

Dissertation

**Molecular allergy diagnostics is highly sensitive and
prevents misdiagnosis in patients sensitized to
aeroallergens**

submitted by

**Dr. med. univ.
Lukas Dietmar KOCH**

for the Academic Degree of
Doctor of Medical Science
(Dr. scient. med.)

at the
Medical University of Graz

Otto Loewi Research Center, Division of Pharmacology

under the Supervision of
Assoz. Prof.ⁱⁿ Priv.-Doz.ⁱⁿ Mag.^a Dr.ⁱⁿ rer. nat. Eva STURM

2022

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 organizations that have contributed to the research for this thesis. Due acknowledgment 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"."

22.05.2022

Dr. Lukas Koch e. h.

Disclosures

This doctoral thesis was the basis for the preparation of a manuscript, which has been published under the following title and journal:

IgE multiplex testing in house dust mite allergy is utile, and sensitivity is comparable to extract-based singleplex testing. Lukas Koch¹, Karin Laipold¹, Lisa Arzt-Gradwohl¹, Urban Čerpes¹, Eva Maria Sturm², Werner Aberer¹, Gunter J Sturm^{1,3}. *Allergy* 2020;75(8):2091-4.

¹Department of Dermatology and Venerology, Medical University of Graz, Austria

²Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Austria

³Allergy Outpatient Clinic Reumannplatz, Vienna, Austria

The manuscript was published as an open-access article by John Wiley and Sons under the terms of the <http://creativecommons.org/licenses/by-nc/4.0/> License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes. It was drafted by the author of this doctoral thesis, which is the reason why significant parts of this doctoral thesis are similar to the manuscript. All co-authors of the manuscript agreed to the reproduction of data and images in this dissertation.

The second part of this doctoral thesis, dealing with seasonal molecular allergy diagnosis, is unpublished/under submission at the time of submission of this doctoral thesis. These parts are marked with ¹⁶¹ throughout the thesis. In addition to the authors listed above, Martina Aumayr⁴ and Wolfgang Hemmer⁵ will be co-authors of future publications. All co-authors agreed to the reproduction of data and images in this dissertation.

⁴MacroArray Diagnostics GmbH, Vienna, Austria

⁵Allergy Outpatient Clinic Reumannplatz, Vienna, Austria

Foreword

"What we know is a drop. What we don't know is an ocean."

Isaac Newton

Acknowledgements

I thank my supervisors, Prof.ⁱⁿ Eva Sturm, Prof. Gunter Sturm and Prof. Werner Aberer, for their permanent and unceasing support, discussion and consultation at any time.

Special thank goes to Karin Laipold for her help performing thousands of sIgE measurements and Dr.ⁱⁿ Lisa Arzt, MSc, for her help with statistics and graph production.

Laboratory analyses were performed using the research budget of the Department of Dermatology, Medical University of Graz. In addition, some of the ALEX² test kits were provided free of charge by the manufacturer MacroArray Diagnostics GmbH, Vienna, Austria.

I am grateful to the Doctoral School “General and Clinical Pathophysiology” and especially its chairperson Prof.ⁱⁿ Adelheid Kresse.

My particular thank goes to my parents, Dr.ⁱⁿ Anna Koch and Dr. Dietmar Koch, for making it possible for me to study medicine and to Prof. Werner Aberer for the opportunity to become a dermatologist.

Finally, I thank my family, especially my wonderful wife Klara, because performing research or scientific work is frequently in exchange for spare time.

Table of Contents

Declaration	II
Disclosures	III
Foreword	IV
Acknowledgements	V
Table of Contents	VI
Abbreviations	1
List of Figures	2
List of Tables.....	3
Zusammenfassung.....	4
Abstract	5
1. Introduction	6
1.1. Allergy.....	6
1.2. Aeroallergens.....	7
1.3. Diagnosis of type I hypersensitivity	8
1.3.1. Medical history.....	9
1.3.2. Skin prick testing.....	10
1.3.3. Intracutaneous testing.....	11
1.3.4. IgE determination.....	11
1.3.5. Basophil activation test	13
1.3.6. Molecular Allergology	13
1.4. The problem of extracts: cross-reactivity	14
1.4.1. Cross-reactive carbohydrate determinants.....	15
1.4.2. The panallergens profilin and polcalcin	18
1.5. Seasonal molecular marker allergens	20
1.6. House dust mite allergy	21
1.7. Therapy.....	23
1.8. Aim of the dissertation & hypotheses	25
2. Materials and Methods	27
2.1. Patients – House dust mite allergy	27
2.2. Patients – Seasonal allergy	27
2.3. Diagnostic tests.....	29
2.3.1. Skin prick tests	29
2.3.2. Singleplex sIgE testing.....	29
2.3.3. Multiplex sIgE testing	29
2.3.4. Molecular house dust mite allergens	29

2.3.5. Molecular seasonal allergens.....	30
2.4. Statistical analysis	31
2.5. Institutional review board approval.....	31
3. Results	32
3.1. Results I – Molecular diagnosis of house dust mite allergy	32
3.1.1. Overall sensitization to Der p and Der f.....	32
3.1.2. Demographical and clinical characterization of the study population.....	32
3.1.3. Sensitization to Der p and Der f extracts.....	33
3.1.4. Correlation of prick test and specific IgE extract results.....	34
3.1.5. Sensitivity of molecular house dust mite allergy diagnosis	34
3.1.6. Molecular house dust mite sensitization pattern	35
3.1.7. Specific IgE levels to molecular house dust mite allergens	38
3.1.8. Correlation of the test systems	38
3.1.9. CCD-reactivity	41
3.1.10. Positivity to other mites.....	41
3.1.11. Total and specific IgE of asthmatics and non-asthmatics.....	42
3.2. Results II – Molecular diagnosis of seasonal allergens.....	43
3.2.1. Demographical and clinical characterization of the study population.....	43
3.2.2. Prevalence of sensitizations.....	43
3.2.3. Correlation of prick test and specific IgE results	44
3.2.4. Sensitizations to CCDs and panallergens	46
3.2.5. Relevance of CCD-reactivity below 0.35 kU _A /l.....	48
3.2.6. Comparison of specific IgE to extracts and molecular allergens	49
3.2.7. Sensitivity of molecular allergy diagnosis.....	49
3.2.8. Specificity of molecular allergy diagnosis	51
3.2.9. Correlation of the molecular test results.....	53
3.2.10. Sensitization patterns.....	57
4. Discussion	59
4.1. House dust mite allergy.....	59
4.2. Seasonal allergy.....	66
5. Conclusion	73
6. Implication for practice.....	75
7. Limitations	77
8. Outlook and suggestions for further work	78
Bibliography.....	79

Abbreviations

AIT	Allergen-specific immunotherapy
ALEX	ALEX® Allergy Explorer
ALEX²	ALEX® Allergy Explorer version 2
CAP	ImmunoCAP™
CCD	Cross-reactive carbohydrate determinant
Der f	<i>Dermatophagoides farinae</i>
Der p	<i>Dermatophagoides pteronyssinus</i>
IgE	Immunoglobulin E
ISAC	ImmunoCAP™ ISAC™
HDM	House dust mite
MAD	Molecular allergy diagnosis
SCIT	Subcutaneous (allergen) immunotherapy
sIgE	Specific Immunoglobulin E
SLIT	Sublingual (allergen) immunotherapy

List of Figures

Figure 1: Sensitization prevalence in the Allergy Outpatient Clinic Reumannplatz

Figure 2: Seasonal allergy analyses performed

Figure 3: Correlation between Der p and Der f extracts

Figure 4: Sensitization to house dust mite molecular allergens

Figure 5: Correlation of molecular house dust mite allergens using different assays

Figure 6: Correlation of molecular Der p allergens between ALEX versions 1 and 2

Figure 7: Pollen sIgE levels

Figure 8: Correlation of seasonal prick test and sIgE results

Figure 9: Sensitizations to CCDs and panallergens

Figure 10: Correlation of the two molecular allergy test systems

Figure 11: Correlation between seasonal extracts and molecular allergens I

Figure 12: Correlation between seasonal extracts and molecular allergens II

Figure 13: Sensitization to molecular timothy grass pollen allergens

List of Tables

Table 1: Molecular house dust mite allergens investigated

Table 2: Additional seasonal molecular marker allergens investigated

Table 3: Characterization of the house dust mite study population

Table 4: Sensitivity of molecular test systems

Table 5: Differences in molecular Der p sensitization

Table 6: Percentages of patients sensitized to multiple molecular Der p allergens

Table 7: Specific IgE levels to molecular house dust mite allergens

Table 8: Positivity to other mites in Der p molecular negative patients

Table 9: Characterization of the seasonal allergen study population

Table 10: Frequency of multiple cross-reactivities

Table 11: Clinical relevance of CCD-reactivity below 0.35 kU_A/l

Table 12: Sensitivity of seasonal molecular allergy diagnosis

Table 13: “False-negative” molecular results

Table 14: Mean sIgE levels of seasonal molecular marker allergens

Table 15: Different sIgE profiles recommended for routine use

Zusammenfassung

Hintergrund: Die allergenspezifische Immuntherapie ist die einzige kausale Therapie inhalativer Soforttypallergien, vorausgesetzt das krankheitsauslösende Allergen wird korrekt identifiziert. Bedauerlicherweise wird die Spezifität der Diagnose mittels Extrakten durch Kreuzreaktionen unter anderem aufgrund von Sensibilisierungen gegenüber kreuzreagierenden Kohlenhydrat-Determinanten (CCDs), Profilinen und Polcalcinen beeinflusst. In Folge kann es zu Fehldiagnosen und Verschreibung falscher Immuntherapien kommen. Die molekulare Allergiediagnostik (MAD) könnte diese Fehler verhindern. Ziel dieser Dissertation war es, die Verlässlichkeit der MAD zur Diagnose von Allergien gegenüber Hausstaubmilbe (HSM) und den sechs häufigsten saisonalen Allergenquellen in Österreich (Alternaria sowie Pollen von Esche, Birke, Gräsern, Beifuß und Ambrosia) zu untersuchen.

Methodik: Im ersten Teil wurden anhand von 215 Patient*innen die Sensitivität molekularer Hausstaubmilbenallergene mit verschiedenen Testsystemen analysiert. Im zweiten Teil wurde zuerst die Häufigkeit einer Pollensensibilisierung bei 2.948 Patient*innen evaluiert. Bei 600 dieser Patient*innen wurde daraufhin die Prävalenz von Kreuzreaktionen studiert. Anschließend wurde anhand 742 Patient*innen die Sensitivität und Spezifität saisonaler molekularer Allergene untersucht.

Resultate: Die molekulare Sensitivität der Diagnose einer HSM-Allergie mit zumindest Der p 1, 2, und 23 betrug je nach Methode zwischen 93,0% und 94,9%; bei Extraktwerten $> 3.5 \text{ kU}_A/\text{L}$ betrug sie sogar 99,3%. 1.627 Patient*innen (55,2%) waren positiv auf zumindest eine und 1.002 (34,0%) waren positiv auf mehrere der fünf untersuchten Pollenquellen. In etwa jede/r Zehnte (10,2%) aller zur Allergiediagnostik Überwiesenen und beinahe jede/r fünfte (18,5%) pollensensibilisierte Patient*in zeigte Reaktivität auf mindestens ein kreuzreagierendes Allergen (CCDs, Profilin oder Polcalcin) und war deshalb gefährdet fehldiagnostiziert zu werden. Die Sensitivität der saisonalen MAD betrug zwischen 96,2% und 100% mittels ImmunoCAP und zwischen 91,5% und 100% mittels ALEX². Außer Art v 1 bestimmt mit ALEX² in Sera mit sehr niedrigen Beifußextraktwerten waren alle molekularen Markerallergene (Alt a 1, Amb a 1, Art v 1, Bet v 1, Fra / Ole e 1, Phl p 1 and 5) statistisch gleichwertig zur extraktbasierten Diagnose in beiden Testsystemen. Die Spezifität war 100% für alle saisonalen Allergene in beiden Testsystemen.

Schlussfolgerung: Die MAD mittels kommerziell verfügbarer Testsysteme ist sensitiv und zuverlässig in der Diagnose von Allergien gegenüber HSM und saisonalen Aeroallergenen. Sie hilft primäre Allergenquellen korrekt zu ermitteln und dadurch Fehldiagnosen zu vermeiden.

Abstract

Background: Allergen immunotherapy is the only causal treatment of respiratory allergy. Identifying the correct allergens is necessary to prescribe effective immunotherapies; however, the specificity of extract-based allergy diagnosis is impaired by cross-reactivity due to cross-reactive carbohydrate determinants (CCDs), profilins, or polcalcins. Consequently, cross-reactivity may lead to misdiagnosis and the prescription of wrong immunotherapies. Molecular allergy diagnostics (MAD) may avoid the confounding effects of cross-reactivity. This thesis aimed to investigate the reliability of MAD with different commercially available methods to diagnose allergy to house dust mite (HDM) and the six most common seasonal allergens in Austria: *Alternaria* and pollen of ash, birch, timothy grass, mugwort and ragweed.

Methods: First, 215 patients were investigated to determine the sensitivity of molecular HDM allergy diagnosis using four different test methods. In the second part, the incidence of multiple pollen-sensitization was analyzed in 2,948 patients. In 600 of these patients, the prevalence of cross-reactivity was explored. In addition, sensitivity and specificity of seasonal molecular allergy diagnosis were investigated in 742 patients using two different test methods.

Results: The sensitivities of molecular tests were correlated to allergen-specific immunoglobulin E (sIgE) levels to the extract. The overall sensitivity to diagnose HDM allergy using at least Der p 1, 2 and 23 ranged between 93.0% and 94.9%, depending on the method performed. A sensitivity of 99.3% was observed in sera with extract levels > 3.5 kU_A/l. Pollen allergy was prevalent in our study population. 1,627 patients (55.2%) were positive to at least one, and 1,002 patients (34.0%) were positive to more than one of the five pollen allergens investigated. About one tenth (10.2%) of all patients referred to allergy diagnosis, and nearly one-fifth (18.5%) of all pollen-allergic patients had reactivity to at least one cross-reactive allergen (CCDs, profilin or polcalcin) and were therefore at potential risk of misdiagnosis. The sensitivity of seasonal MAD was high, with sensitivities between 96.2% and 100% using ImmunoCAP and 91.5% and 100% using ALEX². Apart from Art v 1 determined with ALEX² in sera with low mugwort-extract levels, all molecular allergens (Alt a 1, Amb a 1, Art v 1, Bet v 1, Fra / Ole e 1, Phl p 1 and 5) performed statistically equally to extract-based diagnosis in both methods. Specificity was 100% for both platforms and all seasonal molecular marker allergens.

Conclusions: MAD using commercially available methods is a sensitive and reliable technique to diagnose allergy to HDM and seasonal aeroallergens. MAD helps to avoid misdiagnosis and to correctly select primary allergen sources for immunotherapy.

1. Introduction

1.1. Allergy

The term allergy was introduced by Clemens von Pirquet in 1906 to create awareness that some individuals show unusual reactions when exposed to foreign substances ¹. Today, the definition of allergy and allergic diseases is an abnormal adaptive immune response against innocuous, non-infectious external substances called allergens ². In recent decades, the scientific community has discovered several different types of allergic diseases. In 1963, about 60 years after Pirquet's initial observation, Philip George Houthem Gell and Robert Royston Amos Coombs categorized the main different reaction patterns into four categories: immediate (type I hypersensitivity), cytotoxic (type II hypersensitivity), immune complex-mediated (type III hypersensitivity) and delayed (type IV hypersensitivity) ³. Today, more than 50 years later, the main principles of their classification are still valid, although some reaction types, especially type IV hypersensitivity, have been elaborated and further categorized ⁴.

This thesis focuses on immediate-type or type I hypersensitivity reactions, defined by Immunoglobulin E-mediated (IgE-mediated) reactions with the support of T cells (especially T helper 2 cells), mast cells and basophils ². Type I hypersensitivity has two stages: the initial sensitization and the effector stage ⁵.

Contact of the adaptive immune system of a genetically susceptible individual with an allergen leads to sensitization (primary immune response). Sensitization, which may happen at the first contact of the immune system with a specific allergen or later, leads to the formation of sIgE antibodies. These antibodies circulate in the blood and can be bound onto the surface of immune cells such as mast cells or basophils. The effector stage of type I hypersensitivity is triggered upon repeated allergen contact and occurs within minutes ⁶. The IgE antibodies bound onto high-affinity IgE receptors on mast cells and basophils are crosslinked by the allergen, resulting in activation of mast cells and basophils ². Stimulation of these cells leads to the release of preformed (histamine, heparin, serine proteases such as tryptase and others) and de novo synthesized (e.g. prostaglandins, leukotrienes, platelet-activating factor) mediators ⁷. These mediators cause vasodilatation, increased vascular permeability and functional changes ². Symptoms of type I hypersensitivity reactions range from acute and severe (e.g. a fatal anaphylactic reaction after a Hymenoptera sting) to chronic and mild (e.g. rhinoconjunctivitis due to pollinosis).

1.2. Aeroallergens

Aeroallergens are allergens primarily spread via air. Inhalation of aeroallergens induces inhalant symptoms such as rhinitis and lower respiratory tract inflammation like bronchitis or asthma. In addition, they cause conjunctivitis and, more rarely when in higher concentrations, dermatitis. Allergy to aeroallergens is a prevalent chronic disease. It has been estimated that more than 300 million and around 400 million individuals worldwide are affected by asthma and rhinoconjunctivitis, respectively ⁸. In Austria, 24% of residents more than 14 years old suffer from allergy, a slightly higher proportion of females (27%) than males (22%) ⁹. The distribution and relevance of aeroallergens vary according to a multitude of different factors like geographical region, season, temperature or humidity. In addition, climate change may lead to various distribution and seasonal patterns of some allergens in the future ¹⁰. Some aeroallergens, such as house dust mites, cause problems throughout the year (“perennial allergens”), whereas others, such as tree pollen, peak at different times in the seasons of the year (“seasonal allergens”).

At the planning stage of this thesis, we first focused on the most relevant aeroallergens in Austria. A previous evaluation of prick test sensitization rates in patients visiting the allergy centre “Floridsdorf Allergy Centre” in Vienna because of suspected allergy problems assessed between 1997-2007 showed the following prevalence: 56.3% grass pollen, 41.7% birch pollen, 37.2% house dust mite, 33.1% cat, 21.9% mugwort pollen, 17.7% ash pollen, 16.4% dog, 12.3% plantain pollen, 11.1% ragweed pollen and 8.1% *Alternaria* ¹¹. To exclude a potential prick test bias, we investigated specific Immunoglobulin E (sIgE) analysis results from patients referred to the Allergy Outpatient Clinic Reumannplatz in Vienna between 2014-2017 and observed a slightly different allergen order and lower prevalence rates (Figure 1).

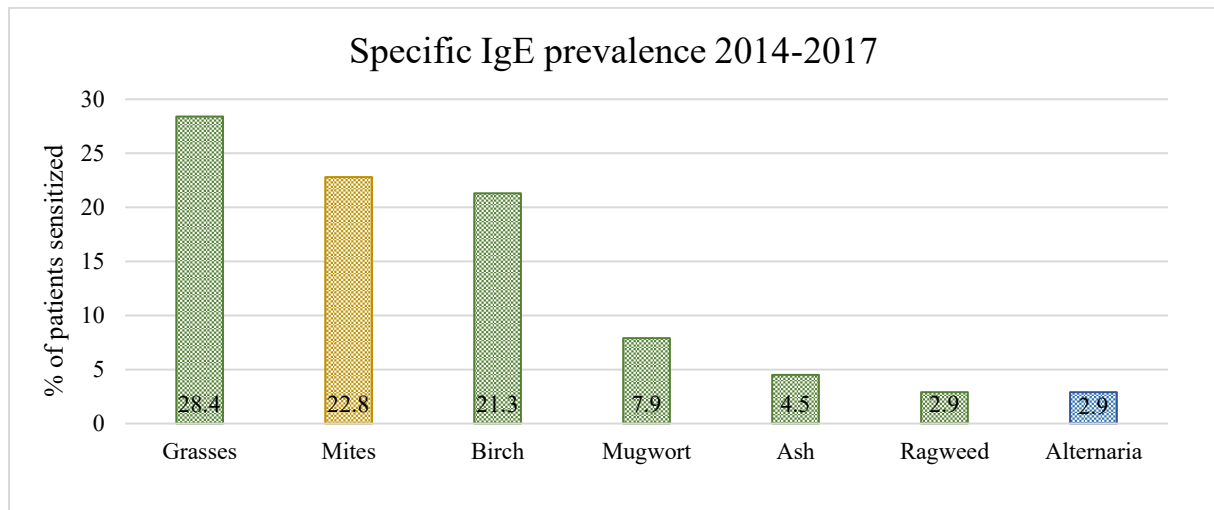


Figure 1: Sensitization prevalence in the Allergy Outpatient Clinic Reumannplatz

The seven most common aeroallergens in this IgE analysis were: pollen from timothy grass (28.4%), birch (21.3), mugwort (7.9%), ash (4.5%) and ragweed (2.9%), as well as house dust mites (22.8%) and Alternaria (2.9%). For this reason, these seven allergens were selected for further studies.

Plantain pollen was not considered particularly clinically relevant in our patient population as *in-vivo* and *in-vitro* tests against plantain are typically blurred due to cross-reactivity¹². In addition, allergy to cats and dogs was not investigated because molecular sensitization patterns in animal allergy in Austria have already been addressed elsewhere¹³.

1.3. Diagnosis of type I hypersensitivity

In general, the diagnosis of any disease is based on three firm foundations: medical history obtained from the patient, physical signs noticed upon examination and diagnostic tests. In allergic diseases, patient reported symptoms upon allergen contact and sensitization based on diagnostic tests are conditions sine quibus non. In case of symptoms without sensitization, non-allergic diseases have to be considered. On the other hand, a patient who is sensitized in a test but without symptoms is not allergic per definition, although sensitization can be preclinical, and symptoms may develop at any time later.

Thus, expressed as a simple formula: **Allergy = Symptom(s) × Sensitization**

Diagnosis of type I allergy is always based on medical history (to evaluate symptoms) and diagnostic tests, for example, skin prick testing and *in-vitro* testing (to assess sensitization). But

again, positive test results alone, especially *in-vitro*, represent sensitization only and not an allergy. Every positive test result must, therefore, always be correlated with the patient's clinical history¹⁴. Nowadays, an offer is made to send a blood sample to a laboratory where an allergen chip test with hundreds of allergens is performed. The test can be bought online; however, results can be confusing for the patient as the meaning of the results is not explained by a specialist. Ideally, the relevance of sensitization determined with *in-vitro* tests should be evaluated with specific provocation tests; or at least additional *in-vivo* testing.

The two core characteristics describing the reliability of any diagnostic test are sensitivity and specificity. The sensitivity of a test is the percentage of patients correctly identified from all patients with the disease. For example, if 90 out of 100 allergic patients are correctly diagnosed with an allergy test, the sensitivity would be 90%. In this case, 10% of patients with the disease would be missed due to false-negative results.

On the other hand, the specificity of a test indicates whether a test can correctly identify healthy people not suffering from the disease. For example, if a test is correctly negative in 95 out of 100 healthy individuals, the specificity would be 95%; however, in this case, 5% of the healthy individuals tested would receive false-positive results.

Diagnostic allergy tests, especially sIgE determinations, are prone to error due to decreased specificity because of cross-reactivity (see below).

1.3.1. Medical history

First, the medical history is necessary to evaluate whether the patients' symptoms fit allergic disease. It has been reported that approximately one-half of patients presenting with suspected allergy symptoms in primary care may be non-allergic¹⁵. Medical history is, therefore, the essential filter before performing any allergy test. Second, conducting a medical history helps identify and narrow down potential allergens for further diagnostic.

Typical questions include (i) symptoms such as conjunctivitis, rhinitis, cough or dyspnea, (ii) onset and time course, for example, seasonal vs. perennial, (iii) prior diseases and regular drug intake, (iv) already-used therapies and their effect, (v) family history, (vi) occupation and hobbies, (vii) smoking habits, (viii) symptoms linked to food, and others.

Nevertheless, taking a medical history has limitations. Sometimes patients suspect wrong elicitors, forget details or even lie deliberately. For these reasons, a medical history can also point in the wrong direction, and repeated interviews are frequently necessary to confirm or rule out positive test results. Repetition is especially critical when a predefined panel of

(hundreds of) allergens is tested because inevitably, positive results will arise with the need for differentiation between allergy and sensitization.

Because allergic and non-allergic diseases may reveal similar symptoms, for example, rhinitis, a medical history alone without a confirming allergy test is always insufficient for a reliable allergy diagnosis.

1.3.2. Skin prick testing

Skin prick testing represents the primary diagnostic test for type I allergy diagnosis ¹⁴. Prick testing is minimally invasive and the most frequently performed *in-vivo* test for IgE-mediated allergy. In adults, the test is usually performed at the volar aspects of the forearms because these skin areas are easily accessible. Skin tests can also be performed in infants ¹⁶. Here, the back skin is commonly used, which has the advantage that the potential test area is more extensive, and the children cannot see what is happening.

The basic principle of a skin prick test is inserting allergens into the skin using a pricking needle to make them accessible for immune cells; therefore, one drop of a solution containing allergen(s) is applied to the skin. By pricking into the skin through the drop, small amounts of allergens are transported into the skin. Standardized solutions are commercially available for the essential type I allergens such as pollen, mites, moulds and some foods. A negative control (saline solution – no reaction expected) and positive control (e.g. histamine hydrochloride solution – positive response expected) are necessary when performing skin prick tests to detect conditions of overreaction or underreaction of the patient's skin ¹⁴. After a waiting time of 15 minutes, the prick test is evaluated. Usually, a wheal larger than 3 mm at a respective test area implies a positive reaction ¹⁴.

Skin prick testing has some limitations. Firstly, commercially available extracts contain not only single allergens but a mixture of allergens of the respective allergen sources and are therefore prone to cross-reactivity.

The second limitation is that some allergens of the respective allergen source may be underrepresented in commercially available allergen extracts; for example, either due to the production process or because the allergen is lipophilic – the latter is a problem because prick test extracts are aqueous solutions. This underrepresentation of different allergens has been shown, for example, for house dust mite or hazelnut allergens ^{17,18}. Furthermore, extracts for some foods and rare allergens are not commercially available or poorly standardized leading to

less reliable prick test results; therefore, the prick-to-prick technique is frequently utilized, where the lancet is plunged several times into the allergen source (e.g. an apple) immediately before pricking the patient's skin. For some food allergens, it has been shown that prick-to-prick testing has higher sensitivity than testing with commercial food extracts ¹⁹⁻²¹.

Thirdly, interpretation of prick test results can be difficult or altered in patients with skin diseases, such as eczema or dermatographism, or after intake of certain medications. Especially oral H1-antihistamines, but also some other drugs such as anxiolytics, decrease diagnostic sensitivity ^{14,22}. For example, a recent study showed strong suppression of skin prick tests by mirtazapine and quetiapine ²³.

Fourthly, the quality of the skin test depends greatly on the performing and interpreting healthcare professional, implicating a risk of subjectiveness bias compared to objective laboratory analyses.

1.3.3. Intracutaneous testing

The intracutaneous test or intradermal test is similar to the skin prick test. The difference is that the allergen-containing solution is directly injected intradermally using a syringe with a small needle. It has been reported that intracutaneous testing is more sensitive and reproducible than skin prick testing ²⁴; however, because of different factors such as time, patient comfort or extract composition needed, prick tests are more practicable ²⁵.

As in prick testing, an intradermal test is assessed after a waiting time of 15 minutes after allergen application. In addition, the intradermal test can also be used for late assessments to evaluate potential type IV allergies. This possibility is especially interesting for diagnosing drug allergies when the type of reaction (type I versus type IV) cannot be fully differentiated based on the medical history; therefore, drug allergy is the main indication for this test. Furthermore, it is used for diagnosing Hymenoptera allergy. It is not regularly applied for diagnosing aeroallergens, however. When analyzing aeroallergens, it has been shown that prick testing has better specificity and similar sensitivity ²⁶⁻²⁸.

1.3.4. IgE determination

The determination of specific IgE (sIgE) against the suspected allergen(s) in a patient's serum is the classical *in-vitro* test of type I allergy. Different read-outs can quantify the number of antibodies in the individual serum. Using radioallergosorbent tests (RAST), quantification used to be semiquantitatively in RAST classes from 0 to 6. With modern fluorescence enzyme-linked immunoassays, such as the ImmunoCAP™ 1000 platform (CAP, Thermo Fisher Scientific,

Waltham, Massachusetts, USA), quantification is more precise and results are stated in kilounits of allergen-specific IgE per litre (kU_A/l) ²⁹.

Accurate assessment of sIgE is critical for the identification of the causative allergen and may predict the probability of disease severity ¹⁵. In addition, sIgE measurements and the cut-offs used (e.g. 0.35 kU_A/l using CAP) are not dichotomously positive or negative. Although very low sIgE, for example, 0.25 kU_A/l, is considered “negative”, it may be relevant in some patients. It is, therefore, necessary to interpret sIgE in relation to the total IgE of the patient; 0.25 kU_A/l of sIgE to a particular allergen is probably irrelevant in a patient with, for instance, 2000 kU_A/l total IgE but may be relevant in a patient with only, for example, 20 kU_A/l total IgE.

To date, two different types of *in-vitro* sIgE tests are commercially available: singleplex assays to quantify sIgE against a single allergen and multiplex assays which allow the simultaneous measurement of multiple sIgE antibodies in one test run.

Here is an example of a routinely performed singleplex test profile for the screening of inhalant allergy in Central Europe:

Extract	Name	Botanical name
Ash pollen	European ash pollen	<i>Fraxinus excelsior</i>
Birch pollen	Common silver birch pollen	<i>Betula verrucosa</i>
Grass pollen	Timothy grass pollen	<i>Phleum pratense</i>
Alternaria	Alternaria alternata spores	<i>Alternaria alternata</i>
Mugwort pollen	Mugwort pollen	<i>Artemisia vulgaris</i>
Ragweed pollen	Common ragweed pollen	<i>Ambrosia artemisiifolia</i>
House mite	European house dust mite	<i>Dermatophagoides pteronyssinus</i>

Thus, using singleplex analysis, seven single test runs must be performed to analyze sIgE against these allergens. On the other hand, multiplex arrays explore reactivity to hundreds of allergens simultaneously and thereby generate a comprehensive allergen profile. To avoid paralysis by analysis, however, modern multiplex assays allow electronic customizations of the panels. Besides cost, the main disadvantage of such multiplex tests is that their diagnostic sensitivity may be decreased in patients with low sIgE levels because of higher detection limits, higher coefficients of variation and potential inhibition due to antigen-specific IgG ³⁰.

Skin prick tests and sIgE measurements are not fully interchangeable and may be considered complementary³¹; however, a good correlation between skin prick testing and IgE assays has been shown for some allergens³².

1.3.5. Basophil activation test

For selected allergens, especially insect venoms or some drug allergens, the basophil activation test is used as an additional *in-vitro* test³³⁻³⁵. After stimulation of a patient's blood sample with an allergen, the degree of basophil degranulation, as assessed by degranulation-dependent CD63 surface expression, is measured by flow cytometry. The basophil activation test is a functional test and may reduce the need for *in-vivo* procedures³⁶; however, this test is rarely used to diagnose allergy to aeroallergens because (i) it is not available in many laboratories, (ii) it is not reimbursed by insurance companies, (iii) it is too laborious for very prevalent allergies such as pollen allergy, and (iv) prick tests and sIgE determinations work well for the diagnosis of pollen allergy despite the possibility of cross-reactivity (see below).

1.3.6. Molecular Allergology

Contrary to extract-based *in-vivo* and *in-vitro* testing, where extracts containing several potential allergens of an allergen source are tested, the principal idea of molecular allergology is to test for individual allergens of an allergen source. These individual molecular allergens, sometimes called molecular components, are highly purified proteins, either isolated directly from the natural allergen source or produced recombinantly. Tests using these molecular allergens inform about the “reactivity against a single antigen of an allergen source” rather than the “reactivity against the whole allergen source”. Thus, this technique enables a highly differentiated diagnosis and defines more detailed sensitization profiles of single individuals or groups.

Due to significant advances in discovering distinct allergens in allergen sources, a standardized allergen nomenclature was introduced in 1986³⁷ and has been updated and revised several times^{38,39}. This official terminology provided by the Allergen Nomenclature Sub-Committee of the World Health Organization and the International Union of Immunological Societies is available up-to-date online (www.allergen.org). It lists all currently defined allergens according to the following structure:

first three letters of the genus - first letter of the species - order of discovery
for example

Phl p 1

Phleum pratense 1

The origin of an allergen can be further denoted by adding an n (for natural) or r (for recombinant) in front of the allergen name, for example, nPhl p 1 or rPhl p 1, indicating that the allergen was derived from a natural allergen source or was produced by genetic engineering. This discrimination is relevant because despite purification procedures, naturally derived allergens may have cross-reactive carbohydrate determinants (CCDs) which recombinant allergens lack. The reason is that recombinant allergens are mainly produced in bacteria, for example, in *Escherichia coli*, and post-translational protein modification in bacteria is limited compared to eukaryotic cells ⁴⁰.

For the last forty years, an allergen has been classified as a major allergen if the IgE binding frequency to a molecular allergen was >50% in a defined population. If below, it has been termed a minor allergen ⁴¹. This classification has helped to sort allergens by potential relevance in a population. Still, it has to be remembered that IgE reactivity to allergens (major allergens as well) is not always clinically relevant – IgE sensitization is not synonymous with allergy. On the other hand, “minor” allergens can elicit severe and clinically relevant allergenic activity ⁴². In addition, different members of an allergen family can be minor allergens in some allergen sources but major allergens in others. For example, the timothy grass pollen profilin Phl p 12 is a minor grass pollen allergen but the melon profilin Cuc m 2 is a major melon allergen ⁴³. For a single allergic individual, it is of little importance whether the majority of the population has IgE reactivity to the same allergen or not. As a result, the terms major and minor allergens are not very useful for precision allergology, and it has recently been proposed that use of this major and minor classification should be ceased ⁴⁴.

1.4. The problem of extracts: cross-reactivity

To date, prick testing and sIgE-based *in-vitro* testing using extracts are still the two mainstays of routinely performed type I allergy diagnostics. The main problem with any extract-based test, especially in the diagnosis of pollen allergy, is that test extracts may contain not just one

or a few but several allergens of the respective source. Some of these allergens are specific for the allergen source but unfortunately, some also exist in other allergen sources. It has been shown that pollen allergens belong to 29 of 7868 known protein families, and expansins, profilins, and calcium-binding proteins are the major pollen allergens⁴⁵. These proteins, especially profilin and calcium-binding proteins (polcalcins), are found in virtually every pollen, leading to cross-reactivity. Two allergens are cross-reactive when one can bind the specific IgE induced by the other. Cross-reactivity frequently occurs between allergens with similar sequences and three-dimensional structures, especially between highly conserved “panallergens” (such as profilin or polcalcin). Cross-reactivity usually requires more than 70% sequence identity and it is rarely observed below 50% sequence identity⁴⁶. Because profilins and polcalcins from different pollen sources share sequence identities between 70% to 85% and between 64% to 92%⁴⁵, they are typical candidates responsible for cross-reactivity.

Discriminating between genuine sensitization or cross-reactivity can be challenging. For example, cross-reactivity based on panallergens such as profilins or polcalcins can be observed in prick and *in-vitro* testing when using extracts. In addition, when using extracts for sIgE determination, results can additionally be hampered by IgE antibodies against cross-reactive carbohydrate determinants (CCDs - see below). In general, cross-reactivity does not alter the diagnostic sensitivity of extract-based allergy tests (sensitized or allergic patients are still diagnosed correctly); however, cross-reactivity affects and lowers the specificity of these tests (patients may be misdiagnosed as sensitized or allergic, although they are not genuinely sensitized or allergic to the respective allergen(s)).

1.4.1. Cross-reactive carbohydrate determinants

Protein glycosylation is one of the major post-translational modifications of proteins. It alters the genetically determined primary structure of proteins by adding various carbohydrate structures. These carbohydrate modifications range from monosaccharides to highly complex branched polysaccharides. The two most important forms of protein glycosylation in plants and invertebrates are N-linked and O-linked glycosylation^{47,48}. A typical (initial) pathway of N-glycosylation is conserved in eukaryotes. Still, the specific modifications of N-linked oligosaccharides in plants and invertebrates (insects) differ from those in vertebrates (humans)^{47,48}. This difference can result in immunogenic structures such as β 1,2-linked xylose and, most importantly, α 1,3-linked fucose^{47,48}. The immunogenic effect that IgE antibodies in human sera can bind to many different plant and insect antigens was first detected in 1981. It was proposed

that these antigens were carbohydrates; thus, the term cross-reactive carbohydrate determinant (CCD) was first suggested ⁴⁹.

Nevertheless, it took more than a decade before intensive research identified the negative consequence of CCD cross-reactivity on routine allergy diagnosis using extracts. CCDs are widely distributed and found on proteins of plants, including pollens and food, as well as in insect venoms ⁵⁰; therefore, IgE against CCDs is relevant due to interference with extract-based sIgE diagnostics, whereas skin tests seem to be unaffected. Initially, evidence of *in-vitro* activity of CCDs led to a controversial discussion in the literature regarding their *in-vivo* relevance ^{51,52}. It is now common knowledge, however, that anti-CCD IgE to plants and insect venoms have poor biological activity and are considered clinically irrelevant ⁵³⁻⁶⁰. The two probably most striking sentences highlighting the clinical irrelevance of anti-CCD IgE are by Friedrich Altmann published in 2007:

“It is highly unlikely that physicians would fail to notice if up to 20% of their patients had allergic episodes after they had ingested virtually every kind of vegetable food. A glycoprotein which binds IgE by CCDs only must therefore be rated as just a clinically irrelevant allergen.” ⁵⁸

One of the most promising hypotheses to explain why anti-CCD IgE is biologically inactive is the presence of blocking CCD-specific IgG₄ antibodies. As in patients treated with allergen-immunotherapy, the constant natural exposure to ubiquitous CCDs, for example, via plant food, may lead to the formation of blocking anti-CCD IgG ^{58,61}.

It is worthy of mention, however, that clinically relevant IgE-antibodies directed to some unique glycoproteins exist, as it has been shown for sIgE-mediated allergy against Galactose- α -1,3-Galactose (α -Gal). Patients sensitized to this glycoprotein suffer from anaphylactic reactions after contact with certain drugs, for example, cetuximab, or after ingestion of mammalian meat and offal ^{62,63}.

Sensitization rates to classical CCDs vary enormously depending on the patient populations investigated. In addition, sensitization rates differ between the CCD-specific IgE antibodies tested (e.g. MUXF3, Bromelain, horseradish peroxidase) ⁶⁴. Still, an overall rate of approximately 20–25% seems reasonable, as the most extensive studies so far have shown CCD reactivity in 22 and 23% ^{59,65}. In contrast, another study investigating 1,025 adults showed reactivity to MUXF3 ≥ 0.35 kU_A/l in only 5.6% of the study population; however, considering

a cut-off of ≥ 0.1 kU_A/l, 18.0% would have been positive⁶⁶. It is worthy of note that it seems that the cut-off used for routine diagnostics, 0.35 kU_A/l is not helpful for differentiating whether or not a serum has clinically relevant CCD-reactivity regarding cross-reactivity. In routine diagnostics, we have observed several times that MUXF3 below the cut-off of 0.35 kU_A/l could induce false-positive extract results to pollen higher than 0.35 kU_A/l, which would be considered positive. The current thesis will investigate this finding as a secondary research question: how prevalent is CCD sensitization below the 0.35 kU_A/l cut-off and is it clinically relevant?

Importantly, cellulose used as a solid-phase allergen carrier in the CAP platform can contain varying amounts of CCDs. As a consequence, false-positive test results up to 2 kU_A/l may arise in patients with high levels of sIgE to CCDs, even when using nonglycosylated recombinant allergens⁶⁷.

Unfortunately, many physicians are still unaware of CCDs and anti-CCD-antibodies and their negative effect on extract-based sIgE diagnostics (decreased specificity leading to false-positive test results). Consequently, patients are still at risk of being misdiagnosed based on wrong blood test results and undergoing inappropriate treatment and dietary restrictions.

There are two approaches to dealing with the diagnostic CCD problem: CCD-IgE determination or CCD-IgE blocking/inhibition. The presence of CCD-IgE in a patient's serum can be proved with commercially available assays, for example, by determining MUXF3 using CAP; if MUXF3 is positive, CCD reactivity and false-positive sIgE results have to be considered. On the other hand, CCD-antibody blocking/inhibition can be performed by incubating the patient's serum with CCDs before sIgE analysis to bind existing anti-CCD-antibodies and avoid later CCD-reactivity and false-positive sIgE results. Although not always complete, CCD-IgE inhibition is reliable and enhances the accuracy of *in-vitro* diagnosis without influencing sIgE against relevant (protein) epitopes^{65,68}. The newest commercially available multiplex assay on the market, the ALEX® Allergy Explorer version 2 (ALEX², MacroArray Diagnostics, Vienna, Austria), combines both approaches by first blocking CCD IgE and then testing for CCD to indicate a potential incomplete inhibition.

1.4.2. The panallergens profilin and polcalcin

Panallergens are structurally highly conserved, strongly cross-reactive allergens present in different allergen sources, even in unrelated sources, throughout the plant kingdom. Importantly, cross-reactive IgE to panallergens does not always result in clinical symptoms but frequently interferes with extract-based sIgE diagnosis by decreasing diagnostic specificity. Examples for panallergens are profilins (e.g. Bet v 2, Phl p 12), polcalcins (e.g. Bet v 4, Phl p 7), non-specific lipid transfer proteins (e.g. Art v 3) or pathogenesis-related-10-proteins (Bet v 1). This thesis concentrates on profilins and polcalcins, which have the most significant impact on extract-based allergy diagnosis.

Since the discovery of the first allergenic profilin from birch pollen (Bet v 2) in 1991⁶⁹, profilins have been isolated from a variety of pollen, plant-based food sources and latex. Profilins represent a family of small (12–15 kDa), highly conserved molecules sharing sequence identities of more than 75% even between members of distantly related organisms⁷⁰. Sensitization to profilins has been reported in about 13–33% of patients⁷¹⁻⁷⁴, depending on allergens, geographical regions and patient populations investigated. In Spain, for example, sensitization rates have been reported from below 10% along the Mediterranean coast up to 50% in some western regions⁷⁴.

Profilins are present in all eukaryotic cells; however, primary sensitization to profilin seems to arise mainly from pollen sensitization⁷⁵. There is no real evidence of primary sensitization to plant food profilins, which was exclusively seen in patients with sIgE against pollen profilins in one study⁷⁵. Furthermore, it has been reported that sensitization to grass pollen profilin (and polcalcin) develops late in the molecular spreading of grass pollen allergy⁷⁶. Additionally, profilin sensitization seems to be a marker of a more severe grass pollen sensitization⁷⁴.

IgE against profilin was shown to have biologic activity in histamine release- and skin prick tests⁷⁷. In certain studies, up to 57% of profilin reactors suffered from food allergy⁷⁸. Profilin sensitization is the reason why some grass pollen-allergic patients cross-react to food allergens⁷⁹. Allergy to profilins from plant-based food, primarily to exotic fruits (kiwi, litchi, melon, orange or others) but also more local food such as tomatoes or apples, is mainly associated with mild clinical symptoms, such as oral allergy syndrome⁷⁸. Typical symptoms of oral allergy syndrome are oral itching, lip swelling, labial angioedema and glottal oedema¹⁵. Food processing (cooking) cannot explain these localized oral rather than systemic symptoms as food processing does not seem to modify profilin allergenicity⁷⁵. It can, however, be explained because profilins are susceptible to pepsin digestion, which has been demonstrated, for

example, for melon or apple profilin^{43,80}. Nevertheless, profilins have also been shown to act as severe food allergens, for example, in patients overexposed to grass pollen, and it has been suggested that intake through the oral mucosa may be sufficient to elicit systemic anaphylactic reactions⁴².

On the other hand, the clinical relevance of sensitizations to profilin from pollen is still a matter of discussion⁷⁵. There is, however, growing evidence that profilins can elicit respiratory symptoms⁸¹. For example, using purified profilin from date palm (Pho d 2), a positive conjunctival and bronchial provocation test was demonstrated in 90% and 95% of profilin-sensitized patients, respectively⁸¹. Another study showed that 65% of profilin-sensitized patients had a positive conjunctival allergen challenge using the same profilin⁸². Importantly, none of the non-profilin-sensitized controls showed reactivity to the provocation tests performed in both studies. In contrast, the authors of another article concluded that sole sensitization to profilin appears to be not clinically relevant in allergic rhinoconjunctivitis⁸³. Although many profilin-sensitized patients do not experience clinical symptoms, patient monitoring and differentiation between cross-reactivity and genuine sensitization seem advisable⁷⁰.

Polcalcins were discovered as panallergens in the 1990s^{84,85}. Their name reveals that they are members of the calcium-binding protein family but only expressed in pollen. Thirty-two distinct subfamilies of calcium-binding proteins exist and they are ubiquitous and additionally found in fish and mammals⁸⁶. Intense cross-reactivity has been reported between polcalcins found in pollen or between parvalbumins found in fish⁸⁶; however, there is no evidence for cross-reactivity between calcium-binding proteins of plants, fish or mammals⁸⁷. With sensitization rates of between 8–10%^{71,85,88}, polcalcins are minor allergens in pollen allergy, again with variability regarding geographical regions, patient populations and allergens investigated. Polcalcins such as Phl p 7 were demonstrated to have biological activity by inducing basophil histamine release and positive skin prick tests in sensitized individuals⁸⁵; however, literature dealing with the ability of anti-polcalcin IgE to elicit clinical symptoms is scarce. It was recently stated that sole sensitization might not have clinical relevance in allergic rhinoconjunctivitis⁸³. Sensitization to polcalcins, however, can decrease the specificity of extract-based tests. The most suitable polcalcin molecule available for diagnosis seems to be Phl p 7, which contained the most relevant IgE epitopes of the four polcalcins investigated in one study (Phl p 7, Aln g 4, Jun o 4 and Bet v 3)⁸⁹.

1.5. Seasonal molecular marker allergens

Contrary to panallergens, most patients genuinely allergic to a respective allergen source are sensitized to molecular marker allergens. Significantly elevated sIgE against a marker allergen confirms a primary sensitization, whereas negative sIgE rules it out. Importantly, marker allergens also have a variable potential to cross-react, especially in botanically related allergen sources. Examples are Amb a 1 of ragweed (cross-reacting with Art v 6, both pectate lyases) or Art v 1 from mugwort (cross-reacting with Amb a 4, both defensin-like proteins).

The seasonal molecular marker allergens investigated in this thesis are:

Allergen	Allergen source	Botanical Name
Alt a 1	Alternaria	<i>Alternaria alternata</i>
Amb a 1	Ragweed pollen	<i>Ambrosia artemisiifolia</i>
Art v 1	Mugwort pollen	<i>Artemisia vulgaris</i>
Bet v 1	Birch pollen	<i>Betula verrucosa</i>
Fra / Ole e 1	Ash / Olive pollen	<i>Fraxinus excelsior / Olea europaea</i>
Phl p 1 and 5	Timothy grass pollen	<i>Phleum pratense</i>

Ole e 1 was used as a surrogate marker using CAP because Fra e 1 is only available on ALEX.

To serve as appropriate marker allergens, these molecular allergens should have high sensitivity. In fact, available literature supports this qualification. Sensitization rates reported were up to 80.7%–98.4% for Alt a 1⁹⁰⁻⁹³, 90%–100% for Amb a 1⁹⁴⁻⁹⁸, >70%–>95% for Art v 1⁹⁸⁻¹⁰², 88.4%–97% for Bet v 1¹⁰³⁻¹⁰⁶, 97.7% for Fra e 1¹⁰⁷ and 87.6%–88% for Ole e 1^{108,109}, 74.8%–98% for Phl p 1¹¹⁰⁻¹¹⁵, and 27.2%–80% for Phl p 5^{74,110-113,116}. It must, however, be considered that these sensitization rates differed considerably between the various studies depending on the following study characteristics: cohort size (small vs. large), age (children vs. adults), methods investigated (immunoblots, ELISA, prick test), allergen origin (natural vs. recombinant) and by definition of sensitized and allergic patients in the respective study (extract cut-offs, patient history, allergen challenge, et cetera).

Two of these considerations are especially relevant. Firstly, unfortunately, many of these data were gathered by immunoblotting, ELISA or skin prick testing with molecular allergens; therefore, they are not directly comparable to daily routine allergy diagnostics using commercially available sIgE test platforms. Secondly, the selection of allergic patients seems to be inconsistent between some of these studies. Many of the mentioned studies have found high reactivity rates to profilin and polcalcin, which indicates that cross-reactivity may have

played an essential role in the investigated sera. For example, in one study investigating molecular diagnosis of birch pollen allergy in different European countries, two subgroups only showed 62% and 65% reactivity to Bet v 1, but 33% as well as 43% and 27% as well as 7% reactivity to Bet v 2 and Bet v 4¹⁰³. It is questionable whether all of these “birch pollen-allergic patients” were genuinely sensitized to birch pollen. Some may have only cross-reacted to birch pollen extract due to sensitization to Bet v 2 (birch profilin), Bet v 4 (birch polcalcin) or both. For this reason, one can assume that the sensitivities of molecular marker allergens in those studies were biased and probably underestimated.

To achieve correct and practically applicable sensitivity rates of a molecular marker allergen, however, a study should i) use commercially available methods and ii) include genuinely sensitized patients rather than cross-reactive sera. For this reason, we have only used commercially available sIgE test methods and included only mono-allergic patients for sensitivity analyses in this thesis.

In addition to the molecular marker allergens mentioned above, the following molecular allergens were investigated to characterize their value for MAD: Alt a 6, Amb a 4; Art v 3; Bet v 2, 4 and 6; Phl p 2, 4, 6 and 11.

1.6. House dust mite allergy

The second part of this thesis addressed the evaluation of molecular allergy diagnostics of house dust mite (HDM) allergy. Allergy to HDM is a significant health burden, as it is the most prevalent cause of inhalant allergy worldwide. Sensitization rates in the general population of up to 21.7% in Europe and 27.5% in the United States have been reported^{117,118}. In a large pan-European study with patients referred to allergology centres, percentages were even higher, with 31.3% and 28.9% of the study population sensitized to *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f), respectively¹¹⁹. In all of these studies, house dust mites were the most common perennial indoor allergen and overall inhalant allergen. The pyroglyphid mites Der p (historically also known as the European HDM) and Der f (also known as the American HDM) are the most relevant mite species in developed countries. They cohabit regularly, and mite-allergic patients in temperate countries are classically co-exposed and co-sensitized to both mites^{120,121}. In addition, other house or storage mites may also be relevant in some geographical regions¹²².

Diagnosis of HDM allergy is based on clinical symptoms, skin prick testing and determination of sIgE. Sometimes, conjunctival or nasal provocation tests are performed to test the clinical relevance of positive test results. In the case of sIgE testing, singleplex test systems detecting sIgE to Der p or Der f extracts are widely used. In addition, multiplex tests have been developed as an individualized diagnostic approach providing sIgE determination to a wide range of molecular allergens and extracts at reasonable cost. The sensitivity of multiplex tests for the diagnosis of HDM allergy was, however, reported lower compared to singleplex testing^{123,124}.

The only causative treatment of HDM allergy is allergen-specific immunotherapy, as a complete allergen avoidance is difficult to achieve. In recent years, significant advances have been made in the development of HDM immunotherapies, particularly in the sublingual treatment of HDM allergy. These tablets contain a 1:1 mixture of Der p and Der f characterized by a constant 1:1:1:1 ratio between the four major marker allergens Der p 1, Der p 2, Der f 1 and Der f 2, with promising efficacy and safety profiles reported^{116,125,126}. The standardization to group 1 and 2 allergens has a potential disadvantage, however, as this “small allergen spectrum” could eventually decrease efficacy in HDM-allergic patients sensitized to further allergens; therefore, determining molecular sensitization patterns could help to select the best eligible patients for immunotherapy.

Der p 1 and 2 have been well known as major HDM allergens for a long time^{127,128}; however, it is doubtful that these two allergens are sufficient to diagnose and treat all HDM-allergic patients correctly. Based on the available literature, 83.5–97% of HDM allergic patients can be detected with Der p 1 and Der p 2^{122,129-132}. These percentages indicate that: firstly, the diagnosis and treatment solely based on group 1 and 2 allergens is not able to detect and treat between 3% and 16.5% of all HDM-allergic patients, and secondly, other allergen groups are relevant for HDM allergy in these latter patients.

The most promising allergen candidate to improve diagnosis and treatment is Der p 23, first described in 2013¹³³. High allergenic activity and sensitization rates of up to 74% were reported¹³³. For this reason, Der p 23 may be essential for HDM allergy diagnosis and immunotherapy. Once in the spotlights, mono-sensitization rates to Der p 23 between 4.2% and 5.3% were published^{121,134,135}. These mono-sensitization rates are remarkable, and Der p 23 is probably the most critical allergen to close the diagnostic and therapeutic gap; however, even with Der p 1, 2 and 23, not all patients may be correctly diagnosed (and treated). Perhaps other allergens could play an important role, as 32 Der p molecular allergens have been described in the

WHO/IUIS Allergen Nomenclature Database (retrieved from www.allergen.org, 22th of May, 2022).

Molecular allergy diagnosis has evolved and is now a recognized part of routine diagnosis; however, its contribution in diagnosing house dust mite allergy and the sensitivity of the different molecular singleplex and multiplex assays available is still unclear. Since 2017, the multiplex platform ALEX® Allergy Explorer (ALEX, MacroArray Diagnostics, Vienna, Austria) has been commercially available, measuring sIgE to seven Der p molecular allergens (Der p 1, 2, 5, 7, 10, 11 and 23), several mite extracts and 282 molecular allergens and extracts in total. In addition, the ALEX² (ALEX version 2) was recently launched, testing for two additional Der p allergens (Der p 20 and 21) and 295 molecular allergens and extracts in total.

1.7. Therapy

There are three possibilities to treat type I allergic diseases: (a) allergen avoidance, (b) symptomatic treatment and (c) allergen-specific immunotherapy (AIT).

Allergen avoidance may be possible for some allergens (e.g. drugs, exotic animals, etc.); however, strict avoidance can be difficult or nearly impossible for inhalant allergens such as pollens or house dust mites. Measures to reduce HDM allergen quantity, such as encasings, acaricides or vacuum cleaners with HEPA filters ¹³⁶, have been proposed and are commercially available; however, two systematic reviews indicate that these measures, especially when applied solitarily, only have a limited effect on rhinitis and no effect on asthma and can, therefore, not be generally recommended ^{137,138}.

Symptomatic treatment only reduces symptoms (conjunctivitis, rhinitis, bronchial obstruction) without targeting the underlying pathophysiological process. The most frequently used drugs are antihistamines, corticosteroids and β 2- adrenergic receptor agonists. These drugs have clear clinical benefits but unfortunately, they only suppress allergic symptoms and do not change the allergic disease state, stop disease progression or cure an allergy. Furthermore, many patients misuse symptomatic treatments and have a significant burden of residual symptoms ¹³⁹.

Allergen-specific immunotherapy (AIT) is the only causal treatment altering the natural course of IgE-mediated allergic disease ^{136,140}. Recurrent low-dose application of allergens induces immunological tolerance. Initially, subcutaneous allergen immunotherapy (SCIT) was performed. Nowadays, sublingual allergen immunotherapies (SLIT) are also commercially available. Both modalities are effective; however, it has been recently shown that subcutaneous allergen immunotherapy had higher adherence rates than sublingual administration in a real-world setting ¹⁴¹.

The ideal allergen immunotherapy should have disease-modifying effects and:

- (i) reduce symptoms and the use of symptomatic drugs
- (ii) have a long-term impact even after cessation of the treatment
- (iii) prevent new sensitizations to other allergens, and
- (iv) prevent progression of the disease (prevent asthma in patients with rhinitis).

All four effects could be demonstrated in clinical studies:

- (i) A meta-analysis of sublingual immunotherapy studies showed better efficacy than standard symptomatic treatment ¹⁴². Even in patients with partially or uncontrolled asthma, HDM SLIT could reduce time to first asthma exacerbation during the reduction phase of inhalant corticosteroids ¹²⁶. Another study showed about 60% and 10% reduction of rhinitis- and asthma medication using HDM SCIT ¹⁴³.
- (ii) AIT had long-term clinical effects for several years (e.g. 7 or 12 years) after treatment cessation ¹⁴⁴⁻¹⁴⁷.
- (iii) AIT prevented new sensitizations in children with rhinitis and/or asthma mono-sensitized to house dust mites ¹⁴⁸ or sensitized to grass pollen ¹⁴⁵.
- (iv) SCIT had a long-term preventive effect on the development of seasonal and perennial asthma in children up to 7 years after treatment termination ¹⁴⁴. The prevention of asthmatic symptoms could also be confirmed in a large, double-blind placebo-controlled study using grass pollen SLIT in children ¹⁴⁹ as well as two retrospective database analyses of patients receiving grass pollen SLIT ¹⁵⁰ or HDM SCIT ¹⁴³.

As with most other effective drugs, AIT has side effects; however, modern immunotherapies are tolerated well. Common side effects are usually mild to moderate, localized to the application site, temporary and resolve spontaneously ^{116,125,126,151,152}. Typical side effects are erythema, swelling, pruritus and pain of the arm in SCIT and oral pruritus, irritated mouth and throat and oral oedema in SLIT.

Importantly, safety has also been shown in children¹⁵³ and patients with partially or uncontrolled asthma ¹²⁶. In the latter study, side effects were not increased in patients with uncontrolled asthma compared to the complete analysis set.

AIT is, however, a potential trigger for systemic reactions (anaphylaxis). For example, a survey performed in the United States between 2008 and 2016 based on the data of 54.5 million SCIT injection visits observed systemic reactions in about one per 1000 injection visits (0.1%) and a fatality rate of one per 9.1 million injection visits (0.000011%) ¹⁵⁴. Although the risk for

immunotherapy-induced fatalities is extremely low, it is crucial to select and treat only patients truly allergic and with appropriate drugs containing the individually relevant allergens.

At the moment, AIT is performed with standardized extracts. These extracts do not necessarily contain (all) the allergens to which a patient is sensitized. Such a treatment is contrary to precision medicine, one of the most important developments in medicine in the 21st century. At the present time, however, it is not known whether an individualized treatment would have a benefit over standardized therapy. Actually, one study failed to show a significant effect of AIT consisting of a mix of recombinant grass allergens¹⁵⁵. Furthermore, it is unclear how long it will take (if ever) until individualized therapies are available, considering the required approval procedures and cost of licensing these drugs. Thus, precision molecular allergology is more advanced in a diagnostic than in a therapeutic view; however, reliable, sensitive, and specific diagnostic methods are essential for prescribing immunotherapies and the future development of individualized treatments.

1.8. Aim of the dissertation & hypotheses

For decades, prick and *in-vitro* testing using allergen extracts have been the two mainstays of routine allergy diagnostics; however, considering the potential influence of cross-reactivity or the various allergens present in some allergen sources, it is questionable whether this standard is still appropriate. Several studies have shown that the use of multiplex tests using molecular allergology lead to different therapeutic decisions compared to extract-based testing¹⁵⁶⁻¹⁵⁸. In addition, a recent review has demonstrated the benefit of molecular allergology and its impact on specific allergy diagnosis and therapy¹⁵⁹. In this article, however, relatively low sensitization rates (>90% for Phl p 1, >90% for Bet v 1, >70% for Art v 1, > 90% for Der p 1 and 2, no data on Fra e 1 or Amb a 1 or Der p 23) have been reported. Furthermore, as mentioned above, many previous studies investigating sensitivities have little practical value due to selection bias or the (not routinely available) test methods used. Thus, with this thesis, (i) we want to emphasize the impact of cross-reactivity on extract-based sIgE diagnostic of seasonal allergens and (ii) identify how many patients are at risk of being misdiagnosed. Furthermore, (iii) we want to answer whether molecular allergology can solve the problem of cross-reactivity and (iv) clarify whether MAD is applicable in allergen sources with multiple relevant allergens, such as HDM allergy.

Based on these questions, this thesis had the following primary hypotheses:

Molecular allergy diagnosis using commercially available methods can replace the extract-based sIgE diagnosis because:

- (i) it is sensitive
- (ii) it is specific
- (iii) it helps to prevent misdiagnosis
- (iv) it aids to select appropriate allergens for immunotherapy

Secondary research questions of this thesis were:

- (i) How many patients referred to allergy diagnosis are allergic to seasonal allergens or house dust mites?
- (ii) How common is pollen poly-sensitization?
- (iii) Is cross-reactivity between pollen allergens prevalent and clinically relevant?
- (iv) How prevalent is sensitization to CCDs and the panallergens profilin and polcalcin?
- (v) How common is CCD sensitization below the 0.35 kU_A/l cut-off and is it relevant?
- (vi) What are the molecular sensitization patterns of aeroallergens in Austria?
- (vii) Are minor allergens such as Bet v 6, Phl p 2, 6, 11, Alt a 6, Amb a 4 or Art v 3 relevant in mono-allergic patients?
- (viii) Is the surrogate molecular allergen Ole e 1 using CAP appropriate for diagnosing ash pollen allergy?
- (ix) How cross-reactive are Der p and Der f extracts?
- (x) Is Der p 1 and 2 sufficient to diagnose HDM allergy?
- (xi) How important is Der p 23?
- (xii) Can additional Der p allergens (on ALEX²) increase sensitivity?
- (xiii) Do HDM allergic asthmatics and non-asthmatics have sensitization differences?
- (xiv) Are there any differences between the molecular test systems available?
- (xv) Is molecular diagnostics always better?

2. Materials and Methods

Two studies have been performed to investigate the hypotheses mentioned above. The first study evaluated the molecular diagnosis of house dust mite allergy. The second study examined the molecular diagnosis of Austria's six most relevant seasonal allergens, namely *Alternaria* and the pollen of ash, birch, timothy grass, mugwort and ragweed.

2.1. Patients – House dust mite allergy

In a first step, we have explored medical reports of patients with positive skin prick tests to analyze sensitization rates to the two most relevant house dust mite species in Austria, *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f). 28,572 patients had a positive skin prick test to Der p and/or Der f from the 1st of January 2005 to the 31st of December 2018 ¹⁶⁰.

In a second step, we included 215 HDM-allergic patients to compare the different molecular diagnostic methods available. Inclusion criteria were a clear-cut history of HDM allergy (chronic rhinitis, conjunctivitis and/or bronchial asthma), positive sIgE to Der p extract (≥ 0.35 kU_A/l), and a positive HDM skin prick test ¹⁶⁰. All three inclusion criteria had to be positive. None of these 215 patients had previously received immunotherapy to any allergen. We have investigated Der p-sensitized patients because Der p sensitization was dominant in our study population, and more molecular allergens were available for Der p than for Der f.

2.2. Patients – Seasonal allergy

In this study, four different data analyses have been performed (Figure 2).

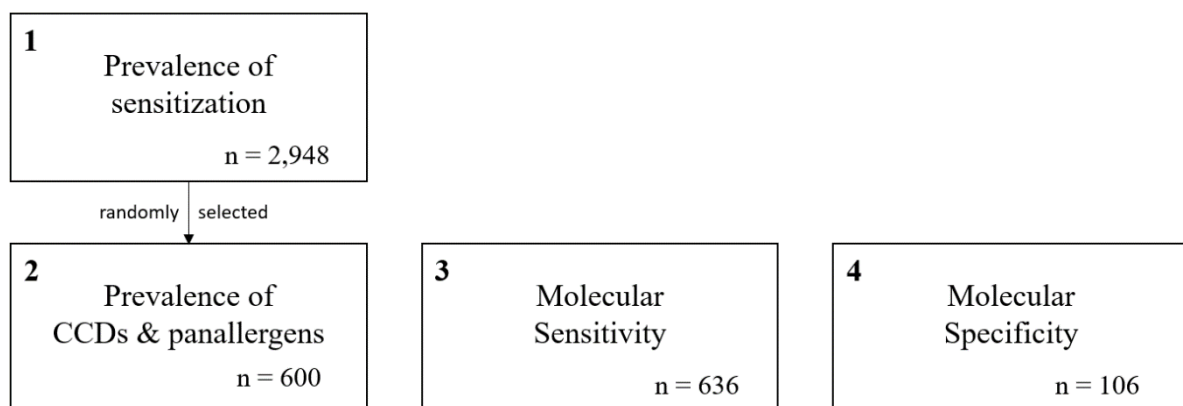


Figure 2: Seasonal allergy analyses performed

In total, data from 3,590 patients were explored. The first analysis investigated the prevalence of multiple (pollen) sensitizations. For this reason, 2,948 patients referred to the outpatient clinic between 1st of January and 31st of December 2020 with rhinitis, conjunctivitis and/or bronchial asthma were included (analysis 1). Then, patients were grouped according to their number of pollen (ash, birch, timothy grass, mugwort and ragweed) sensitizations, from one to five ¹⁶¹.

Second, to explore sIgE to CCDs, polcalcins and profilins, 100 patients from each of these five groups were randomly selected. Sera of these 500 patients and a control group of 100 non-allergic subjects were evaluated for sIgE to MUXF3, Phl p 7 and Phl p 12 ¹⁶¹. MUXF3 was determined using CAP, whereas Phl p 7 and 12 were tested using ALEX². All controls had negative sIgE to the investigated six seasonal allergen extracts and *Dermatophagoides pteronyssinus* in the ImmunoCAP system and negative prick tests to 13 aeroallergens (pollen, pets, mites and moulds) ¹⁶¹.

In the third analysis, the sensitivities of the major molecular marker allergens of *Alternaria* (Alt a 1) and pollen of ash (Fra e 1 / Ole e 1), birch (Bet v 1), timothy grass (Phl p 1 and Phl p 5), mugwort (Art v 1) and ragweed (Amb a 1) were investigated ¹⁶¹. After sample size estimation, 636 allergic patients (106 per allergen) visiting the outpatient clinic between 2013 and 2020 were included ¹⁶¹. The majority of the subjects were mono-allergic to the respective allergen investigated (defined by positive prick and sIgE tests to the respective extract plus history of symptoms in the corresponding season of the respective allergen) ¹⁶¹. Solely 25% of the mugwort- and ragweed-allergic patients were double-sensitized. Cross-reactivity between these two pollen allergens is very common using extracts; therefore, we could not find enough patients mono-sensitized to these allergens – although we screened medical reports from a period of 8 years ¹⁶¹. In these double-sensitized cases, primary sensitization was assumed if the specific IgE of one allergen was at least threefold higher than the other ¹⁶¹. In the 636 sera used for sensitivity analyses, minor molecular allergens of the respective allergen sources were also determined to explore their diagnostic value compared to the major molecular marker allergens. The fourth analysis investigated the specificity of Alt a 1, Amb a 1, Art v 1, Bet v 1, Fra/Ole e 1, Phl p 1 and Phl p 5 in 106 non-allergic subjects (the control group from analysis 1 and six additional persons) using ALEX² ¹⁶¹.

None of the patients in any of the four analyses received allergen-specific immunotherapies prior to or at the time of blood sampling.

2.3. Diagnostic tests

2.3.1. Skin prick tests

All skin prick tests were performed using extracts from ALK-Abelló (Hørsholm, Denmark). Test results were considered positive in the case of erythema and a wheal larger than 3 mm in diameter^{160,161}.

2.3.2. Singleplex sIgE testing

All extract-based and molecular sIgE levels were analyzed with the ImmunoCAP™ 1000 platform (CAP, Thermo Fisher Scientific, Waltham, Massachusetts, USA), which expresses sIgE levels in kU_A/l (kilo units per litre) at a positivity cut-off of equal to or larger than 0.35 kU_A/l^{160,161}. Values higher than 100 kU_A/l were classified as 100 kU_A/l for statistical analyses.

2.3.3. Multiplex sIgE testing

Three multiplex systems were used for the house dust mite analysis: ISAC™ (ISAC, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and version 1 and 2 of the ALEX® Allergy Explorer (ALEX and ALEX², MacroArray Diagnostics, Vienna, Austria). Assays were performed based on the instructions of the producers. ISAC uses ISU (ISAC Standardized Units), and values of equal to or larger than 0.30 ISU are rated as positive. In contrast, ALEX and ALEX² state sIgE levels in kU_A/l with a positivity cut-off ≥ 0.30 kU_A/l¹⁶⁰. In the study investigating seasonal allergens, ALEX² (the ALEX® Allergy Explorer version 2) was used for multiplex analyses¹⁶¹.

2.3.4. Molecular house dust mite allergens

Molecular house dust mite allergens determined are listed in Table 1. All molecular allergens investigated were recombinant, except nDer p 1 and 2 using ISAC. All four test methods were performed in all 215 sera.

Table 1: Molecular house dust mite allergens investigated

n = naturally derived allergen; r = recombinant allergen; empty fields indicate that the allergen was not available using the respective method.

Allergen	CAP	ISAC	ALEX	ALEX ²
Der p 1	r	n	r	r
Der p 2	r	n	r	r
Der p 5			r	r
Der p 7			r	r
Der p 10	r	r	r	r
Der p 11			r	r
Der p 20				r
Der p 21				r
Der p 23	r		r	r

2.3.5. Molecular seasonal allergens

The seasonal molecular marker allergens of *Alternaria* (Alt a 1) and the pollen of ash (Fra e 1 / Ole e 1), birch (Bet v 1), timothy grass (Phl p 1 and Phl p 5), mugwort (Art v 1) and ragweed (Amb a 1) were investigated. All determinations were performed using the singleplex platform CAP and the multiplex assay ALEX². Ole e 1 was used as a surrogate marker for ash pollen using CAP because Fra e 1 is not commercially available on the CAP platform.

Furthermore, these six allergen sources' additional major and minor allergens were determined to create sensitization profiles (Table 2). Again, Ole e 7 and 9 were investigated as potential surrogate markers for ash pollen as no other *Fraxinus excelsior* allergens despite Fra e 1 were available / identified to date (22th of May, 2022).

Table 2: Additional seasonal molecular marker allergens investigated

Allergen source	CAP	ALEX ²
Alternaria		Alt a 6
Ash	Ole e 7, 9	Ole e 1, 7, 9
Birch	Bet v 2, 4, 6	Bet v 2, 6
Timothy grass	Phl p 2, 4, 6, 7, 11, 12	Phl p 2, 6, 7, 12
Mugwort	Art v 3	Art v 3
Ragweed		Amb a 4

2.4. Statistical analysis

For the house dust mite analysis, Pearson's chi-squared test, McNemar's test, Cochran's Q test, and Spearman's rank correlation coefficient were performed using IBM SPSS Statistics 26 (IBM, Somers, USA). Graphs were drawn using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, USA).

For the seasonal allergen analysis, a sample size calculation to investigate molecular sensitivity was performed using R version 3.6.1 (The R Foundation, Vienna, Austria). With a sample size of 100 and an expected sensitivity of 0.96 (alternatively 0.98), an exact two-sided 95% confidence interval (Clopper and Pearson) ranged from 0.90 to 0.99 (alternatively 0.93 to 1.00). With an assumed drop-out rate of 5%, 106 patients were needed per group to show that the sensitivity is indeed above 90%, respectively. Statistical analysis was performed using McNemar's test, Wilcoxon signed-rank test and Spearman's rank correlation with IBM SPSS Statistics 27. Graphs were generated using GraphPad Prism 9.0.

The level of significance was set at 0.05 in both studies.

2.5. Institutional review board approval

The institutional review board of the Medical University of Graz approved this doctoral thesis under approval no. 29-398 ex 16/17.

3. Results

3.1. Results I – Molecular diagnosis of house dust mite allergy

3.1.1. Overall sensitization to Der p and Der f

Skin prick test data from a period of 14 years (2005-2018) were analyzed. In total, 28,572 patients had positive skin tests to Der p and/or Der f ¹⁶⁰. Of these, 27,142 (95.0%) were sensitized to Der p whereas 25,360 (88.8%) were sensitized to Der f. In detail, 83.8% had positive skin prick test to Der p and Der f, 11.2% were mono-sensitized to Der p, and 5.0% were mono-sensitized to Der f ¹⁶⁰.

3.1.2. Demographical and clinical characterization of the study population

Two hundred fifteen HDM-allergic patients were included to investigate molecular HDM allergy diagnostics ¹⁶⁰; 119 (55.3%) were females, and 64 (29.8%) were younger than 18 years old. Rhinitis was the leading symptom, with 192 (89.3%) of all patients affected. Lower airway tract symptoms (chronic bronchitis and/or asthma) and conjunctivitis were observed in 69 (32.1%) and 64 (29.8%) patients, respectively. HDM-related food allergy (cross-reactivity to shellfish) was reported in only 2 (0.9%) patients ¹⁶⁰. Demographic data and details of clinical symptoms can be found in Table 3. We did not detect any statistically significant differences in symptoms between sex or age ¹⁶⁰.

Table 3: Characterization of the house dust mite study population

Reproduced from Koch L et al ¹⁶⁰ with permission of publisher John Wiley and Sons. Age is stated in years as median + interquartile range; bronchitis = patient-reported chronic cough; asthma = established diagnosis by a pulmonologist; food allergy = cross-reactivity to shellfish

	Adults n = 151	Children n = 64	Females n = 119	Males n = 96	Total n = 215
Relative percent	70.2%	29.8%	55.3%	44.7%	100.0%
Age	31 (24–42)	12 (10–15)	25 (17–35)	25.5 (13–41)	25 (16–36)
Rhinitis	88.7%	90.6%	87.4%	91.7%	89.3%
Conjunctivitis	30.5%	28.1%	33.6%	25.0%	29.8%
Bronchitis	17.9%	18.8%	17.6%	18.8%	18.1%
Asthma	14.6%	12.5%	16.0%	11.5%	14.0%
Food allergy	1.3%	0.0%	0.8%	1.0%	0.9%

3.1.3. Sensitization to Der p and Der f extracts

All 215 patients were positive to Der p extract tested with CAP, because this was the main inclusion criterion. Additionally, a high cross-reactivity to Der f could be observed using CAP: 210 of 215 (97.7%) patients were positive to Der p and Der f. In the five Der f “negative” patients, low sIgE levels to Der f between 0.16 and 0.29 kU_A/l could be detected. Specific IgE levels to Der p were also low in these patients (between 0.36 and 0.85 kU_A/l). The sensitivity between Der p and Der f did not differ statistically ($p = 0.063$). Furthermore, the correlation between Der p and Der f extract was strong (Spearman’s rho = 0.765, $p < 0.001$, Figure 3).

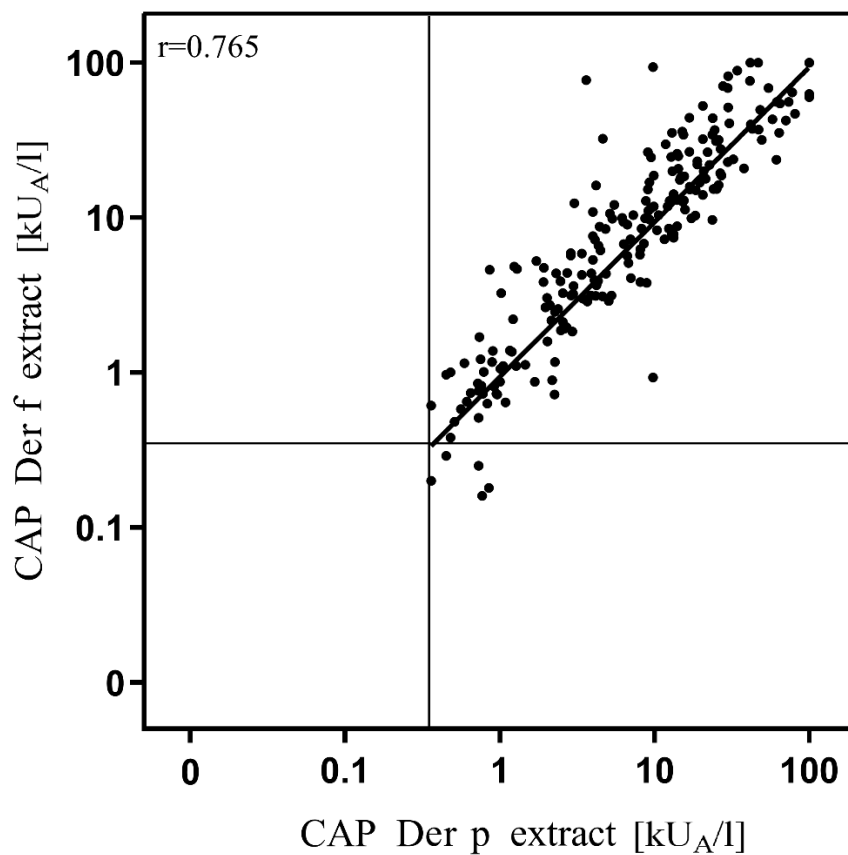


Figure 3: Correlation between Der p and Der f extracts

3.1.4. Correlation of prick test and specific IgE extract results

All 215 (100%) patients had a positive prick test to Der p or Der f; 94% and 87% (n = 202 and 187) were prick positive to Der p and Der f, respectively. Both prick tests were positive in 174 patients (80.9%), compared to 41 patients (19.1%) in which either Der p (28 patients – 13%) or Der f (13 patients – 6.1%) were positive.

Of the 174 patients with positive prick tests to both allergens, two had equal sIgE levels of Der p and Der f extracts. Of the remaining patients, however, 75 patients had higher levels against Der p and 97 had higher levels against Der f; therefore, Der f levels were higher than Der p levels. In the 28 Der p prick-positive, Der f prick-negative patients, IgE levels to Der p or Der f were higher in 50% of the cases; however, in the Der p prick-negative, Der f prick-positive patients, seven compared to five had higher levels against Der p than Der f extracts.

3.1.5. Sensitivity of molecular house dust mite allergy diagnosis

In the overall analysis, the sensitivity of molecular HDM allergy diagnosis was lower than extract-based diagnosis ¹⁶⁰. Overall sensitivities were 88.8% (ISAC), 93.0% (CAP using molecular allergens), 93.5% (ALEX version 1) and 94.9% (ALEX²), and therefore increased with the number of molecular allergens tested ¹⁶⁰. The results of molecular-based CAP, ALEX and ALEX² did not differ statistically ¹⁶⁰. In contrast, the sensitivity of ISAC was significantly lower compared to ALEX and ALEX² (p = 0.006 and p < 0.001) as well as to the molecular-based CAP (p = 0.022) ¹⁶⁰. This difference was mainly due to the unavailability of Der p 23 using ISAC because omission of Der p 23 using CAP, ALEX and ALEX² resulted in lower sensitivity rates of 87.9%, 88.4% and 90.2%, respectively, which were all statistically similar to the 88.8% of the ISAC (p = 0.392) ¹⁶⁰.

The sensitivity of the molecular test systems correlated to levels of sIgE to Der p extract: the higher the Der p extract levels, the better the sensitivity of molecular testing performed ¹⁶⁰. Table 4 demonstrates the test sensitivities of all four molecular test methods with and without excluding patients with low IgE levels.

Table 4: Sensitivity of molecular test systems

Reproduced from Koch L et al ¹⁶⁰ with permission of publisher John Wiley and Sons. The higher the sIgE levels to Der p extract, the better the sensitivities of the four molecular test methods studied. ALEX² performed statistically equally to the extract-based diagnosis in the case of extract sIgE levels ≥ 1.0 kU_A/l, whereas all other test methods performed statistically equally in the case of extract sIgE levels ≥ 3.5 kU_A/l. All p-values listed are direct comparisons to extract-based singleplex diagnosis using CAP.

	All patients n = 215	Der p extract ≥ 0.7 kU _A /l n = 204	Der p extract ≥ 1.0 kU _A /l n = 186	Der p extract ≥ 3.5 kU _A /l n = 141
ISAC	88.8%	89.7%	93.6%	97.9%
	p < 0.001	p < 0.001	p < 0.001	p = 0.250
CAP	93.0%	94.6%	96.2%	99.3%
	p < 0.001	p = 0.001	p = 0.016	p = 1.000
ALEX	93.5%	94.6%	96.8%	99.3%
	p < 0.001	p = 0.001	p = 0.031	p = 1.000
ALEX ²	94.9%	95.6%	97.3%	99.3%
	p = 0.001	p = 0.004	p = 0.063	p = 1.000

3.1.6. Molecular house dust mite sensitization pattern

With ALEX², it was possible to create a sensitization pattern using nine recombinant Der p molecular allergens (Figure 4) ¹⁶⁰. Overall, 94.9% of the patients were sensitized to at least one of the nine molecular allergens determined; however, only three allergens, namely Der p 1, 2 and 23, were major allergens in our study population, with sensitization rates of 55.3%, 77.7% and 54.0%, respectively ¹⁶⁰.

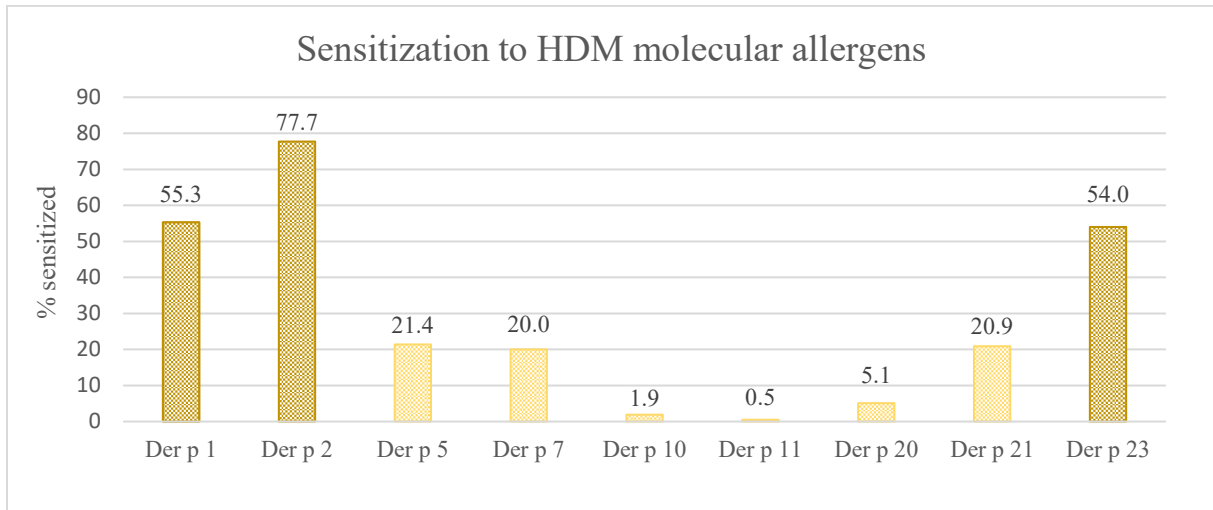


Figure 4: Sensitization to house dust mite molecular allergens

Reproduced from Koch L et al ¹⁶⁰ with permission of publisher John Wiley and Sons. This figure shows the molecular sensitization pattern of 215 HDM-allergic patients.

Sensitization rates between the four molecular methods investigated were similar (Table 5); however, the sensitivity of Der p 1 was slightly lower, and Der p 23 was markedly higher in ALEX² compared to all others. Interestingly, Der p 11 was rarely positive in ALEX² compared to ALEX version 1.

Table 5: Differences in molecular Der p sensitization

All numbers are percentages. Empty fields indicate that the allergen was not available using the respective method.

Allergen	CAP	ISAC	ALEX	ALEX ²
Der p 1	58.1	61.4	58.6	55.3
Der p 2	77.2	76.7	75.8	77.7
Der p 5			20.9	21.4
Der p 7			20.0	20.0
Der p 10	1.9	2.8	2.3	1.9
Der p 11			8.4	0.5
Der p 20				5.1
Der p 21				20.9
Der p 23	46.5		46.5	54.0

Of 215 patients, 87.9% and 88.4% (n = 189 and 190) had sIgE reactivity to Der p 1, Der p 2 or both determined with ALEX² and CAP, respectively. Importantly, these percentages indicate that between 11.6% and 12.1% of the patients were not sensitized to the two major allergens Der p 1 and 2, to which modern sublingual immunotherapies are standardized.

In addition, from the 189 Der p 1 and/or Der p 2 positive patients defined by ALEX², only 65 (34.4%) were solely sensitized to these allergens. On the other hand, 124 (65.6%) were additionally sensitized to Der p 5, 7, 10, 11, 20, 21 or 23. From these 124 patients, 56 (29.6%) had one, 33 (17.5%) had two, 19 (10.1) had three, 14 (7.4%) had four, 1 (0.5%) had five and 1 (0.5%) had six additional sensitizations.

Reactivity to one or more of the three major allergens Der p 1, 2 and 23 was observed in 94.0% and 93.0% (n = 202 and 200 for ALEX² and CAP, respectively) ¹⁶⁰.

Mono-sensitization to Der p 23 was found in 4.7% with both methods ¹⁶⁰. On the other hand, using ALEX², mono-sensitization could rarely be detected to Der p 10 and Der p 20 (0.4% each). Interestingly, we did not see any mono-sensitization to Der p 5, Der p 7, Der p 11 or Der p 21, which indicates that these allergens did not increase the overall sensitivity of ALEX² in comparison with ALEX version 1 ¹⁶⁰.

The majority of patients were sensitized to few molecular allergens. No patient was sensitized to all nine allergens, and 5.1% did not react to any of these nine allergens investigated (Table 6) ¹⁶⁰.

Table 6: Percentages of patients sensitized to multiple molecular Der p allergens

X-positive	1x	2x	3x	4x	5x	6x	7x	8x	9x
Relative %	27.4%	21.4%	19.5%	13.0%	6.5%	6.0%	0.5%	0.5%	0%

3.1.7. Specific IgE levels to molecular house dust mite allergens

Specific IgE levels to the different molecular Der p allergens are listed in Table 7. The highest levels in all test systems were found for Der p 2, followed by Der p 1 and Der p 23. Mean levels to Der p 10, 11 and 20 were lower than to Der p 5, 7 and 21.

Table 7: Specific IgE levels to molecular house dust mite allergens

CAP: kU_A/l; ISAC: ISU; ALEX and ALEX²: kU_A/l

Allergen	CAP		ISAC		ALEX		ALEX ²	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Der p 1	6.97	13.72	9.06	18.30	5.27	8.08	4.23	6.47
Der p 2	12.68	17.46	14.68	18.99	7.90	10.28	15.04	13.07
Der p 5					1.23	3.62	1.56	4.69
Der p 7					0.94	3.15	1.16	3.90
Der p 10	0.22	2.86	15.68	19.99	0.21	2.24	0.20	2.28
Der p 11					0.27	2.16	0.00	0.02
Der p 20							0.34	2.63
Der p 21							2.47	7.16
Der p 23	2.57	6.68			2.71	5.96	5.52	9.03

3.1.8. Correlation of the test systems

All three molecular test systems correlated strongly with Spearman's rho ranging between 0.940 and 0.955 (Der p 1), between 0.959 and 0.973 (Der p 2), as well as 0.953 (Der p23), ($p < 0.001$, Figure 5)¹⁶⁰. Due to the low number of Der p 10 sensitizations, correlations for Der p 10 were unreliable¹⁶⁰. Interestingly, correlations between CAP and ISAC were more linear than those between these two methods and ALEX², which were more curved.

ALEX versions 1 and 2 correlated strongly (Figure 6). Der p 1, 5 and 7 had quite linear scatter plots compared to Der p 2 and 23, which were broader. Furthermore, the scatter plot of Der p 23 indicates a slightly higher sensitivity of Der p 23 using ALEX² (54.0%) versus version 1 (46.5%). On the other hand, Der p 11 had only one positive result using ALEX² compared to several using version 1.

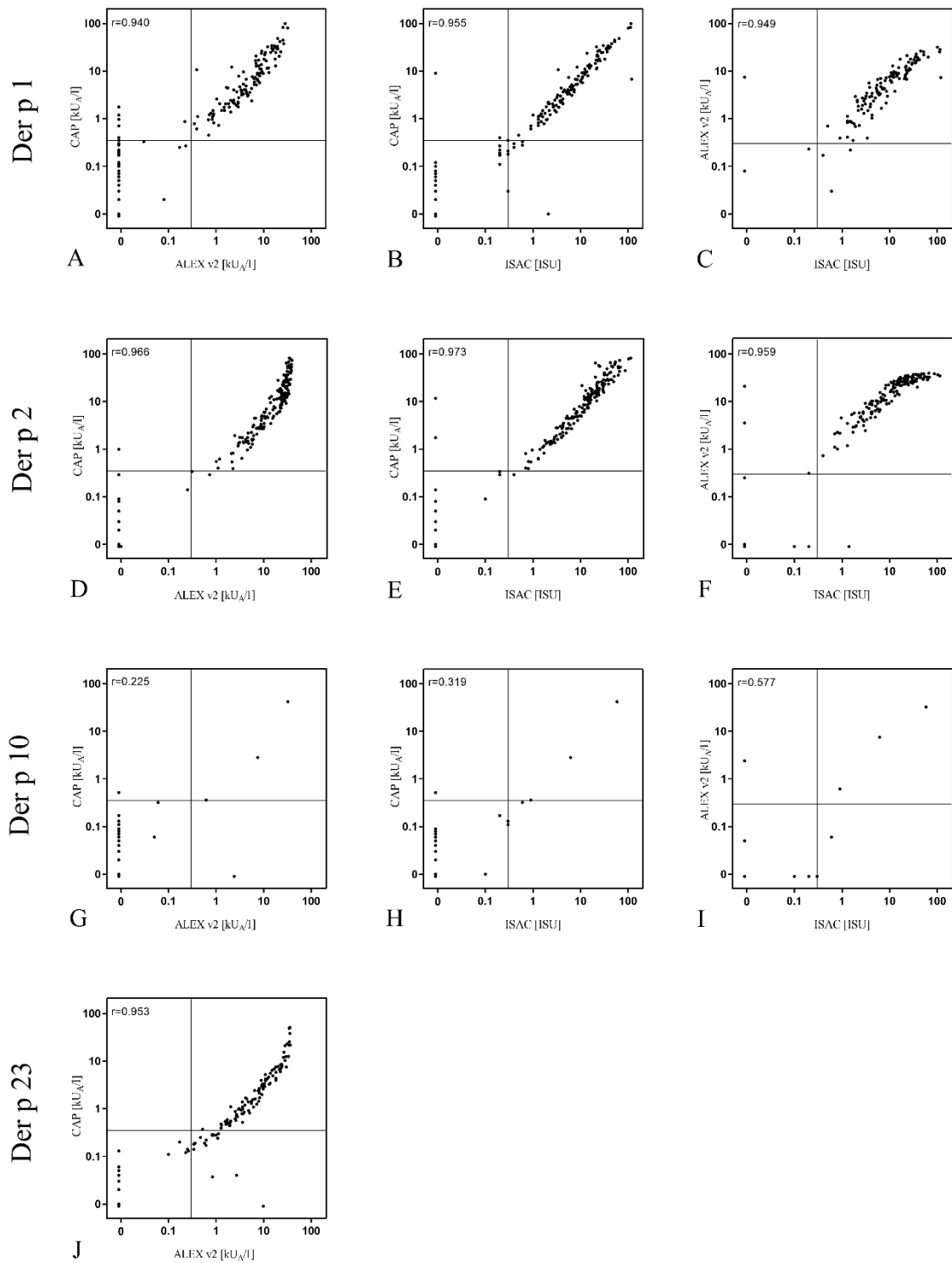
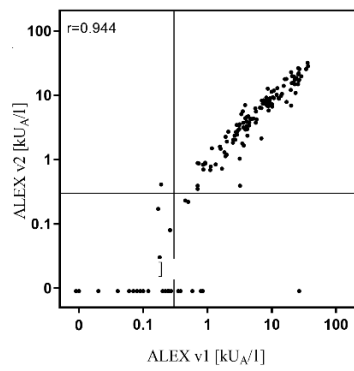
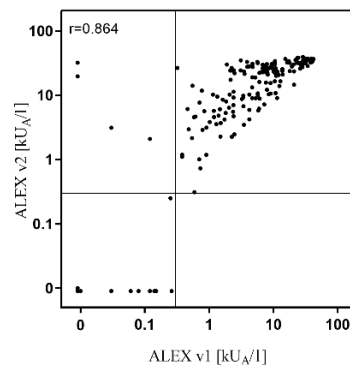


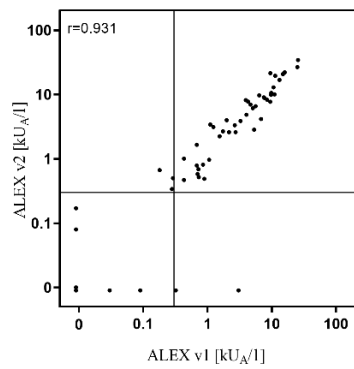
Figure 5: Correlation of molecular house dust mite allergens using different assays
 Reproduced from Koch L et al ¹⁶⁰ with permission of publisher John Wiley and Sons.



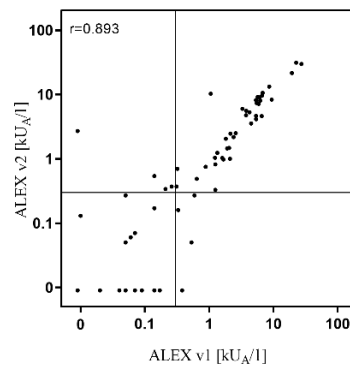
Der p 1



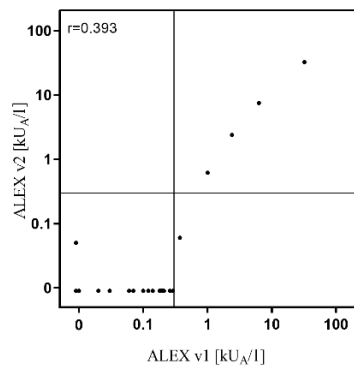
Der p 2



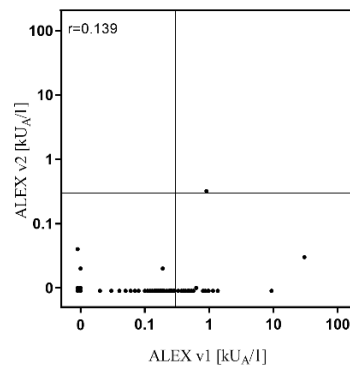
Der p 5



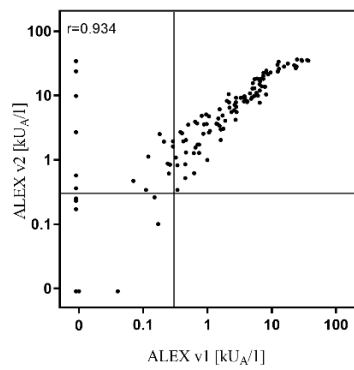
Der p 7



Der p 10



Der p 11



Der p 23

Figure 6: Correlation of molecular Der p allergens between ALEX versions 1 and 2

3.1.9. CCD-reactivity

None of the eleven Der p molecular negative patients reacted to MUXF3 determined with the CAP platform.

3.1.10. Positivity to other mites

All 215 patients were screened for reactivity to the house dust mite *Blomia tropicalis* and the storage mites *Acarus siro*, *Glycophagus domesticus*, *Lepidoglyphus destructor* and *Tyrophagus putrescentiae* using ALEX version 1. Eighteen of the 215 patients (8.4%) were positive to at least one of these mites. Sensitization was found in 7.0% (*Blomia tropicalis*), 3.3% (*Acarus siro*), 0.9% (*Glycophagus domesticus*), 2.3% (*Lepidoglyphus destructor*) and 0.9% (*Tyrophagus putrescentiae*)¹⁶⁰; however, none of the 11 Der p molecular negative patients reacted to any of these mites¹⁶⁰.

Because these sensitization rates appeared to be quite low, we repeated the determination of other house dust and storage mites in the eleven Der p molecular negative patients using CAP. Surprisingly, all 11 patients were sensitized to all mites mentioned above (*Blomia tropicalis*, *Acarus siro*, *Glycophagus domesticus*, *Lepidoglyphus destructor* and *Tyrophagus putrescentiae*). In addition, 100% and 45.5% of the patients were also positive to the house dust mites *Dermatophagoides microceras* and *Euroglyphus maynei*. Interestingly, nine out of eleven patients showed (slightly) higher sIgE values to other mites than to Der p, possibly indicating a primary sensitization to these mites (Table 8).

Table 8: Positivity to other mites in Der p molecular negative patients

Specific IgE levels (kU_A/l) to extracts of the house dust mites *Dermatophagoides pteronyssinus* (Der p), *Blomia tropicalis* (Blo t), *Dermatophagoides microceras* (Der m) and *Euroglyphus maynei* (Eur m), as well as the storage mites *Acaris siro* (Aca s), *Glycophagus domesticus* (Gly d), *Lepidoglyphus destructor* (Lep d) and *Tyrophagus putrescentiae* (Tyr p), of the 11 Der p molecular negative patients, are shown. Specific IgE values higher to other mites than the Der p extract are highlighted in grey.

Serum	Der p	Blo t	Der m	Eur m	Aca s	Gly d	Lep d	Tyr p
1	0.48	0.71	0.56	0.06	1.03	0.62	0.92	1.77
2	1.00	1.09	1.02	0.79	1.10	0.96	1.05	0.72
3	4.17	4.79	2.97	1.17	3.98	2.81	2.98	2.22
4	1.01	1.28	1.13	0.64	1.20	1.03	1.11	0.92
5	2.49	2.38	1.50	0.42	2.01	1.62	2.00	1.36
6	0.92	0.47	0.54	0.24	0.55	0.37	0.39	0.40
7	0.95	1.04	0.65	0.32	0.83	0.64	0.67	0.72
8	1.20	0.78	1.48	0.20	0.59	0.63	0.83	0.71
9	0.76	0.81	0.57	0.12	0.69	0.51	0.62	0.60
10	0.73	0.94	0.70	0.45	0.87	0.72	0.82	0.60
11	0.65	0.92	0.69	0.23	0.79	0.69	0.80	0.52

3.1.11. Total and specific IgE of asthmatics and non-asthmatics

Total IgE levels were significantly higher in asthmatics than non-asthmatic patients (mean total IgE 315 kU_A/l vs. 144 kU_A/l, $p = 0.022$)¹⁶². Asthmatics had higher sensitization rates to Der p 1 and Der p 10 than non-asthmatics, $p = 0.039$ and $p = 0.044$, respectively¹⁶². Positivity to Der p 2 and Der p 23 and sIgE levels to these four molecular allergens did not significantly differ between the two groups¹⁶².

3.2. Results II – Molecular diagnosis of seasonal allergens

3.2.1. Demographical and clinical characterization of the study population

Table 9 summarizes the demographical and clinical information on the four study populations investigated.

Table 9: Characterization of the seasonal allergen study population

Analyses 1 investigated the prevalence of sensitizations. Analysis 2 examined sensitization to CCDs, profilins and polcalcins. In addition, analyses 3 and 4 explored the sensitivity and specificity of seasonal molecular allergy diagnosis. Age is listed as median and interquartile ranges. The 600 patients from analysis 2 were randomly selected from analysis 1 ¹⁶¹.

	Analysis 1 Prevalence of sensitizations	Analysis 2 CCDs & panallergens	Analysis 3 Molecular sensitivity	Analysis 4 Molecular specificity
Patients	2,948	600	636	106
Sex	52.9% female	50.2% female	56.3% female	67.9% female
Age	29 (17–43) years	28 (16–41) years	32 (20–43) years	36 (25–54) years
Rhinitis	79.5%	84.5%	93.7%	61.3%
Conjunctivitis	32.1%	50.0%	63.8%	26.4%
Bronchitis	20.1%	15.5%	16.8%	23.6%
Asthma	8.8%	8.8%	5.2%	7.5%

3.2.2. Prevalence of sensitizations

1,660 (56.3%) of the 2,948 patients had IgE reactivity to one or more of the six seasonal allergens investigated (Alternaria or pollen of ash, birch, timothy grass, mugwort and ragweed) ¹⁶¹. In addition, 837 patients (28.4%) had positive sIgE to Der p extract. 1,627 (55.2%) reacted to one or more of the five pollen allergens. Overall, poly-sensitization to pollen allergens was detected in 34.0% of the patients ¹⁶¹. In the population sensitized to pollen, sensitization to all five pollens was detected in 22.4%. Furthermore, 6.7%, 12.8%, 19.8% and 38.4% were sensitized to four, three, two or only one pollen source, respectively ¹⁶¹. Interestingly, many of the pollen sIgE levels were low. Overall, 16.9% of the pollen sIgE extract results were ≤ 0.7 kU/l ¹⁶¹. Depending on the pollen allergen, between 8.9% and 30% of the sIgE values ranged between 0.35 and 0.70 kU_A/l (Figure 7); consequently, applying cut-offs above 0.7 kU_A/l or 3.5 kU_A/l, merely 15.4% and 5.3% or 4.5% and 3.0% of the patients were positive to five or four pollen, respectively.

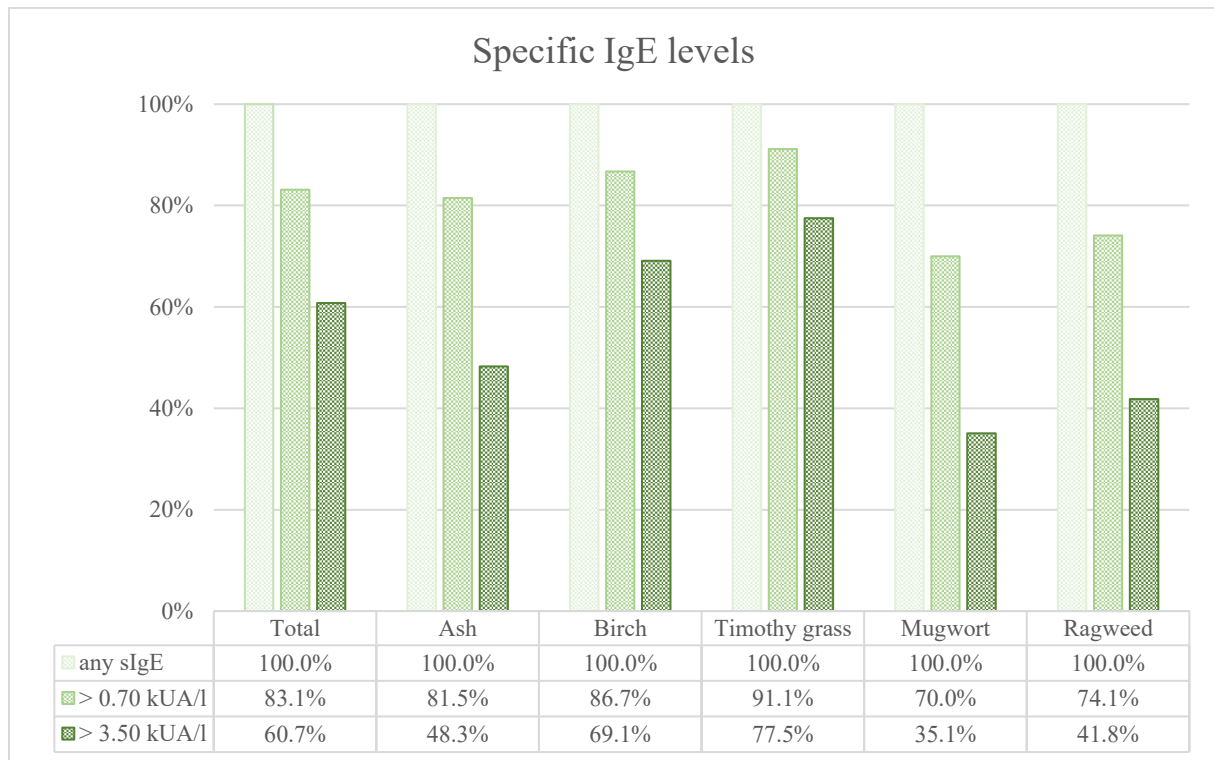


Figure 7: Pollen sIgE levels

Total lists average percentages of all positive pollen extract sIgE results. Total (n = 4,148), ash (n = 826), birch (n = 889), timothy grass (n = 1,299), mugwort (n = 517), ragweed (n = 617). Many of the patients had low sIgE levels to pollen: depending on the pollen allergen, between 8.9% and 30% of the sIgE values ranged between 0.35 and 0.70 kU_A/l.

3.2.3. Correlation of prick test and specific IgE results

Patients were grouped into four categories regarding their prick test and sIgE extract results: “true-positive results” (prick and extract positive), “true-negative results” (prick and extract negative), “false-positive prick test, or sIgE < 0.35 kU_A/l” (prick positive, extract negative) and “false-negative prick test, or false-positive sIgE” (prick negative, extract positive) (Figure 8). Interestingly, the prick positive, extract negative category was seldom in all allergens investigated, and the prick negative, extract positive category was frequently in pollen allergens but not *Alternaria*.

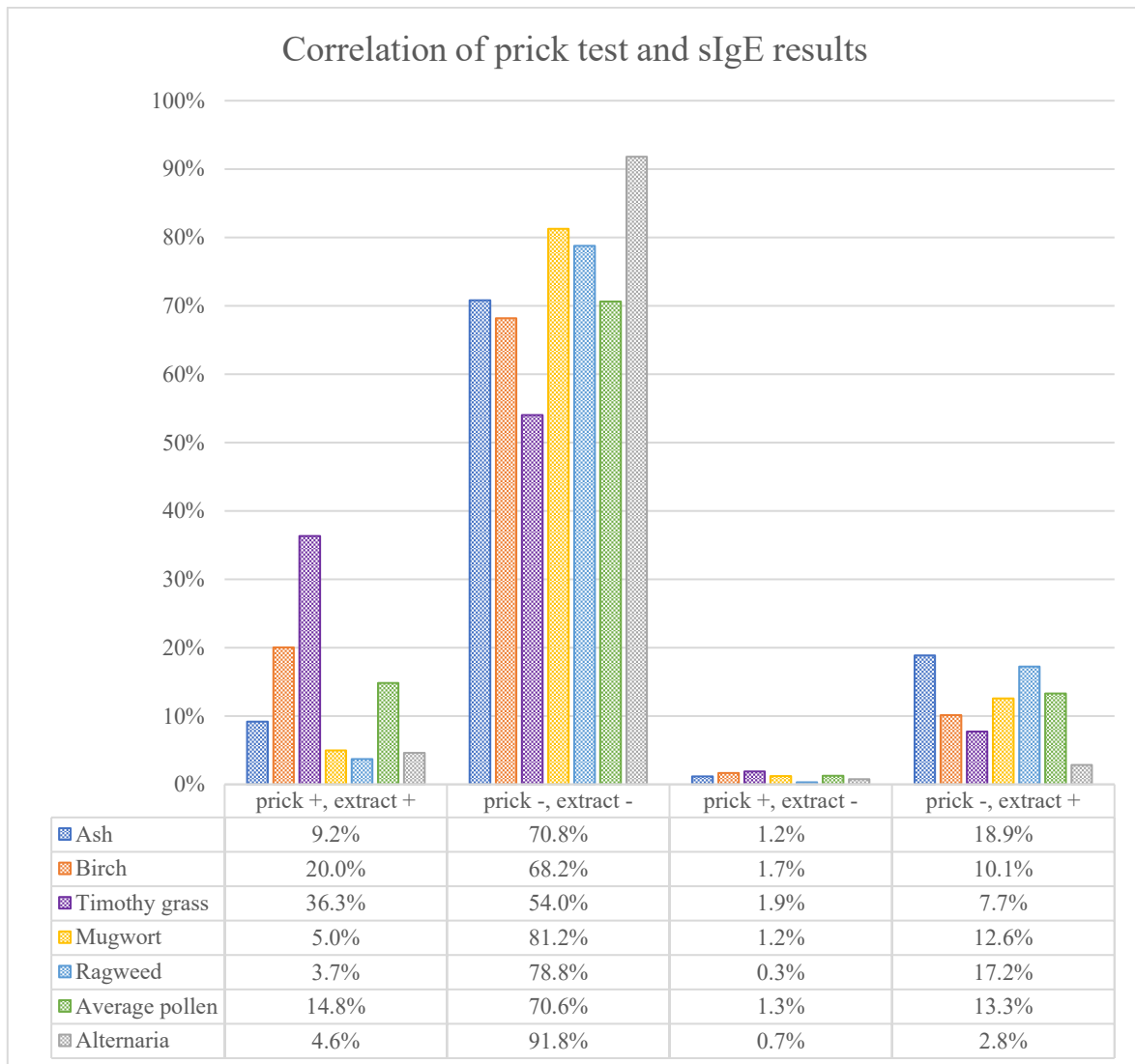


Figure 8: Correlation of seasonal prick test and sIgE results

Overall, 17,688 prick tests were correlated to sIgE results (6 allergens in 2948 patients). Average pollen represents the average of the five pollen species investigated ¹⁶¹.

In total, 2.8% of the patients with positive sIgE to Alternaria extracts had negative prick tests. On the other hand, an average of 13.3% of the patients with positive sIgE to pollen allergens had negative corresponding prick tests. The difference of 10.5% may be explained by cross-reactivity via anti-CCD IgE antibodies, reacting with pollen but not with Alternaria extract ¹⁶¹.

3.2.4. Sensitizations to CCDs and panallergens

The 2,948 patients were grouped according to their number of pollen extract positivity (from one to five pollen positive). One hundred patients of each pollen-positive group were randomly selected (5 x 100 patients). In these 500 patients, the CCD marker MUXF3 (using CAP) and the polcalcin and profilin markers Phl p 7 and Phl p 12 (using ALEX²) were determined (Figure 9). In addition, these three parameters were analyzed in a control group of one hundred randomly selected non-allergic patients without any reactivity in prick tests or sIgE determinations to the five pollen investigated. Table 10 lists the frequency of patients with reactivity to multiple cross-reactive allergens / panallergens.

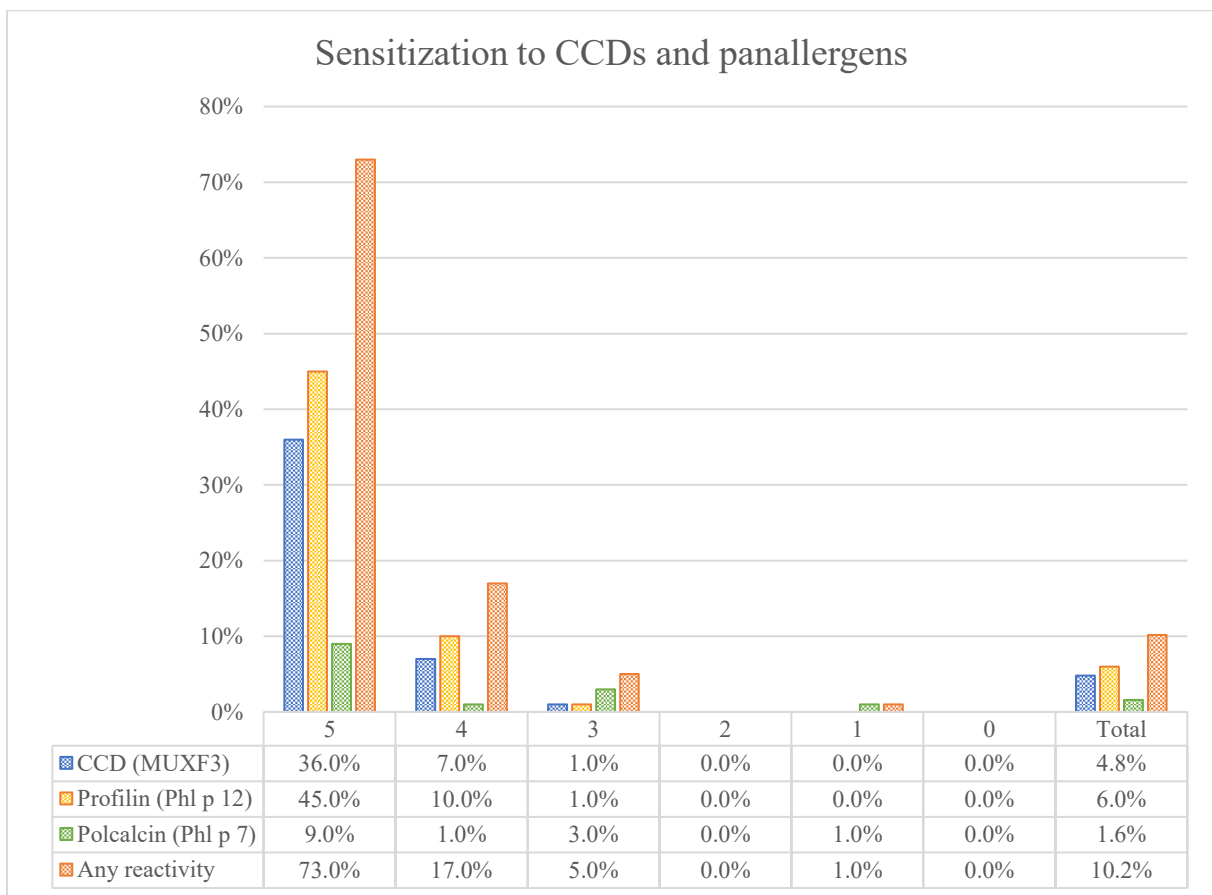


Figure 9: Sensitizations to CCDs and panallergens

Patients were grouped from 5 to 0 pollen sensitizations – 600 patients were investigated (100 per group). Total percentages were extrapolated for the overall study population (n = 2,948) ¹⁶¹.

Table 10: Frequency of multiple cross-reactivities

Table 10 shows the coincidence of reactivity to CCDs, profilin and polcalcin, n = 100 per group. Total percentages were extrapolated for the overall study population (“Total”, n = 2,948) ¹⁶¹.

Cross-reactivities	x-fold pollen positive sera						Total
	5	4	3	2	1	0	
One	56.0%	16.0%	5.0%	0.0%	1.0%	0.0%	8.1%
Two	17.0%	1.0%	0.0%	0.0%	0.0%	0.0%	2.1%
Three	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Any reactivity	73.0%	17.0%	5.0%	0.0%	1.0%	0.0%	10.2%

Sensitizations to CCDs and panallergens were most commonly observed in patients with reactivity to all five pollen extracts; in this group, 73% were sensitized to any cross-reactive allergen, and 17% even showed sIgE to two panallergens or one panallergen and CCDs ¹⁶¹. In the patients with reactivity to four pollen extracts, pan-sensitization was 17% and still relevant ¹⁶¹, whereas this was rarely seen from group 3 downwards. In none of the 500 pollen-positive patients was reactivity to all three, CCDs, profilin and polcalcin, detected.

After extrapolating from 500 to 1,627 patients sensitized to pollen, 18.5% could have reacted to at least one of the three, CCDs profilin or polcalcin ¹⁶¹. In detail, sIgE to CCDs, profilins and polcalcins could be expected in 8.7%, 10.9% and 2.9% of pollen-sensitized patients, respectively ¹⁶¹.

After including the 100 patients from the control group and extrapolating from 600 patients to the overall study population referred to our outpatient clinic with respiratory symptoms for allergy diagnosis (n = 2,948), reactivity to CCDs, profilin or polcalcin could be expected in 4.8%, 6.0% and 1.6%, respectively, with an overall sensitization rate of 10.2% ¹⁶¹.

Interestingly, we did not observe any sensitization to profilin or polcalcin without simultaneous reactivity to at least one molecular pollen marker allergen in any of the 600 patients investigated. Furthermore, only three out of 600 patients reacted to CCDs without simultaneous sensitization to molecular pollen or plant food allergens; however, these patients had sIgE reactivity to wasp venom as a possible explanation for their CCD reactivity ¹⁶¹.

3.2.5. Relevance of CCD-reactivity below 0.35 kU_A/l

MUXF3 is commonly determined to analyze the CCD reactivity of a serum. In our cohort, 43 (8.8%) of the 500 pollen-allergic patients were positive (sIgE \geq 0.35 kU_A/l) to it. In addition, 63 patients (12.6%) showed reactivity to MUXF3 of between 0.10 and 0.34 kU_A/l. Interestingly, the cut-off of 0.35 kU_A/l may be inappropriate to establish whether relevant CCD reactivity is present. For this reason, six patients with MUXF3 reactivity below the cut-off were investigated for potential interference with extract-based diagnosis. MUXF3 values in these patients ranged between 0.17 and 0.32 kU_A/l. Importantly, these patients had no reactivity to profilin or polcalcin (Phl p 7 and Phl p 12 sIgE results were 0.00 kU_A/l). All six patients, however, had weakly positive sIgE to multiple pollen extracts (without clinical symptoms), which turned negative after the CCD block (Table 11).

Table 11: Clinical relevance of CCD-reactivity below 0.35 kU_A/l

Many weakly positive pollen extract results turned negative after the CCD block.

Grey colour = primary sensitizers with positive corresponding molecular allergens; yellow = positive extract sIgE before CCD block; green = negative extract sIgE after CCD block

Extract		1	2	3	4	5	6
	MUXF3	0.28	0.19	0.31	0.17	0.22	0.32
Birch	before	14.10	0.37	26.20	0.00	0.00	0.00
	after block	11.9	0.06	24.0	0.08	0.05	0.02
Ash	before	0.51	0.49	9.16	0.44	0.37	0.40
	after block	0.05	0.05	8.50	0.07	0.03	0.01
Timothy grass	before	70.20	74.00	0.46	100.00	100.00	33.6
	after block	60.10	66.40	0.09	78.00	100.00	39.1
Mugwort	before	0.55	0.59	0.00	0.45	0.56	0.41
	after block	0.08	0.11	0.05	0.09	0.25	0.08
Ragweed	before	0.63	0.60	0.39	0.54	0.47	0.64
	after block	0.06	0.15	0.05	0.09	0.17	0.07

3.2.6. Comparison of specific IgE to extracts and molecular allergens

In the 500 pollen-allergic patients described above, sIgE to extracts and molecular allergens were compared. From 1,500 positive extract results, only 985 (65.7%) had positive corresponding molecular marker allergens (Alt a 1, Amb a 1, Art v1, Bet v 1, Fra e 1, Phl p 1 + 5). The other way round, this indicates that 34.3% of the positive extracts results could not be confirmed by molecular allergology ¹⁶¹.

The correlation between extracts and molecular marker allergens decreased inversely with the number of positive extract results: in the one to five-fold positive pollen patients, 92.0%, 85.0%, 79.7%, 59.3% and 49.4% of the positive extract results had positive corresponding molecular marker allergens, respectively ¹⁶¹. Correlation also differed between the pollen species investigated: overall, 91.4% of the timothy grass, 77.7% of the birch, 74.0% of the ash, 29.6% of the mugwort and 21.3% of the ragweed pollen extracts showed positive corresponding molecular marker allergens, respectively ¹⁶¹.

3.2.7. Sensitivity of molecular allergy diagnosis

The sensitivity of molecular allergy diagnosis using marker allergens ranged between 91.5% and 100% using ALEX² and between 96.2% and 100% using CAP ¹⁶¹. As with molecular diagnosis of HDM allergens (see above), sensitivities correlated to extract sIgE levels and increased with higher IgE values to the respective pollen extracts (Table 12) ¹⁶¹.

Table 12: Sensitivity of seasonal molecular allergy diagnosis

The sensitivities of molecular allergy diagnosis using ALEX² and CAP are shown. With CAP, Ole e 1 was used as a substitute marker for ash pollen allergy, as no Fra e 1 was available. All p-values are direct comparisons to extract-based singleplex diagnosis using CAP; if the molecular allergen had 100% sensitivity, no statistical test could be performed due to constant values (-)¹⁶¹.

Allergen	Extracts ≥ 0.35 kU _A /l	ALEX ²	p-value	CAP	p-value
Ash pollen	Fra e 1 (n = 106)	98.1%	0.500		
	Ole e 1 (n = 106)			98.1%	0.500
Birch pollen	Bet v 1 (n = 106)	100%	-	100%	-
Timothy grass pollen	Phl p 1 (n = 106)	98.1%	0.500	97.2%	0.250
	Phl p 1+5 (n = 106)	100%	-	100%	-
Mugwort pollen	Art v 1 (n = 106)	91.5%	0.004	96.2%	0.125
Ragweed pollen	Amb a 1 (n = 106)	97.2%	0.250	97.2%	0.250
Alternaria	Alt a 1 (n = 106)	100%	-	100%	-

Allergen	Extracts ≥ 0.70 kU _A /l	ALEX ²	p-value	CAP	p-value
Ash pollen	Fra e 1 (n = 104)	98.1%	0.500		
	Ole e 1 (n = 104)			98.1%	0.500
Birch pollen	Bet v 1 (n = 102)	100%	-	100%	-
Timothy grass pollen	Phl p 1 (n = 106)	98.1%	0.500	97.2%	0.250
	Phl p 1+5 (n = 106)	100%	-	100%	-
Mugwort pollen	Art v 1 (n = 89)	94.4%	0.063	96.6%	0.250
Ragweed pollen	Amb a 1 (n = 103)	99.0%	1.000	98.1%	0.500
Alternaria	Alt a 1 (n = 106)	100%	-	100%	-

Allergen	Extracts ≥ 3.50 kU _A /l	ALEX ²	p-value	CAP	p-value
Ash pollen	Fra e 1 (n = 74)	100%	-		
	Ole e 1 (n = 74)			100%	-
Birch pollen	Bet v 1 (n = 89)	100%	-	100%	-
Timothy grass pollen	Phl p 1 (n = 96)	99.0%	1.000	99.0%	1.000
	Phl p 1+5 (n = 96)	100%	-	100%	-
Mugwort pollen	Art v 1 (n = 23)	95.7%	1.000	100%	-
Ragweed pollen	Amb a 1 (n = 84)	98.8%	1.000	100%	-
Alternaria	Alt a 1 (n = 75)	100%	-	100%	-

The molecular marker allergens of birch pollen and *Alternaria*, Bet v 1 and Alt a 1, already showed 100% sensitivity in the overall analysis, which is why no statistical test could be performed as results were identical to the extract-based analysis¹⁶¹. The major molecular allergen of ash pollen, Fra e 1, showed a sensitivity of 98.1% using ALEX². Interestingly, Ole e 1, used as a substitute marker for ash pollen using CAP as no Fra e 1 is available on the CAP system, showed identical sensitivity¹⁶¹. The essential marker allergen of timothy grass pollen allergy, Phl p 1, showed a high sensitivity in both test systems and was statistically equal to extract-based diagnosis (97.2% CAP vs. 98.1% ALEX²); however, adding sIgE determination to Phl p 5 increased sensitivity of MAD to 100% in both test systems¹⁶¹. In addition, Amb a 1 showed good sensitivity; 97.2% of the patients were positive in both test platforms in the overall analysis¹⁶¹.

Only Art v 1 differed significantly from the extract-based analysis. Art v 1 had a higher overall sensitivity using CAP compared to ALEX² (96.2% vs. 91.5%) and Art v 1 using CAP performed statistically equally to the mugwort extract ($p = 0.125$), whereas Art v 1 using ALEX² performed statistically worse ($p = 0.004$)¹⁶¹. Including the mugwort extract analysis present on ALEX², sensitivity increased to 94.3%, which was still statistically inferior to the extract-based analysis using CAP ($p = 0.001$). In patients with sIgE to the extracts greater than 0.7 kU_A/l, however, Art v 1 performed statistically equally to extract-based diagnosis when determined using ALEX² as well ($p = 0.063$)¹⁶¹.

3.2.8. Specificity of molecular allergy diagnosis

Initially, the prevalence of “false-positive” molecular results of Amb a 1, Alt a 1, Art v 1, Bet v 1, Ole e 1, Phl p 1 and Phl p 5 were tested in 50 controls in each of the six mono-allergic patient populations (from the sensitivity analyses) in both test systems.

Out of 1,750 measurements per assay, which were expected to be negative, none were positive using CAP, and 15 were positive using ALEX², leading to “false-positivity” rates of 0.0% and 0.9%, respectively. By definition, this was not a specificity analysis because all these patients were pollen-allergic, although to different pollen sources; however, specificity would be 100% for CAP and 99.1% for ALEX².

Most of the “false-negative” molecular results using ALEX² had low sIgE levels; however, it was remarkable that the correlating allergens using CAP, although lower than the cut-off of 0.35 kU_A/l and therefore “negative”, reacted in all 15 sera as well. None of the patients reported symptoms, and extracts and prick tests to these allergens were negative; therefore, these positive results only constituted clinically irrelevant sensitizations, not allergic conditions; however,

these numbers indicate that ALEX² was more sensitive than CAP, although these patients showed low sIgE levels (Table 13).

Table 13: “False-negative” molecular results

Specific IgE levels (kU_A/l)

Category	ALEX ²		CAP	
Alternaria	Phl p 1	0.39	Phl p 1	0.17
		0.32		0.10
		0.31		0.17
	Phl p 5	1.47	Phl p 5	0.28
Ash	Phl p 1	0.53	Phl p 1	0.15
Birch	Fra e 1	1.45	Ole e 1	0.20
		0.69		0.07
		0.52		0.12
		0.48		0.11
	Phl p 1	0.52	Phl p 1	0.34
Timothy grass	Fra e 1	0.90	Ole e 1	0.16
		0.61		0.08
Ragweed	Fra e 1	0.96	Fra e 1	0.12
		0.87		0.14
	Alt a a1	0.72	Alt a 1	0.18

In addition, a true specificity analysis was performed in 106 sera from non-allergic subjects with negative prick tests to 13 aeroallergens (pollen, pets, mites and moulds) and negative sIgE to extracts of the six seasonal allergens investigated.

Alt a 1, Amb a 1, Art v 1, Bet v 1, Ole e 1, Phl p 1 and Phl p 5 were tested using ALEX² (7 x 106 = 742 determinations). None of these 106 subjects reacted to any of these seven molecular allergens, resulting in a 100% specificity for each of the seven molecular allergens investigated

161.

3.2.9. Correlation of the molecular test results

With Spearman's rho ranging between 0.790 and 0.940, determination of the six molecular allergens using CAP and ALEX² correlated strongly (Figure 10) ¹⁶¹. Correlations were slightly curved for most allergens, indicating that maximum values of ALEX² were lower; however, this observation that sIgE levels with ALEX² were capped did not result in lower mean levels because only a minority of patients showed very high sIgE levels. Interestingly, ALEX² even had a higher mean sIgE compared to the CAP for most allergens (Table 14).

Table 14: Mean sIgE levels of seasonal molecular marker allergens

Only mean sIgE levels of Amb a 1 were higher using CAP. For all other allergens, ALEX² had higher mean values ¹⁶¹.

Allergen		ALEX ²	CAP	p-value
Alt a 1	[kU _A /l]	21.3	15.3	< 0.001
Amb a 1	[kU _A /l]	10.9	22.4	< 0.001
Art v 1	[kU _A /l]	3.4	2.9	< 0.001
Bet v 1	[kU _A /l]	21.9	16.5	< 0.001
Fra e 1 / Ole e 1	[kU _A /l]	25.2	16.0	< 0.001
Phl p 1	[kU _A /l]	20.0	18.6	< 0.001
Phl p 5	[kU _A /l]	10.9	7.4	< 0.001

In addition, Figures 11 and 12 demonstrate direct comparisons of Alternaria and pollen of ash, birch, timothy grass, mugwort and ragweed between extracts (CAP) and the molecular allergens detected by CAP (left side) and ALEX² (right side). Interestingly, correlations on the left side of both figures (CAP extract vs. CAP molecular) were quite linear. In contrast, correlations on the right side (CAP extract vs. ALEX² molecular) were curved, indicating that ALEX² values were capped.

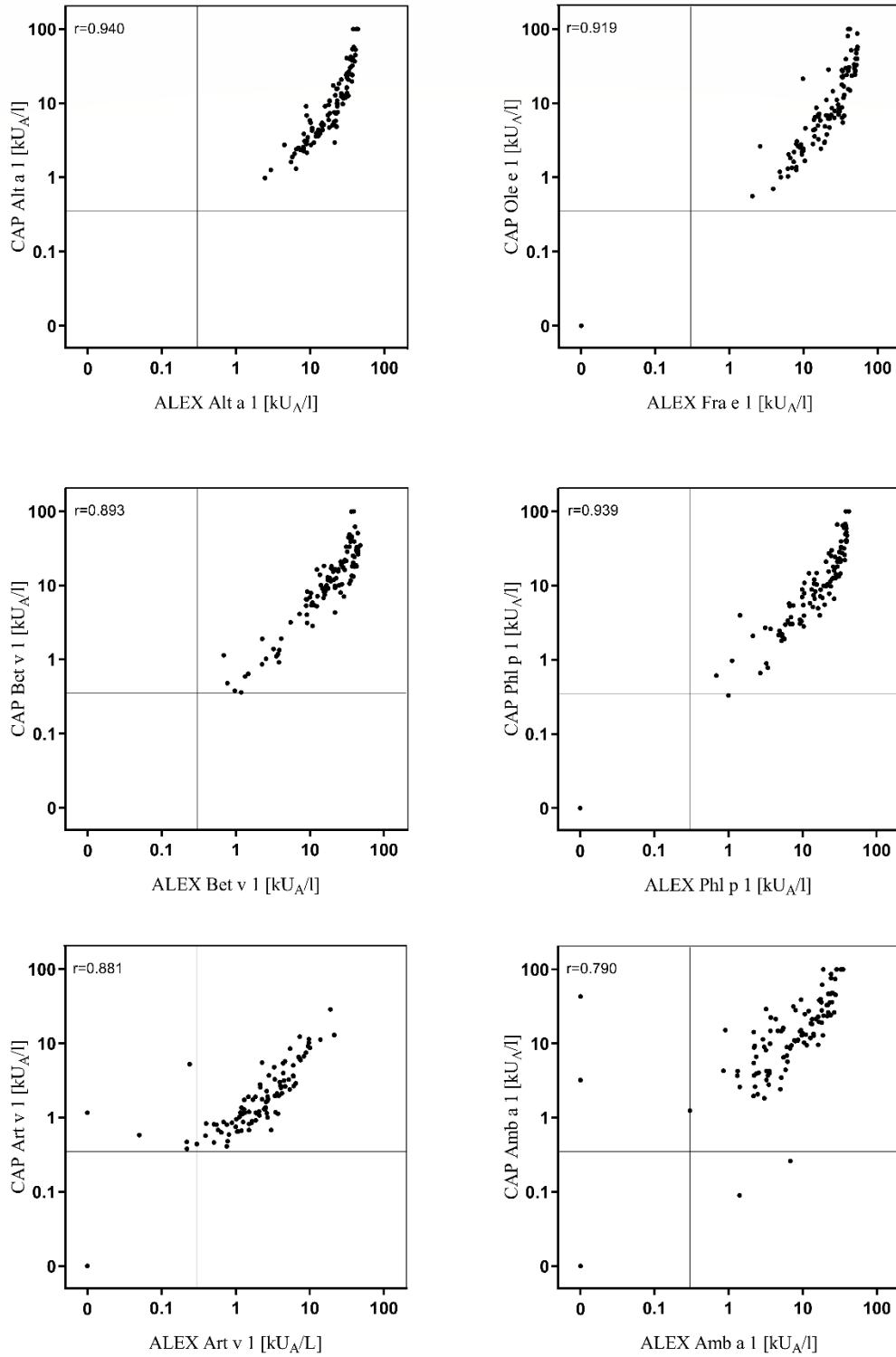
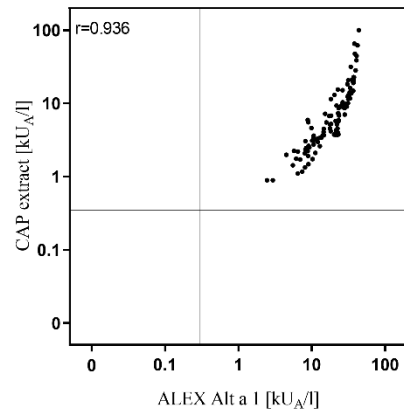
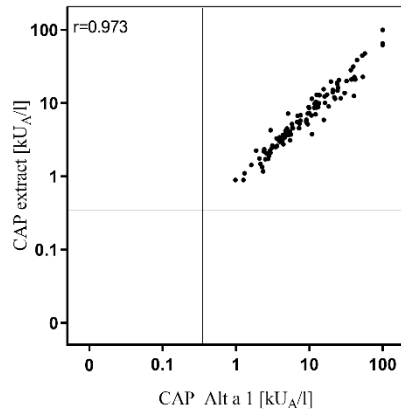


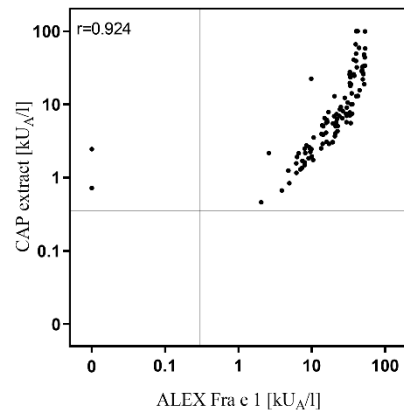
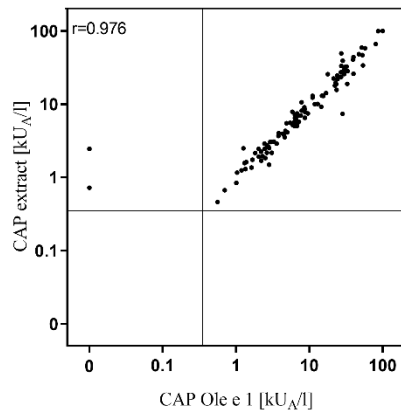
Figure 10: Correlation of the two molecular allergy test systems

Figure 10 shows scatter blots and Spearman's rhos of the molecular allergens Alt a 1, Fra e 1 / Ole e 1, Bet v 1, Phl p 1, Art v 1, Amb a 1 in comparison between ALEX² and CAP¹⁶¹.

Alternaria



Ash



Birch

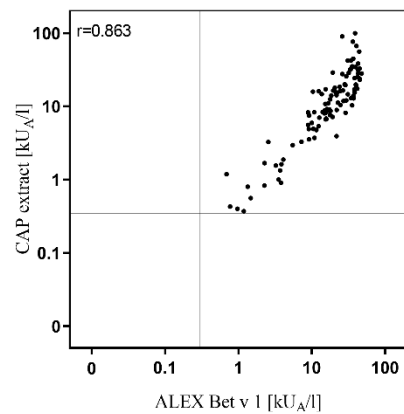
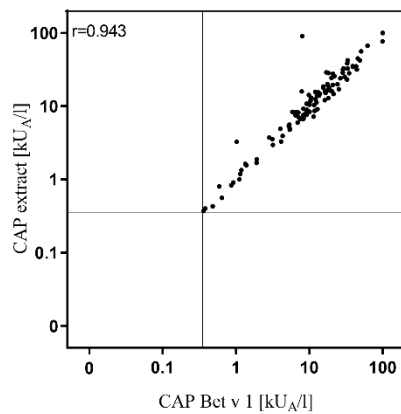
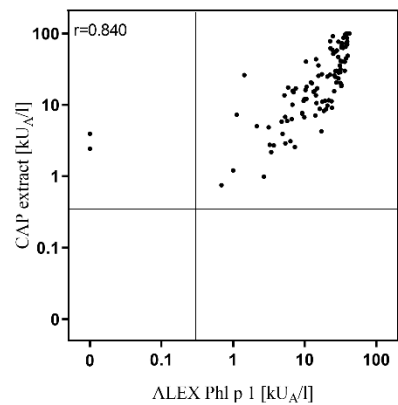
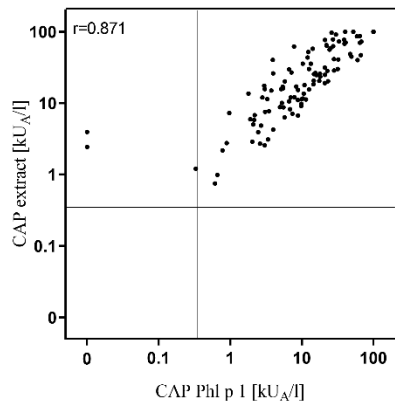


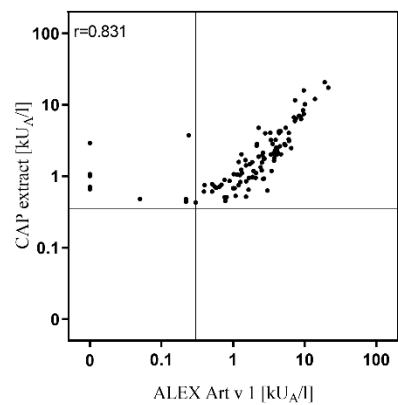
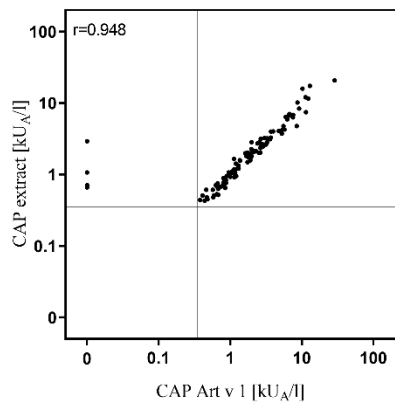
Figure 11: Correlation between seasonal extracts and molecular allergens I

Figure 11 shows scatter blots and Spearman's rhos between the extracts and molecular allergens of Alternaria (Alt a 1), ash (Fra e 1 / Ole e 1) and birch (Bet v 1) determined using CAP (left side) and ALEX² (right side).

Grasses



Mugwort



Ragweed

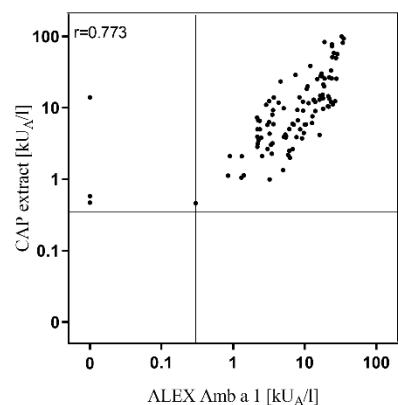
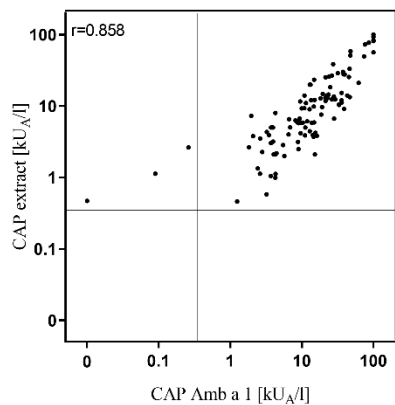


Figure 12: Correlation between seasonal extracts and molecular allergens II

Figure 12 shows scatter blots and Spearman's rhos between the extracts and molecular allergens of timothy grass (Phl p 1), mugwort (Art v 1) and ragweed (Amb a 1) determined using CAP (left side) and ALEX² (right side).

3.2.10. Sensitization patterns

Besides the molecular marker allergens, additional allergens (Alt a 6, Amb a 4; Art v 3; Bet v 2, 4 and 6; Phl p 2, 4, 6 and 11) were tested in the 636 patients used for sensitivity analyses.

Only Alt a 6 was available as an additional *Alternaria* allergen; however, the determination of Alt a 6 did not show any benefits compared to Alt a 1, as only 7/106 patients were positive compared to 106/106 patients positive to Alt a 1.

For the diagnosis of ragweed pollen allergy, besides Amb a 1, Amb a 4 was available but was only positive in 1/106 patients. No sIgE to Art v 1 was detectable in this patient (Amb a 4 and Art v 1 are both defensin-like proteins with potential cross-reactivity).

The determination of Art v 3 in the mugwort pollen-allergic patients did not reveal any benefits. Only 1/106 using CAP and 0/106 using ALEX² were positive to this allergen.

Additional allergens tested in the birch pollen-allergic patients were Bet v 2, 4 and 6 using CAP and Bet v 2 and 6 using ALEX². The profilin Bet v 2 was positive in 0/106 using CAP and 1/106 using ALEX²; the polcalcin Bet v 4 was negative in all 106 sera with both test methods. Both results are not surprising, as these were mono-allergic patients defined by prick and sIgE reactivity. Furthermore, Bet v 6 was only positive in 1/106 patients in both test systems; however, Bet v 1 levels in this patient were about threefold higher than the Bet v 6 results in both assays.

Ole e 9 was negative in all 106 ash pollen-allergic patients. On the other hand, Ole e 7 reacted in 14 patients using CAP and one patient using ALEX². The patient, who was positive in both methods, was one of the two patients negative to Ole e 1; therefore, one could assume a possible primary sensitization to olive tree pollen rather than ash tree pollen in this patient. It is of note that Ole e 7 is a putative non-specific lipid transfer protein – all other lipid transfer proteins available on ALEX², such as Art v 3 from mugwort pollen or Pru p 3 from peach, were negative in the respective patient.

A sensitization profile to timothy grass pollen allergens of 106 patients determined by CAP and ALEX, respectively, is depicted in Figure 13. Both test systems revealed similar results for Phl p 1, 2, 5, 6, 7 and 12. Interestingly, only Phl p 1 and Phl p 4 constituted major allergens by definition, as Phl p 5 sensitization was below 50% in both test systems. 94.3% were sensitized to Phl p 4 using CAP, an allergen not available on ALEX². The polcalcin Phl p 7 and the profilin Phl p 12 were negative in all patients in both test systems, which again is not surprising as these 106 patients were mono-allergic to timothy grass pollen.

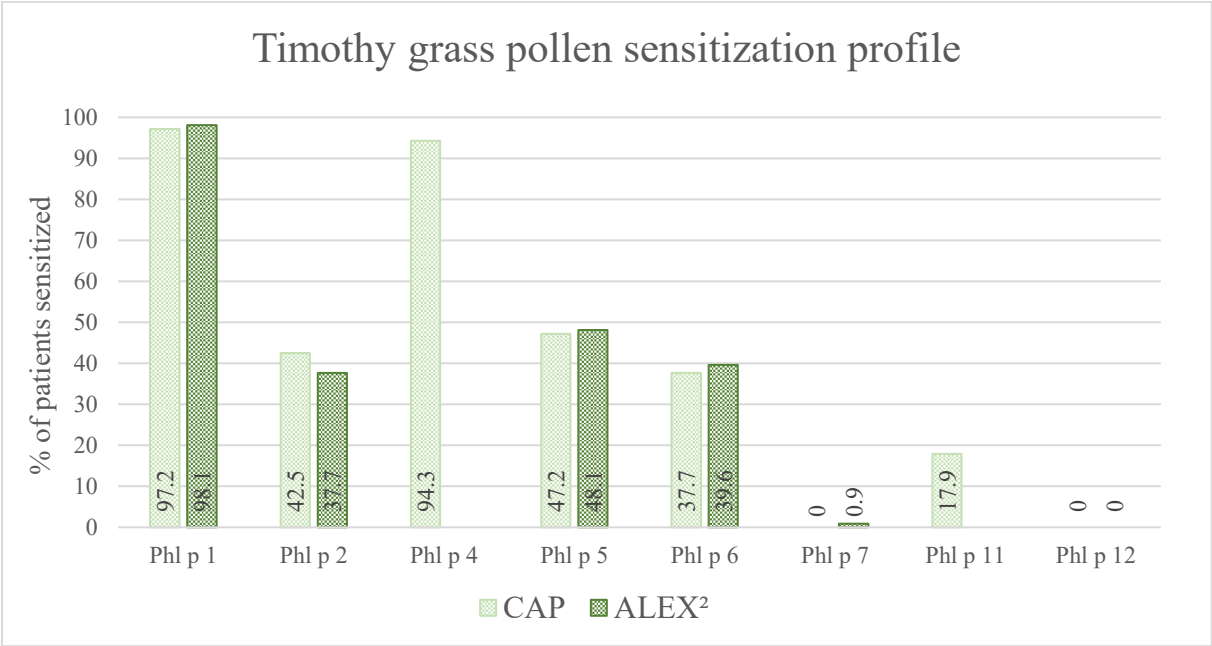


Figure 13: Sensitization to molecular timothy grass pollen allergens

By definition, only Phl p 1 and Phl p 4 constituted major allergens.

4. Discussion

4.1. House dust mite allergy

HDM allergy-induced rhinitis, conjunctivitis and asthma impact the quality of life directly during the day and dramatically reduce sleep quality with indirect effects during daytime due to chronic fatigue. The prevalence of symptoms reported by our patients was in agreement with previous studies ^{163,164}. No differences between females and males or adults and children were observed in our study population. Rhinitis was the leading symptom, with 89.3% affected. In addition, almost one-third of the patients also reported conjunctivitis (29.8%) and, more importantly, lower respiratory symptoms such as bronchitis or asthma. The high prevalence of respiratory symptoms (18.1% bronchitis, 14.0% asthma) observed is in line with a previously reported prevalence of 16.2% for asthma in atopic patients with rhinitis ¹⁶⁵. Similarly, the prevalence of respiratory symptoms was between 22% and 31% in our patients with seasonal allergies. For this reason, performing a medical history regarding lower respiratory symptoms (e.g. cough, dyspnoea, wheezing) is essential and lung function tests should routinely be performed if patients report these symptoms. We could observe that total IgE levels and sensitization rates to Der p 1 and 10 were higher in asthmatics than in non-asthmatics; however, these findings have no impact on daily patient care. Furthermore, HDM-related food allergy was rare in our study population, as only 0.9% of patients (cross-)reacted to shellfish. This rate is even lower than the reported 2.2% in a Bavarian HDM-allergic patient cohort in Germany ¹⁶⁶. Still, it might be partially caused due to an intended selection bias since the inclusion criterium in our study was a respiratory, not food allergy.

837 (28.4%) of our 2,948 patients referred to the outpatient clinic with rhinitis, conjunctivitis and/or bronchial asthma had sIgE reactivity to Der p extract, which is similar to the 31.3% reported in a large pan-European study with patients referred to allergology centres ¹¹⁹. Der p and Der f are the two most relevant HDM species in developed countries. Der f tends to predominate in areas with a prolonged period of dry weather ¹⁶⁷, but both species cohabit regularly ¹²⁰. All 215 HDM-allergic patients investigated showed sIgE reactivity to Der p extract as the main inclusion criterion. Surprisingly, although Der p is the predominant mite in our geographical area, 210 patients (97.7%) were also positive to the Der f extract (sIgE to Der f \geq cut-off of 0.35 kU_A/l). More precisely, all patients reacted to Der f, as the five Der f “negative” patients had sIgE values between 0.16 and 0.29 kU_A/l. Interestingly, determination

of Der f extract was statistically equal to Der p extract ($p = 0.063$) and more sensitive than any molecular IgE approach we have investigated.

This finding may be explained by a high cross-reactivity between the two mite species. Based on cDNA sequences, a homology of 81% between Der p 1 and Der f 1 and 88% between Der p 2 and Der f 2 has been shown^{168,169}. These percentages fit perfectly with a previous report that more than 70% sequence identity is usually required for cross-reactivity⁴⁶. In addition, the difference between 88% and 81% may explain why cross-reactivity between Der p 2 and Der f 2 was higher than between Der p 1 and Der f 1 in one previous study¹²².

Alternatively, besides cross-reactivity, it has been proposed that most Der p and Der f double-sensitized patients may be due to a “*bona fide* co-exposure and sensitization”¹²¹. Probably, it is a combination of both.

The sensitivity of single HDM prick tests with Der p or Der f was lower than *in-vitro* sIgE testing. Although 100% of the patients were positive to any of the two prick tests, only 94% and 87% were pricked positive to Der p and Der f, respectively. Using skin prick tests, we observed mono-sensitization to Der p in 13%, which is more than the previously reported 5.3%¹⁷⁰ and a considerably higher mono-sensitization rate compared to sIgE determinations. The reason for this difference remains unclear, but the potential explanations may be: (i) a selection bias as sIgE to Der p was the main inclusion criterion; (ii) a lower sensitivity of the more error-prone prick testing compared to the more accurate sIgE diagnosis and (iii) an eventual underrepresentation of some cross-reactive allergens in the prick extracts.

Der p 1 and 2 have been well known as major HDM allergens for a long time^{127,128}. Since their discovery, several studies have shown that most HDM allergic patients might be correctly diagnosed based on molecular allergology using these group 1 and 2 allergens. For example, it was reported that between 87.5 and 97% of HDM-allergic patients could be correctly diagnosed with Der p 1 and Der p 2^{122,129-132}. Nevertheless, these studies show a wide sensitivity range and have some limitations. Many of these studies have investigated a selected patient population and excluded patients with low sIgE levels to Der p extract; for example, only patients with sIgE to Der p extract ≥ 0.7 kU_A/l^{122,131} or ≥ 1.0 kU_A/l^{129,132} were included. In addition, one study selected patients solely based on case history and skin prick test without detailed information on extract sIgE levels¹³⁰.

Interestingly, older studies showed higher sensitivities with 95% (2004)¹³⁰, 97% (2008)¹³¹ and 94% (2012)¹²⁹, whereas more recent data showed lower sensitivities of 90% (2012)¹²² and 87.5% (2016)¹³².

The highest sensitivity with 97% was reported in a study that included only patients with sIgE to Der p extract ≥ 0.7 kU_A/l¹³¹. Additionally, in this study, the measurements of Der p 1 and Der p 2 were done using RAST-based dot blot assays with purified mite allergens rather than with commercially available molecular IgE assays¹³¹. Moreover, several studies, including ours, have reported mono-sensitization rates to Der p 23 of between 4.2% and 5.3%^{121,134,135,160}, which indicates that a sensitivity of 97% with Der p 1 and 2 alone cannot be expected with routine diagnostic methods.

In our patient population, 87.9% using ALEX² and 88.4% using CAP were sensitized to either Der p 1, Der p 2 or both¹⁶⁰. Our percentages are lower than most of the above numbers, which may be explained by different patient populations/selection criteria. We intentionally did not exclude patients with low levels of sIgE to Der p extract to reflect an unbiased random sample out of daily practice¹⁶⁰; therefore, we also included patients with sIgE between 0.35 and 0.70 kU_A/l. Overall, 13.5% of our study population had low (≤ 1.0 kU_A/l) sIgE levels to Der p¹⁶⁰, in contrast to almost all studies mentioned above.

Nevertheless, despite slightly varying sensitivities, all these studies, including ours, have two key messages in common: firstly, molecular test methods solely based on group 1 and 2 allergens are not able to correctly diagnose all HDM-allergic patients (between 3 and 12.5% remain undetected), and secondly, some other allergen groups are probably relevant for correct diagnosis of HDM allergy in these patients.

In 2013, a sensitization rate of 74% to Der p 23 was described, indicating Der p 23 as a new major allergen¹³³. Interestingly, using CAP, only 46.5% of our study population was sensitized to Der p 23. Nevertheless, using ALEX², 54.0% of our patients tested positive for Der p 23 and we could confirm Der p 23 as the third major allergen; however, it is irrelevant whether Der p 23 is a major allergen per definition or not, as it has no impact on the single allergic individual. The prevalence of Der p 23 mono-sensitization was more important because the additional determination of Der p 23, besides Der p 1 and 2, increased overall sensitivity.

Our observed mono-sensitization rate to Der p 23 of 4.7% was identical using ALEX² and CAP¹⁶⁰, and aligns with previously reported rates of between 4.2% and 5.3%^{121,134,135}. It is, therefore, essential to include Der p 23 in any molecular HDM allergy diagnostic workup. For example, ISAC had the lowest sensitivity in our study because Der p 23 was missing; however,

it has been added to the array in the current version which is why one can assume that ISAC is now more comparable to other tests on the market.

Adding Der p 23 to the diagnostic panel was still insufficient to diagnose all HDM-allergic patients, however, indicating that other allergens may be needed for a complete diagnosis. For example, Der p 10 and Der p 20 are other minor allergens that slightly increased molecular sensitivity. On the other hand, we detected no mono-sensitization to Der p 5, 7, 11 and 21; therefore, the diagnostic benefit of additional testing with those allergens was limited ¹⁶⁰.

The overall sensitivity of the MAD to diagnose HDM allergy was good, ranging from 88.8% to 94.9% in our analyses ¹⁶⁰; nevertheless, no commercially available molecular test could detect all HDM-allergic patients. For example, with the most comprehensive analysis method, ALEX², 11 out of 215 (5.1%) patients were negative for the nine molecular allergens investigated ¹⁶⁰. There are several explanations:

Firstly, although nine molecular allergens have been tested ¹⁶⁰, maybe more allergens are needed, as 32 Der p molecular allergens have been described in the WHO/IUIS Allergen Nomenclature Database up to now (retrieved from www.allergen.org, 22th of May, 2022). Nevertheless, it is questionable whether adding even more molecular allergens to a multiplex test panel is beneficial. On the one hand, even on multiplex systems, capacities are limited, and the benefit of adding allergens like Der p 20 with a 0.4% increase in sensitivity is little. Furthermore, the added value of exploring additional rare allergens seems questionable since immunotherapy vaccines available are standardized only to major allergens, most of them only to group 1 and 2 allergens. The situation appears to be similar to that with bee venom allergy. Bee venom-allergic patients usually show a broad sensitization pattern, and the available molecular allergen panel has a low overall sensitivity of 71.6% ¹⁷¹.

Secondly, one may speculate that some Der p molecular negative patients were not genuinely sensitized to Der p. Instead, they may have only shown cross-reactivity to Der p due to a primary sensitization to other house dust mites or storage mites. Possible candidates would be the house dust mites *Blomia tropicalis* and *Dermatophagoides microceras*, or storage mites such as *Acaris siro*, *Glycophagus domesticus*, *Lepidoglyphus destructor* or *Tyrophagus putrescentiae*. Initially, we thought this was not the case, as extracts of all these allergens (except *Dermatophagoides microceras*, which was not available) tested negative in all eleven Der p molecular negative patients using ALEX version 1; however, additional analyses with the CAP platform revealed that all eleven patients showed IgE reactivity to other mites. Nine out of

eleven Der p molecular negative patients even had (slightly) higher sIgE levels to other mites than Der p. These results raise the question of whether ALEX results were false-negative or CAP results false-positive.

In the literature, co-sensitization between house dust and storage mites was frequently reported as 85.4% of patients sensitized to storage mites were positive to Der p¹⁷², and 46% of patients sensitized to Der p were positive to storage mites¹⁷³. On the other hand, our overall analysis using ALEX version one only showed sIgE reactivity to one of the five mites mentioned above in 8.4%; therefore, the sensitivity of ALEX version 1 was considerably lower than previously reported, possibly indicating that ALEX results to other mites in the 11 Der p molecular negative patients were wrong and the CAP results were correct. Based on the sensitization profiles and sIgE levels shown in table 8, primary sensitization to other mites than Der p may have been possible. Likely candidates would be the house dust mites *Blomia tropicalis* and *Dermatophagoides microceras*, and the storage mites *Acaris siro*, *Lepidoglyphus destructor* or *Tyrophagus putrescentiae*.

The evidence about cross-reactivity between Der p and non-pyroglyphid mites is conflicting. Older articles indicate some degree of cross-reactivity based on inhibition studies¹⁷³⁻¹⁷⁸. On the other hand, other studies reported high cross-reactivity between storage mites but only limited cross-reactivity to Der p^{122,130,131,179-182}. These differences are the reason why it was hypothesized that simultaneous sensitization to house dust and storage mites is more likely to be induced by co-sensitization rather than cross-reactivity^{122,131}.

In general, it is difficult to differentiate between co-sensitization and cross-reactivity of house dust and storage mites. Due to the retrospective design and because no prick tests to storage mites nor any provocation tests were performed in our patients, we cannot clarify whether our eleven Der p molecular negative patients were genuinely sensitized to Der p or other mites. Extensive inhibition studies would be needed but were not feasible due to the limited serum available. In addition, it is unclear whether IgE-cross-reactivity between storage mites and Der p is clinically relevant.

Thirdly, technical issues may explain why not all Der p-allergic patients could be diagnosed with molecular allergology. It has been reported that compared to singleplex methods, the sensitivity of multiplex assays may be decreased in patients with low sIgE levels because of higher limits of detection, higher coefficients of variation and potential inhibition due to antigen-specific IgG³⁰. Our data clearly showed that the sensitivity of molecular multiplex arrays (but also of molecular singleplex analysis) was impaired in patients with low sIgE levels

¹⁶⁰. Nevertheless, under ideal conditions – in patients with sIgE levels to Der p extract greater than 3.5 kU_A/l – sensitivities of all molecular allergy test systems were very high, ranging from 97.9 to 99.3% ¹⁶⁰. Furthermore, the newest multiplex assay, ALEX², already performed statistically equally to extract-based diagnosis in patients with Der p sIgE values greater than 1.0 kU_A/l (sensitivity of 97.3%, p = 0.063) ¹⁶⁰.

Finally, a possible CCD-interaction can be excluded based on our data. We did not observe any reactivity to CCDs (MUXF3) in any of our 215 HDM-allergic patients, including the eleven Der p molecular negative patients. This observation is contrary to previous data, in which sIgE to MUXF3 was reported in 25% of the patients ¹²⁹. Nevertheless, the reported 25% reactivity to MUXF3 was probably independent of the HDM sensitization and reflected the general CCD-reactivity of the study population investigated. The authors stated, however, that "*CCD reactivity was unexpectedly prevalent, with an average of 25% MUXF3 IgE+ sera. However, serum levels of MUXF3 IgE were extremely low (0.12–3.31 kU_A/l, median 0.56 kU_A/l) and therefore unlikely to interfere with Dp IgE measurements*" and they also mentioned "*CCD reactivity, a confounding factor in mite IgE testing*" ¹²⁹, citing two other papers from Altmann et al. (2007) ⁵⁸ and Malandain et al. (2007) ¹⁸³. These two papers do not justify adding MUXF3 to the HDM diagnostic panel, though. In the first paper, mites are only mentioned once, stating that "*proteins from house dust mites, despite their phylogenetic relationship, are probably not core 3-fucosylated as they fail to bind rabbit anti-CCD antibodies*" ⁵⁸. The second paper investigated CCD reactivity to 52 extract and recombinant allergens using two sera with significant levels of CCD IgE reactivity. They "unexpectedly" found a slight influence of glycan-related reactivity in several non-plant, non-insect allergens, such as domestic mites, amongst others ¹⁸³. Nevertheless, this unexpected influence may be attributed retrospectively to CCD reactivity to the cellulose of the CAP system used in the study rather than a direct reaction with the mite extract. CCD-antibodies can induce false-positive CAP results due to interference with the cellulose of the CAP system, as mentioned above ⁶⁷.

There is no evidence that house dust mites contain MUXF3, a typical N-glycan found in plants, and adding MUXF3 to a diagnostic panel will not help detect more HDM-allergic patients.

The correlation between the molecular HDM test systems was strong in our study. The most significant difference was that correlations between CAP and ISAC were more linear than those between these two methods with ALEX, which were more curved in the scatter plots (see Figure 5 above). Technical reasons may cap IgE levels of ALEX; however, from a diagnostic and

therapeutic view, it is irrelevant whether the individual patient's sIgE levels to a respective allergen are 40 kU_A/l or 100 kU_A/l.

The most noticeable difference between ALEX versions 1 and 2 was that ALEX² was more sensitive to detect Der p 23 but less sensitive to determine sIgE to Der p 11.

Modern HDM immunotherapies are often standardized to group 1 and 2 allergens^{116,125,126}; however, our numbers indicate that only about one-third (34.4%) of all HDM-allergic patients are treated with standardized allergens to which they are solely sensitized. On the other hand, nearly two-thirds (65.6%) of HDM-allergic patients may receive underpowered immunotherapies not containing all allergens to which these patients are sensitized. It would be interesting if patients with broad sensitization patterns were less responsive to therapy. A recent study based on a post hoc analysis showed that patients sensitized to additional allergens besides Der p 1 and 2 may have a reduced benefit when treated with standardized HDM immunotherapies¹⁸⁴. In addition, another study showed that patients sensitized only to Der p 1 and/or Der p 2 benefited more from AIT than patients sensitized to additional allergens¹⁸⁵. Thus, stratification of patients with HDM allergy according to their molecular sensitization profiles may enhance the success of AIT¹⁸⁵.

On the other hand, data for grass pollen SLIT were different. For example, treatment was efficacious in one study irrespective of whether patients were sensitized to single or multiple grass pollen allergens¹⁸⁶; however, this study used a 5-grass-pollen-extract, not a vaccine standardized to single allergens.

Taken together, molecular HDM allergy diagnostics, using singleplex analysis and modern multiplex assays, showed high sensitivity, comparable to extract-based testing¹⁶⁰. Multiplex assays are an individualized diagnostic approach determining sensitization patterns of HDM-allergic patients at reasonable cost¹⁶⁰. Individual sensitization profiles based on molecular allergology can help to select patients for allergen immunotherapy. In addition, they are a step towards personalized medicine. In patients with low sIgE levels, however, there is a minimal residual risk of underdiagnosing HDM allergy¹⁶⁰. For this reason, it is advisable to perform molecular HDM allergy diagnosis combined with prick tests.

4.2. Seasonal allergy

More than half of the patients (56.3%) admitted to allergy diagnosis due to rhinoconjunctivitis and/or bronchial asthma were sensitized to (at least one of) the six seasonal allergens investigated ¹⁶¹. *Alternaria* only played a minor role (1.1% were mono-sensitized), whereas 55.2% were sensitized to pollen allergens ¹⁶¹. About one-third (34%) of these patients were sensitized to multiple pollen allergens ¹⁶¹; therefore, pollen allergy was prevalent in our patient population and a reliable diagnosis is indispensable to select the appropriate vaccine for immunotherapy. Determination of sIgE and prick testing, both using extracts, are the two most frequently performed diagnostic procedures; however, extract-based pollen allergy diagnosis, especially *in-vitro*, can be hampered by pan-sensitization-induced cross-reactivity.

Overall, the sIgE levels were low in up to 16.9% of the extract determinations, ranging between 0.35 and 0.70 kU_A/l (Figure 7) ¹⁶¹, with even higher rates of up to 30% for certain pollen allergens (Figure 7). A small proportion of these test results may present weak genuine sensitizations; however, many were solely based on cross-reactivity.

Furthermore, the correlation between sIgE and prick test results of 2,948 patients revealed that extract-positive/prick-negative results were about 4.5 times more likely for pollen than *Alternaria* (13.3% vs. 2.8%). Most of these “false-positive” pollen sIgE results were probably due to cross-reactivity by anti-CCD antibodies and only a minority due to false-negative prick tests. Cross-reacting sIgE to profilin and polcalcin could be excluded here because these antibodies usually induce positive prick test results ¹⁶¹.

In addition, in the 500 pollen-allergic patients investigated, 34.3% of the positive pollen extract sIgE could not be confirmed with molecular allergology using marker allergens ¹⁶¹. Consequently, extract-based pollen sIgE determinations are prone to error by cross-reactivity, leading to decreased diagnostic specificity. The main reason for cross-reactivity is sIgE to CCDs and the panallergens profilin and polcalcin.

Overall, about one tenth (10.2%) of all patients referred to allergy diagnosis and nearly one-fifth (18.5%) of the pollen-allergic patients revealed pan-sensitizations via CCDs or the panallergens profilin and polcalcin ¹⁶¹. As a result, these patients were at risk of being incorrectly diagnosed and prescribed the inaccurate immunotherapies ¹⁶¹. Considering the hundreds of millions affected by allergic rhinoconjunctivitis and asthma worldwide ⁸, these percentages translate into millions at risk.

In our pollen-sensitized patients, the sensitization rates were higher for profilin and CCDs (10.9% and 8.7%) than for polcalcin (2.9%)¹⁶¹ and were in line with previously published data^{59,65,71-73,85,88}; however, clinically relevant reactivity may be even higher than these rates. For example, for CCDs, we could show that “negative” CCD values below the cut-off of 0.35 kU_A/l can still elicit clinically relevant false-positive pollen extract sIgE results.

Interestingly, about 80% of the cross-reactive patients were only sensitized to one panallergen, and 20% were simultaneously sensitized to two of them, but none was sensitized to all three (CCDs, profilin and polcalcin). Overall sensitization to two panallergens was rare, however, as only 2.1% of our study population was sensitized to two panallergens (out of three, CCDs, profilin and polcalcin). This percentage is in line with a previous paper, which assumed that sensitization to both panallergens (profilin and polcalcin) in the same individual is approximately <2%¹⁸⁷.

The negative impact of CCDs and panallergens such as profilin and polcalcin on IgE diagnosis using extracts has been known for years. The dimension may have been underestimated, though, because blocking of sIgE to CCDs or determining molecular allergens was not regularly done. The main impediment to their widespread use in routine allergy diagnostics may have been the additional cost due to expensive reagents and more work expenditure.

There is a general consensus that IgE antibodies to CCDs are clinically irrelevant for pollen and insect venom allergy despite negatively influencing extract-based diagnostics by decreasing diagnostic specificity^{53,55,58,60,188}. Only three out of 600 patients reacted to CCDs without simultaneous sensitization to molecular pollen or plant food allergens in our study population. These patients were sIgE positive to wasp venom as a possible explanation for CCD reactivity. Although differences between CCDs in Hymenoptera (MMF3F6) and pollen (e.g. MUXF3) exist⁵⁸, it has been shown that CCD antibodies elicited by Hymenoptera venom can distort pollen IgE diagnosis using extracts¹⁸⁹, which our data confirms.

In contrast, profilins and polcalcins are true minor aeroallergens and clinically relevant at times, especially in some geographical areas or some allergens. In addition, although their role in triggering respiratory allergic symptoms is still controversial, there is growing evidence that some profilins can elicit respiratory symptoms^{75,81}.

In our patients, sensitizations to profilin and polcalcin were consistently associated with genuine pollen sensitization. None had primary sensitization to profilin or polcalcins. In

addition, affected patients were sensitized to at least one molecular pollen marker allergen and reported symptoms during the pollen season of the primary sensitizer(s). This finding indicates that sensitizations to profilin and polcalcin are usually not direct and genuine but bystander phenomena that arise later in the course of pollen allergy. This theory is in line with previous data from Germany showing that sensitization to profilin and polcalcin from grass pollen occurs late in the molecular spreading of grass pollen allergy ⁷⁶. In addition, a previous study with smaller cohorts reported that patients mono-sensitized to birch pollen did not show sensitizations to bromelain-type CCD or profilin ¹⁹⁰, which is identical to all our patients with mono-sensitization.

The most common mono-sensitizers inducing profilin sensitization were grass pollen (18.2%), ash pollen (3%) and birch pollen (3%). In addition, multiple true pollen sensitizations were detected in 75.8% of the patients sensitized to profilin. Overall, genuine sensitization to Amb a 1, Art v 1, Bet v 1, Fra e 1 or Phl p 1/5 could be found in 19.7%, 24.2%, 50.0%, 54.5% or 86.4% of the profilin- and polcalcin-sensitized patients, respectively. Interestingly, although the diagnosis of mugwort and ragweed pollen allergy was affected by cross-reactivity, their contribution to profilin or polcalcin sensitization was negligible. One could speculate that the environmental pollen load of mugwort and ragweed is low compared to other allergens, such as grass pollen; however, it is also possible that we may have missed patients with profilin and polcalcin sensitizations due to primary mugwort or ragweed allergy pollen because allergies to mugwort and ragweed pollen are rare compared to, for example, birch or grass pollen.

There are two options for dealing with the influence of cross-reactivity on extract-based pollen sIgE diagnosis: (i) The consequent determination of the different cross-reactive allergens and (ii) molecular allergy diagnostics to avoid or minimize their influence.

The first option, determining all cross-reactive allergens in every patient (CCDs, profilin, and polcalcin), is cost-intensive; still, fortunately, the risk of involvement can be anticipated indirectly by determining sIgE to a representative pollen extract panel in every patient. For Central Europe, we recommend the pollen of ash, birch, timothy grass, mugwort and ragweed. Poly-sensitization to pollen has to be expected in about one-third (34.0%) of patients referred to allergy diagnosis due to rhinoconjunctivitis and/or bronchial asthma and poly-sensitization to pollen extracts is a warning sign for cross-reactivity.

Our data indicate that 73% of the sera with five and 17% of the sera with four positive pollen results were influenced by cross-reactivity ¹⁶¹. Consequently, 50.7% and 40.7% of the pollen extract results in the 5-fold and 4-fold pollen positive group had negative corresponding

molecular marker allergens, implying that these pollen extract results were probably only positive due to cross-reactivity; therefore, multiple positive extract sIgE results have to be interpreted with caution. An exact correlation to the patient's history and prick tests is mandatory, and before prescription of immunotherapy, additional determination of sIgE to molecular allergens is still advisable. On the other hand, if these five pollen extracts were determined and only three or fewer were positive, the risk of cross-reactivity was marginal (5%) or negligible ($\leq 1\%$)¹⁶¹.

The second option is the use of molecular allergology. Depending on the platform used, MAD can strongly reduce or even completely avoid the negative influence of cross-reactivity on pollen allergy diagnostics.

Molecular allergology with single major marker allergens has been highly sensitive for diagnosing the six most common seasonal inhalant allergens in Central Europe, with sensitivities of between 96.2% and 100% using CAP and 91.5% and 100% using ALEX²¹⁶¹. Similarly to house dust mite allergy, however, single marker allergens may not be perfect for every allergen source. Our study group has reported previously that Phl p 1 is sufficient to diagnose timothy grass pollen allergy with a sensitivity of 98%¹¹⁵. Based on our current data, with sensitivities of 97.2% and 98.1% in the overall analysis using CAP and ALEX²¹⁶¹, we can confirm the results of our previous study; nevertheless, additional testing of Phl p 5 could further increase sensitivity¹⁶¹. In our current analysis, simultaneous determination of Phl p 1 and Phl p 5 was able to diagnose 100% of the mono-allergic timothy grass pollen patients correctly. In other geographical regions, though, additional allergens may also be relevant. For example, mono-sensitization to Phl p 2 or Phl p 4 has rarely been observed¹⁹¹.

For this reason, and because of the 94.3% positivity rate in our 106 timothy grass pollen-allergic patients using CAP, it is odd that Phl p 4 is not available on ALEX². Nevertheless, with 100% sensitivity in our cohort, the combination of Phl p 1 and 5 is a good compromise, at least for Central Europe. An additional benefit of this combination is its availability for single determinations with the CAP platform, where both molecular allergens can be determined for the cost of one analysis (allergen g213: rPhl p 1, rPhl p 5b).

The diagnosis of *Alternaria* (Alt a 1–100%), ash (Fra / Ole e 1–98.1%), birch (Bet v 1–100%) and ragweed pollen allergy (Amb a 1–97.2%) with single major marker allergens showed excellent sensitivity with both test systems (ALEX² and CAP) as well.

Interestingly, sIgE determination to Ole e 1 from olive pollen on the CAP platform served well as a surrogate marker for Fra e 1 of ash pollen, which is only available on ALEX², indicating

strong cross-reactivity between these two members of the Oleaceae family. Specific IgE determination to Ole e 1 and Fra e 1 showed 98.1% sensitivity in our analysis. On the other hand, two previous reports only showed positivity to Ole e 1 in 87.6%¹⁰⁸ and 88%¹⁰⁹. The latter publication additionally showed, in agreement with our data, that the determination of Ole e 7 and 9 does not increase the sensitivity¹⁰⁹.

Also, Alt a 6, Amb a 4, Bet v 6, Phl p 2, Phl 6 or Phl p 11 did not increase the sensitivity of diagnosis in the respective mono-allergic patients, indicating that it is sufficient to determine only molecular marker allergens.

The weakest allergen in our analysis was Art v 1 to diagnose mugwort pollen allergy. Overall sensitivity using CAP was 96.2% and better than 91.5% using ALEX². Determination with CAP performed statistically equal to extract-based diagnosis ($p = 0.004$), whereas determination with ALEX² did not ($p = 0.125$). Adding Art v 3 did not increase the sensitivity in both assays; the mugwort pollen extract on ALEX² did increase the test's overall sensitivity to 94.3%, though¹⁶¹. These slightly lower percentages compared to all other allergens investigated cannot be explained solely by technical problems of the ALEX², however, but rather by the selection of our mugwort patients¹⁶¹.

Genuine mono-sensitization to mugwort was rare in our patients, and we had to screen our database from 2013 to 2020 to include a sufficient number of patients. Because mono-sensitized patients were rarely seen, most had low levels of sIgE compared to the other allergens. For example, only 89 out of 106 patients (84.0%) had sIgE to mugwort pollen extract ≥ 0.7 kU_A/l, whereas with all other allergens, at least 102 out of 106 (96.2%) had extract levels ≥ 0.7 kU_A/l. Consequently, mean sIgE levels to Art v 1 were low – about three times lower than Amb a 1 or Phl p 5, and more than six times lower than the other marker allergens (Table 14).

Mugwort is not a rare allergen per se, and with reported prick test positivity rates of 21.9%, it is the third most common seasonal aeroallergen in Austria after grass and birch pollen¹¹; however, patients are rarely mono-sensitized to mugwort pollen, and many positive extract test results are due to cross-reactivity rather than genuine sensitization. Not only CCDs, profilin and polcalcin are relevant here. Mugwort and ragweed cross-react intensively because these two botanically related plants share multiple similar allergens¹⁹². The minor allergen of mugwort pollen, Art v 6, shares sequence homology with the major marker allergen Amb a 1 of ragweed, both being pectate lyases⁹⁵. Furthermore, the structure of the minor ragweed allergen Amb a 4 is similar to the major marker allergen Art v 1 of mugwort, both defensin-like proteins¹⁹³. In addition, both allergen sources have a lipid-transfer protein allergen (Art v 3 and Amb a 6)¹⁹⁴.

These shared allergens are the reason why many patients are double-sensitized to both extracts, mugwort and ragweed pollen, despite genuine sensitization to only one of both allergen sources. For example, in a study of 372 weed pollen-allergic patients, only 7% of the 147 mugwort pollen-sensitized patients were not sensitized to ragweed⁹⁴. Considering this percentage, it is not surprising why it was difficult for us to find 106 patients mono-sensitized mugwort for the sensitivity analysis.

It has been reported that sensitivity of multiplex systems may be decreased in patients with low sIgE levels because of higher limits of detection, higher coefficients of variation and a potential inhibition by antigen-specific IgG³⁰. As observed with house dust mite allergy, this is also valid for our patients allergic to mugwort pollen. The higher the sIgE to these allergen extracts, the better was the sensitivity of molecular allergy diagnosis.

For this reason, the slightly decreased sensitivity of Art v 1 compared to the other seasonal allergens can mostly be explained by a selection bias of our mugwort-allergic patients with low sIgE levels to mugwort extracts. In patients with sIgE levels greater than 0.7 kU_A/l, however, Art v 1 showed statistically comparable results to extract-based diagnosis in both test systems¹⁶¹.

Nevertheless, our data also demonstrate that it is not a general characteristic of multiplex systems to have higher detection limits than singleplex assays. In the molecular “false-positivity” analysis, 1,750 molecular values of patients with negative prick tests and sIgE to extracts were expected to be negative; however, ALEX² showed 15 slightly elevated molecular values (sIgE between 0.31 and 1.47 kU_A/l). All of the corresponding determinations using CAP were below the cut-off (molecular IgE levels between 0.07 and 0.34 kU_A/l), but none was truly negative. These sensitizations were clinically silent at the time of blood sampling; however, the high correlation between both test systems indicates that (at least some of) these results were due to genuine sensitizations, and ALEX² was able to detect them earlier.

Besides being sensitive, molecular allergy diagnostics using single major allergens was also highly specific to diagnosing the six most common seasonal inhalant allergens (*Alternaria*, ash, birch, timothy grass, mugwort and ragweed) in Central Europe.

Our molecular “false-positivity” analysis revealed the first impression on specificity. CAP and ALEX showed a “false-positivity rate” of 0 and 0.9% here; however, these percentages cannot be translated into a “specificity” of 100% and 99.1% because the sera investigated belonged to actual pollen-allergic patients, although to other pollen and, as mentioned above, we assume that these “false-positive” results were not false-positive but probably true sensitizations.

The specificity analysis in 106 non-allergic subjects showed very similar results, however. Our data revealed 100% specificity for Amb a 1, Alt a 1, Art v 1, Bet v 1, Fra/Ole e 1, Phl p 1 and Phl p 5 using ALEX². In general, specificity analyses were rarely presented in the literature; however, for example, 100% specificity has been reported for Bet v 1, which is in line with our results ¹⁰⁴.

The molecular correlations between the multiplex assay ALEX² and the singleplex assay CAP were strong, with Spearman's rho ranging between 0.790 and 0.940 (Figure 10). As shown above, for HDM diagnosis as well, sIgE values of ALEX² were again limited; however, this had no impact on routine diagnostic care, and mean sIgE levels were still higher for most allergens using ALEX². Furthermore, besides Art v 1 in patients with very low mugwort pollen extract levels, both systems using molecular allergens produced almost similar results.

Nevertheless, both test systems have potential disadvantages. Cost is probably the most significant barrier to the broad use of molecular allergy diagnosis. Using CAP, molecular analyses are more expensive than extract determinations, and performing extensive molecular profiles is more cost-intensive by means of CAP than with multiplex chips such as ALEX or ISAC. An additional consideration is potential false-positive test results up to 2 kU_A/l even with nonglycosylated recombinant allergens in patients with high levels of sIgE to CCDs due to varying amounts of CCDs in the cellulose used as a solid-phase allergen carrier in CAP ⁶⁷.

CCDs are no longer an issue utilizing ALEX², as a CCD block is performed routinely before every analysis; however, one must consider a slightly decreased sensitivity of ALEX² in patients with very low extract levels, at least for some allergens such as *Dermatophagoides pteronyssinus* or mugwort pollen. Furthermore, although ALEX² determines a comprehensive allergen panel, both extract-based and molecular, with 295 allergens from 165 sources with one test run at reasonable cost, ALEX² is more expensive than some single CAP analyses. It is a matter for discussion whether every patient needs a comprehensive laboratory workup – and a thorough workup in every patient with allergy symptoms could eventually be limited not only by cost but also by physicians' capacities to interpret all these gathered results; however, a broader application of molecular pollen allergy diagnosis could improve the specificity and quality of allergy diagnosis and, consequently, the effectiveness of allergen immunotherapy ¹⁶¹.

5. Conclusion

Most HDM-allergic patients can be diagnosed correctly using a few molecular marker allergens. Besides Der p 1 and 2, we could confirm that Der p 23 is a major allergen, and with mono-sensitization rates of 4.7%, Der p 23 has to be part of any molecular work-up. Although the most modern multiplex assay, ALEX², had slightly better sensitivity, all three commercially available platforms are suitable for molecular HDM allergy diagnosis; however, a routine determination of three molecular allergens (Der p 1, 2, 23) to screen for one allergen source may be cost-intensive, especially when performing singleplex analyses. Moreover, no cross-reactivity exists between HDM and MUXF3, profilin or polcalcins. Molecular allergology is more precise but not necessarily better for diagnosing HDM allergy; thus, it is also suitable to screen with HDM extracts. Der p and Der f extracts showed high cross-reactivity and performed statistically equal; therefore, the determination of sIgE to one of these extracts is sufficient; however, we recommend performing additional MAD before prescribing HDM immunotherapy.

Pollen allergy is prevalent in Austria. 55.2% of all patients referred to allergy diagnosis were sensitized to pollen, and 34% reacted to multiple pollen species. In addition, sensitization to CCDs and panallergens such as profilin or polcalcin was common in patients allergic to pollen, especially in patients reacting to multiple pollen allergens. Cross-reactivity induced by these sensitizations frequently confounded extract-based diagnosis by decreasing diagnostic specificity. Consequently, about one tenth (10.2%) of all patients referred to allergy diagnosis, and nearly one-fifth (18.5%) of pollen-allergic patients were at potential risk of misdiagnosis. Misdiagnosis may lead to the prescription of immunotherapies with the wrong primary allergen sources, resulting in ineffective therapies with potential side effects.

We recommend two options for avoiding misdiagnosis: determining a routine pollen extract panel in every patient (in Central Europe: pollen extracts of ash, birch, timothy grass, mugwort and ragweed) or performing molecular pollen allergy diagnostics. In option one, if these five pollens are determined, and four or five are positive, the high risk of cross-reactivity must be considered; however, if only three or fewer are positive, it is negligible. Option two, performing molecular allergy diagnostics, avoids the influence of cross-reactivity via profilin and polcalcin, and – when performed using ALEX² – also of anti-CCD antibodies.

Both molecular test systems investigated, CAP and ALEX², showed reliable results to diagnose pollen allergy. These commercially available molecular diagnostic methods are highly sensitive and specific, prevent misdiagnosis and help to select primary allergen sources for

immunotherapy ¹⁶¹. Molecular allergy diagnosis of *Alternaria* is reliable as well; however, determination of sIgE to *Alternaria* extract is also suitable for diagnosis because no cross-reactivity exists via CCDs, profilin or polcalcin.

6. Implication for practice

Our data imply that the determination of sIgE to pollen extracts is prone to error due to false-positive results elicited by cross-reactivity; therefore, molecular marker allergens should preferably be used if sIgE to only individual pollen allergens are determined. In addition, several sIgE profiles could be used to minimize the risk of cross-reactivity (Table 15). On the other hand, *Alternaria* and house dust mite extracts are not influenced by cross-reactivity due to antibodies against CCDs, profilin and polcalcin and are suitable for screening purposes; however, we recommend determination of molecular allergens before the prescription of house dust mite immunotherapy.

Table 15. Different sIgE profiles recommended for routine use

1 Extracts only	2 Extracts+Indicators	3 Hybrid	4 Molecular only
Ash extract	Ash extract	Ole e 1	Ole e 1
Birch extract	Birch extract	Bet v 1	Bet v 1
Timothy grass extract	Timothy grass extract	Phl p 1 + 5	Phl p 1 + 5
Mugwort extract	Mugwort extract	Art v 1	Art v 1
Ragweed extract	Ragweed extract	Amb a 1	Amb a 1
<i>Alternaria</i> extract	<i>Alternaria</i> extract	<i>Alternaria</i> extract	Alt a 1
Der p extract	Der p extract	Der p extract	Der p 1
	MUXF3		Der p 2
	Phl p 12		Der p 23
	Phl p 7		(MUXF3)

Profile 1 is the most cost-effective option and should always be combined with prick testing. Importantly, all five pollen extracts should always be determined to indicate cross-reactivity. If three or more of the five pollen extracts are positive, cross-reactivity must be considered. In this case, if numerous prick tests are positive, profilin or polcalcin sensitization is probably the reason; if the corresponding prick tests are negative, CCD involvement is likely.

Profile 2 is similar to profile 1, with the difference that CCDs, profilin and polcalcins are always determined. Using this method, the interpreting physician knows directly whether cross-reactivity is present. For this reason, it is not strictly necessary to determine all five pollen

allergens in every patient; however, identifying the genuine sensitizations using extracts in the presence of cross-reactivity may still be difficult and need further molecular workup.

Profile 3 is not influenced by profilins or polcalcins and indicates genuine sensitizations if sIgE results are positive; however, multiple-positive results may point to possible CCD involvement via interference with the cellulose matrix if this profile is performed using CAP. Nevertheless, correlation with prick tests helps in this case: if all or many corresponding prick tests are negative, CCD involvement is likely.

Profile 4 is the most sophisticated approach, again not influenced by profilins or polcalcins. Still, if performed using CAP, correlation with prick tests is necessary in case of multiple positive results to exclude CCD involvement; however, this panel determined with the multiplex assay ALEX² (CCD inhibition integrated) detects true sensitizations only.

All four profiles, the cost-effective profile 1, the intermediate-cost profiles 2 and 3, and the sophisticated profile 4, are suitable for routine care, as long as the interpreting physician considers their potential disadvantages.

7. Limitations

The most significant limitation of this study may be a selection bias due to the retrospective study design. Statistical planning revealed that 106 patients per allergen were necessary to determine the sensitivity of the molecular seasonal allergens with certainty. The data collection period in a prospective single-centre study would have been approximately eight years because mono-allergic patients to ash, *Alternaria*, ragweed and especially mugwort are rare; therefore, we chose to conduct this study in a retrospective design.

8. Outlook and suggestions for further work

Our data confirm that molecular allergy diagnosis of the investigated allergens using commercially available methods is sensitive and specific; therefore, physicians can rely on this technique.

Further investigations of molecular allergy therapies are the next essential step. Firstly, additional data should be collected if patients with specific molecular allergen profiles treated with immunotherapy have different outcomes. For example, two recent studies showed that patients sensitized to additional allergens besides Der p 1 and 2 may receive a limited benefit when treated with standardized HDM immunotherapies^{184,185}. These observations need to be confirmed by further studies. If the difference can be established also for other allergens, it should be evaluated whether patients profit from individualized therapies. If so, precision diagnostic followed by precision therapy may be the future of allergy care.

Bibliography

1. von Pirquet C. Allergie. Münch Med Wochenschr. 1906;53:1457–1458.
2. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature*. 2008;454(7203):445-54.
3. Coombs RRA, Gell PGH. The classification of allergic reactions underlying disease. In: Coombs RRA and Gell PGH, eds. *Clinical Aspects of Immunology* Philadelphia: Davis. 1963;317.
4. Dispenza MC. Classification of hypersensitivity reactions. *Allergy Asthma Proc*. 2019;40(6):470-3.
5. Abbas M, Moussa M, Akel H, . Type I Hypersensitivity Reaction (Updated 2021 Jul 21). In: StatPearls [Internet]. StatPearls Publishing: Treasure Island, USA. 2021: <https://www.ncbi.nlm.nih.gov/books/NBK560561/> accessed 22.05.2022.
6. Bax HJ, Keeble AH, Gould HJ. Cytokinergic IgE Action in Mast Cell Activation. *Front Immunol*. 2012;3:229.
7. Crivellato E, Travan L, Ribatti D. Mast cells and basophils: a potential link in promoting angiogenesis during allergic inflammation. *Int Arch Allergy Immunol*. 2010;151(2):89-97.
8. Pawankar R, Canonica G, Holgate S, Lockey R, Bleiss M. The WAO White Book on Allergy (Update 2013). Wisconsin, USA. 2013: <https://www.worldallergy.org/UserFiles/file/WhiteBook2-2013-v8.pdf> accessed 22.05.2022.
9. Österreichischer Gesundheitsbericht 2016. Statistik Austria, Austria. 2017: <https://jasmin.goeg.at/328/1/gesundheitsbericht2016.pdf> accessed 22.05.2022.
10. Lake IR, Jones NR, Agnew M, Goodess CM, Giorgi F, Hamaoui-Laguel L, et al. Climate Change and Future Pollen Allergy in Europe. *Environ Health Perspect*. 2017;125(3):385-91.

11. Hemmer W. Hausstaubmilben, Tiere und Co. *J Pneumolog* 2016;4:9-13.
12. Stemeseder T, Metz-Favre C, de Blay F, Pauli G, Gadermaier G. Do *Plantago lanceolata* Skin Prick Test-Positive Patients Display IgE to Genuine Plantain Pollen Allergens? Investigation of Pollen Allergic Patients from the North-East of France. *Int Arch Allergy Immunol.* 2018;177(2):97-106.
13. Hemmer W, Sestak-Greinecker G, Braunsteiner T, Wantke F, Wöhrl S. Molecular sensitization patterns in animal allergy: Relationship with clinical relevance and pet ownership. *Allergy.* 2021;76(12):3687-96.
14. Bousquet J, Heinzerling L, Bachert C, Papadopoulos NG, Bousquet PJ, Burney PG, et al. Practical guide to skin prick tests in allergy to aeroallergens. *Allergy.* 2012;67(1):18-24.
15. Ahlstedt S, Murray CS. In vitro diagnosis of allergy: how to interpret IgE antibody results in clinical practice. *Prim Care Respir J.* 2006;15(4):228-36.
16. Ménardo JL, Bousquet J, Rodière M, Astruc J, Michel FB. Skin test reactivity in infancy. *J Allergy Clin Immunol.* 1985;75(6):646-51.
17. Casset A, Mari A, Purohit A, Resch Y, Weghofer M, Ferrara R, et al. Varying allergen composition and content affects the in vivo allergenic activity of commercial *Dermatophagoides pteronyssinus* extracts. *Int Arch Allergy Immunol.* 2012;159(3):253-62.
18. Zuidmeer-Jongejan L, Fernández-Rivas M, Winter MG, Akkerdaas JH, Summers C, Lebens A, et al. Oil body-associated hazelnut allergens including oleosins are underrepresented in diagnostic extracts but associated with severe symptoms. *Clin Transl Allergy.* 2014;4(1):4.
19. Ortolani C, Ispano M, Pastorello EA, Ansaloni R, Magri GC. Comparison of results of skin prick tests (with fresh foods and commercial food extracts) and RAST in 100 patients with oral allergy syndrome. *J Allergy Clin Immunol.* 1989;83(3):683-90.

20. Rosen JP, Selcow JE, Mendelson LM, Grodofsky MP, Factor JM, Sampson HA. Skin testing with natural foods in patients suspected of having food allergies: is it a necessity? *J Allergy Clin Immunol.* 1994;93(6):1068-70.
21. Rancé F, Juchet A, Brémont F, Dutau G. Correlations between skin prick tests using commercial extracts and fresh foods, specific IgE, and food challenges. *Allergy.* 1997;52(10):1031-5.
22. Rusznak C, Davies RJ. ABC of allergies. Diagnosing allergy. *BMJ.* 1998;316(7132):686-9.
23. Kjaer HF, Mortz CG, Bindslev-Jensen C. Does treatment with antidepressants, antipsychotics, or benzodiazepines hamper allergy skin testing? *Clin Transl Allergy.* 2021;11(7):e12060.
24. Indrajana T, Spieksma FT, Voorhorst R. Comparative study of the intracutaneous, scratch and prick tests in allergy. *Ann Allergy.* 1971;29(12):639-50.
25. Oppenheimer J, Nelson HS. Skin testing. *Ann Allergy Asthma Immunol.* 2006;96(2):S6-12.
26. Brusasco V, Crimi E, Crimi P, Valenti S. Comparison between intracutaneous and prick tests in detecting sensitizations to common Mediterranean allergens. *Respiration.* 1980;40(6):317-22.
27. Nelson HS, Oppenheimer J, Buchmeier A, Kordash TR, Freshwater LL. An assessment of the role of intradermal skin testing in the diagnosis of clinically relevant allergy to timothy grass. *J Allergy Clin Immunol.* 1996;97(6):1193-201.
28. Wood RA, Phipatanakul W, Hamilton RG, Eggleston PA. A comparison of skin prick tests, intradermal skin tests, and RASTs in the diagnosis of cat allergy. *J Allergy Clin Immunol.* 1999;103(5):773-9.

29. Kleine-Tebbe J, Poulsen LK, Hamilton RG. Quality management in IgE-based allergy diagnostics. *J Lab Med.* 2016;40(2):81-96.
30. Jakob T, Forstenlechner P, Matricardi P, Kleine-Tebbe J. Molecular allergy diagnostics using multiplex assays: methodological and practical considerations for use in research and clinical routine: Part 21 of the Series Molecular Allergology. *Allergo journal international.* 2015;24:320-32.
31. Droste JH, Kerhof M, de Monchy JG, Schouten JP, Rijcken B. Association of skin test reactivity, specific IgE, total IgE, and eosinophils with nasal symptoms in a community-based population study. The Dutch ECRHS Group. *J Allergy Clin Immunol.* 1996;97(4):922-32.
32. Cho JH, Suh JD, Kim JK, Hong SC, Park IH, Lee HM. Correlation between skin-prick testing, individual specific IgE tests, and a multiallergen IgE assay for allergy detection in patients with chronic rhinitis. *Am J Rhinol Allergy.* 2014;28(5):388-91.
33. Sturm GJ, Böhm E, Trummer M, Weiglhofer I, Heinemann A, Aberer W. The CD63 basophil activation test in Hymenoptera venom allergy: a prospective study. *Allergy.* 2004;59(10):1110-7.
34. Sturm GJ, Kranzelbinder B, Sturm EM, Heinemann A, Groselj-Strele A, Aberer W. The basophil activation test in the diagnosis of allergy: technical issues and critical factors. *Allergy.* 2009;64(9):1319-26.
35. Bokanovic D, Arzt-Gradwohl L, Schwarz I, Schrautzer C, Laipold K, Aberer W, et al. Possible utility of basophil activation test in dual honeybee and vespid sensitization. *J Allergy Clin Immunol Pract.* 2020;8(1):392-4.e5.
36. Santos AF, Alpan O, Hoffmann HJ. Basophil activation test: Mechanisms and considerations for use in clinical trials and clinical practice. *Allergy.* 2021;76(8):2420-32.

37. Marsh DG, Goodfriend L, King TP, Lowenstein H, Platts-Mills TA. Allergen nomenclature. *Bull World Health Organ.* 1986;64(5):767-74.
38. King TP, Hoffman D, Lowenstein H, Marsh DG, Platts-Mills TA, Thomas W. Allergen nomenclature. *Allergy.* 1995;50(9):765-74.
39. Radauer C, Nandy A, Ferreira F, Goodman RE, Larsen JN, Lidholm J, et al. Update of the WHO/IUIS Allergen Nomenclature Database based on analysis of allergen sequences. *Allergy.* 2014;69(4):413-9.
40. Macek B, Forchhammer K, Hardouin J, Weber-Ban E, Grangeasse C, Mijakovic I. Protein post-translational modifications in bacteria. *Nat Rev Microbiol.* 2019;17(11):651-64.
41. Lowenstein H. Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. *Prog Allergy.* 1978;25:1-62.
42. Alvarado MI, Jimeno L, De La Torre F, Boissy P, Rivas B, Lázaro MJ, et al. Profilin as a severe food allergen in allergic patients overexposed to grass pollen. *Allergy.* 2014;69(12):1610-6.
43. Rodriguez-Perez R, Crespo JF, Rodríguez J, Salcedo G. Profilin is a relevant melon allergen susceptible to pepsin digestion in patients with oral allergy syndrome. *J Allergy Clin Immunol.* 2003;111(3):634-9.
44. Caraballo L, Valenta R, Acevedo N, Zakzuk J. Are the Terms Major and Minor Allergens Useful for Precision Allergology? *Front Immunol.* 2021;12:651500.
45. Radauer C, Breiteneder H. Pollen allergens are restricted to few protein families and show distinct patterns of species distribution. *J Allergy Clin Immunol.* 2006;117(1):141-7.
46. Aalberse RC. Structural biology of allergens. *J Allergy Clin Immunol.* 2000;106(2):228-38.

47. Fötisch K, Vieths S. N- and O-linked oligosaccharides of allergenic glycoproteins. *Glycoconj J*. 2001;18(5):373-90.
48. Wilson IB. Glycosylation of proteins in plants and invertebrates. *Curr Opin Struct Biol*. 2002;12(5):569-77.
49. Aalberse RC, Koshte V, Clemens JG. Immunoglobulin E antibodies that crossreact with vegetable foods, pollen, and Hymenoptera venom. *J Allergy Clin Immunol*. 1981;68(5):356-64.
50. Wilson IB, Harthill JE, Mullin NP, Ashford DA, Altmann F. Core alpha1,3-fucose is a key part of the epitope recognized by antibodies reacting against plant N-linked oligosaccharides and is present in a wide variety of plant extracts. *Glycobiology*. 1998;8(7):651-61.
51. Iacovacci P, Afferni C, Butteroni C, Pironi L, Puggioni EM, Orlandi A, et al. Comparison between the native glycosylated and the recombinant Cup a1 allergen: role of carbohydrates in the histamine release from basophils. *Clin Exp Allergy*. 2002;32(11):1620-7.
52. Foetisch K, Westphal S, Lauer I, Retzek M, Altmann F, Kolarich D, et al. Biological activity of IgE specific for cross-reactive carbohydrate determinants. *J Allergy Clin Immunol*. 2003;111(4):889-96.
53. van der Veen MJ, van Ree R, Aalberse RC, Akkerdaas J, Koppelman SJ, Jansen HM, et al. Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. *J Allergy Clin Immunol*. 1997;100(3):327-34.
54. van Ree R, Aalberse RC. Specific IgE without clinical allergy. *J Allergy Clin Immunol*. 1999;103(6):1000-1.
55. Mari A, Iacovacci P, Afferni C, Barletta B, Tinghino R, Di Felice G, et al. Specific IgE to cross-reactive carbohydrate determinants strongly affect the in vitro diagnosis of allergic diseases. *J Allergy Clin Immunol*. 1999;103(6):1005-11.

56. Aalberse RC, van Ree R. Crossreactive carbohydrate determinants. *Clin Rev Allergy Immunol.* 1997;15(4):375-87.
57. Aalberse RC. Clinical relevance of carbohydrate allergen epitopes. *Allergy.* 1998;53(45):S54-7.
58. Altmann F. The role of protein glycosylation in allergy. *Int Arch Allergy Immunol.* 2007;142(2):99-115.
59. Mari A. IgE to cross-reactive carbohydrate determinants: analysis of the distribution and appraisal of the in vivo and in vitro reactivity. *Int Arch Allergy Immunol.* 2002;129(4):286-95.
60. Mari A, Ooievaar-de Heer P, Scala E, Giani M, Pirrotta L, Zuidmeer L, et al. Evaluation by double-blind placebo-controlled oral challenge of the clinical relevance of IgE antibodies against plant glycans. *Allergy.* 2008;63(7):891-6.
61. Jin C, Hantusch B, Hemmer W, Stadlmann J, Altmann F. Affinity of IgE and IgG against cross-reactive carbohydrate determinants on plant and insect glycoproteins. *J Allergy Clin Immunol.* 2008;121(1):185-90.
62. Chung CH, Mirakhur B, Chan E, Le QT, Berlin J, Morse M, et al. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. *N Engl J Med.* 2008;358(11):1109-17.
63. Jappe U. Update on meat allergy. α -Gal: a new epitope, a new entity? *Hautarzt.* 2012;63(4):299-306.
64. Yokoi H, Yoshitake H, Matsumoto Y, Kawada M, Takato Y, Shinagawa K, et al. Involvement of cross-reactive carbohydrate determinants-specific IgE in pollen allergy testing. *Asia Pac Allergy.* 2017;7(1):29-36.

65. Holzweber F, Svehla E, Fellner W, Dalik T, Stubler S, Hemmer W, et al. Inhibition of IgE binding to cross-reactive carbohydrate determinants enhances diagnostic selectivity. *Allergy*. 2013;68(10):1269-77.
66. Vidal C, Sanmartín C, Armisén M, Rodríguez V, Linneberg A, Gonzalez-Quintela A. Minor interference of cross-reactive carbohydrates with the diagnosis of respiratory allergy in standard clinical conditions. *Int Arch Allergy Immunol*. 2012;157(2):176-85.
67. Hemmer W, Altmann F, Holzweber F, Gruber C, Wantke F, Wöhrl S. ImmunoCAP cellulose displays cross-reactive carbohydrate determinant (CCD) epitopes and can cause false-positive test results in patients with high anti-CCD IgE antibody levels. *J Allergy Clin Immunol*. 2018;141(1):372-81.e3.
68. Aberer W, Holzweber F, Hemmer W, Koch L, Bokanovic D, Fellner W, et al. Inhibition of cross-reactive carbohydrate determinants (CCDs) enhances the accuracy of in vitro allergy diagnosis. *Allergol Select*. 2017;1(2):141-9.
69. Valenta R, Duchêne M, Pettenburger K, Sillaber C, Valent P, Bettelheim P, et al. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science*. 1991;253(5019):557-60.
70. Hauser M, Roulias A, Ferreira F, Egger M. Panallergens and their impact on the allergic patient. *Allergy Asthma Clin Immunol*. 2010;6(1):1.
71. Asero R, Jimeno L, Barber D. Preliminary results of a skin prick test-based study of the prevalence and clinical impact of hypersensitivity to pollen panallergens (polcalcin and profilin). *J Investig Allergol Clin Immunol*. 2010;20(1):35-8.
72. Cuesta-Herranz J, Barber D, Blanco C, Cistero-Bahíma A, Crespo JF, Fernández-Rivas M, et al. Differences among pollen-allergic patients with and without plant food allergy. *Int Arch Allergy Immunol*. 2010;153(2):182-92.

73. Asero R, Tripodi S, Dondi A, Di Rienzo Businco A, Sfika I, Bianchi A, et al. Prevalence and Clinical Relevance of IgE Sensitization to Profilin in Childhood: A Multicenter Study. *Int Arch Allergy Immunol*. 2015;168(1):25-31.
74. Barber D, de la Torre F, Feo F, Florido F, Guardia P, Moreno C, et al. Understanding patient sensitization profiles in complex pollen areas: a molecular epidemiological study. *Allergy*. 2008;63(11):1550-8.
75. Santos A, Van Ree R. Profilins: mimickers of allergy or relevant allergens? *Int Arch Allergy Immunol*. 2011;155(3):191-204.
76. Hatzler L, Panetta V, Lau S, Wagner P, Bergmann RL, Illi S, et al. Molecular spreading and predictive value of preclinical IgE response to *Phleum pratense* in children with hay fever. *J Allergy Clin Immunol*. 2012;130(4):894-901.e5.
77. van Ree R, Fernández-Rivas M, Cuevas M, van Wijngaarden M, Aalberse RC. Pollen-related allergy to peach and apple: an important role for profilin. *J Allergy Clin Immunol*. 1995;95(3):726-34.
78. Asero R, Monsalve R, Barber D. Profilin sensitization detected in the office by skin prick test: a study of prevalence and clinical relevance of profilin as a plant food allergen. *Clin Exp Allergy*. 2008;38(6):1033-7.
79. van Ree R, Voitenko V, van Leeuwen WA, Aalberse RC. Profilin is a cross-reactive allergen in pollen and vegetable foods. *Int Arch Allergy Immunol*. 1992;98(2):97-104.
80. Ma Y, Zuidmeer L, Bohle B, Bolhaar ST, Gadermaier G, Gonzalez-Mancebo E, et al. Characterization of recombinant Mal d 4 and its application for component-resolved diagnosis of apple allergy. *Clin Exp Allergy*. 2006;36(8):1087-96.
81. Ruiz-Hornillos J, López-Matas MA, Berges Jimeno P, Henríquez A, Blanco S, Seoane-Rodríguez M, et al. Profilin is a marker of severity in allergic respiratory diseases. *Allergy*. 2020;75(4):853-61.

82. Núñez R, Carballada F, Lombardero M, Jimeno L, Boquete M. Profilin as an aeroallergen by means of conjunctival allergen challenge with purified date palm profilin. *Int Arch Allergy Immunol.* 2012;158(2):115-9.
83. San Nicolás M, Braun T, Eder K, Berghaus A, Gröger M. Clinical Relevance of IgE to Profilin and/or Polcalcin in Pollen-Sensitized Patients. *Int Arch Allergy Immunol.* 2016;169(2):101-7.
84. Seiberler S, Scheiner O, Kraft D, Lonsdale D, Valenta R. Characterization of a birch pollen allergen, Bet v III, representing a novel class of Ca²⁺ binding proteins: specific expression in mature pollen and dependence of patients' IgE binding on protein-bound Ca²⁺. *EMBO J.* 1994;13(15):3481-6.
85. Niederberger V, Hayek B, Vrtala S, Laffer S, Twardosz A, Vangelista L, et al. Calcium-dependent immunoglobulin E recognition of the apo- and calcium-bound form of a cross-reactive two EF-hand timothy grass pollen allergen, Phl p 7. *FASEB J.* 1999;13(8):843-56.
86. Wopfner N, Dissertori O, Ferreira F, Lackner P. Calcium-binding proteins and their role in allergic diseases. *Immunol Allergy Clin North Am.* 2007;27(1):29-44.
87. Valenta R, Hayek B, Seiberler S, Bugajska-Schretter A, Niederberger V, Twardosz A, et al. Calcium-binding allergens: from plants to man. *Int Arch Allergy Immunol.* 1998;117(3):160-6.
88. Kowal K, Pampuch A, Sacharzewska E, Swiebocka E, Siergiejkó Z, Siergiejkó G, et al. Serum immunoglobulin E reactivity to cross-reacting panallergen components in north-eastern Poland patients pollen sensitized. *Allergy Asthma Proc.* 2020;41(3):183-91.
89. Tinghino R, Twardosz A, Barletta B, Puggioni EM, Iacovacci P, Butteroni C, et al. Molecular, structural, and immunologic relationships between different families of recombinant calcium-binding pollen allergens. *J Allergy Clin Immunol.* 2002;109(2):314-20.

90. De Vouge MW, Thaker AJ, Curran IH, Zhang L, Muradia G, Rode H, et al. Isolation and expression of a cDNA clone encoding an *Alternaria alternata* Alt a 1 subunit. *Int Arch Allergy Immunol.* 1996;111(4):385-95.
91. Twaroch TE, Focke M, Fleischmann K, Balic N, Lupinek C, Blatt K, et al. Carrier-bound Alt a 1 peptides without allergenic activity for vaccination against *Alternaria alternata* allergy. *Clin Exp Allergy.* 2012;42(6):966-75.
92. Asturias JA, Ibarrola I, Ferrer A, Andreu C, López-Pascual E, Quiralte J, et al. Diagnosis of *Alternaria alternata* sensitization with natural and recombinant Alt a 1 allergens. *J Allergy Clin Immunol.* 2005;115(6):1210-7.
93. Rodríguez D, Tabar AI, Castillo M, Martínez-Gomariz M, Dobski IC, Palacios R. Changes in the Sensitization Pattern to *Alternaria alternata* Allergens in Patients Treated with Alt a 1 Immunotherapy. *J Fungi (Basel).* 2021;7(11).
94. Asero R, Wopfner N, Gruber P, Gadermaier G, Ferreira F. Artemisia and Ambrosia hypersensitivity: co-sensitization or co-recognition? *Clin Exp Allergy.* 2006;36(5):658-65.
95. Jahn-Schmid B, Hauser M, Wopfner N, Briza P, Berger UE, Asero R, et al. Humoral and cellular cross-reactivity between Amb a 1, the major ragweed pollen allergen, and its mugwort homolog Art v 6. *J Immunol.* 2012;188(3):1559-67.
96. Bordas-Le Floch V, Le Mignon M, Bouley J, Groeme R, Jain K, Baron-Bodo V, et al. Identification of Novel Short Ragweed Pollen Allergens Using Combined Transcriptomic and Immunoproteomic Approaches. *PLoS One.* 2015;10(8):e0136258.
97. Adolphson C, Goodfriend L, Gleich GJ. Reactivity of ragweed allergens with IgE antibodies. Analyses by leukocyte histamine release and the radioallergosorbent test and determination of cross-reactivity. *J Allergy Clin Immunol.* 1978;62(4):197-210.

98. Gadermaier G, Wopfner N, Wallner M, Egger M, Didierlaurent A, Regl G, et al. Array-based profiling of ragweed and mugwort pollen allergens. *Allergy*. 2008;63(11):1543-9.
99. de la Hoz F, Polo F, Moscoso del Prado J, Sellés JG, Lombardero M, Carreira J. Purification of Art v I, a relevant allergen of *Artemisia vulgaris* pollen. *Mol Immunol*. 1990;27(7):651-7.
100. Oberhuber C, Ma Y, Wopfner N, Gadermaier G, Dedic A, Niggemann B, et al. Prevalence of IgE-binding to Art v 1, Art v 4 and Amb a 1 in mugwort-allergic patients. *Int Arch Allergy Immunol*. 2008;145(2):94-101.
101. Deng S, Yin J. Mugwort Pollen-Related Food Allergy: Lipid Transfer Protein Sensitization and Correlation With the Severity of Allergic Reactions in a Chinese Population. *Allergy Asthma Immunol Res*. 2019;11(1):116-28.
102. Himly M, Jahn-Schmid B, Dedic A, Kelemen P, Wopfner N, Altmann F, et al. Art v 1, the major allergen of mugwort pollen, is a modular glycoprotein with a defensin-like and a hydroxyproline-rich domain. *FASEB J*. 2003;17(1):106-8.
103. Movérare R, Westritschnig K, Svensson M, Hayek B, Bende M, Pauli G, et al. Different IgE reactivity profiles in birch pollen-sensitive patients from six European populations revealed by recombinant allergens: an imprint of local sensitization. *Int Arch Allergy Immunol*. 2002;128(4):325-35.
104. Tresch S, Holzmann D, Baumann S, Blaser K, Wüthrich B, Cramer R, et al. In vitro and in vivo allergenicity of recombinant Bet v 1 compared to the reactivity of natural birch pollen extract. *Clin Exp Allergy*. 2003;33(8):1153-8.
105. Ciprandi G, Comite P, Mussap M, De Amici M, Quaglini S, Barocci F, et al. Profiles of Birch Sensitization (Bet v 1, Bet v 2, and Bet v 4) and Oral Allergy Syndrome Across Italy. *J Investig Allergol Clin Immunol*. 2016;26(4):244-8.

106. Valenta R, Duchene M, Vrtala S, Birkner T, Ebner C, Hirschwehr R, et al. Recombinant allergens for immunoblot diagnosis of tree-pollen allergy. *J Allergy Clin Immunol.* 1991;88(6):889-94.
107. Barderas R, Purohit A, Papanikolaou I, Rodríguez R, Pauli G, Villalba M. Cloning, expression, and clinical significance of the major allergen from ash pollen, Fra e 1. *J Allergy Clin Immunol.* 2005;115(2):351-7.
108. Imhof K, Probst E, Seifert B, Regenass S, Schmid-Grendelmeier P. Ash pollen allergy: reliable detection of sensitization on the basis of IgE to Ole e 1. *Allergo J Int.* 2014;23(3):78-83.
109. Eder K, Gellrich D, Meßmer C, Canis M, Gröger M. Component resolved analysis of ash pollen allergy in Bavaria. *Allergy Asthma Clin Immunol.* 2018;14:76.
110. Barber D, de la Torre F, Lombardero M, Antépara I, Colas C, Dávila I, et al. Component-resolved diagnosis of pollen allergy based on skin testing with profilin, polcalcin and lipid transfer protein pan-allergens. *Clin Exp Allergy.* 2009;39(11):1764-73.
111. Andersson K, Lidholm J. Characteristics and immunobiology of grass pollen allergens. *Int Arch Allergy Immunol.* 2003;130(2):87-107.
112. Laffer S, Spitzauer S, Susani M, Pairleitner H, Schweiger C, Grönlund H, et al. Comparison of recombinant timothy grass pollen allergens with natural extract for diagnosis of grass pollen allergy in different populations. *J Allergy Clin Immunol.* 1996;98(3):652-8.
113. Cipriani F, Mastroilli C, Tripodi S, Ricci G, Perna S, Panetta V, et al. Diagnostic relevance of IgE sensitization profiles to eight recombinant *Phleum pratense* molecules. *Allergy.* 2018;73(3):673-82.
114. Valenta R, Vrtala S, Ebner C, Kraft D, Scheiner O. Diagnosis of grass pollen allergy with recombinant timothy grass (*Phleum pratense*) pollen allergens. *Int Arch Allergy Immunol.* 1992;97(4):287-94.

115. Bokanovic D, Aberer W, Hemmer W, Heinemann A, Komericki P, Scheffel J, et al. Determination of sIgE to rPhl p 1 is sufficient to diagnose grass pollen allergy. *Allergy*. 2013;68(11):1403-9.
116. Nolte H, Maloney J, Nelson HS, Bernstein DI, Lu S, Li Z, et al. Onset and dose-related efficacy of house dust mite sublingual immunotherapy tablets in an environmental exposure chamber. *J Allergy Clin Immunol*. 2015;135(6):1494-501.e6.
117. Bousquet PJ, Chinn S, Janson C, Kogevinas M, Burney P, Jarvis D. Geographical variation in the prevalence of positive skin tests to environmental aeroallergens in the European Community Respiratory Health Survey I. *Allergy*. 2007;62(3):301-9.
118. Arbes SJ, Jr., Gergen PJ, Elliott L, Zeldin DC. Prevalences of positive skin test responses to 10 common allergens in the US population: results from the third National Health and Nutrition Examination Survey. *J Allergy Clin Immunol*. 2005;116(2):377-83.
119. Heinzerling LM, Burbach GJ, Edenharter G, Bachert C, Bindslev-Jensen C, Bonini S, et al. GA(2)LEN skin test study I: GA(2)LEN harmonization of skin prick testing: novel sensitization patterns for inhalant allergens in Europe. *Allergy*. 2009;64(10):1498-506.
120. Arlian LG, Confer PD, Rapp CM, Vyszynski-Moher DL, Chang JC. Population dynamics of the house dust mites *Dermatophagoides farinae*, *D. pteronyssinus*, and *Euroglyphus maynei* (Acari: Pyroglyphidae) at specific relative humidities. *J Med Entomol*. 1998;35(1):46-53.
121. Batard T, Baron-Bodo V, Martelet A, Le Mignon M, Lemoine P, Jain K, et al. Patterns of IgE sensitization in house dust mite-allergic patients: implications for allergen immunotherapy. *Allergy*. 2016;71(2):220-9.
122. Barber D, Arias J, Boquete M, Cardona V, Carrillo T, Gala G, et al. Analysis of mite allergic patients in a diverse territory by improved diagnostic tools. *Clin Exp Allergy*. 2012;42(7):1129-38.

123. Wöhrl S, Vigl K, Zehetmayer S, Hiller R, Jarisch R, Prinz M, et al. The performance of a component-based allergen-microarray in clinical practice. *Allergy*. 2006;61(5):633-9.
124. Huss-Marp J, Gutermuth J, Schäffner I, Darsow U, Pfab F, Brockow K, et al. Comparison of molecular and extract-based allergy diagnostics with multiplex and singleplex analysis. *Allergo J Int*. 2015;24:46-53.
125. Demoly P, Emminger W, Rehm D, Backer V, Tommerup L, Kleine-Tebbe J. Effective treatment of house dust mite-induced allergic rhinitis with 2 doses of the SQ HDM SLIT-tablet: Results from a randomized, double-blind, placebo-controlled phase III trial. *J Allergy Clin Immunol*. 2016;137(2):444-51.e8.
126. Virchow JC, Backer V, Kuna P, Prieto L, Nolte H, Villesen HH, et al. Efficacy of a House Dust Mite Sublingual Allergen Immunotherapy Tablet in Adults With Allergic Asthma: A Randomized Clinical Trial. *JAMA*. 2016;315(16):1715-25.
127. Chapman MD, Platts-Mills TA. Purification and characterization of the major allergen from *Dermatophagoides pteronyssinus*-antigen P1. *J Immunol*. 1980;125(2):587-92.
128. Heymann PW, Chapman MD, Aalberse RC, Fox JW, Platts-Mills TA. Antigenic and structural analysis of group II allergens (Der f II and Der p II) from house dust mites (*Dermatophagoides* spp). *J Allergy Clin Immunol*. 1989;83(6):1055-67.
129. Bronnert M, Mancini J, Birnbaum J, Agabriel C, Liabeuf V, Porri F, et al. Component-resolved diagnosis with commercially available *D. pteronyssinus* Der p 1, Der p 2 and Der p 10: relevant markers for house dust mite allergy. *Clin Exp Allergy*. 2012;42(9):1406-15.
130. Pittner G, Vrtala S, Thomas WR, Weghofer M, Kundi M, Horak F, et al. Component-resolved diagnosis of house-dust mite allergy with purified natural and recombinant mite allergens. *Clin Exp Allergy*. 2004;34(4):597-603.

131. Weghofer M, Thomas WR, Kronqvist M, Mari A, Purohit A, Pauli G, et al. Variability of IgE reactivity profiles among European mite allergic patients. *Eur J Clin Invest.* 2008;38(12):959-65.
132. Becker S, Schleiderer T, Kramer MF, Haack M, Vrtala S, Resch Y, et al. Real-Life Study for the Diagnosis of House Dust Mite Allergy - The Value of Recombinant Allergen-Based IgE Serology. *Int Arch Allergy Immunol.* 2016;170(2):132-7.
133. Weghofer M, Grote M, Resch Y, Casset A, Kneidinger M, Kopec J, et al. Identification of Der p 23, a peritrophin-like protein, as a new major *Dermatophagoides pteronyssinus* allergen associated with the peritrophic matrix of mite fecal pellets. *J Immunol.* 2013;190(7):3059-67.
134. Jiménez-Feijoo R, Pascal M, Moya R, Riggioni C, Domínguez O, Lózano J, et al. Molecular Diagnosis in House Dust Mite-Allergic Patients Suggests That Der p 23 Is Clinically Relevant in Asthmatic Children. *J Investig Allergol Clin Immunol.* 2020;30(2):127-32.
135. Matos Semedo F, Dorofeeva Y, Pires AP, Tomaz E, Taborda Barata L, Inácio F, et al. Der p 23: Clinical Relevance of Molecular Monosensitization in House Dust Mite Allergy. *J Investig Allergol Clin Immunol.* 2019;29(4):314-6.
136. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy.* 2008;63(86):S8-160.
137. Sheikh A, Hurwitz B, Nurmatov U, van Schayck CP. House dust mite avoidance measures for perennial allergic rhinitis. *Cochrane Database Syst Rev.* 2010;2010(7):Cd001563.
138. Gøtzsche PC, Johansen HK. House dust mite control measures for asthma. *Cochrane Database Syst Rev.* 2008(2):Cd001187.

139. White P, Smith H, Baker N, Davis W, Frew A. Symptom control in patients with hay fever in UK general practice: how well are we doing and is there a need for allergen immunotherapy? *Clin Exp Allergy*. 1998;28(3):266-70.
140. Calderon MA, Demoly P, Gerth van Wijk R, Bousquet J, Sheikh A, Frew A, et al. EAACI: A European Declaration on Immunotherapy. Designing the future of allergen specific immunotherapy. *Clin Transl Allergy*. 2012;2(1):20.
141. Vogelberg C, Brüggjenjürgen B, Richter H, Jutel M. Real-World Adherence and Evidence of Subcutaneous and Sublingual Immunotherapy in Grass and Tree Pollen-Induced Allergic Rhinitis and Asthma. *Patient Prefer Adherence*. 2020;14:817-27.
142. Durham SR, Creticos PS, Nelson HS, Li Z, Kaur A, Meltzer EO, et al. Treatment effect of sublingual immunotherapy tablets and pharmacotherapies for seasonal and perennial allergic rhinitis: Pooled analyses. *J Allergy Clin Immunol*. 2016;138(4):1081-8.e4.
143. Jutel M, Brüggjenjürgen B, Richter H, Vogelberg C. Real-world evidence of subcutaneous allergoid immunotherapy in house dust mite-induced allergic rhinitis and asthma. *Allergy*. 2020;75(8):2050-8.
144. Jacobsen L, Niggemann B, Dreborg S, Ferdousi HA, Halken S, Høst A, et al. Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma: 10-year follow-up on the PAT study. *Allergy*. 2007;62(8):943-8.
145. Eng PA, Borer-Reinhold M, Heijnen IA, Gnehm HP. Twelve-year follow-up after discontinuation of preseasonal grass pollen immunotherapy in childhood. *Allergy*. 2006;61(2):198-201.
146. Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, et al. Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med*. 1999;341(7):468-75.
147. Durham SR, Emminger W, Kapp A, Colombo G, de Monchy JG, Rak S, et al. Long-term clinical efficacy in grass pollen-induced rhinoconjunctivitis after treatment with

- SQ-standardized grass allergy immunotherapy tablet. *J Allergy Clin Immunol*. 2010;125(1):131-8.e1-7.
148. Inal A, Altintas DU, Yilmaz M, Karakoc GB, Kendirli SG, Sertdemir Y. Prevention of new sensitizations by specific immunotherapy in children with rhinitis and/or asthma monosensitized to house dust mite. *J Investig Allergol Clin Immunol*. 2007;17(2):85-91.
149. Valovirta E. Effect of AIT in children including potential to prevent the development of asthma. *Allergy*. 2011;66(95):S53-4.
150. Zielen S, Devillier P, Heinrich J, Richter H, Wahn U. Sublingual immunotherapy provides long-term relief in allergic rhinitis and reduces the risk of asthma: A retrospective, real-world database analysis. *Allergy*. 2018;73(1):165-77.
151. Nelson HS. Oral/sublingual Phleum pretense grass tablet (Grazax/Grastek) to treat allergic rhinitis in the USA. *Expert Rev Clin Immunol*. 2014;10(11):1437-51.
152. Biedermann T, Kuna P, Panzner P, Valovirta E, Andersson M, de Blay F, et al. The SQ tree SLIT-tablet is highly effective and well tolerated: Results from a randomized, double-blind, placebo-controlled phase III trial. *J Allergy Clin Immunol*. 2019;143(3):1058-66.e6.
153. Ibañez MD, Kaiser F, Knecht R, Armentia A, Schöpfer H, Tholstrup B, et al. Safety of specific sublingual immunotherapy with SQ standardized grass allergen tablets in children. *Pediatr Allergy Immunol*. 2007;18(6):516-22.
154. Epstein TG, Liss GM, Berendts KM, Bernstein DI. AAAAI/ACAAI Subcutaneous Immunotherapy Surveillance Study (2013-2017): Fatalities, Infections, Delayed Reactions, and Use of Epinephrine Autoinjectors. *J Allergy Clin Immunol Pract*. 2019;7(6):1996-2003.e1.

155. Niederberger V, Neubauer A, Gevaert P, Zidarn M, Worm M, Aberer W, et al. Safety and efficacy of immunotherapy with the recombinant B-cell epitope-based grass pollen vaccine BM32. *J Allergy Clin Immunol*. 2018;142(2):497-509.e9.
156. Sastre J, Landivar ME, Ruiz-García M, Andregnette-Rosigno MV, Mahillo I. How molecular diagnosis can change allergen-specific immunotherapy prescription in a complex pollen area. *Allergy*. 2012;67(5):709-11.
157. Passalacqua G, Melioli G, Bonifazi F, Bonini S, Maggi E, Senna G, et al. The additional values of microarray allergen assay in the management of polysensitized patients with respiratory allergy. *Allergy*. 2013;68(8):1029-33.
158. Stringari G, Tripodi S, Caffarelli C, Dondi A, Asero R, Di Rienzo Businco A, et al. The effect of component-resolved diagnosis on specific immunotherapy prescription in children with hay fever. *J Allergy Clin Immunol*. 2014;134(1):75-81.
159. Barber D, Diaz-Perales A, Escribese MM, Kleine-Tebbe J, Matricardi PM, Ollert M, et al. Molecular allergology and its impact in specific allergy diagnosis and therapy. *Allergy*. 2021;76(12):3642-58.
160. Koch L, Laipold K, Arzt-Gradwohl L, Čerpes U, Sturm EM, Aberer W, et al. IgE multiplex testing in house dust mite allergy is utile, and sensitivity is comparable to extract-based singleplex testing. *Allergy*. 2020;75(8):2091-4.
161. Koch L, Laipold K, Arzt-Gradwohl L, Sturm EM, Aberer W, Aumayr M, Hemmer W, Cerpes U, Sturm GJ. unpublished data under submission. 2022.
162. Koch L, Laipold K, Schrautzer C, Arzt L, Cerpes U, Sturm EM, Aberer W, Binder B, Sturm GJ. Comparison of sIgE levels to mite extracts and allergen components between asthmatic and non-asthmatic house dust mite allergic patients. *Allergy Special Issue: Abstracts from the EACCI 2018*. 2018;73:551.

163. Frati F, Scurati S, Dell'Albani I, Puccinelli P, Incorvaia C, Passalacqua G. Evaluation of house dust mite allergy in real life: patients' characteristics and satisfaction with treatment. *Eur Ann Allergy Clin Immunol.* 2014;46(1):17-21.
164. Molina-Saenz MM, Villa-Arango AM, Cardona-Villa R. [Safety of subcutaneous immunotherapy with tyrosine-adsorbed house dust mite extracts in patients with allergic disease]. *Rev Alerg Mex.* 2017;64(1):52-65.
165. Leynaert B, Bousquet J, Neukirch C, Liard R, Neukirch F. Perennial rhinitis: An independent risk factor for asthma in nonatopic subjects: results from the European Community Respiratory Health Survey. *J Allergy Clin Immunol.* 1999;104(2):301-4.
166. Becker S, Groger M, Canis M, Pfrogner E, Kramer MF. Tropomyosin sensitization in house dust mite allergic patients. *Eur Arch Otorhinolaryngol.* 2012;269(4):1291-6.
167. Platts-Mills TA, Chapman MD. Dust mites: immunology, allergic disease, and environmental control. *J Allergy Clin Immunol.* 1987;80(6):755-75.
168. Dilworth RJ, Chua KY, Thomas WR. Sequence analysis of cDNA coding for a major house dust mite allergen, Der f I. *Clin Exp Allergy.* 1991;21(1):25-32.
169. Trudinger M, Chua KY, Thomas WR. cDNA encoding the major mite allergen Der f II. *Clin Exp Allergy.* 1991;21(1):33-7.
170. Wörner A, Christoph J, Mahler V. Typ-I-Allergien auf Vorratsmilben bei beruflich exponierten und nichtexponierten Patienten. *Arbeitsmed Sozialmed Umweltmed.* 2014;49:844-9.
171. Arzt L, Bokanovic D, Schrautzer C, Schwarz I, Laipold K, Aberer W, et al. Questionable diagnostic benefit of the commercially available panel of bee venom components. *Allergy.* 2017;72(9):1419-22.

172. Müsken H, Fernández-Caldas E, Marañón F, Franz JT, Masuch G, Bergmann KC. In vivo and in vitro sensitization to domestic mites in German urban and rural allergic patients. *J Investig Allergol Clin Immunol*. 2002;12(3):177-81.
173. van der Heide S, Niemeijer NR, Hovenga H, de Monchy JG, Dubois AE, Kauffman HF. Prevalence of sensitization to the storage mites *Acarus siro*, *Tyrophagus putrescentiae*, and *Lepidoglyphus destructor* in allergic patients with different degrees of sensitization to the house-dust mite *Dermatophagoides pteronyssinus*. *Allergy*. 1998;53(4):426-30.
174. Luczynska CM, Griffin P, Davies RJ, Topping MD. Prevalence of specific IgE to storage mites (*A. siro*, *L. destructor* and *T. longior*) in an urban population and crossreactivity with the house dust mite (*D. pteronyssinus*). *Clin Exp Allergy*. 1990;20(4):403-6.
175. Johansson E, Borgå A, Johansson SG, Van Hage-Hamsten M. Immunoblot multi-allergen inhibition studies of allergenic cross-reactivity of the dust mites *Lepidoglyphus destructor* and *Dermatophagoides pteronyssinus*. *Clin Exp Allergy*. 1991;21(4):511-8.
176. Johansson E, Johansson SG, Van Hage-Hamsten M. Allergenic characterization of *Acarus siro* and *Tyrophagus putrescentiae* and their crossreactivity with *Lepidoglyphus destructor* and *Dermatophagoides pteronyssinus*. *Clin Exp Allergy*. 1994;24(8):743-51.
177. Ebner C, Feldner H, Ebner H, Kraft D. Sensitization to storage mites in house dust mite (*Dermatophagoides pteronyssinus*) allergic patients. Comparison of a rural and an urban population. *Clin Exp Allergy*. 1994;24(4):347-52.
178. Park JW, Ko SH, Yong TS, Ree HI, Jeoung BJ, Hong CS. Cross-reactivity of *Tyrophagus putrescentiae* with *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* in urban areas. *Ann Allergy Asthma Immunol*. 1999;83(6):533-9.
179. Arias-Irigoyen J, Lombardero M, Arteaga C, Carpizo JA, Barber D. Limited IgE cross-reactivity between *Dermatophagoides pteronyssinus* and *Glycyphagus domesticus* in patients naturally exposed to both mite species. *J Allergy Clin Immunol*. 2007;120(1):98-104.

180. Gafvelin G, Johansson E, Lundin A, Smith AM, Chapman MD, Benjamin DC, et al. Cross-reactivity studies of a new group 2 allergen from the dust mite *Glycyphagus domesticus*, Gly d 2, and group 2 allergens from *Dermatophagoides pteronyssinus*, *Lepidoglyphus destructor*, and *Tyrophagus putrescentiae* with recombinant allergens. *J Allergy Clin Immunol*. 2001;107(3):511-8.
181. van Hage-Hamsten M, Johansson SG, Höglund S, Tüll P, Wirén A, Zetterstrom O. Storage mite allergy is common in a farming population. *Clin Allergy*. 1985;15(6):555-64.
182. Tee RD, Gordon DJ, van Hage-Hamsten M, Gordon S, Nunn AJ, Johansson SG, et al. Comparison of allergic responses to dust mites in U.K. bakery workers and Swedish farmers. *Clin Exp Allergy*. 1992;22(2):233-9.
183. Malandain H, Giroux F, Cano Y. The influence of carbohydrate structures present in common allergen sources on specific IgE results. *Eur Ann Allergy Clin Immunol*. 2007;39(7):216-20.
184. Chen KW, Ziegelmayer P, Ziegelmayer R, Lemell P, Horak F, Bunu CP, et al. Selection of house dust mite-allergic patients by molecular diagnosis may enhance success of specific immunotherapy. *J Allergy Clin Immunol*. 2019;143(3):1248-52.e12.
185. Rodríguez-Domínguez A, Berings M, Rohrbach A, Huang HJ, Curin M, Gevaert P, et al. Molecular profiling of allergen-specific antibody responses may enhance success of specific immunotherapy. *J Allergy Clin Immunol*. 2020;146(5):1097-108.
186. Baron-Bodo V, Horiot S, Lautrette A, Chabre H, Drucbert AS, Danzé PM, et al. Heterogeneity of antibody responses among clinical responders during grass pollen sublingual immunotherapy. *Clin Exp Allergy*. 2013;43(12):1362-73.
187. Kleine-Tebbe J, Matricardi PM, Hamilton RG. Allergy Work-Up Including Component-Resolved Diagnosis: How to Make Allergen-Specific Immunotherapy More Specific. *Immunol Allergy Clin North Am*. 2016;36(1):191-203.

188. Hemmer W, Focke M, Kolarich D, Wilson IB, Altmann F, Wohrl S, et al. Antibody binding to venom carbohydrates is a frequent cause for double positivity to honeybee and yellow jacket venom in patients with stinging-insect allergy. *J Allergy Clin Immunol.* 2001;108(6):1045-52.
189. Kochuyt AM, Van Hoeyveld EM, Stevens EA. Prevalence and clinical relevance of specific immunoglobulin E to pollen caused by sting- induced specific immunoglobulin E to cross-reacting carbohydrate determinants in Hymenoptera venoms. *Clin Exp Allergy.* 2005;35(4):441-7.
190. Ebo DG, Hagendorens MM, Bridts CH, De Clerck LS, Stevens WJ. Sensitization to cross-reactive carbohydrate determinants and the ubiquitous protein profilin: mimickers of allergy. *Clin Exp Allergy.* 2004;34(1):137-44.
191. Tripodi S, Frediani T, Lucarelli S, Macrì F, Pingitore G, Di Rienzo Businco A, et al. Molecular profiles of IgE to *Phleum pratense* in children with grass pollen allergy: implications for specific immunotherapy. *J Allergy Clin Immunol.* 2012;129(3):834-9.e8.
192. Wopfner N, Gadermaier G, Egger M, Asero R, Ebner C, Jahn-Schmid B, et al. The spectrum of allergens in ragweed and mugwort pollen. *Int Arch Allergy Immunol.* 2005;138(4):337-46.
193. Léonard R, Wopfner N, Pabst M, Stadlmann J, Petersen BO, Duus J, et al. A new allergen from ragweed (*Ambrosia artemisiifolia*) with homology to art v 1 from mugwort. *J Biol Chem.* 2010;285(35):27192-200.
194. Gadermaier G, Hauser M, Ferreira F. Allergens of weed pollen: an overview on recombinant and natural molecules. *Methods.* 2014;66(1):55-66.