.

Species	**Zymographic pattern**	**PAGE (Pollen)**	**PAGE (Pollen + Tears)**
Birch	+	+	+*
Common hazel	+	+	+*
Common timothy	+	+	+*
Corkscrew hazel	+	+	+*
Elder	+	+	+*
European alder	+	+	+*
European ash	+	+	+*
European chestnut	+/-	+/-	+/-
European hornbeam	+/-	+/-	+/-
European silver fir	+/-	+	+*
Golden foxtail grass	+	+	+*
Greater plantain	+	+	+*
Horse chestnut	+	+	+*
Korean fir	+	+	+*
Lime tree	+	+	+*
Maple	+/-	+/-	+/-
Orchard grass	+	+	+*
Scots pine	+/-	+	+*
Walnut	+	+	+*
White willow	+	+	+*

+… clear bands visible

+/- … weak bands visible

* … destruction of tear fluid proteins

8.3 Microscopic analysis of cell-cultures following incubation with pollen extract

Assessment of the cells under the inverse microscope revealed that cells treated with pollen extract showed severe structural changes (Figure 23-27) compared to the control (Figure 28).

Figure 23. CHANG-cells incubated with Elder pollen extract at a concentration of 25 mg/ml after 5 hours.

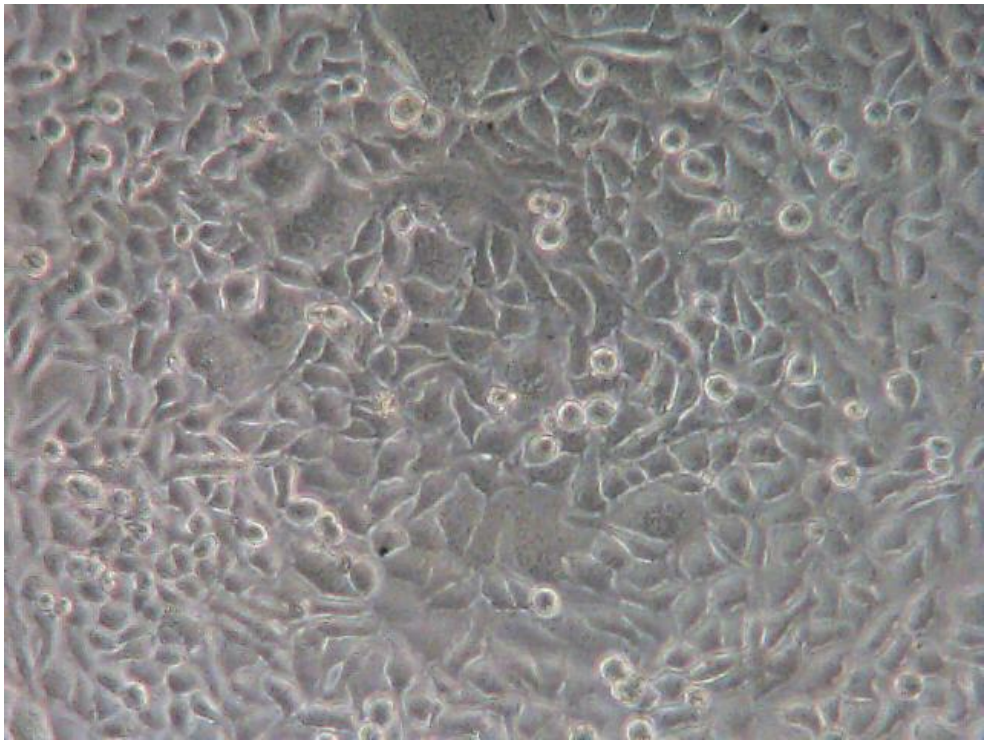


Figure 24. CHANG-cells incubated with European ash pollen extract at a concentration of 5 mg/ml after 5 hours.

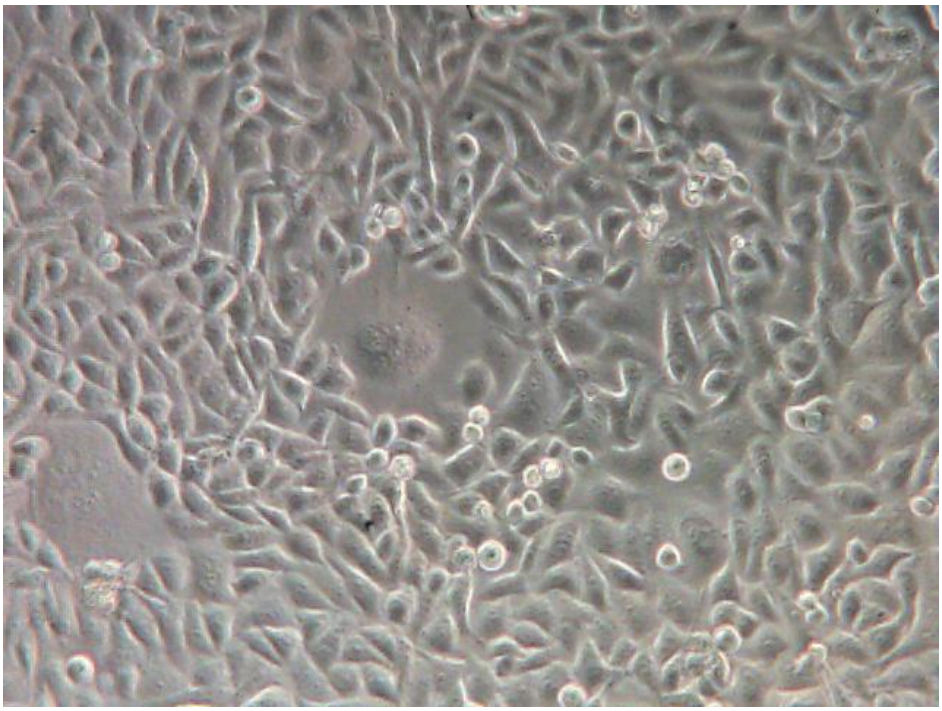


Figure 25. CHANG-cells incubated with European ash pollen extract at a concentration of 25 mg/ml after 5 hours.

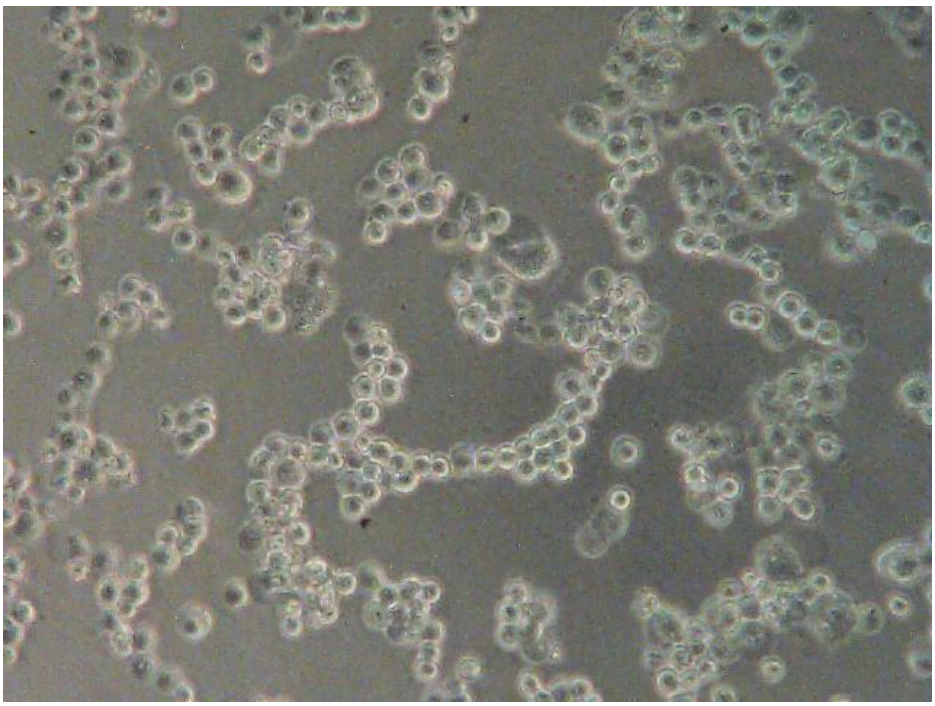


Figure 26. CHANG-cells incubated with Greater plantain pollen extract at a concentration of 25 mg/ml after 5 hours.

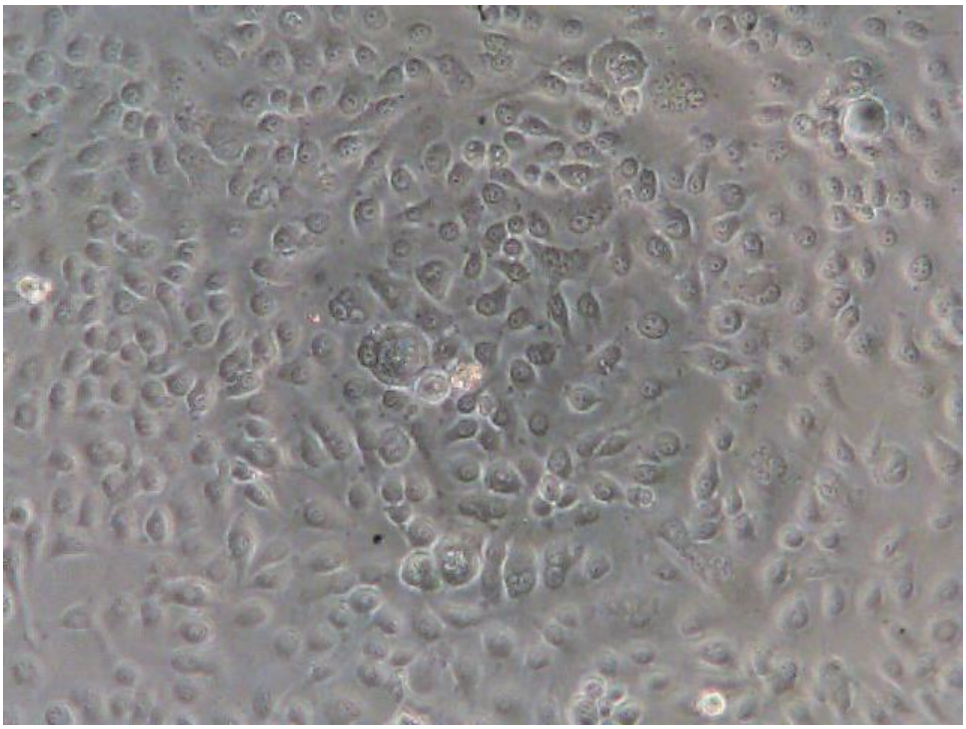


Figure 27. CHANG-cells incubated with Korean fir pollen extract at a concentration of 25 mg/ml after 5 hours.

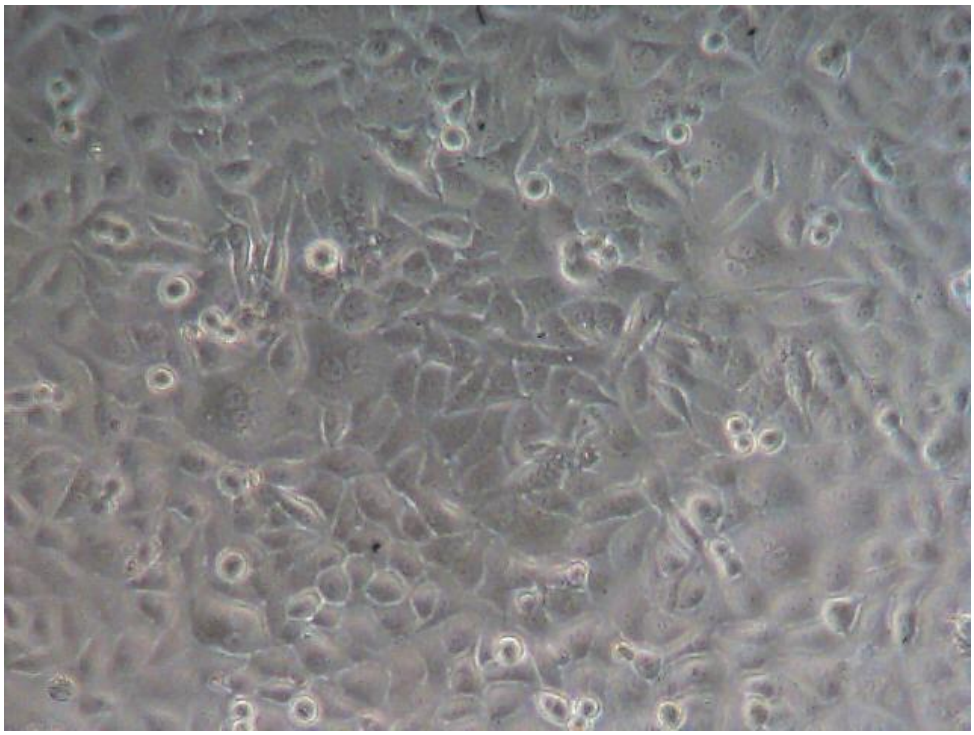
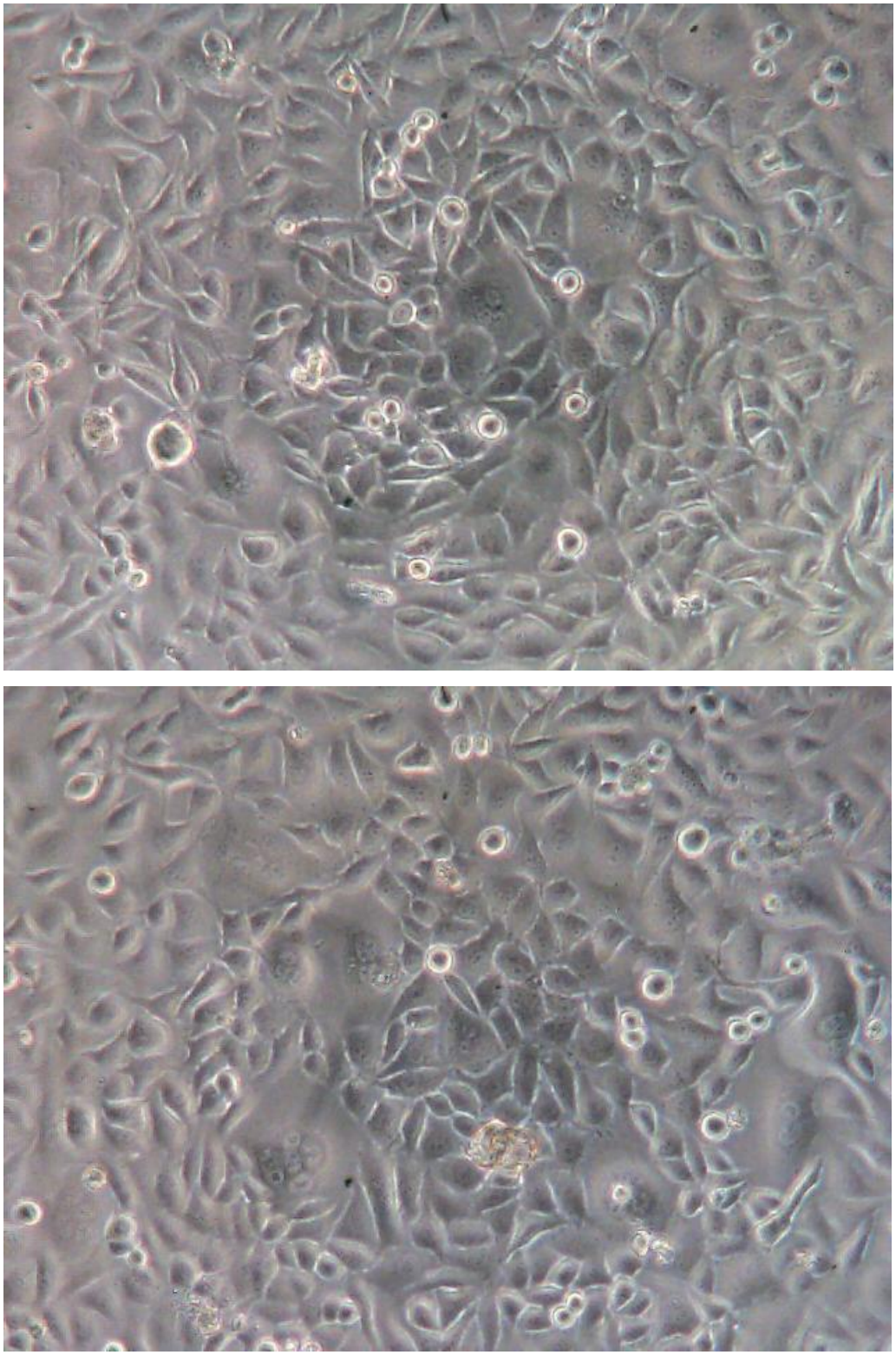


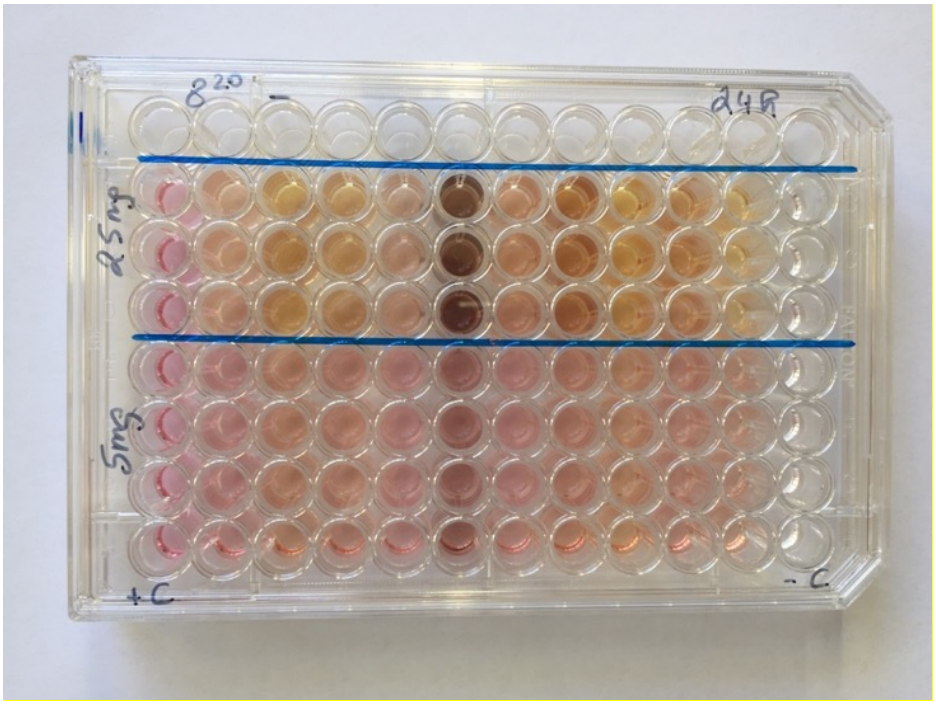
Figure 28. CHANG-cells without pollen addition (control).



8.4 MTS and WST-1 assay

The analysis of pollen extracts by the MTS- and WST-1 assay exposed, that pollen possess spontaneous dehydrogenase activity. So, it was necessary to use two different blanks in order to cut out the reagent basic and pollen dehydrogenase activity, respectively. Both of the blanks were subtracted from the corresponding control and sample wells [1].

Figure 29. 96-well-microtiterplate of the WST-1 assay.



We could observe changes in cell viability of human conjunctival cells incubated with 5 mg/mL or 25 mg/ml of pollen extracts for 5 and 24 hours, respectively. Depending on the concentration of the pollen extract and incubation time applied, the extend of the effects varied [1]. Table 3 gives a summary of the MTS and WST-1 results for the different pollen species.

Table 3. Cell viability results of human conjunctival cells incubated with 5 mg/ml or 25 mg/ml of pollen extracts for 5 and 24 hours, assessed by MTS and WST-1 assays.

			MTS			WST-1		
Species	**Conc.**	**Dur.**	**Mean**	**SD**	**CV%**	**Mean**	**SD**	**CV%**
Control	0 mg/ml	24 h	1.002	0.087	8.6%	1.008	0.136	13.5%
Control	0 mg/ml	5 h	1.000	0.105	10.5%	1.000	0.096	9.6%
Elder	25 mg/ml	24 h	0.575	0.052	9.0%	< 0.1		
Elder	25 mg/ml	5 h	1.042	0.030	2.9%	0.732	0.090	12.3%
Elder	5 mg/ml	24 h	0.676	0.051	7.6%	1.479	0.079	5.4%
Elder	5 mg/ml	5 h	1.035	0.018	1.7%	0.931	0.212	22.8%
European ash	25 mg/ml	24 h	< 0.1			< 0.1		
European ash	25 mg/ml	5 h	0.445	0.250	56.2%	0.256	0.118	46.1%
European ash	5 mg/ml	24 h	0.904	0.068	7.5%	0.994	0.253	25.5%
European ash	5 mg/ml	5 h	1.082	0.073	6.7%	0.969	0.155	16.0%
Greater plantain	25 mg/ml	24 h	0.341	0.027	7.8%	< 0.1		
Greater plantain	25 mg/ml	5 h	0.657	0.141	21.4%	0.438	0.075	17.2%
Greater plantain	5 mg/ml	24 h	0.630	0.045	7.1%	0.708	0.099	14.0%
Greater plantain	5 mg/ml	5 h	0.560	0.045	8.1%	0.469	0.175	37.4%
Common timothy	25 mg/ml	24 h	0.233	0.089	38.1%	< 0.1		
Common timothy	25 mg/ml	5 h	0.848	0.125	14.7%	0.571	0.055	9.6%
Common timothy	5 mg/ml	24 h	0.705	0.054	7.7%	0.621	0.165	26.6%
Common timothy	5 mg/ml	5 h	1.071	0.069	6.5%	0.833	0.103	12.4%
Golden foxtail grass	25 mg/ml	24 h	< 0.1			< 0.1		
Golden foxtail grass	25 mg/ml	5 h	0.650	0.224	34.4%	< 0.1		
Golden foxtail grass	5 mg/ml	24 h	0.698	0.116	16.6%	0.827	0.081	9.7%
Golden foxtail grass	5 mg/ml	5 h	0.982	0.099	10.1%	0.570	0.212	37.1%
Orchard grass	25 mg/ml	24 h	0.429	0.197	45.9%	< 0.1		
Orchard grass	25 mg/ml	5 h	0.953	0.116	12.2%	0.646	0.115	17.9%
Orchard grass	5 mg/ml	24 h	0.858	0.074	8.6%	1.241	0.222	17.9%
Orchard grass	5 mg/ml	5 h	1.056	0.104	9.8%	0.755	0.223	29.5%
European silver fir	25 mg/ml	24 h	1.115	0.053	4.8%	0.748	0.292	39.0%
European silver fir	25 mg/ml	5 h	1.245	0.203	16.3%	0.900	0.268	29.8%
European silver fir	5 mg/ml	24 h	0.965	0.203	21.0%	0.727	0.279	38.3%
European silver fir	5 mg/ml	5 h	1.083	0.163	15.0%	0.927	0.267	28.8%

			MTS			WST-1		
Species	**Conc.**	**Dur.**	**Mean**	**SD**	**CV%**	**Mean**	**SD**	**CV%**
Korean fir	25 mg/ml	24 h	0.994	0.032	3.2%	0.809	0.172	21.3%
Korean fir	25 mg/ml	5 h	1.227	0.209	17.0%	1.115	0.190	17.0%
Korean fir	5 mg/ml	24 h	0.996	0.090	9.0%	0.822	0.310	37.7%
Korean fir	5 mg/ml	5 h	1.156	0.091	7.9%	1.111	0.140	12.6%
Scots pine	25 mg/ml	24 h	0.590	0.033	5.6%	< 0.1		
Scots pine	25 mg/ml	5 h	1.012	0.206	20.4%	0.466	0.079	16.9%
Scots pine	5 mg/ml	24 h	0.950	0.104	10.9%	1.012	0.158	15.6%
Scots pine	5 mg/ml	5 h	1.171	0.109	9.3%	1.104	0.185	16.8%
Birch	25 mg/ml	24 h	< 0.1			< 0.1		
Birch	25 mg/ml	5 h	0.180	0.064	35.3%	< 0.1		
Birch	5 mg/ml	24 h	0.722	0.078	10.8%	0.554	0.100	18.1%
Birch	5 mg/ml	5 h	1.000	0.156	15.6%	1.044	0.095	9.1%
Common hazel	25 mg/ml	24 h	0.952	0.055	5.8%	0.609	0.260	42.7%
Common hazel	25 mg/ml	5 h	1.176	0.149	12.7%	0.832	0.061	7.3%
Common hazel	5 mg/ml	24 h	1.088	0.112	10.3%	1.153	0.363	31.5%
Common hazel	5 mg/ml	5 h	1.158	0.125	10.8%	1.045	0.266	25.4%
Corkscrew hazel	25 mg/ml	24 h	0.894	0.100	11.2%	0.389	0.032	8.2%
Corkscrew hazel	25 mg/ml	5 h	0.921	0.073	7.9%	0.462	0.068	14.8%
Corkscrew hazel	5 mg/ml	24 h	0.917	0.035	3.9%	0.782	0.120	15.3%
Corkscrew hazel	5 mg/ml	5 h	1.002	0.155	15.5%	0.258	0.231	89.6%
European alder	25 mg/ml	24 h	0.119	0.052	44.0%	< 0.1		
European alder	25 mg/ml	5 h	0.873	0.134	15.4%	< 0.1		
European alder	5 mg/ml	24 h	0.880	0.110	12.5%	0.614	0.104	16.9%
European alder	5 mg/ml	5 h	1.111	0.110	9.9%	1.067	0.132	12.4%
European hornbeam	25 mg/ml	24 h	0.879	0.083	9.4%	0.378	0.068	18.0%
European hornbeam	25 mg/ml	5 h	1.107	0.188	17.0%	0.779	0.142	18.3%
European hornbeam	5 mg/ml	24 h	0.944	0.144	15.3%	0.773	0.222	28.7%
European hornbeam	5 mg/ml	5 h	1.165	0.126	10.8%	1.060	0.329	31.0%
European chestnut	25 mg/ml	24 h	0.427	0.127	29.7%	< 0.1		
European chestnut	25 mg/ml	5 h	0.752	0.105	14.0%	0.456	0.133	29.1%
European chestnut	5 mg/ml	24 h	0.778	0.087	11.2%	0.964	0.258	26.8%
European chestnut	5 mg/ml	5 h	0.970	0.047	4.8%	0.906	0.122	13.5%

			MTS			WST-1		
Species	**Conc.**	**Dur.**	**Mean**	**SD**	**CV%**	**Mean**	**SD**	**CV%**
Walnut	25 mg/ml	24 h	0.665	0.148	22.3%	< 0.1		
Walnut	25 mg/ml	5 h	0.974	0.194	19.9%	< 0.1		
Walnut	5 mg/ml	24 h	0.881	0.135	15.4%	0.824	0.267	32.4%
Walnut	5 mg/ml	5 h	1.138	0.147	12.9%	0.967	0.221	22.8%
White willow	25 mg/ml	24 h	0.448	0.173	38.6%	0.383	0.042	11.0%
White willow	25 mg/ml	5 h	0.808	0.207	25.7%	0.581	0.063	10.8%
White willow	5 mg/ml	24 h	0.749	0.122	16.3%	0.799	0.172	21.6%
White willow	5 mg/ml	5 h	0.980	0.030	3.1%	0.601	0.191	31.7%
Lime tree	25 mg/ml	24 h	< 0.1			< 0.1		
Lime tree	25 mg/ml	5 h	0.453	0.205	45.2%	0.417	0.184	44.1%
Lime tree	5 mg/ml	24 h	0.539	0.120	22.2%	0.139	0.138	99.3%
Lime tree	5 mg/ml	5 h	0.865	0.252	29.2%	0.559	0.341	61.0%
Horse chestnut	25 mg/ml	24 h	< 0.1			< 0.1		
Horse chestnut	25 mg/ml	5 h	< 0.1			< 0.1		
Horse chestnut	5 mg/ml	24 h	0.705	0.075	10.7%	0.791	0.203	25.6%
Horse chestnut	5 mg/ml	5 h	0.935	0.105	11.2%	0.959	0.198	20.7%
Maple	25 mg/ml	24 h	0.174	0.025	14.5%	< 0.1		
Maple	25 mg/ml	5 h	0.319	0.100	31.4%	< 0.1		
Maple	5 mg/ml	24 h	0.418	0.138	33.0%	0.144	0.064	44.6%
Maple	5 mg/ml	5 h	1.114	0.173	15.5%	0.636	0.143	22.5%

By the MTS assay, after an incubation time of 24 hours and at a concentration of 25 mg/ml, a decrease of cell viability compared to control could be detected for all of the different pollen species, except for European silver fir and Korean fir. Whereas, the WST-1 assay showed a reduction in cell viability for these species compared to the control [1].

In summary, the results of the MTS and WST-1 assays were comparable, while the WST-1 results for cell viability at a concentration of 25 mg/mL were by trend lower than the MTS results (Figures 30-33) [1].

Figure 30. Cell viability after incubation with 5 mg/ml pollen extracts for 5 hours. Statistically significant changes compared to the viability of the control are marked with an asterisk. Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom.

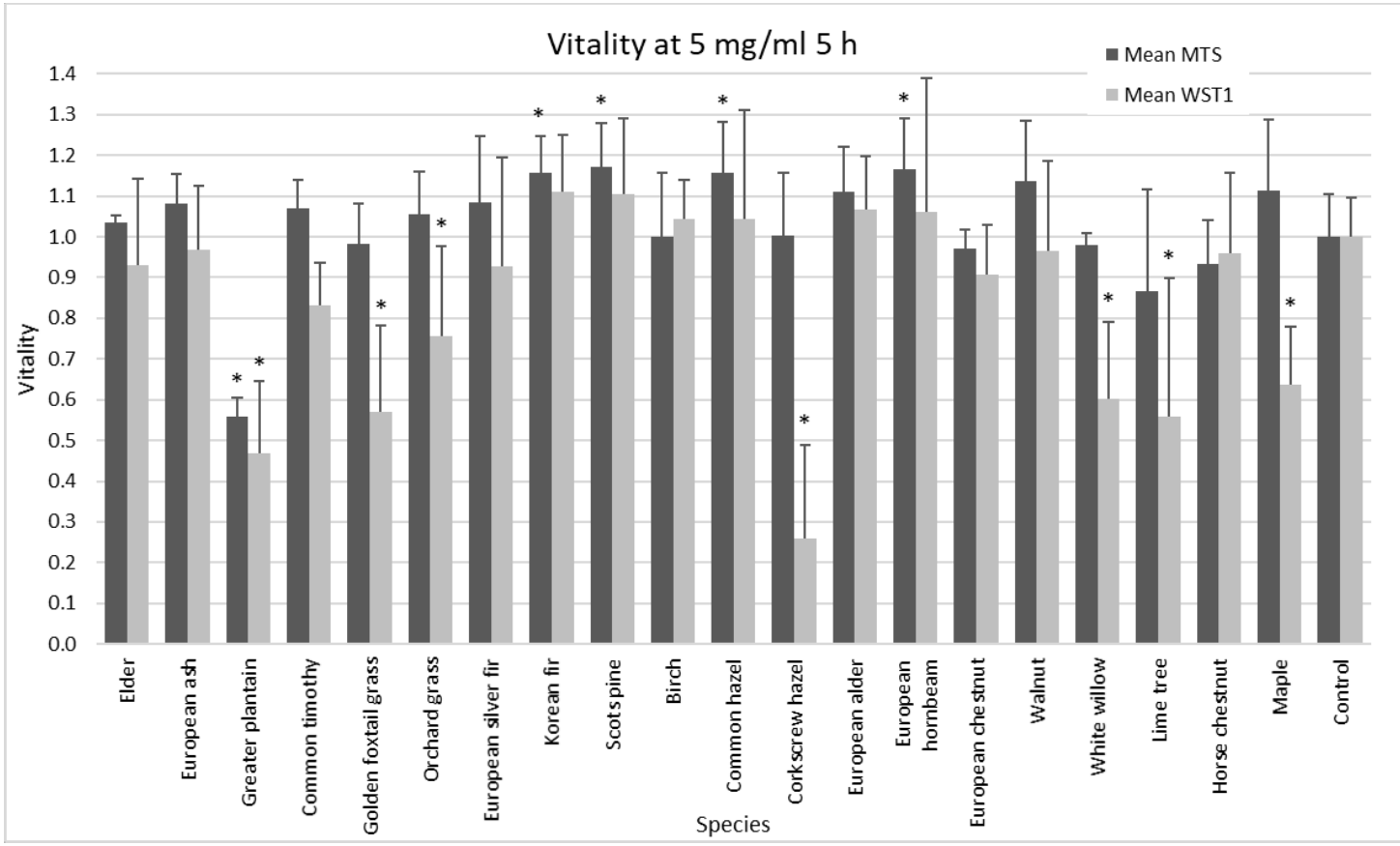


Figure 31. Cell viability after incubation with 5 mg/ml pollen extracts for 24 hours. Statistically significant changes compared to the viability of the control are marked with an asterisk. Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom.

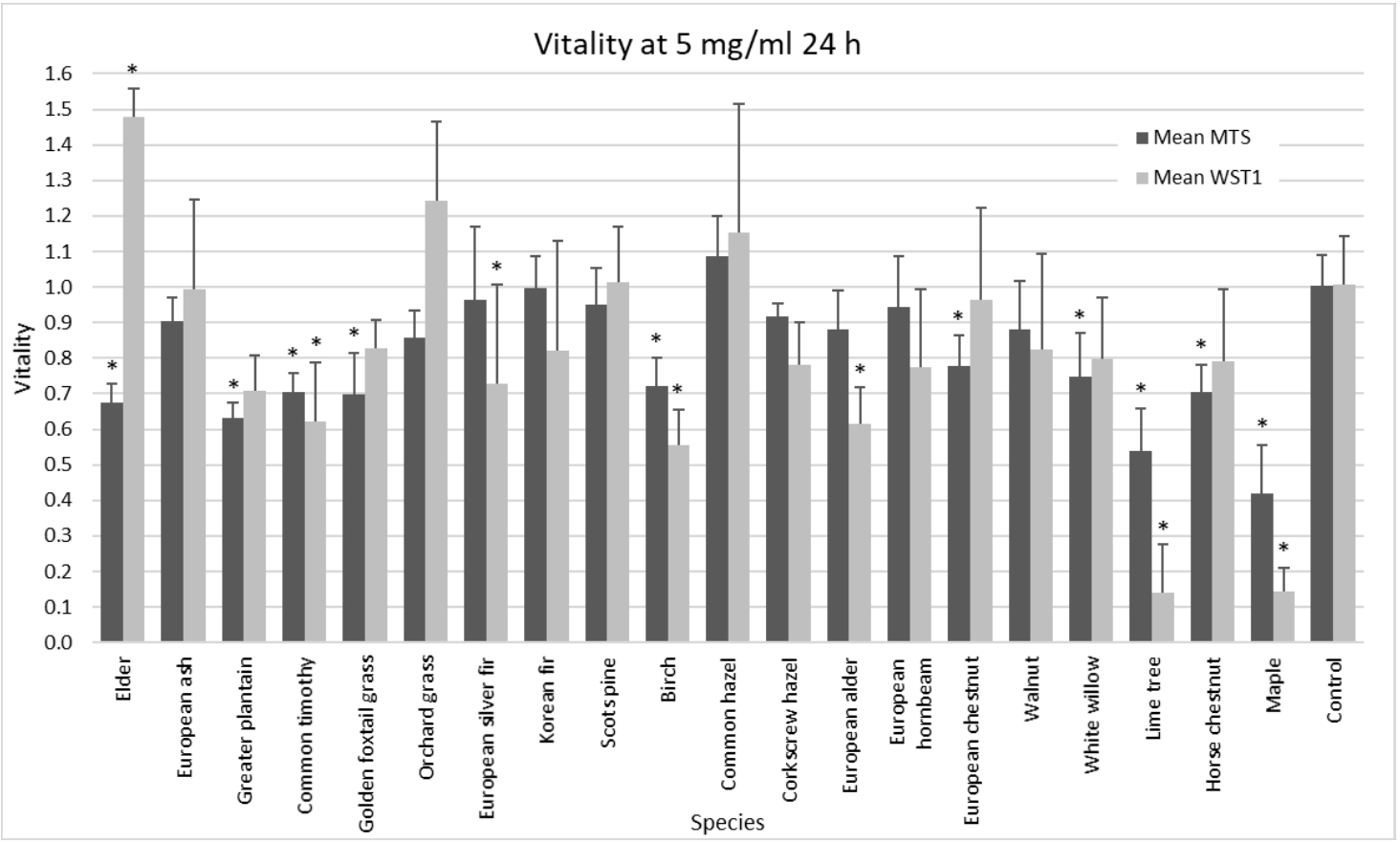


Figure 32. Cell viability after incubation with 25 mg/ml pollen extracts for 5 hours. Statistically significant changes compared to the viability of the control are marked with an asterisk. Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom.

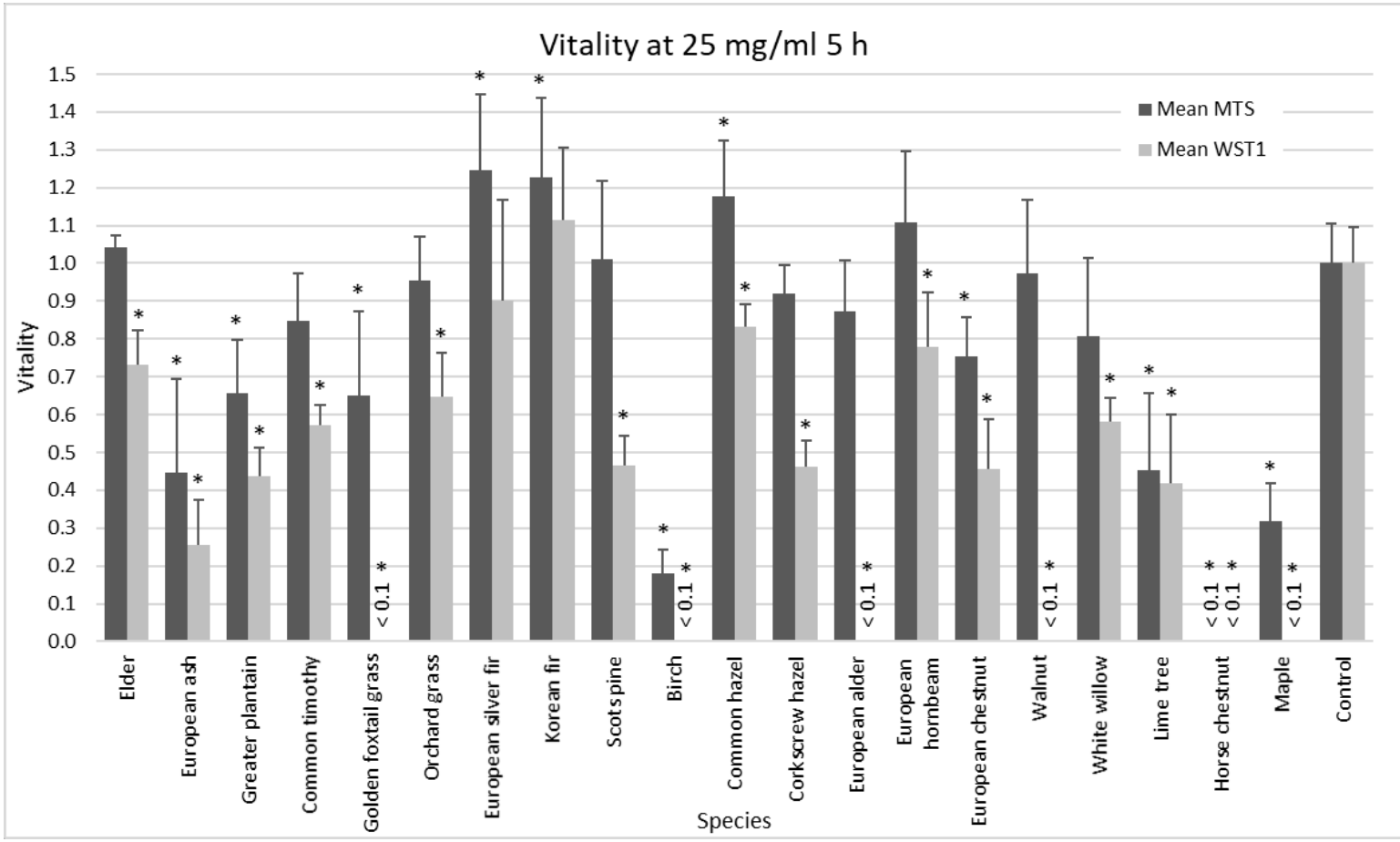
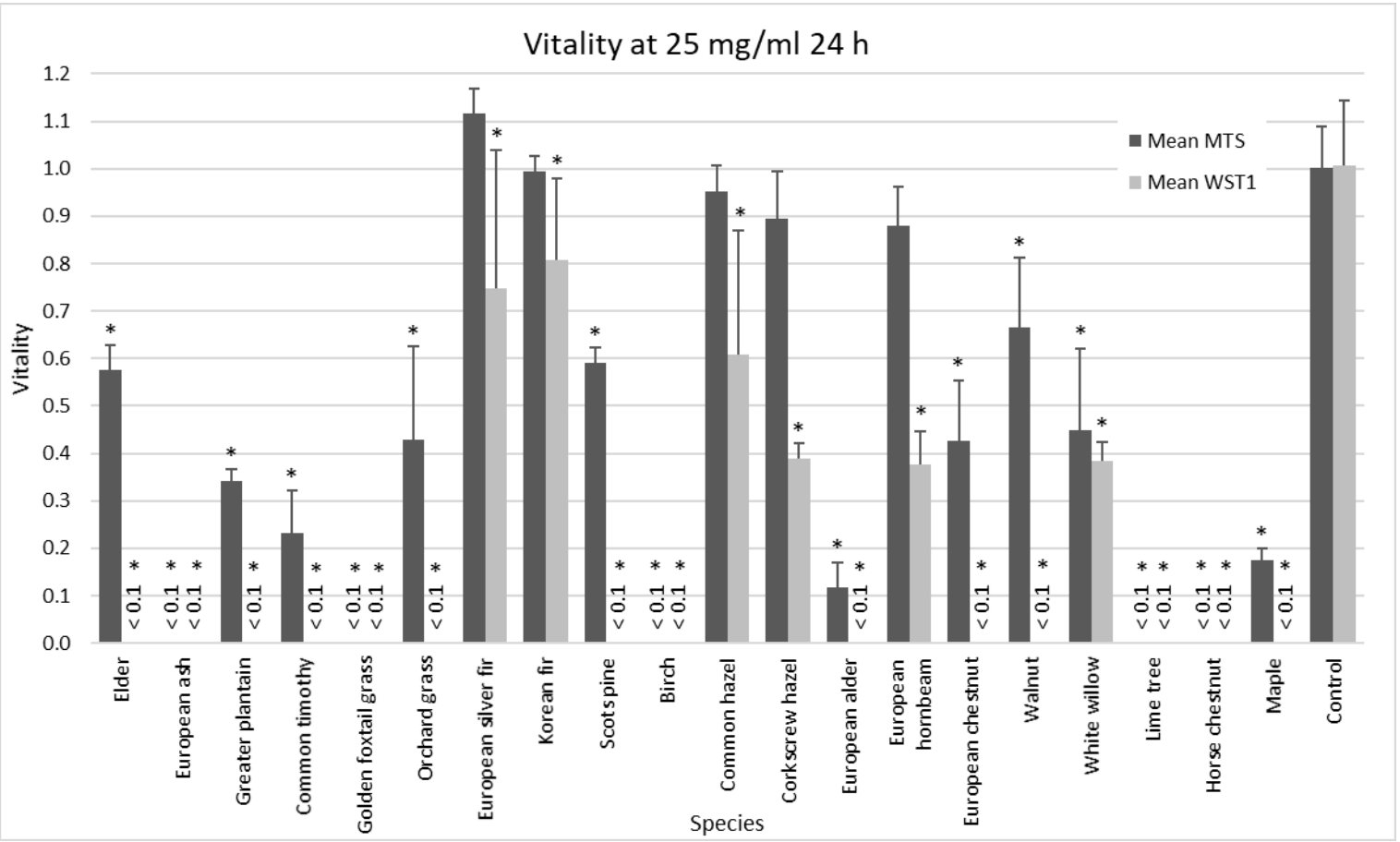


Figure 33. Cell viability after incubation with 25 mg/ml pollen extracts for 24 hours. Statistically significant changes compared to the viability of the control are marked with an asterisk. Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom.



When pooling the results of the 20 pollen species into their 11 different plant families as a biological classification, the incubation of human epithelial cells with pollen extracts from Pinaceae (p=0.001), Betulaceae (p=0.009) and Juglandaceae (p=0.048) at a concentration of 5 mg/ml statistically significantly increased cell viability after 5 hours of incubation (Table 4). This effect did not remain significant with an increased pollen concentration of 25 mg/ml or 24 hours of incubation (Table 5) [1].

Table 4. Cell viability assessed by MTS assay after a 5-hour incubation with 5mg/ml pollen extract. Post Hoc Tests: Dunnett t (2-sided).

(I) Family	(J) Family	Mean Diff (I-J) MTS	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Adoxaceae	Control	.034857	.081586	>.999	-.19811	.26782
Oleaceae	Control	.081945	.081586	.978	-.15102	.31491
Plantaginaceae	**Control**	-.440300	.098805	**<.001**	-.72244	-.15816
Poaceae	Control	.036462	.050146	.998	-.10673	.17965
Pinaceae	**Control**	.127739	.032034	**.001**	.03627	.21921
Betulaceae	**Control**	.101105	.029617	**.009**	.01653	.18568
Fagaceae	Control	.010778	.059582	>.999	-.15936	.18091
Juglandaceae	**Control**	.137748	.048036	**.048**	.00058	.27492
Salicaceae	Control	-.020257	.081586	>.999	-.25322	.21271
Malvaceae	Control	-.134918	.048036	.057	-.27209	.00225
Sapindaceae	Control	.072456	.043329	.634	-.05127	.19618

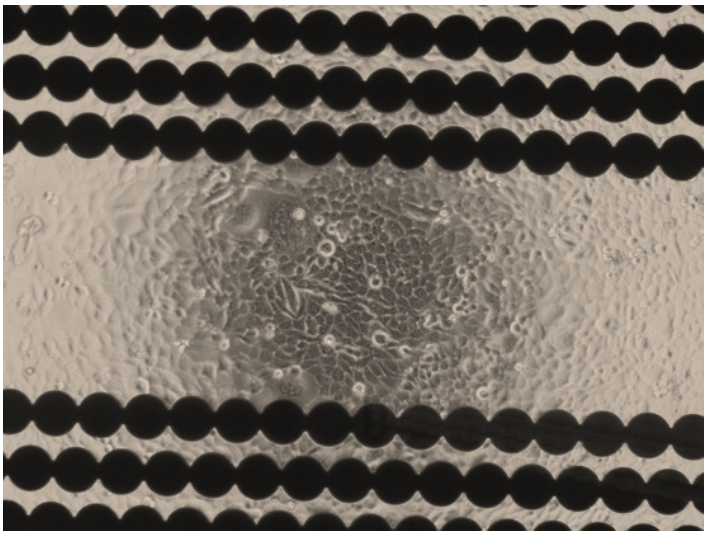
Table 5. Cell viability assessed by MTS assay after a 24-hour incubation with 25mg/ml pollen extract. Post Hoc Tests: Dunnett t (2-sided).

(I) Family	(J) Family	Mean Diff (I-J) MTS	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Adoxaceae	**Control**	-.426839	.120224	**.006**	-.77243	-.08125
Oleaceae	**Control**	-.985372	.120224	**<.001**	-1.33096	-.63978
Plantaginaceae	**Control**	-.661702	.120224	**<.001**	-1.00729	-.31611
Poaceae	**Control**	-.778621	.078705	**<.001**	-1.00486	-.55238
Pinaceae	Control	-.124422	.074863	.630	-.33962	.09078
Betulaceae	**Control**	-.415436	.067399	**<.001**	-.60918	-.22170
Fagaceae	**Control**	-.514947	.090881	**<.001**	-.77619	-.25371
Juglandaceae	Control	-.337654	.120224	.060	-.68324	.00793
Salicaceae	**Control**	-.554463	.120224	**<.001**	-.90005	-.20888
Malvaceae	**Control**	-.945599	.120224	**<.001**	-1.29119	-.60001
Sapindaceae	**Control**	-.921360	.090881	**<.001**	-1.18260	-.66012

8.5 RTCA

Cell proliferation assessed by the electrical impedance measurements of the xCELLigence RTCA system revealed a marked influence of the different pollen extracts on cultivated conjunctival cells [1]. The control cells showed a normal proliferation (Figure 34).

Figure 34. Control cells after 48 hours in a cell culture E-plate with interdigitated micro-electrodes integrated into the bottom.



Alterations of cell adhesion, proliferation and viability could be observed after the incubation of conjunctival cells with extracts from different pollen species (Figures 35-37) [1].

Figure 35. Cells incubated with 5mg/ml birch pollen extract after 48 hours in a cell culture E-plate with interdigitated micro-electrodes integrated into the bottom.

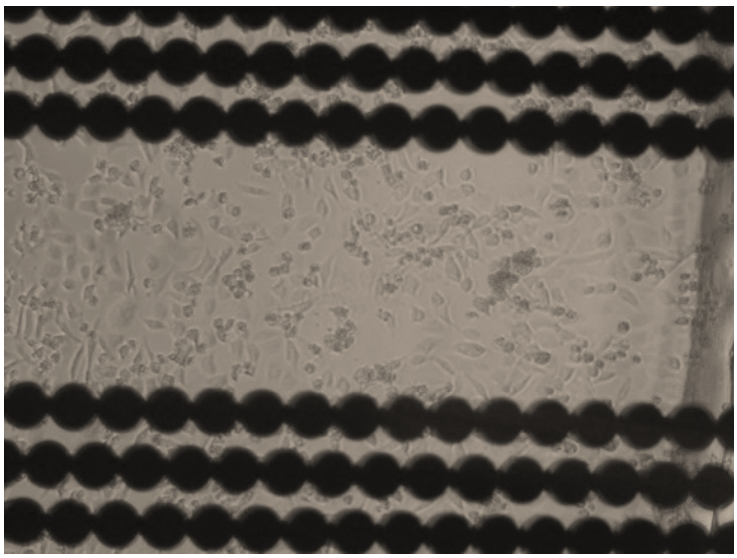


Figure 36. Cells incubated with 25mg/ml European alder pollen extract after 48 hours in a cell culture E-plate with interdigitated micro-electrodes integrated into the bottom.

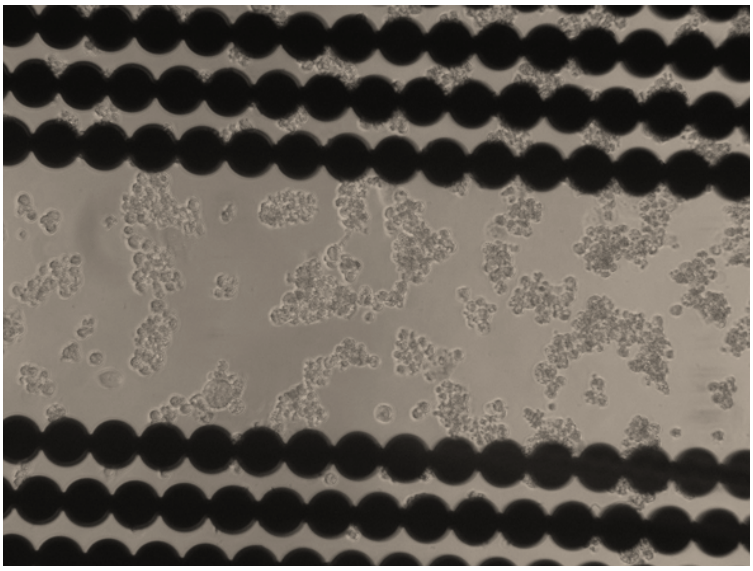
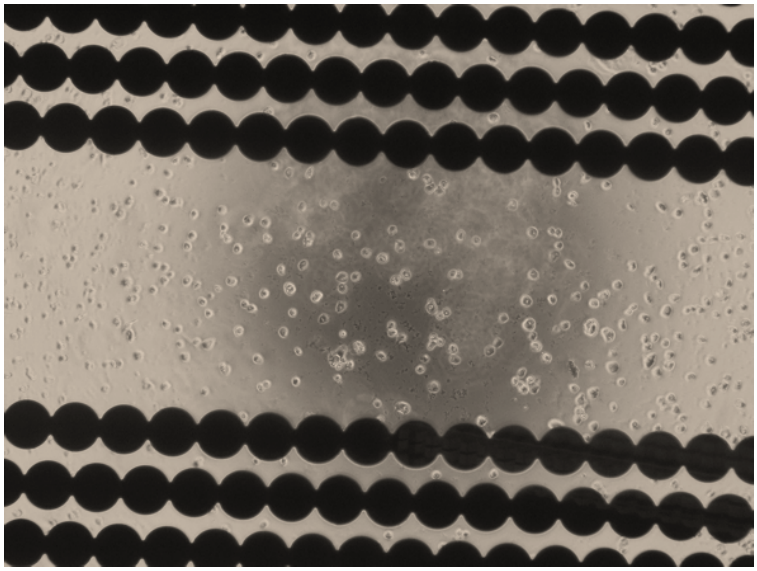


Figure 37. Cells incubated with 25mg/ml Walnut pollen extract after 48 hours in a cell culture E-plate with interdigitated micro-electrodes integrated into the bottom.



Dose depended cytostatic and cytotoxic effects of the different pollen species could be observed. European silver fir pollen showed the weakest, whereas Lime tree pollen showed the strongest effect on the cell growth curves and CI [1]. Table 6 provides a summary of the RTCA results. (Figures 38-43).

Table 6. Effects of different pollen species on CI according to RTCA. Reproduced from [1] with permission of publisher Taylor & Francis Group, London, United Kingdom.

Species	**Clade**	**Order**	**Family**	**5 mg/mL Pollen extract**	**25 mg/mL Pollen extract**
Birch	Rosids	Fagales	Betulaceae	cytotoxic	cytotoxic
Common hazel	Rosids	Fagales	Betulaceae	none	cytotoxic
Common timothy	Commelinids	Poales	Poaceae	cytotoxic	cytotoxic*
Corkscrew hazel	Rosids	Fagales	Betulaceae	cytostatic	cytostatic*
Elder	Asterids	Dipsacales	Adoxaceae	cytostatic	cytotoxic
European alder	Rosids	Fagales	Betulaceae	cytotoxic	cytotoxic*
European ash	Asterids	Lamiales	Oleaceae	cytostatic	cytotoxic*
European chestnut	Rosids	Fagales	Fagaceae	cytostatic*	cytotoxic
European hornbeam	Rosids	Fagales	Betulaceae	cytostatic	cytotoxic
European silver fir	Pinopsida	Pinales	Pinaceae	none	cytostatic*
Golden foxtail grass	Commelinids	Poales	Poaceae	cytostatic*	cytotoxic
Greater plantain	Asterids	Lamiales	Plantaginaceae	cytostatic	cytotoxic
Horse chestnut	Rosids	Sapindales	Sapindaceae	cytostatic*	cytotoxic*
Korean fir	Pinopsida	Pinales	Pinaceae	none	cytostatic*
Lime tree	Rosids	Malvales	Malvaceae	cytotoxic*	cytotoxic*
Maple	Rosids	Sapindales	Sapindaceae	cytotoxic	cytostatic*
Orchard grass	Commelinids	Poales	Poaceae	cytostatic*	cytotoxic*
Scots pine	Pinopsida	Pinales	Pinaceae	cytostatic*	cytotoxic*
Walnut	Rosids	Fagales	Juglandaceae	cytostatic	cytotoxic*
White willow	Rosids	Malpighiales	Salicaceae	cytostatic*	cytotoxic

* … pronounced effect

Figure 38. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - Birch 25mg/ml, blue - Birch 5mg/ml, pink - Common hazel 25mg/ml, turquoise - Common hazel 5mg/ml, purple - European alder 25mg/ml, brown - European alder 5mg/ml, dark green - European hornbeam 25mg/ml).

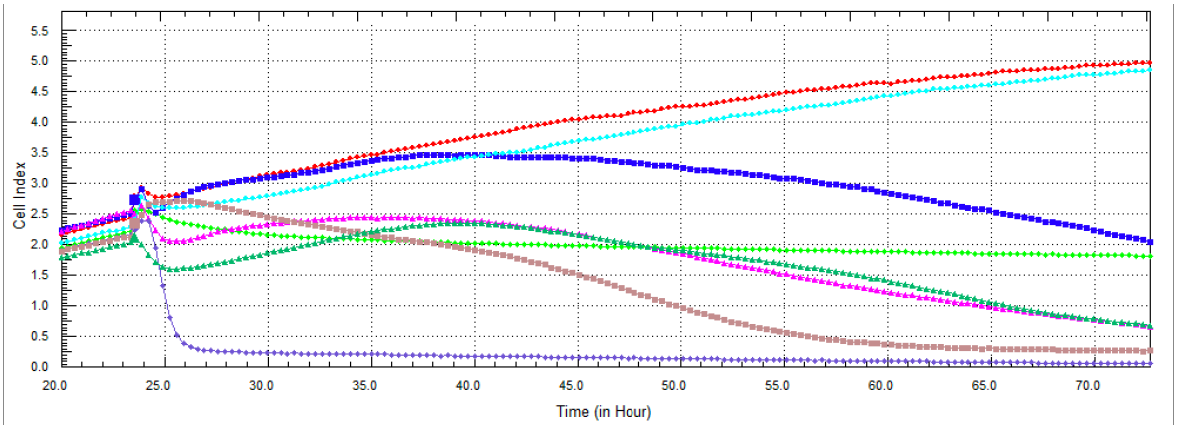


Figure 39. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - European hornbeam 5mg/ml, blue - Korean fir 25mg/ml, pink - Korean fir 5mg/ml, turquoise - Scots pine 25mg/ml, purple - Scots pine 5mg/ml, brown - European silver fir 25mg/ml, dark green - European silver fir 5mg/ml).

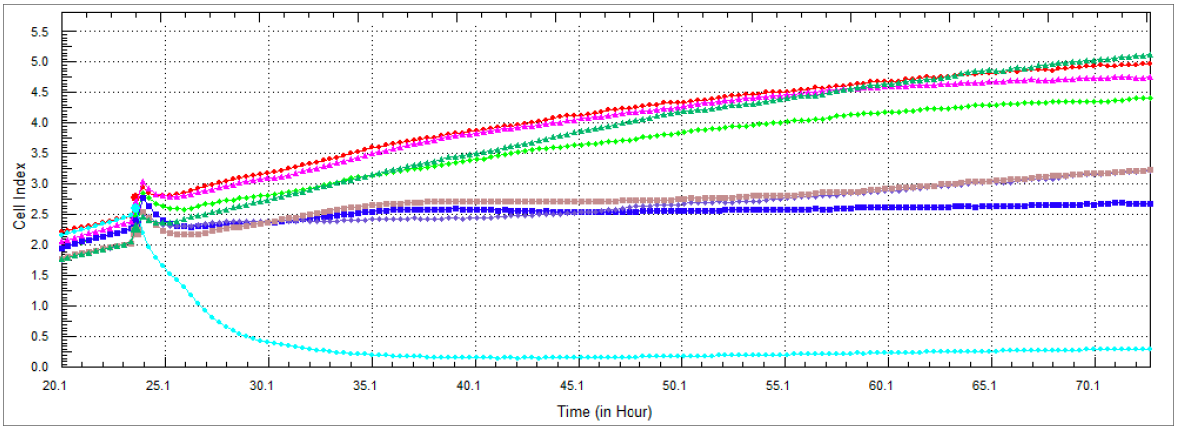


Figure 40. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - Walnut 25mg/ml, blue - Walnut 5mg/ml, pink - Maple 25mg/ml, turquoise - Maple 5mg/ml, purple - Lime tree 25mg/ml, brown - Lime tree 5mg/ml).

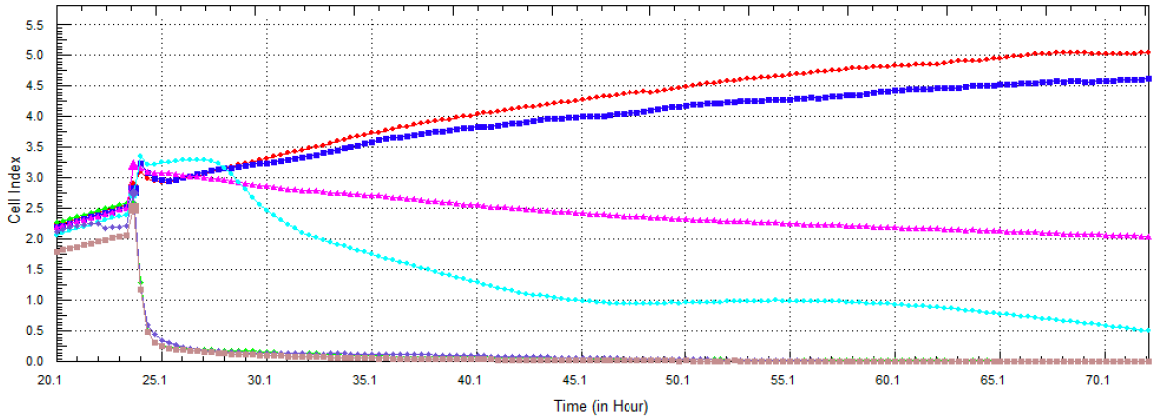


Figure 41. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - Horse chestnut 25mg/ml, blue - Horse chestnut 5mg/ml, pink - European ash 25mg/ml, turquoise - European ash 5mg/ml, purple - European chestnut 25mg/ml, brown - European chestnut 5mg/ml, dark green - Elder 25mg/ml).

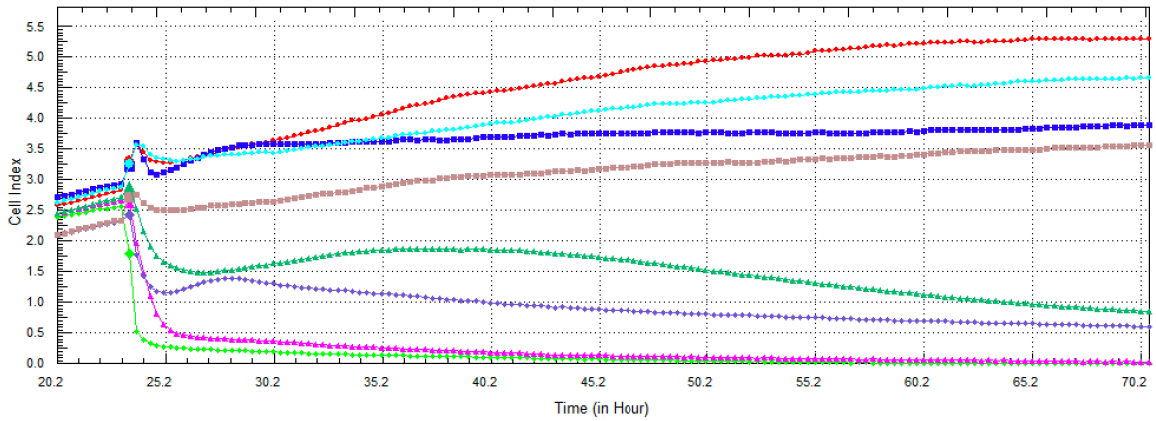


Figure 42. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - Elder 5mg/ml, blue - Common timothy 25mg/ml, pink - Common timothy 5mg/ml, turquoise - Orchard grass 25mg/ml, purple - Orchard grass 5mg/ml, brown - Golden foxtail grass 25mg/ml, dark green - Golden foxtail grass 5mg/ml).

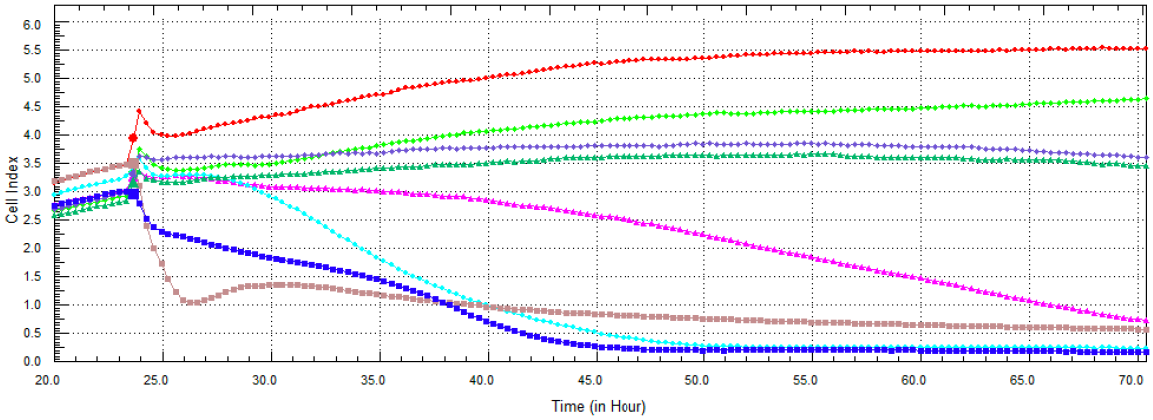
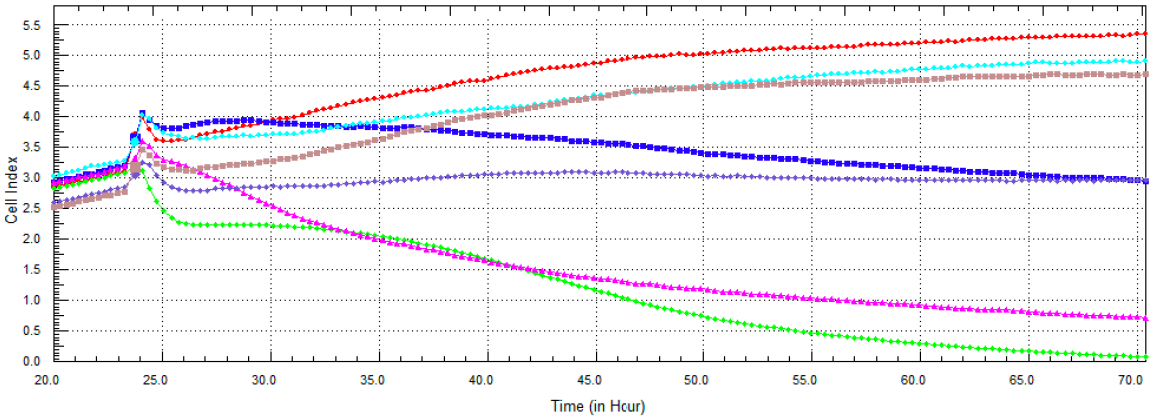


Figure 43. xCELLigence growth curves of control (red) and cells incubated with different pollen extracts (green - White willow 25mg/ml, blue - White willow 5mg/ml, pink - Greater plantain 25mg/ml, turquoise - Greater plantain 5mg/ml, purple - Corkscrew hazel 25mg/ml, brown - Corkscrew hazel 5mg/ml).



8.6 LDH-assay

Our attempt to supplement the results of the MTS and WST-1 assays by the additional performance of a LDH release assay to quantify cell death and cell lysis provided highly variable results, which could not be reproduced from one assay to another.

9. Discussion

Pollen were identified to act as a significant trigger for various diseases and therefore have an important impact on health care systems world-wide. They are thought to induce an important group of diseases (e.g. allergic asthma, conjunctivitis, bronchitis and rhinitis), which become more prevalent during the last decades and affect approximately 40% of the people [1,7,8].Unfortunately, there is still limited data on the prevalence of pollen reactions affecting the eye.

Our biochemical investigation of pollen species from different plant families confirmed the presence of various pollen proteins among other substances. Zymography identified some of the proteins as proteolytically active proteases. Depending on the type of proteases within the different pollen species, the enzymatic activity varied. PAGE revealed the degradation of tear fluid proteins by pollen species from different plant families. This reaction caused a destruction of tear fluid proteins and is thought to destabilize the tear-film, which is built through interaction between proteins, lipids and mucous-substances. Tears are no longer able to moisten the affected areas, leading to dry sites on the ocular surface and causing unpleasant irritations [91]. Interestingly, there were almost no visible bands in the zymography and PAGE of European chestnut, European hornbeam and Maple. We also could not detect a destruction of tear fluid proteins by these pollen species. A possible explanation might be the increased presence of lipids in the coating of some of the pollen species compared to others, thus making it more difficult to obtain the content of the pollen [1].

In many parts of the world grass pollen is the most important cause of pollen allergy. Although the frequency of pollinosis due to grass pollen differs from area to area, it is known to be the most common European pollen allergy. The Gramineae include at least 600 types and more than 10.000 species. In Europe, there are known to be over 400 herbaceous plants pollinated by wind. Meadow foxtail (Alopecurus pratensis), Orchard grass (Dactylis glomerata) and Timothy (Phleum pratense), which are also called Tall meadow grasses, are the source for most of the Gramineae pollen in the atmosphere. Another potent source is cultivated Rye (Secale cereal), having a very high pollen production. There is a substantial cross reactivity between the different types of grass

pollen, although some exceptions occur. Typically, conjunctival and nasal symptoms are induced by grass-pollen allergens [8,20,21,121].

The Fagales comprise three different families: the Betulaceae - with the genera Alnus (Alder) and Betula (Birch); the Corylaceae - containing the genera Carpinus (Hornbeam), Corylus (Hazel) and Ostrya (Hopbeam); last but not least the Fagaceae - Castanea (Sweet chestnut), Fagus (Beech) and Quercus (Oak). Birch pollen are often referred to as the ultimate allergenic tree-pollen of central, eastern as well as northern Europe. Whereas in the Mediterranean regions, pollen of Olive (Olea europaea) and Cypress (Cupressus) trees are commonly known as highly allergenic [8,22].

The most important genus of the Urticaceae (nettle) family in terms of allergenic potential is Parietaria. Parietaria judaica and officinalis are the most important species. Parietaria judaica can primarily be found at the Mediterranean coasts. Colombo and colleagues found, that both species possess small, highly cross-reacting glycoprotein allergens [8,45,122]. Cortes et al. were able to purify an aminopeptidase out of Parietaria judaica pollen. The enzyme was able to disrupt epithelial barriers, paving the way of pollen allergens to dendritic cells [8,94].

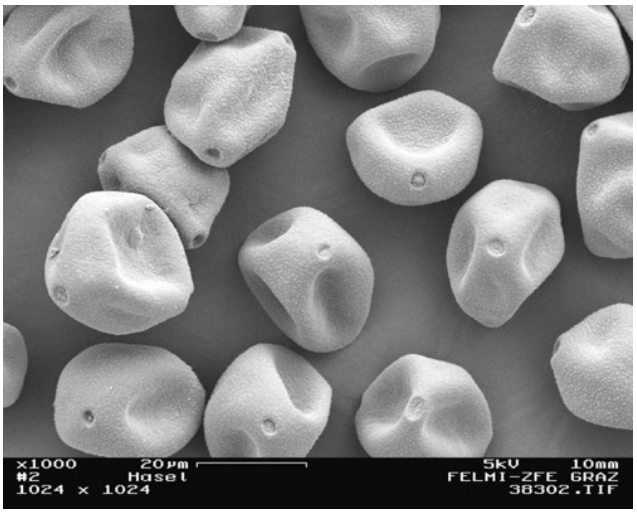
The largest plant family, comprising nearly 20.000 species is the Compositae (Asteraceae). Most involved in pollinosis are Ragweed (Ambrosia) and Mugwort (Artemisia). Almost every single Ambrosia artemisiifolia plant is able to release millions of pollens. These grains are able to travel very long distances, due to their small size [8,12,21].

Especially in central and eastern European regions, allergists are getting increasingly interested in Ambrosia pollen. In 1960 Ambrosia artemisiifolia, was noted in Europe for the first time. The distribution of common ragweed is usually around a medium latitude and has started its growth from Hungary, being affected most by Ambrosia species, Croatia, distinct areas of France, as well as Italy [8,123-125]. Meanwhile, Ambrosia has been found in Austria, Bulgaria, Czechia, Poland, Slovakia, Sweden and Switzerland [8,126,127].

Ophthalmologists primarily face pollen reactions affecting the tear film and the ocular surface of the human eye. Usually, the ocular surface acts as protective barrier against different kinds of environmental influences. Due to its close connection to the vascular and

lymphatic system, it also is an immune-competent wall against infectious agents. The lacrimal system resembles an outstanding permanent cleaning system for the ocular surface. Loss of only one of the physiological functions of the ocular surface can immediately lead to potentially harmful disorders and often consecutively lead to visual impairment [1,71-73]. Dried pollen getting in contact with any fluid, like tear fluid, immediately bloat and release their content through small pores in their outer membrane (Figure 44) into the environment, e.g. the ocular surface.

Figure 44. Pores of pollen.



Ocular surface disorders can be connected to the presence of pollen as well. Due to an overlap of symptoms, like conjunctival chemosis, discharge, dryness, foreign body sensation, inflammation, itching, photophobia and even visual impairment, it is often very difficult to distinguish ocular surface disorders from each other by the clinical picture only (e.g. dry eye disease) [5].

There are distinctive subtypes of dry eye disease due to different pathophysiological pathways [5,71,101-103]. Generally, these occur when inadequate tear volume or function (e.g. hyperevaporation) results in an unstable tear film with hyperosmolarity and inflammation, consecutively leading to an ocular surface disease [71,101-103]. While pollen enzymes can trigger dry eye disease by destroying important tear film components,

dry eye disease itself can also facilitate the contact of pollen allergens and enzymes with the ocular surface, when there is only little or non-functioning tear film left [5]. A disruption of the corneal epithelial barrier has been found in dry eye as well, which might make those patients even more susceptible to inflammatory reactions caused by pollen (e.g. SNAC-syndrome) [1,128].

It seems feasible that an accumulation of etiological factors of dry eye such as exposure to wind, ultraviolet light and ozone for example may lead to an increased sensibility to pollen proteases as well [1,2,129-131]. Recently, it could also be shown that ultraviolet light irradiation increased the histamine level of Alder and Hazel pollen in a dose-dependent manner [132].

Furthermore, one has to keep in mind, that people are rather exposed to pollen grains or granules derived from them than to pure allergens alone. For a very long time pollen grains have just been deemed as the transporters of allergens. Only very few payed attention to the non-protein ingredients of pollen [8,133-135]. Traidl-Hoffmann and colleagues analysed the exine as well as exsudate of pollen. They found, that lipids represent chief components of these [8,134]. Additionally, multiple different pollen plant hormones need long chain unsaturated fatty acids as precursors for their biosynthesis. These dinor isoprostanes are also referred to as phytoprostanes. They emerge by autoxidation, without the help of enzymes and concerning their structure, they look like human prostaglandins or isoprostanes [8,136-138]. Phytoprostanes could resemble an evolutionary ancient host defence function in plants [8,139,140].

While more and more functions of phytoprostanes in the life of the plants are understood, we still lack knowledge about their role in the human immune response. Recently, it has been revealed that bioactive lipids are released by pollen grains, too. In vitro these lipids can lead to an activation of human eosinophils and neutrophils [8,141-143]. Besides, in vitro dendritic cells can be activated and maturated even by getting in contact with intact pollen grains. Allakhverdi et al. suggest, that pollen act as a transporter for allergens as well as play a key role during the induction of an allergic response of the immune system [8,144].

15-40 µm is the aerodynamic size of intact pollen grains. Therefore, it should not be possible for them to enter the lower respiratory tract. This could explain, why the

characteristic hay fever symptoms primarily affect the conjunctiva and the nasal or nasopharyngeal mucous membrane [8,145,146].

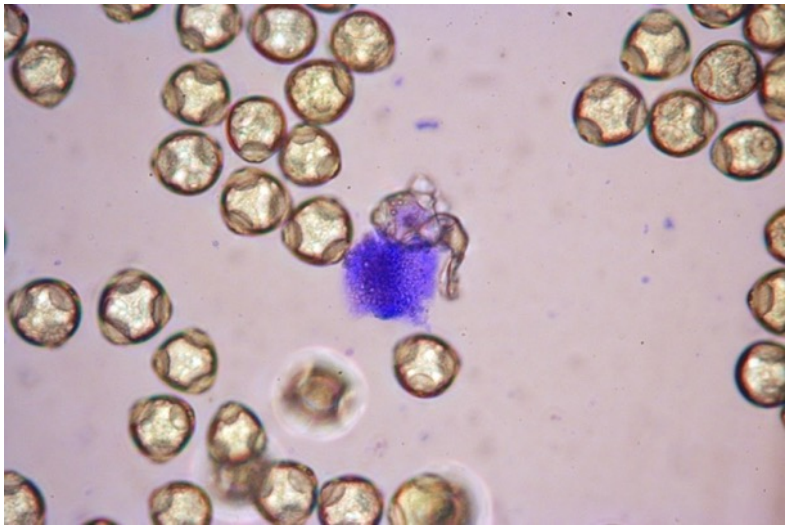
Though, cough or wheezing are normally symptoms of the bronchial regions, they can be observed in patients suffering from pollen allergies. There exist several hypotheses for the occurrence of thoracic complaints in hay fever. For example, pollen grains might be able to reach the lower airways with mouth breathing [8,147].

By fractionating collected particulate matter into different classes according to size, recent studies found particles sizes ranging from 2 to 10 micrometres (paucimicronic), to submicronic sizes [8,148-152]. Evidence suggests that these particles, being smaller than intact pollen, are carrying pollen allergen. They might be able to penetrate the lower respiratory tract and seem to be responsible for symptoms of pollinosis in the lower airways by direct action [8,153,154]. Already Blackley postulated about airborne “free granular matter” inducing “hay asthma” in 1873 [8]. In the year 1972 researches proved the occurrence of Ambrosia allergens transported by smaller particles than pollen grains [8,155]. This observation could be confirmed and extended by different techniques, to other ragweed-pollen allergen, pollen allergens of Gramineae, Red oak, Japanese cedar and Betula [8,148-152,156-158]. The localization of pollinosis-related symptoms seems to be partly defined by the type and concentration of these airborne small particles, in association with the amount of pollen present in the atmosphere [8].

It is unlikely that pollen-grain fragments make a major contribution to the pollen-allergen-carrying particulate matter in the atmosphere since pollen grains usually hardly fragmentize and therefore, are probably not a significant part of particulate matter loaded with pollen allergens in the air. However, it was proposed, that pollen rupture under special circumstances, like during thunderstorms by osmotic shock. Abrasion from pollen grains might be possible as well [8,156].

A chief component of pollen grains are so-called starch granules. Their sizes range from 0.5µm up to 5µm and specific pollen allergens can be found in them [8,159,160]. They might be released directly into the air in rare cases, when pollen grains rupture (Figure 45) [8].

Figure 45. Ruptured pollen, releasing starch granules (Coomassie blue).



Several studies report, that also other parts from plants are able to transport pollen antigens. Examples might be inflorescences, leaves and orbicules or Ubisch bodies as well [8,149,161]. Despite, Ubisch bodies have already been discovered in 1865 by Rosanoff [162], their exact purpose has yet to be determined. They might function as a transporter for sporopollenin, an inert biopolymer, promoting pollen resistance against biological, chemical and physical attacks [163].

It is thought, that a transfer of allergens from pollen grains to different kinds of other particles, being present in the atmosphere, is possible. This might be possible by physical contact or leaching, occurring at the ground or in the atmosphere [8,159].

Especially, in areas with a high concentration of particulate matter in the air, this process of transfer might be clinically relevant [8,153]. It is thought to explain the extensive variety of sizes of these secondary pollen-allergen carriers, too [8,164].

People living in urban regions appear to be affected by respiratory allergies due to pollen exposition more often than subjects living in rural regions. Urbanization, as well as increased levels of traffic emissions seem to facilitate the development of these disorders [8,68,70,165-167].

Besides the air pollutants, like nitrogen dioxide (NO_2) and inhalable particulate matter (PM2.5 and PM10), often high concentrations of ozone and ultraviolet irradiation are involved. The formation of O_3 depends on the supply of enough substrate, usually vehicle emissions of NO_2 and on the weather in the respective area. The rate of conversion of NO_2 into O_3 is especially high on days with a lot of sunshine [8].

According to the current literature, when breathing the nasal mucosa absorbs about 40 to 60% of O_3. Whereas, the rest will reach the small bronchial system. An increased contact to O_3 induces a reduction of the lung function, leads to an amplified reactivity of the airways to bronchoconstrictors promoting an increased risk of an exacerbation in asthmatic patients [8,168,169]. Furthermore, ozone increases the number of hospital admissions for respiratory diseases, including asthma [8,169-171]. It is also able to enhance inflammation and epithelial permeability in the airways [8,170-172].

Exposure to O_3 also triggers the appearance of inflammatory cells and mediators, like granulocyte-macrophage colony-stimulating factor (GM-CSF) as well as interleukin (IL)-6 and IL-8 and fibronectin, in asthmatic subjects [8,172,173].

O_3 and other pollutants may mediate an increased vulnerability to the antigen that allergic subjects are sensitized to [8,173,174]. Even the incidence of asthma has found to be connected when preforming heavy exercises in regions with high O_3 concentrations [8,175].

A possible explanation could be the accumulation of small particles carrying allergens as well as particulate matter in the lower bronchi. Another mechanism behind this reaction may be an increase in epithelial permeability and enhanced oxidative stress in the airways, acting as kind of a primer for the subsequent allergen-induced responses [8].

Air pollutants affect pollen in the atmosphere itself, as well as people's airways directly. By interaction with pollen grains, they might play a key role in atopic sensitization and possibly exacerbate allergic diseases [8,176,177].

The relationship of air pollution and respiratory allergic diseases in pollen allergy has been studied extensively [8,70,176-179]. Air pollution leads to mucosal damage and impaired mucociliary clearance in the airways and thereby may pave the way for pollen allergens to immune cells [8,176]. Additionally, the allergenic potential of plants is influenced by the

reaction of the vegetation with air pollutants and climate changes. There are various factors influencing this reaction, like the type of air pollution, climatic factors, as well as the species of the plant and its nutrition [8].

Interestingly, in atopic subjects there are also certain air pollutants, which might possess immunological effects on the synthesis of IgE as well. Recent studies especially identified certain particles in the exhaust of diesel combustion engines. The interact with airborne paucimicronic particles and pollen [8].

Climate changes are known to have potential effects on the start and length of the flowering season, rate of pollen assembly, allergenic potential and the distribution of the plant as well as its attributes [8,180]. The data from the International Phenological Gardens in Europe has revealed, that spring events, such as flowering, have advanced by 6 days, and that autumn events have been delayed by 4.8 days, compared with the early 1960s [8].

Local rises in temperature and exposure to a higher CO_2 concentration are accountable for an increased number of pollen during the last century [8,181,182]. Further attributes of allergenic plants reacting to higher CO_2 levels or changes in temperature, are the extension of the flowering period, particularly in summer and late pollinating species, and a stronger allergenic potential in tree pollen growing at higher temperatures [8,183-185].

The spatial distribution of pollen has already been altered by changes in climate. This might be due to changes in European air circulation, facilitating periods, where pollen are carried for long distances, which promotes additional pollen sensitizations among subjects suffering from allergic disease [8,186].

Meanwhile, many cases of plants colonising new geographic areas due to climate changes have been observed. Nevertheless, one might argue, that the impact of the predicted amount of global warming might be less noticeable than changes due to the use of land and an increase in international transportation [8,187].

D'Amato and colleagues recently published a review on asthma and its association with thunderstorms [8,188]. "The outflow of colder air occurring during thunderstorms is thought to sweep up pollen grains and particles, concentrating them at the ground level." [8] Consequently, sensitized subjects are exposed to an increased concentration of allergenic matter, promoting severe allergic reactions [8,189].

Usually wind transports allergenic matter. Meanwhile there are various studies suggesting, that pollen is able to travel for huge distances. Turbulences, especially in the lower layers of the atmosphere disperse pollen grains and allergen carrying particles. This phenomenon mainly depends on the altitude the particles and grains reach and might explain, why people get sensitized to certain pollen allergens, while actually living at a great distance to the actual pollen sources [8,190].

The incubation of human conjunctival cells with extracts of different pollen species caused changes of cytomorphology resulting in cell damage. After the incubation with pollen extracts the normally adherently growing cells detached from each other and floated freely in the supernatant [1]. In our opinion, this might be due to the proteolytic destruction of intercellular contact proteins [1,95]. According to Cunha, the incubation of human alveolar epithelial cells with pollen extracts and isolated serin-endopeptidases of Parietaria judaica lead to a loss of cell coherency. This is thought to occur due to the cleavage of a cytoplasmatic tight junction protein, zonula occludens 1 [1,191]. Unfortunately, it has yet to be determined whether pollen proteases degrade tight junctions directly or via an indirect mechanism [191]. Proteolytic damage of the epithelial barrier promotes the development of further inflammatory and allergic reactions on mucosal surfaces [95]. Allergens crossing epithelial barriers are starting the sensitization process at subepithelial dendritic cells. These are presenting allergenic peptides to naive T-lymphocytes, causing the production of specific IgE [1,92,93].

In our study, the pollen extracts of regional pollen species from different plant families (Adoxaceae, Betulaceae, Fagaceae, Juglandaceae, Malvaceae, Oleaceae, Pinaceae, Plantaginaceae, Poaceae, Salicacea, Sapindaceae) induced microscopically visible cell damage in cultivated conjunctival cells [1].

Additionally, we found a significant reduction of cell viability as assessed by MTS, WST-1 assay and RTCA. The in-vitro data suggest, that patients exposed to pollen-polluted surroundings might not only suffer from destruction of tear fluid proteins, but also from damage to ocular surface cells as well.

Unfortunately, we could not supplement our results by the additional performance of a LDH release assay. The quantification of cell death and cell lysis provided highly variable

results, which could not be reproduced from one assay to another. In our opinion, this might be due to interactions of the LDH release assay with pollen ingredients.

Data concerning the residence time of pollen on the ocular surface, revealed that pollen can last up to five hours on the ocular surface after the initial exposure. For most of the pollen this should be enough time to perform their enzymatic reactions [192].

During the pollen-seasons allergy like symptoms can be observed among allergic as well as non-allergic subjects. Since the pathomechanism of enzymatic pollen reactions is independent of a possible allergic sensitization, it seems reasonable, that allergic as well as non-allergic subjects might suffer from those. The typical anti-allergic treatments, (e.g., H1-antagonists, cromoglycates and desensitization) can probably not protect from proteolytic tissue damage. Additionally, a deficiency of lacrimal antiproteases might lead to an intensification of symptoms [1].

Different levels of tear fluid and ocular surface antiproteases might also be an answer to the question, why not all humans are affected by SNAC-syndrome in the same way. Normally antiproteases are present in different body fluids and protect against enzymatic tissue damage. Examples are α1-antitrypsin, α1-antichymotrypsin and α2-macroglobulin, which have been identified in human tear fluid and nasal secrete as well [1,87]. The most important role, as far as quantity is concerned, plays α_1-antitrypsin. It is an acute phase protein. A sufficient amount of these antiproteases protects against trypsin and other serinproteases, which are present in pollen [193,194].

The number of people affected by enzymatic pollen reactions may be underestimated. Whether these reactions, affecting body fluids and mucosal surfaces, represent a separate entity or a subtype of an allergic disease has still to be determined. It seems possible, that mixed forms of SNAC and allergic reactions might occur as well [1,6,87,91].

Further studies on possible treatment options are warranted. Theoretically, the development of antiprotease eye drops or enzyme inhibitors sounds promising [195]. Unfortunately, different pollen species containing a variety of enzymes, some not even specified yet, might complicate this [196]. Therefore, specification of these enzymes would be the basis for the development of new treatment options. In the meantime, prevention measures to reduce pollen exposure can be recommended. Ideally, during

pollen seasons, patients should try to stay outside only at times, when the airborne pollen concentrations are low, e.g. in the morning, during the night hours and after rain. Outdoor working and exercising should be avoided during high airborne pollen concentrations. Patients should be instructed to wash their face and hair often, to keep the windows in the bed room closed and their pillows free of pollen. Eye flushes with different rinsing-solutions lead to a substantial removal of pollen from the tear film and ocular surface, following pollen exposure [4]. Through these means the pollen-elicited allergic as well as enzymatic reactions may be minimized [1].

Our investigations of pollen species from different plant families could show similar effects on human tear fluid and epithelial cells as observed with Hazelnut and Birch pollen [6]. The present data provides a broad basis for further research on pollen reactions affecting the tear film and ocular surface and underlines the importance of enzymatic pollen reactions affecting secretions lining mucosal membranes and mucosal epithelia of the human body. Thus, further studies on the pollen composition and their reactions' exact pathomechanisms, e.g. the identification of the lysed proteins in tear fluid, diagnosis and therapy are warranted [1].

10. Bibliography

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