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Identification and characterization of house dust mite allergens influencing the allergic phenotype and improvement of allergy diagnosis in clinical routine

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Dissertation

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ΔMFI	Fold change of median fluorescence intensity
AA	Allergic asthma
AD	Atopic dermatitis
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APC	Allophycocyanin (fluorophore)
Api m	*Apis mellifera* (WHO/IUIS nomenclature for allergens of the honey bee)
APS	Ammonium persulfate
AR	Allergic rhinitis
Ara h	*Arachis hypogaea* (WHO/IUIS nomenclature for allergens of peanuts)
Asp f	*Aspergillus fumigatus* (WHO/IUIS nomenclature for allergens of the common mold)
ATCC	American Type Culture Collection
AUC	Area under the curve
BAT	Basophil activation test
BCIP	5-Bromo-4-chloro-3'-indolyphosphate
Bet v	*Betula verrucosa* (WHO/IUIS nomenclature for allergens of the European white birch
Bla g	*Blattella germanica* (WHO/IUIS nomenclature for allergens of the German cockroach)
bp	Base pairs
Can f	*Canis familiaris* (WHO/IUIS nomenclature for allergens of the domestic dog)
CAP-FEIA	Carrier-Polymer-System-Fluorescence-Enzyme-Immunoassay
CCLE	Cancer Cell Line Encyclopedia
CD	Circular dichroism
CI	Confidence interval
CoFAR	Consortium for Food Allergy Research
CRD	Component-resolved diagnosis
CSTS	Combined symptom and tolerance score
CSTC	Combined symptom and tolerance class
DC	Dendritic cell
ddH_2O	Ultrapure water, deionized and filtered
Der f	*Dermatophagoides farinae* (WHO/IUIS nomenclature for allergens of the American house dust mite)

Der p	*Dermatophagoides pteronyssinus* (WHO/IUIS nomenclature for allergens of the European house dust mite)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	1,4-Dithiothreitol
EAACI	European Association of Allergy and Clinical Immunology
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
EKB	Elbe Klinikum Buxtehude
ESI	Electrospray ionization
FcεRI	High affinity IgE receptor
Fel d	*Felis domesticus* (WHO/IUIS nomenclature for allergens of the domestic cat)
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FPLC	Fast protein liquid chromatography
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
HDM	House dust mite
His-Tag	Hexahistidine-Tag
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSD	Honest significant difference
IEX	Ion-exchange chromatography
Ig	Immunoglobulin
IL	Interleukin
IMAC	Immobilized metal ion affinity chromatography
IP-10	Interferon-gamma induced protein 10 kD
IPTG	Isopropyl-ß-D-thiogalactopyranosid
IQR	Interquartile range
IUIS	International Union of Immunological Societies
LAL	Limulus amebocyte lysate
LB	Lysogeny broth

LPS	Lipopolysaccharide
(k)Da	(Kilo)Dalton
mA	Milliampere
mAu	Milli-absorbance units
MD-2	Myeloid differentiation factor 2
MES	2-(N-Morpholino)ethane sulfonic acid
MHC	Major histocompatibility complex
MHH	Hannover Medical School
ML	MD-2-related lipid-recognition
MS	Mass spectrometry
MSD	Mesoscale Discovery
MWCO	Molecular weight cut-off
m/z	Mass-to-charge ratio
NBT	4-Nitro blue tetrazolium chloride
NC	Nitrocellulose
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NHS	N-hydroxysulfosuccinimid
NIR	Near-infrared
nm	Nanometer
OD	Optical density
OFC	Oral food challenge
OR	Odds ratio
P_n	Number of passages
PAGE	Polyacrylamide gel electrophoresis
Pen m	*Penaeus monodon* (WHO/IUIS nomenclature for allergens of the black tiger shrimp)
PEX	Peanut extract
PCR	Polymerase chain reaction
PE	Phycoerythrin (fluorophore)
PE-Cy7	Phycoerythrin-Cyanine7 (tandem fluorophore)
pET	Plasmid expression by T7-RNA-polymerase (vector)
pI	Isoelectric point
PMF	Peptide mass fingerprinting
PRR	Pattern recognition receptor

RBL	Rat basophil leukemia
RCB	Research Center Borstel
RH	Relative humidity
RMU	Relative multiplex units
RNA	Ribonucleic acid
ROC	Receiver-operator-characteristics
RR	Relative risk
RT	Room temperature
SCIT	Specific subcutaneous immunotherapy
SCORAD	SCORing Atopic Dermatitis
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SG	Subgroup
sIgE	(Antigen-/Allergen-) Specific IgE
SIT	Specific immunotherapy
SLIT	Specific sublingual immunotherapy
SP	Study population
SPT	Skin prick test
SSC	Side scatter
TB	Terrific broth
TBS	Tris-buffered saline
TEMED	*N,N,N',N'*-Tetramethylethylendiamin
TGF-β	Transforming growth factor beta
Th	T-helper cell
TIM-4	T-cell immunoglobulin mucin-4
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethan
Tween-20	Polyoxyethylen(20)-sorbitan-monolaurat
Tyr p	*Tyrophagus putrescentiae* (WHO/IUIS nomenclature for allergens of the storage mite)
UKSH	University Hospital Center Luebeck
UniProt	Universal Protein Resource
UV	Ultraviolet

VEGF	Vascular endothelial growth factor
v/v	Volume per volume
WHO	World Health Organization
w/v	Weight per volume
x g	Times gravity (centrifugal force)
YT	Yeast extract-tryptone

1 Introduction

1.1 Allergic Diseases — History and classification

While descriptions of symptoms that would correspond to today's allergic diseases date back to ancient sources such as inscriptions from the ancient Egypt and Greco-Roman texts, the Viennese pediatrician Clemens von Pirquet was the first to use the term "allergy" in 1906 to describe altered reactivity of the immune system to exogenous substances, thus including both hyper- but also hyposensitivity [1, 2]. In 1963, the British immunologists Philip Gell and Robin Coombs proposed the system most widely used today to classify allergic reactions, which restricted the definition of "allergy" to hypersensitivity reactions only [3]. Their scheme distinguishes four types of hypersensitivity reactions (type I–IV), with type I to III being antibody-mediated and type IV being a cell-mediated immune response. The different reaction types are further specified in Table 1 (without subtypes). Type I and type IV, also referred to as immediate and delayed hypersensitivity reactions, respectively, are the most relevant forms of allergy today [4]. Even though the classes appear to be clearly divided, multiple types of hypersensitivity reactions can be mediated by a single antigen, which is commonly observed in drug hypersensitivity but also others such as inhalant allergens like house dust mites (HDM), that have been reported to elicit type I and type IV reactions [4-6]. The immunoglobulin E (IgE) mediated type I or immediate hypersensitivity is what is popularly called today a "classic" allergic reaction, including but not limited to atopy, anaphylaxis and allergic asthma [7]. This thesis focuses on type I hypersensitivity reactions induced by direct or indirect repeated exposure to certain antigens, also commonly known as allergens.

Table 1: Classification of hypersensitivity reactions

Class	Mediators	Immunological Reaction	Clinical Presentation
Type I	IgE, mast cells	Fast response IgE-cross linking on mast cell and basophil surface	Anaphylaxis Asthma Angioedema
Type II	IgM, IgG, complement, MAC	Cytotoxicity through activation of membrane attack complex (MAC) Antibody-dependent cell-mediated cytotoxicity (ADCC)	Thrombocytopenia Chronic idiopathic urticaria
Type III	IgG	Soluble antibody-antigen immune complex	Serum sickness Rheumatoid arthritis
Type IV	Th_1, Th_2	T-cell activation via antigen-presenting cell	Persistent allergic rhinitis Contact dermatitis Drug hypersensitivity

Modified from [8, 9].

1.2 Pathophysiology of type I hypersensitivity reactions

All allergic reactions are preceded by the sensitization phase. Upon exposure to environmental antigens, a dysfunctional immune response in persons with a respective genetic predisposition also known as atopy can ultimately lead to the production of antigen-specific IgE (sIgE) antibodies mediating the further allergic response.

While the term “atopic” is often used synonymously with allergic, it actually is defined by the European Academy of Allergy and Clinical Immunology (EAACI) to be a constitutional trait describing a mainly inherited predisposition to develop an abnormal immune response with IgE antibody production in response to allergen exposure [10]. Atopic individuals usually exhibit atopic dermatitis (AD) in childhood and develop allergic rhinitis and/or asthma later in life, frequently to multiple allergen sources. This progression of allergic diseases throughout life is also called the atopic march. However, individuals considered as non-atopic can develop an IgE-mediated allergy at any time of life as well, often restricted to a specific allergen rather than multiple sensitizations [11-14].

It is important to note that not all encounters with allergens lead to a sensitization and neither do sensitizations always progress to symptomatic allergic diseases. The determinants of why and when allergic sensitization occurs and what the crucial factors for further progression are, is thought to be a mix of genetic predisposition and environmental factors, wherein genetics are reported to account for 50–70% of the individual risk to develop an allergic disease [15, 16].

The sensitization phase is initiated by the encounter of an environmental antigen. Depending on the allergen source and its route of exposure, the potentially allergenic molecules — usually proteins — have to cross the respiratory, intestinal or skin epithelial barrier before they can provoke an immune response. An impaired barrier as seen in AD patients can therefore facilitate the development of allergies [17, 18]. Although many different proteins can induce allergic reactions, those with enzymatic activity in particular are often considered important sensitizing agents, as they are at advantage when penetrating the epithelial barrier [19]. For example, the cysteine protease Der p 1, a clinically highly relevant allergen of the European HDM *Dermatophagoides pteronyssinus*, is able to disrupt intercellular tight junctions in airway epithelial cells by cleaving occludin, an important protein for barrier function and tight junction stability [20, 21]. However, many allergens do not exhibit enzymatic activity and in rare cases they can even be protease inhibitors [22, 23].

After crossing the epithelial barrier, immature dendritic cells (DC) in peripheral tissues can phagocytose the allergens, degrade them and present the respective peptides on their cell surface

by MHC II (major histocompatibility complex II). The maturated, antigen-bearing DC subsequently migrates to the lymph nodes or local mucosa and acts as an antigen-presenting cell by priming naïve T-cells (Th_0 cells) [24, 25]. The further differentiation of these cells is determined by their local environment. In the presence of IL-4, IL-5, IL-9 and/or IL-13, which can be derived from a range of cells including e.g. natural killer T cells or basophils, the differentiation into Th_2 cells is favored. But under different conditions, the same cells could instead differentiate into other T cell subsets such as regulatory T-cells (T_{reg}) cells in the presence of TGF-β (Transforming growth factor beta), which are rather associated with tolerance and protection from allergies [26]. Upon activation, the Th_2 cells themselves release IL-4 and IL-13 and therefore maintain the environment for further differentiation of Th_0 into Th_2 cells (clonal expansion) [27, 28]. When naïve mature B-cells encounter an allergen, peptides of it are presented on the cells' surface by MHC II. Upon interaction of a Th_2 cell with MHC II and further with either CD40, CD86 or CD80 on the surface of an allergen-specific B-cell, usually happening within a lymph node or lymphoid follicle, the activated B-cell proliferates and undergoes class-switching to produce sIgE antibodies rather than IgM and IgD (clonal selection) [27, 29]. The B-cell now further differentiates into its final form, a long-lived plasma cell able to produce large quantities sIgE antibodies. These antibodies circulate in the blood stream where they can bind to the high-affinity IgE receptor FcεRI present on the surface of basophils and/or mast cells, which concludes the sensitization phase [30].

Upon re-exposure, the same antigen can now interact with the sIgE bound to the FcεRI on mast cells and basophils, which is called the effector phase. When two or more IgE antibodies and their associated FcεRI are cross-linked by one antigen, the cell's degranulation is triggered, during which histamine, prostaglandins and further proinflammatory mediators responsible for the symptoms of the acute phase type-I hypersensitivity reaction are released [31]. Due to the rapid distribution of allergens in the blood stream and lymphoid system, allergic reactions are not limited to their local place of entry, such as the airways or gut, but can be systemic as well. In this case, multiple organ systems uninvolved in the initial allergen contact such as the skin, airways or cardiovascular system can be severely affected and lead to a potentially life-threatening shock state also referred to as anaphylaxis, a phenomenon often observed in food and drug allergy [32].

However, a sensitization does not always translate to a clinically relevant allergy. Different studies have investigated why some individuals exhibit so called "silent sensitizations", and while there is evidence that the affinity of IgE antibodies to FcεRI, protective IgG4 antibodies as well as genetic predisposition might play a role in this process, there also are studies with

conflicting evidence. Overall, it is not yet well-understood which factors determine the "breach of tolerance" and development of actual allergic symptoms following a sensitization [33, 34].

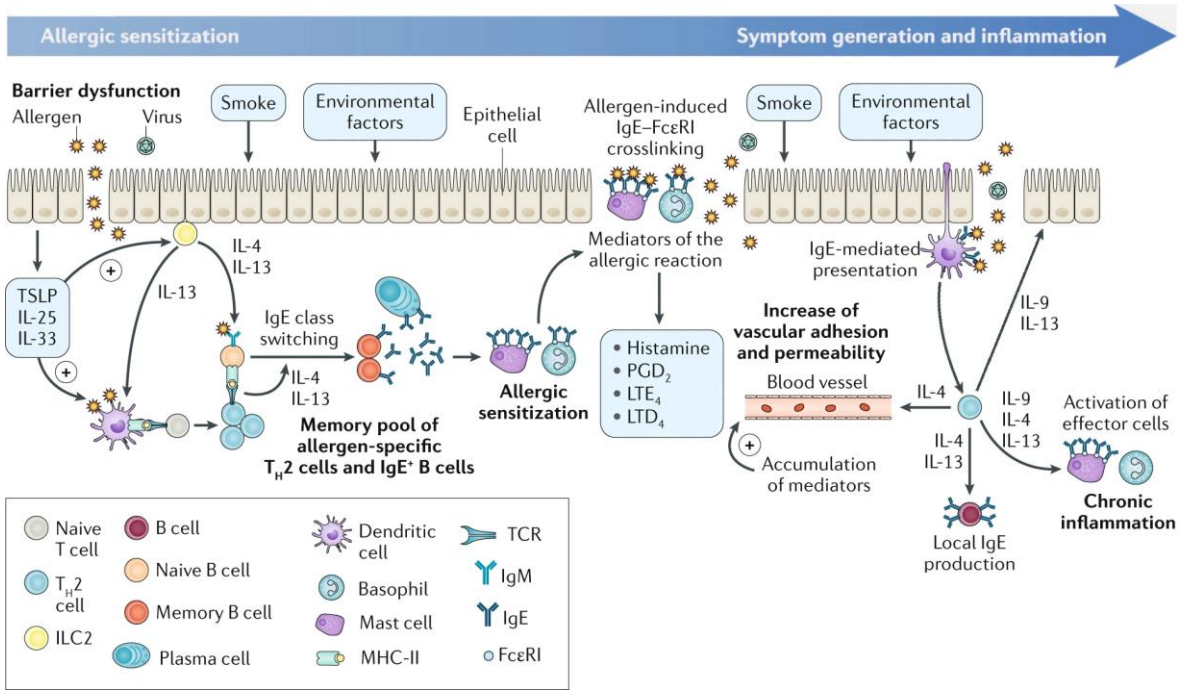


Figure 1.1: Pathophysiology of Type-I hypersensitivity

Modified from [35].

1.3 Epidemiology of allergic diseases in Europe

According to the EAACI, 150 million EU citizens suffered from chronic allergic diseases in 2015, which corresponds to approximately 30% of the EU population at that time [36]. In 2016, the Robert-Koch-Institute released a position paper revealing that more than 50% of the German population were sensitized to at least one allergen [37]. This makes allergy the most prevalent chronic disease in Europe, and it is even estimated that by 2025, every second European will suffer from allergy [36].

While allergic reactions have been known for a long time (refer to chapter 1.1), their prevalence in industrialized areas such as Europe and America has grown exponentially within the last decades [38, 39]. Various hypotheses have been postulated as to what the cause could be. One of the most popular approaches is the "hygiene hypothesis" first proposed in 1989 by Strachan et al. It is based on the idea that rising hygiene standards lead to a decline of exposure to microorganisms, parasites and infections and therefore facilitate misdirected immune responses

as seen in allergies or, a more recent addition to this theory, autoimmune disorders. However, this theory, albeit popular, is not without controversy [40, 41].

Overall, the reasons for the rapid emergence of allergic diseases worldwide and in westernized countries in particular are not fully elucidated, although it seems to be general consensus that it must be mainly environmental causes due to the time frame being too short for genetic changes [42].

1.4 House dust mite allergy

1.4.1 House dust mites and their allergens

The term “house dust mites” describes over 20 species of arachnids mostly belonging to the *Pyroglyphidae* family. Despite several potentially allergy-causing species such as *Blomia tropicalis* or *Dermatophagoides microceras*, in the following work the term house dust mite (HDM) only entails *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, commonly referred to as the European and American HDM, since these are the most relevant species [43, 44].

The preferred living environment of HDM is of moderate temperature and high surface humidity. Since almost 75% of their body weight is water and they rely on water vapor in the ambient air for hydration, they are highly susceptible to drought and excessive heat [45, 46]. Their viable temperature and relative humidity (RH) ranges as well as reported global occurrence are listed in Table 2.

Table 2: Living conditions of house dust mites

	D. pteronyssinus	***D. farinae***
Temperature Optimum	15–20°C	25–30°C
Humidity Optimum	75–80% RH	70–75% RH
Critical Humidity	60–65% RH	47–50% RH
Occurrence	Commonly all over Europe Most frequent species	Common in America and Korea Second most frequent

Data retrieved from [46-48].

HDM mainly feed on skin flakes of humans or animals and fungi and are therefore found in places where the accumulation of these substances is promoted. In particular, they are universally present in carpets, curtains, mattresses and any kind of upholstered furniture [46].

Allergens of the HDM can either be derived from their feces or the mite body. Dry air caused by heating during the colder months leads to the death of a majority of the HDM population,

and large quantities of mite allergens are released from their decomposing bodies, often exacerbating allergic symptoms [49, 50]. To this date, 32 different proteins are registered as allergens in the official database established by the WHO/IUIS Allergen Nomenclature Sub-committee (World Health Organization and International Union of Immunological Societies) for *D. pteronyssinus* and 37 for *D. farinae* [51].

Table 3: Classification of HDM allergens

Der p / Der f	Biochemical function	Biological activity (proposed)	Location	Seroprevalence
Major allergens				
1	Cysteine protease	Proteolysis	Feces	80–100%
2	ML[a] domain protein	Lipid binding	Feces	80–100%
23	Peritrophin	Chitin binding, dung ball stabilizing	Feces	74%
Mid-tier allergens				
4	Amylase	Glycoside hydrolase	Feces	40%
5	Unknown	Hydrophobic binding	Feces	30–40%
7	Similar to LPS-binding protein	Hydrophobic binding	Unknown	40%
21	Unknown	Hydrophobic binding	Gut	30%
Minor allergens				
3, 6, 9	Serine proteases	Proteolysis	Feces	9–97%
10	Tropomyosin	Muscle protein	Gut	6–28%
11	Paramyosin	Muscle protein	Gut	8–87%
13	Fatty acid binding protein	Lipid transfer	Gut	10–20%
15	Chitinase	Glycosyl hydrolase	Gut	15–30%
18	Chitin-binding	Non-catalytic chitinase like	Gut	15–30%
20	Arginine kinase	ATP maintenance	Gut	14–44%

Modified from [52-54]. Not included: allergens of unknown importance (Der p 14, 24–40)

[a] ML = MD-2-related lipid-recognition.

The known house dust mite allergens are a heterogeneous group of proteins with various structures and biological functions that are in part not fully elucidated yet. The serodominant allergens, also labeled as major allergens, that more than 50% of patients are sensitized to, are Der p 1, 2, and 23, although the latter was discovered rather recently and more than 30 years after Der p 1 and 2 [55-58]. Other, non-major, allergens are often referred to as minor allergens or they can be further categorized according to their IgE binding frequency into mid-tier and minor allergens as listed in Table 3 [52, 59]. However, these classifications are not official, and their definition has varied over time. It is an ongoing topic of controversy as Carabello et

al. have criticized that the association of sensitization frequency with clinical relevance is not scientifically evident and the term “minor allergen” is often wrongfully associated with lack of importance [60].

1.4.2 Epidemiology and symptoms

Due to their ubiquitous and perennial presence, HDM are one of the most prominent airborne allergen sources worldwide. Approximately 20% of the general population in industrialized European countries is sensitized to HDM, and in asthmatic individuals the prevalence rises up to 85%. However, the GA^{2}LEN study demonstrated that even within Europe the sensitization rates are highly variable between countries, ranging from approximately 9% in Sweden to more than 30% in Spain. In general, house dust mite sensitization is usually higher in countries with a Mediterranean climate [61-63]. According to several studies, 16–24% of the general German population is sensitized to HDM. As mentioned before, sensitization does not always translate to a clinically relevant allergy. Among sensitized individuals, the rate of symptomatic HDM allergy is estimated around 75%. It can therefore be assumed that the prevalence of actual HDM allergy in Germany is 12–18% with a tendency to further increase [63-66].

The phase of sensitization often happens during childhood, wherein a high exposure to indoor allergens seems to be a crucial factor, since approximately 7% of children with high exposure develop HDM sensitization within their first three years of life, compared to 2% with low exposure [67]. Furthermore, HDM allergens are even found in breast milk, so newborns and in particular their gastrointestinal systems are frequently in contact with them. Gastrointestinal exposure is generally thought to induce tolerance rather than allergy, but it has also been proposed that the gut can be a place of sensitization as well. Similar phenomena are known in e.g. peanut allergy, where sensitization can occur via the skin even though the subsequent symptoms are related to oral ingestion, which could explain why the risk of allergy is increased in children breastfed with milk containing higher than average concentrations of HDM allergens [45, 68, 69].

The allergic symptoms elicited by HDM exposure can affect different organs such as the eyes, skin as well as the nasal and bronchial part of the respiratory system, which can ultimately result in AD and exacerbations thereof, allergic rhino-conjunctivitis (AR) or allergic asthma (AA) (Figure 1.2). Particularly the association of HDM with asthma has been well-studied and documented during the last decades [53].

While HDM allergy, in contrast to food allergies, usually does not present with life-threatening symptoms, rare cases of anaphylaxis caused by HDM often following an ingestion of substantial amounts of mites or massive inhalative exposure are reported in the literature as well [70-72].

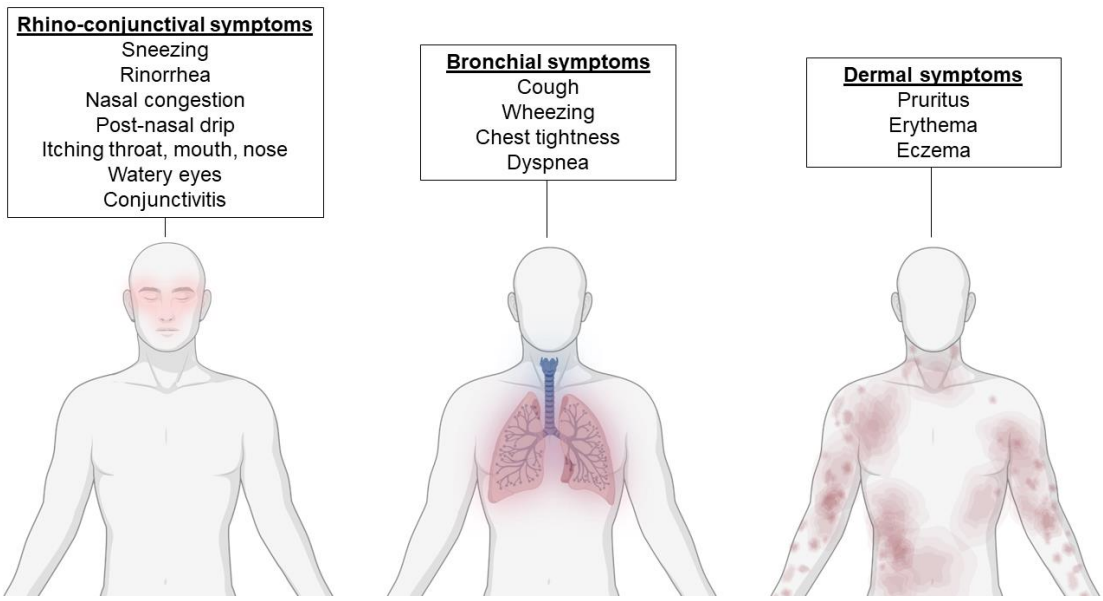


Figure 1.2: Symptoms of HDM allergy

Data derived from [73, 74]. Parts of the figure were created with Biorender.com and pictures were exported under a paid license.

1.4.3 The role of house dust mites in allergic diseases

The constant exposure to HDM and their allergens, which, contrary to popular belief, mainly occurs during the daytime and not in bed, makes them one of the most relevant allergens in airway and skin-related allergies [75].

The importance of HDM in the elicitation of allergic diseases has been widely studied [20, 62, 76-87]. In a recent, longitudinal study, mite sensitization during childhood was reported as one of the strongest risk factors for the development of allergic asthma, rhinitis and atopic eczema [88]. In patients with AD, mite-specific T-cells can be found more often in lesions of the skin than in the blood, the majority of which produce a Th_2-like cytokine profile. Moreover, skin lesions can be induced by external contact as well as inhalation of HDM allergens, which can be reproduced during an allergen challenge using diagnostic HDM extracts [89-91]. HDM are furthermore considered a dominant risk factor in the development of allergic asthma, which is more frequently uncontrolled and more severe in HDM-sensitized patients compared to non-

sensitized asthmatics [76, 92]. However, even in non-sensitized asthmatic individuals a bronchial response can be provoked when the patients are exposed to high levels of HDM. Reversely, avoidance measures such as specialized mattress covers with pores smaller than the mites' fecal pellets can lessen inflammation and improve clinical appearance of skin lesions in AD as well as bronchial hyperreactivity in asthmatic patients [62, 79, 93-96].

Several mechanisms by which HDM and single allergens thereof can elicit an allergic response and inflammation have been investigated in the last decades. As already indicated in chapter 1.2, proteases often play a major role in allergic diseases. Several proteins with proteolytic activity are present in the fecal pellets of HDM, such as the trypsin-like major allergen Der p 1, which has been thoroughly investigated and found to not only disrupt tight junctions in the epithelial airway and skin barrier but also to interact with PAR-2 (protease-activated-receptor-2), which is associated with both allergic asthma and AD [21, 84, 97]. PAR-2 can cause a non-histamine mediated pruritus as well as delayed epidermal recovery, which could be part of the reason why pruritus in AD patients is often not responsive to treatment with antihistamines [45, 85, 98]. Further immunological interactions of Der p 1 include but are not limited to airway remodeling, activation of DCs and promotion of a Th_2-mediated immune response [53, 99-101].

Other HDM allergens with proteolytic activity include Der p 3, 6 and 9, a group of serine proteases reportedly involved in disruption of epithelial tight junctions, cleavage of occludin in epithelial cells and airway remodeling similar to Der p 1 albeit with less seroprevalence [53, 102]. HDM proteases do, however, not only play a key role in the development of HDM allergy, but can furthermore facilitate sensitization to other allergen sources through the weakened epithelial barrier and promote Th_2-type immune responses [100, 101, 103, 104].

However, non-proteolytic HDM allergens can also influence the immune system in various ways. In many commonly occurring allergies, major allergens often exhibit lipid-binding properties such as Bet v 1 (*Betula verrucosa*, European white birch), Fel d 1 (*Felis domesticus*, domestic cat) or Can f 1 (*Canis familiaris*, domestic dog) in birch pollen, cat and dog allergy, respectively [105]. It has been proposed that binding of lipids can enhance the allergenicity of proteins due to the lipids acting as adjuvants or altering the biochemical properties of the allergen [106]. The best studied allergen in this group in regards to HDM allergens is the major allergen Der p 2, which, together with Der p 1, was one of the first discovered single allergens in mites. Der p 2 has been reported to exhibit a hydrophobic binding cavity potentially carrying LPS and therefore acting as an adjuvant. It can furthermore interact with TLR4 (toll-like receptor 4) via molecular mimicry of its coreceptor MD-2 (Myeloid differentiation factor 2) due to their similar structures, which subsequently leads to the release of proinflammatory cytokines [105, 107, 108]. Further allergens with proposed lipid-binding capacities are Der p 5,

7, 13, 14 and 21 although their mode of interaction with lipids and immunological properties are little investigated [106].

The various mechanisms by which HDM allergens can provoke allergic responses besides IgE reactivity and their ability to activate and alter adaptive as well as innate immune responses and inflammation is why they are considered a crucial factor in the atopic march [45].

While the majority of existing research is centered on the two longest-known major allergens Der p 1 and Der p 2, recent advancements have demonstrated capability of other non-major allergens to induce immune responses by for example activation of pattern recognition receptors (PRR) such as TLR2 [45, 106]. Nevertheless, the clinical relevance and pathomechanistic properties of many non-major allergens remain uncertain as of yet.

1.4.4 Climate change influencing house dust mites

In HDM allergy, *D. pteronyssinus* is usually reported as the most relevant species and also is the one most studied in the literature [46, 109]. Studies involving Der f allergens often originate from regions were *D. farinae* is the predominant HDM species such as Korea [110]. In Europe, Der f allergens only play a minor role in research as well as clinical routine, which explains the lack of single Der f allergens included in routine diagnostic platforms.

However, a fact not yet considered is how mite populations and their preferred habitats might change during the course of climate change. As elaborated in chapter 1.4.1, the viability of HDM is highly dependent on humidity and temperature with different preferences and critical humidity limits. In general, *D. pteronyssinus* is more susceptible to heat and dry air [47, 48].

In Middle Europe specifically, temperatures are rising, and the relative surface humidity has decreased over the last four decades as depicted in Figure 1.3 — a trend that is predicted to continue [48, 111]. When comparing the preferred living conditions of house dust mite species, it becomes obvious that *D. farinae* is better equipped to deal with higher temperatures and drought compared to *D. pteronyssinus* (Table 2). A study in which dust samples in Dutch homes were collected over a time period of 8 years revealed that the amount of Der f allergens on beds and floors was constantly rising while Der p allergen content stagnated or even decreased [112]. Studies like these are rare and the effects of climate change on mite population is highly dependent on the local conditions. It nevertheless seems plausible to assume that *D. farinae* and its allergens will gain importance in Middle Europe as the climate changes progresses further.

However, not only the geographical distribution and local dominance of HDM is subject to change due to climate change but also the metabolism of mites, which might influence the proportions and abundance of proteins produced by HDM in yet unknown ways [48].

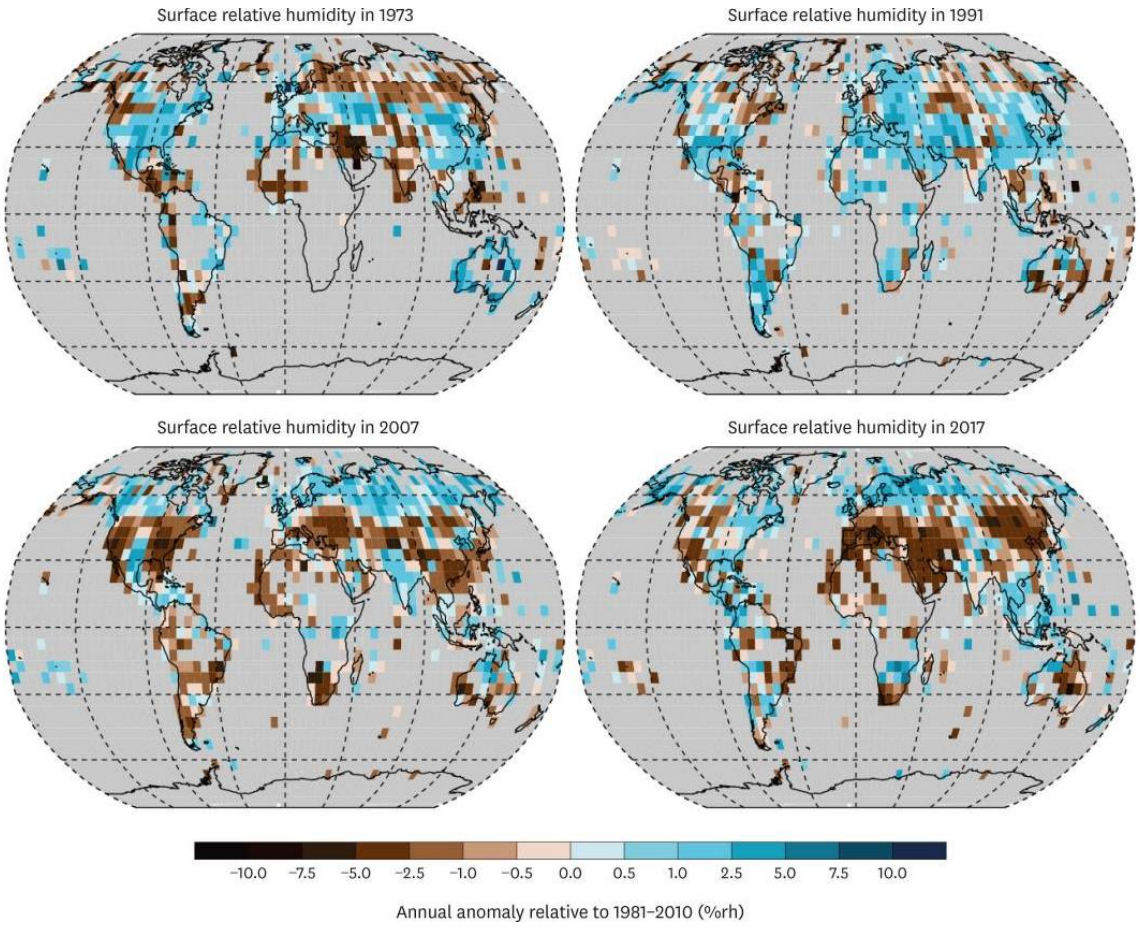


Figure 1.3: Development of relative humidity worldwide between 1973 and 2017

Obtained from [111].

1.5 Allergy in clinical routine

1.5.1 Medical history

The diagnosis of every allergic disease is driven by the medical history of patients, which is documented in detail by the treating physician. The symptoms described by the patients are essential to verify their relation to an allergic disease or unrelated factors. In case of HDM allergy, this entails for example sneezing, rhinorrhea or itching eyes after waking up in the morning and during hoovering. Key symptoms for the diagnosis of allergic asthma are the prevalence of recurrent paroxysmal dyspnea during the night, wheezing, tightness of the chest or persistent cough with or without discharge. Furthermore, potential risk factors such as family predisposition, previously existing allergies or atopic eczema during childhood are documented [113]. The suspected diagnosis is then further verified by either the *in vivo* detection of sIgE through skin prick tests (SPT) or determination of sIgE concentrations in the patient's serum by *in vitro* diagnostic tests.

1.5.2 Skin prick test and specific IgE detection

Both SPT and determination of allergen-specific serum IgE are commonly used to further support the evidence that an allergy is the cause of the patient's symptoms.

Skin tests are an important and widely used tool to support the diagnosis of the different types of allergies such as the epicutaneous patch test for type IV hypersensitivity reactions like contact dermatitis (Table 1) or the SPT for immediate hypersensitivity reactions including inhalant allergies such as HDM and other airborne allergen sources but also food, drug or insect venom allergy [114]. A representative SPT procedure is depicted in Figure 1.4A. Different allergen extracts as well as a histamine solution as a positive control and a negative control, usually a 0.9% saline solution, are applied intradermally on the inner forearm with a lancet. Other localizations can be chosen as well in case of tattoos, dermographism or sun damage. At the peak of reaction, the wheals induced by the respective stimuli are measured in size. A diameter 3–4 mm larger than the negative control, depending on the lancet used for pricking, is interpreted as positive [115]. The advantages of SPT include its cheap, fast and relatively simple execution. However, they do come with a considerable failure rate and numerous contraindications for certain usually skin-associated conditions. False-positive results can be caused by unspecific mast cell degranulation, contaminants in the extracts as well as used technique and devices. Reasons for false-negative results are anti-allergy medications such as

antihistamines that have not been discontinued before testing or if the used allergen extracts are of poor quality [115].

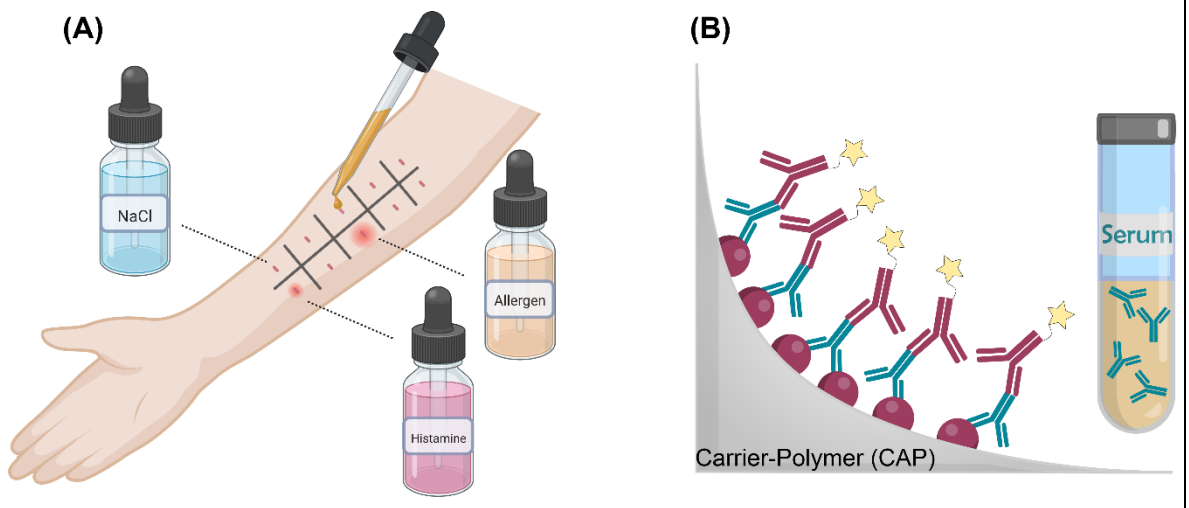


Figure 1.4: Detection of allergen-specific IgE by skin prick test and serum analysis

(A) Illustration of skin prick test procedure. Parts of the figure were created with Biorender.com and pictures were exported under a paid license.
(B) Measurement of sIgE in serum by ImmunoCAP

According to the EAACI, the gold-standard of *in vitro* determination of sIgE concentrations is the fluorescence-based CAP-FEIA (Carrier-Polymer-System-Fluorescence-Enzyme-Immunoassay) assay, more commonly referred to under its commercial name ImmunoCAP (Thermo Fisher Scientific/Phadia, Uppsala, SE). In this fully automated FEIA-system, serum samples of patients are incubated with an excess amount of antigen bound to a polymer carrier matrix and the sIgE is quantified by fluorescence detection (Figure 1.4B). Since this method is calibrated against the WHO 75/502 or WHO 11/234 standard, the amount of IgE can be exactly determined. The results are classified into so called CAP classes with a cut-off of 0.35 kU/l to be considered sensitized with potential clinical relevance [116].

Table 4: Classification of specific IgE concentrations measured by ImmunoCAP

CAP class	Specific IgE concentration
0	< 0.35 kU/l
1	0.35–< 0.7 kU/l
2	0.7–< 3.5 kU/l
3	3.5–< 17.5 kU/l
4	17.5–< 50 kU/l
5	50–< 100 kU/l
6	≥ 100 kU/l

Data derived from [117].

Aside from being independent of any patient's conditions and anti-allergy medications, the ImmunoCAP's biggest advantage lies within its comparably wide range of provided allergens as it not only contains allergen extracts but furthermore recombinant or natural purified molecular allergens, which allows for component-resolved diagnosis (CRD). CRD allows for targeted inquiries regarding cross-reactivity or dedicated marker allergens, such as Ara h 2 or Ara h 6 (*Arachis hypogaea*, peanut) as marker of peanut allergy severity, or Api m 10 (*Apis mellifera*, honey bee) for a prediction of insect venom immunotherapy failure [118]. In addition, the clinical significance of *in vitro* IgE diagnostics and CRD is increasing because more and more SPT-solutions are taken from the market.

Furthermore, CRD allows to establish individual sensitization profiles for the allergic patient. The ImmunoCAP does however only include a selection of molecular allergens which, in contrast to other routine diagnostic options, is comparably wide-ranged but still depicts only a small proportion of known and WHO/IUIS-registered allergens. Four single Der p allergens, namely Der p 1, 2, 10 and 23, are contained in the ImmunoCAP even though 32 allergens are known to date and only extract-based measurement is possible for determination of Der f-specific IgE. Even though multiplex-systems like ImmunoCAP-ISAC or ALEX2 AllergyExplorer with broader allergen ranges are emerging, they are not routinely indicated, and costs are often not reimbursed by health insurances [116, 119, 120].

For most aeroallergens, SPT and ImmunoCAP lead to matching results. However, there are also studies reporting differing results between the two. The quality of SPT is highly dependent on the stability and quality of used allergens extracts, the individual patient and the expertise of the executing person [121]. Especially aqueous diagnostic extracts have been reported to have a high batch-to-batch variety regarding their allergen content as well as big discrepancies between different manufacturers [122]. It therefore can be necessary that both tests are used when the results are inconclusive regarding the medical history.

1.5.3 Allergen challenges

As described in chapter 1.2, the presence of sIgE alone is not obligatory indicative of a clinically relevant allergy but stands for the presence of sensitization only. If the medical history together with SPT or *in vitro* IgE detection does not allow a clear diagnosis, an allergen challenge can be performed, wherein an allergic reaction is provoked by exposing the patient to the allergen source in question. Challenges can take place in nasal, bronchial or conjunctival form for inhalant allergies, in oral form for food or epicutaneously for contact allergies [115]. In clinical routine, allergen challenges are the only option to undoubtedly prove the causal association of

IgE sensitization and allergy symptoms. However, they are laborious and time-consuming for both the patient and practitioner and furthermore bear the risk of severe allergic symptoms or even an anaphylactic shock, which is why they have to be carried out under constant medical / intensive care monitoring. The national German guidelines for diagnosis and treatment of asthmatic diseases (Nationale Versorgungsleitlinie Asthma) recommends to perform nasal and bronchial allergen challenges only for extraordinary inquiries and not routinely [113].

In contrast, oral food challenges (OFC) are still considered the gold standard and are commonly used for the diagnosis of food allergy if the medical history only allows a vague association between symptoms and the ingested foods. Especially children require a clear diagnosis with known clinical relevance due to their common adverse but non-IgE related reactions to food and the potentially life-threatening nature of food allergies [123, 124]. The challenge can be carried out openly as well as double- or single-blinded, wherein the flavor and texture of the allergen source is masked. During the provocation, the respective food is administered in half-logarithmically increasing doses after intervals of 30–40 min each until either clinically objectifiable symptoms occur or a predefined dose, usually corresponding to 3 g of protein from the respective allergen source, is ingested without symptoms. In the latter case, the challenge is repeated the following day before the result can be regarded as negative [124, 125].

1.5.4 Basophil activation test

Despite being a powerful tool to mimic the allergic reaction *ex vivo*, the basophil activation test is not yet routinely used for allergy diagnosis. It is currently only indicated for targeted inquiries after previous tests were inconclusive, or under extraordinary circumstances when IgE detection by skin or serum tests are not possible [126].

This method is based on the degranulation of basophil granulocytes in human blood samples after stimulation with allergen extracts or molecular allergens under physiological conditions and thus imitates the reaction of the human body when exposed to allergens. Basophils are, together with mast cells, a key factor of the allergic reaction happening in the effector phase. As described in chapter 1.2, they express the high affinity IgE receptor FcεRI on their surface, which, in case of a sensitization, can carry sIgE antibodies. When an allergen cross-links two or more antibodies and the adjunct FcεRI receptors, degranulation of the basophils is triggered [127]. Granules inside the basophils, filled with histamine and other proinflammatory mediators, fuse with the cell membrane and release their contents. Thereby, the membrane protein CD63, usually exclusively present on the intracellular histamine vesicle membranes, reaches the cell surface, where it is then accessible for detection by fluorophore-coupled

antibodies and subsequent measurement via flow-cytometry. Unstimulated basophils usually do not express CD63 on their surface and therefore the upregulation of CD63 is highly correlated to histamine release [127-129]. CD203c is the second commonly applied marker for identification and activation assessment of basophils. While it is consecutively present on the cells surface, its expression is further upregulated during basophil activation [130].

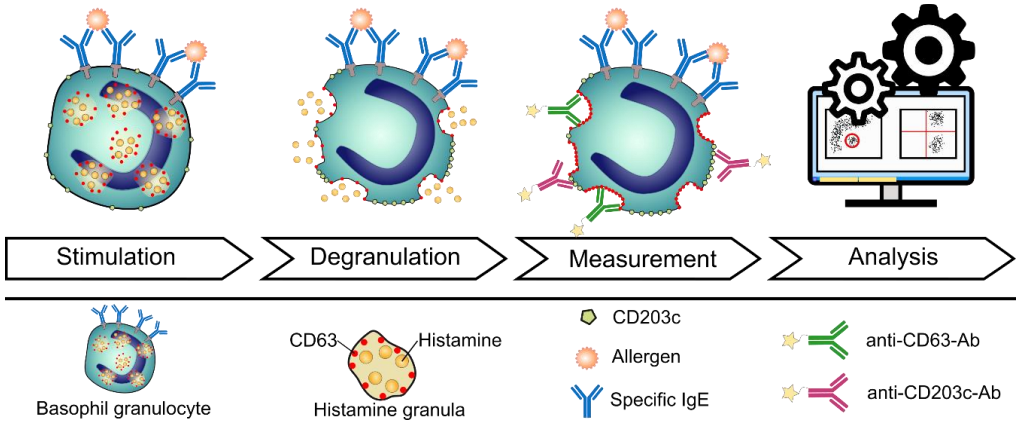


Figure 1.5: Mechanistic illustration of the basophil activation test

One of its biggest advantages over older mediator-release assays such as mast cell or rat basophil leukemia (RBL) assays is its use of whole human blood, which entails not only sIgE but also the donor-specific intrinsic activity and releasability of the effector cells. However, for use in clinical routine, the commercial options are still limited and require a complex establishing process and thorough knowledge of flow-cytometric techniques [115].

Overall, despite recent advancements including a reliable, cost-effective protocol developed within the research group of Clinical and Molecular Allergology, a one-fits-all approach and definitive proof of the basophil activation test to not only differentiate between sensitized and allergic but also allowing to reliably rate the severity of the allergic reaction is missing to make it an unconditionally suitable tool for routine diagnosis [131, 132].

1.5.5 Therapy

While there are numerous options for the symptomatic treatment of allergic diseases such as antihistamines, corticosteroids, or biologicals, the only available causal treatment is allergen-specific immunotherapy (SIT), whereby tolerance shall be induced by a long-term low-dose exposure to the allergen source itself. Forms of SIT available for the treatment of HDM allergy include subcutaneous SIT (SCIT) by quarterly administration of a higher-dosed allergen depot or sublingual SIT (SLIT) with daily self-administered drops or tablets. Other types of

immunotherapies mainly used for the treatment of food allergies include oral and, a rather recent development, the epicutaneous form of administration [133, 134].

The induction of tolerance is a complex mechanism involving several cellular and humoral adaptions. The most notable ones entail the "exhaustion" and subsequent anergy of effector cells by negative feedback loops, IL-10 induced production of protective IgG4 antibodies and normalization of Th_2 immune responses [135-141].

While several meta-analyses confirm the efficacy of SIT for indications such as allergic rhinitis and asthma, the high heterogeneity of available products and patient populations do not allow a generalized statement [142-145]. Just like extracts used for diagnosis, the active ingredients of SIT undergo highly variable production processes which are poorly regulated by authorities and not standardized across different manufacturers, as e.g. the guidelines from the European Medicines Agency (EMA) only propose the analysis of major allergen content but leave the choice of "relevant allergens" for the products to the manufacturers [133, 146].

1.6 Aims of the thesis

Allergic diseases in general and house dust mite (HDM) allergy in particular are a worldwide problem that will continue to gain importance due to steadily increasing prevalences. Adequate diagnosis and therapy options are therefore an essential prerequisite to provide optimal healthcare for the increasing number of patients. Despite over 30 allergens of HDM being known today, the clinical relevance and immunological impact of many of them remain elusive, as the majority of available research is still centered on the major allergens Der p 1 and Der p 2. As a result, available extracts for the diagnosis and therapy of allergic patients are often poorly monitored for their non-major allergen content and might therefore lack potentially important allergens. Furthermore, most non-major allergens are not available for component-resolved diagnosis in clinical routine. This thesis therefore sought to investigate the clinical relevance of several non-major allergens, their involvement in eliciting allergic responses and the promotion of proinflammatory environments in the lung on a cellular level to identify potentially important allergens that subsequently can be integrated in diagnostic tools used in clinical routine in the future to overall improve the diagnosis of allergic patients. In particular the effects of lipid-associated allergens were of interest, as lipophilicity in other allergies is often connected to enhanced allergenicity and augmentation of the allergic response, which constitutes a little-investigated phenomenon in HDM allergy although several allergens with lipid-association have been reported in HDM.

To address this wide knowledge gap in non-major HDM allergens, a step-wise approach shall be followed in this thesis:

For the study of individual IgE sensitization patterns of patients, a selection of house dust mite allergens, containing major, mid-tier as well as minor allergens, shall first be recombinantly produced and tested for purity and proper folding.

Subsequently, these allergens shall be tested with sera from both children and adults to establish individual IgE sensitization profiles and use those to identify allergens that are associated with different allergic diseases such as allergic asthma or atopic dermatitis, and might thus act as marker allergens for these phenotypes, as well as allergens that play a role particularly in the initiation of sensitization and asthma in children, so-called "initiator allergens". For the analysis of children's sera in particular, Der f homologues of the previously identified potential biomarker allergens shall be included to evaluate the potentially increasing relevance of these allergens. Due to the global climate change, *D. farinae* populations seem to increase in Middle Europe, which may result in Der f allergens dominating the initial sensitization phase of children rather than Der p allergens.

Basic pathomechanistic insight shall be gained through investigating the potential of these allergens to promote allergic responses aside from the IgE-mediated immune response but furthermore through interactions with the epithelium of the lung. In this part of the project, the allergens shall be applied as stimuli in a bronchial epithelial cell culture model, measuring the allergens' influence on the release of various proinflammatory, asthma-associated mediators in the lung, which may allow for evaluating a possible "organ specificity" of these allergens.

Moreover, the direct interaction of the different allergens with primary cells during the IgE-mediated allergic response, more specifically with basophil granulocytes in the blood of the patients, shall be investigated using the basophil activation test (BAT). Since allergen challenges are not routinely indicated for HDM-allergic patients and due to the SARS-CoV-19-related hygiene restrictions, the originally planned monitoring of basophil reactivity profiles of HDM-allergic patients with the indication of an allergen-specific immunotherapy was not feasible. Instead, after conducting a proof-of-principle study in HDM-allergic patients, peanut allergy shall be chosen as a model disease in this part of the project to evaluate the potential of the BAT to replace allergen challenges in order to improve the diagnosis of allergic patients in clinical routine by utilizing the ability of single allergens to imitate the allergic reaction with fresh whole blood basophils *ex vivo* and use this assay to predict the prevalence of a clinical manifest allergy as well as its severity without the need of invasive and laborious allergen challenges.

2 Material and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals used in this thesis were, if not stated otherwise, purchased in research grade or analytic grade (*per analysis*, p.a.) from the following manufacturers: AppliChem (Darmstadt, DE), Bio-Rad (Hercules, USA), Carl Roth (Karlsruhe, DE), Merck (Darmstadt, DE), Serva Elektrophoresis (Heidelberg, DE), Sigma-Aldrich (Steinheim, DE) and Thermo Fisher Scientific (Freiburg, DE). All solutions and buffers were prepared with ultra-purified water from a Milli-Q Reference system (Merck Millipore, Darmstadt, DE), in the following abbreviated as ddH_2O.

2.1.2 Consumables

Consumables used in the experiments within the thesis were purchased from Capricorn Scientific (Ebsdorfergrund, DE), Corning Inc. (Corning, USA), Cytiva/ex. GE Healthcare (Chalfont St. Giles, UK), Greiner BioOne (Kremsmünster, AT), Merck Millipore (Darmstadt, DE), PolyAn (Berlin, DE), Sarstedt (Nümbrecht, DE), Sigma-Aldrich (Steinheim, DE), Thermo Fisher Scientific (Freiburg, DE) and VWR (Darmstadt, DE).

2.1.3 Materials obtained through material transfer agreements

Natural (n)Der p 1 and recombinant (r)Der f 1 were obtained from Allergopharma GmbH (Reinbek, DE) supported by a material transfer agreement.

2.1.4 Buffers

Commonly used buffers and their composition are listed in Table 5.

Table 5: Commonly used buffers and their composition

Phosphate-buffered saline (PBS, pH 7.2)	1,4 mM	KH_2PO_4
	2,7 mM	KCl
	8 mM	Na_2HPO_4 x 2 H_2O
	122 mM	NaCl
Phosphate-buffered saline with Tween-20 (PBST, pH 7.2)	PBS with 0.05% (v/v) Tween-20	
Tris-buffered saline, 10x (TBS, pH 7.2)	1 M	Tris
	1 M	NaCl
	50 mM	$MgCl_2$ x 6 H_2O
Tris-buffered saline with Tween-20 (TBST, pH 7.4)	TBS with 0.05% (v/v) Tween-20	
Tris-buffered saline, 5x (TBS, pH 9.5)	500 mM	Tris
	500 mM	NaCl
	25 mM	$MgCl_2$ x 6 H_2O

2.1.5 Bacterial strains

Three different strains of *Escherichia coli* (*E. coli)* were used in the experiments (Table 6). Molecular biology techniques were carried out exclusively in BL21 cells. Rosetta gami, SHuffle and BL21 cells were used for protein expression. When the cells carry a suitable plasmid after transformation, all of the used strains express recombinant proteins in an isopropyl-ß-D-thiogalactopyranosid (IPTG)-dependent manner. Furthermore, IPTG-independent induction is possible in specialized autoinduction media that rely on glucose for temporary suppression of induction until the culture has grown sufficiently, and lactose for subsequent automatic induction [147, 148]. Both ways lead to expression of T7 RNA polymerase by either directly inducing the lac promoter (autoinduction) controlling the T7 gene with the addition of lactose or by inhibiting the lac repressor (IPTG). This polymerase catalyzes the transcription of the inserted gene of interest controlled by the T7 promoter and is therefore essential for recombinant protein expression.

Table 6: Strains of *E. coli* for cloning and expression of HDM allergens

Strain	Genotype	Manufacturer
E. coli BL21(DE3)	BF^- *dcm ompT hsdS* ($r_B^- m_B^-$) *gal λ* (DE3)	New England BioLabs (Frankfurt/Main, DE)
E. coli Rosetta gami(DE3)	*Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL* (DE3) *F'[lac+ lacIq pro] gor522::Tn10 trxB pRARE2 (CamR, StrR, TetR)*	New England BioLabs (Frankfurt/Main, DE)
E. coli SHuffle T7	F´ *lac, pro, lacI*^{q} */ Δ(ara-leu)7697 araD139 fhuA2 lacZ::T7 gene1 Δ(phoA)PvuII phoR ahpC* galE (or U) galK λatt::pNEB3-r1-cDsbC* ($Spec^R$, *lacI*^{q}) *ΔtrxB rpsL150*(Str^R) *Δgor Δ(malF)3*	New England BioLabs (Frankfurt/Main, DE)

2.1.6 Recombinant protein expression media

All media were made from ultrapure water (ddH_2O) and autoclaved. Cultivation and selection of *Escherichia coli* (*E. coli)* was performed in LB (lysogeny broth), 2x YT (yeast-extract tryptone) or TB (terrific broth) media supplemented with either 0.01% (w/v) ampicillin or 0.01% (w/v) of ampicillin, chloramphenicol and tetracycline each (Carl Roth GmbH & Co. KG), dependent on the used strain of *Escherichia coli* (*E. coli*). Details of used strains and respective media are listed in Table 7. The additives for TB autoinduction medium were prepared like the ZYM-5052 medium described by F.W. Studier [148].

Table 7: Overview of growth media, agar plate media and *E. coli* strains.

Medium	Components	Additives	Use
LB	1% (w/v) NaCl 1% (w/v) Peptone 0.5% (w/v) Yeast extract	Ampicillin	Growth
		Ampicillin + Agar	Plating
		50% (v/v) Glycerol	Cryoconservation
2x YT	0.5% (w/v) NaCl 1.6% (w/v) Peptone 1% (w/v) Yeast extract	Ampicillin	Growth of BL21
		Ampicillin + Chloramphenicol + Tetracyclin	Growth of Rosetta gami
TB	0.5% (w/v) Glycerol 2.4% (w/v) Peptone 1.2% (w/v) Yeast extract	Ampicillin 17 mM KH_2PO_4 72 mM K_2HPO_4	Growth of SHuffle
		Ampicillin 50 mM Na_2HPO_4 50 mM KH_2PO_4 25 mM $(NH_4)_2SO_4$ 0.5% (v/v) glycerol 0.05% (w/v) glucose 0.2% (w/v) α-lactose 2 mM $MgSO_4$	Autoinduction of SHuffle

All antibiotic additives were added at 0.01% (w/v) each.

2.1.7 Vectors

The vectors listed in Table 8 were used to carry out cloning and expression of the recombinant HDM allergens.

Table 8: Used vectors with respective size and antibiotic resistance

Vector	Resistance	Size	Application	Manufacturer
pMAT	Ampicillin	2274 bp	Supplied by the manufacturer containing the ordered gene sequence of the respective HDM allergen	GeneArt AG / Thermo Fisher Scientific (Regensburg, DE)
pET-17b	Ampicillin	3306 bp	Expressions vector for HDM allergen expression	Merck Millipore (Darmstadt, DE)
pET-23b	Ampicillin	3365 bp	Expression vector for HDM allergen including an optional C-terminal His-tag	Merck Millipore (Darmstadt, DE)

2.1.8 Human materials

The human materials analyzed in this thesis were collected within studies that were approved by the ethics committee of the University of Luebeck (Reference no. 13-136 and 10-126) or Hannover Medical School (MHH, reference no. 5582 and 9329_BO_K_2020).

All patients, or their legal guardians in case of underage participants, were provided with a thorough study information sheet outlining planned tests and collected samples. Following the declaration of Helsinki, all participants signed written informed consent before enrolment after talking to the recruiting physician and being informed about all possible risks.

HDM-allergic patients were recruited during visits in the specialized allergy outpatient clinics at Research Center Borstel (RCB) or the University Hospital Center Luebeck (UKSH), both Depts. of Pneumology. AA was diagnosed according to the official current German guideline on asthma (“Nationale Versorgungsleitlinie Asthma” / National Disease Management Guide Asthma). In addition to assessment by trained allergologists during visits and medical history, all study participants filled out a detailed questionnaires documenting HDM-related allergic symptoms, including but not limited to: symptom development or exacerbation after start of the heating season and/or while dusting the flat, development of bronchial or skin related symptoms after house dust exposure in different scenarios, prevalence of medically confirmed AA, medication and history of allergen-specific immunotherapy (SIT). sIgE concentrations against Der p extract, Der f extract, rDer p 1 and rDer p 2 were measured routinely by CAP-FEIA. sIgE concentrations against rDer p 10 were determined if indicated by suspected *Crustaceae* cross-

reactivity. sIgE concentrations against rDer p 23 were only determined for a small part of study participants.

Furthermore, sera from HDM-allergic AD patients were obtained from Hannover Medical School (MHH). These participants were *post hoc* provided with the same questionnaire as UKSH/RCB recruited patients. AD was diagnosed by trained dermatologists based on the U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis and patients had to have at least a 1-year history of AD [149]. Severity grades of AD were assessed by trained dermatologists based on an investigator's global assessment equivalent to Simpson et al. [150]. Mild AD was defined as slight erythema, induration and/or lichenification. Moderate AD included clear erythema, induration and/or lichenification with possible oozing and crusting, whereas severe AD was defined as deep or bright red erythema, severe induration and/or lichenification as well as a widespread manifestation. sIgE concentrations against Der p extract were measured routinely by CAP-FEIA.

Sera of asthmatic and wheezing children during an acute bronchial obstruction as well as of non-asthmatic children were obtained from the Helios University Hospital Wuppertal (UHW).

Heparinized blood from children with a suspected peanut allergy was obtained from the Department of Dermatology, Elbe Klinikum Buxtehude (EKB) before undergoing an OFC. The children had a possible history of peanut-induced allergic reactions and increased levels of serum IgE determined by ImmunoCAP against at least one of the analytes listed in Table 9.

Table 9: Overview of study populations

Study Population	No. of patients	Allergic Disease	Recruitment	ImmunoCAP measurements
SP1	199	HDM allergy with predominant airway symptoms	RCB / UKSH	Der p extract Der f extract rDer p 1 rDer p 2 rDer p 10 rDer p 23
SP2	185	HDM allergy with predominant AD	MHH	Der p extract
SP3	143	Acute asthmatic exacerbation	UHW	Aeroallergen mix sx1 Der p extract [a]
SP4	15	Suspected peanut allergy	EKB	Ara h extract rAra h 1 rAra h 2 rAra h 3 rAra h 8 rAra h 9

[a] Measurement only conducted when sx1 screening turned out positive

2.1.9 Instruments

Documentation systems

- Chemiluminescence/fluorescence/UV imager, ChemiDoc MP (Bio-Rad, Hercules, USA)
- Scanner, Canoscan 5600F (Canon, Mississauga, CA)
- Gel documentation system, Gel Doc XR+ (Bio-Rad, Hercules, USA)
- Infrared imager, Odyssey CLx (Li-Cor, Lincoln, USA)

Electrophoresis and blotting

- Elektrophoresis chamber, model 45-2010-1 (PeqLab, Erlangen, DE)
- Elektrophoresis chamber, Novex Mini-Cell (Invitrogen, Carlsbad, USA)
- Elektrophoresis chamber, Wide Mini-Sub Cell (Bio-Rad, Hercules, USA)
- Semi-dry blotter, Pegasus (Phase, Lübeck, DE)
- Voltage supply, Modell 200/2.0 (Bio-Rad, Hercules, USA)
- Voltage supply, Modell E835 (Consort, Turnhout, B)

Liquid chromatography systems

- ÄKTAstart (GE Healthcare, Chalfont St. Giles, UK)
- ÄKTApurifier (GE Healthcare, Chalfont St. Giles, UK)
- ÄKTApure (GE Healthcare, Chalfont St. Giles, UK)

Mass spectrometry

- Hybrid quadrupole-orbitrap mass spectrometer, QExactive (Thermo Fisher Scientific, Freiburg, DE)

Flow cytometry

- Flow cytometer, LSR II (BD, Heidelberg, DE)
- Flow cytometer, FACSymphony A1 cell analyzer (BD, Heidelberg, DE)

Microscopy

- Inverted microscope, Wilovert (Will, Wetzlar, DE)

Scales

- Laboratory scale, Sartorius Universal U3600P (Sartorius AG, Göttingen, DE)
- Precision scale, Sartorius Analytic A200S (Sartorius AG, Göttingen, DE)

Centrifuges

- Cooling centrifuge, Avanti J-26S XPI (Beckman Coulter, Jersey City, USA)
- Cooling centrifuge, 5910 Ri (Eppendorf AG, Hamburg, DE)
- Cooling centrifuge, Rotanta 460R (A. Hettich, Tuttlingen, DE)
- Cooling centrifuge, Sigma 3-18K (Sigma Laborzentrifugen, Osterode, DE)
- Centrifuge, Mikro 200 (A. Hettich, Tuttlingen, DE)
- Centrifuge, Modell 5424 (Eppendorf AG, Hamburg, DE)

Other Instruments

- Class II safety cabinet, Mars (ScanLaf, Allerød, DK)
- Horizontal shaker, SM 30 (Edmund Bühler GmbH, Hechingen, DE)
- Incubation chamber, BBD 6220 (Heraeus Holding, Hanau, DE)
- Laminar air safety bench, HLB 2448 (Heraeus Holding, Hanau, DE)
- Magnetic hotplate stirrer, C-MAG HS 7 (IKA Labortechnik, Staufen, DE)
- Microarray printer, Nano-Plotter NP2 (GeSiM, Radeberg, DE)
- Microwave, Duostar (Privileg, Stuttgart, DE)
- Multi-mode microplate reader, SpectraMax M5 (Molecular Devices, San Jose, CA, USA)
- Orbital incubation shaker (refrigerated), Innova 44R (Eppendorf, Hamburg, DE)
- Orbital incubation shaker, ThermoForma 420 (Thermo Fisher Scientific, Freiburg, DE)
- Orbital shaker, KS501 digital (IKA Labortechnik, Staufen, DE)
- pH meter, CG 842 (Schott-Geräte, Mainz, DE)
- Pipettes, Eppendorf Research (plus), 10/100/1000 µl (Eppendorf, Hamburg, DE)
- Roller-mixer, Modell rm5.40 (Karl Hecht, Sondheim, DE)
- Rocking shaker, WT16 (Biometra, Göttingen, DE)
- Spectral photometer, BioPhotometer (Eppendorf, Hamburg, DE)
- Spectral photometer, NanoDrop 1000 (Thermo Fisher Scientific, Freiburg, DE)
- Thermal mixer, Thermomixer 5436 (Eppendorf AG, Hamburg, DE)
- Thermocycler, T Personal (Biometra, Göttingen, DE)
- Ultrasonic bath, Sonorex Super RK102 H (Bandelin Electronic GmbH, Berlin, DE)
- UV-Photometer, Infinite 200m (Tecan, Männedorf, CH)
- Vacuum Concentrator, SpeedVac (Thermo Fisher Scientific, Freiburg, DE)
- Vortex-Mixer, Vortex Genie 2 (Scientific Industries, New York, USA)
- Warming chamber, UN 30 (Memmert, Schwabach, DE)

2.1.10 Software

Among standard software such as Microsoft Office the following software listed in Table 10 were also part of this work.

Table 10: Used Software

Software	Manufacturer/Reference	Location
AlphaFold	[151, 152]	-
BLAST	National Library of Medicine	Bethesda, USA
CDToolx	[153]	-
Clustal Omega	[154]	-
FACSDiva v8.0.1	BD Biosciences	San Jose, USA
FCS Express v7.0	De Novo Software	Glendale, USA
FindPept tool	[155]	-
Image Lab v5.2.1	Bio-Rad	Hercules, USA
Image Studio v4.0	Li-Cor Biosciences	Lincoln, USA
K2D3	[156]	-
MSD Discovery Workbench	Mesoscale Discovery	Rockville, USA
PDBsum	[157]	-
Prism v9.0	GraphPad Software	San Diego, USA
Quantity One v4.6.1	Bio-Rad	Hercules, USA
RStudio	R Studio Inc.	Boston, USA
SequencePro v2.10	Applied Biosystems	Weiterstadt, DE
Serial Cloner v2.6.1	[158]	-
SignalP	[159]	-
STRIDE	[160]	-
Unicorn Control Software v5.01	GE Healthcare	Chalfont St. Giles, UK
UniProt	[161]	-
Xcalibur v4.027.19	Thermo Fisher Scientific	Bremen, DE

v: Version

2.2 Methods

2.2.1 Production of recombinant HDM allergens

During the course of this work, several HDM allergens of Der p (Der p 2, Der p 5, Der p 7, Der p 10, Der p 13, Der p 20, Der p 21, Der p 23) and Der f (Der f 2, Der f 5, Der f 20, Der f 21) were recombinantly expressed and purified. Expression of Der p allergens was already established in *E. coli* Rosetta gami at the start of the thesis. However, yields were low and DNA constructs were not optimized for the host organism. Therefore, the constructs were host-adapted, the expressing *E. coli* strain was switched to SHuffle cells and the expression method was optimized. Expression of Der f allergens was newly established within the course of the thesis. All allergens were selected from the official WHO/IUIS database.

2.2.1.1 Design of DNA sequences

Non-optimized DNA constructs for Der p allergens were already present at the start of the thesis. These constructs were utilized for protein expression in *E. coli* Rosetta gami and did not have a C-terminal His-tag as this was provided by the used pET-23b vector.

DNA sequences were retrieved from the NCBI database. All sequences were checked for possible signal peptides with SignalP (Table 10) and if indicated, signal peptides were clipped from the sequence. DNA constructs were designed in the pMAT vector with the GeneArt Online tool (GeneArt AG/Thermo Fisher Scientific, Regensburg, DE) including the cleaving sites for the restriction enzymes EcoRI and NdeI. A C-terminal His-tag was added to enable affinity purification. The sequences were subsequently optimized to *E. coli* as the host organism to improve expression effectivity and yield.

2.2.1.2 Amplification of DNA insert by PCR

DNA polymerase, buffer, primers and water were combined as indicated in Table 11. The lyophilized DNA inserts were dissolved in 50 µl ddH_2O. 1 µl of this solution was combined with 19 µl of the PCR mixture. The DNA was then amplified with a PCR cycler, the detailed program is described in Table 12. The annealing temperature was optimized to match the melting temperatures of forward and reverse primers.

Table 11: Composition of PCR master mix

Dream Taq Hot Star Green PCR Master Mix	50 µl
T7 Primer (50 pmol/µl)	3 µl
pMATrev-Primer (50 pmol/µl)	3 µl
ddH2O	44 µl

Table 12: PCR program

94°C	2 min		Initial Denaturation
94°C	30 sec		Denaturation
57°C	45 sec	35 cycles	Annealing
72°C	30 sec		Extension
73°C	5 min		Final Extension
Cooling			

2.2.1.3 Analytic agarose gel electrophoresis

Successful amplification and restriction of the genetic sequence (refer to chapter 2.2.1.2 and 2.2.1.6) was verified by an analytic agarose gel electrophoresis, the method of which has been described by Sambrook and Russel [162]. To cast a 1.2% (w/v) agarose gel, 0.6 g agarose were weighed in and suspended in 50 ml TAE buffer (Tris-acetate EDTA buffer pH 8.5, 1 mM EDTA, 40 mM Tris-acetate). After heating in the microwave, 50 µl GelRed dye (10,000x concentrate, Merck, Darmstadt, DE) was added and the solution was poured in a 15 x 7 cm gel chamber. When the gel was fully set, the wells were loaded with the PCR samples, a standard-sized DNA marker (100–10,000 bp) and PCR-grade H_2O (free from RNAse and DNAse) as control. The electrophoresis was carried out at 100 V for 60 min, after which the DNA bands were detected by UV (ultraviolet) light and documented via a GelDoc XR+.

2.2.1.4 Preparative agarose gel electrophoresis

To isolate the DNA of interest after amplification and restriction of the constructs (refer to chapter 2.2.1.2 and 2.2.1.6), the agarose gel electrophoresis was carried out on a preparative scale and UV-visualized bands indicating DNA of the desired size were excised with a scalpel. The contained DNA was extracted with a GeneJET Gel Extraction Kit (Thermo Fisher Scientific). After solubilization of the agarose gel bands by addition of the chaotropic agent guanidine thiocyanate, the contained DNA was bound to a silica-based membrane and eluted by 2x 25 µl PCR-grade H_2O after several washing steps.

2.2.1.5 Determination of DNA concentration and purity

A spectral photometer (NanoDrop) was used to determine concentration and purity of DNA isolated from preparative agarose gels (refer to chapter 2.2.1.4) or of plasmid DNA purified from transformed *E. coli* (refer to chapter 2.2.1.9). 1.5 µl of ddH_2O was loaded onto the sample pedestal for blank subtraction. Subsequently, the absorption of each sample was measured from 230–320 nm. The DNA concentration was calculated by the photometer based on the Lambert-Beer law. Further measures were absorption ratios at 260 nm/280 nm, corresponding to the absorption maxima of DNA and proteins, respectively, as well as 260 nm/230 nm, which is a secondary purity measure indicating impurities caused by chaotropic agents and organic compounds. Both ratios should result in a value around 1.8, whereas a higher value indicates contamination by RNA, and a lower value suggests contamination by salts or proteins.

2.2.1.6 Restriction of DNA

After amplifying the DNA of HDM allergen constructs, the PCR products were cut at the designed restriction sites NdeI and EcoRI in preparation to subsequently insert them into the pET-17b vector. Briefly, 25 µl of the DNA isolated from preparative agarose gel electrophoresis was combined with 19 µl PCR-grade H_2O, 5 µl sample buffer (10x Fast Digest Green Buffer, Thermo Fisher Scientific) and 0.5 µl each of the restriction enzymes NdeI and EcoRI. The sample was incubated for 60 min at 37°C on a thermal shaker, after which the reaction was stopped by heat inactivation at 80°C for 5 min. Success of restriction was verified by agarose gel electrophoresis, and the desired bands were excised and purified as described in chapter 2.2.1.4. The vector pET-17b was treated the same way to cut open the plasmid backbone for subsequent insertion of the DNA construct. The vector map is depicted in Appendix 1.

2.2.1.7 DNA ligation

The isolated and cut DNA constructs were ligated into the restricted pET-17b vector. The plasmid is generated by annealing of the compatible overhangs (“sticky ends”) that were generated by the restriction enzymes. The ends were covalently and enzymatically linked by T4-DNA-Ligase (Rapid DNA Ligation Kit, Thermo Fisher Scientific). The proportions of used reagents were calculated according to the method of R. Cranenburgh [163]. Ligation was carried out at a vector/insert ratio of 1:3 for 1 h at room temperature (RT) or at 4°C overnight.

Variation of the method:

The vector/insert ratio for the ligation of the rDer f 5 DNA construct was 1:5 to ensure a successful ligation.

2.2.1.8 Production of competent *E. coli* cells

Since *E. coli* cells are not naturally able to take up foreign DNA, they were made competent for transformation by using the calcium chloride method [164]. 5 ml of LB medium were inoculated with BL21 or SHuffle cells and grown overnight at 37°C on an orbital shaker at 200 rpm. This culture was used to further inoculate 20 ml of LB medium that was grown to mid-log phase (OD 0.6–0.8) under the same conditions. The bacteria suspension was incubated on ice for 10 min and subsequently centrifuged at 5000 x g for 10 min at 4 °C. Afterwards, the supernatant was discarded, the bacterial pellet resuspended in 10 ml ice-cold calcium chloride buffer (100 mM $CaCl_2$) and incubated on ice for additional 30 min. The now competent cells were centrifuged as before and the pellet was resuspended in calcium chloride-glycerol buffer (100 mM $CaCl_2$, 10% (v/v) glycerol) for cryoconservation. 200 µl aliquots were dispensed in pre-cooled 1.5 ml reaction tubes and immediately frozen in liquid nitrogen. Aliquots were stored at -80°C for further use.

2.2.1.9 Transformation of competent cells by heat shock

To transform *E. coli* BL21 cells with the respective ligated DNA plasmid containing the desired allergen sequence, an aliquot of competent cells was thawed for 10 min on ice and subsequently incubated for 30 min on ice with all of the reaction mixture obtained after ligation (refer to chapter 2.2.1.7). Afterwards, the cells were transformed by heat shock at 42°C for 60 sec, and cooled down for 10 min on ice before 800 µl of LB medium was added. The cells were incubated for 60 min at 37°C on a thermal shaker. Afterwards 50 µl, 100 µl, 250 µl and the rest of the resulting pellet after brief centrifugation at 5000 x g were plated on agar petri dishes (LB medium supplemented with ampicillin, Table 7) with a Drigalski spatula in a safety cabinet. Incubation of the plates was carried out at 37°C overnight.

Variation of the method:

After successful transformation of BL21 cells and confirmation that the inserted plasmid was correct, isolated plasmid DNA from these cells was used to subsequently transform SHuffle cells. The method was carried out as described with the exception that only 1 µl of isolated plasmid DNA was added to the thawed competent cells.

2.2.1.10 Selection and colony PCR

After transformation of BL21, five different clones from each of the resulting agar plate were picked with a pipette tip and dissolved in 50 µl of PCR-grade water in separate 1.5 ml reaction tubes. 1 µl of each tube was combined with 19 µl of the PCR master mix (Table 11) and stored

on ice. The PCR was carried out as described in chapter 2.2.1.2 and the correct size of the expected DNA amplicon was verified through analytical agarose gel electrophoresis (refer to chapter 2.2.1.3). The rest of the bacteria suspension from verified clones was used to inoculate 10 ml of LB medium supplemented with ampicillin (Table 7), which subsequently was grown overnight at 37°C on an orbital shaker at 200 rpm.

2.2.1.11 Preparation of glycerol stocks

In order to cryoconserve transformed and verified *E. coli* clones, 2 ml of bacterial culture grown overnight were centrifuged at 5000 x g for 2 minutes in a 2 ml PCR-grade reaction tube (RNAse-free, DNAse-free). The resulting bacterial pellet was resuspended in 1 ml LB medium supplemented with 50% glycerol (Table 7) and immediately frozen in liquid nitrogen. The tubes were stored at -80°C for further use.

2.2.1.12 Isolation of plasmid DNA

The plasmid DNA of the selected BL21 clones was isolated from the remaining 8 ml of overnight cultured *E. coli* (refer to chapter 2.2.1.10 and chapter 2.2.1.11). After centrifuging at 1000 x g for 10 minutes, the supernatant was discarded and the pellet was treated according to the instructions of the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Freiburg, DE). After alkaline lysis, the cell debris was precipitated by addition of SDS, immediately neutralized and the plasmid DNA was bound to a silica-based membrane. The DNA was eluted with 20 µl PCR-grade H_2O after several washing steps and a second elution step was performed with 15 µl PCR-grade H_2O. Concentration and purity of the eluted DNA was determined as described in chapter 2.2.1.5. If the concentration exceeded 100 ng/µl, the sample was diluted with PCR-grade H_2O. If the concentration was below 50 ng/µl, it was concentrated in a SpeedVac Vacuum Concentrator. Plasmids were stored in a PCR-grade reaction tube at -20°C.

2.2.1.13 DNA sequencing as ligation control

In order to ensure that a plasmid with a correct and complete DNA insert was taken up by the transformed clone and to verify that no errors occurred during the PCR by the Taq polymerase, which can e.g. lead to point mutations in the DNA sequence, 15 µl of the purified plasmid DNA was sent to a third-party laboratory (Eurofins Genomics, Köln, DE) for DNA Sanger sequencing. Results were analyzed with SerialCloner (Table 10). Clones with faulty inserts were discarded, and plasmid DNA from clones carrying the correct insert were used to transform *E. coli* SHuffle cells as described in the variation of chapter 2.2.1.9.

Variation of the method:

For inserts longer than 1000 bp (rDer f 20), two tubes were sent for forward and reverse sequencing.

2.2.1.14 Expression of recombinant HDM allergens

The expression of recombinant Der p allergens in Rosetta gami cells was already established at the beginning of this work and was carried out to produce further amounts of protein. Because of low yields, the expression was altered and established in SHuffle cells after the DNA constructs underwent host-specific optimization described in chapter 2.2.1.1.

All Der f allergens were expressed simultaneously in BL21, SHuffle cells in regular growth medium and SHuffle cells in autoinduction medium. Optimized Der p allergens were expressed in SHuffle cells in regular growth medium.

For the expression of recombinant allergens, clones of BL21 cells with a correct insert, verified by DNA sequencing, and/or clones of SHuffle cells after transformation with the respective plasmid DNA and ampicillin selection were chosen. Frozen glycerol stocks of the clones carrying the desired insert (refer to chapter 2.2.1.11) were scraped with a sterile pipette filter tip to inoculate 15 ml of the growth media for the specific strains listed in Table 7. After culturing overnight at 37°C on an orbital shaker (200 rpm), the 15 ml were transferred into 1 l of medium in a shake-flask with baffled bottoms for improved oxygen supply. Culture conditions remained unaltered, and growth was monitored by periodical measurement of OD_{600}, for which 1 ml of bacterial suspension was transferred into a cuvette (Eppendorf AG), and optical density was measured at 600 nm on a spectral photometer (BioPhotometer, Eppendorf AG). Cells were cultured until they reached the mid-log growth phase (OD_{600} = 0.6–0.8), at which point SHuffle cells were temperature shocked in an ice bath for 15 min and subsequently transferred to a 16°C environment. BL21 cells remained at 37°C. At this point, expression was induced by the addition of 400 µl 1 M IPTG for BL21 and SHuffle cells in regular growth medium. SHuffle cells in autoinduction medium were not supplemented with IPTG. Culturing was continued overnight and bacteria were harvested the next day.

Variation of the method:

In the following the non-optimized expression method in Rosetta gami cells for Der p allergens is described. The respective glycerol stocks were thawed and a sterile metal loop was used to inoculate 5 ml of the growth medium (Table 7). Cells were cultured overnight at 37°C on an orbital shaker at 250 rpm, and the bacterial suspension was transferred into 100 ml of growth medium the next day. After culturing for 6 h at the same conditions, the bacterial suspension

was divided into 2 flasks containing 2 l of growth medium each. The flasks were incubated overnight (37°C, 190 rpm) and expression was induced the next day with 2 ml 1 M IPTG each when OD_{600} reached 2.0–3.0. Cells were harvested after 4 h of expression.

2.2.1.15 Lysis of *E. coli* cells

After expression was complete, 500 ml of the bacterial suspension were centrifuged in 1000 ml plastic screw cap bottles each at 5000 x g for 10 min. The pellet of each bottle was resuspended in 15 ml of binding buffer (300 mM NaCl, 50 mM Na_2HPO_4, pH 8.0), and the contents of matching screw cap bottles were joined together in a 50 ml conical centrifuge tube. The cell membranes were lysed by the addition of 0.4 g octylthioglucoside (Goldbio, St. Louis, MO, USA), and free nucleic acids were degraded with 0.025% (v/v) endonuclease (MoBiTec Molecular Biotechnology, Göttingen, DE). After incubation for 60 min at RT on a roller-mixer, the tubes were centrifuged at 4000 x g for 20 min at RT. The supernatant containing the desired proteins was used for manual immobilized metal affinity chromatography (IMAC) as described in chapter 2.2.2.1 and the bacterial pellet was discarded.

2.2.2 Purification of recombinant HDM allergens

2.2.2.1 Immobilized metal affinity chromatography

As the first purification step, the His-tag of expressed proteins was utilized to perform manual immobilized metal affinity chromatography (IMAC). This specialized form of chromatography is based on the affinity of histidine towards metal ions to bind them on a resin matrix with covalently bound metal chelates such as Ni^{2+} and Co^{2+}.

After washing 10 ml of cobalt resin material (HisPur™ Cobalt Resin, Thermo Fisher Scientific) in a 50 ml conical centrifugation tube with 3x 20 ml ddH_2O and 2x 20 ml binding buffer (Table 13), the resin was added to the bacterial lysate supernatant obtained in chapter 2.2.1.15 and incubated on a roller-mixer for 1 h at RT. Afterwards, the tube's content was transferred into an sealed empty column. When the resin had settled at the bottom, the column was opened and the flow-through was collected. To eliminate remaining debris and impurities, the column was rinsed with 40 ml of binding buffer as the first step. Afterwards, 40 ml each of wash buffer I and wash buffer II with rising imidazole concentrations were applied to elute bound proteins with low affinity to Co^{2+}, often consisting of unspecific *E. coli* proteins. The final elution step was carried out with 5x 2 ml of elution buffer with high imidazole content. Composition of all used buffers is listed in Table 13. All fractions including flow-through of the lysate were

collected and elution of desired proteins in each fraction was subsequently analyzed via SDS-PAGE and Western blot (refer to chapter 2.2.3 and 2.2.4.).

Table 13: Buffers used for immobilized metal affinity chromatography

Binding buffer (pH 8.0)	300 mM	NaCl
	50 mM	NaH_2PO_4
Wash buffer I (pH 8.0)	10 mM	Imidazole
	300 mM	NaCl
	50 mM	NaH_2PO_4
Wash buffer II (pH 8.0)	40 mM	Imidazole
	300 mM	NaCl
	50 mM	NaH_2PO_4
Elution buffer (pH 8.0)	250 mM	Imidazole
	300 mM	NaCl
	50 mM	NaH_2PO_4

Variation of the method:

An unoptimized protocol of this method used at the beginning of this thesis included a single elution step with 25 ml of elution buffer instead of 5x 2 ml of elution buffer.

Der p 13 was eluted with a pH gradient instead of an imidazole gradient. After collecting the flow-through and washing with binding buffer, elution buffers (50 mM sodium acetate, 300 mM NaCl) at the following pH values were subsequently used for elution: 15 ml at pH 6.3, 2x 15 ml at pH 5.0, 2x 15 ml at pH 4.0, 2x 15 ml at pH 3.0, 2x 15 ml at pH 2.0.

Washing of rDer p 23 with binding buffer was performed three times.

2.2.2.2 Ion exchange chromatography

As IMAC often did not purify the desired proteins to a sufficient degree, the fractions of IMAC containing the desired allergen, as indicated by SDS-PAGE and western blot, were pooled and used for ion exchange chromatography (IEX) via HPLC (ÄKTApure and ÄKTApurifier, GE Healthcare/Cytiva). IEX relies on the affinity of a positively or negatively net charged compound to interact with molecules of the opposite charge. The charged matrix of IEX columns will reversibly bind oppositely charged proteins while compounds with the same or a neutral net surface charge will pass through the column without interaction. Proteins with different isoelectric points (pI) can be eluted and separated with a salt or pH gradient due to the different strengths of interaction with the column matrix.

To concentrate and change the buffer of the pooled sample, ultra-centrifugal filters with a molecular weight cut-off (MWCO) of 3 kDa (Merck Millipore, Darmstadt, DE) were used as

advised in the manufacturer's instructions. The exchange buffer depended on the form of ion exchange the protein underwent. Proteins with a pI above 6.5–7.0 were purified via cation exchange whereas anion exchange was applied for allergens with a lower pI. Used buffers for each chromatography type and the used HPLC columns are listed in Table 14. The final volume after ultracentrifugation was 2 ml in buffer A, and 1 ml was injected onto the column for each run after equilibrating the column with buffer A. Duration of a run was 120 minutes at 1.0 ml/min with a linear gradient starting with 100% buffer A to 30% buffer B within minutes 20–80 and increasing to 100% buffer B after 100 minutes. Fractions were collected at a volume of 1 ml per tube and protein elution was detected by UV (280 nm). A tricine-SDS-PAGE of selected fractions with UV signals indicating protein elution revealed the fractions containing the desired protein.

Table 14: Buffer and columns used for ion exchange chromatography

Ion Exchange	Column	Buffer A	Buffer B
Anion Exchange (AIEX)	SOURCE 15Q 4.6 / 100 PE	20 mM BisTris pH 8.8	20 mM BisTris 1 M NaCl pH 8.8
Cation Exchange (CIEX)	SOURCE 15S 4.6 / 100 PE	50 mM sodium acetate pH 5.5	50 mM sodium acetate 1 M NaCl pH 5.5

Variation of the method:

As an alternative buffer for anion exchange chromatography, 20 mM L-histidine was used for buffer A, 20 mM L-histidine + 1 M NaCl for buffer B. These buffers were only used in the beginning during purification of proteins expressed in Rosetta gami. For the purification of rDer p 10 the runtime was prolonged to 200 min.

2.2.2.3 Size exclusion chromatography

If the SDS-PAGE analysis still revealed impurities in all or a part of the eluted fractions after IEX, these fractions were subsequently pooled and further purified through size exclusion chromatography (SEC), which separates proteins according to the speed with which they pass through a porous column matrix. Since proteins are only retained if they fit into the pores of the matrix, large proteins elute first as interactions are less likely.

The samples' buffer was exchanged to 20 mM NH_4HCO_3, and they were simultaneously concentrated through centrifugal filters as before (refer to chapter 2.2.2.2) to a final volume of 4–12 ml, depending on the amount of protein and the number of fractions from the IEX eluate pool. 1 ml of the concentrate was applied to a Superdex 75 10/300 GL (Cytiva/GE Life

Sciences, Little Chalfont, UK) column with a 20 mM NH_4HCO_3 running buffer at 0.5 ml/min. Running time per application was 75 minutes, 0.4 ml fractions were collected and protein elution was detected by an UV cell (280 nm). All runs were performed on a HPLC system (ÄKTApure and ÄKTApurifier, GE Healthcare/Cytiva). Tricine-SDS-PAGEs of the fractions were analyzed as described in chapter 2.2.3. If the purity was still insufficient, an optional second IEX was performed as described in chapter 2.2.2.2, and purity was verified by tricine-SDS-PAGE.

Table 15: Parameter and conditions of size exclusion chromatography.

Parameter	Condition
Running Buffer	20 mM NH_4HCO_3
Column	Superdex 75 (GE Healthcare)
Flow rate	0.5 ml/min
Runtime	75 min
Detection	UV (280 nm)
Fractions	0.4 ml

2.2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.2.3.1 Tricine-SDS-PAGE

Proteins of different sizes were separated and purity of expressed allergens was evaluated after each purification step with help of electrophoresis. Herein, proteins move through a polyacrylamide gel at different speeds solely based on their mass. The tricine-SDS-PAGE method by Schägger and Jagow modified by Haider et al. was applied in this work [165, 166]. This protocol is best suited in regards of separation of proteins with masses smaller than 30 kDa.

Separation gels with an acrylamide content of 12% and stacking gels with 4% acrylamide were composed as listed in Table 17 with the gel buffer prepared as listed in Table 16. After mixing all components, leaving the APS (ammonium persulfate) catalyst for last, the gel was carefully poured between two glass plates so as not to introduce air bubbles. The still fluid gel was then immediately topped by isopropanol and left to polymerize for 30 min. After polymerization was complete, isopropanol was discarded, and the stacking gel was poured on top. To create wells, a sample comb sprayed with isopropanol was inserted. When the stacking gel was set, the comb was removed and sample wells were rinsed with ddH_2O. The gel cassettes were inserted into a vertical double gel system (model no. 45-2010-1, PeqLab) and filled with running buffer (Table 16).

Samples were prepared by mixing 40 µl of sample with 10 µl of concentrated sample buffer (Table 16) and incubating for 5 min at 95°C in order to fully unfold the proteins. After administering the samples and a prestained standard-sized protein marker (PageRuler Prestained Protein Ladder 10–180 kDa, Thermo Fisher Scientific) into the respective wells, the electrophoresis started at a voltage of 80 V until the blue buffer front hit the separation gel, at which point the voltage was raised to 200 V. Electrophoresis was stopped shortly before the blue buffer band would have exited the separation gel, after around 60 minutes total.

The finished gels were either directly stained with Coomassie dye solution or used to transfer proteins onto a membrane via western blotting (refer to chapter 2.2.3.3 and 2.2.4.1).

Table 16: Buffers for Tricine-SDS-PAGE.

Gel buffer pH 8.8	2,5 M	Tris
Sample buffer pH 6.8 (5x)	0.02% (w/v)	Bromophenol blue
	2.5% (w/v)	DTT (dithiothreitol)
	5 mM	EDTA
	25% (v/v)	Glycerol
	5% (w/v)	Sodium dodecylsulfate
	500 mM	Tris-HCl
Running buffer	0.05% (w/v)	Sodium dodecylsulfate 10% (w/v)
	25 mM	Tricine
	25 mM	Tris

Table 17: Composition of Tricine-SDS-gels

Compound	**12% separation gel**	**4% stacking gel**
Ammonium persulfate (APS)	50 µl	50 µl
Gel buffer	5.6 ml	0.76 ml
ddH_2O	1.3 ml	3.62 ml
Pyronin 1% (v/v)	-	8 µl
Rotiphorese® Gel 40 (40% Acrylamid/Bisacrylamid 19:1)	3.0 ml	0.5 ml
N,N,N',N'-Tetramethylethylendiamine (TEMED)	6 µl	5 µl

2.2.3.2 SDS-PAGE using precast gels

Additional to casting tricine-SDS-gels, commercial precast gels (4–12% NuPAGE Bis-Tris gels, Thermo Fisher Scientific) were used according to the manufacturer's instructions. Because of their superior protein separation and sensitivity to staining, these gels can be advantageous for samples that are difficult to separate or with low protein content. A MES-based buffer (2-(N-Morpholino)ethane sulfonic acid) was used as running buffer, the composition of which is listed in Table 18.

Table 18: Composition of running buffer for precast Bis-Tris-gels.

MES running buffer	50 mM	MES
	50 mM	Tris
	1 mM	EDTA
	0.1% (w/v)	SDS

2.2.3.3 Coomassie staining of SDS gels

Due to the interaction of aromatic or alkaline amino acids with Coomassie dye, this highly sensitive staining method can be applied to detect as little as 0.1 µg of protein and is therefore well suited for purity assessment [167, 168].

After the gel electrophoresis was finished, SDS residues were washed away with ddH_2O, and the gel was transferred into the Coomassie dye solution. After heating in the microwave for 1 min, the gel was further incubated in the hot Coomassie dye on a platform rocker for 15 min, after which the dye was filtered for reuse and the gel was covered with destaining solution. The gel was heated in the microwave and further incubated as described before, and the destaining solution replaced by fresh solution after incubation on a platform rocker for 15 min. This step was repeated until the gel's background was fully destained and protein bands were clearly visible. Composition of dye and destaining solution is listed in Table 19. Coomassie stained gels were documented using a flatbed scanner (CanoScan 5600F).

Table 19: Solution for Coomassie staining of SDS gels.

Staining solution	2% (w/v)	Coomassie brilliant blue R-250
	10% (v/v)	Glacial acetic acid
	45% (v/v)	Methanol
Destaining solution	9% (v/v)	Glacial acetic acid
	18% (v/v)	Isopropanol

2.2.4 Electrophoretic transfer of proteins

2.2.4.1 Semi-dry western blot

For confirmation of successful expression, the proteins separated by gel electrophoresis (refer to chapter 2.2.3) were transferred onto a nitrocellulose (NC) membrane by electroblotting, wherein the proteins are driven from the gel onto a membrane by an electric current that pulls the compounds, negatively charged by SDS, towards the anode.

The used protocol followed in principle the method described by P. Matsudaira [169] who describes a semi-dry blotting protocol using a continuous buffer system that does not rely on different buffers for anode and cathode. However, the method was modified by using a different, methanol-free and therefore less hazardous and environmentally friendly transfer buffer, wherein methanol content is replaced by isopropanol as suggested by M.A. Villanueva [170]. The buffer composition is listed in Table 20.

Table 20: Transfer buffer for semi-dry western blotting

Transfer buffer (pH 8.3)	25 mM	Tris
	19.2 mM	Glycine
	20% (v/v)	Isopropanol

Six pieces of protein-free filter paper (GE Healthcare) were cut to exceed the size of the gel by approximately 1 cm on each side. The NC membrane, filter papers and gel were soaked thoroughly in the transfer buffer, then they were carefully stacked to avoid air bubbles as depicted in Figure 2.1. Electroblotting was carried out at 75 mA/cm^2 for 60 min. After finishing, the empty gel was rinsed with ddH_2O and stained with Coomassie dye as described in chapter 2.2.3.3 to confirm the success of the protein transfer.

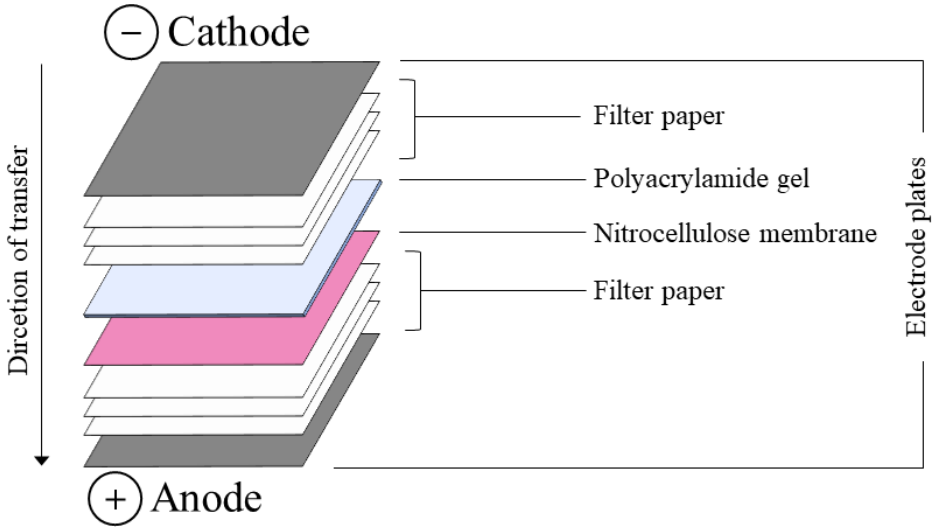


Figure 2.1: Composition of a continuous semi-dry blotting system.

Modified from [171].

2.2.4.2 Polyhistidine staining on nitrocellulose membranes

The transferred proteins were visualized on the NC membrane with a histidine staining to confirm that the fraction collected during IMAC did in fact contain the desired protein by staining its unique affinity His-tag, even though the method can also result in unspecific staining of histidine-rich proteins, which usually exhibit less prominent bands. The NC membrane was rinsed in ddH_2O and the membrane was subsequently incubated in blocking buffer (Table 21) for 30 min to block any unoccupied binding sites of the membrane and therefore reduce unspecific binding of detecting antibodies.

Table 21: Buffer and solution for polyhistidine staining.

Blocking buffer pH 7.4	0.1 M 0.1 M 2.5 mM 0.05% (v/v)	Tris NaCl $MgCl_2$ x 6 H_2O Tween-20
Blocking buffer pH 9.5	0.1 M 0.1 M 2.5 mM 0.05% (v/v)	Tris NaCl $MgCl_2$ x 6 H_2O Tween-20
NBT solution	0.033% (w/v)	Nitro-blue tetrazolium chloride
BCIP solution	0.5% (w/v)	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt

The primary antibody was diluted in 30 ml blocking buffer as indicated in Table 22 and incubated with the membrane overnight at RT on a platform shaker. The following day, three

washing steps with 10 ml blocking buffer for 10 min each were performed before incubating for 30 min with the secondary antibody diluted in blocking buffer and washed again as described. The last washing step was carried out with blocking buffer pH 9.5 to optimize the pH value for subsequent colorimetric detection, wherein the prewarmed (37°C, light protected) NBT and BCIP solutions (Table 21) were combined, shortly mixed and immediately poured over the membrane. After incubation for 5–10 min or until the bands were clearly visible, the NBT/BCIP mixture was discarded, and the membrane was dried after a brief rinse with ddH_2O to stop the enzymatic reaction with the alkaline phosphatase (AP). Polyhistidine-stained membranes were documented using a flatbed scanner (CanoScan 5600F).

Table 22: Antibodies used for polyhistidine staining.

	Species	Target	Label	Dilution	Type	Manufacturer
Primary Antibody	Mouse	penta His	---	1:5000	monoclonal	Qiagen (San Diego, USA)
Secondary Antibody	Goat	Mouse IgG	AP	1:5000	polyclonal	Dianova (Birmingham, USA)

2.2.5 Protein identification by peptide mass fingerprinting

To verify the identity of the expressed allergens after purification, stained gel bands were enzymatically digested and the resulting peptides were analyzed on a mass spectrometer for their peptide mass fingerprint, in which the sample's mass list is aligned with that of a chosen *in silico* digested protein or a database of multiple proteins. While this method does neither indicate the sample's purity nor its quantity, a high recovery is a strong indicator of substantial presence of the desired protein within the analyzed gel band [172-175].

2.2.5.1 Protein digestion

After gel electrophoresis and Coomassie staining/destaining (refer to chapter 2.2.3), the gels were washed in ddH_2O for 30 min and the desired bands, either those indicating a protein of the correct size or bands remaining after the last purification step that were presumably di- or oligomers, were excised using a razor blade (Schreiber GmbH, Fridingen, DE) and transferred into a 1.5 ml reaction tube containing 200 µl of destaining solution (Table 23). The samples were incubated for 40 minutes on a thermal shaker at 37°C and 150 rpm. If the gel bands were not fully destained by then, the destaining solution was replaced with fresh one and the step was repeated. Afterwards, the supernatant was discarded, and the gel piece was dehydrated with 100 µl of acetonitrile for 5 min at RT before the volatile acetonitrile was fully removed by drying the sample in a vacuum concentrator (SpeedVac). 30 µl of a 10 mM DTT solution were pipetted onto the dried gel bands and incubated for 45 min at 37°C before the supernatant was

replaced with 30 µl of a 10 mM iodoacetamide solution and incubated as before. Afterwards, 60 µl of ddH_2O were added and the entire supernatant was discarded before incubating in 150 µl destaining solution for 5 min and repeating the dehydrating and drying steps as above. 20 µl of porcine trypsin solution (Trypsin Gold, Mass Spectrometry Grade, Promega, Mannheim, DE) was added into the tube and left to incubate for 1 h at 4°C. After the addition of 45 µl of reaction buffer (Table 23), the sample was incubated overnight on a thermal shaker at 37°C and 150 rpm. The following day, 150 µl of ddH_2O was pipetted into the tube and further incubated for 10 min as described, before the supernatant was transferred into a new reaction tube. The solution was fully evaporated in a vacuum concentrator and re-dissolved in 20 µl of 0.1% (w/v) trifluoroacetic acid. The sample was prepared for mass spectrometry analysis by using ZipTip protein concentration pipette tips (Merck Millipore, Darmstadt, DE) as per the manufacturer's instructions. The samples were eluted in 10 µl of elution buffer (Table 23).

Table 23: Solutions and buffers for protein digestion by trypsin.

Destaining solution	50% (v/v)	Acetonitrile
	100 mM	NH_4HCO_3
Reaction buffer	10% (v/v)	Acetonitrile
	40 mM	NH_4HCO_3
Elution buffer	50% (v/v)	Acetonitrile
	0.1% (v/v)	Trifluoroacetic acid

2.2.5.2 Mass spectrometry

The digested proteins were analyzed by LC-MS/MS on a Q-Exactive Plus Hybrid Quadrupole-Orbitrap tandem mass spectrometer (Thermo Fisher, Freiburg, DE) which was externally calibrated with a peptide mixture (Peptide calibration standard II, Bruker LabScape). Samples were ionized with a Triversa Advion NanoMate ion source (Advion Bioscience, Ithaca, USA) utilizing nano-electrospray ionization (nano-ESI) and detected by a quadrupole mass filter and orbitrap mass analyzer. The obtained spectra were deconvoluted with XCalibur Software (Table 10), and the 400 most abundant masses were used for further analysis. Masses equivalent to protein-specific fingerprint peptides were identified with ExPASy FindPept (Table 10) with the following settings: maximum mass tolerance ± 0.5 Da, up to 1 missed cleavage, possible N-terminal acetylation or formylation, possible methionine oxidation. Protein recovery of less than 30% was considered as not successful.

2.2.6 Determination of protein concentration by spectral photometry

To determine the concentration of the purified allergens for further experiments, their absorbance at 280 nm was measured using a UV-Vis spectrophotometer (NanoDrop 1000,

Thermo Fisher Scientific). This method is based on the absorbance of tyrosine, tryptophan and cysteine disulfide bonds in pure protein solutions. The concentration was calculated with the Lambert-Beer law utilizing the respective mass extinction coefficient of the respective proteins, that were calculated beforehand with the ExPasy ProtParam tool (Table 10).

2.2.7 Investigation of protein folding by circular dichroism spectroscopy

To investigate if the recombinant Der p allergens adopted a correct folding during expression and purification and to also investigate the folding of nDer p 1, far-UV circular dichroism (CD) spectroscopy was utilized. This method is based on the fact that oppositely polarized waves of lights are absorbed differently by optically active substances, such as amino acids [176]. In preparation, buffer of all samples was exchanged to CD buffer (Table 24) as many substances and ions absorb light at wavelengths < 200 nm and are therefore unsuitable for far-UV CD spectroscopy. Buffer exchange was performed by using ultrafiltration centrifuge tubes with a MWCO of 3 kDa or 10 kDa (Amicon, Merck Millipore, Darmstadt, DE) as instructed by the manufacturer. Concentration of the samples was measured by spectral photometry (refer to chapter 2.2.6) and 1.5 ml reaction tubes containing 200 µl at a concentration of 0.15 mg/ml and 0.25 mg/ml were prepared for each allergen.

Spectra (190–260 nm) were recorded in a 0.1 cm quartz cuvette at 1 nm resolution and 50 nm/min scanning speed on a Jasco-715A spectrometer (Japan Spectroscopic Co., Tokyo, Japan) equipped with a temperature controller. Triplicates of each sample were measured, and the blank buffer spectrum was subtracted. The raw spectra were processed and smoothed using CDToolX software (Table 10) by applying Savitzky-Golay filters [177]. These resulting spectra were used to determine the secondary structure contents using the K2D3 tool (Table 10), which is based on theoretically derived spectra from reference proteins of a similar size to determine the proportions of β-strands and α-helices in the given sample. For reference, PDBsum data (Table 10) from crystal structures of the respective proteins were used, which is a tool for calculating the secondary structure contents of crystal structures in the PDB database. Due to their highly similar structures, the matching Der f allergen was used as a reference if no Der p crystal structure was available for the respective protein. If neither was available, the closest match by PDBsum's sequence search was used as well as the secondary structure contents of the actual allergen calculated by STRIDE (Table 10) using their 3D structure computed by AlphaFold (Table 10), which is a very recent and highly reliable tool using neural networks to predict the folding of proteins based on their sequence. For rDer p 23, the available crystal structure was only of a partial sequence. However, as the missing residues were predicted to be random-coil structure, the fraction of β-strands obtained from PDBsum was

recalculated with the total residues of the whole protein. Additionally, the structure contents from the AlphaFold model as described above were used as a second reference.

Table 24: Buffer for CD spectroscopy

CD buffer (pH 7.4)	7 mM	K_2HPO_4
	3 mM	KH_2PO_4

2.2.8 IgE detection via multiplex dot test

In order to analyze sera of study patients (SP1 and SP2, Table 9) for the presence of sIgE, binding to the previously expressed and purified Der p allergens (refer to chapter 2.2.1 and 2.2.2), a multiplex dot test was used. This method is superior to commonly used immunoblotting as it keeps the allergens in its native state, which is especially important for airborne allergens, as their epitopes are mainly discontinuous [178]. The essential method was developed within the research group, and a previous version of it has been published by Jappe et al. [179]. The method used in this work follows the published one in principle but has been adapted to HDM allergens, further optimized and was published within the course of this thesis [180]. All used analytes are listed in Table 25. Natural Der p 1 was obtained from Allergopharma (Reinbek, DE) supported by a material transfer agreement and was not produced within this thesis. A schematic summary of the method is illustrated in Figure 2.2.

Table 25: Allergens and controls for IgE detection via dot test.

Type	Analyte	Origin	Host
Negative control	2.5% (w/v) Tris	---	---
Positive control / Standard	Human IgE	monoclonal	hybridoma
Allergen	Der p 1	natural	*D. pteronyssinus*
Allergen	Der p 2	recombinant	*E. coli*
Allergen	Der p 5	recombinant	*E. coli*
Allergen	Der p 7	recombinant	*E. coli*
Allergen	Der p 10	recombinant	*E. coli*
Allergen	Der p 13	recombinant	*E. coli*
Allergen	Der p 20	recombinant	*E. coli*
Allergen	Der p 21	recombinant	*E. coli*
Allergen	Der p 23	recombinant	*E. coli*

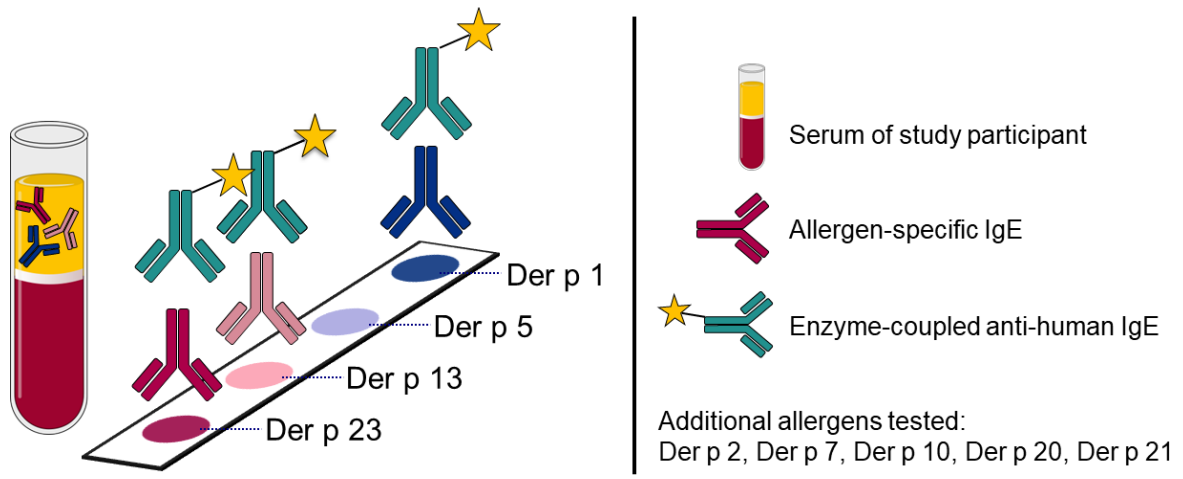


Figure 2.2: Schematic illustration of multiplex dot test for allergen-specific IgE detection

A NC membrane (0.45 µm pore size, Amersham, Thermo Fisher Scientific, Freiburg, DE) was prepared by cutting it into 5 mm wide strips. Concentrations of the purified protein solutions were determined by UV absorption at 280 nm (refer to chapter 2.2.6), and two 1.5 ml reaction tubes with concentrations of 1 mg/ml and 2.5 mg/ml, respectively, were prepared for each allergen. 1 µl of each tube was applied to the membrane strips in a row, corresponding to 1 µg and 2.5 µg of allergen each, as well as 4x 1 µl Tris control (Table 25).

For standardization, human IgE (non-immune, DIANOVA, Hamburg, DE) was applied on three separate membrane strips, each in decreasing concentrations corresponding to 250, 200, 150, 100, 75, 50, 25, 10, 5, 2.5, and 1 ng, respectively. After having dried completely, the strips were incubated with blocking buffer (Table 26) at RT for 2 h to saturate unoccupied protein binding sites and therefore reduce unspecific binding of the sera and secondary antibody. The strips were rinsed with ddH_2O until all skim milk residues were removed. Subsequently, the strips were incubated with 100 µl of patients' sera diluted 1:20 in TBST, including sera from healthy controls, overnight at RT on a platform rocker. Additional negative control strips were incubated with 2.5% Tris (w/v) without serum.

Table 26: Buffers for multiplex dot test.

TBST pH 7.4	0.1 M	Tris
	0.1 M	NaCl
	2.5 mM	$MgCl_2$ x 6 H_2O
	0.05% (v/v)	Tween-20
Blocking buffer pH 7.4	0.1 M	Tris
	0.1 M	NaCl
	2.5 mM	$MgCl_2$ x 6 H_2O
	0.05% (v/v)	Tween-20
	5% (w/v)	Skim milk powder

2.2.8.1 Chemiluminescence detection

The bound IgE antibodies in the samples were detected by chemiluminescence, which measures the light emitted during the enzymatic oxidation of a luminol substrate induced by a horseradish peroxidase (HRP)-coupled secondary detection antibody.

After the membrane strips had incubated over night, they were washed 3 x 10 minutes with washing buffer (Table 27) and subsequently incubated for 2 h at RT with a HRP-conjugated mouse anti-human IgE Fc antibody (Southern Biotech, Birmingham, AL, USA) diluted 1:10,000 in antibody diluent (Table 27). For detection, the HRP substrate was added according to manufacturer's protocol (Clarity Western ECL Substrate Kit, Bio-Rad, Munich, DE), and signals were measured on a Chemidoc readout system (Bio-Rad, Hercules, CA, USA). Signals of the IgE standards were used to create a standard curve by a logit-log-function using Graph Pad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). All sample signals were normalized to the standard curve, generating semi-quantitative results within 0–100 relative multiplex units (RMU) correlating with sIgE concentrations present in the patients' sera. Normalized signals of > 0.5 RMU were considered positive.

Table 27: Buffer and solution for chemiluminescence detection.

Washing buffer pH 7.4	0.1 M	Tris
	0.1 M	NaCl
	2.5 mM	$MgCl_2$ x 6 H_2O
	0.05% (v/v)	Tween-20
Antibody diluent pH 7.4	0.1 M	Tris
	0.1 M	NaCl
	2.5 mM	$MgCl_2$ x 6 H_2O
	0.05% (v/v)	Tween-20
	2.5% (w/v)	Skim milk powder

2.2.8.2 Statistical analysis

All analyses were conducted in GraphPad Prism 9 (Table 10) except for logistic regression, for which R 4.2.1 in RStudio (Table 10) was utilized. Study patients were grouped according to their clinical phenotypes, namely AA, AR and/or AD, by reviewing self-reported symptoms in the questionnaires as well as medical history. Discrepancies between symptoms indicated in the questionnaire and medically confirmed symptoms led to exclusion of the patient from allocation to the respective subgroups. Comparisons of the patient population regarding age, sex, allergic diseases as well as total and specific IgE concentrations were calculated by Chi square test for prevalence and Mann-Whitney or Kruskal-Wallis tests for metric variables of two or more than two groups, respectively. Differences in prevalence of sensitizations to the tested HDM allergens (Table 25) were analyzed with two-tailed Fisher's exact test as well as calculation of

relative risk (RR) and 95% confidence interval (95%CI). The data were also analyzed unstratified by univariate and multiple logistic regression including subsequent calculation of odds ratio (OR) and 95%CI to adjust for confounding factors such as age, sex and the simultaneous prevalence of asthma and AD. Performance and sensitivity of the dot test was evaluated by comparing the results of nDer p 1 and rDer p 2 sensitizations to the respective ImmunoCAP results with one-tailed Fisher's exact test. Linear regression was applied to correlate the number of individual HDM sensitizations, in the following named sensitization count, with the sIgE concentrations against Der p extract. To determine the relationship between sensitization count and disease phenotype, One-way ANOVA (Analysis of variance) with *post hoc* Tukey HSD (honest significant difference) test was used.

2.2.9 Development of a novel microarray for IgE detection

In order to downscale the previously used multiplex dot test described in chapter 2.2.8 to reduce the amounts of required patient serum and recombinant protein while enhancing sensitivity, a novel microarray utilizing near-infrared (NIR) based detection was developed within the course of this work. A schematic overview of the method is depicted in Figure 2.3.

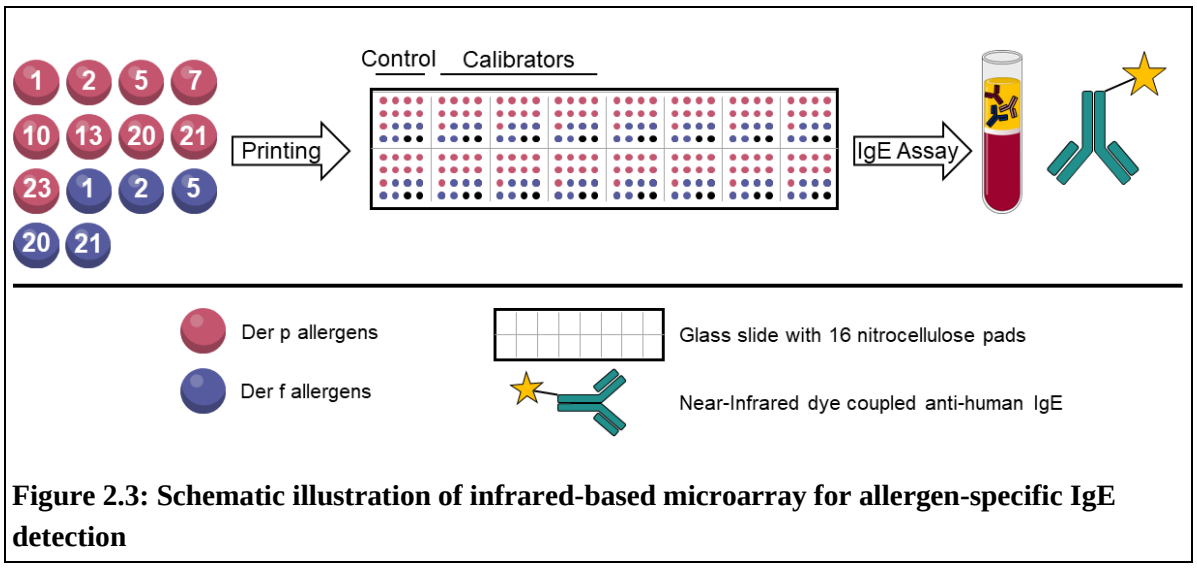


Figure 2.3: Schematic illustration of infrared-based microarray for allergen-specific IgE detection

2.2.9.1 Microarray printing

As a first step, the purified allergens underwent a buffer exchange into ddH_2O by using ultra-centrifugal filters with a molecular weight cut-off (MWCO) of 3 kDa or 10 kDa (Merck Millipore, Darmstadt, DE) to avoid crystallization during the printing process. Afterwards, the concentration of the protein solutions was determined by spectral photometry as described in chapter 2.2.6 and adjusted to 1 mg/ml for all proteins corresponding to the highest recommended spotting solution by the microarray slide manufacturer.

All allergens listed in Table 28 were printed contactless in a 4x4 array design on a glass slide coated with 16 4x4 mm NC membrane pads (OnCyte SuperNova, Grace BioLabs, Bend, OR, USA) by a NanoPlotter NP2 (GeSiM, Radeberg, DE) with a piezoelectric pipette head (Nano-Tip A-R-J, GeSiM, Radeberg DE). Spots were printed with 50 droplets consisting of 350 pl allergen solution each. After printing, the slides were air-dried and stored at 4°C protected from light and dust until use. Natural Der p 1 and recombinant Der f 1 were obtained from Allergopharma (Reinbek, DE) supported by a material transfer agreement.

Table 28: Allergens for microarray printing.

Type	Analyte	Origin	Host
Allergen	Der f 1	recombinant	*P. pastoris*
Allergen	Der f 2	recombinant	*E. coli*
Allergen	Der f 5	recombinant	*E. coli*
Allergen	Der f 20	recombinant	*E. coli*
Allergen	Der f 21	recombinant	*E. coli*
Allergen	Der p 1	natural	*D. pteronyssinus*
Allergen	Der p 2	recombinant	*E. coli*
Allergen	Der p 5	recombinant	*E. coli*
Allergen	Der p 7	recombinant	*E. coli*
Allergen	Der p 10	recombinant	*E. coli*
Allergen	Der p 13	recombinant	*E. coli*
Allergen	Der p 20	recombinant	*E. coli*
Allergen	Der p 21	recombinant	*E. coli*
Allergen	Der p 23	recombinant	*E. coli*

2.2.9.2 Standardization

The standardization of the microarray followed in principle a 3-point calibration method. A negative control serum of a non-allergic patient that underwent detailed testing for sIgE beforehand was spiked with anti-Der p 2 antibodies that were isolated from an allergic donor (Indoor Biotechnologies, Cardiff, UK) corresponding to a concentration of 1 kU/l, 6 kU/l and 30 kU/l, respectively. To validate the sIgE concentration, the spiked sera were sent to a third-party routine diagnostic laboratory to test the rDer p 2-specific IgE concentration via ImmunoCAP. A linear standard curve was established using the signal intensities of the calibrator sera as well as the negative control serum and the obtained values were normalized accordingly from using the slope-intercept form of the standard curve equation ($y = mx + b$). Sample signals for each allergen were evaluated for their individual signal-to-noise-ratio prior to standardization and only signals exceeding the respective highest background signal in any

of the control and calibrator sera at least three-fold were normalized, with the exception of rDer p 2 and rDer f 2, in which only the control serum was included as background for the pretest. After passing the pretest and normalization, values of more than 0.15 normalized units were considered as positive.

Variation of the method:

At the beginning of method development, an alternative approach for standardization was used that followed in principle the standardization described for the multiplex dot test (refer to chapter 2.2.8). Additional to the allergen panels, a dilution series of human IgE was printed onto a separate NC membrane slot that was blocked and detected simultaneously with the other samples using NIR-conjugated anti-human IgE dyes as described in chapter 2.2.9.3. None of the results presented in this thesis followed this method of standardization.

2.2.9.3 IgE detection assay

The dried microarray slides were assembled with a 16-well chamber system (ProPlate, Grace BioLabs, Cardiff, UK) to create incubation chambers holding up to 300 µl for each NC pad. The NC membrane was then incubated with 200 µl of blocking buffer (Table 29) for 2 h at RT without agitation to reduce unspecific antibody binding. The slides were then washed three times with 200 µl of wash buffer each (Table 29) for 5 min on a platform rocker before the buffer was discarded. Subsequently, 25 µl of antibody diluent was pipetted into each well and topped with 25 µl of patients' sera or the respective control and standardization sera (refer to chapter 2.2.9.2). The incubation chambers were sealed with adhesive aluminum foil and incubated on a platform rocker at RT for 90 min before the samples were discarded and the wells were washed 3 times with 200 µl wash buffer each as described before. Bound IgE was detected by incubating with 200 µl of a NIR dye-conjugated anti-hIgE antibody (DyLight 800, ImmunoReagents, Raleigh, NC, USA) diluted 1:10,000 in antibody diluent for 30 min at RT on a platform rocker. Lastly, the slides were washed two times with 200 µl wash buffer and ddH_2O each, rinsed with ddH2O from a top-dispensing wash bottle after removing the incubation chamber top, and left to dry in the dark at room temperature for 30 min. IgE signals were measured with an infrared imager at 800 nm (Odyssey CLx, Licor, Bad Homburg, DE), and analyzed using imageStudio 4.0 (Table 10).

Table 29: Buffers and reagents used for the IgE microarray

Name	Composition	Components	Manufacturer
Blocking buffer	50% (v/v)	Super G blocking buffer	Grace BioLabs
	43.45% (v/v)	PBS pH 7.2 (Table 5)	---
	4% (w/v)	Bovine serum albumin	Merck Millipore
	2.5% (w/v)	Skim milk powder	Carl Roth
	0.05% (v/v)	Tween-20	Carl Roth
	100 mM	DL-Arginine	Sigma Aldrich
Wash buffer pH 7.2	99.9% (v/v)	PBS pH 7.2 (Table 5)	---
	0.1% (v/v)	Tween-20	Carl Roth
Antibody diluent	93.9% (v/v)	PBS pH 7.2 (Table 5)	---
	4% (w/v)	Bovine serum albumin	Merck Millipore
	2% (w/v)	Skim milk powder	Carl Roth
	0.1% (v/v)	Tween-20	Carl Roth
	300 mM	DL-Arginine	Sigma Aldrich

2.2.10 Implementation of HDM allergens in IgE routine diagnostic platform

In order to quantitate sIgE concentrations against recombinant allergens unavailable for routine diagnostics in sera of study patients, selected purified allergens were implemented in the routine diagnostic platform ImmunoCAP by applying the streptavidin CAP technique, which utilizes an ImmunoCAP-compatible streptavidin matrix to bind previously biotinylated allergens with a strong, non-covalent bond.

2.2.10.1 Biotinylation of allergens

Buffer of rDer p 5, rDer p 20 and rDer p 21 was exchanged from 20 mM storage buffer to biotinylation buffer (Table 30) by using ultra-centrifugal filters with a molecular weight cut-off (MWCO) of 3 kDa or 10 kDa (Merck Millipore, Darmstadt, DE) since ammonium and primary amines interfere with the highly reactive NHS (N-hydroxysulfosuccinimid) ester used for coupling. After buffer exchange, the concentration of the protein solutions was determined by spectral photometry as described in chapter 2.2.6 and the concentration was adjusted to 2 mg/ml for all proteins. A 10 mM solution of EZ-Link NHS-LC-Biotin (Thermo Fisher Scientific, Freiburg, DE) in dimethyl sulfoxide (DMSO) was prepared freshly before each use. Enough biotinylation solution (Table 30) to create a 20x molar excess was added into each sample tube and incubated for 30 min at RT resulting in 3–5 bound molecules of biotin per protein molecule according to the manufacturer's instructions. Afterwards, excess biotinylation reagent was removed by ultrafiltration buffer exchange with PBS as described above. The biotinylated proteins were stored at 4°C for at most 24 h before further use.

Table 30: Buffers and reagents used for allergen biotinylation

Name	Composition	Components	Manufacturer
Storage buffer	20 mM	NH_4HCO_3	---
Biotinylation buffer	Dulbecco's PBS w/o Ca^{2+} & Mg^{2+}; w/o phenol red		Capricorn
Biotinylation solution	10 mM EZ-Link NHS-LC-Biotin in DMSO		Thermo Fisher Carl Roth

2.2.10.2 Preparation of streptavidin CAPs

Concentration of the biotinylated allergen solution was determined as described in chapter 2.2.6, and the concentrations were adjusted to 0.33 mg/ml for each allergen solution. The streptavidin CAPs (o212 CAP, Thermo Scientific/Phadia, Uppsala, SE) were washed 3 times with 250 µl of wash buffer that was prepared as listed in Table 31. Afterwards, 30 µl each of the allergen solutions, corresponding to 10 µg of allergen per CAP, was pipetted carefully onto the center of the streptavidin matrix and left to incubate at RT for 30 minutes before washing again as described. The prepared streptavidin CAP storage pens were sealed shut and kept at 4°C until measurement.

Table 31: Buffers and reagents used for streptavidin CAP coupling

Name	Composition	Components	Manufacturer
Wash buffer	8% (v/v)	Washing solution concentrate	Phadia / Thermo Fisher
	1.72% (v/v)	Washing solution additive	Phadia / Thermo Fisher
	90.28% (v/v)	ddH_2O	---

2.2.10.3 Measurement of IgE concentrations

The coupled streptavidin CAPs were used to determine sIgE concentrations in sera of study patients against the respective allergens. The serum samples were measured undiluted as well as, if necessary, diluted 1:5 and 1:25 with ImmunoCAP IgE/ECP/Tryptase Sample Diluent (Thermo Fisher Scientific/Phadia, Uppsala, SE). All measurements were carried out by a third-party routine diagnostic laboratory using the ImmunoCAP 250 (Thermo Fisher Scientific/Phadia, Uppsala, SE) according to the manufacturer's instructions. The resulting IgE concentrations were classified as indicated by the manufacturer.

2.2.10.4 Statistical analysis

One-Way ANOVA with *post hoc* Tukey HSD test was applied to detect differences of measured IgE concentrations against rDer p 5, 20 and 21 in patients from SP1 and SP2 using GraphPad Prism 9 (Table 10).

2.2.11 Human airway cell stimulation assay

In order to investigate the pathomechanistic and immunological properties of recombinant HDM allergens of interest and their potential involvement in proinflammatory pathways that could ultimately lead to the development of allergic asthma, the allergens were used to stimulate Calu-3 cells, a commonly used human bronchial cell line that has been described as a suitable model for the investigation of bronchial epithelial function. It was originally derived from the lung carcinoma of a white, male patient [181, 182].

2.2.11.1 Determination of LPS content by Limulus amebocyte lysate assay

To determine the lipopolysaccharide (LPS) content of the recombinant allergens, which is inherent to proteins produced in *E. coli* if not using a specialized LPS-deficient strain, a chromogenic endotoxin quantification assay was used as per the manufacturer's instructions (Pierce Chromogenic Endotoxin Quant Kit, Thermo Fisher Scientific, DE). This widely-used method is based on the endotoxin-dependent activation of an enzyme present in amebocytes of the horseshoe crab *Limulus polyphemus* [183]. The supplied standard originated from the *E. coli* strain O111:B4. The sample's absorbance was measured at 405 nm on a SpectraMax M5 plate reader (Molecular Devices, San Jose, CA, USA) and LPS concentrations were determined by generating a standard curve.

2.2.11.2 Culture of Calu-3 bronchial cells

The Calu-3 cell line (American Type Culture Collection, ATCC, HTB-55) was cultured as a submerged cell culture model in 75 cm^3 culture flasks for adherent cells. Cells were only handled openly in a class II safety cabinet (Mars, Scanlaf, DK). Used media and cell culture reagents are listed in Table 32.

After thawing shortly in a 37°C water bath, $9x10^6$ cells were seeded into a culture flask filled with 13 ml of prewarmed growth medium (Table 32). The cells were cultured at 37°C, 5% CO_2 and 95% relative humidity. Medium was exchanged every other day until the cells reached 80% confluency in inspection under a microscope (Wilovert, Will GmbH, Wetzlar, DE). At this point, the cells were split by removing the growth medium and thoroughly rinsed with 10 ml of prewarmed wash buffer (Table 32) before adding 7 ml of prewarmed detachment buffer and incubating at 37°C for 10–12 min. The cells were inspected under a microscope and if necessary, further 5 ml of detachment buffer was added and incubated for 5 min. To stop the enzymatic detachment, 3 ml of growth medium was added, and the detached cell suspension was then pipetted up and down in the flask to separate lumps and detach remaining cells before

it was transferred into a 15 ml conical centrifuge tube and centrifuged at 300 x g for 5 min at RT. The supernatant was discarded, and the remaining pellet was resuspended in 9 ml of growth medium. 3 ml of the suspension was pipetted into a new culture flask with 10 ml of prewarmed growth medium and the cells were cultured as described above. The number of passages (P) was noted on every flask. Cells were not used for stimulation assays until they had reached P4.

Table 32: Media and reagents used for cell culture.

Name	Composition	Components	Manufacturer
Growth medium	89% (v/v)	RPMI 1640 with stable glutamine	Capricorn
	10%	Fetal bovine serum advanced	Capricorn
	1%	Penicillin-streptavidin mix	Capricorn
Wash buffer	Dulbecco's PBS w/o Ca^{2+} & Mg^{2+}; w/o phenol red		Capricorn
Detachment buffer	0.25% Trypsin-EDTA in HBSS		Capricorn

2.2.11.3 Test for mycoplasma contamination by PCR

To exclude an infection of Calu-3 cells by mycoplasmas, a PCR-based mycoplasma detection test was carried out (MycoSPY Master Mix, Biontex, Munich, DE). When the cells reached 80% confluency, 100 µl of the cell culture supernatant was transferred into a 1.5 ml reaction tube and incubated at 94°C for 5 min. After centrifugation for 1 min at 13 000 x g, the PCR mixture was composed with 22 µl of Master Mix, 1 µl of internal control and 2 µl of the supernatant. The PCR was carried out using the parameters listed in Table 33. Afterwards, the sample was analyzed using analytical agarose gel electrophoresis as described in chapter 2.2.1.3. If a band with a size of 500 bp was visible, the test was considered positive.

Table 33: PCR program for mycoplasma detection

94°C	1 min		Initial Denaturation
94°C	30 sec		Denaturation
62°C	30 sec	35 cycles	Annealing
72°C	30 sec		Extension
72°C	6 min		Final Extension
Cooling			

2.2.11.4 Stimulation of Calu-3 cells and inhibition assay

Buffers of all allergens were exchanged to sterile PBS by using an ultrafiltration centrifuge tube with a MWCO of 3 kDa or 10 kDa (Amicon, Merck Millipore, Darmstadt, DE) as instructed by the manufacturer. Concentration of the samples was measured by spectral photometry (refer

to chapter 2.2.6) and 10x stock solutions in PBS of all stimuli were prepared as listed in Table 34 and stored at -80°C until use.

After passaging the cells for at least 4 times, the density of cells in the detached cell suspension was manually counted in a Neubauer Improved chamber under a microscope. In a 24-well plate, $2x10^5$ cells were seeded in 1 ml of growth medium per well and cultured as described before. After 48 h, the growth medium was exchanged, and cells were incubated for further 24 h. The stock solutions of stimuli were diluted 1:10 in growth medium to a final volume of 250 µl/well. All allergens and controls used for stimulation are listed in Table 34.

Additionally, allergens and the respective inhibitor controls were tested after inhibition, in which the respective wells were preincubated for 1 h at 37°C with the desired inhibitor (Table 34) before removing the well's supernatant carefully, washing and dispensing the stimuli on them. All tests were measured as triplicates and experiments were performed at least twice at different passage numbers. After 24 h of stimulation, the supernatants were carefully transferred and stored in a 96-well low-binding plate at -80°C until analysis.

Table 34: Stimuli for cell stimulation assay.

Type	Compound	Origin	Final concentration	Manufacturer
Negative control	Sterile PBS	---	---	Capricorn
LPS negative control	Ultrapure LPS *E. coli* O111:B4	natural	50 EU/ml	Invivogen
Positive control	Ultrapure LPS *E. coli* O111:B4	natural	100,000 EU/ml	Invivogen
TLR2 control	Pam3CSK4	synthetic	2000 ng/ml	Invivogen
TLR4 control	Ultrapure LPS *E. coli* O111:B4	natural	100,000 EU/ml	Invivogen
TLR2 inhibitor	Anti-hTLR2 neutralizing	monoclonal	10 µg/ml	Invivogen
TLR4 inhibitor	Anti-hTLR4 neutralizing	monoclonal	10 µg/ml	Invivogen
Allergen	Der p 5	recombinant	50, 100, 200 µg/ml	---
Allergen	Der p 10	recombinant	50, 100, 200 µg/ml	---
Allergen	Der p 20	recombinant	50, 100, 200 µg/ml	---
Allergen	Der p 21	recombinant	50, 100, 200 µg/ml	---

2.2.11.5 Selection of analytes

To determine which analytes to include in the multiplex cytokine assay used to measure the stimulation assay sample, an approach of literature research and data mining was applied to identify suited candidates. The analytes had to fulfill all of the following criteria: 1. Associated with AA in lung epithelial cells, 2. Available for analysis on the MSD U-plex platform (Mesoscale Discovery, Rockville, MD, USA), 3. Expressed by Calu-3 cells. A list of curated candidates was generated mainly utilizing the work of Zissler et al. [184]. Analytes not available for the assay were excluded. However, as cell lines do not exhibit the same cell metabolism as primary cells, it was not guaranteed that all of the potential analytes would be expressed by Calu-3 cells at all. Therefore, the DepMap portal of the Cancer Cell Line Encyclopedia (CCLE) was used to obtain the RNA sequencing gene expression data for 1019 cell lines total. The data were analyzed with the help of RStudio (Table 10) to extract the expression of relevant genes coding for the potential analytes in Calu-3 cells. Of the remaining candidates, 12 were chosen after the strength of evident association to AA in the literature and the level of expression [185-196]. The final candidates and their expression level in Calu-3 cells are listed in Table 35.

Table 35: Analytes for cell stimulation assay

Analyte	Gene expression (RSEM)	Contribution to asthma (proposed)
IL-1α	2.05	Promotion of Th_2 immune response Activation of fibroblasts and airway hyperplasia
IL-1β	1.75	Acute airway inflammation Promotes infiltration of eosinophils and activation of mast and T cells
IL-6	3.13	Promotion of Th_2 immune response
IL-8	7.04	Neutrophil recruitment and activation
IL-15	1.8	Eosinophilia Growth and differentiation of T cells
IL-18	6.0	Activates natural killer cells, monocytes, basophils, mast cells Promotes IgE production and Ig-class switch
IP-10	1.26	Airway hyperreactivity and inflammation
Eotaxin-3	0.24	Eosinophil and basophil recruitment
G-CSF	0.5	Promotes neutrophil-mediated airway inflammation
GM-CSF	0.35	Chronic airway inflammation Release of leukotrienes
TNF-α	0.33	Activates epithelium, endothelium, antigen presenting cells, monocytes/macrophages
VEGF-A	6.0	Airway remodeling Th_2-mediated airway inflammation

Data derived from [185-196].

2.2.11.6 Multiplex cytokine assay

The analytes chosen in chapter 0 were measured with the U-PLEX assay platform (Mesoscale Discovery, Rockville, MD, USA) in 96-well plates. This platform utilizes designated spots with unique linkers at the bottom of each well, which can be coupled with the desired analytes' detection antibodies after coupling the allergen to the respective linker agent first. The samples can then be detected by electrochemiluminescence.

The assay was carried out according to the manufacturer's instructions. Briefly, the plate was prepared by combining 200 µl of the supplied biotinylated detection antibodies with 300 µl of the linking agent for 30 min at RT before adding 200 µl of Stop solution and incubating for further 30 min. Afterwards, 600 µl of each linked antibody solution was pooled into a master mix and, if necessary, stop solution was added to reach a total volume of 6 ml. The 96-well plate was coated with 50 µl per well for 1 h at RT on a thermal shaker at 300 rpm. After washing with 3x 150 µl of PBST (Table 5) per well, 50 µl each of sample or calibrator was added to the wells, the plate was sealed with adhesive aluminum foil and incubated overnight at 4°C. The following day, the plate was washed as before and 50 µl each of the supplied detection antibody solution were pipetted into the plate. After sealing it again, it was incubated for 1 h at RT on a thermal shaker at 300 rpm. The plate was washed as before and prepared for measurement by adding 150 µl of the supplied reading buffer.

Samples were measured using a MESO QuickPlex SQ 120 (Mesoscale Discovery, Rockville, MD, USA) and analyzed with the MSD Discovery Workbench software (Table 10).

2.2.11.7 Statistical analysis

The raw data exported from MSD Discovery Workbench were processed and analyzed in GraphPad Prism 9. Changes in concentration of the analytes were analyzed using One-way ANOVA with *post hoc* Šídák test for preselected pairings. All controls and stimuli were compared to PBS, and inhibition tests and controls were additionally compared to the corresponding uninhibited values to determine differences in analyte release.

2.2.12 Basophil activation test

2.2.12.1 Stimulation of whole blood basophils

Heparinized blood (Vacutainer LH 170, BD, Heidelberg, DE / S-Monovette® 9 ml LH, Sarstedt) from study patients was obtained and stored at 4°C after blood sampling. The samples were further processed within 48 h. The essential method has been developed in a cooperation of the research group Clinical and Molecular Allergology with the flow-cytometric unit of the Research Center Borstel [131]. It is based on the *ex vivo* stimulation of vital basophil granulocytes in whole blood with different stimuli and subsequent following flow cytometric analysis. All used stimuli are listed in Table 36.

For a general assessment of the potential of molecular HDM allergens to induce a basophil response, all available Der p allergens were combined in a BAT design for HDM-allergic patients.

For the further evaluation of the BAT as a replacement for allergen challenges, peanut allergy was used as a model disease due to sample availability and the possibility to test patients that were simultaneously challenged in a controlled setting provided by an OFC. A comprehensive panel of peanut allergens was chosen for analysis in this part of the project (Table 36). Alkaline PEX and dialyzed oleosins isolated from roasted peanuts, the production and clinical relevance of which is described in detail in a previous thesis by C. Schwager, were already present in the research group and were not produced in this work [171, 197, 198]. The recombinant peanut allergens Ara h 2 and Ara h 8 were purchased commercially (Indoor Biotechnologies, Cardiff, UK). The stimulation assay was carried out under a clean safety bench.

Stock solutions of all stimuli were prepared with PBS at a concentration 10x higher than the final concentration indicated in Table 36. fMLP and allergens were then serially diluted 1:10 so that three concentrations per stimulus were obtained. 10 µl of each stimulus at each concentration was pipetted into a round-bottom 96-well microtiter plate, topped by 90 µl of the heparinized blood that was gently agitated beforehand, and mixed by carefully pipetting up and down. The plate was incubated in a closed stainless-steel box on a wetted paper towel at 37°C for 30 min.

Table 36: Controls and allergens used as stimuli in basophil activation test.

Stimulus	Final Concentration	Function
PBS	---	Negative control
fMLP (Formyl-Methionyl-Leucyl-Phenylalanine)	1 µM, 0.1 µM, 0.01 µM	Positive control (IgE-independent)
Anti-IgE A	1 µg/ml	Positive control (IgE-dependent)
Anti-IgE B	1 µg/ml	Positive control (IgE-dependent)
Anti-IgE A + B	1 µg/ml	Positive control (IgE-dependent)
BAT design for HDM allergy		
Natural Der p 1	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Single allergen
Recombinant Der p 2	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Single allergen
Recombinant Der p 5	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Single allergen
Recombinant Der p 7	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Single allergen
Recombinant Der p 10	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Single allergen
Recombinant Der p 13	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Single allergen
Recombinant Der p 20	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Single allergen
Recombinant Der p 21	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Single allergen
Recombinant Der p 23	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Single allergen
BAT design for peanut allergy		
Alkaline peanut extract (PEX)	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Allergen mix
Oleosins from roasted peanuts	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Allergen mix
Recombinant Ara h 2	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Single allergen
Recombinant Ara h 8	10 µg/ml, 1 µg/ml, 0.1 µg/ml	Single allergen

After stimulation, the blood was stained in a darkened room with 35 µl each of a mix of fluorophore-conjugated antibodies as listed in Table 37. The plate was incubated for 40 min at 4°C under light protection. The red blood cells were subsequently lysed by adding 200 µl 1-Step Fix/Lyse Solution (eBioscience Inc., San Diego, USA) and incubated for 10 min protected from light at RT. Following a centrifugation step at 200 x g for 5 min at 4°C, the supernatant was decanted by a swinging motion and the lysis step was repeated. After washing two times with 200 µl of PBS each, the pellet was resuspended in 280 µl of MacsQuant Running Buffer (Miltenyi Biotec, Hamburg, DE). The samples were stored at 4°C and analyzed by flow cytometry within 24 h.

Table 37: Composition of the staining antibody mix used for basophil activation test.

Antibody	Conjugate	Dilution	Manufacturer
Anti-Human CD63 (clone H5C6)	APC	1:16	BioLegend
Anti-Human CD203c (clone NP4D6)	PE	1:8	BioLegend
Anti-Human FcεRIα (clone AER-37)	PE/Cy7	1:16	BioLegend

2.2.12.2 Flow cytometry

Basophils contain intracellular histamine granules with the CD63 antigen associated to the vesicle membrane. If histamine release occurs, the antigen is relocated to the cell surface and can be measured as an activation marker via flow cytometry. After stimulation, the lysed and fixed samples were measured on a LSR II or Symphony A1 flow cytometer (BD Biosciences, Heidelberg, DE) with an adjacent HTS loader (high throughput sampler). The sample processing settings are listed in Table 38. Signals were recorded via FACSDiva Software (Table 10) and the gating strategy identified basophils as FcεRIα$^+$/ CD203c$^+$ cells after doublet exclusion. Activation was defined as CD63$^+$ within the gated CD203c$^+$ cells and the proportion of activated basophils was calculated percentage of CD63$^+$ cells (%CD63$^+$). The detailed gating strategy is depicted in Appendix 2. The recorded data were analyzed using FCS Express 7 (Table 10) and exported data were processed with GraphPad Prism (Table 10).

Table 38: HTS loader settings for flow cytometry

Throughput mode	Standard
Flow	2.5 µl/sec
Sample volume	250 µl
Mixing volume	100 µl
Mixing speed	250 µl/sec
Mixes	5
Wash volume	800 µl
Recording delay (BLR)	Yes, 5 sec

3 Results

Parts of these results have been published during the course of this thesis [180]. Figures, sentences, small passages, or whole paragraphs adopted from the published article may be quoted verbatim or in spirit of this work without being highlighted or cited separately in the following section.

3.1 Production of recombinant HDM allergens

For use in the subsequent assays, several Der p and Der f allergens including major, mid-tier and minor allergens as well as several lipid-associated allergens were recombinantly expressed in *E. coli* and purified via high-performance liquid chromatography (HPLC). Pre-existing expression strategies were optimized and the folding of the allergens was investigated after successful purification.

3.1.1 Cloning of recombinant HDM allergens

For Der p allergens, gene constructs were already present in the research group at the start of the thesis. However, these constructs yielded only little amounts of protein during expression, part of which could have been caused by the non-optimized gene sequences. Therefore, the constructs were host-adapted for *E. coli*, a process in which base triplets rarely occurring in the host organism are replaced by common ones, leading to faster and more reliable translation as well as higher protein yields.

Control of restriction by analytical agarose gel electrophoresis revealed bands corresponding to the size of the desired constructs. After isolating them by preparative gel electrophoresis and ligating with the pET-17b vector, BL21cells were transformed with the ligation end product. After selection on ampicillin agar plates, colony PCR of 5 clones each confirmed that the cells had taken up a plasmid of the desired size (Figure 3.1). To confirm that the insert was correct and no errors were introduced during PCR by the Taq polymerase, the thermostability of which comes with the drawback of low fidelity that can lead to substitution of single bases and therefore change the amino acid sequence, the isolated plasmids were sent to a third-party laboratory for DNA sequencing [199, 200]. Analysis of the results revealed that approximately 20–30% of clones had faulty inserts with 1–5 substituted nucleotides per insert and these clones were therefore discarded. SHuffle cells were transformed with isolated plasmid DNA of a chosen BL21 clone carrying a correct insert.

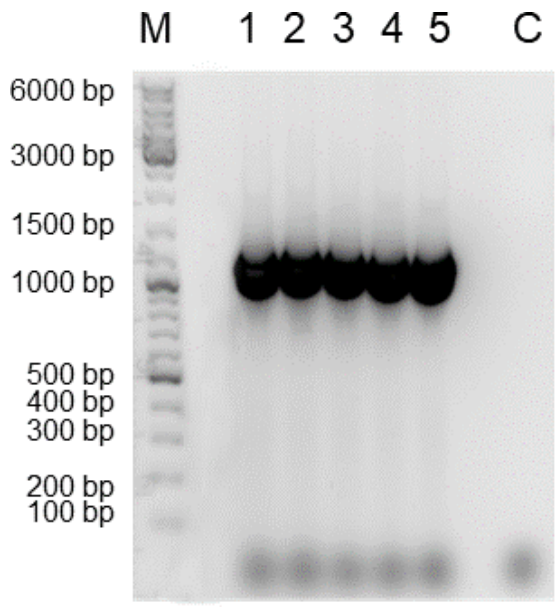


Figure 3.1: Exemplary analysis after colony PCR of transformed BL21 clones.
Verification of correct insert size for rDer f 20 (1098 bp) in transformed BL21 cells after ampicillin selection via analytical agarose gel electrophoresis after PCR. (1.2% agarose gel, GelRed dye)
M: Marker; C: Negative control; 1–5: Amplified plasmids of clones I–V

3.1.2 Expression of recombinant allergens

At the beginning of this work, recombinant Der p allergens were expressed in Rosetta gami cells. The yields, however, were poor, as for example only 3 mg of rDer p 23 could be obtained out of an expression in 4 l culture medium. With the optimized constructs, switching strains to SHuffle cells and altered expression protocol, the yields could be greatly improved up to for example 20 mg of rDer p 20 in 1 l culture medium. Sufficient amounts of rDer p 7 and rDer p 21 were already present in the research group and these allergens were not produced during the course of this thesis.

The results of Der f allergen expression in SHuffle cells with autoinduction media were very variable for each allergen and only the expression of rDer f 2 led to considerable amounts of allergen produced via autoinduction. Therefore, even though autoinduction was convenient, as cell density did not have to be monitored and IPTG addition could be omitted, this method was not suitable for a lot of the expressed proteins. An overview of expression outcomes in SHuffle cells with IPTG induction and autoinduction for Der f allergens is depicted in Appendix 3.

Expression of rDer f 2 was not successful in BL21 but only SHuffle cells. However, this was expected as it was known from previous expressions of Der p allergens before the start of this thesis that BL21 cells were unable to express soluble group 2 mite allergens, presumably

because this *E. coli* strain has a very low capacity to correctly form disulfide bonds in expressed proteins.

Overall, expression of recombinant Der p and Der f proteins worked best in SHuffle cells in TB growth medium with optimized gene constructs and the altered expression protocol described in chapter 2.2.1.14.

3.1.3 Purification of recombinant HDM allergens

The purification process is exemplified for rDer f 20. Overview of the purification workflow and results for each of the other HDM allergens produced during the course of this thesis with the respective results are listed in Appendix 4–Appendix 12.

3.1.3.1 Immobilized metal affinity chromatography

The first purification step was carried out via IMAC utilizing the affinity of the His-tag of the expressed proteins towards a cobalt resin column as described in chapter 2.2.2.1. Analysis of 40 µl each of the flow-through and collected fractions via SDS-PAGE and western blot (refer to chapter 2.2.3 and 2.2.4) revealed successful expression of a protein of the desired size at approximately 40 kDa in the respective wash buffer fractions of BL21 and SHuffle cells with IPTG induction. Only small amounts of protein were found in the fractions collected from SHuffle cells with autoinduction, therefore these samples were discarded. The wash buffer fractions I and II were pooled for each expression, keeping the proteins expressed in different *E. coli* strains separate, and samples were used for further purification.

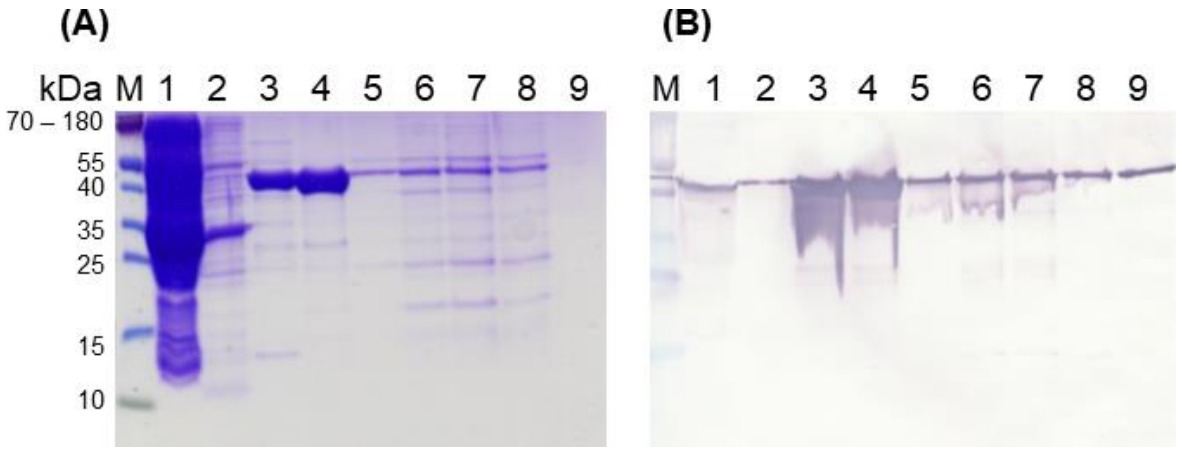


Figure 3.2: Purification of rDer f 20 after cell lysis by IMAC.

A–B: Analysis of flow-through and fractions collected during IMAC purification by (A) SDS-PAGE (12% Tricine gel, Coomassie dye) and (B) subsequent Western Blot (NC membrane, polyhistidine stain). Shown is expression in SHuffle cells with regular IPTG induction.

M: Marker; 1: Flow-through; 2: Binding buffer; 3 Wash buffer I; 4: Wash buffer II; 5–9: Elution I–V

3.1.3.2 Ion exchange chromatography

The pooled, concentrated and re-buffered samples were further purified via cation exchange chromatography (CIEX) as described in chapter 2.2.2.2. Since the volume before concentration of the pooled wash buffers was 80 ml and the SDS-PAGE analysis indicated a substantial amount of protein, the samples were concentrated to a final volume of 10 ml and runs were performed consecutively via the HPLC system's scouting function with 1 ml of sample each to avoid exceeding the column capacity. Fractions containing rDer f 20 were eluted after approximately 145 ml and confirmed by SDS-PAGE analysis. As the purity of the allergen was sufficient, a subsequent size exclusion chromatography was not necessary.

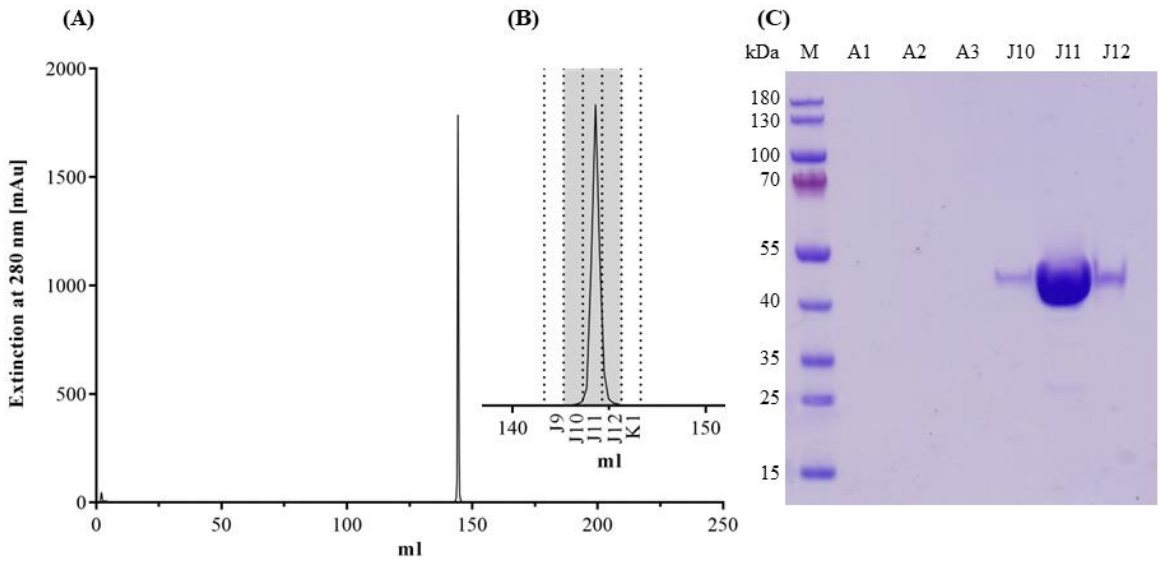


Figure 3.3: Purification of rDer f 20 via ion exchange chromatography.

A: Chromatogram and collected fractions with suspected target protein after CIEX.

B: Magnification of peak and respective fractions (marked grey) presumably containing the target protein.

C: Analysis of protein elution by SDS-PAGE (12% Tricine gel, Coomassie dye). M: Marker; A1–J12: Collected HPLC fractions

3.1.4 Final purity assessment and identity verification

To assess the purity of the expressed proteins, they were analyzed via SDS-PAGE by applying samples in increasing concentration from 1 µg to 10 µg per lane. If the purity was sufficient, the band of the highest concentration was excised and analyzed by peptide mass fingerprinting to verify the identity of the protein, which could be confirmed for all allergens with a very high sequence coverage of at least 70% up to 94%. An exemplary spectrum with identification of fingerprint peptides is depicted for Der f 20 in Appendix 13, and the full list of fingerprinting results for expressed all allergens is listed in Appendix 14. Afterwards, the allergens were stored

in 20 mM NH_4HCO_3 at -80°C. In Figure 3.4 and Figure 3.5 the final purity assessment of all recombinant Der f and Der p allergens used in this work is depicted, respectively.

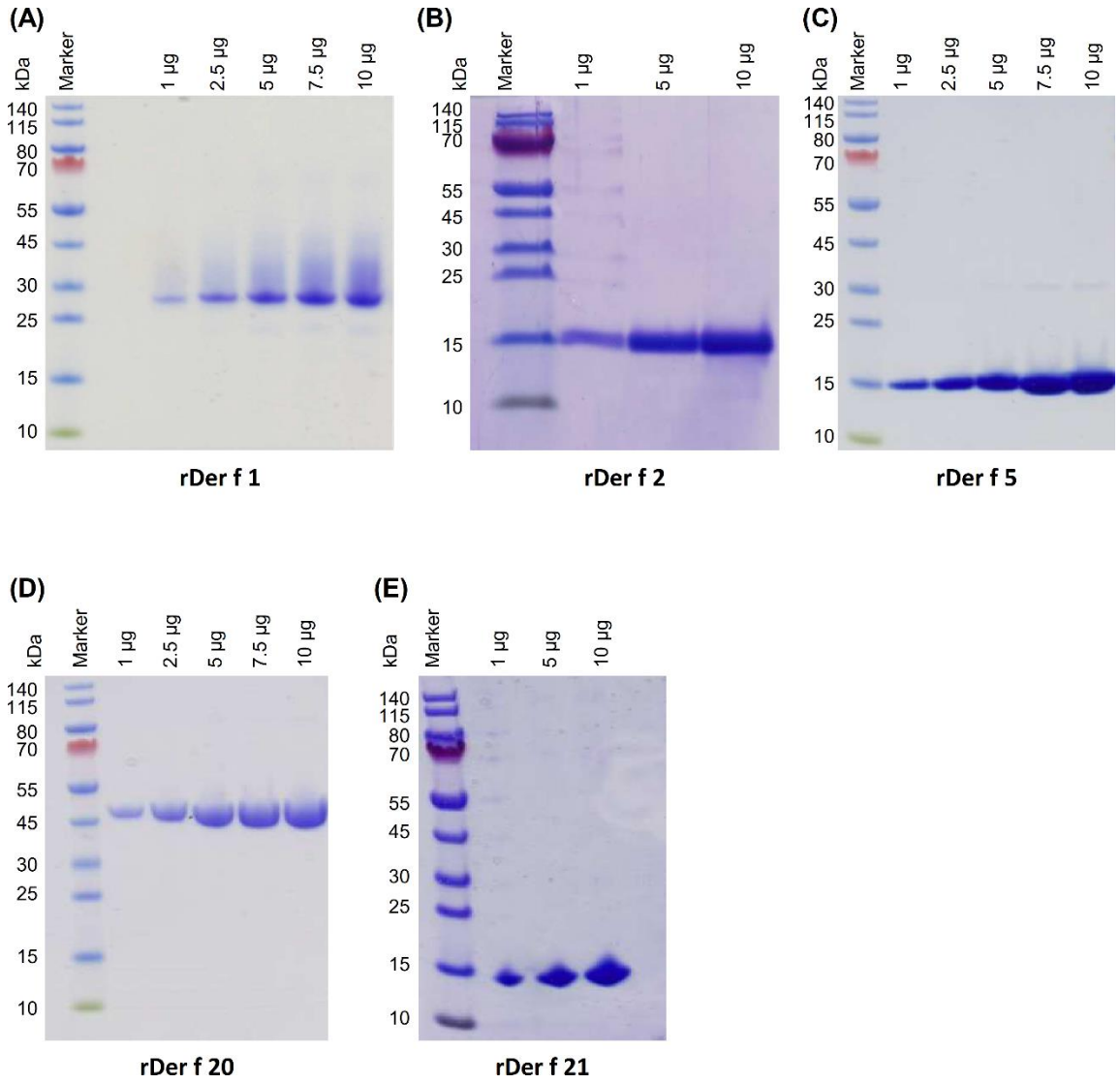


Figure 3.4: Purity assessment of recombinant Der f allergens.

A–E: Tricine-SDS-PAGEs stained with Coomassie dye of rDer f 1 (A), rDer f 2 (B), rDer f 5 (C), rDer f 20 (D) and rDer f 21 (E) after the final purification step. r = recombinant

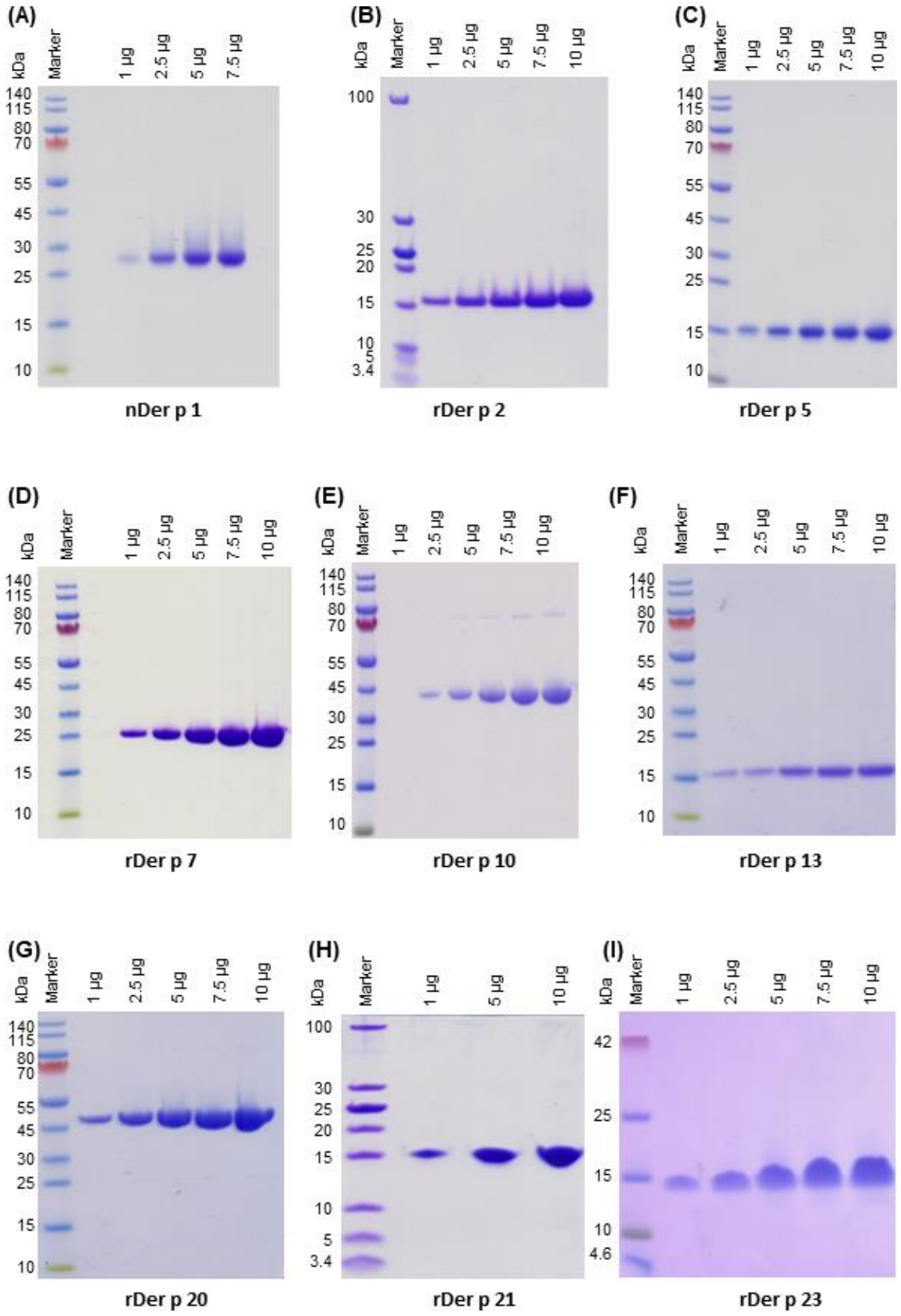


Figure 3.5: Purity assessment of recombinant and natural Der p allergens

A–I: Tricine-SDS-PAGEs stained with Coomassie dye of nDer p 1 (A), rDer p 2 (B), rDer p 5 (C), rDer p 7 (D), rDer p 10 (E), rDer p 13 (F), rDer p 20 (G), rDer p 21 (H), and rDer p 23 (I) after the final purification step. n = natural, r = recombinant

3.1.5 Folding of recombinant HDM allergens

As airborne allergens exhibit mainly discontinuous, conformational epitopes, it is crucial for them to adopt a correct folding during expression since they can otherwise have poor IgE reactivity [201]. Therefore, the folding of all Der p allergens used in this work were investigated by CD spectroscopy as described in chapter 2.2.7.

The spectra after blank subtraction and smoothing for all Der p allergens are depicted in Figure 3.6.

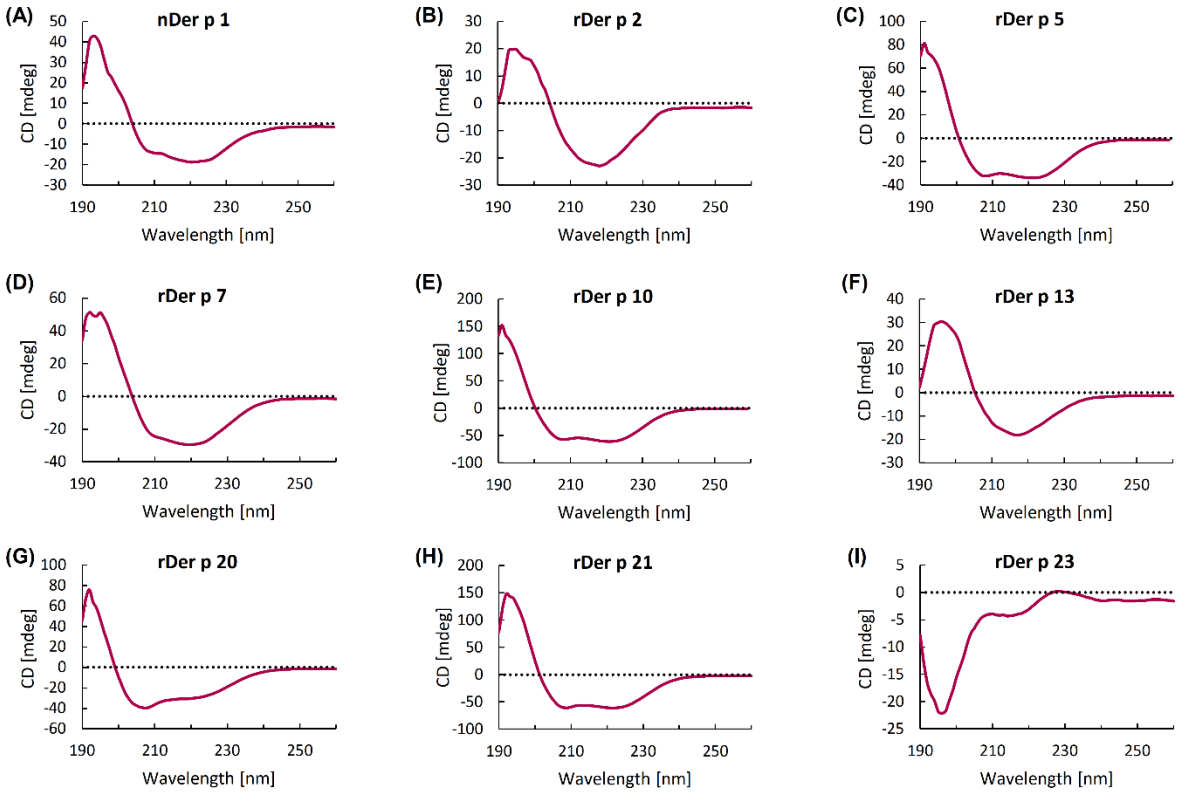


Figure 3.6: Far-UV Circular dichroism spectroscopy data of Der p allergens

A–I: Far-UV CD spectra of nDer p 1 (A), rDer p 2 (B), rDer p 5 (C), rDer p 7 (D), rDer p 10 (E), rDer p 13 (F), rDer p 20 (G), rDer p 21 (H), and rDer p 23 (I) in 10 mM potassium phosphate buffer pH 7.4.
n = natural, r = recombinant

All analyzed protein structures demonstrated folding, and the predicted secondary structure contents of alpha helices and beta strands as calculated by K2D3, based on *in silico*-derived spectra (for details on the functionality of K2D3 refer to chapter 2.2.7), were within 10% total of the respective reference structures for all allergens except rDer p 7, which differed in beta sheet content by 15% (Table 39). However, the spectral data of rDer p 7 (Figure 3.6) were in line with others found in the literature [202], therefore appropriate folding was assumed. The reference spectrum for rDer p 23 calculated by K2D3 was very different from the experimental

data and therefore the computational error in the secondary structure was potentially big. However, the obtained result still was within 10% of the reference structure. In the literature, spectra similar to the one obtained are reported for rDer p 23 and its unique spectrum might be due to the fact that it consists predominantly of random coil structure [203, 204].

Overall, these results showed that *E. coli* is a generally suitable host organism to express HDM allergens that are correctly folded and therefore possess discontinuous epitopes making them fully adequate for use in IgE profiling of allergic patients.

Table 39: Secondary structure content of Der p allergens analyzed by K2D3

Allergen	K2D3 results	Reference	PDB of reference
nDer p 1	α helix: 32% β strand: 21%	α helix: 33% β strand: 15%	1XKG
rDer p 2	α helix: 7% β strand: 36%	α helix: 0% β strand: 32–47%	1KTJ, 1A9V
rDer p 5	α helix: 79% β strand: 1%	α helix: 84% β strand: 0%	3MQ1
rDer p 7	α helix: 26% β strand: 29%	α helix: 28% β strand: 44%	3UV1
rDer p 10	α helix: 95% β strand: 0%	α helix: 94–99% β strand: 0%	A12d[a] / STRIDE + AF
rDer p 13	α helix: 5% β strand: 44%	α helix: 9% β strand: 50%	2A0A
rDer p 20	α helix: 43% β strand: 12%	α helix: 36–40% β strand: 14–15%	5ZHQ[a] / STRIDE + AF
rDer p 21	α helix: 79% β strand: 1%	α helix: 79–80% β strand: 0%	5YNY, 5YNX
rDer p 23	α helix: 2% β strand: 24%	α helix: 0% β strand: 29%–32%	4ZCE[b] / STRIDE + AF

[a] No crystal structure of the respective Der p or Der f allergens available. Closest match by PDBsum sequence search used for reference.

[b] Only partial crystal structure available, fraction of strands was recalculated with the number of residues of the mature protein.

AF = AlphaFold model

3.2 Identification of potential HDM marker allergens in adults

To further elucidate the role and clinical relevance of single Der p allergens, the sensitization patterns of 384 HDM-allergic adult study patients (SP1 and SP2, Table 9) were measured with the multiplex dot test as described in chapter 2.2.8. An exemplary dot test membrane of 15 patients is shown in Figure 3.7.

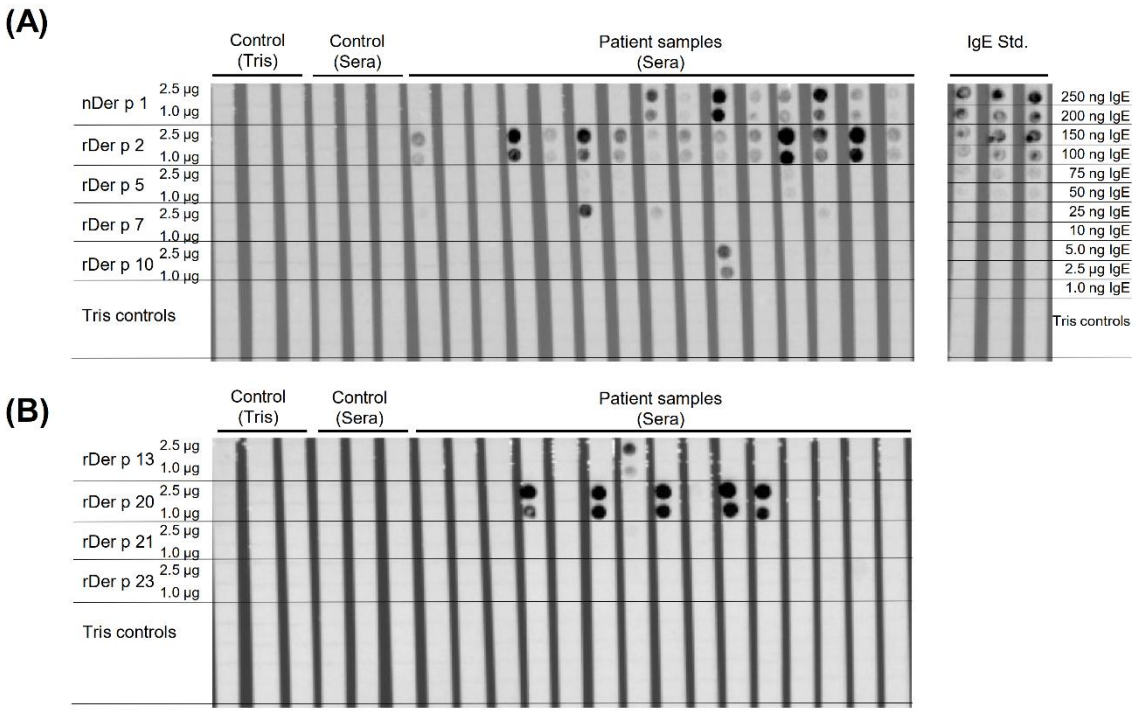


Figure 3.7: Exemplary nitrocellulose membrane strips used in the multiplex dot test

A–B: Example of an HDM-multiplex test after detection via HRP on a Chemidoc readout system for 15 patients. Includes human IgE standard serial dilution (right), negative controls with buffer and three different non-allergic sera (left).

3.2.1 Study cohort

The investigated study collective consisted of 199 patients with predominant allergic airway symptoms (SP1, Table 9) and 185 patients with predominant atopic dermatitis (AD) (SP2, Table 9). Their clinical characteristics are displayed in Table 40. The study participants were further divided into five subgroups (SG1–SG5) according to self-reported and medically confirmed symptoms, the detailed characteristics of which are listed in Appendix 15. Discrepancies between self-reported symptoms and medical history led to exclusion from subgroup allocation. Mean total serum IgE was considerably higher for patients with predominant AD symptoms (Table 40). The combination of AD with other allergic diseases depicts the extrinsic atopic phenotype, as described by Akdis et al. among others, which is by definition associated with

high total and specific serum IgE [205-207]. To still ensure comparability between groups, 87 patients (SG5, Appendix 15) with predominant AD were specifically selected with a low to moderate (0.70–50.0 kU/l) sIgE concentration against whole HDM extract.

Table 40: Characteristics of adult HDM-allergic patients.

	Patients (Total)	**SP1 Predominant airway**	**SP2 Predominant AD**	**P**
N	384	199	185	
Sex (m / w)	187 / 197	76 / 123	111 / 74	< 0.0001
Age [years] mean (min-max)	42 (18–87)	42 (18–87)	43 (23–86)	0.9945
total serum IgE mean, IU/ml (95% CI)	2691,9 (± 727.9)	562.2 (± 186.5)	3702.2 (± 1318.8)	< 0.0001
total serum IgE median, IU/ml (IQR)	622.0 (151.0–2500.0)	222.0 (106.7–533.3)	2039.0 (594.0–4452.0)	
Specific IgE concentrations [kU/l]				
sIgE Der p extract median, kU/l (IQR)	22.0 (4.48–124.5) [a]	9.1 (2.8–36.6) [a]	89.3 (13.5–354.0) [a]	< 0.0001
sIgE Der f extract median, kU/l (IQR)	N/A	11.0 (2.8–34.6)	N/A	
Allergic diseases				
Rhino-conjunctivitis Yes (%)	223 (82%) [b]	165 (83%)	58 (75%) [b]	0.9046
Allergic asthma Yes (%)	149 (55%) [b]	108 (54%)	41 (57%) [b]	0.6960
Atopic dermatitis Yes (%)	134 (49%) [b]	62 (31%)	72 (100%) [b]	< 0.0001
Hay fever Yes (%)	203 (75%) [b]	141 (71%)	62 (86%) [b]	0.0105
Animal dander allergy Yes (%)	123 (45%) [b]	83 (42%)	40 (56%) [b]	0.0431
Food allergy Yes (%)	132 (49%) [b]	86 (43%)	46 (64%) [b]	0.0026
Crustaceae allergy Yes (%)	22 (8%) [b]	12 (6%)	10 (14%) [b]	0.0364

Comparisons between predominant airway and predominant AD patients by Chi-square (prevalence) or Mann-Whitney (metric variables) tests. N/A = not available

[a] 2 patients from RCB/UKSH and 7 patients from MHH had CAP class 6 (> 100 kU/l) with unknown actual sIgE concentrations. 5 patients from MHH exceeded 2,500 kU/l sIgE whose sera were not further diluted.

[b] Only 72 of 185 patients from MHH completed a *post hoc* questionnaire regarding their HDM-specific symptoms other than AD. All patients suffered from AD as assessed by trained dermatologists

3.2.2 Performance of the multiplex dot test

In order to verify results of the dot test and evaluate if it was suited for the detection of sIgE antibodies, the results obtained for patients of SP1 (Table 40) were compared to the respective results of the ImmunoCAP for sIgE against n/rDer p 1 and rDer p 2 .The analysis revealed that results of sera with sIgE concentrations in CAP class 3 and higher were comparable between both methods. However, dependent of the used allergen, the multiplex dot test was significantly less sensitive at lower IgE-concentrations than the commercial test (Figure 3.8). The dot test detected significantly less samples as IgE-positive within CAP class 2 for both allergens ($P = 0.006$ (nDer p 1, positive rate 31% vs 16%), $P = 0.004$ (rDer p 2, positive rate 21% vs 7%)). Out of 113 patients tested IgE-positive for rDer p 1 in the ImmunoCAP, 88 were also tested IgE-positive for nDer p 1 in the multiplex dot test. Overall, the dot test was able to identify 85% (169/199) of the participants in SP1 as HDM-IgE-positive. The sensitivity of the multiplex test was not significantly inferior for samples with IgE-concentrations ranging in other CAP classes, even though there was a trend indicating a lower sensitivity of the multiplex for rDer p 2 within CAP class 3 ($P = 0.09$). Despite drawbacks regarding sensitivity, this analysis shows that the dot test is a suitable tool to detect sIgE antibodies nevertheless as it detected 85% of the patients' sensitizations correctly.

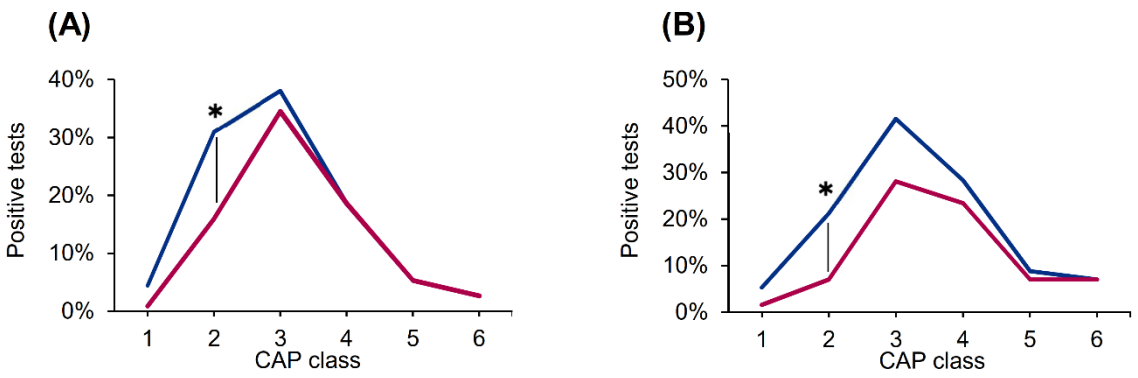


Figure 3.8: Performance of the multiplex dot test compared to ImmunoCAP for nDer p 1 and rDer p 2

A–B: Comparison of 199 HDM-allergic patients tested IgE-positive for n/rDer p 1 (A) or rDer p 2 (B) in the ImmunoCAP (blue line) with the respective multiplex results (red line). Prevalence of positive tests were compared with Fisher's exact test. Y-axis: percentage of population.
* $P < 0.01$

3.2.3 Sensitization count and its correlation to allergic multimorbidity

In clinical routine, high concentrations of sIgE detected in the patients' sera is often automatically associated with the severity of allergic disease. However, prevalence of sIgE against e.g. Der p extract can also be not clinically relevant at all, and studies report conflicting results whether IgE levels can be associated with prevalence and severity of allergic diseases [208-210]. A hypothesis developed within this thesis was that the severity and phenotype of the allergic disease is rather dependent on the number of recognized allergens within a single allergen source, hereafter referred to as "sensitization count", rather than IgE levels.

An analysis of the cumulative sensitization counts between patients exhibiting allergic asthma (AA) and allergic rhinitis (AR) as well as AD and non-AD, respectively, revealed that the frequency of sensitization to more than three allergens was significantly increased in individuals with predominant AD compared to non-AD patients (P = 0.0002; RR = 2.60 [1.62–4.18], Figure 3.9B). A similar trend was observed for asthmatic patients, even though this was not statistically significant (Figure 3.9A).

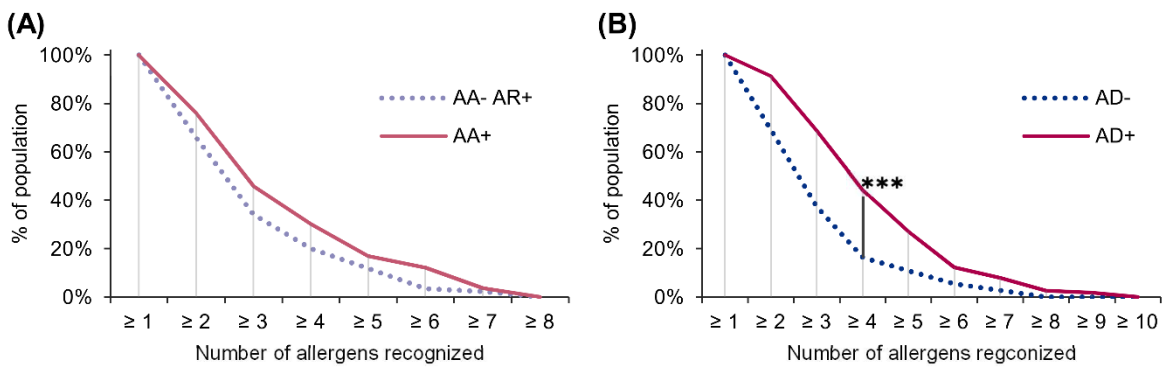


Figure 3.9: Sensitization counts in HDM-allergic patients with predominant AD or predominant airway symptoms

A–B: Cumulative sensitization counts in (A) AR (SG1, N = 84) and AA (SG2, N = 85) as well as (B) non-AD (SG3, N = 103) and predominant AD (N = 115) patients were compared with Fisher's exact test for ≥ 4 allergens (P = 0.15 (A); P < 0.001 (B)). Patients with > 100 kU/l sIgE against Der p extract were excluded to ensure comparability. * P < 0.05; ** P < 0.01; *** P < 0.001

Subsequently, the participants were grouped according to their clinical phenotypes, namely the single or combined presence of AR, AA and AD, and ranked after sensitization count and disease severity in Figure 3.10A. Supporting the results from Figure 3.9B, the ranking demonstrates that AD patients generally had a higher sensitization count than phenotypes without AD. Analysis of the ranking of the patients with phenotypes from isolated AR to

combined AR, AA and AD demonstrated a significant trend that sensitization count increased with clinical multimorbidity ($P < 0.0001$, Figure 3.10A).

Unlike sensitization count, the mean CAP class of Der p extract-specific IgE did not change between the phenotypes in CAP classes 1–5 (Figure 3.10B). Throughout CAP class 1–5 the distribution of phenotypes did not change, whereas patients exhibiting multiple allergic diseases were particularly more represented in CAP class 6 than in other classes (Figure 3.10C). A comparison of respective slopes and intercepts between CAP class 1–5 revealed no significant differences ($P = 0.59$) in phenotype composition, whereas CAP class 6 was significantly different from the others ($P < 0.0001$, Figure 3.10C). Therefore, contrary to the sensitization count, CAP classes other than CAP class 6 were not correlated with phenotype severity.

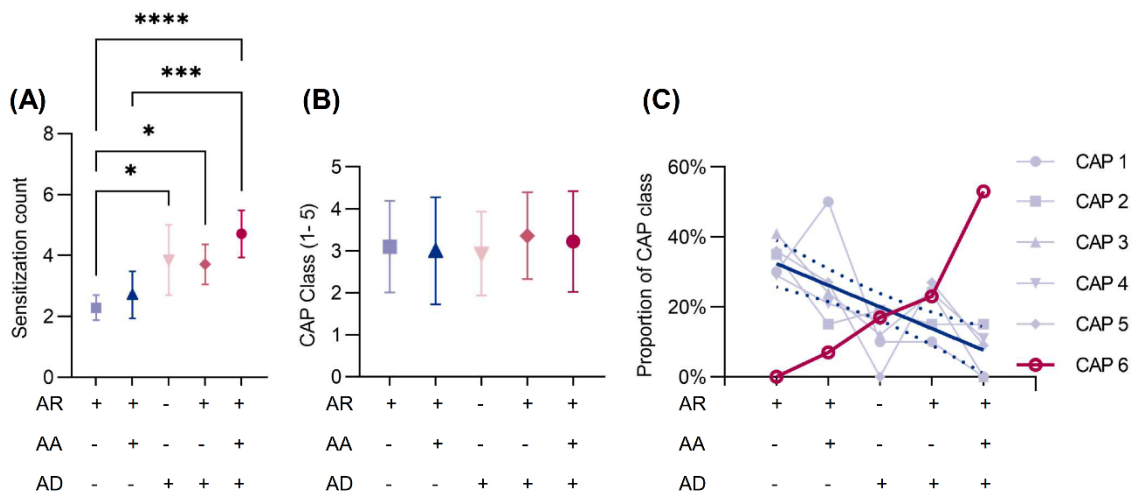


Figure 3.10: The influence of sensitization count and CAP class on allergy phenotype

A–B: Different phenotypes of HDM allergy were compared regarding (A) mean sensitization counts (Mean± 95%CI) and (B) mean CAP class 1–5 (Mean± 95%CI) of Der p extract-specific IgE measured by ImmunoCAP using One-Way ANOVA ($P < 0.0001$ (A); n.s. (B)); *Post hoc* tests: Tukey HSD (shown (A), n.s. (B)), Test for trend ($P < 0.0001$ (A); n.s. (B)). P values are multiplicity adjusted.

C: Determination of the association of each CAP class with allergic phenotypes via analysis of the phenotype distribution within the respective CAP class population by linear regression and comparison of respective slopes and intercepts. Proportions of phenotypes did not change between CAP class 1–5 ($P = 0.59$), whereas CAP class 6 was significantly different from the others ($P < 0.0001$) and was associated with the prevalence of multiple allergic diseases. Y-axis = Proportion of respective CAP class population. Straight blue line = best-fit curve of CAP class 1–5 with dotted lines = 95%CI

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; n.s. = not significant

After demonstrating the correlation of increasing sensitization count to phenotype severity, the sensitization counts were correlated with the respective ImmunoCAP results for IgE against Der p extract of the patients (Figure 3.11) by linear regression. Patients with sIgE concentrations between 500–2500 kU/l sIgE were excluded from this analysis as only few data

points were available for this large range. While the slope of the best-fit line after linear regression was significantly non-zero ($P < 0.0001$), the correlation coefficient ($r^2 = 0.093$) only indicated a weak correlation between sIgE concentration and sensitization count according to J. Cohen or even negligible according to Falk and Miller [211, 212]. This could explain the lack of correlation between CAP class and disease phenotypes presented in Figure 3.10B. Therefore, sIgE concentrations cannot be used as a surrogate parameter for sensitization count and cannot, with the exception of CAP class 6, be correlated with the allergic disease severity.

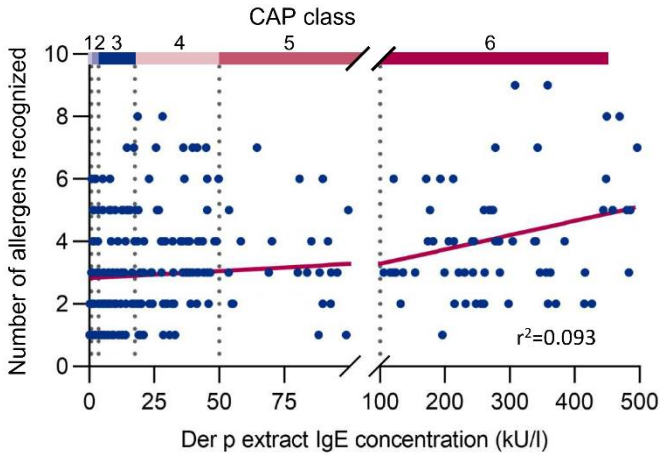


Figure 3.11: Correlation between sensitization count and specific IgE concentration

Correlation between sensitization count in multiplex-testing and sIgE concentrations against Der p extract as measured by ImmunoCAP in 312 HDM-allergic patients analyzed by linear regression ($r^2 = 0.093$); deviation from zero $P < 0.0001$.

The clinical relevance of the preceding results was assessed in Figure 3.12 by separating the participants into two groups with sensitization counts either higher than three or a maximum of three. Patients from SP2 were not included in this analysis, since they were specifically selected for their phenotypes and, therefore, were not representative for the general HDM-allergic population. Patients suffering from AR but no other HDM-related symptoms constituted the predominant phenotype in the group reacting to three or less allergens, the proportion of which significantly declined in patients reacting to more than three allergens (57%/29%, $P = 0.03$; RR = 0.51 [0.27–0.99]). The combination of AA and AR without AD was approximately the same for both groups. However, the frequency of combined AA and AD was significantly higher in patients sensitized to more than 3 allergens (9%/29%, $P = 0.02$; RR = 3.08 [1.20–7.90]) and was the most frequent phenotype together with isolated AR. Combined AR and AD was also more prevalent in this group (8%/21%) but the difference was not significant ($P = 0.13$; RR = 2.57 [0.86–7.67]).

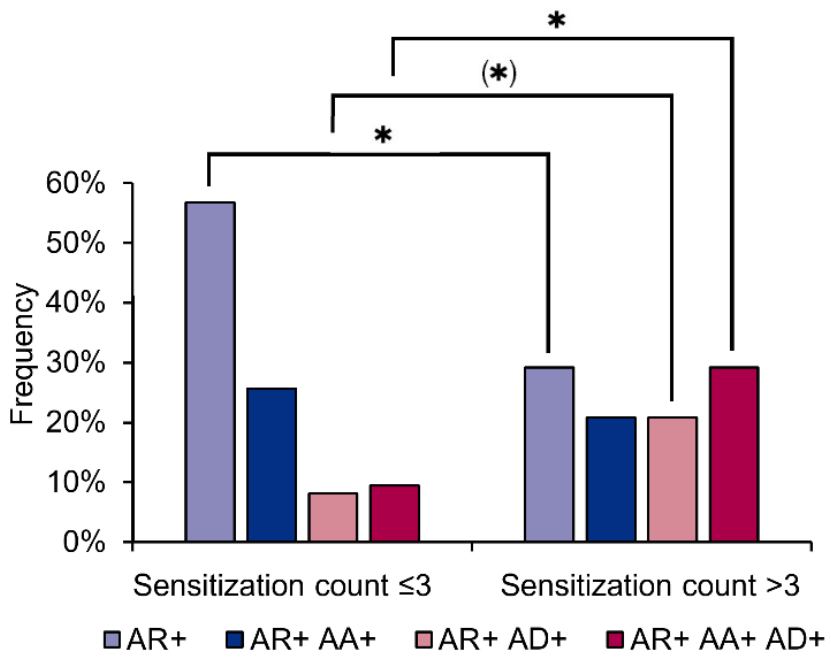


Figure 3.12: Potential clinical relevance of sensitization count as a diagnostic parameter

The disease phenotype distribution between patients with a sensitization count > 3 or ≤ 3 HDM allergens was compared with Fisher's exact test, significant differences between the subgroups are shown. * $P < 0.05$

All of these results confirm the initial hypothesis that the disease severity was associated with the sensitization count in form of the simultaneous presence of multiple allergic diseases, particularly if they were AD-associated. Furthermore, the Der p extract-specific IgE concentrations did not reflect this and with the exception of very high sIgE levels they were not correlated with disease severity.

3.2.4 Identification of potential marker allergens

The results obtained from the multiplex dot test by investigating the presence of sIgE against a panel of Der p allergens as described in chapter 2.2.8 were further analyzed to establish individual sensitization profiles of HDM-allergic adults (SP1 and SP2, Table 9 and Table 40) in order to identify allergens that were connected to different allergic diseases, namely AA or AD, and could therefore serve as potential biomarkers in the future.

3.2.4.1 Stratified molecular sensitization profiling in atopic diseases

The frequency of sensitizations against single Der p allergens was first investigated in stratified analyses by comparing the predefined groups (refer to Appendix 15) of patients regarding their sensitization profiles detected with the multiplex dot test. A representative multiplex membrane is depicted in Figure 3.7.

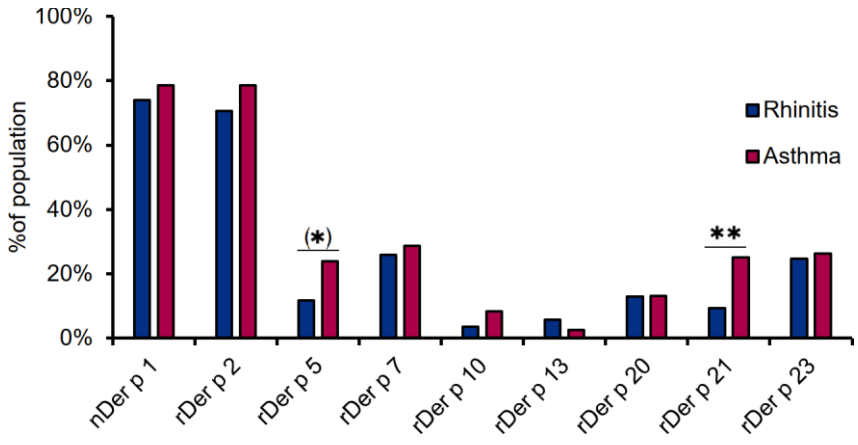


Figure 3.13: Sensitization profiles of patients with predominant airway symptoms

Prevalence of sensitization to nine different HDM-allergens of asthmatic (red bars, N = 84) and rhinitis-only (blue bars, N = 85) adults with medically confirmed HDM allergy. y-axis: prevalence in the respective group. Multiplex-signals > 0.5 RMU were considered to be positive. All comparisons by Fisher's exact test. Significant differences between the subgroups are shown. (*) $P < 0.1$, * $P < 0.05$ and ** $P < 0.01$

To identify potential marker allergens in AA patients (SG2, Appendix 15), study participants from the predominant airway allergy group were compared to patients with isolated AR (SG1, Appendix 15). As expected, sensitizations to the well-studied major allergens nDer p 1 and rDer p 2 were most common in both groups, followed by sensitizations to rDer p 7, 23 or 20 in rhinitic patients (Figure 3.13). Albeit rDer p 23 being reported as a major allergen, the sensitization prevalence did not reach 50% in the patients of this cohort [58]. Sensitization to rDer p 21 was significantly more frequent in asthmatic individuals with a more than two-fold increase ($P = 0.008$; RR = 2.65 [1.25–5.66]), and a similar trend was observed for rDer p 5, even though this effect was not quite significant ($P = 0.069$; RR = 1.92 [0.95–3.84]).

The sensitization profiles of patients with predominant AD (SG4 + 5, Appendix 15) were compared to those of non-AD patients (SG3, Appendix 15). The latter group consisted of study patients from SP1 (Table 9 and Table 40) who had no medical history of AD and who stated in the questionnaire that they had never suffered from AD neither during childhood nor as an adult. Results of sensitization profiling are shown in Figure 3.14A. Similar to the results of AA patients, adults exhibiting AD were significantly more often sensitized to rDer p 5 ($P < 0.0001$;

RR = 3.46 [1.91–6.27]) and rDer p 21 (P = 0.0023; RR = 2.20 [1.29–3.79]) compared to non-AD patients. Additionally, prevalence of rDer p 2 (P < 0.0001; RR = 1.56 [1.31–1.84]) and rDer p 20 (P < 0.0001; RR = 2.93 [1.74–4.95]) sensitization was significantly higher in AD patients as well. Remarkably, there were also significant, more than 4-fold increases in the otherwise rare sensitizations to rDer p 10 and rDer p 13 (P = 0.004; RR = 4.28 [1.73–10.58]).

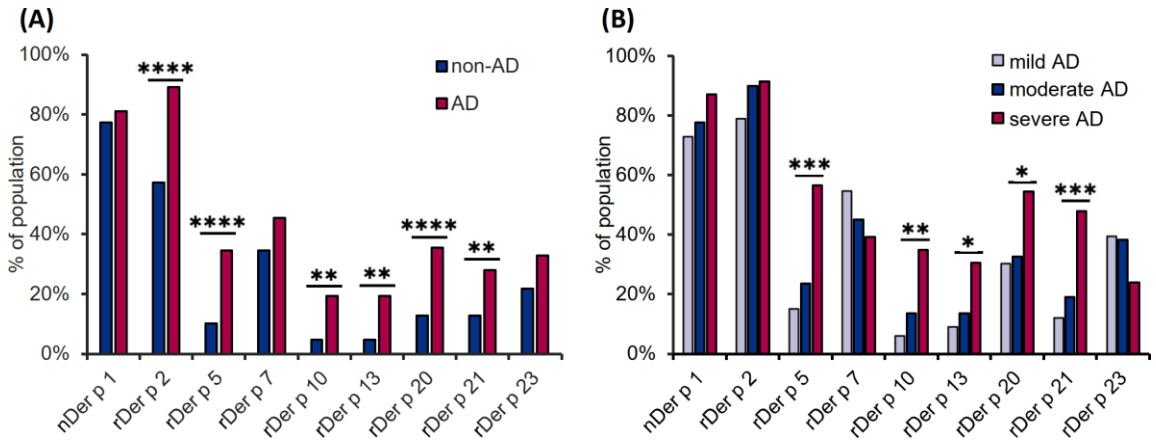


Figure 3.14: Sensitization profiles of patients with predominant atopic dermatitis

Prevalence of sensitization to nine different HDM-allergens of HDM-allergic adults with (red bars, N = 185, SG4 + 5) and without (blue bars, N = 110, SG3) atopic dermatitis (A) as well as sensitization prevalence of adults with mild (light blue bars, N = 33), moderate (blue bars, N = 89) or severe (red bars, N = 46) atopic dermatitis as assessed by trained dermatologists (B). Multiplex-signals > 0.5 RMU were considered to be positive. y-axis: prevalence in respective group. All comparisons by Fisher's exact test. Significant differences between the subgroups are shown. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001

In order to further elucidate how the sensitization patterns observed in AD patients were influenced by the severity of their AD, 165 patients with AD were subsequently divided according to disease severity in mild, moderate and severe cases as assessed by trained dermatologists (Figure 3.14B). Patients lacking severity assessment and six patients falling in-between severity grades (e.g. "mild to moderate") were excluded from the subgroups. No significant differences between mild and moderate phenotype subsets were found. Patients with severe AD had numerous significantly increased sensitization prevalences compared to moderate cases, namely rDer p 5 (P = 0.0002; RR = 2.40 [1.52–3.76]), rDer p 10 (P = 0.0065; RR = 2.58 [1.34–4.98]), rDer p 13 (P = 0.022; RR = 2.26 [1.14–4.47]), rDer p 20 (P = 0.017; RR = 1.67 [1.12–2.49]) and rDer p 21 (P = 0.0007; RR = 2.50 [1.48–4.23]). Interestingly, prevalence of rDer p 7 and rDer p 23 sensitizations did slightly decline with increasing disease severity. These changes were, however, not significant. Since, unlike all others, changes in rDer p 2 sensitization prevalence shown in Figure 3.14A are not reflected here, the difference in Figure 3.14A might be an artefact from the multiplex dot test due to low sIgE of non-AD patients and lower multiplex-sensitivity for rDer p 2 demonstrated in Figure 3.8.

3.2.4.2 Unstratified analysis of sensitization patterns

As sensitization frequency to rDer p 5 and rDer p 21 was increased in both AA and AD patients, particularly severe AD, in the stratified analyses, the question arose whether one or both of these effects were a result of confounding due to multimorbidity of patients exhibiting both AA and AD.

Therefore, the association of Der p allergens with AA and AD was determined by unstratified analysis of data from 241 patients, which equated to all patients from SG1–5 with completed questionnaires, using univariate and subsequently multiple logistic regression models. This combination allowed to first identify confounding factors and subsequently determine the associations of variables, such as different Der p allergens, with defined outcomes, in this case the prevalence of AA or AD, after adjustment for these confounders.

Univariate effects for all allergens as well as the potential confounders age, sex and prevalence of AA or AD were determined for the outcomes AD or AA as independent variables, respectively. Results are listed in Table 41. While age and sex had no significant effect on the outcomes, AA and AD were associated with each other in the univariate analyses, respectively, which confirmed the hypothesis that these effects were confounded by each other in the stratified analyses. Therefore, allergens with a significant effect on the outcome AA or AD were each adjusted for the occurrence of AD or AA, respectively, in multivariate logistic regression analyses. After adjustment, rDer p 5 and rDer p 21 were associated with AA ($P = 0.0080$, $OR = 2.48$ [1.28–4.95]; $P = 0.0060$, $OR = 2.63$ [1.34–5.37]) and a similar trend was observed for rDer p 10 even though this did not reach statistical significance ($P = 0.0823$, $OR = 2.29$ [0.93–6.18]). rDer p 2, 5, 10, 20, 21 and 23 were associated with AD, respectively (rDer p 2: $P = 0.0022$, $OR = 2.84$ [1.48–5.64]; rDer p 5: $P = 0.0010$, $OR = 3.24$ [1.64–6.72]; rDer p 10: $P = 0.0485$, $OR = 2.66$ [1.06–7.64]; rDer p 20: $P = 0.0048$, $OR = 2.76$ [1.39–5.76]; rDer p 21: $P < 0.0001$, $OR = 4.68$ [2.27–10.48]; rDer p 23: $P = 0.0002$, $OR = 3.21$ [1.78–5.93]).

This essentially reflects the results observed in the stratified analyses (Figure 3.13 and Figure 3.14A). However, sensitization to rDer p 23 was not significantly increased in the stratified analyses of AD patients. In the logistic regression model, only 72 patients from the predominant AD group were included due to missing or incomplete questionnaires from other participants. Sensitization to rDer p 23 was increased in this subpopulation (43% in the subpopulation / 33% in the whole population) and might not be representative for the whole group, which could have caused these differences.

Due to their biological similarities in sequence and folding, rDer p 5 and rDer p 21 were combined in separate multivariate models with AA or AD as outcomes, respectively, as well.

The effect estimates were substantially different (23–38% change in estimates) in these models compared to the adjusted effects of the individual allergens, indicating a further relationship between rDer p 5 and rDer p 21 themselves.

Overall, the unstratified data analysis could confirm the essential results from chapter 3.2.4.1 and further substantiate the finding that rDer p 5 and rDer p 21 were associated with both AA and AD independently of allergic multimorbidity. The association of rDer p 20 with AD was confirmed as well. Furthermore, a separate multivariate model indicated that an additional relationship between rDer p 5 and rDer p 21 exists.

Table 41: Results of univariate and multiple logistic regression analyses of molecular sensitization patterns

Univariate analyses (Outcome: AD)

Variable	Estimate	Error	z value	P	OR [95 CI]
Age	-0.0103	0.009	-1.149	0.2510	0.98[0.97–1.01]
Sex	0.1069	0.2647	0.403	0.6870	1.11[0.66–1.87]
AA	0.6234	0.2623	2.377	**0.0175**	1.87[1.12–3.13]
nDer p 1	0.5997	0.3152	1.903	0.0571	1.82[0.98–3.41]
rDer p 2	1.1028	0.3369	3.2273	**0.0011**	3.01[1.58–5.95]
rDer p 5	1.2879	0.3504	0.3675	**0.0002**	3.62[1.87–7.44]
rDer p 7	0.4951	0.2784	1.778	0.0754	1.64[0.95–2.85]
rDer p 10	1.071	0.4951	2.183	**0.0290**	2.92[1.17–8.30]
rDer p 13	0.6204	0.4822	1.23	0.1980	1.85[0.74–5.07]
rDer p 20	1.063	0.3553	2.992	**0.0028**	2.89[1.47–5.99]
rDer p 21	1.62	0.381	4	**< 0.0001**	5.03 [2.47–11.15]
rDer p 23	1.1301	0.3011	3.753	**0.0002**	3.09[1.74–5.68]

Univariate analyses (Outcome: AA)

Variable	Estimate	Error	z value	P	OR [95 CI]
Age	0.0041	0.009	0.452	0.6510	1.00[0.99–1.02]
Sex	0.0759	0.2658	0.256	0.7750	1.08[0.64–1.82]
AD	0.6234	0.2623	2.377	**0.0175**	1.87[1.12–3.13]
nDer p 1	0.4279	0.3166	1.352	0.1770	1.53[0.83–2.88]
rDer p 2	0.4393	0.3215	1.367	0.1720	1.55[0.83–2.94]
rDer p 5	1.0325	0.3335	3.096	**0.0020**	2.81[1.48–5.52]
rDer p 7	0.2277	0.2758	0.826	0.4090	1.26[0.73–2.16]
rDer p 10	0.9524	0.4694	2.029	**0.0424**	2.59[1.07–6.93]
rDer p 13	0.5039	0.4693	1.074	0.2830	1.65[0.67–4.33]
rDer p 20	0.3418	0.3284	1.041	0.2980	1.41[0.74–2.70]
rDer p 21	1.11	0.3396	3	**0.0011**	3.02 [1.58–6.03]
rDer p 23	0.1203	0.281	0.43	0.6670	1.13[0.65–1.96]

Multivariate analyses (Outcome: AD)

Variable	Estimate	Error	z value	P	OR [95 CI]
rDer p 2	1.0429	0.3404	3.064	**0.0022**	2.84[1.48–5.64]
AA	0.5742	0.268	2.143	**0.0321**	1.78[1.05–3.01]
rDer p 5	1.1744	0.357	3.29	**0.0010**	3.24[1.64–6.72]
AA	0.4597	0.2717	1.692	0.0906	1.58[0.93–2.70]
rDer p 10	0.9791	0.4962	1.973	**0.0485**	2.66[1.06–7.64]
AA	0.5597	0.2657	2.107	**0.0351**	1.75[1.04–2.96]
rDer p 20	1.0152	0.36	2.82	**0.0048**	2.76[1.39–5.76]
AA	0.5931	0.267	2.221	**0.0263**	1.80[1.07–3.07]
rDer p 21	1.5443	0.3866	3.995	**< 0.0001**	4.68[2.27–10.48]
AA	0.4089	0.2755	1.484	0.1378	1.50[0.88–2.59]
rDer p 23	1.1656	0.3054	3.816	**0.0002**	3.21[1.78–5.93]
AA	0.6361	0.2714	2.344	**0.0191**	1.88[1.11–3.23]
rDer p 5	0.6906	0.3934	1.756	0.0792	1.99[0.93–4.40]
rDer p 21	1.2614	0.4156	3.035	**0.0024**	3.53[1.61–8.31]
AA	0.3482	0.2795	1.246	0.2128	1.42[0.82–2.45]

Multivariate analysis (Outcome: AA)

Variable	Estimate	Error	z value	P	OR [95 CI]
rDer p 5	0.9078	0.342	2.654	**0.0080**	2.48[1.28–4.95]
AD	0.4597	0.2717	1.692	0.0906	1.58[0.93–2.70]
rDer p 10	0.8266	0.4757	1.738	0.0823	2.29[0.93–6.18]
AD	0.5597	0.2657	2.107	**0.0351**	1.75[1.04–2.96]
rDer p 21	0.9674	0.3522	2.746	**0.0060**	2.63[1.34–5.37]
AD	0.4089	0.2755	1.484	0.1378	1.51[0.88–2.59]
rDer p 5	0.629	0.3743	1.68	0.0929	1.87[0.91–0.96]
rDer p 21	0.7057	0.3844	1.836	0.0664	2.03[0.96–4.38]
AD	0.3544	0.2787	1.272	0.2035	1.42[0.82–2.46]

3.2.4.3 Analysis of rDer p 20-specific IgE levels in atopic dermatitis

During analysis of the results presented in chapter 3.2.4.1 for AD patients with high Der p extract-specific IgE concentrations (SG4, Appendix 15), a distinct group of samples with particularly high signals for rDer p 20-specific IgE stood out in the raw data of the multiplex dot test. These samples clustered around the upper limit of the detection scale between 90–100 RMU, and were remarkably distinct from sera with lower signals to rDer p 20, which did not exceed 55 RMU (Figure 3.15).

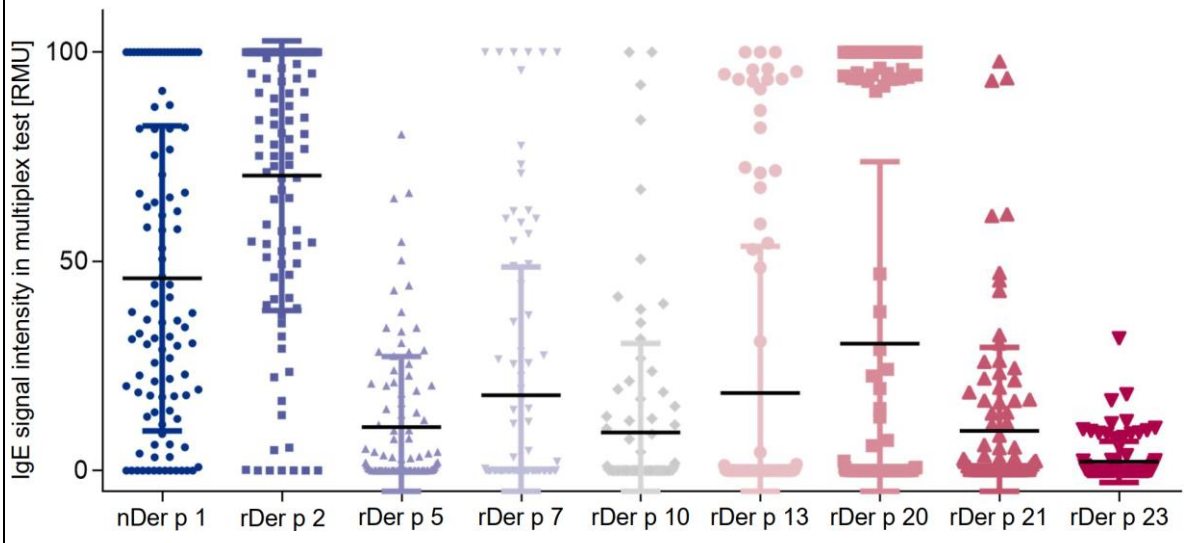


Figure 3.15: Results of rDer p 20-IgE detection in patients with atopic dermatitis

Multiplex dot test signal intensities measured in RMU against 9 different HDM allergens in sera of patients with AD and high Der p extract-specific IgE levels as measured by ImmunoCAP (SG4, Appendix 15). Depicted is mean ± standard deviation.

Since the multiplex-assay only determines semi-quantitative sIgE results, rDer p 20-IgE positive patients were subsequently divided into "rDer p 20-high IgE" and "rDer p 20-low IgE" subgroups: The prevalence of severe AD in the two subsets and additionally "rDer p 20-IgE negative" patients was compared (Figure 3.16). As a result, more than 70% of "rDer p 20-high IgE" patients suffered from severe AD, which was more than two-fold higher than in the "rDer p 20-low IgE" group ($P = 0.027$; RR = 2.62 [0.97–7.07]) and in the "rDer p 20-IgE negative" group ($P = 0.0008$; RR = 2.33 [1.44–3.78]). There were no significant differences in the severities of AD between the rDer p 20-low IgE and rDer p 20-IgE negative subsets.

Overall, 50% (18/36) of the participants with severe AD and high IgE concentrations against Der p extract (CAP class 5/6) were identified as rDer p 20-high IgE patients.

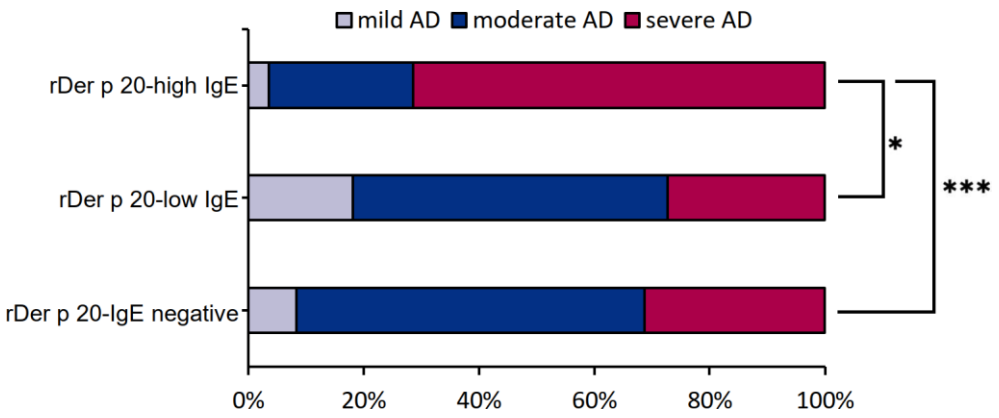


Figure 3.16: Association of high IgE against rDer p 20 with severe atopic dermatitis

All rDer p 20-IgE positive (N = 39) individuals with predominant AD and high Der p extract-specific IgE levels (SG5, Appendix 15) were divided into high and low IgE-responders, and AD severity was compared to rDer p 20-IgE-negative (N = 48) patients. All comparisons by Fisher's exact test. Significant differences between the subgroups are shown. * P < 0.05 ** P < 0.01

3.2.5 Integration of potential marker allergens in routine diagnostic platforms

The previously identified potential marker allergens for AA (rDer p 5, rDer p 21) and AD (rDer p 20) were subsequently integrated into the routine diagnostic platform ImmunoCAP by applying the streptavidin CAP technique as described in chapter 2.2.10.

A particular intention of this was to further specify the presumably high sIgE levels against rDer p 20 observed in chapter 3.2.4.3, since the multiplex dot test can only generate semi-quantitative results. Therefore, the sIgE concentrations of all rDer p 20-IgE-positive patients from SG3 (N = 7) and SG4 (N = 39) (refer to Appendix 15) with enough material left for further analyses were quantified via ImmunoCAP.

The results revealed that 75% of individuals with sIgE concentrations of more than 80 kU/l exhibited severe AD, which represents an over two-fold increase in prevalence when compared to patients with less than 80 kU/l sIgE (P = 0.012; RR = 2.25 [1.06–4.77]) or rDer p 20-IgE negative patients (P = 0.0009; RR = 2.35 [1.44–3.83]) (Figure 3.17A). Comparison of patients with less than 80 kU/l sIgE and rDer p 20-IgE-negative subsets resulted in no significant difference between the two. The ImmunoCAP measurements (Figure 3.17A) therefore confirmed and further specified the results obtained with the multiplex-test (Figure 3.16).

Accordingly, rDer p 20-IgE concentrations were significantly higher in patients with severe AD compared to moderate (P = 0.029), mild (P = 0.026) or no AD (P = 0.0045) (Figure 3.17B), reaching concentrations of up to over 1000 kU/l in some patients. Overall, 50% (18/36) of study

participants with severe AD and high Der p extract-specific IgE concentrations (SG4, Appendix 15) had more than 80 kU/l of sIgE against rDer p 20. The concentration of sIgE against rDer p 20 accounted for an average of 5.9% of Der p extract-specific IgE for AD patients below 80 kU/l and 57.1% for AD patients above 80 kU/l of rDer p 20-specific IgE, respectively.

In conclusion, these results verified those obtained by the dot test. It confirmed the role of a high sIgE concentration against rDer p 20 in severe cases of AD and further substantiated its biomarker potential.

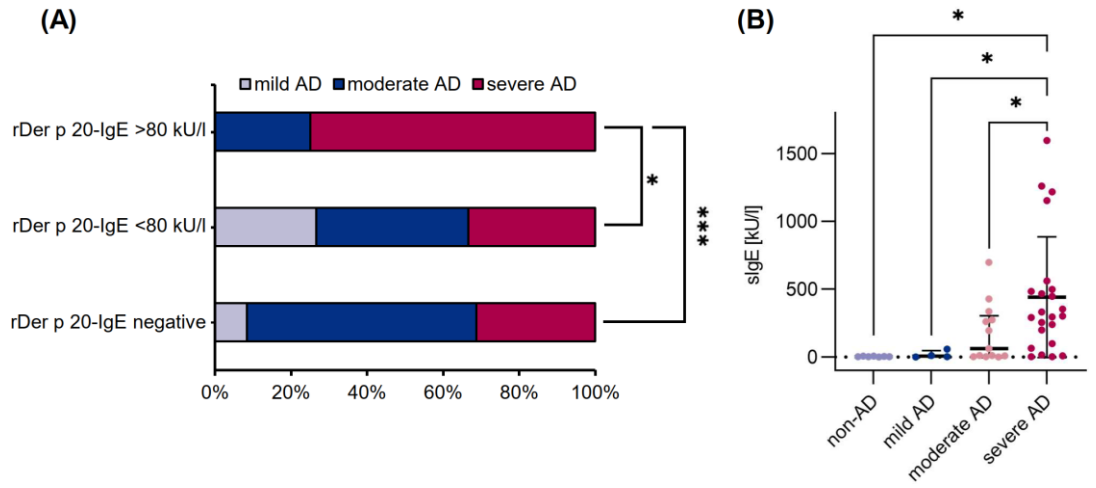


Figure 3.17: Verification of biomarker potential of rDer p 20 via ImmunoCAP

A: rDer p 20-IgE-negative (N = 48) and rDer p 20-IgE-positive patients (N = 39) with mild, moderate, or severe AD and high sIgE against Der p extract were investigated for severity of AD in individuals with sIgE against rDer p 20 above or below 80 kU/l by Fisher's exact test.
B: Derp 20-specific IgE concentrations measured by ImmunoCAP were directly compared between the different severity groups and rDer p 20-IgE-positive non-AD patients (N = 7) using One-Way ANOVA (P = 0.008); *Post hoc* test: two-stage step-up method of Benjamini, Krieger and Yekutieli (shown). Error bars depict median and interquartile ranges. Significant differences between the groups are shown.
* P < 0.05, ** P < 0.01, *** P < 0.001

Furthermore, the concentrations of sIgE against rDer p 5 and rDer p 21 were measured by ImmunoCAP for all study participants of SP1 (Table 9 and Table 40) with a positive result for these allergens in the multiplex dot test as described before, too. The results in Figure 3.18 demonstrate that asthmatic patients did not exhibit elevated serum IgE concentrations against neither rDer p 5 nor rDer p 21 compared to rhinitic patients. In patients with AD the sIgE concentrations against rDer p 5 were significantly increased, and a similar trend was observed for rDer p 21 although this was not significant. In both cases, only few patients were available for the measurements, which was a disadvantage in the statistical analyses and it was not possible to further differentiate the severity grades of AD. Overall, in comparison to rDer p 20, the sIgE concentrations were moderate and only 2 (Der p 5) or 3 (rDer p 21) samples exceeded

the quantification limit of the ImmunoCAP. It therefore appears that for rDer p 5 and rDer p 21 the prevalence of sensitization itself is the decisive risk factor for AA and not the sIgE levels.

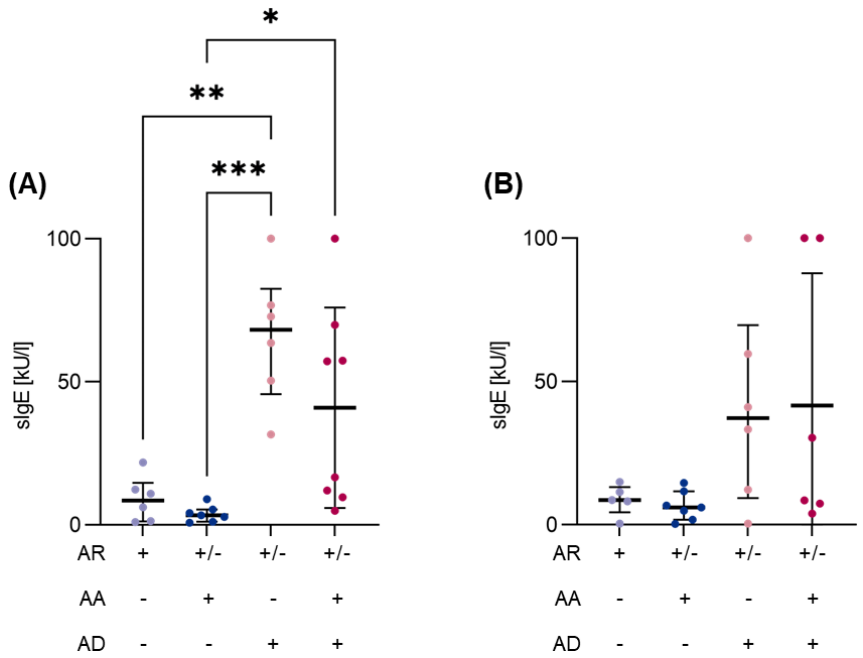


Figure 3.18: Quantification of rDer p 5- and rDer p 21-specific IgE concentrations by ImmunoCAP.

A–B: sIgE concentrations against rDer p 5 (A, N = 27) and rDer p 21 (B, N = 24) measured by ImmunoCAP were directly compared between the allergic phenotypes using One-Way ANOVA ($P < 0.0001$ (A); $P = 0.0725$ (B)); Post hoc test: Tukey HSD (shown). Error bars depict median and interquartile ranges. Significant differences between the groups are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (multiplicity adjusted)

3.3 Molecular sensitization profiling of asthmatic, wheezing and non-asthmatic children

After identifying potential marker allergens associated with AA in adults in the previous chapter, the next step was to identify allergens associated with the development of asthma in children. The initiator allergens, defined as the first allergen recognized by an individual during their course of sensitization, were of special interest as they may give insight into the relevance of single allergens for the development of asthma and might further be a helpful guidance in the development of preventive strategies. The aim was to identify “high risk initiator allergens”, that indicate a risk of asthma during childhood if a sensitization to one or several of these allergens is present.

3.3.1 Study cohort

The group of study participants included in this part of the project consisted of 143 children in total with 38 non-asthmatic controls, 53 wheezing children (age 0–4) and 52 asthmatic children recruited during an acute exacerbation of bronchial obstruction (SP3, Table 9). Wheezing is a term used to describe children of preschool age with transient or persistent episodes of bronchial obstruction that can potentially evolve to clinically manifest asthma later on. Children were screened for sIgE against an aeroallergen mix containing Der p extract among others (6 missing values) and in case of a positive result, the concentration of sIgE against Der p extract was additionally determined (3 missing values). While medical history of AR, AD and conjunctivitis were documented for most study participants, it was unclear whether these symptoms were causally related to HDM exposure.

Table 42: Characteristics of asthmatic, wheezing and non-asthmatic children.

	Non-Asthma	Wheeze	Asthma	P
N	38	53	52	
Sex (m / w)	21/17	34/19	37/15	0.2986
Age [years] mean (min-max)	8 (1–16)	2 (0–4)	10 (5–17)	< 0.0001
total serum IgE mean, IU/ml (95% CI)	148.5 (± 79.7)	186.0 (± 136.9)	661.3 (± 208.1)	< 0.0001
total serum IgE median, IU/ml (IQR)	43.1 (14.0–212.3)	37.0 (8.7–93.4)	354.5 (98.3–944.8)	
Specific IgE measurements				
sIgE sx1 positive N (%)	12 (32%)	13 (25%)	42 (81%)	< 0.0001
sIgE sx1 [kU/l] median (IQR)	1.4 (0.9–10.5)	21.0 (6.8–60.7)	52.0 (15.3–98.9)	
sIgE d1 positive N (%)	10 (26%)	11 (21%)	36 (69%)	0.0941
sIgE d1 [kU/l] median (IQR)	2.2 (1.4–6.0)	65.3 (11.5–99.4)	58.2 (12.7– > 100.0)	
Allergic diseases				
Atopic dermatitis: unknown	4	3	12	0.2016
Atopic dermatitis: Yes (%) [a]	6 (18%)	15 (30%)	7 (18%)	
Rhinitis: unknown	2	3	11	< 0.0001
Rhinitis: Yes (%) [a]	2 (6%)	7 (14%)	28 (68%)	
Conjunctivitis: unknown	2	3	11	< 0.0001
Conjunctivitis: Yes (%) [a]	2 (6%)	7 (14%)	19 (46%)	

Comparisons between subgroups by Chi-square (prevalence) or Kruskal-Wallis (metric variables) tests.

sx1 = Aeroallergen mix containing cat and dog dander, HDM (Der p), timothy grass, birch, rye, mugwort and *Cladosporium herbarum*. d1 = Der p extract. IQR = interquartile range

[a] Percentage calculated without individuals with unknown status of the respective parameter

3.3.2 Development of a novel infrared-based microarray for IgE detection

While the multiplex dot test used in the previous chapters proved to be an effective tool for detection of sIgE in adults, it had limitations regarding sensitivity, included a labor-intensive logit-log plot standardization and required 50 µl of serum per analysis. Since biomaterial collection in young children is often only possible to a very limited extent, and the resulting samples are valuable, a chip-based microarray utilizing fluorescence detection in the near infrared (NIR) range was developed as described in chapter 2.2.9, which offered superior sensitivity compared to fluorophores absorbing at UV wavelengths due to low background fluorescence at NIR wavelengths while only requiring 25 µl of serum. The use of nitrocellulose-coated chips additionally provided high protein-binding capacity in comparison to regular surface-activated glass slides, and further enhanced the sensitivity of the array. Developing this method included extensive optimization of the composition of the blocking buffer and antibody diluent as well as incubation times and spot size (not shown), which for example included the addition of arginine to the blocking buffer and antibody diluent to combat unspecific signals caused by the arginine kinases rDer p 20 and rDer f 20 interacting with arginine residues of the detection antibody. An exemplary microarray slide is depicted in Figure 3.19A. The final protocol resulted in a highly sensitive assay that was able to reliably detect IgE at a concentration of less than 1 kU/l with an average signal-to-noise ratio of 10.8 (95%CI ± 5.5, N = 12) and high linearity of the standard curve with a mean correlation coefficient of 0.9936 (95%CI ± 0.0075, N = 12), which additionally enabled a simplified normalization without the need of a logit-log plot (Figure 3.19B) resulting in semi-quantitative units. A comparison between the children identified as sensitized against Der p extract by the ImmunoCAP and children with at least one detected sensitization in the microarray revealed no significant differences for any of the CAP classes (Figure 3.19C). Still, the microarray only identified a part of children with CAP class 1 (0.35–0.70 kU/l sIgE) as positive. This could be caused by the fact that, in contrast to the comparison made in chapter 3.2.2, the ImmunoCAP parameter used for reference was Der p extract-specific IgE rather than sIgE against a single allergen, resulting in the effect that low-level sensitizations to multiple allergens can add up in the extract-based measurement, which is not possible in the component-resolved microarray. Overall, the method developed within this thesis demonstrated high capability of detecting even low levels of sIgE while simultaneously the required amount of serum was reduced by half. The high linearity allowed for easy standardization and might even be a base to allow a true quantitative measurement of sIgE concentrations in the future, which, however, will have to be validated individually for each allergen.

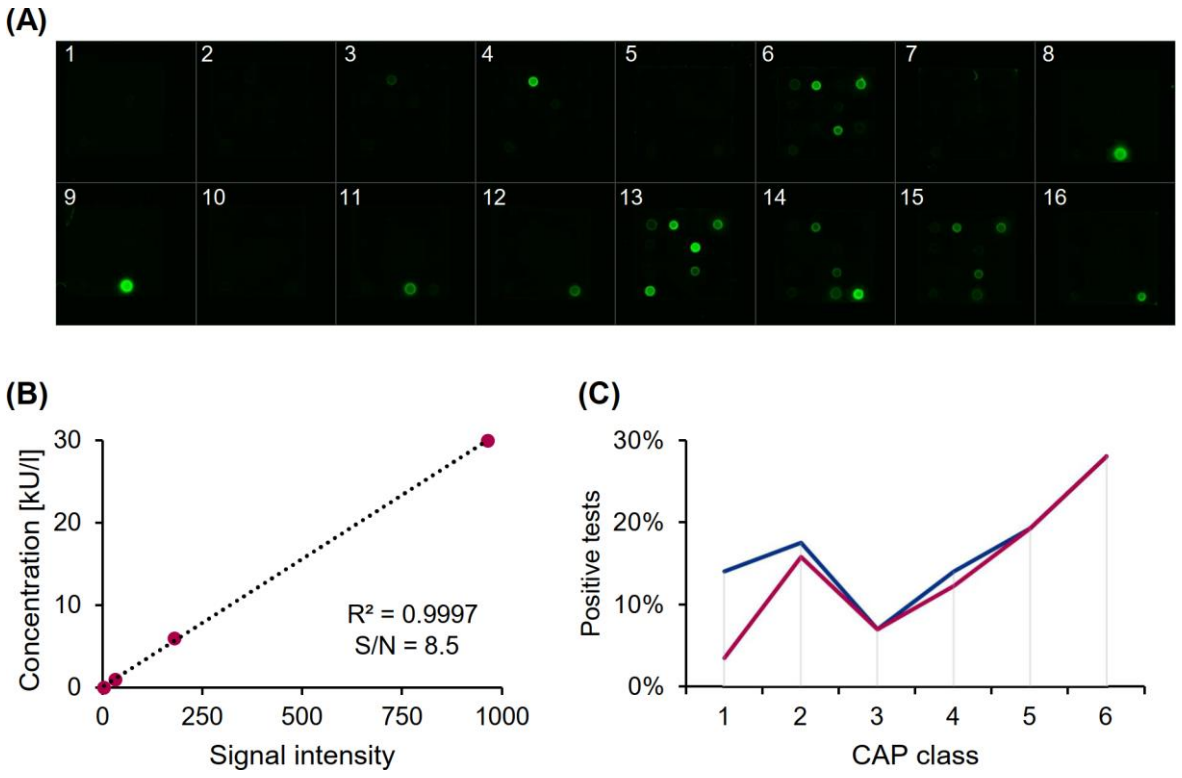


Figure 3.19: Sensitivity and linearity of the novel infrared-based microarray

A: Exemplary microarray chip with negative control serum (1), calibrator sera consisting of negative serum spiked with 1, 6 and 30 kU/l of anti-Der p 2-IgE respectively (2–4), and samples of 12 asthmatic children (5–16).
B: Exemplary standard curve generated by linear regression from negative and calibrator control sera used to normalized sample signals. S/N = signal to noise ratio.
C: Comparison of samples detected as HDM-sensitized in the ImmunoCAP (Der p extract, blue line) and the microarray (Sensitization count ≥ 1, red line). Comparison for each CAP class by Fisher's exact test, no significant differences were found.

3.3.3 Sensitization patterns and initiator allergens

In contrast to adult patients, the asthmatic children reacted significantly more often to more than three allergens than the non-asthmatic controls (P = 0.0318; RR = 3.95 [1.00–15.58]) as shown in Figure 3.20A. This could be due to the fact that these children were recruited during a time of exacerbation of their bronchial symptoms and therefore overall exhibited a possibly more severe and uncontrolled disease than the adult asthmatic patients analyzed in the previous chapters. It could also be an indicator that the sensitization count in asthmatic individuals rises faster than in non-asthmatic individuals, a difference that might be less prominent in adulthood where the patients have often been sensitized since many years. Furthermore, the control group for the asthmatic adults in Figure 3.9 were rhinitic patients whereas the prevalence of an HDM-induced rhinitis is unclear in the non-asthmatic children. The sensitization count was not significantly different between young (5–10 years) and adolescent asthmatic children (11–17

years) in Figure 3.20B, demonstrating that the complex IgE response to HDM allergens is most likely already developed in the beginning of the disease. In wheezing children, the sensitization count seemed to be in a transient state between non-asthmatic and asthmatic individuals.

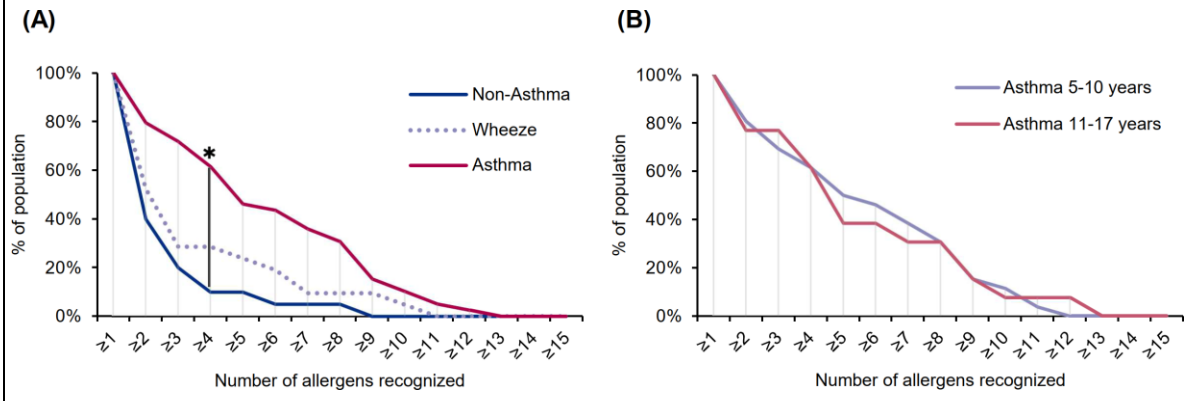


Figure 3.20: Sensitization counts of asthmatic, wheezing and non-asthmatic children

A–B: Cumulative sensitization counts in (A) asthmatic (N = 39), wheezing (N = 21) and non-asthmatic (N = 20) children as well as (B) asthmatic children aged 5–10 years (N = 26) and 11–17 years (N = 13) were compared with Fisher's exact test for ≥ 4 allergens (P = 0.0318 (A); n.s. (B)). * P < 0.05

The detailed analysis of the molecular sensitization profiles of the different subgroups (Figure 3.21A) revealed that asthmatic children were significantly more often sensitized to the major allergens nDer p 1 (P = 0.0175; RR = 2.63 [1.06–6.54]), rDer f 1 (P = 0.0035; RR = 4.87 [1.27–18.72]), rDer p 2 (P = 0.0207; RR = 1.71 [1.02–2.85]) and rDer p 23 (P = 0.008; RR = 4.61 [1.19–17.94]) but also rDer p 5 (P = 0.0114; RR = 7.69 [1.09–54.13]) and rDer p 7 (P = 0.0219; RR = 6.67 [0.94–47.39]), the former of which was already identified as a potential asthma biomarker in the previous chapters, compared to non-asthmatic children. Additionally, sensitization prevalence of rDer f 5 (P = 0.0218; RR = N/A) and rDer f 21 (P = 0.0422; RR = 5.79 [0.80–41.69]), but not rDer p 21, was significantly increased as well. Interestingly, only 45% of non-asthmatic children were sensitized to rDer p 2 but 65% to rDer f 2, the latter of which was a similar degree of sensitization as seen in asthmatic children. The sensitization rates of wheezing children ranged, as before, between those of non–asthmatic and asthmatic children with no significant differences to either group except for rDer f 1 (P = 0.0341; RR = 4.07 [1.00–16.53]), which was significantly more often recognized compared to non-asthmatic children. No sensitizations against rDer p 13 were detected in any group.

Furthermore, the sensitization patterns between young and adolescent children were compared in Figure 3.21B to detect age-related differences in sensitizations to the different HDM species. While the sensitization rates to major Der p and Der f allergens as well as rDer p/f 20 were similar in both age groups, young children were more often sensitized to rDer f 5 and rDer f 21 whereas adolescent children rather exhibited sensitization to rDer p 5 and rDer p 21. However, these changes were not significant, possibly due to the low number of patients in the subgroups.

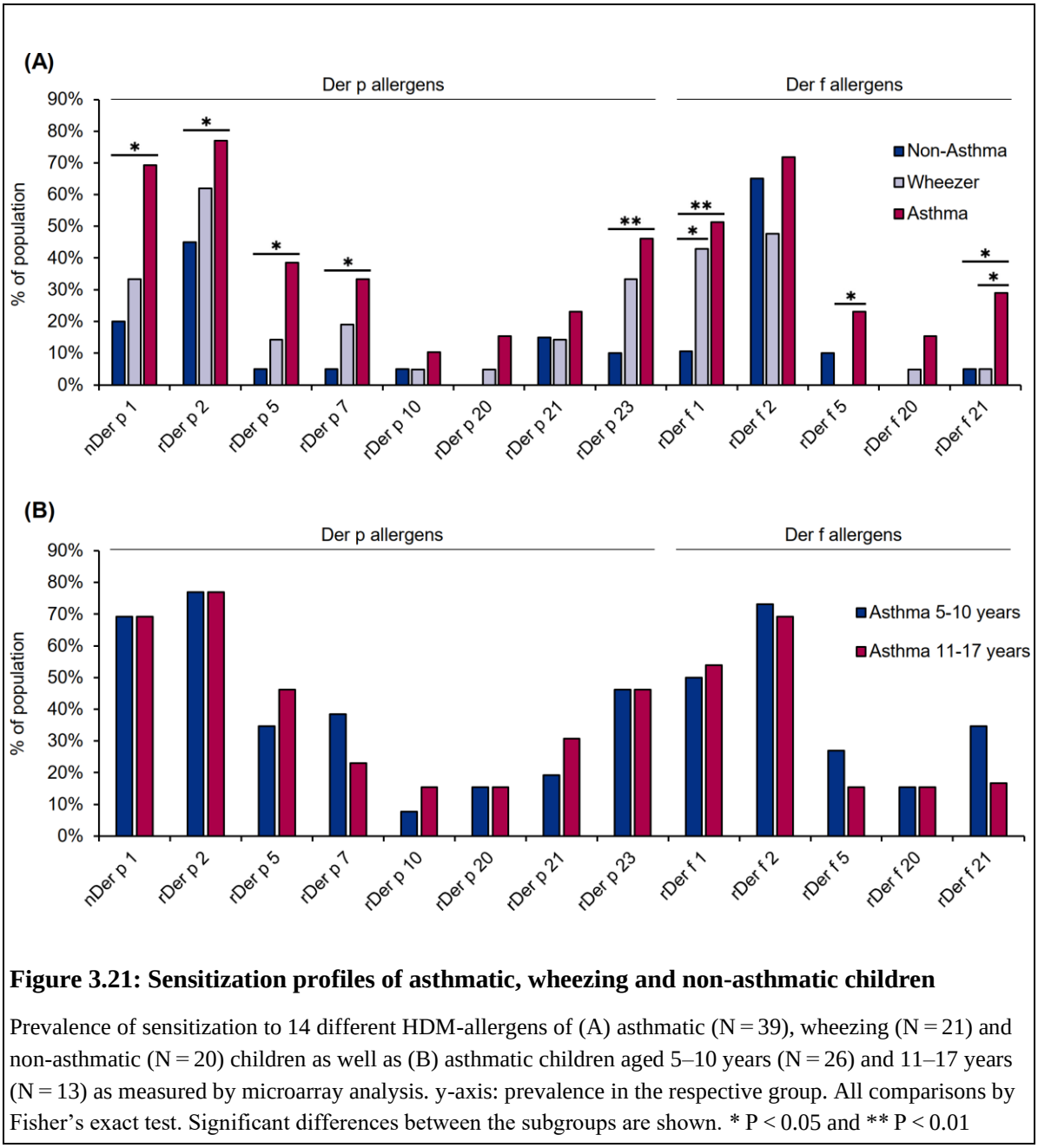


Figure 3.21: Sensitization profiles of asthmatic, wheezing and non-asthmatic children

Prevalence of sensitization to 14 different HDM-allergens of (A) asthmatic (N = 39), wheezing (N = 21) and non-asthmatic (N = 20) children as well as (B) asthmatic children aged 5–10 years (N = 26) and 11–17 years (N = 13) as measured by microarray analysis. y-axis: prevalence in the respective group. All comparisons by Fisher's exact test. Significant differences between the subgroups are shown. * $P < 0.05$ and ** $P < 0.01$

Investigation of the degree and nature of mono- and polysensitization to HDM allergens of the different study groups showed that, in accordance with the results presented in Figure 3.20, asthmatic children were significantly less often monosensitized than wheezing ($P = 0.0403$; RR = 0.43 [0.20–0.92]) and non-asthmatic ($P = 0.00038$; RR = 0.34 [0.17–0.70]) study

participants (Figure 3.22A). However, closer examination of the nature of monosensitizations revealed that, in case of monosensitization, asthmatic children were significantly more often monosensitized to non-major allergens compared to the other two subgroups, wherein the monosensitization usually was caused by major allergens ($P = 0.0015$; RR = N/A and $P = 0.0194$; RR = 4.5 [1.19–16.96], Figure 3.22B). The analysis of single allergens responsible for monosensitizations, in order to identify possible initiator allergens in asthmatic children, showed that in wheezing (70%) and non-asthmatic (59%) children, group 2 mite allergens were the main source of sensitization whereas group 1 allergens were only responsible for approximately 20% of the monosensitizations (Figure 3.22C). Monosensitization to rDer p 7 was observed in non-asthmatic as well as asthmatic children. In asthmatic children, only 25% of monosensitizations were triggered by major allergens and 50% were caused by group 5 and group 21 mite allergens, which further emphasizes their importance for AA. The monosensitizations to these allergens were already present at a young age, as the youngest children with monosensitization to Der p 7 or group 21 mite allergens were 5 years old while the group 5 mite allergens were first recognized as monosensitizations at age 7. Children monosensitized to these allergens were asthmatic in 70% of the cases, which furthermore demonstrates that these allergens had a high potential of being “initiator allergens”, meaning that they were able to initiate allergic sensitization without the involvement of major allergens, and this monosensitization was furthermore associated with the risk of asthma.

Overall, these results show that a complex sensitization pattern involving a high sensitization count is associated with asthma in children. Furthermore, the identified marker allergens rDer p 5 and rDer p 21 as well as their equivalents in *D. farinae* are already relevant and potent initiator allergens by causing monosensitizations in asthmatic children. rDer p 7 was furthermore able to initiate HDM sensitization in asthmatic as well as non-asthmatic children. Although there were hints that particularly young children might be sensitized to Der f rather than Der p allergens compared to adolescents, the study cohort was too small to statistically assess these results with sufficient computational power.

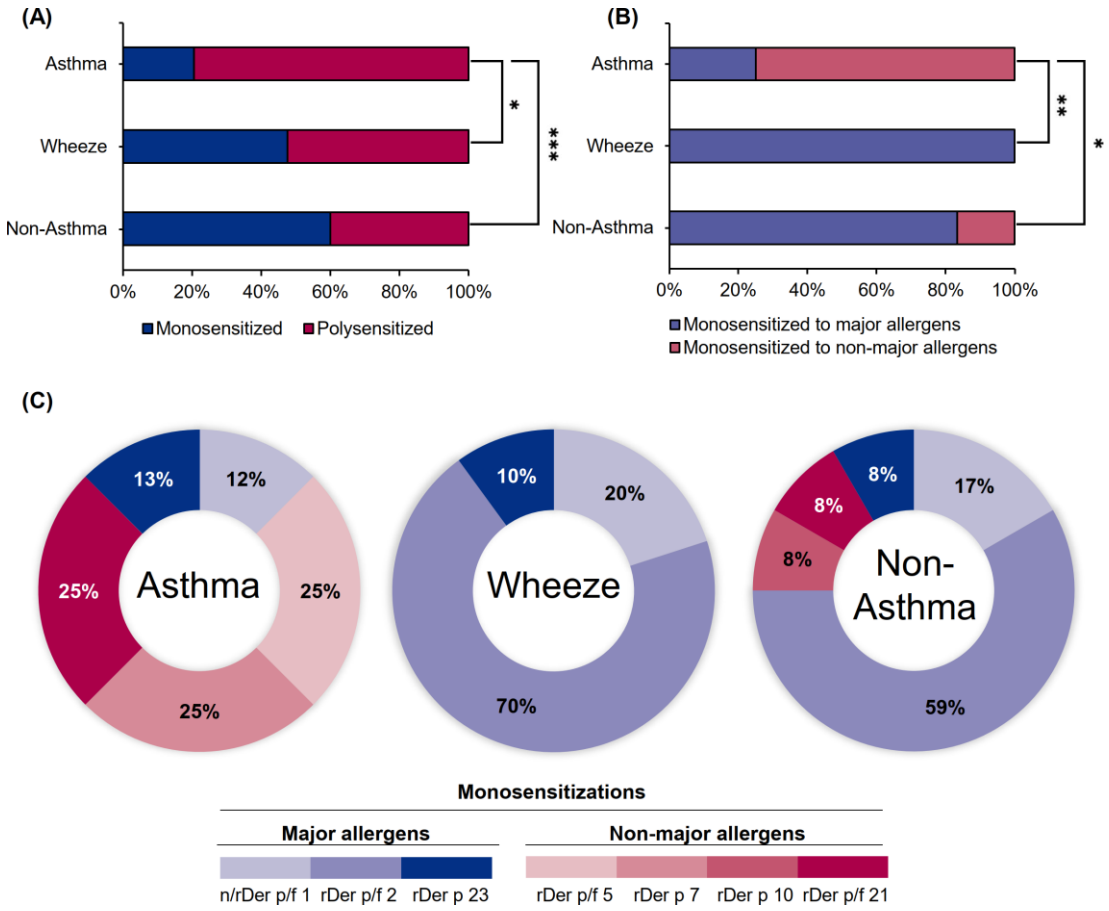


Figure 3.22: Mono- and polysensitization in asthmatic, wheezing and non-asthmatic children

A–B: Comparisons of mono- and polysensitization prevalence (A) as well as cause of monosensitization (B) in asthmatic (N = 39), wheezing (N = 21) and non-asthmatic (N = 20) children. All comparisons by Fisher's exact test. Significant differences between the subgroups are shown. * P < 0.05, ** P < 0.01, *** P < 0.001
C: Molecular HDM allergens recognized as monosensitizations in the sera of asthmatic (N = 8, A), wheezing (N = 10, B), and non-asthmatic children (N = 12, C). Der p and Der f allergens were grouped into group 1, 2, 5 and 21 mite allergens.

3.4 Stimulation of bronchial epithelial cells by HDM allergens

After having identified potential marker allergens in chapter 3.2.4, they were subsequently further immunologically characterized with the help of cell culture models using the human bronchial epithelial cell line Calu-3. The aim was to elucidate the ways in which these allergens can partake in the elicitation of inflammation, as they seemingly had a high clinical relevance in severe forms of allergic diseases. After culturing the Calu-3 cells as described in chapter 2.2.11.2, they were tested for mycoplasma infection by PCR (refer to chapter 2.2.11.3), since these can influence not only the physiology but also metabolism of infected cells and therefore distort the results of stimulation experiments [213]. The absence of a band at 500 bp in Figure 3.23 confirmed that the cells were healthy and not infected by mycoplasmas.

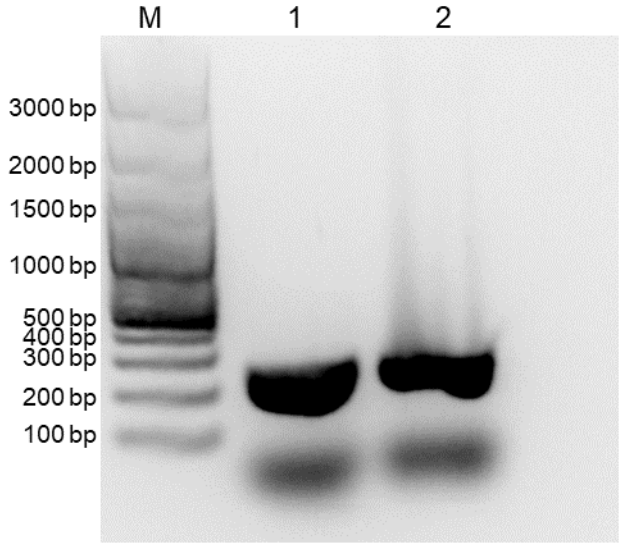


Figure 3.23: Test for mycoplasma contamination of Calu-3 cells by PCR

Confirmation of absence of mycoplasmas from cell culture supernatant indicated by lack of a band with a size of 500 bp. M: Marker, 1: Cell culture supernatant, 2: Internal control

In addition to the recombinant allergens rDer p 5, 20 and 21, the importance of which became evident in chapter 3.2.4, recombinantly produced rDer p 10 was chosen as a stimulus. Even though sensitizations in the general HDM-allergic population are relatively rare, it was associated with severe AD as shown in Figure 3.14 and associated with AA in the univariate logistic regression model (Table 41).

A further pre-test for the stimulation experiments was the determination of LPS content in the recombinant allergens. While LPS is unavoidable in protein expression in *E. coli*, it can skew results with its TLR4 activity and subsequent cytokine release. The LPS concentrations determined with an LAL assay as described in chapter 2.2.11.1 ranged between 0.4–10 EU/ml at a protein concentration of 0.1 mg/ml (for detailed results refer to Appendix 16). Therefore, an internal LPS control of 50 EU/ml was added to the stimulation assay design to ensure the observed signals were not a result of LPS contamination. Since preliminary experiments indicated that the Calu-3 cells had a high tolerance for LPS, a concentration of 100,000 EU/ml was chosen as the positive control and as an inhibition control for the neutralizing anti-TLR4 antibody. The TLR2/TLR1 agonist Pam3CSK4 served as a positive and inhibition control for TLR2-dependent stimulation.

In the stimulation assay, the concentration of various proinflammatory mediators, all associated with AA (Table 35), in response to the HDM allergens or controls were determined in the cell culture supernatant with an electrochemiluminescence-based multiplex assay. All three recombinant allergens that were associated with AA in the previous chapters, namely rDer p 5,

rDer p 10 and rDer p 21, had a significant impact on the release of at least three of the analytes, whereas recombinant rDer p 20, which was found to be associated with AD, did not influence any of the cytokine concentrations in the cell culture supernatant (Figure 3.26).

Among the other allergens, rDer p 5 had the lowest capability of activating the Calu-3 cells. In its highest tested concentration, it was able to increase the release of IL-6 ($P = 0.023$), IL-8 ($P = 0.043$) and GM-CSF ($P = 0.025$) between two- to three-fold compared to PBS but none of the other analytes (Figure 3.24). rDer p 10 stimulated the release of these mediators as well and to a much higher degree with approximately 10- to 15-fold change at 200 µg/ml compared to PBS (Figure 3.25). It moreover stimulated the release of various other cytokines, namely eotaxin-3 ($P = 0.0064$), G-CSF ($P < 0.0001$), IL-1α ($P = 0.0007$), IL-1β ($P < 0.0001$), IL-15 ($P < 0.0001$), IP-10 ($P < 0.0001$) and TNF-α ($P < 0.0001$). Particularly the concentration of IL-15 was notably increased over 50-fold compared to PBS and 9-fold compared to the positive control Pam3CSK4, which was the highest value induced by any of the stimuli. However, the most potent activator overall was rDer p 21, as it induced the broadest range of cytokine releases often resulting in analyte concentrations multiple times higher than induced by the other allergens and positive controls (Figure 3.27). As an example, at 200 µg/ml it led to an approximately 26- to 28-fold increase of IL-6 and IL-8 production compared to PBS, respectively, which corresponded approximately to more than ten times (Der p 5) or two times (Der p 10) higher concentrations than in cells that were stimulated with the other allergens. In addition to the cytokine release induced by rDer p 10, rDer p 21 furthermore significantly increased the release of IL-18 ($P = 0.0001$). None of the allergens or controls significantly affected the production of VEGF-A by the Calu-3 cells.

The TLR4- and TLR2-dependent positive controls LPS and Pam3CSK4 did, as expected, induce the release of multiple cytokines, and the inhibition by the neutralizing antibodies worked well for both substances. Nevertheless, none of the allergens showed a partial or full inhibition of its epithelial-activating ability when the cells were pretreated with the TLR2- and TLR4-neutralizing antibodies. Since both TLR-dependent positive controls did however lead to a similar pattern of cytokine releases, in particular compared to rDer p 10 and rDer p 21, it seems possible that pathways involving other TLRs or heterodimers thereof could be involved.

Overall, these results demonstrate that all asthma-associated allergens, with rDer p 21 as the most potent one, led to an increased release of proinflammatory mediators whereas the AD-associated allergen rDer p 20 did apparently not interact with the bronchial epithelial cells. rDer p 20 might therefore exhibit an organ specificity in regards to the skin. However, this hypothesis is of speculative nature as of now and will have to be further investigated using skin-specific cell models in the future.

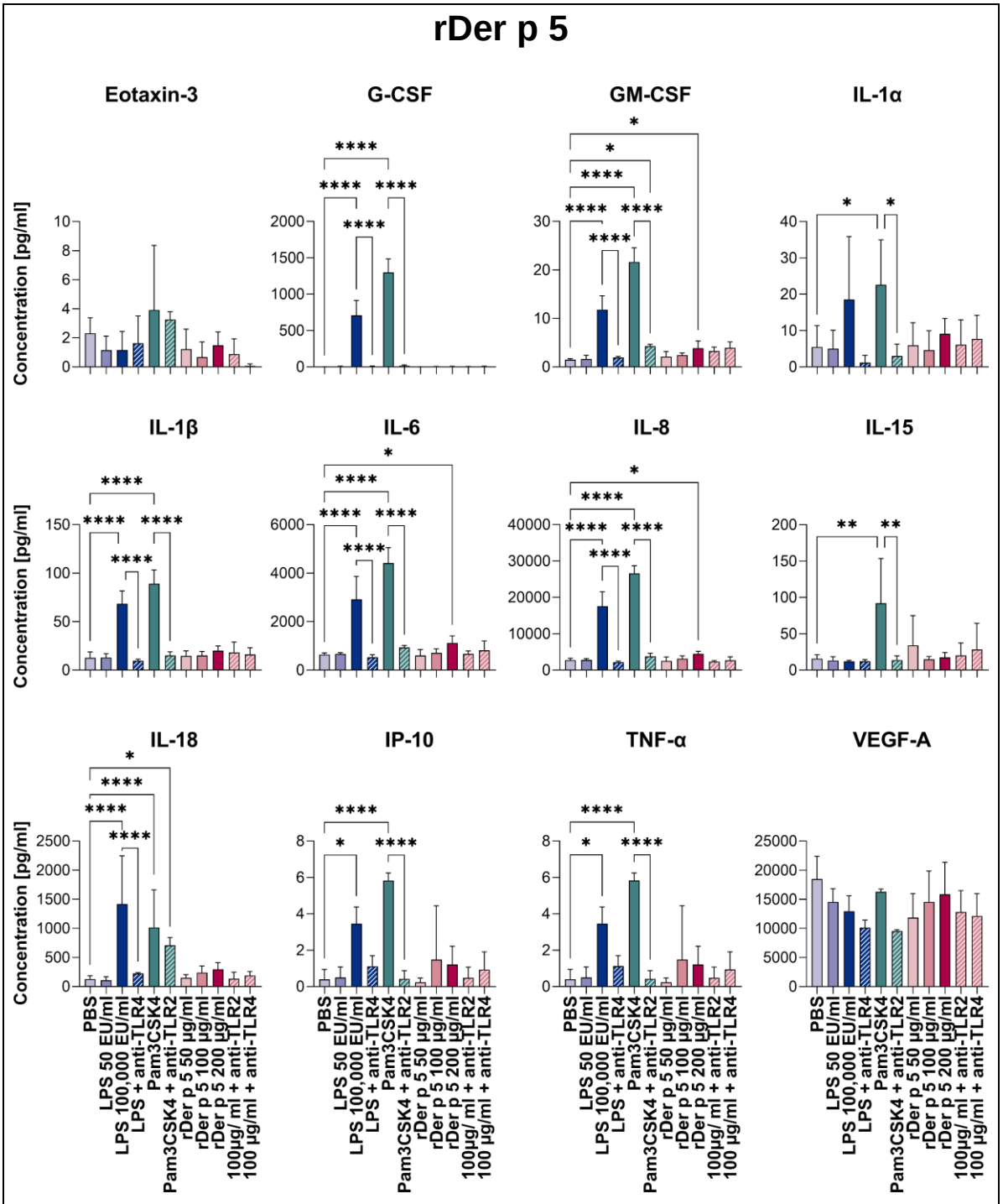


Figure 3.24: Cytokine release of Calu-3 cells after stimulation with rDer p 5

Negative controls: PBS, LPS 50 EU/ml. Positive controls: LPS 100,000 EU/ml (TLR4-dependent), Pam3CSK4 (TLR2-dependent). Inhibition controls: anti-TLR4 (neutralizing) + LPS 100,000 EU/ml, anti-TLR2 (neutralizing) + Pam3CSK4. Stimuli: rDer p 5 50 µg/ml, 100 µg/ml (stimulation and inhibition tests) and 200 µg/ml. Statistical analysis was carried out with One-Way ANOVA and *post hoc* Šídák test for preselected pairings. All controls and stimuli were compared to PBS, and inhibition tests were additionally compared to the corresponding uninhibited samples or controls.

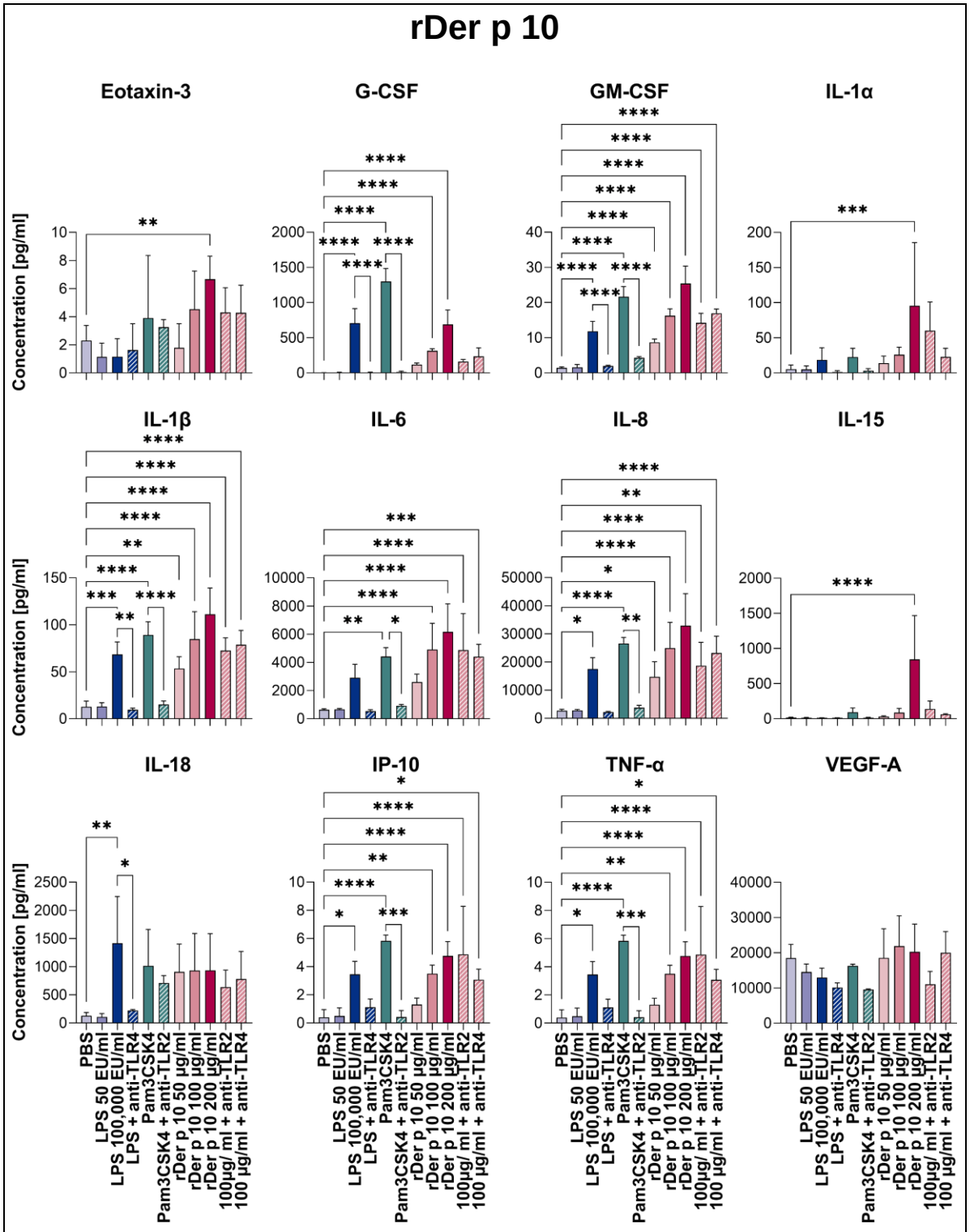


Figure 3.25: Cytokine release of Calu-3 cells after stimulation with rDer p 10

Negative controls: PBS, LPS 50 EU/ml. Positive controls: LPS 100,000 EU/ml (TLR4-dependent), Pam3CSK4 (TLR2-dependent). Inhibition controls: anti-TLR4 (neutralizing) + LPS 100,000 EU/ml, anti-TLR2 (neutralizing) + Pam3CSK4. Stimuli: rDer p 10 50 µg/ml, 100 µg/ml (stimulation and inhibition tests) and 200 µg/ml. Statistical analysis was carried out with One-Way ANOVA and *post hoc* Šídák test for preselected pairings. All controls and stimuli were compared to PBS, and inhibition tests and controls were additionally compared to the corresponding uninhibited values.

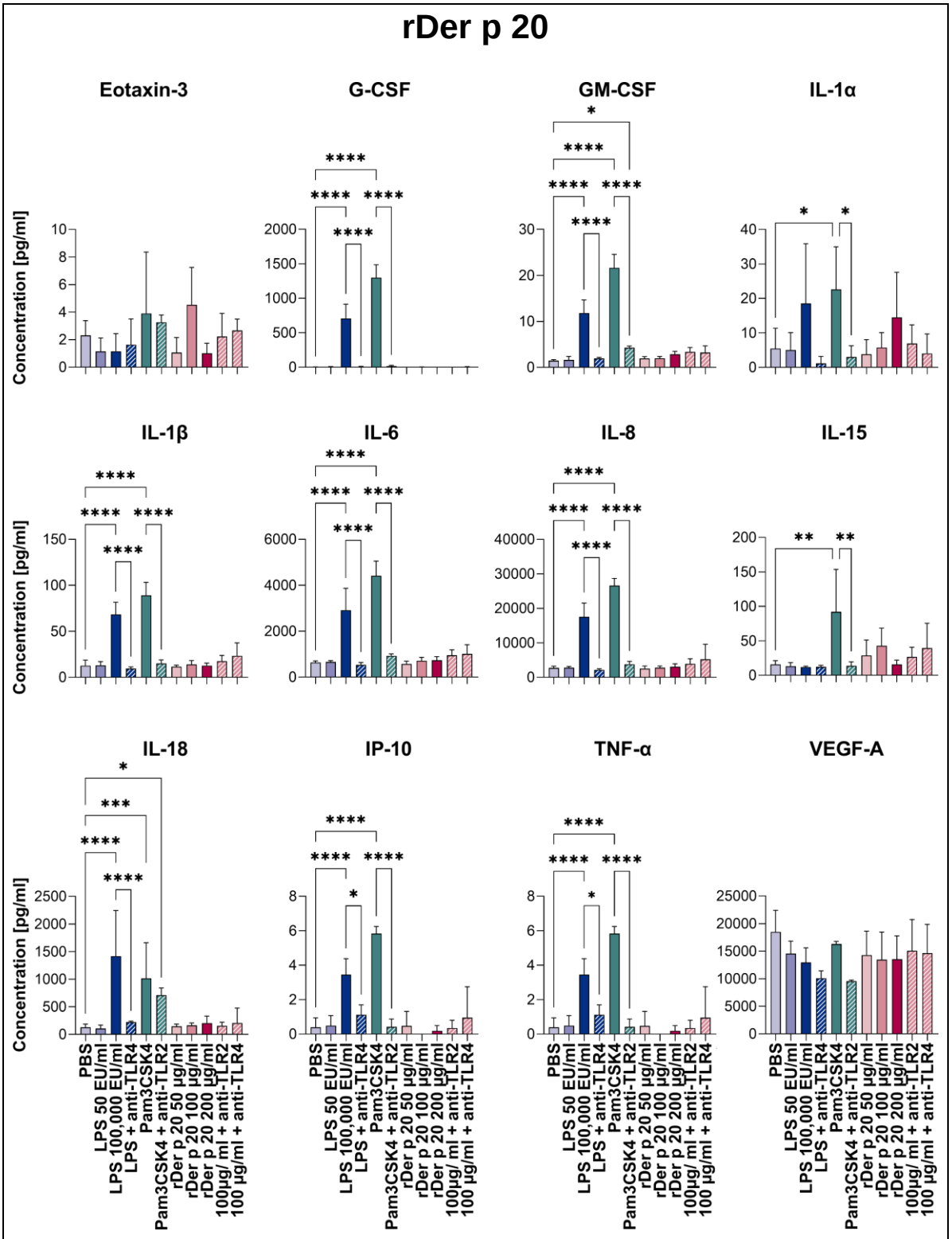


Figure 3.26: Cytokine release of Calu-3 cells after stimulation with rDer p 20

Negative controls: PBS, LPS 50 EU/ml. Positive controls: LPS 100,000 EU/ml (TLR4-dependent), Pam3CSK4 (TLR2-dependent). Inhibition controls: anti-TLR4 (neutralizing) + LPS 100,000 EU/ml, anti-TLR2 (neutralizing) + Pam3CSK4. Stimuli: rDer p 20 50 µg/ml, 100 µg/ml (stimulation and inhibition tests) and 200 µg/ml. Statistical analysis was carried out with One-Way ANOVA and *post hoc* Šídák test for preselected pairings. All controls and stimuli were compared to PBS, and inhibition tests and controls were additionally compared to the corresponding uninhibited values.

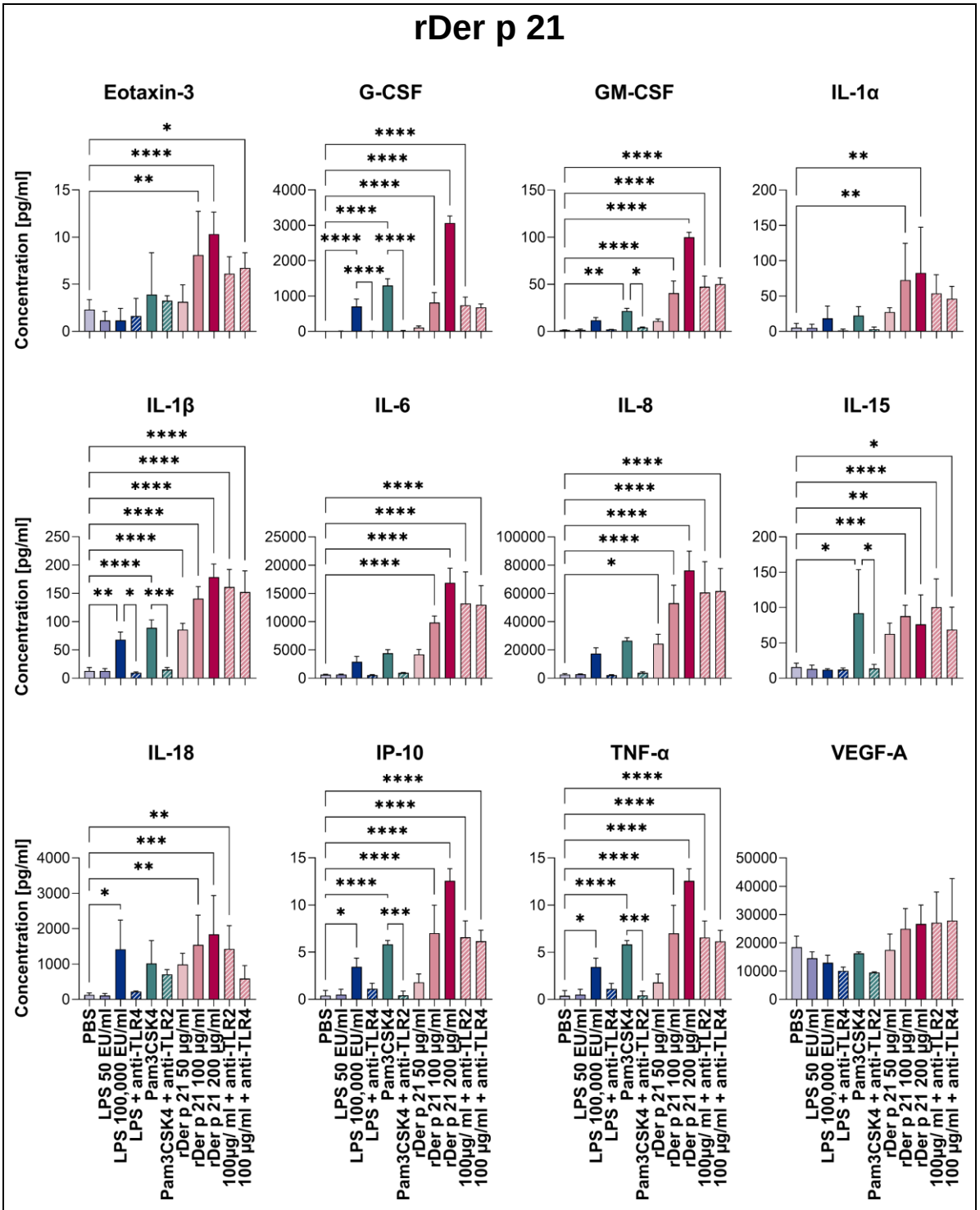


Figure 3.27: Cytokine release of Calu-3 cells after stimulation with rDer p 21

Negative controls: PBS, LPS 50 EU/ml. Positive controls: LPS 100,000 EU/ml (TLR4-dependent), Pam3CSK4 (TLR2-dependent). Inhibition controls: anti-TLR4 (neutralizing) + LPS 100,000 EU/ml, anti-TLR2 (neutralizing) + Pam3CSK4. Stimuli: rDer p 21 50 µg/ml, 100 µg/ml (stimulation and inhibition tests) and 200 µg/ml. Statistical analysis was carried out with One-Way ANOVA and *post hoc* Šídák test for preselected pairings. All controls and stimuli were compared to PBS, and inhibition tests and controls were additionally compared to the corresponding uninhibited values.

3.5 Replacement of allergen challenges by basophil activation test

In the previous chapters, direct IgE-detection with the identification of biomarker allergens and sensitization count was described as a tool to identify patients at risk for allergic multimorbidity, AA or severe forms of AD. After confirming the importance of certain single allergens in the sensitization profiles of HDM-allergic patients, their interaction and possible organ specificity with the lung epithelium was further elucidated in a bronchial cell culture model. In the following, the interaction of Der p allergens with patients' primary cells, namely whole blood basophil granulocytes, will be investigated using the basophil activation test (BAT) to address another aim of this thesis, which was to improve the diagnosis of allergic patients in clinical routine. As this method is used by few experienced centers as an additional allergy *in vitro* diagnostic test for indirect IgE-detection, the aim was to evaluate not just simply the IgE reactivity but moreover the potential of the BAT to replace allergen challenges, which can be both time-consuming and unpleasant for patients as they may experience severe allergic reactions such as an asthma attack during a bronchial allergen challenge. Therefore, a component-resolved BAT containing single allergens identified as important in the allergic disease, such as the marker and initiator allergens identified in the previous chapters, may help to make these challenges unnecessary to diagnose a clinical manifest allergy in the future as well as give implications for immunotherapy as a preventative measure if a patient is e.g. reactive to the marker allergens previously identified in this thesis.

As a first step, the general reactivity of basophils to HDM allergens was assessed in patients exhibiting different phenotypes of HDM allergy. For the further investigation of the BAT's potential to replace allergen challenges, peanut allergy was chosen as a model disease in this part of the project due to sample availability and because the controlled and well-monitored setting provided by an oral food challenge (OFC) is suited best for a proof-of-principle study in this matter. In addition, symptoms experienced in food allergy, especially mild ones, are often better objectifiable than those of a rhino-conjunctival or airway allergy.

3.5.1 Allergen-cell interaction: Basophil activation test in HDM allergy

To further elucidate the impact of HDM allergens on allergic diseases, their potential to elicit an allergic reaction was evaluated in a basophil activation test in three patients exhibiting different allergic phenotypes, namely AR, AR with *Crustaceae* cross-reactivity and AA. All patients had a medical history of HDM allergy and confirmed sensitization via ImmunoCAP.

Activation of basophils was determined by isolating the basophils with the gating strategy shown in Appendix 2 and calculating the proportion of $CD63^+$ cells, which corresponds to the

percentage of basophils that have released histamine in response to a stimulus. A positive response was defined as a signal with over 2.5-fold increase compared to PBS and at least 1.5% degranulated basophils.

As shown in Figure 3.28A and C, not only the major allergens nDer p 1 and rDer p 2 but also further non-major allergens, namely rDer p 5, 7 and 13, were able to trigger basophil degranulation in both patients with predominant airway symptoms, which was reproducible in a repeated test. However, the patients showed interindividual differences regarding the strength of the reaction, and rDer p 20, rDer p 21 as well as rDer p 23 were furthermore able to activate the basophils in the patient exhibiting AA in contrast to the patient with AR. Another study participant suffering from HDM-induced rhinitis and shellfish allergy demonstrated only reactivity to the tropomyosin rDer p 10, which also is responsible for cross-reactivity with *Crustaceae* allergens, and is therefore in line with the experienced clinical symptoms (Figure 3.28B). Overall, the data showed that also non-major allergens can be substantially involved in the allergic response and that the basophil activation test is a suitable tool to not only detect sensitizations, which is its current application when it is used in clinical settings, but also to assess the individual allergic response induced by molecular allergens in the patient tested.

This proof-of-principle regarding the capacity of the recombinant allergens produced in this thesis to interact with human basophils completed, the next steps aimed at further exploring the potency of the BAT as a tool for the replacement of allergen challenges. Due to the SARS-CoV-2-pandemic, HDM-allergic patients for whom the indication of an allergen-specific immunotherapy was given could not be invited to the RCB-study center because of hygiene restrictions. For this particular part, it was therefore decided to choose a different allergic disease characterized by distinct phenotypes and certain allergens that are considered to be marker allergens for the severity of the reaction and for which the BAT was shown to differentiate between truly allergic and merely sensitized [198]. Thus, the following chapter investigates the BAT in peanut-allergic patients who underwent an OFC before inclusion into a treatment study for peanut-specific immunotherapy. As peanut allergy can be fatal, the clinical situation was different from that of HDM patients in the pandemic since the HDM immunotherapy could be postponed in contrast to the treatment of potentially life-threatening food allergy. This choice of study design furthermore brought the advantage of a setting that was better controlled and monitored than nasal challenges carried out for HDM allergy.

The implications of this shall, however, be adapted to HDM allergy in the future when the respective patients are available again.

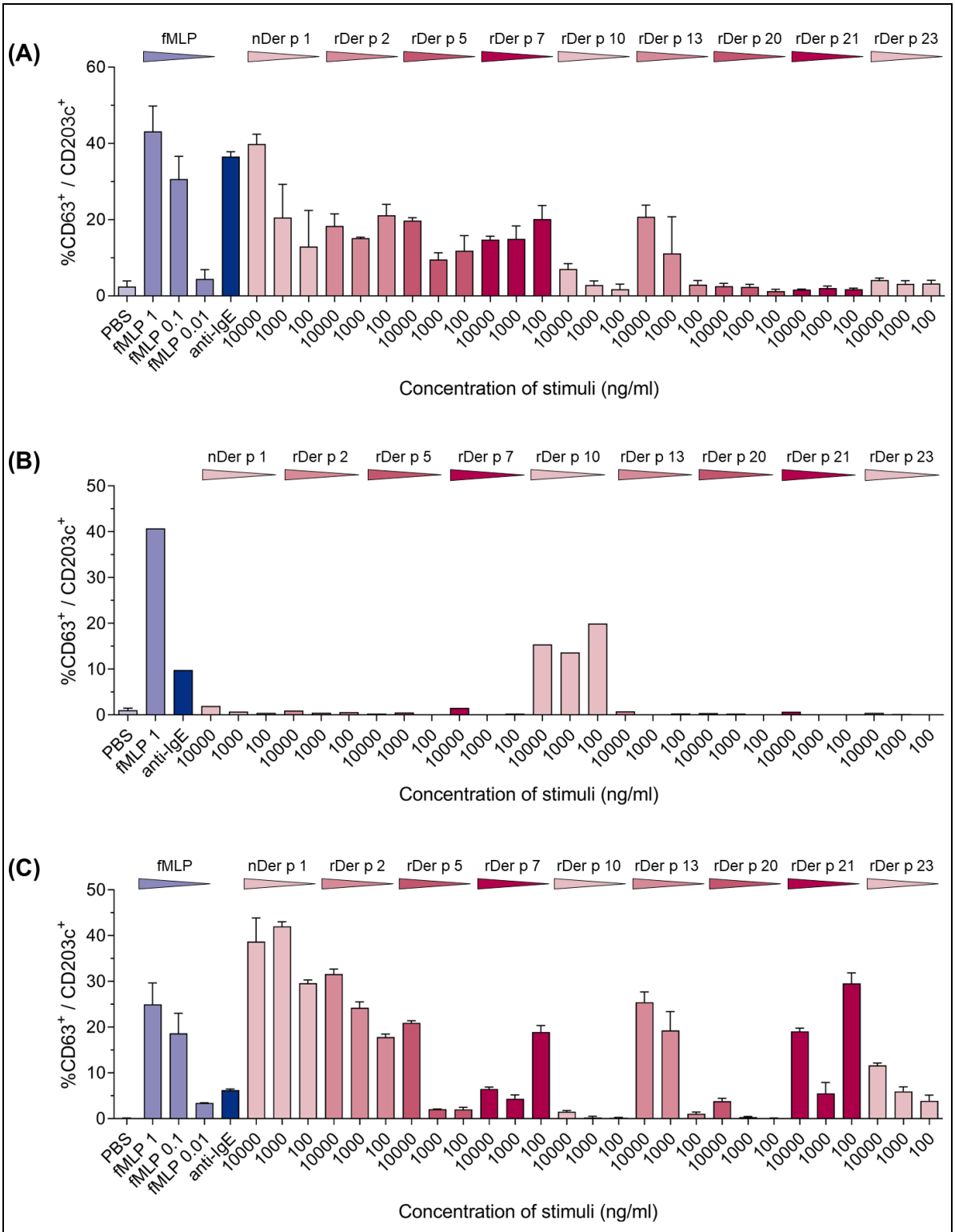


Figure 3.28: Basophil activation in whole blood induced by HDM allergens

Proportion of degranulated cells ($CD63^+$) after gating basophils ($CD203c^+$) by flow-cytometric analysis after performing a basophil activation test in the blood of study participants with confirmed HDM allergy and rhinitis (A), *Crustaceae* cross-reactivity (B) and asthma (C). Tests in panels A and C were repeated independently. Whiskers depict standard deviation.

3.5.2 Development of an analysis algorithm to replace allergen challenges: Component-resolved basophil activation test in peanut allergy

3.5.2.1 Study cohort

In total, 15 children with a suspected peanut allergy and a positive test for sIgE against at least one peanut allergen that were scheduled for an oral allergen challenge were recruited in the EKB by Dr. Andreas Kleinheinz and Amely Brückner in cooperation with Prof. Dr. Uta Jappe. Heparinized blood for the BAT was acquired before the start of the OFC. The challenge was carried out single-blinded, and partially defatted peanut flour was administered in half-logarithmically increasing doses with a total of 7 doses. The symptoms developed during the challenge were documented in detail, and the challenge was terminated as soon as objective symptoms occurred. Children were classified as peanut-allergic or peanut-asymptomatic (sensitized but non-allergic) based on their challenge results, wherein non-allergic was defined as successful ingestion of 9.8 g of partially defatted peanut flour on two subsequent days each without symptoms (negative food challenge). The clinical characteristics of the study groups are listed in Table 43.

Table 43: Characteristics of children with suspected peanut allergy.

Patients	Peanut-allergic n = 9		Peanut-asymptomatic n = 6
Age mean (range)	8 (1–17)		2 (1–4)
Sex (M/W)	7/2		5/1
Total IgE mean (95%CI)	273.4 (± 254.2)		548.9 (± 557.7)
Specific IgE concentrations			
Peanut extract, mean (95%CI)	37.0 (± 28.4)		1.29 (± 0.48)
rAra h 2, mean (95%CI)	24.7 (± 19.7)		0.35 (± 0)
Allergic diseases			
Allergic asthma, N (%)	1 (11%)		0 (0%)
Atopic eczema, N (%)	5 (56%)	6	6 (100%)

3.5.2.2 Cross-evaluation of basophil activation test and oral food challenge results

The results of the BAT were compared to the OFC outcomes for each patient, and the children were grouped according to their allergy status. 13% of the participants were non-responders in the basophil activation test, which is indicated by non-responsiveness to anti-IgE positive controls or any allergen stimuli but normal response to fMLP, which leads to a non-IgE-mediated degranulation by G-protein coupled receptor (GPCR) signaling [214]. All of the following analyses only entail the responsive patients of which 5 exhibited an asymptomatic peanut sensitization and 8 were diagnosed with peanut allergy according to the OFC results.

The parameters monitored in the flow cytometric analysis of the BAT after isolating the basophils with the gating strategy shown in Appendix 2 entailed the percentage of CD63-positive cells in the gated basophils (%$CD63^+$, Figure 3.29), which corresponds to the proportion of degranulated basophils, as well as fold change of median fluorescence intensity (ΔMFI) compared to the negative control PBS for CD63 and CD203c (Figure 3.30 and Figure 3.31), respectively.

While in theory, degranulation of the basophils should only occur if the tested individual is truly allergic, as it mimics the allergic reaction *ex vivo*, 60% of the study participants with negative food challenge results reacted to PEX with substantial degranulation and upregulation of CD63 and CD203c expression as demonstrated in Figure 3.29A, Figure 3.30A and Figure 3.31A. However, none of the other allergens led to activation of the basophils in sensitized-only study participants.

In addition to PEX, patients with positive outcome of the OFC often reacted to recombinant Ara h 2 and naturally purified, roasted oleosins, the latter of which caused the highest proportion of CD63-positive cells with up to 80% degranulated basophils (Figure 3.29B). Recombinant Ara h 8, which is described in the literature as a marker for cross-reactivity with the birch pollen allergen Bet v 1 and thus indicates pollen-associated peanut allergy, only led to basophil activation in three patients and only one of those showed a substantial response of 17% $CD63^+$ basophils whereas the others did not exceed 4% of activated basophils [215].

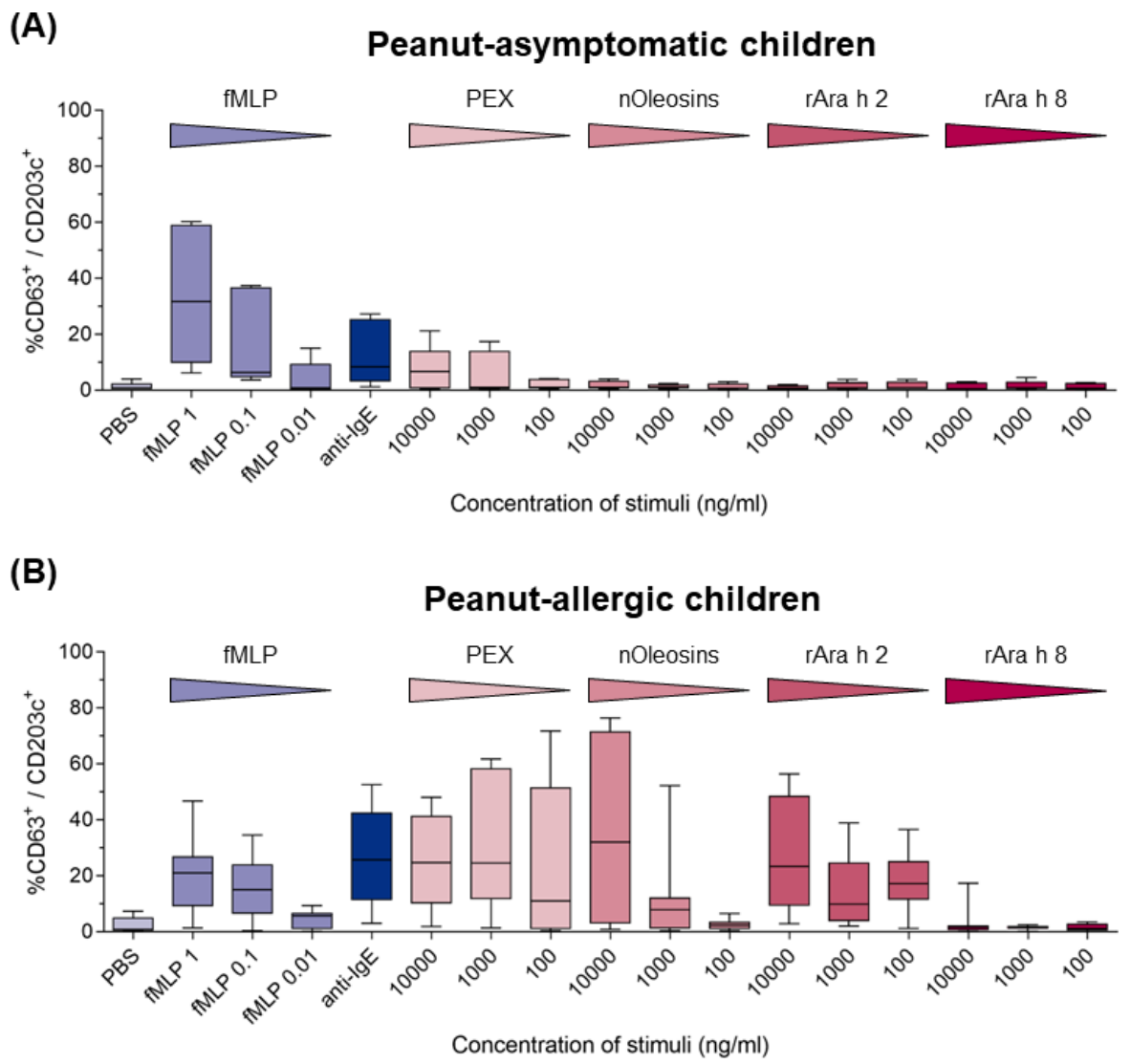


Figure 3.29: Proportion of CD63-positive basophils in basophil activation test

A–B: Activation of basophils in response to stimuli and controls in basophil activation test for peanut sensitized-only patients (N = 4, A) and peanut-allergic patients (N = 8, B). Visualized as box-whisker plot, boxes depict median and IQR, whiskers depict minimum and maximum. PEX = peanut extract

The fold change of CD203c expression on the basophil surface was higher in patients with peanut allergy than in merely sensitized patients, but overall the observed changes were moderate with a less than 3-fold increase as the maximum response (Figure 3.31), whereas the expression of CD63 increased up to over 100-fold in some patients, even though these extreme cases were rather rare (Figure 3.30). Notably, this hyperresponsiveness occurred only in response to allergens and not the anti-IgE positive control, and not all of the allergens that led to a basophil degranulation did necessarily induce a hyperresponsive CD63 ΔMFI in the same patient. However, the ΔMFI of CD63 was generally more responsive when the basophils were activated by stimulation with allergens, which was expected as it is, in contrast to CD203c, not constitutively present on the cell surface and its upregulation therefore depends on

degranulation of the basophils. Overall, all three of the monitored parameters showed substantial differences between allergic and sensitized patients, which confirmed their potential for differentiating true allergy from a silent sensitization.

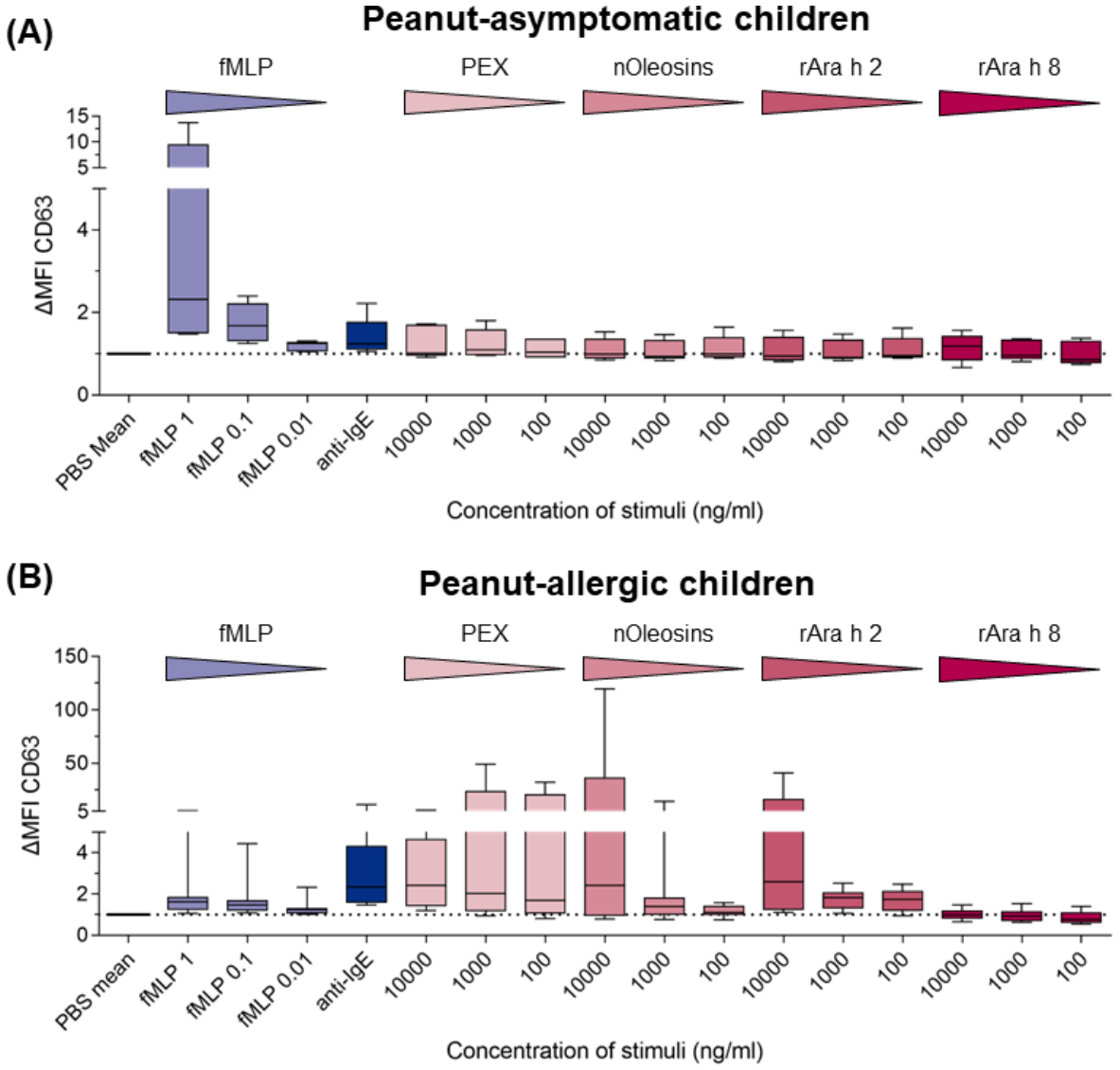


Figure 3.30: Fold change of CD63 median fluorescence intensity in basophil activation test

A–B: Upregulation of CD63 expression on the cell surface of basophils in response to stimuli and positive controls compared to PBS in basophil activation test for peanut sensitized-only patients (N = 4, A) and peanut-allergic patients (N = 8, B). Visualized as box-whisker plot, boxes depict median and IQR, whiskers depict minimum and maximum. PEX = peanut extract

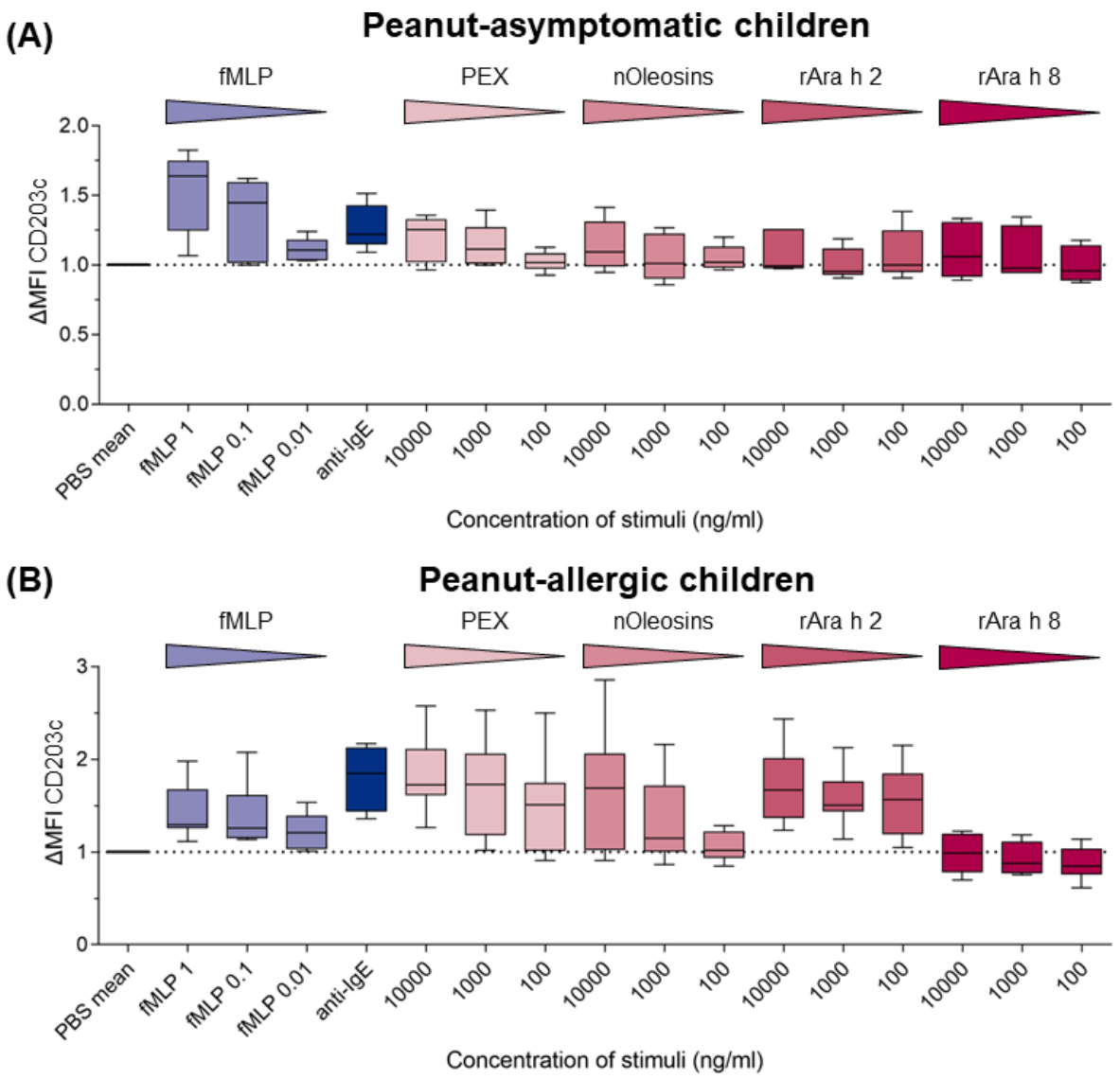
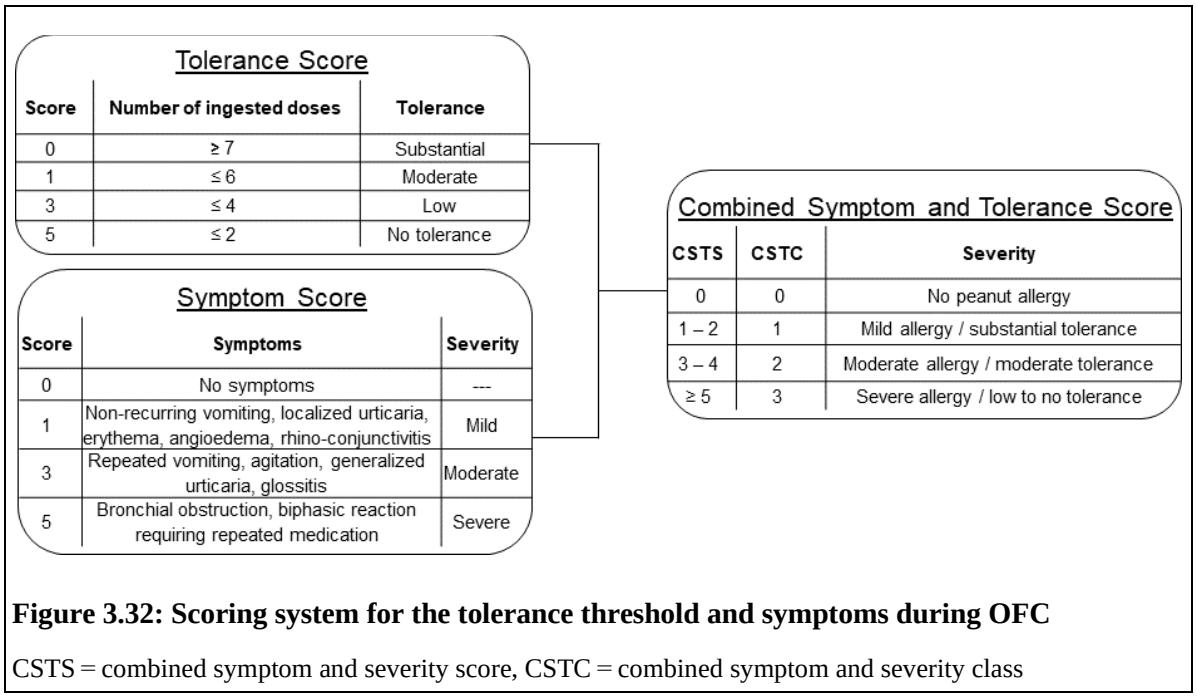


Figure 3.31: Fold change of CD203c median fluorescence intensity in basophil activation test

A–B: Upregulation of CD203c expression on the cell surface of basophils in response to stimuli and positive controls compared to PBS in basophil activation test for peanut sensitized-only patients (N = 4, A) and peanut-allergic patients (N = 8, B). Visualized as box-whisker plot, boxes depict median and IQR, whiskers depict minimum and maximum. PEX = peanut extract

3.5.2.3 Development of a scoring system and BAT analysis algorithm for prediction of allergy prevalence and severity

Since regular scores to assess the severity of food allergy usually only entail the experienced symptoms, a scoring system that included both the tolerance threshold and symptoms occurring during the OFC was developed in this thesis to classify the patients according to their OFC results and to provide a basis for decision-making with regards to the BAT becoming a reliable substitute for OFCs. Separate scores for tolerance and symptom severity, only including objective symptoms as these are pivotal for terminating the OFC, were integrated into a combined symptom and tolerance score (CSTS), wherein the symptom scale was based on the CoFAR Grading Scale for Systemic Allergic Reactions and the official German guidelines for the management of anaphylaxis [32, 216]. This approach followed in principle the combined symptom and medication scores often used to assess the success of immunotherapy of inhalant allergies. Patients were further classified into combined symptom and tolerance classes (CSTC) according to their scores, wherein CSTC 0 corresponded to a negative food challenge result, and CSTC 1–3 to positive food challenge results with increasing symptom severity and/or decreasing tolerance threshold. The detailed scoring system is summarized in Figure 3.32. A single score was used to assess symptom severity, which corresponded to the score of the most severe symptom experienced during the OFC. While CSTC 1 was the threshold of a diagnosed allergic reaction, it was associated with mild symptoms and a substantial peanut tolerance, e.g. corresponding to the amount of peanut in a snickers bar. These patients were advised to maintain small amounts of peanut in their diet to sustain the tolerance, whereas strict peanut abstinence was recommended for patients with CSTC 2–3.



Tolerance Score

Score	Number of ingested doses	Tolerance
0	≥ 7	Substantial
1	≤ 6	Moderate
3	≤ 4	Low
5	≤ 2	No tolerance

Symptom Score

Score	Symptoms	Severity
0	No symptoms	---
1	Non-recurring vomiting, localized urticaria, erythema, angioedema, rhino-conjunctivitis	Mild
3	Repeated vomiting, agitation, generalized urticaria, glossitis	Moderate
5	Bronchial obstruction, biphasic reaction requiring repeated medication	Severe

Combined Symptom and Tolerance Score

CSTS	CSTC	Severity
0	0	No peanut allergy
1 – 2	1	Mild allergy / substantial tolerance
3 – 4	2	Moderate allergy / moderate tolerance
≥ 5	3	Severe allergy / low to no tolerance

Figure 3.32: Scoring system for the tolerance threshold and symptoms during OFC

CSTS = combined symptom and severity score, CSTC = combined symptom and severity class

A visualization of the individual CSTS values of participants and their corresponding BAT parameters revealed that all of the monitored parameters (%CD63$^+$, ΔMFI CD63 and ΔMFI CD203c) increased with the scores and therefore confirmed the relation of the BAT results to both tolerance threshold and symptom severity (Figure 3.33). In particular the proportion of CD63-positive basophils and the respective fold change in MFI was often massively increased in patients with the highest scores. In contrast, only utilizing either the symptom or the tolerance score as shown in Appendix 17 was less suitable, especially for patients with high tolerance but severe symptoms and vice versa during the OFC. Overall, the results attested each parameter a promising correlation to the CSTS that could be used to classify the patients according to their BAT outcomes.

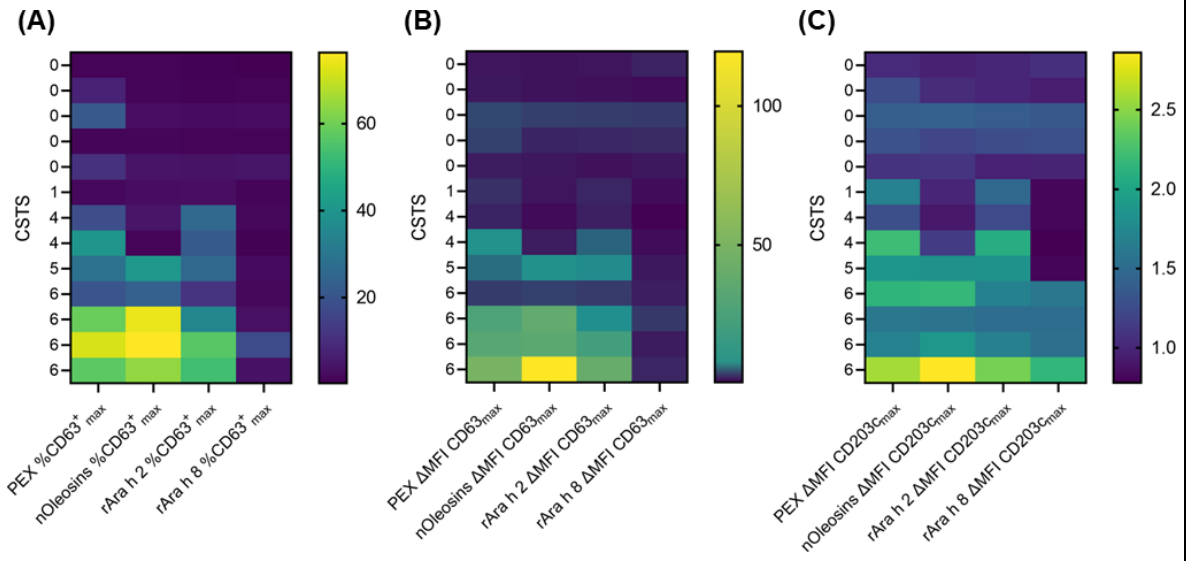


Figure 3.33: Heat map of BAT parameters and combined symptom and tolerance score

A: Maximum percentage of activated basophils observed during BAT for each stimulus and study participant
B–C: Maximum fold change of CD63 (B) or CD203c (C) MFI compared to PBS observed during BAT for each stimulus and study participant.
CSTS = combined symptom and tolerance score

By matching the CSTC of the children with the proportion of activated basophils and fold-change of the MFI of CD63 and CD203c, thresholds of all three parameters were established that corresponded best to the observed outcomes of the food challenge. An analysis algorithm for all patients that were reactive to at least one of the stimuli in BAT was developed to differentiate between the different CST classes by taking into account the number of allergens the patients reacted to as well as the individual proportion of activated basophils and increases in MFI of CD63 and CD203c. The algorithm is outlined in detail in Figure 3.34.

The thresholds were only applied to allergens the individual reacted to, wherein a positive reaction was defined as a signal exceeding the negative control by 2.5 times and at least 1.5% of degranulated basophils in total. Therefore, even if a single threshold was exceeded, such as "< 5% $CD63^+$ cells" when stimulated with natural oleosins, the value was not taken into account for the algorithm if it did not fulfill the criteria for a positive reaction. As soon as one of the parameters' values to differentiate CSTC 1–3 was exceeded, the patients were assigned to the next-higher level until the criteria were met. For the placement in CSTC 3, at least one of the criteria needed to match.

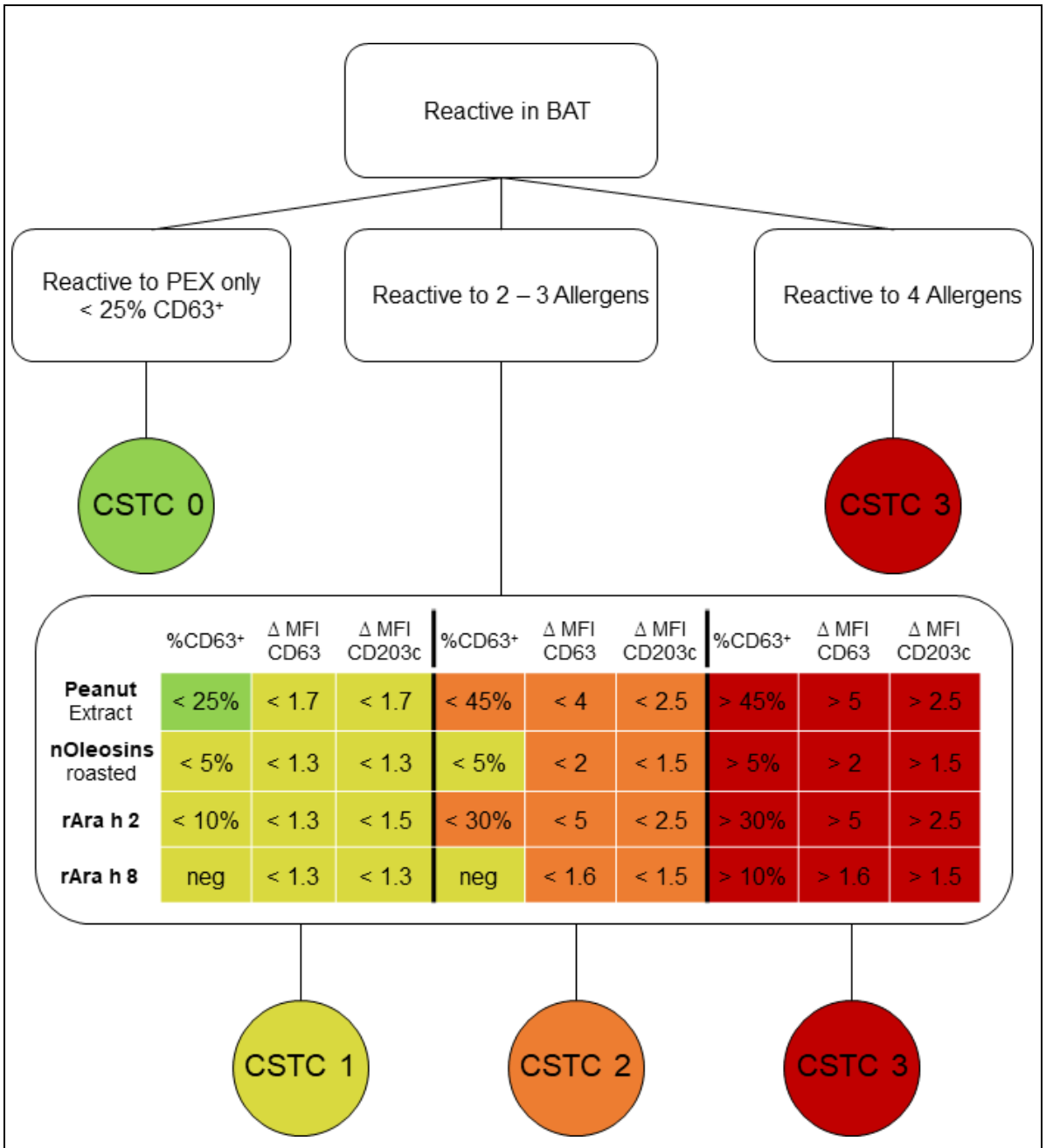
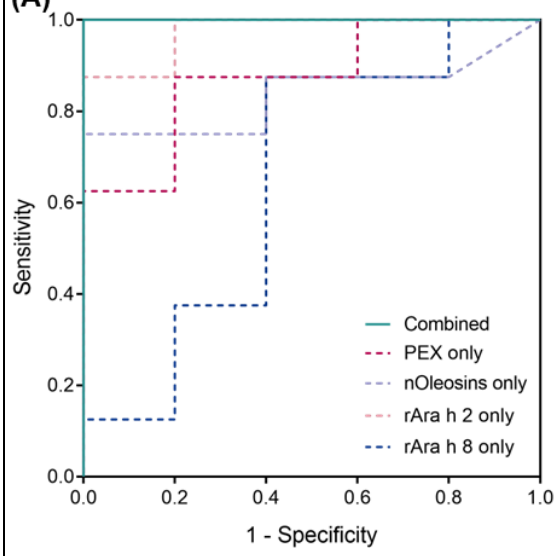


Figure 3.34: Algorithm for BAT analysis to predict peanut allergy and severity

PEX = peanut extract. MFI = median fluorescence intensity. CSTC = combined symptom and tolerance class.

For performance evaluation regarding the ability to differentiate between silent sensitization and actual peanut allergy, each allergen and the combined algorithm were analyzed using a receiver-operator-characteristics (ROC) curve and rated after the respective area under the curve (AUC) according to the criteria established by Luna-Herrera et al. (Figure 3.35A-B) [217]. Overall, rAra h 2 performed best as a single parameter to predict the prevalence of peanut allergy. While oleosins were highly specific for severe cases of allergy, the lack of reactions by patients with mild and moderate symptoms towards them, as can be seen in Figure 3.33, rendered them unsuitable for the differentiation between silent sensitization and allergy. Overall, none of the single parameters reached the sensitivity and specificity of the analysis algorithm in terms of diagnosing the prevalence of a clinically manifest allergic reaction. Furthermore, the developed algorithm was able to reliably differentiate between the different severity grades established by the CST scoring system (Figure 3.35C). All patients that were responsive in BAT were classified correctly by the analysis algorithm, leading to a positive and negative predictive value of 100% for all responders, which corresponded to 87% of the total study population. Overall, the herein developed CSTC scoring system combined with the BAT analysis algorithm proved highly effective in the prediction of allergy prevalence and severity of all patients with the only drawback of a non-responder rate of 13% (refer to chapter 3.5.2.2).

(A)



(B)

Analysis	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Performance
Combined	1.0 (1.0 – 1.0)	1.0 (0.63 – 1.0)	1.0 (0.48 – 1.0)	perfect
PEX-only	0.88 (0.68 – 1.0)	0.88 (0.47 – 0.99)	0.80 (0.28 – 0.99)	good
nOleosins-only	0.84 (0.61 – 1.0)	0.75 (0.41 – 0.96)	0.80 (0.38 – 0.99)	good
rAra h 2-only	0.98 (0.90 – 1.0)	1.0 (0.63 – 1.0)	0.80 (0.28 – 0.99)	excellent
rAra h 8-only	0.65 (0.31 – 0.99)	0.88 (0.53 – 0.99)	0.60 (0.23 – 0.93)	poor

(C)

CSTC	n	Positive predictive value (95% CI)	Negative predictive value (95 % CI)
0	5	1.0 (1.0 – 1.0)	1.0 (1.0 – 1.0)
1	1	1.0 (1.0 – 1.0)	1.0 (1.0 – 1.0)
2	2	1.0 (1.0 – 1.0)	1.0 (1.0 – 1.0)
3	5	1.0 (1.0 – 1.0)	1.0 (1.0 – 1.0)

Figure 3.35: Performance of BAT for prediction of allergy prevalence and severity

A: ROC curve for each BAT stimulus and the combined algorithm to differentiate between peanut allergy and asymptomatic sensitization.
B: Performance evaluation of ROC to predict prevalence of an allergic reaction.
C: Evaluation of algorithm for predicting the CSTC of study participants
PEX = peanut extract, CSTC = combined symptom and tolerance class

4 Discussion

This thesis aimed to identify single house dust mite (HDM) allergens that could serve as marker allergens for certain phenotypes of allergic diseases, in particular allergic asthma and atopic dermatitis. Despite the association of HDM with the development of both of these diseases being well known, the literature on the particular relevance of single HDM allergens in this regard is sparse. This is due to the fact that only four HDM allergens are commercially available for routine diagnostic tests and therefore for the respective population studies, which often utilize commercial, non-customized tests. In addition, it was investigated whether lipophilic HDM allergens in particular were of clinical importance as has been previously observed in the research group for food allergen sources [179, 198]. Therefore, potentially relevant HDM allergens unavailable for commercial routine tests were recombinantly expressed and purified to analyze the individual sensitization profiles of HDM-allergic individuals exhibiting different allergic diseases. The sensitization patterns as well as polysensitizations were analyzed in-depth and possible clinical implications were identified. In the process, IgE antibody- and cell-based-diagnostic measures were optimized and evaluation algorithms developed to ultimately improve the diagnosis of allergic patients. Additionally, the pathomechanistic properties of the allergens identified as important for severe allergic diseases in this thesis were further elucidated using a human bronchial epithelial cell model.

Parts of the results were discussed in a publication during the course of this thesis [180]. Sentences, small passages, or whole paragraphs adopted from the published article may be quoted verbatim or in spirit of this work without being highlighted or cited separately in the following section.

4.1 Production of recombinant house dust mite allergens

At first, several HDM allergens had to be produced recombinantly in order to be able to perform sensitization profile analyses. The panel of Der p allergens that were selected for expression in this thesis was based on the known Der p allergens at the time, in particular major and mid-tier allergens but also several minor allergens such as Der p 10, 13 and 20 (Table 3). Several other allergens were attempted to express before and during the course of this thesis, such as Der p 6, 11 and 18, but the production was not successful, mainly due to the formation of inclusion bodies instead of soluble proteins. The selection of the posterior produced Der f allergens was oriented towards the potential marker allergens of Der p allergens already identified in this thesis, namely the Der f homologues of Der p 5, 20 and 21.

Production of recombinant HDM allergens has been described in various host organisms including prokaryotes (*E. coli*) and several eukaryotic organisms such as yeast (*Pichia pastoris*) or insect cells (*Spodoptera frugiperda*, Sf9 strain) [218, 219]. Despite the advantages of eukaryotic expression systems such as enhanced folding capability and post-translational modifications, *E. coli* still is one of the most frequently used hosts in the production of recombinant proteins due to its fast growth rate, inexpensiveness, simple handling and high scalability of the expression process [220, 221].

When using *E. coli* for the expression of eukaryotic proteins, the differences in codon usage of donor and host organisms can be overcome by codon-optimizing the DNA constructs, a process in which rare base triplets are substituted by ones more common in the host organism, to enhance effectivity of the translation [222]. During this work, the yield of proteins could be greatly improved after introduction of optimized DNA sequences. Furthermore, the expression parameters such as temperature, growth medium, point of induction and inductor concentration can substantially influence the yield and solubility of the expressed proteins. Lower inductor concentration and temperature during expression slow down the production rate, which leaves more time to correctly translate and fold the target proteins [223, 224]. Implementing these considerations has positively influenced the success and yield of protein expression during the course of this thesis. Even though an increase of protein yield when the cells are induced during the late log phase is reported in the literature, which corresponds to the expression technique established in the research group before the start of this work, inducing the cells during their mid-log growth phase led to superior results for the variety of proteins expressed within this project [225]. A further method for recombinant protein expression in *E. coli* utilizes a medium supplemented with a lactose source for IPTG-independent induction also known as autoinduction. Addition of glucose represses the induction until the bacteria have, depending on the amount, grown to mid- or late log phase and completely consumed the glucose, leaving lactose as the only carbon source. This autoinduction technique first described by F.W. Studier bears advantages such as easy handling, no need of monitoring and growth to high bacterial density [148]. For successful autoinduction, an intact lacZ gene and therefore active LacZ β-galactosidase in the used *E. coli* strain is obligatory as it converts lactose to allolactose, the proposed natural inducer of the lac promoter [226, 227]. As the SHuffle strain used in this thesis has an inactivated lacZ gene, autoinduction should in theory not be possible (Table 6). Nevertheless, it is reported that protein yields of SHuffle cells grown in autoinduction medium were superior to regular IPTG induction concluding that this induction must follow a different, β-galactosidase-independent pathway that the authors coined “autoexpression”, the mechanism of which is not further researched [228]. The outcomes of autoexpression carried out in SHuffle

cells for Der f allergens during this thesis were very variable as it resulted in high expression of Der f 2, minor expression of Der f 20 and no expression of Der f 5 and Der f 21 (refer to Appendix 3). Therefore, even though the method of autoinduction is convenient and can lead to improved protein expression even in lacZ-deficient SHuffle cells, the results are highly dependent on the target protein and the method does not seem to be universally suited for this *E. coli* strain.

However, the arguably greatest disadvantage of *E. coli* cells is their limited capacity to fold eukaryotic proteins. Especially the presence of disulfide bonds, as e.g. seen in Der p 2, can often be the cause of difficulties during expression due to the reducing environment in the cytoplasm resulting in insoluble protein aggregates also referred to as inclusion bodies [229, 230]. Today, a variety of methods are available that try to combat this handicap including the co-expression of chaperones to aid in the folding process or the use of different mutant strains such as SHuffle, which lacks both glutathione and thioredoxin reductase to favor the formation of disulfide bonds compared to its parental strain BL21 [228, 231-233]. The production of the allergen Der p 2 had failed in BL21 before and during the course of this thesis, presumably due to its structure containing three disulfide bonds. However, it was successfully expressed in SHuffle cells which confirmed the added value of the SHuffle strain in the production of recombinant proteins.

Overall, SHuffle cells with regular IPTG induction had the most favorable outcome for most HDM allergens due to reliable expression with high to very high yields and low background expression of host proteins which allowed for a fast two-step purification process by carrying out a single ion exchange chromatography step after IMAC to achieve sufficient purity.

Due to the nature of the encounter of airborne allergens with the immune system, usually by inhalation of or skin contact with the native proteins, the B-cell epitopes recognized by sIgE antibodies are often discontinuous and of conformational nature, in contrast to food allergens, which, due to their processing during digestion or cooking, exhibit far more often linear epitopes [201]. For HDM allergens in particular, a study that aimed to produce hypoallergenic peptides found that only 2 of the 6 investigated allergens, namely Der p 5 and Der p 23, displayed any linear IgE epitopes [178]. Therefore, it was crucial for the recombinant HDM allergens produced in this work to adopt a correct folding during expression in order to exhibit adequate IgE reactivity for subsequent experiments. The analysis of the secondary structure of Der p allergens by CD spectroscopy and subsequent structure modeling via K2D3 suggests that the allergens adopted a correct folding. The spectrum of rDer p 23 indicated little amount of folded protein and its curve resembled rather that of a degraded protein, which is likely caused by its high content of random-coil structure, and spectra found in the literature resemble the one

recorded in this work [203, 204]. The largest difference between reference and recorded spectra was observed in the beta strand content of rDer p 7, which differed from its reference structure by 15%. However, it is well known that particularly the prediction of beta strand content is less accurate compared to alpha-helical content, as different angles of twisted beta strands result in a large range of different spectra [156, 234, 235]. As the other spectra of Der p 7 found in the literature resemble the recorded one, sufficient folding could still be assumed [202]. Overall, the experiments suggested a correct folding of all Der p allergens and confirmed the suitability of *E. coli* as a host organism in the expression system. Since there were no indications of shortcomings in regards to folding in any of the investigated proteins, the Der f allergens that were produced later during the course of this thesis were not analyzed by CD spectroscopy again, as their sequences were highly similar to their Der p counterparts.

4.2 The association of sensitization count, allergen-specific IgE concentration and allergic phenotype

A particular emphasis in this thesis lies on the analysis of molecular sensitization profiles in HDM allergy and the connection of single HDM allergens to specific allergic phenotypes such as allergic asthma (AA), allergic rhinitis (AR) or atopic dermatitis (AD).

Determination of sIgE concentrations is performed routinely in the diagnosis of HDM-allergic patients for Der p extract or the available molecular allergens Der p 1, 2, 10 and/or 23. Even though the results are categorized in CAP classes 1–6 (Table 4), the actual clinical implication of a high sIgE concentration is not clear. While there is evidence that the amount of total serum IgE could correlate with the prevalence of AA, although this is controversial, this seems not to be the case for sIgE [208-210]. Except for a few specific approaches such as the prediction of outcome in oral food challenges (OFC), a prominent example being Ara h 2 in peanut allergy although there is conflicting evidence from several studies regarding its correlation to disease severity, the sIgE levels generally cannot be connected to disease prevalence or severity other than that the observed sensitization is more likely to be clinically relevant in patients with a high sIgE level [215, 236-240].

In HDM allergy in particular, there is evidence from the PARIS birth cohort that IgE concentrations > 3.5 kU/l against Der p extract in children is associated with the subsequent development of a clinically manifest allergy in the form of allergic rhinitis or asthma. A study investigating atopy phenotypes in children with different sensitizations found an association of severe atopy in children with sIgE levels against seasonal inhalant allergens, defined as any sensitization to grass or tree pollen, but no association with mite-specific IgE levels. Otherwise,

the clinical relevance of HDM-specific IgE concentrations is mostly unexplored [241, 242]. Therefore, it appears that factors apart from mite-specific IgE levels influence the phenotype and severity of HDM allergy. Instead, the theory was established in this thesis that the severity of the allergic disease could be connected to the complexity of the sIgE patterns present in the patients' sera rather than the concentration thereof. This hypothesis was further investigated by analyzing the sensitization profiles of HDM-allergic adults exhibiting different allergic phenotypes.

The results from chapter 3.2.3 demonstrated that the number of recognized allergens, which has been termed "sensitization count" in this work, was associated with the phenotypes and multimorbidity of HDM-allergic patients in particular (Figure 3.10A). Study participants with a sensitization count > 3 suffered more frequently from AD in combination with AA and/or AR whereas isolated AR was dominant among individuals reacting to three or less HDM allergens (Figure 3.12). The routinely measured sIgE concentration against Der p extract was only weakly correlated with the sensitization count, which could explain the lack of association of sIgE concentration with disease phenotype observed in the literature (Figure 3.11). The phenotype distribution of the study participants remained the same throughout CAP classes 1–5, and only very high concentrations of sIgE (CAP class 6) were associated with severe phenotypes (Figure 3.10B-C).

Although the few available studies that investigated the association of HDM-specific IgE and allergy phenotype or severity were, other than in this work, performed in children, the results observed in this thesis confirm that sIgE concentration against Der p extract only has a very limited impact on the disease phenotype in HDM allergy. While there was an association of allergic multimorbidity with the prevalence of sIgE > 100 kU/l (CAP class 6), individuals with this trait constituted less than 10% of all patients recruited in the outpatient clinics, and therefore, a majority of patients could benefit from utilizing the sensitization count in addition to sIgE measurement in clinical routine. However, to fully elucidate its potential as a parameter for risk assessment in clinical routine, longitudinal studies would be necessary to investigate how the disease progression is linked to the sensitization count.

4.3 Identification of potential biomarker allergens by molecular profiling in HDM-allergic adults

The detection of molecular HDM sensitization patterns was moreover used for the stratified analysis of groups with different allergic diseases, namely AA, AR and AD, to identify potential marker allergens for each phenotype and thereby assess the allergens' possible organ specificity.

The sensitization patterns indicated that rDer p 5 and rDer p 21 were associated with both AA and AD. This was subsequently confirmed in an unstratified analysis of the cohort data by multiple logistic regression, which allowed for the adjustment of confounding factors (refer to chapter 3.2.4.1 and 3.2.4.2). Increased sensitization rates to one or both of these allergens have been described previously in stratified analyses of asthmatic children and adults as well as adults with AD [243-245]. Contrary to the observations made in this thesis, one study did not find differences in Der p 5 and Der p 21 sensitization in patients with and without AD [246]. However, the patient group with respiratory allergy in that study was small and lacked differentiation into asthma and/or rhinitis. Furthermore, all AD patients were children with a mean age of 15 years, substantially lower total and specific IgE levels, and their SCORAD (SCORing Atopic Dermatitis) values suggest that more mild and moderate cases were included compared to the study participants in this thesis, all of which could contribute to the differences in sensitization rates between the results observed here and by Banerjee et al.

Both Der p 5 and Der p 21 are proteins found in mite feces with lipophilic properties and yet unknown biological function [52, 247, 248]. With 32% sequence identity and reported cross-reactivity, they exhibit a substantial homology, and crystal structures as well as AlphaFold models reveal that both form a similar three-helix bundle [248-251]. Hydrophobic binding cavities and/or selective lipid interactions have been reported for monomeric and dimeric Der p 5 as well as dimeric Der f 21, the *D. farinae* homologue to Der p 21 with 71% sequence identity. Since the amino acids involved in the reported binding pocket of Der f 21 are either the same (A70, F33) or substituted by ones with strongly similar properties according to Clustal Omega analysis (F23→L, Y66→F), it seems plausible that such a binding pocket might also be present in Der p 21. These hydrophobic binding sites could facilitate proinflammatory immune responses by potentially carrying LPS or other apolar ligands with e.g. TLR-activity, although their ligands in natural environments are yet to be elucidated [154, 250-252].

Their numerous similarities might be part of the explanation why sensitizations to both rDer p 5 and rDer p 21 were increased to a similar degree in this thesis. In the logistic regression models, the effect estimates were substantially influenced when both proteins were combined in the

same model, which indicates an additional association between the two. The findings overall support the initial hypothesis of this project that especially lipid-associated proteins are of great importance for the severity of allergic diseases, as both Der p 5 and Der p 21 possess lipophilic properties. However, more in-depth mechanistic research is needed to further clarify their role in development and progression of HDM allergy.

The study conducted within this thesis is the largest one to date comparing the sensitization patterns of not only asthmatic and rhinitic or atopic and non-atopic patients but of all these phenotypes in one single cohort. Moreover, it is also the first study that includes an unstratified analysis of the data to adjust for confounders and in particular the simultaneous presence of asthma and AD. Using this approach, the independent association of rDer p 5 and rDer p 21 with both AD and AA was confirmed as well as the association of rDer p 20 with AD. This study also is the first one to integrate these allergens into routine diagnostic platforms and to analyze differences in the IgE concentrations in the sera of patients exhibiting different allergic phenotypes, which demonstrated the extraordinarily high titers of sIgE directed against rDer p 20 in sera of patients suffering from severe AD with IgE concentrations of up to 1500 kU/l, exceeding the upper limit of quantification of the ImmunoCAP by 15 times. Overall, 75% of patients with rDer p 20-sIgE above 80 kU/l exhibited severe AD, a more than two-fold increase compared to patients with low or no sIgE against rDer p 20.

The occurrence of Der p 20 sensitization has also been linked to AD in a very recent screening of 100 patients with AD and it furthermore has been associated with AA in 98 HDM-allergic patients in a study by Sarzsinszky et al. [245, 253]. While the association of sensitization to Der p 20 with AD was also observed in this thesis as shown in Figure 3.14, the link of Der p 20 to AA could not be confirmed. A reason for this might be that the analyses in this thesis included considerably more participants than the one by Sarzsinszky et al. Additionally, the authors did not report the prevalence in asthmatic and non-asthmatic patients but compared only Der p 20-IgE-positive and -negative AA patients. In this thesis there were no alterations in the Der p 20-IgE prevalence in AA patients compared to AR nor did Der p 20-IgE-positive patients suffer from AA more often.

The arginine kinase Der p 20 is usually considered a minor allergen. There is little research revolving around it and therefore only limited data on its clinical relevance is available [52]. However, the screening study of atopic patients by Celakovska et al. also revealed that sensitization to various other arginine kinases originating from cockroach (*Blattella germanica*, Bla g 9), shrimp (*Penaeus monodon*, Pen m 2) and mold (*Aspergillus fumigatus*, Asp f 6) were increased as well, which could indicate a shared pathomechanism of this allergen group.

A potential explanation for the increased rDer p 20-sensitization and sIgE concentration observed in AD could be the skin barrier dysfunction in severe AD patients, which allows allergens to penetrate and induce sensitization more easily. While the feces-originated major allergens Der p 1 and Der p 2 commonly sensitize via the airways, allergens derived from the mite body, such as Der p 20, can potentially induce sensitization via the skin as well [254]. Overall, sIgE against Der p 20 seems a promising biomarker for severe forms of AD but requires further research to elucidate its pathomechanistic properties.

Therefore, while the analysis of sIgE concentrations against HDM extract did not prove useful in the differentiation of allergic phenotypes as demonstrated in the previous chapter, the sIgE concentrations against Der p 20 could actually be advantageous for the diagnosis of HDM-allergic patients and could, for example, be an indication for starting immunotherapy or closely monitoring the disease progression. In contrast, the differences of sIgE concentrations against rDer p 5 and rDer p 21 were not as distinct in patients with AA and AD. While AD patients exhibited higher titers of sIgE against the two allergens, the variance in these data were high and the increased concentrations might have been caused by the fact that AD is by definition often accompanied by high total and specific IgE levels [205-207]. And while in the analysis of rDer p 20-specific IgE levels, differences were found between multiple subsets of AD patients, all of which exhibited high total and Der p extract-specific IgE levels, this was not the case for rDer p 5 and rDer p 21. The clinical relevance of these results is therefore questionable as of now. Overall, it appears that in case of Der p 5 and Der p 21 the prevalence of sensitization itself is crucial for the association with AA and AD rather than sIgE concentrations.

Since Der p 5, Der p 20, and Der p 21 have been demonstrated to be important for severity and phenotype of HDM allergy in this thesis, their presence in commercial HDM extracts used for diagnosis and allergen-specific immunotherapy (SIT) is crucial. As described in chapter 1.5.5, the legal requirements for quality control of the allergen content in therapy extracts are very scarce and do not entail monitoring of non-major allergen content. Especially due to their lipophilic properties, Der p 5 and Der p 21 are at risk to be discriminated in the aqueous extraction process of the water-based extracts. In regards to Der p 21, Casset et al. reported a high batch-to-batch variety of its contents or even a complete lack thereof in different diagnostic extracts [122]. A recent study investigated the range of Der p allergens in the extract used for sublingual immunotherapy tablets by ALK Abello by peptide mass fingerprinting [255]. Unfortunately, the authors do not state the used mass tolerance of their fingerprint analyses conducted with the Mascot engine, which makes it difficult to assess the significance of results with low coverage and whether the cut-off of two detected peptides for presence confirmation is appropriate. Since the default mass tolerance in Mascot of ± 1.2 Da is comparably high, a

cut-off of only two peptides appears to be quite low for presence confirmation, especially if these peptides have no required amino acid length. Notably, among the allergens with the lowest coverages in this analysis were Der p 5 (18%, 3 peptides), Der p 11 (6%, 5 peptides), Der p 21 (9%, 1 peptide) and Der p 37 (13%, 3 peptides), all of which were described to be important for AA or AD in either this thesis or other studies [246, 256]. The almost complete lack of these important allergens in therapeutic extracts may be of potentially huge clinical implication, as it has already been reported that SIT induced the production of protective IgG antibodies against Der p 1 and 2 but neither against Der p 5 nor 21 in HDM-allergic patients, and that furthermore the treatment lacked efficacy in regards to symptom reduction for patients sensitized to HDM allergens other than Der p 1 and 2 after one year of SIT [257, 258].

Limitations of this study include the partially limited number of patients in some subgroup analyses such as the minor allergen Der p 10, which also seemed to be relevant for AA in the univariate logistic regression but this could not be confirmed in the multiple regression model due to its low seroprevalence. Furthermore, only few patients could be included in the subsets of rDer p 5-, rDer p 20- and rDer p 21-IgE-positive patients analyzed in Figure 3.17 and Figure 3.18, which was again caused by the low sensitization rates in the study population and limited sample availability for serum-consuming analyses such as the ImmunoCAP. Particularly the investigation of sIgE concentrations against rDer p 5 and 21 require more patients to allow a sound statistical analysis. Furthermore, this study did not allow a conclusion about how large the collective of patients exhibiting the high IgE concentrations against Der p 20 is in the general population of HDM-allergic patients, since the study group of AD patients was specifically recruited for their AD prevalence. As the study participants were recruited at specialized allergy outpatient clinics in pneumology or dermatology departments at university hospitals, this might have resulted in a patient group biased towards more severe respiratory or skin symptoms. Furthermore, the sex distribution of patients was biased towards male patients in the AD group. Whilst estrogen can induce a type-2 immune response and hypothetically an increase of sIgE**,** other studies suggest higher sensitization rates in male patients whereas again other studies reported no sex-related differences [259-261]. Moreover, the unstratified analyses of the data indicated that age did not influence the outcomes of asthma or AD prevalence and therefore the differences in sex distribution should only depict a minor limitation.

Overall, this study demonstrated the association of certain HDM allergens with clinical phenotypes of atopic patients and thus the importance of component-resolved diagnosis in HDM-allergic patients. Whilst various alterations in the sensitization patterns were detected in the analysis of AD and asthmatic patients, Der p 5, 20 and 21 seem to be the most promising candidates as potential biomarkers for the severity of HDM allergy. In particular high sIgE

concentrations against rDer p 20 were associated with severe forms of AD. This study also adds more weight to the initial hypothesis of this thesis that especially lipophilic or lipid-associated allergens seem to be involved in the elicitation and severity of allergic responses. However, as this analysis is a cross-sectional study, these observations should be further investigated in a prospective design to further assess their value in clinical routine. Moreover, their content in allergen extracts should be monitored as they could be crucial elements for the success of immunotherapy particularly regarding its potential to prevent the progression from upper to lower airway allergic diseases.

4.4 Sensitization patterns and initiator allergens in children

Although the multiplex dot test used for analyzing the sensitization patterns of the adult cohort demonstrated a general suitability for detecting allergen-specific IgE, it had shortcomings in terms of sensitivity in comparison to the ImmunoCAP. Therefore, a nitrocellulose microarray chip with infrared-based detection was developed in this thesis to enhance the sensitivity and further reduce the amount of serum needed per analysis, which made this test method particularly useful in the analysis of children's sera, where the available sample volume is often limited and low sIgE concentrations require a sensitive method of detection. With this microarray, the sensitization profiles of wheezing infants and toddlers (0–4 years), asthmatic children (5–17 years) and non-asthmatic children (1–16 years) were investigated not only using Der p allergens but also allergens of the American HDM *D. farinae* (refer to chapter 3.3). The overall aim of this part of the thesis was to identify potential initiator allergens by analyzing monosensitizations to single allergens but also evaluate the relevance of the previously identified potential marker allergens in children.

The frequency of sensitization to rDer p 5, rDer f 5, rDer p 7, and rDer f 21 was increased in asthmatic children compared to non-asthmatic and wheezing children. This is in line with the literature, where Der p 5 and Der p 7 have been reported as important in asthmatic children in a study by Resch et al., and further confirmed the importance of Der p 5 in the asthmatic phenotype as demonstrated in the previous chapters [243]. Although the sensitization prevalence to rDer f 21 was significantly increased in asthmatic children, this was not the case for rDer p 21, which could, however, also be due to the small size of the study groups.

The analysis of monosensitizations revealed that particularly rDer p/f 5, rDer p 7 and rDer p/f 21 were able to initiate sensitizations in asthmatic children. While it has been proposed by Posa et al. that sensitization to HDM starts with major allergens, as these were detected at the youngest ages, the results obtained in this thesis demonstrate that especially asthmatic

children are more often monosensitized to non-major allergens [262]. In particular group 5 and group 21 mite allergens do not only seem to be associated with AA in adults, but furthermore they appear to be potent initiator allergens for the asthmatic disease, whereas non-asthmatic children were indeed mostly monosensitized to major allergens. However, the IgE response to HDM allergens seems to rapidly expand in asthmatic children as monosensitizations overall were less frequent than in non-asthmatic and wheezing children.

Wheezers, who are defined as children of preschool age with transient or persistent episodes of bronchial obstruction that can evolve to clinically manifest asthma, often placed in-between asthmatic and non-asthmatic children regarding both sensitization count and patterns [263, 264]. Unfortunately, no follow-up data of the study participants were available. It is, however, conceivable that the wheezing children consist of different populations who differ in sensitization counts and prevalence, which might result in a mix of “asthma-like” and “non-asthma-like” profiles leading to results that lie between those two groups of children. These subsets might have different risks of eventually developing asthma (persistent wheezers) or reverting back to normal lung function (transient wheezers). This hypothesis is of speculative nature as of now and will have to be further evaluated in a longitudinal and larger scale study in the future.

A further aspect of this project was to elucidate species-specific differences in mite sensitization patterns to investigate the potential impact of climate change on house dust mite sensitization patterns in Middle Europe.

As described in chapter 1.4.4, climate change can impact the development of various allergen sources, and differences in temperature and relative surface humidity influences growth and metabolism of mites leading to changes in their preferred habitats and rebalancing of locally occurring species. While it usually is reported that *D. pteronyssinus* is the predominantly relevant species in HDM allergy in Middle Europe and the majority of existing research is centered on it, *D. farinae* is better equipped to thrive in the environment created by rising temperatures and decrease in surface humidity during the last decades and even though these phenomena are highly variable between geographical regions, it can be assumed that *D. farinae* and its allergens will gain importance in Middle Europe as the climate changes progress further. Children will be especially affected by this, as their primary sensitization might be elicited by Der f rather than Der p allergens. It can therefore be expected that the biggest influence of *D. farinae* on allergic diseases in Middle Europe is currently in the young. Thus, for the panel of allergens used for the microarray analysis, the Der f equivalents of the Der p allergens identified as important in the adult study patients were recombinantly expressed to investigate their sensitization patterns in children among the major allergens Der f 1 and Der f 2. With that,

this study is the first one to analyze the sensitization patterns with a broad allergen range of both HDM species in children. Overall, Der f allergens were not inferior in regards to sensitization prevalence in any of the study groups, and in particular sensitization to rDer f 2 was more frequent than to rDer p 2 in non-asthmatic children. An analysis of the different age groups of asthmatic children revealed that young children tended to react to rDer f 5 and rDer f 21 more often, whereas adolescents were rather sensitized to rDer p 5 and rDer p 21, indicating that the initial source of sensitization might shift from *D. pteronyssinus* to *D. farinae*, especially since both rDer f 5 and rDer f 21 were also detected as monosensitizations. Meanwhile, all major allergens led to similar sensitization rates. Although the size of this study group was too small to statistically assess these results with sufficient power, they nevertheless suggest that allergens of *D. farinae* are already in the process of and will likely continue gaining more influence in Middle Europe during the next decades, possibly due to ongoing climatic changes. However, since Der f and Der p allergens exhibit substantial sequence homologies, it could be questioned whether an introduction of single Der f allergens would really be necessary for clinical routine and immunotherapy. While in the microarray analysis, all patients reacting to rDer f 20 were also sensitized to rDer p 20 (97% sequence homology), this cross-reactivity was not as clear for other allergens. For the standardization with natural anti-Der p 2 antibodies, only low signals for rDer f 2 were observed. Moreover, especially for the group 5 and 21 mite allergens, which are among the allergens with the lowest sequence homology between the species (Der p 5/Der f 5: 76%, Der p 21/Der f 21: 71%), monosensitizations to either of these allergens were observed and patients reacting to the Der p allergen were not necessarily sensitized to the Der f equivalent and vice versa. In particular, the sensitization prevalence against rDer f 21 but not rDer p 21 was significantly increased in asthmatic children. Therefore, this question cannot be universally answered and must be individually assessed for each allergen. Especially for the potential marker and initiator allergens Der p/f 5 and Der p/f 21 an inclusion in routine diagnostic tools and immunotherapy extracts would be valuable contributions in the clinical diagnosis and treatment of allergic children and future generations.

Overall, the analysis of sensitization patterns further confirmed the importance of the previously identified potential biomarker allergens, namely group 5 and group 21 mite allergens, not only in asthmatic adults but also children and adolescents. Their ability to act as initiator allergens by causing monosensitizations in asthmatic children particularly reinforces their high clinical relevance. Furthermore, there were hints that allergens of *D. farinae* gain importance in the sensitization of young children, a development that should be closely monitored as it can require substantial changes in the way immunotherapy and diagnosis of HDM allergy are conducted in children in the future.

4.5 Interaction of potential marker allergens with the lung epithelium

A further aspect of this thesis focused on the elucidation of the potential of HDM allergens to elicit and augment allergic responses and inflammation in the human body with a particular focus on the potential marker allergens that were identified as important for different clinical manifestations of HDM allergy in the previous chapters. To investigate the pathomechanistic properties and potential pathways by which these allergens can induce immune responses, human bronchial epithelial cells were stimulated with the recombinant purified allergens Der p 5, Der p 10, Der p 20 and Der p 21 and the release of a variety of proinflammatory mediators was analyzed in the cell culture supernatant in chapter 3.4.

While it has been reported that HDM extract can induce the release of a multitude of proinflammatory cytokines in different cell culture models ranging from primary nasal and bronchial epithelial cells in air-liquid-interface culture models to cell lines in submerged culture, the evidence regarding the immunological impact of single allergens is, apart from that of Der p 1 and Der p 2, rarely investigated and not yet understood [53, 265-271].

The tested allergens rDer p 5, rDer p 10, and rDer p 21 were significantly associated with AA in the univariate logistic regression models, and rDer p 5 and rDer p 21 were additionally associated with AA in the multiple logistic regression models as well as in the stratified analyses in the previous chapters. Overall, all three allergens significantly increased the expression of various measured cytokines in a concentration-dependent way although to different degrees, whereof rDer p 21 was the most potent allergen. Most prominent was the upregulation of IL-6 and IL-8 release, both of which are associated with proinflammatory pathways, and they reportedly are connected to airway inflammation and AA. IL-6 levels in circulation and bronchoalveolar lavage fluid are even reported as a potential asthma biomarker, although this is a topic of controversy [272-279]. Moreover, the concentrations of other proinflammatory mediators such as TNF-α, which facilitates the development of early- and late-phase allergic inflammation by e.g. promotion of Th_2-mediated immune responses and recruitment of eosinophils to inflammation sites, and further cytokines that have been described as important in the development of allergic sensitizations, such as IL-1α and GM-CSF, were increased in the cell culture supernatant [271, 280].

In the literature, no studies were available that investigated the cytokine release stimulated by Der p 10 or other mechanistic studies thereof [53]. However, both Der p 5 and Der p 21 have been reported to stimulate the release of IL-6 and/or IL-8 in alveolar (A549) and/or bronchial

(BEAS-2B) cell lines before, the latter of which apparently via a proposed TLR2-dependent pathway as described by Pulsawat et al. [252, 281, 282].

In the experiments conducted in this thesis, the release of IL-6 and IL-8 among other cytokines was confirmed, but in contrast to the results reported by Pulsawat et al. they were not influenced by TLR2 inhibition for neither rDer p 5 nor rDer p 21. These differences could be due to the different cell lines used. BEAS-2B reportedly possess a different baseline metabolism and gene expression than Calu-3 cells, which could be due to their different proposed origin (BEAS-2B: upper airway, Calu-3: lower airway) and immortalization (BEAS-2B: infection with adenovirus 12 / simian virus 40 hybrid, Calu-3: cancer-derived) [283]. Recently, the epithelial origin of BEAS-2B even has been challenged by Han et al., as the cells exhibited characteristics similar to mesenchymal stem cells, and it has been proposed that the cell line was misclassified due to insufficient characterization and misinterpretation of markers that were wrongly thought to be epithelium-specific at the time of its establishment [284]. Misclassification of cell lines is not uncommon, in particular when they have been established several decades ago [285]. Ultimately, this has significant consequences as the results obtained using a wrongly classified cell line cannot simply be applied to the disease that was sought to be investigated if the cells stem from a different tissue.

Although it is possible that allergens can be a ligand to more than one TLR as has been shown for the cat allergen Fel d 1, which can activate TLR2 and TLR4, both BEAS-2B and Calu-3 cells reportedly express all of the surface-bound TLRs 1–6, and therefore exclusively TLR-dependent pathways, even if they involve multiple TLRs, should have theoretically led to similar results in both cell lines despite their potentially different cell types [286-288]. Since Pulsawat et al. have at least for Der p 5 reportedly confirmed the interaction with TLR2 by a HEK cell reporter assay, the involvement of TLR2 cannot be ruled out for now despite the conflicting results obtained in this thesis [252]. An exclusively TLR-dependent pathway could be confirmed in future experiments by inhibition of MyD88 (Myeloid differentiation factor 88), an adaptor protein that, with the exception of TLR3, is involved in the downstream signaling of all membrane-bound TLRs [289]. However, as of now it seems more likely that besides TLR2 other PRRs with differential expression in the two cell lines could be involved, that eventually, in the same way as TLR-signaling, lead to the activation of NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells), and could therefore serve as an alternative pathway to epithelial activation in case of TLR2 inhibition while simultaneously explaining the similar cytokine expression profile to the TLR-dependent controls Pam3CSK4 and LPS.

Even though Der p 5 and Der p 21, as discussed in the previous chapters, exhibit similar structural properties and both led to a comparable increase of sensitization prevalence in

asthmatic individuals, rDer p 21 led to an over ten-fold increase of cytokine concentrations compared to rDer p 5 and overall was more potent in stimulating the epithelial cells. While it seems likely that both allergens would interact with the epithelium via the same pathway, different affinities to the respective receptors could be responsible for the differences in cytokine release observed between the two allergens. Furthermore, both Der p 5 and Der p 21 have proposed lipid-binding properties although their ligands in natural environments are unknown to date. As discussed before, lipids carried by allergens can augment the allergic response and in case of Der p 2 its ligand LPS is obligatory to enable the activation of TLR4 [107]. Since the allergens used to stimulate the bronchial epithelial cells were produced recombinantly, they did not carry their natural lipid ligands and it is therefore possible that both Der p 5 and Der p 21 may exhibit even more potent or different ways of interaction with the lung epithelium in their natural form.

In contrast to Der p 5 and Der p 21, Der p 10 is a non-lipophilic allergen, often the cause of cross-reactivity to *Crustaceae* and mollusks and usually with minor seroprevalence. It is all the more notable that the bronchial epithelial cells were substantially activated after exposure to rDer p 10, even though to a lower degree than rDer p 21 with the exception of IL-15, which reached by far the highest concentrations across all stimuli after stimulation with 200 µg/ml of rDer p 10. However, it has already been reported in the literature that less serodominant allergens are not necessarily inferior in their capability to induce a cytokine response, as Hales et al. described a similar degree of IL-5 production after stimulation of bronchial cells with the natural purified allergens Der p 7 and Der p 1 [290].

In contrast to the other allergens tested in the bronchial epithelial cell model, rDer p 20 had no significant impact on any of the cytokine concentrations measured in the supernatant. This was not unexpected, as it was not associated with asthma in the previous analyses of the sensitization patterns but with AD only. The fact that rDer p 20 apparently did not interact with the bronchial cells adds weight to the initial hypothesis that allergens can possess an organ specificity, and for future analyses it would be highly interesting to test Der p 20 in a different cell culture model suited for the investigation of AD, e.g. in keratinocytes. As Der p 20 is usually considered a minor allergen, it is not well-studied and only limited data on its immunogenic properties are available [52]. However, its homologue in the American HDM, Der f 20, reportedly triggered TIM-4 (T-cell immunoglobulin mucin-4) expression in dendritic cells, a mediator that can induce proinflammatory pathways by promoting Th_2 cell differentiation, and a certain genetic polymorphism of the TIM-4 gene has been associated with AD [291, 292].

Since the aim of this part of the project was to investigate the potential of these allergens to interact with the airway epithelium and elucidate the pathways by which these effects were

mediated, a cell line was chosen since their homogeneity and standardization as well as fast and unlimited proliferation due to immortalization makes cell lines a well-suited model for a first proof-of-principle study. However, cell lines generally diverge from normal human cells in their metabolism and sensitivity to stimuli and are thus farther away from the *in vivo* situation than other models such as primary cells [283, 293]. Since the results obtained with primary cell models can be highly dependent on the donor's characteristics such as if the cells are isolated from an allergic or non-allergic individual, it is less reproducible than using cell lines, which complicates the elucidation of underlying pathways [265]. For future experiments, it would be highly interesting to investigate the individual effects of the HDM allergens presented in this thesis in a primary cell model that is closer to the real-life situation with cells of asthmatic and non-asthmatic donors to see if the cytokine release is dependent on the phenotype of the donors.

The fact that almost all measured analytes were elevated in response to rDer p 10 and 21 led to the question of whether these values could be subject to a systemic error. However, since the experiments were repeated independently with different passages of cells, and all samples were measured in two separate assays containing 8 and 4 of the analytes each, respectively, due to the varying gene expression levels of the analytes in Calu-3 cells (Table 35) requiring different dilutions of the supernatants, a systemic error leading to these results seemed unlikely. Especially the fact that the observed effects were concentration dependent and Der p 20 did not influence the cytokine release in a similar way further strengthens the validity of the data as well as the analyte VEGF-A being unaffected in all stimulation experiments. By checking the recorded image files of the instrument, it was moreover verified that no overexposure effect from IL-6 and IL-8 due to their high concentrations affected any of the other analytes that were included in the multiplex detection assay. The recorded images showed clearly divided spots with no overexposure as can be seen in Appendix 18. It can therefore be assumed that the results depict a genuine upregulation of the analytes' expression during the stimulation with the allergens. For future analyses, it would be helpful to purposefully include an additional "housekeeping" analyte that would likely be unaffected by the stimulation with allergen.

Overall, these results further substantiate the association of the previously identified marker allergens with allergic asthma and allow a first glimpse of the pathomechanistic properties of Der p 5, Der p 10 and Der p 21 even though their pathway signaling could not be fully elucidated during the course of this thesis. All three allergens evoked complex proinflammatory responses in bronchial epithelial cells and they are therefore highly capable of mediating allergic diseases not only through the induction of IgE-production but they furthermore by promotion of an inflammatory environment in the lung. The fact that these effects were not seen when exposing the cells to Der p 20 reinforces the idea of a possible organ specificity for these

potential marker allergens. However, further mechanistic research is necessary to determine the pathways by which the allergens are able to activate bronchial epithelial cells, since the results in this thesis indicate that the immune response by bronchial epithelial cells might involve TLR4- and TLR2-independent signaling.

4.6 Basophil activation test for replacement of allergen challenges

A further aspect of this thesis was to investigate the potential of the basophil activation test (BAT) to replace allergen challenges in order to improve and simplify the diagnosis of allergic patients. However, controlled provocations are not routinely indicated in HDM allergy diagnosis and especially not in children as HDM allergy is usually not of potentially life-threatening severity [113]. Moreover, HDM patients scheduled to start immunotherapy could not attend the outpatient clinics and provide whole blood samples due to hygiene restrictions following the SARS-CoV-2-pandemic.

Therefore, only few patients could be tested for their individual reactivity to HDM allergens in the BAT as a proof-of-principle study. The results showed, in accordance with different clinical phenotypes of the patients, profound interindividual differences and a wide range of allergens involved in eliciting the allergic response rather than only major allergens. The ability of the identified potential marker allergens Der p 5 and Der p 21 to induce basophil activation has furthermore been reported in the literature before [248, 294]. Although no reports about the ability of Der p 20 to activate basophils were available in the literature, another group 20 mite allergen from the storage mite *Tyrophagus putrescentiae* (Tyr p 20) has been reported to elicit basophil degranulation as well [295]. This further solidifies their clinical relevance and is particularly important considering that at least Der p 5 and Der p 21 seem to be insufficiently represented in immunotherapeutic options as discussed in chapter 4.3.

After demonstrating the general suitability of the BAT to detect reactivities in HDM-allergic patients and the ability of the recombinantly produced allergens to elicit an IgE-mediated allergic response, for further investigation in this part of the project peanut allergy was chosen as a model disease in order to verify the performance of the BAT in predicting the allergic response and its severity in particular, since the oral food challenge (OFC) provides a controlled setting with close monitoring of ingested allergen dose and objectifiable symptoms. However, these results can and shall be transferred to other allergic diseases such as HDM allergy as well in the future.

While several attempts have been made in the last decade to utilize the BAT for differentiating between allergic reaction and asymptomatic sensitization as well as predicting allergy severity,

each had its shortcomings. All pre-existing studies used a single stimulus such as peanut extract (PEX) or a maximum of 2 stimuli such as the serodominant allergen Ara h 2 combined with Ara h 6 to assess the allergy status. While the latter approach performed well in predicting the prevalence of an allergic reaction, it was unsuitable for assessing the allergy severity in a study by van Erp et al. [296-298]. This observation was confirmed in this thesis, as Ara h 2 had the best performance of the single stimuli to differentiate allergy from sensitization-only as can be seen in Figure 3.35. Another study utilized only PEX to predict the prevalence of an allergic response. Although the performance of this approach was generally favorable with a 98% sensitivity and a specificity of 96%, there were, just as observed within this thesis, substantial reactions to PEX in asymptomatic patients reaching values as high as 60% of degranulated basophils [297]. This leads to the problem of either false-positive or, if a high cut-off is chosen to combat this, false-negative diagnosis, which therefore requires the confirmation of negative diagnosis by performing an oral food challenge (OFC) nevertheless. Other studies utilizing PEX as the only stimulus in BAT were not able to confirm the performance reported by Santos et al. and resulted in a sensitivity of 79–81% and an 86%–95% specificity [298, 299].

Overall, none of the available studies were able to reliably assess both the allergy status and severity of the study participants. This work is therefore the first with a multifactorial component-resolved approach to basophil activation testing by utilizing four different stimuli and including not only the percentage of activated basophils but also the changes in fluorescence intensity to develop a highly effective and reliable algorithm to both differentiate between allergy and asymptomatic sensitization but to also predict the severity and tolerance threshold of the allergic disease with positive and negative predictive values of 100% each. Although it usually is recommended to include a broad range of allergen concentrations to establish dose-response curves in BAT, in this study reduction of the frequently used six different concentrations tested to three concentrations did not impair the effectivity of the algorithm’s performance and allowed the analysis of a wider allergen range while maintaining a small amount of 2 ml of whole blood needed for a single analysis, which is particularly beneficial in the diagnosis of young children [300].

Even though Santos et al. have already reported a method to independently assess the tolerance threshold and symptom severity by only using PEX as a stimulus, their BAT performed poorly in the ROC curve analysis to differentiate between allergy severity grades [301]. Their chosen parameter to predict severity was the ratio of activated basophils induced by PEX compared to anti-IgE positive control. This is problematic since responses to anti-IgE controls can vary highly depending on the used antibody, possibly due to the binding to different sequences and varying affinities, which can be seen in Appendix 19 wherein the basophil activation induced

by the two antibodies used in this thesis is visualized. This ultimately creates a problem with reproducibility when the setting is applied to clinical routine where a reliable protocol is essential for consistent results between different users. Additionally, their study did not make a distinction between mild and moderate allergy, the differentiation of which is highly clinically relevant, as in this thesis, children with a mild allergy exhibited a substantial tolerance and received different medical recommendations for allergen avoidance than patients with a moderate allergic response in the OFC.

Furthermore, the authors admitted that, while the symptom severity and tolerance threshold in the clinical setting were independent, their chosen parameters to predict both of these did in fact correlate with each other. Their applicability in clinical routine can therefore be questioned, especially since their parameter to predict the tolerance threshold, namely the stimulus concentration at half-maximal basophil activation, only classified 50% of the children correctly in the high-tolerance group. In this thesis, the parameters of the BAT seemed to correlate both with tolerance threshold as well as symptom severity and as of now, there appears to be no parameter to reliably differentiate between the two. Therefore, the CSTC scoring system was developed in this project, which entailed both of these aspects independently.

Another reason for the improved performance of the developed BAT protocol in this work could be the inclusion of natural oleosins as a stimulus, which are a mix of the highly clinically relevant allergens Ara h 14 and Ara h 15 derived from the oil bodies of peanut that have been reported to be associated with severe allergic responses [198]. Although PEX contains a variety of allergens, the poor water-solubility of the lipophilic oleosins leads to insufficient extraction in the aqueous production process. In the separate evaluation of the potential of each stimulus to discriminate asymptomatic and allergic responses, the natural oleosins performed poorly as they were not universally recognized by all peanut-allergic patients, but they proved to be a crucial element when differentiating the severity grades of the allergic disease. Whilst Ara h 8 has been reported as a potentially important allergen for assessing cross-reactivity with birch pollen, reactions to it were rare in this project and there were no patients reactive to rAra h 8 who did not respond to rAra h 2 or oleosins as well, as can be seen in Figure 3.33 [302]. This was to be expected, as the majority of children in this study were younger than 5 years, and at this age the Ara h 2-mediated peanut allergy is dominant, whereas the pollen-associated peanut allergy mediated by Ara h 8 is more frequently seen in older children [303]. Therefore, Ara h 8 provided no benefit to the diagnostic scheme, and oleosins proved to be the crucial element for detecting severe allergic responses. Nevertheless, Ara h 8 was included in the analysis algorithm for now, as the study population was comparably small and Ara h 8 might prove as beneficial to the diagnosis in a larger cohort, especially for children older than 5 years who are

already sensitized to birch pollen and develop sIgE to Ara h 8, the Bet v 1 homologue in peanut, as a pollen-associated food allergy, which can reportedly present as a monosensitization in the absence of Ara h 2-specific IgE [215].

Despite its huge potential to improve the diagnosis of allergic patients by replacing allergen challenges, a commonly observed drawback of the BAT is the rate of non-responders it comes with. In this study, 13% of the study participants were non-responders, which is in line with the values between 10 to 15% that are usually reported in the literature [300]. These patients are not responsive to any IgE-mediated basophil stimulation but show regular degranulation for the GPCR-mediated stimulation by fMLP [214]. It is not exactly determined what leads to the non-responsiveness in patients, although it has been proposed that the individual releasability of basophils is highly variable in regards to the number of cross-linked FcεRI molecules necessary to induce degranulation, and low levels of Syk phosphatase, which is involved in the signal cascade leading to histamine release, might be associated with non-responsiveness as well [304-306]. Interestingly, these patients often show positive responses to skin prick tests nevertheless, suggesting that the allergic reaction is primarily mediated by mast cells and not basophils [296]. For these individuals, an allergen challenge cannot be replaced by basophil activation testing. However, the non-responders are easily identifiable by including IgE-mediated as well as non-IgE-mediated controls [300].

Overall, the BAT protocol and analysis algorithm developed within this project matches a highly unmet clinical need for a reliable replacement of OFCs with excellent performance in distinguishing asymptomatic sensitizations from allergic reactions as well as predicting the severity of the allergic disease. It appears that a multi-factorial approach to basophil activation testing including several allergens is the key for improved diagnostic performance. However, the proposed algorithm has been established with relatively few patients and should therefore be evaluated in a larger study group for refinement and, if necessary, adjustment of the thresholds. Possibly the algorithm could also be further simplified by leaving out Ara h 8 as, at least for now, it did not add an additional benefit to the performance.

The framework of the algorithm moreover has the potential to be transferred to inhalant or other food allergies after identifying the most relevant allergens for these diseases and adjusting the thresholds in a clinical setting. For HDM allergy, especially the marker allergens identified in this thesis appear to be promising candidates to develop such a BAT in the future. This could prove especially useful in the selection and monitoring of patients before and during immunotherapy.

5 Outlook

Within the course of this work, the recombinant production of several HDM allergens was optimized, providing a reliable expression protocol for various allergens in the future by using the SHuffle strain, which is able to produce soluble and correctly folded allergens containing disulfide bonds. These allergens were subsequently used for molecular profiling to identify potential marker allergens and elucidate their organ specificity in a multiplex dot test and a novel infrared-based microarray developed within this project, which will be of great use in the future analysis of valuable patient samples due to its further reduced serum volume requirement and enhanced sensitivity.

The identification of Der p 20 as well as the lipophilic allergens Der p/f 5 and Der p/f 21 as important potential biomarkers for atopic dermatitis and allergic asthma, respectively, in adults as well as in children introduces new and important considerations for clinical routine testing and immunotherapy in the future. During the course of this work, these allergens were furthermore integrated into the routine diagnostic platform ImmunoCAP in a pilot study and are therefore ready for a potential transfer to commercial diagnostic platforms. Their official inclusion as single allergens in routine diagnostic platforms should happen as soon as possible and their content in diagnostic and immunotherapy extracts should be closely monitored, since, as of now, they do seem to be absent in the respective extracts in a lot of cases. Overall, these results add even more weight to the conclusion that stricter and extended quality control guidelines for the manufacturing of HDM extracts used for diagnosis and therapy are necessary, as it appears that this is a currently unmet clinical need in the diagnosis and therapy of HDM-allergic patients. Since group 5 and 21 mite allergens from both *D. pteronyssinus* and *D. farinae* led to monosensitizations and thus may act as initiator allergens in asthmatic children, these patients are currently at risk to receive a false-negative diagnosis with the options available today for routine diagnosis. In particular the development of sensitizations to *D. farinae* should be closely monitored in the future, as the results in this thesis indicate that especially young children are more often sensitized to Der f 5 and Der f 21 than their Der p equivalents, which could be a result of the current effects of climate change. Furthermore, the sensitization count was introduced as a new possible tool for risk assessment in clinical routine in this thesis as well.

The interaction of the identified marker allergens with the lung epithelium was further investigated in a bronchial epithelium cell culture model. These experiments added more weight to the hypothesis of a potential organ specificity, as Der p 20, which was identified as a possible biomarker for atopic dermatitis, did not influence the cytokine release of the cells in contrast to

those allergens associated with asthma, which exhibited a high potential to activate the lung epithelium and create a proinflammatory environment. However, the pathway of these effects could not be fully elucidated during this thesis. As of now, it seems likely that at least one TLR-independent pathway might be involved, which could be further investigated in future experiments with inhibition of the TLR-associated adaptor protein MyD88. This and further testing of the organ specificity of Der p 20 in skin-specific cell models shall be subject of a, currently under revision, DFG-funded research proposal during the next years. The project furthermore provides essential methodological tools for a DFG-funded IRTG-initiative, of which the first phase has recently been reviewed successfully.

The successful development of a highly effective basophil activation test (BAT) design, scoring system and analysis algorithm has a huge potential to improve the clinical routine diagnosis of peanut-allergic children by allowing to predict the prevalence and severity of clinical manifest allergy. This will greatly enhance the current clinical use of the BAT and shall enable to reliably replace allergen challenges, which was not possible with the currently available approaches to BAT design and analysis in the literature. Moreover, its framework may also be transferred to other allergies, such as HDM, in the future.

6 Summary

The ubiquitous presence of house dust mites and their allergens on dust particles on beds, carpets and upholstered furniture makes them one of the most prominent sources for airborne allergens with an approximately 20% sensitization rate of the general population in Europe, which increases up to 80% in asthmatic individuals. Their capability to elicit severe allergic diseases such as asthma and atopic dermatitis becomes an increasing challenge in the healthcare of allergic patients. Even though 32 different allergens of the European house dust mite *D. pteronyssinus* are officially registered in the WHO/IUIS database, only four single house dust mite allergens are available for the routine diagnostic platform ImmunoCAP, which is regarded as the gold standard for *in vitro* IgE diagnostics. And despite various mechanisms by which house dust mites can elicit allergic reactions and inflammation or even promote sensitization to other allergens such as the disruption of epithelial tight-junctions, activation of the innate and adaptive immune system or promotion of a shift towards Th2-responses being known today, the majority of available research is centered on the first discovered major allergens Der p 1 and Der p 2 while investigations of clinical importance and pathomechanistic properties of mid-tier and minor allergens are still limited. This thesis therefore aimed to further elucidate the clinical relevance of various less well-studied allergens and their association with different allergic phenotypes and disease severity, identify potential biomarkers and elucidate their possible organ specificity as well as their potential to act as initiator allergens and activate the epithelium of the lung as part of the allergic sensitization and elicitation process. Furthermore, in order to improve the clinical diagnosis of allergic patients, the potential of the basophil activation test to replace allergen challenges was explored in house dust mite and peanut-allergic patients.

In order to detect the individual sensitization profiles of study participants, various allergens of the European and American house dust mite were recombinantly expressed in *E. coli* and purified. Specific IgE patterns of adult HDM-allergic patients exhibiting different allergic diseases, namely allergic asthma, allergic rhinitis and atopic dermatitis were analyzed with a multiplex dot test including the allergens Der p 1, 2, 5, 7, 10, 13, 20, 21 and 23. The complexity of the individual sensitization profile, named sensitization count, was connected to allergic multimorbidity and might be a valuable tool for risk assessment in allergic patients. Sensitization to the lipid-associated allergens Der p 5 and Der p 21 was associated with the prevalence of allergic asthma/and or atopic dermatitis and high serum IgE concentrations against Der p 20 were identified as a possible biomarker for severe atopic dermatitis.

For the analysis of the sensitization patterns of children, a novel infrared-based microarray with enhanced sensitivity and less required sample volume compared to the previously used multiplex dot test was developed. The sera of asthmatic, non-asthmatic and wheezing children were analyzed for the presence of specific IgE not only against the previously used Der p allergens but furthermore the major allergens and homologues of the identified marker allergens in the American house dust mite, namely Der f 1, 2, 5, 20 and 21. The results further confirmed the importance of group 5 and 21 mite allergens not only in the development of allergic asthma but furthermore as potent initiator allergens alongside Der p 7. Additionally, these data indicated that, particularly in younger children, *D. farinae* might gain importance as the dominating source of sensitization in Middle Europe, presumably due to altered humidity and temperature levels induced by climate change that favor the growth of this species.

The identified potential marker allergens were subsequently used to stimulate bronchial epithelial cells to investigate their organ specificity and further elucidate the pathways by which these allergens can interact with the lung epithelium and thereby promote the allergic sensitization or disease. All asthma-associated allergens, in particular Der p 21, were found to be potent activators of the epithelial cells by triggering the release of various proinflammatory cytokines. These effects were not reduced by TLR2- or TLR4-inhibition, which indicates the involvement of either different TLRs or other pattern recognition receptors. On the other hand, the atopic dermatitis-associated allergen Der p 20 did apparently not interact with the lung epithelium, which further reinforced the idea of its possible organ specificity for the skin, which will have to be further evaluated in skin-specific models in the future.

Following this, the potential of the allergens to directly interact with the primary cells of patients, namely whole blood basophil granulocytes, was further investigated in basophil activation tests of patients with different phenotypes of house dust mite allergy, which revealed profound interindividual differences of basophil reactivity to the single allergens in accordance with their clinical symptoms. The potential of the basophil activation test to be used as a tool in clinical routine to replace allergen challenges was further explored by analyzing the basophils of peanut-allergic children undergoing an oral food challenge. The development of a scoring system entailing both experienced symptoms and tolerance threshold during the food challenge combined with a powerful analysis algorithm allowed to successfully differentiate not only between silent and symptomatic sensitization but furthermore predict the severity of the allergic response, which represents an enormous advance on what was previously possible with basophil activation testing. The framework of this test design and algorithm furthermore has the potential to be transferred to other allergies such as house dust mite allergy.

7 Zusammenfassung

Das ubiquitäre Vorkommen von Hausstaubmilben und deren Allergene auf Staubpartikeln in Betten, Teppichen und Polstermöbeln macht diese zu einer der wichtigsten Quellen für inhalative Allergene weltweit. Schätzungsweise 20% der allgemeinen europäischen Bevölkerung und bis zu 80% der Asthmatiker sind gegen Hausstaubmilben sensibilisiert. Ihr hohes Potenzial, schwere allergische Erkrankungen wie allergisches Asthma oder atopische Dermatitis zu verursachen, stellt eine zunehmende Herausforderung für die heutige Patientenversorgung da. Obwohl mittlerweile bereits 32 verschiedene Allergene der europäischen Hausstaubmilbe *D. pteronyssinus* offiziell als Allergene in der Datenbank der Weltgesundheitsbehörde registriert sind, sind nur vier dieser Allergene für die Bestimmung von spezifischen IgE-Konzentrationen im Serum von allergischen Patienten auf der als Goldstandard der *in vitro* IgE-Diagnostik gewerteten Routineplattform ImmunoCAP verfügbar. Es sind bereits diverse Wege, auf denen Hausstaubmilben zur Entstehung von allergischen Erkrankungen, einer proinflammatorischen Immunantwort und sogar der Sensibilisierung auf andere Allergenquellen beitragen können, bekannt, wie beispielsweise das Spalten von tight-junctions der Epithelzellen, Aktivierung der adaptiven und angeboren Immunzellen oder die Förderung einer Th_2-dominierten Immunantwort. Dennoch stützt sich der Großteil der aktuellen und früheren Forschung auf die zuerst entdeckten Majorallergene Der p 1 und Der p 2, wohingegen die Relevanz und mögliche den Pathomechanismus beeinflussende Eigenschaften von weniger serodominanten Allergenen noch weitestgehend unklar sind. Die vorliegende Arbeit hatte daher das Ziel, die klinische Relevanz von diversen weniger gut untersuchten Hausstaubmilbenallergenen und ihre Assoziation mit verschiedenen allergischen Phänotypen aufzuklären, potentielle Biomarker zu identifizieren und deren mögliche Organspezifität zu untersuchen. Weiterhin sollte das Potential dieser Allergene aufgeklärt werden, als Initiatorallergene bei Kindern zu fungieren und mit dem Lungenepithel zu interagieren. Außerdem wurde im Rahmen dieser Arbeit der Basophilenaktivierungstest im Hinblick auf den Ersatz von Allergenprovokationen untersucht, um die Diagnostik von allergischen Patienten zu verbessern.

Um die Sensibilisierungsprofile der Studienteilnehmer zu untersuchen, wurden diverse Allergene der europäischen und amerikanischen Hausstaubmilbe rekombinant in *E. coli* exprimiert und aufgereinigt. Aus den individuellen IgE-Profilen von erwachsenen Hausstaubmilbenallergikern, die mittels eines Multiplex Dot-Tests, der die Allergene Der p 1, 2, 5, 7, 10, 13, 20, 21 und 23 enthielt, detektiert wurden, wurde die Anzahl der Sensibilisierungen als Parameter für die allergische Multimorbidität identifiziert. Weiterhin konnten Sensibilisierung gegen die lipid-assoziierten Allergene Der p 5 und Der p 21 mit dem

Vorliegen von allergischem Asthma und/oder atopischer Dermatitis in Verbindung gebracht werden. Hohe IgE-Konzentrationen gegen Der p 20 konnten weiterhin als ein Biomarker für schwere atopische Dermatitis identifiziert werden.

Für die Analyse der Sensibilisierungsprofile von Kindern wurde im Rahmen der vorliegenden Arbeit ein neuartiger, infrarot-basierter Microarray entwickelt, der insbesondere durch seine erhöhte Sensitivität und den reduzierten Serumverbrauch eine Verbesserung zum vorher genutzten Multiplex Dot-Test darstellt. Die Seren von asthmatischen und nicht-asthmatischen Kindern sowie von sogenannten „Wheezern“ wurden nicht nur auf die Prävalenz von spezifischen IgE gegen Der p Allergene untersucht, sondern weiterhin auch gegen Allergene der amerikanischen Hausstaubmilbe (Der f 1, 2, 5, 20, 21). Dadurch wurde die Relevanz von Hausstaubmilbenallergenen der Gruppe 5 und 21 für die Entstehung von allergischem Asthma aber auch als potentielle Initiatorallergene zusammen mit Der p 7 bekräftigt. Die Daten lassen zudem den Rückschluss zu, dass *D. farinae* insbesondere bei jungen Kindern als primäre Allergenquelle dominanter zu werden scheint, was möglicherweise an durch den Klimawandel verursachten veränderten Lebensbedingungen liegt, die eher das Wachstum von *D. farinae* fördern.

Die identifizierten potenziellen Markerallergene wurden anschließend zur Stimulation bronchialer Epithelzellen verwendet, um ihre Organspezifität und die Wege, über die diese Allergene mit dem Lungenepithel im Sinne der Förderung von Sensibilisierungen und/oder allergischen Erkrankungen interagieren können, weiter zu untersuchen. Alle Asthma-assoziierten Allergene und insbesondere Der p 21 erwiesen sich als hochpotente Stimuli der Epithelzellen, indem sie die Freisetzung verschiedenster proinflammatorischer Zytokine auslösten. Dieser Effekt wurde durch die Hemmung von TLR2 oder TLR4 nicht beeinflusst, was darauf hindeutet, dass entweder andere TLRs oder andere Pattern-Recognition-Rezeptoren beteiligt sind. Andererseits interagierte das mit atopischer Dermatitis assoziierte Allergen Der p 20 offenbar nicht mit dem Lungenepithel, was die Hypothese einer potentiellen Organspezifität für die Haut weiter untermauert. Dies sollte allerdings in weiteren, hautspezifischen Modellen in der Zukunft näher untersucht werden.

Im darauffolgenden Teil der vorliegenden Arbeit wurde die direkte Interaktion der Hausstaubmilbenallergene mit Primärzellen, den basophilen Granulozyten im Vollblut von Patienten mit verschiedenen Phänotypen der Hausstaubmilbenallergie, mittels des Basophilenaktivierungstests weiter untersucht, wobei sich grundlegende interindividuelle Unterschiede in der Reaktivität auf die Einzelallergene in Übereinstimmung mit den klinischen Symptomen der untersuchten Patienten darstellten. Das weitere Potenzial des Basophilenaktivierungstests, Allergenprovokationen in der klinischen Routine zu ersetzen,

wurde durch die Untersuchungen der Basophilen von erdnussallergischen Kindern ermittelt, bei denen im Rahmen der Diagnostik eine orale Nahrungsmittelprovokation durchgeführt wurde. Die Entwicklung eines Scoring-Systems, das sowohl die auftretenden Symptome als auch die Toleranzschwelle während der Nahrungsmittelprobe umfasst, in Verbindung mit einem leistungsstarken Algorithmus zur Interpretation der durchflusszytometrischen Ergebnisse ermöglichte nicht nur eine erfolgreiche Unterscheidung zwischen asymptomatischer Sensibilisierung und einer klinisch manifesten Erdnussallergie, sondern auch die Vorhersage des Schweregrads der allergischen Reaktion, was einen enormen Fortschritt gegenüber dem darstellt, was bisher mit dem Basophilenaktivierungstest möglich war. Auf Grundlage dieses Designs und des entwickelten Algorithmus ist außerdem das Potenzial gegeben, die Methode zukünftig auch auf andere Allergien wie die Hausstaubmilbenallergie zu übertragen.

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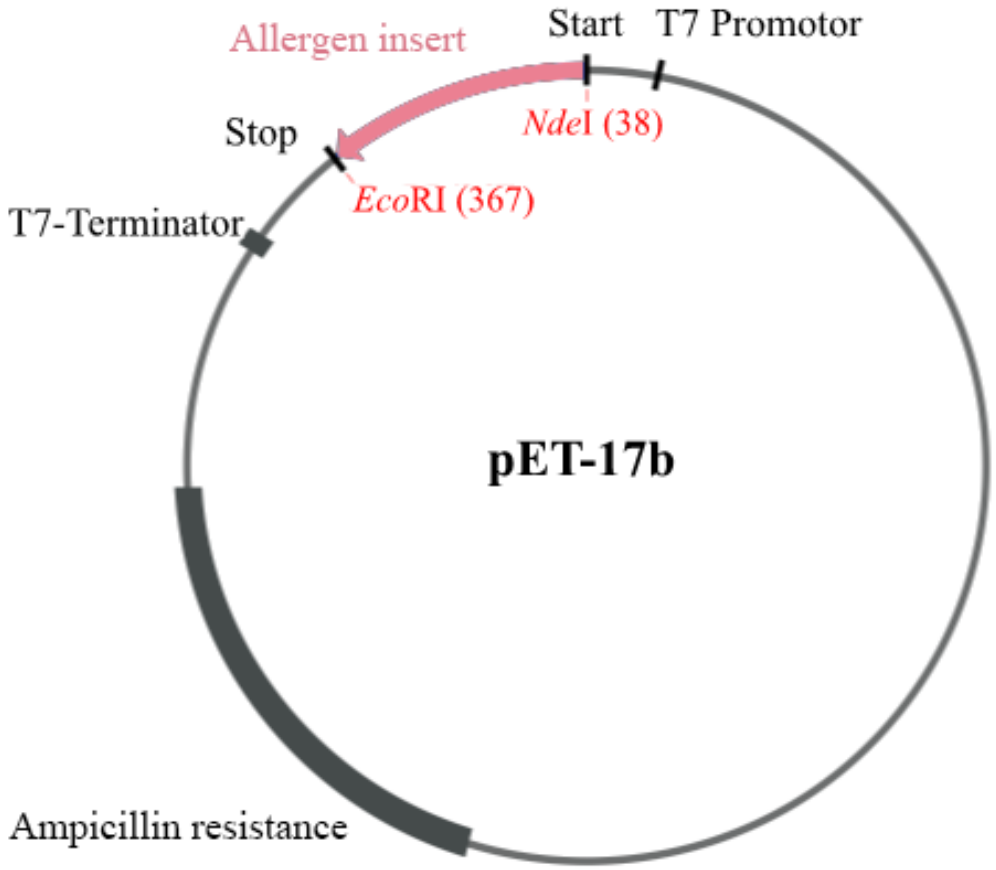
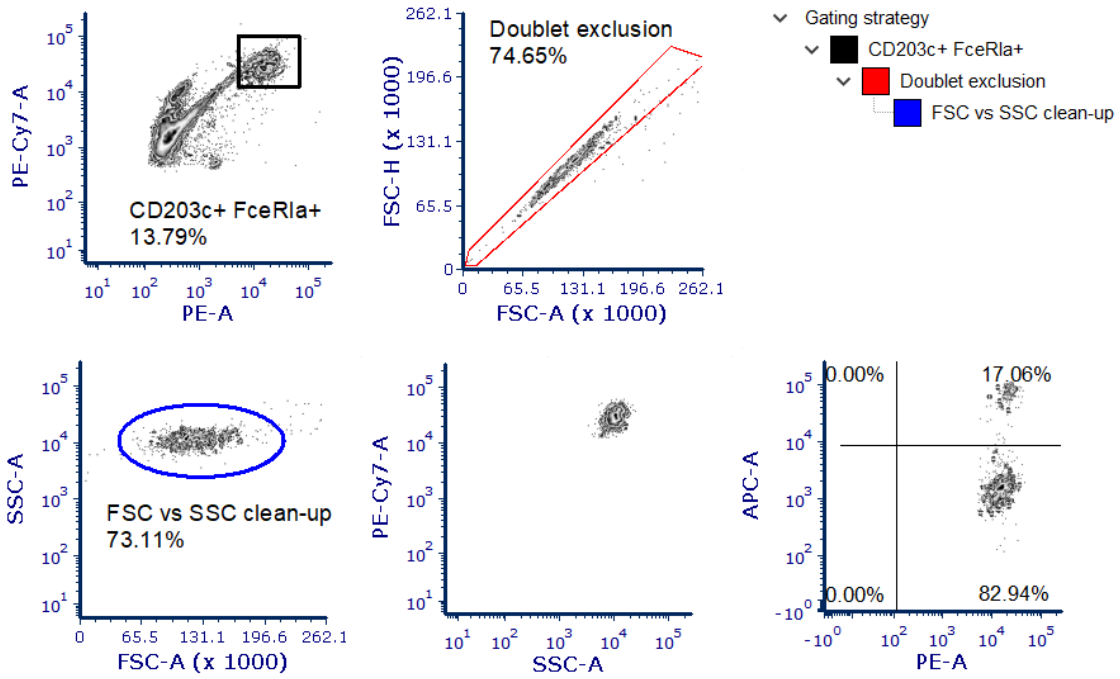
Appendix

Appendix 1: Vector map of pET-17b used for expression of recombinant allergens

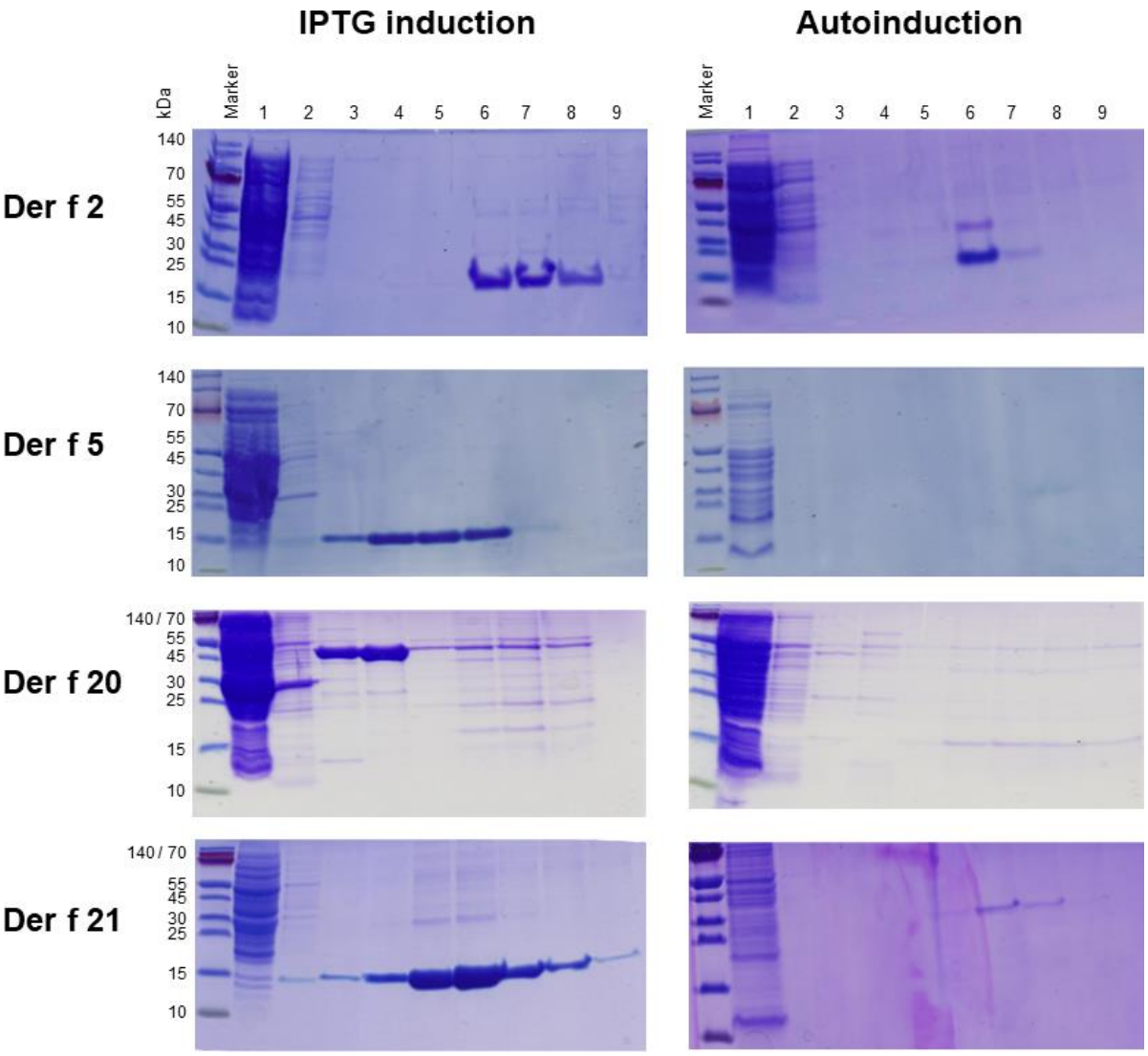


Appendix 2: Exemplary gating strategy used for basophil activation test after flow cytometry



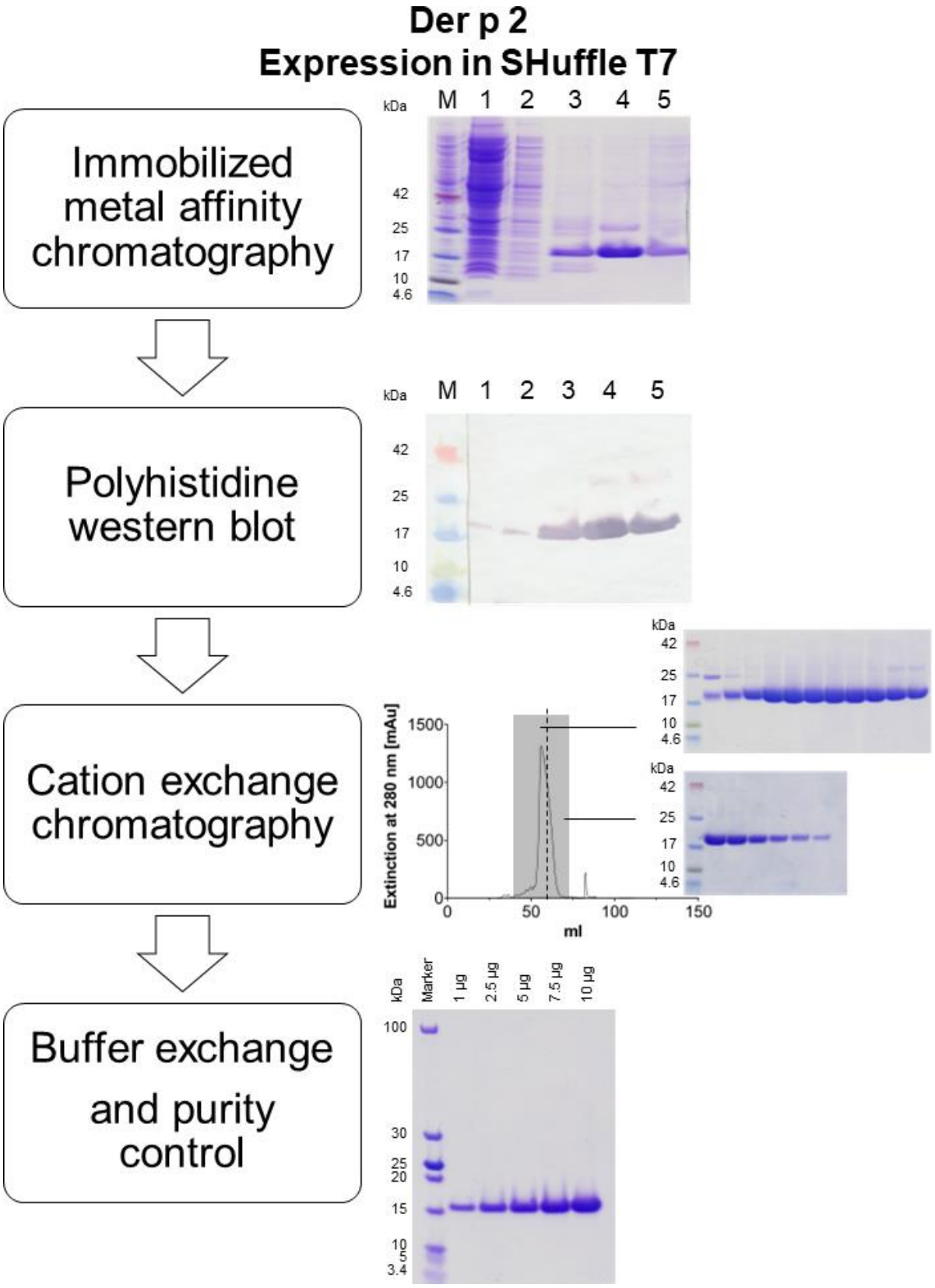
Exemplary gating strategy to identify basophils from whole blood. The shown sample is a positive control stimulated with anti-IgE. Cells were first gated for expression of the basophil markers FcεRIα and CD203c. Forward scatter area (FSC-A) against sideward scatter area (SSC-A) was used to exclude cells other than leukocytes. Doublet exclusion was carried out by side scatter width (SSC-W) vs CD203c gating. The proportion of activated basophils was calculated as $CD63^+$-cells ($FcεRIα^+$, $CD203c^+$).

Appendix 3: Comparison of yields by IPTG-dependent and autoinductive expression



Comparison of expression yields for Der f allergens produced with either IPTG-dependent induction or autoinduction by addition of lactose to the culture medium. Der f 2, Der f 5 and Der f 21 have a size of approximately 15 kDa. Der f 20 has a size of approximately 45 kDa. 1 = Flow-through, 2 = Binding buffer, 3 = Wash buffer I, 4 = Wash buffer II, 5–9 = Eluate I–V

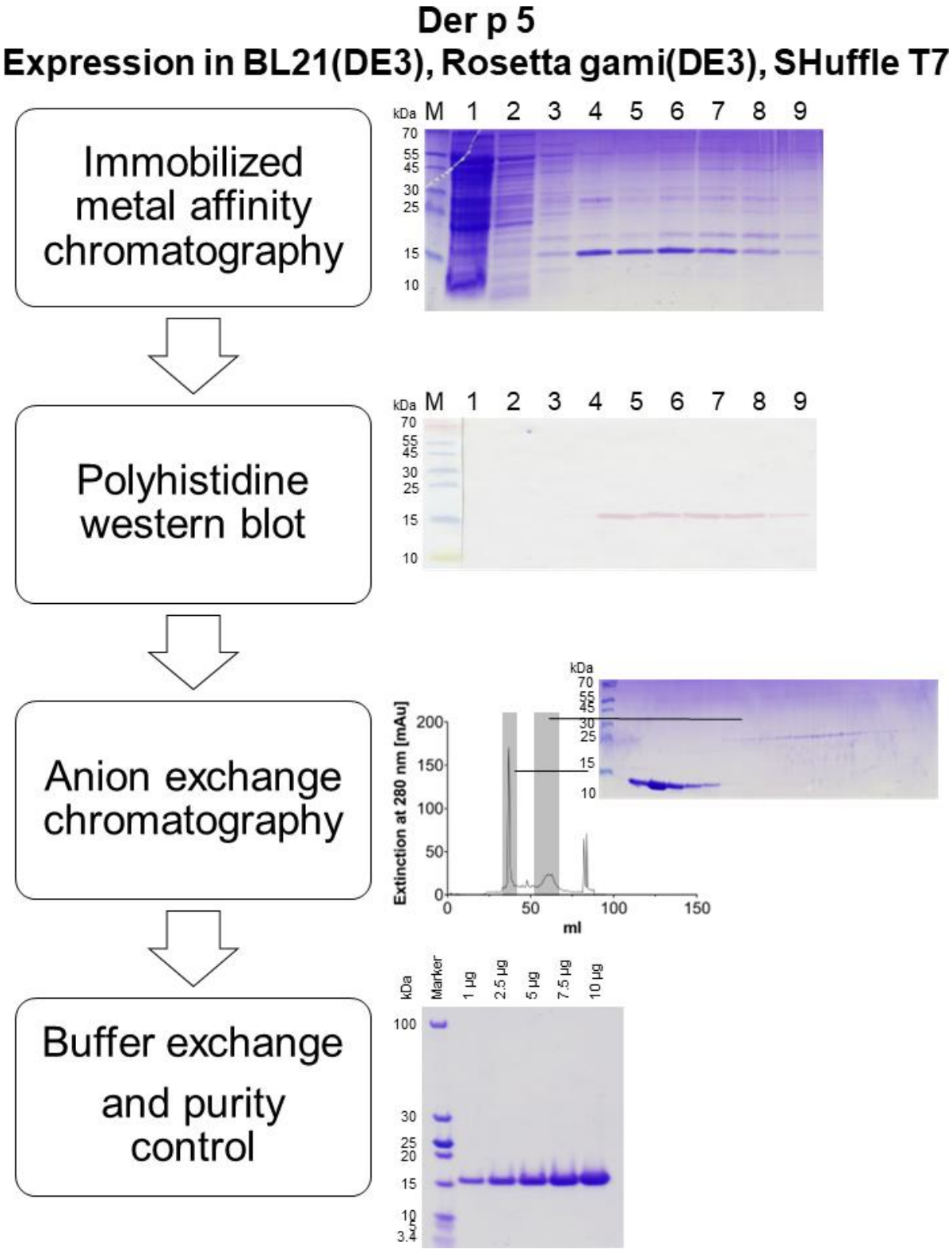
Appendix 4: Purification of recombinant Der p 2



Workflow of the purification of recombinant Der p 5 (approximately 15 kDa)

1 = Flow-through, 2 = Binding buffer, 3 = Wash buffer I, 4 = Wash buffer II, 5 = Eluate

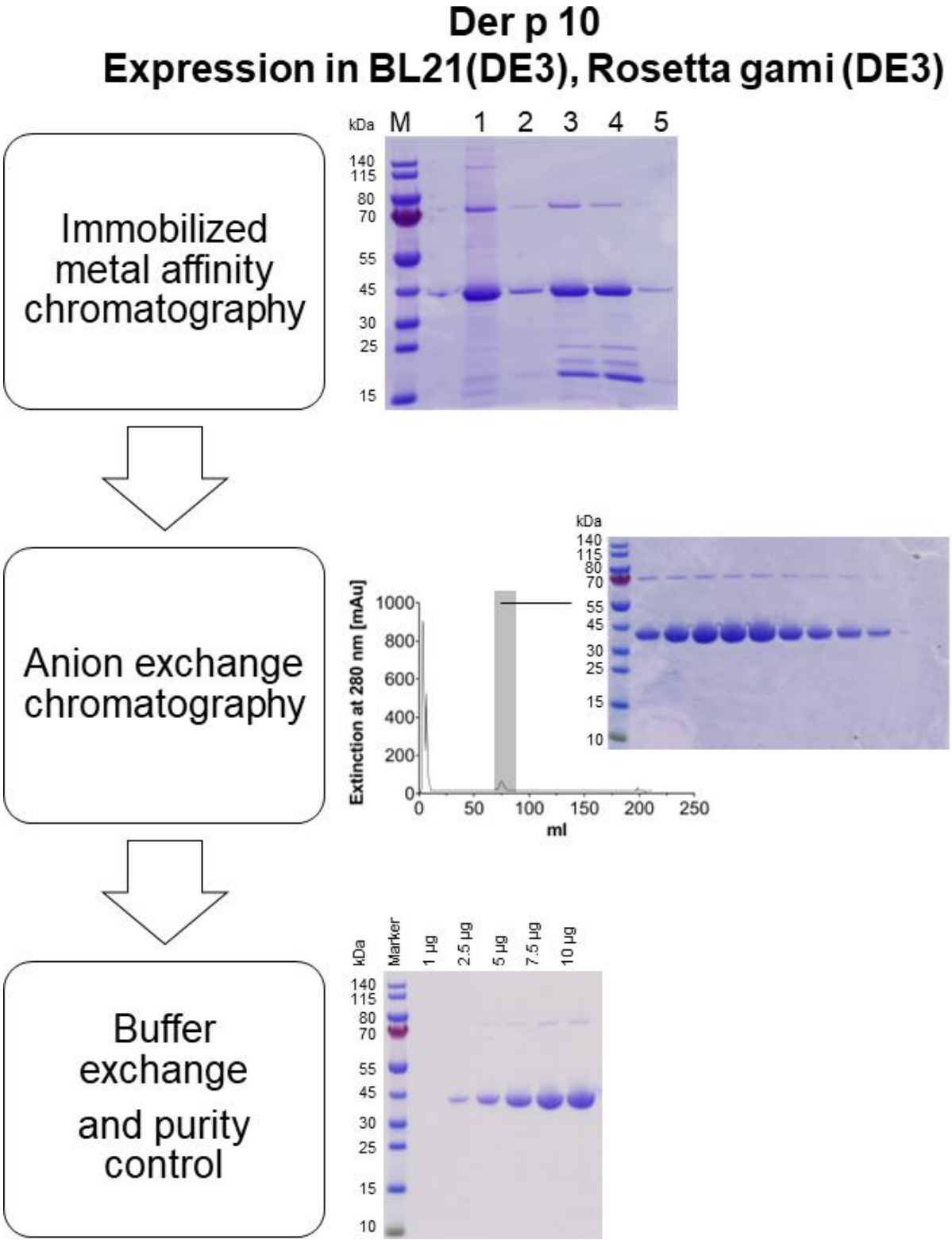
Appendix 5: Purification of recombinant Der p 5



Workflow of the purification of recombinant Der p 5 (approximately 15 kDa)

1 = Flow-through, 2 = Binding buffer, 3 = Wash buffer I, 4 = Wash buffer II, 5–9 = Eluate I–V

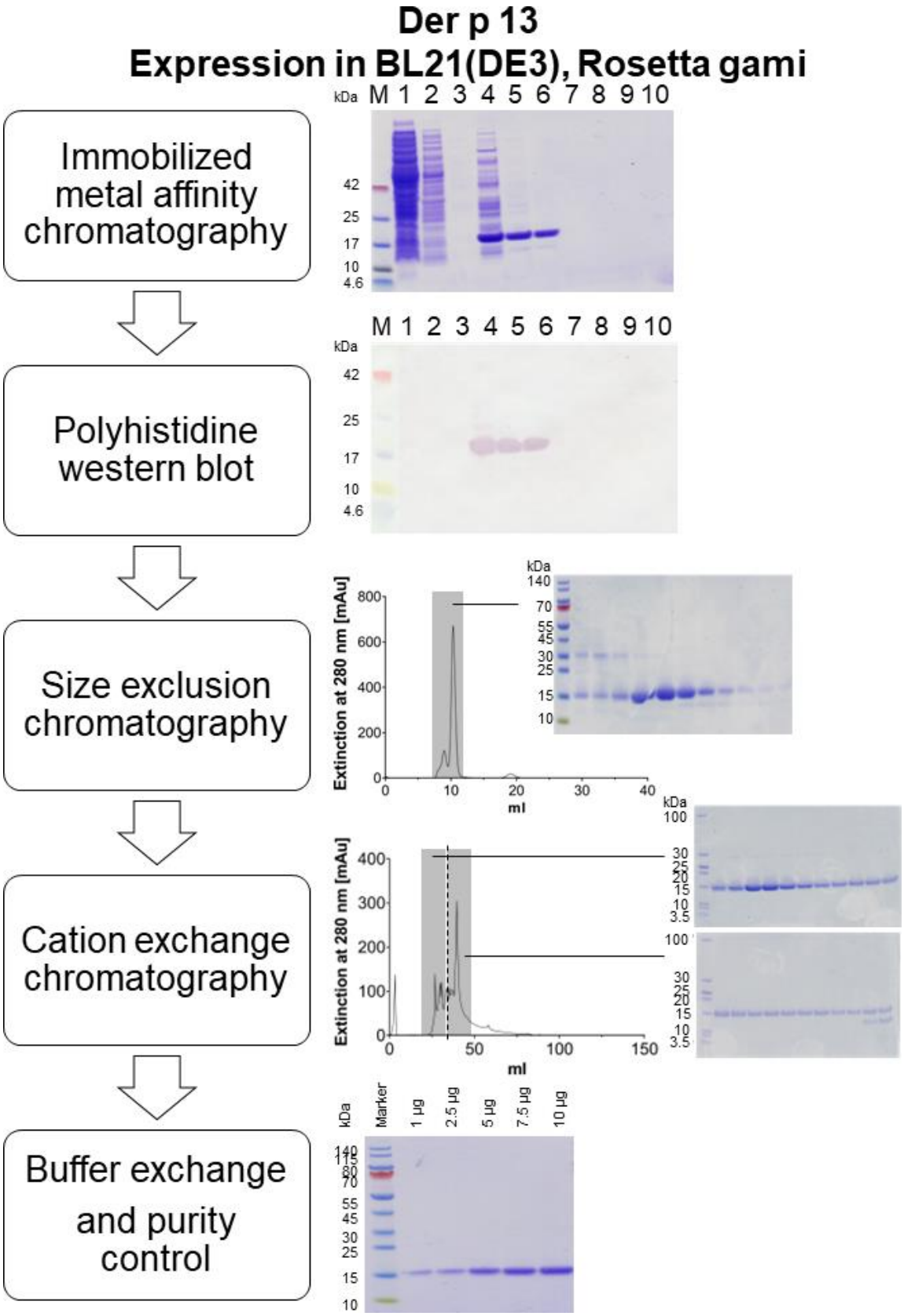
Appendix 6: Purification of recombinant Der p 10



Workflow of the purification of recombinant Der p 10 (approximately 35 kDa)

1 = Flow-through, 2 = Binding buffer, 3 = Wash buffer I, 4 = Wash buffer II, 5 = Eluate

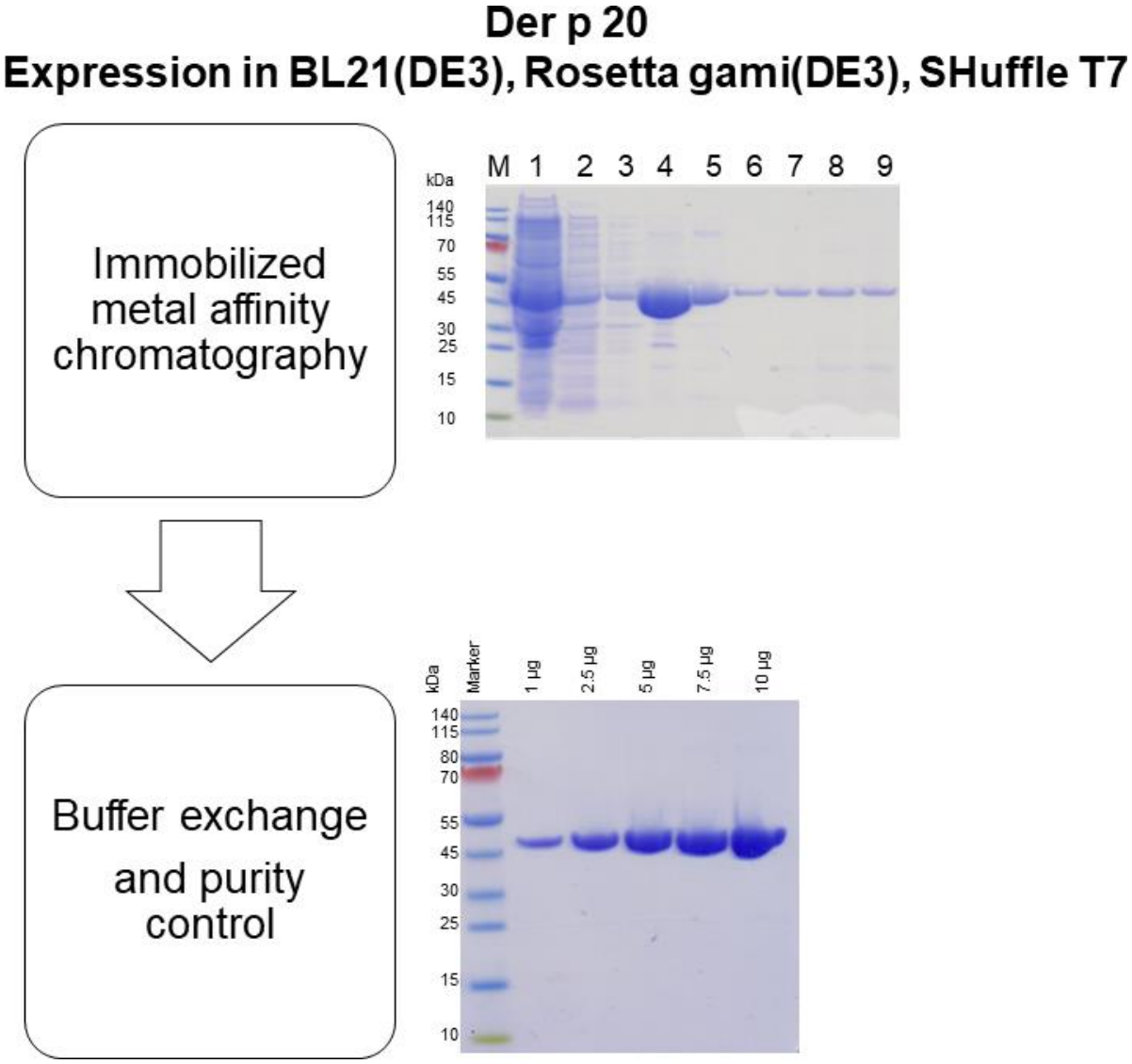
Appendix 7: Purification of recombinant Der p 13



Workflow of the purification of recombinant Der p 13 (approximately 15 kDa)

1 = Flow-through, 2 = Binding buffer, 3 = Eluate pH 6.3, 4–5 = Eluate pH 5.0, 6–7 = Eluate pH 4.0, 8–9 = Eluate pH 3.0, 10 = Eluate pH 2.0

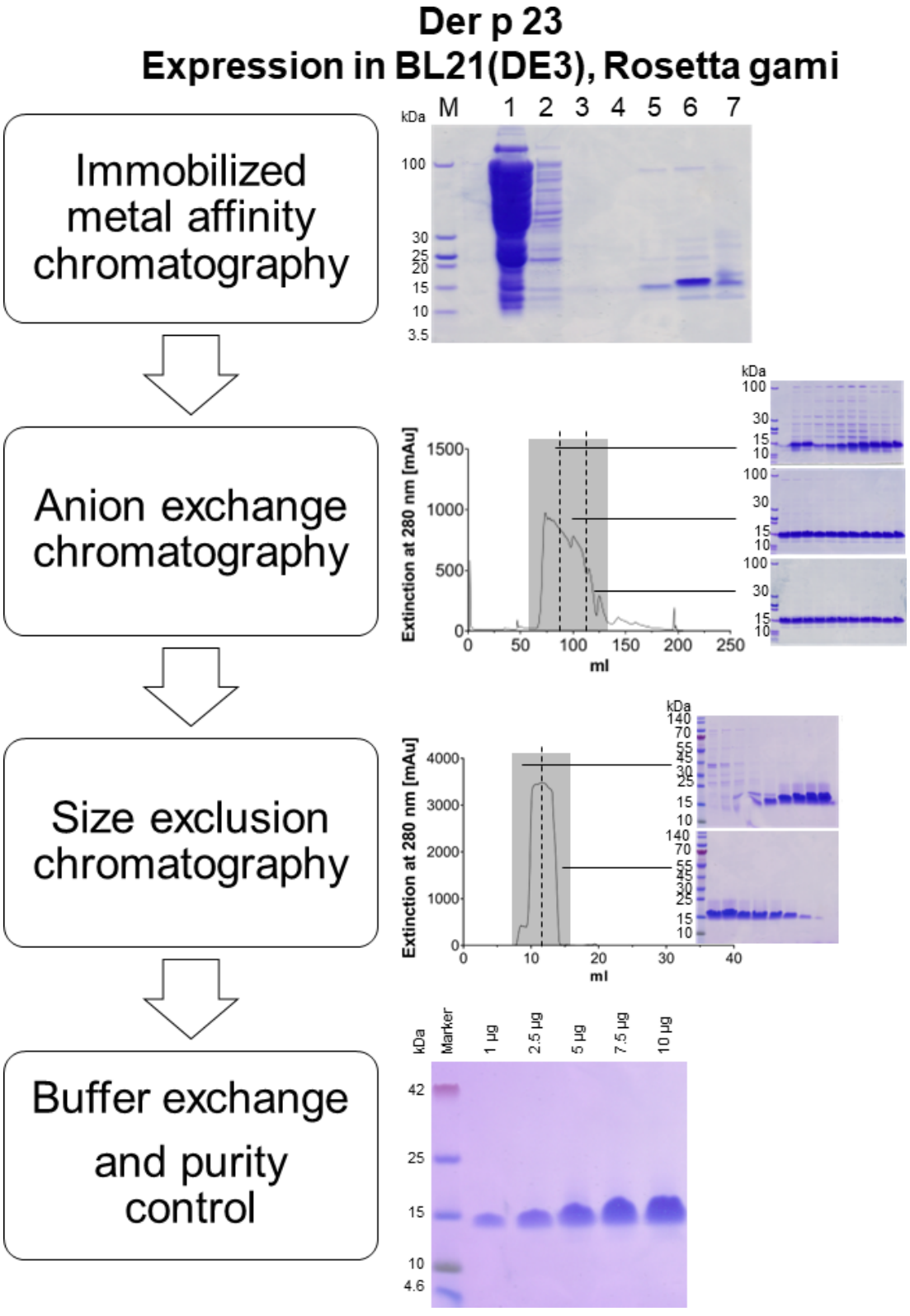
Appendix 8: Purification of recombinant Der p 20



Workflow of the purification of recombinant Der p 20 (approximately 45 kDa)

1 = Flow-through, 2 = Binding buffer, 3 = Wash buffer I, 4 = Wash buffer II, 5–9 = Eluate I–V

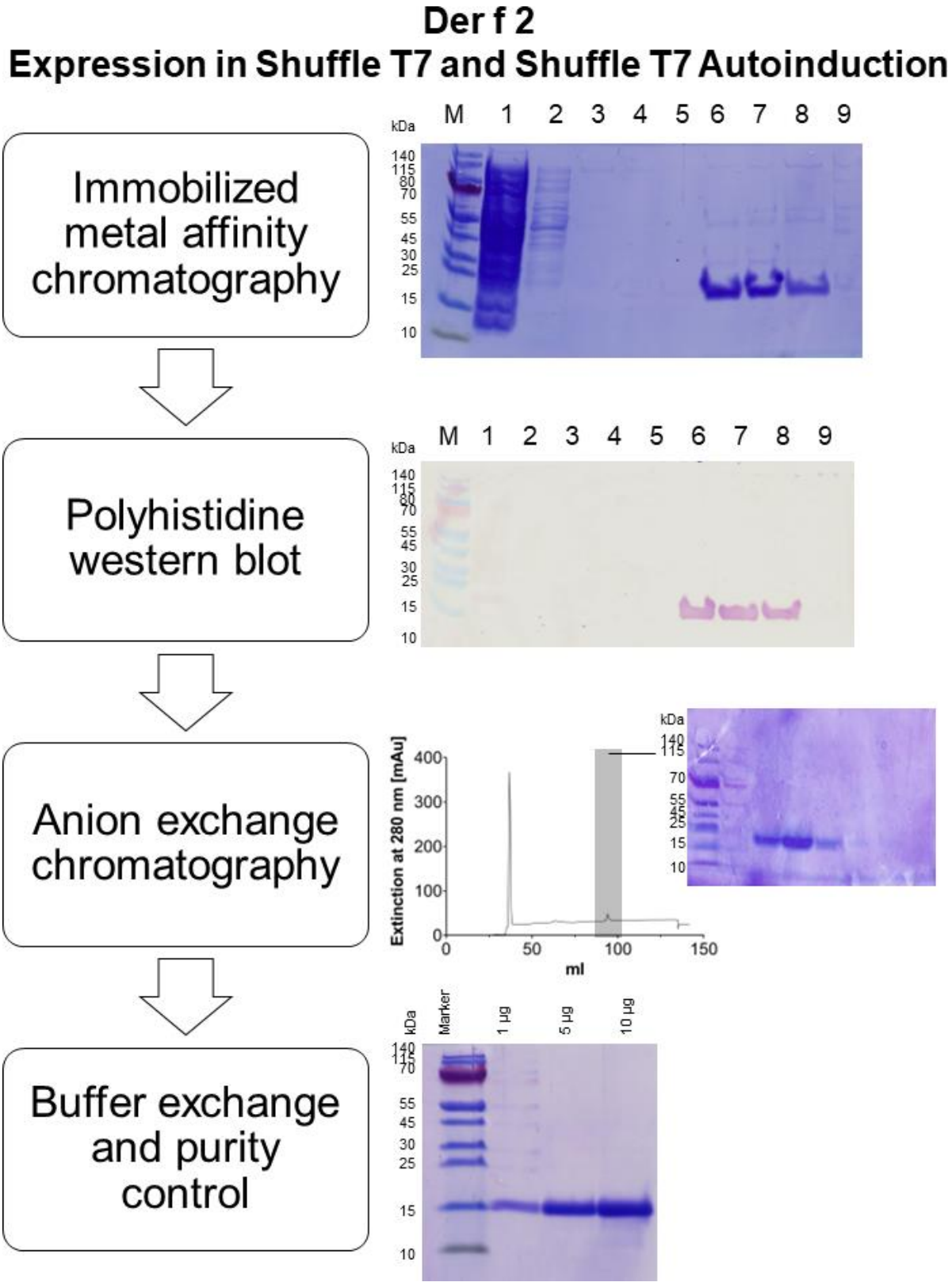
Appendix 9: Purification of recombinant Der p 23



Workflow of the purification of recombinant Der p 23 (approximately 15 kDa)

1 = Flow-through, 2–4 = Binding buffer I–III, 5 = Wash buffer I, 6 = Wash buffer II, 7 = Eluate

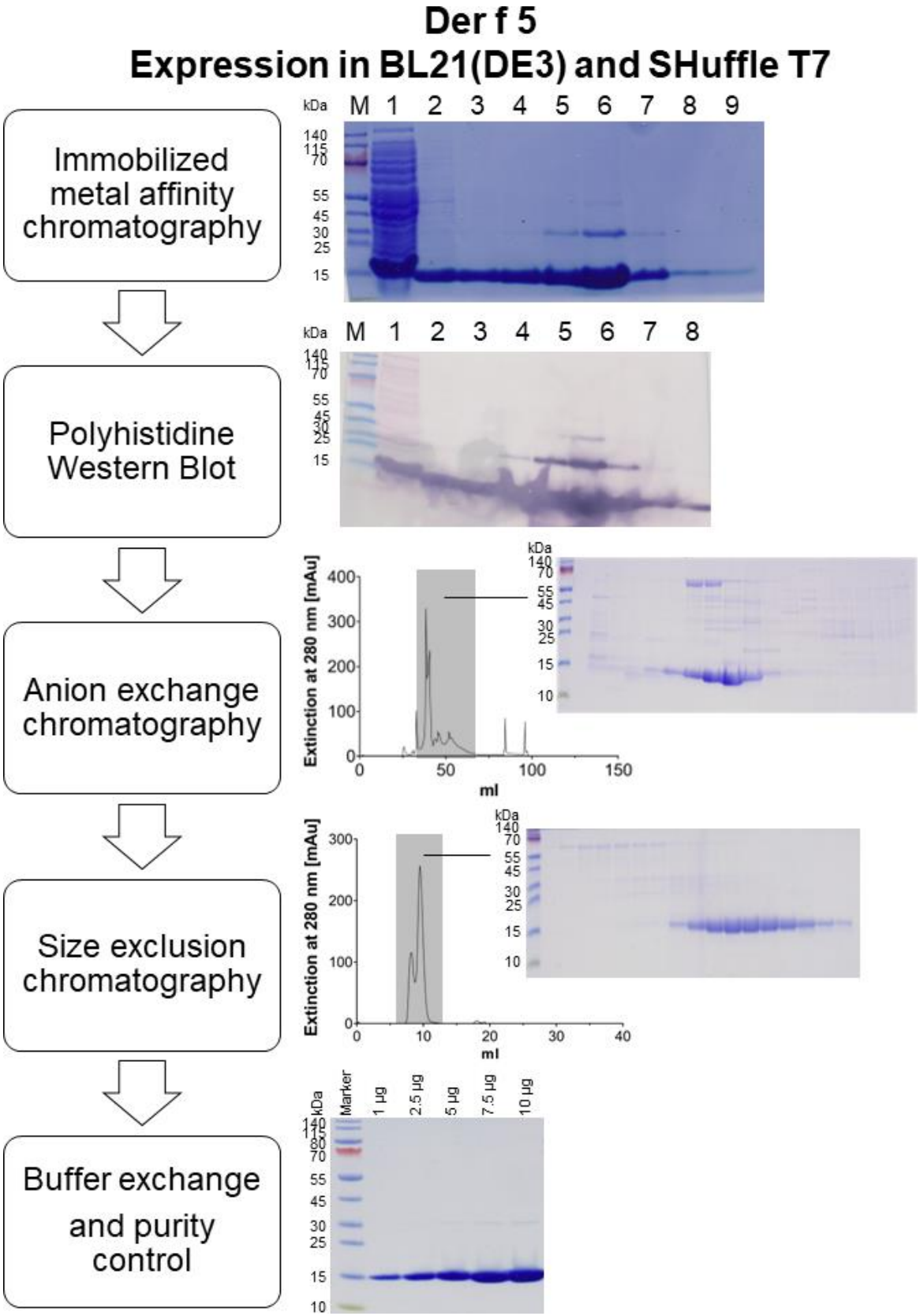
Appendix 10: Purification of recombinant Der f 2



Workflow of the purification of recombinant Der f 2 (approximately 15 kDa)

1 = Flow-through, 2 = Binding buffer, 3 = Wash buffer I, 4 = Wash buffer II, 5–9 = Eluate I–V

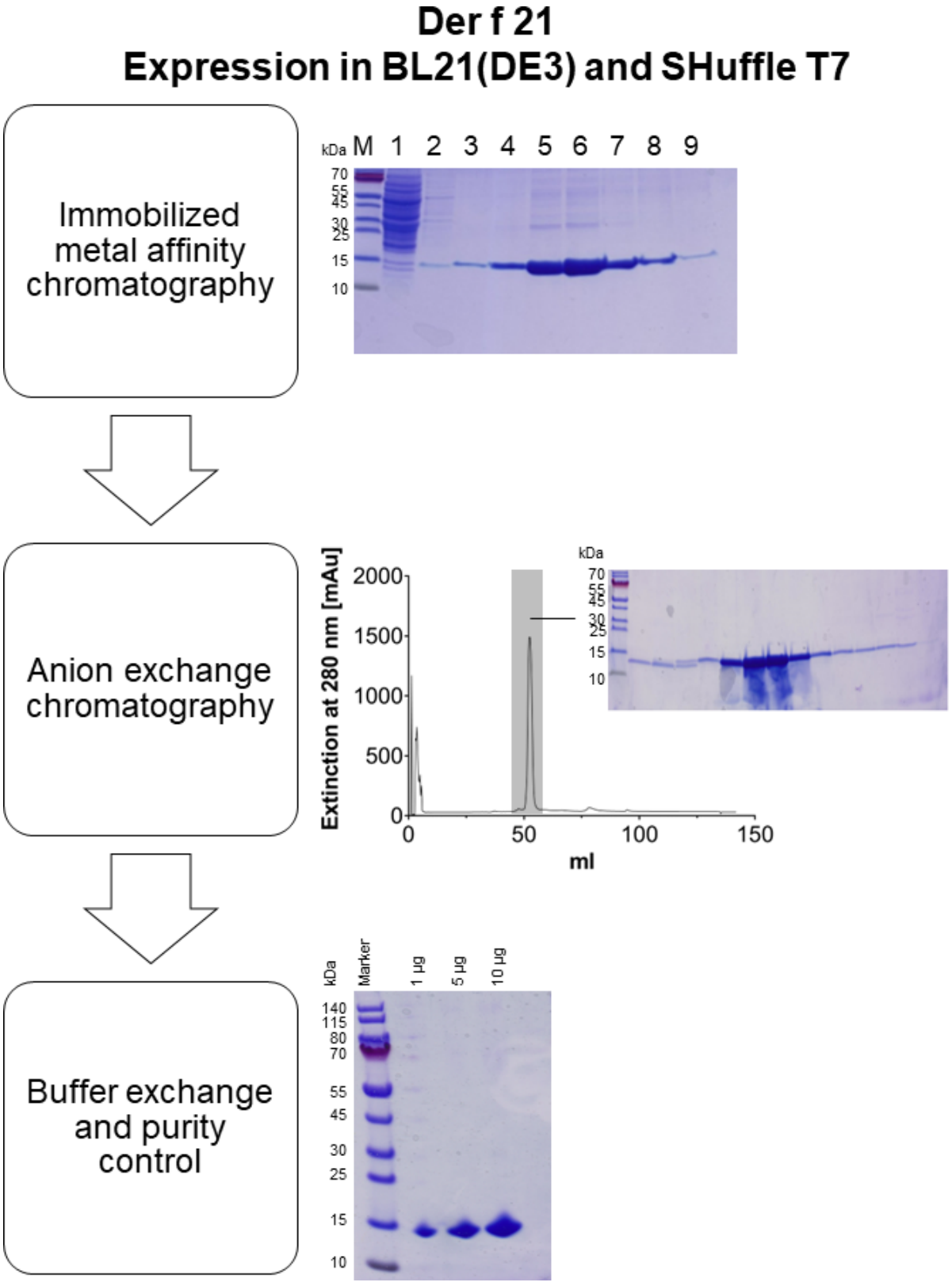
Appendix 11: Purification of recombinant Der f 5



Workflow of the purification of recombinant Der f 5 (approximately 15 kDa)

1 = Flow-through, 2 = Binding buffer, 3 = Wash buffer I, 4 = Wash buffer II, 5–9 = Eluate I–V

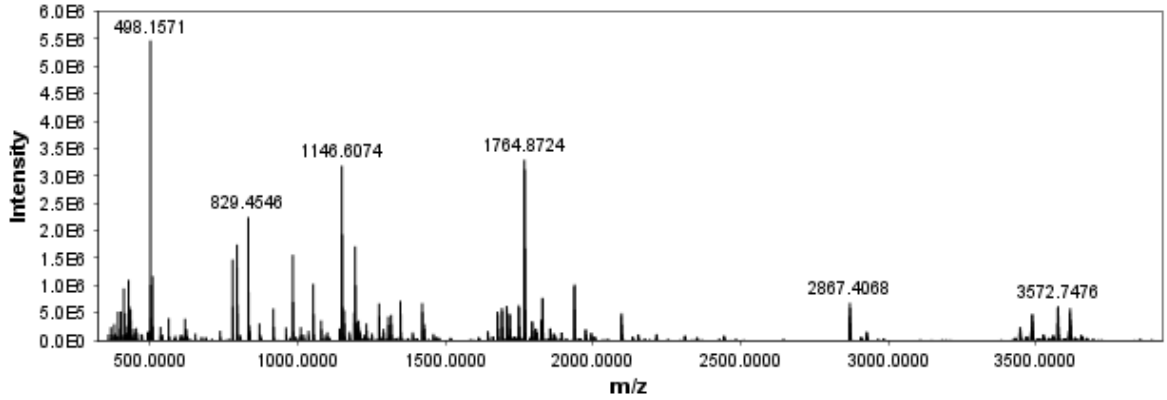
Appendix 12: Purification of recombinant Der f 21



Workflow of the purification of recombinant Der f 21 (approximately 15 kDa)

1 = Flow-through, 2 = Binding buffer, 3 = Wash buffer I, 4 = Wash buffer II, 5–9 = Eluate I–V

Appendix 13: Exemplary identification of Der f 20 by peptide mass fingerprinting



Fragment	$M_{theoretical}$ [m/z]	$M_{detected}$ [m/z]	Sequence	Position	Modification
1	356.242	356.242	(K)/LPK/(L)	287–289	
2	618.349	618.349	(K)/IPFSR/(D)	257–261	
3	734.400	734.400	(R)/IISMQK/(G)	230–235	Oxidized methionine
4	776.397	776.397	(R)/YWPVGR/(G)	203–208	
5	791.418	791.418	(K)/LEAGFQK/(L)	10–16	
6	791.418	791.418	(K)/GFKPTDK/(H)	92–98	
7	791.429	791.429	(R)/YNLQVR/(G)	304–309	
8	829.455	829.455	(R)/DVLDQLK/(T)	34–40	
9	914.518	914.519	(K)/KLEEVAAR/(Y)	296–303	Missed cleavage
10	957.503	957.504	(R)/GIFHNDKK/(T)	209–216	Missed cleavage
11	980.493	980.493	(K)/NEYVISTR/(V)	117–124	
12	991.501	991.501	MVDQATLSK/(L)	1–9	
13	1007.496	1007.496	MVDQATLSK/(L)	1–9	Oxidized methionine
14	1049.508	1049.508	(R)/FLQAANACR/(Y)	194–202	Acetylation
15	1076.561	1076.562	(K)/GGDLKEVFGR/(L)	236–245	Missed cleavage
16	1138.569	1138.570	(R)/MGLTEYQAVK/(E)	331–340	
17	1146.607	1146.607	(K)/LIDDHFLFK/(E)	181–189	
18	1154.564	1154.564	(R)/MGLTEYQAVK/(E)	331–340	Oxidized methionine
19	1191.614	1191.613	(K)/GQLATFEGELK/(G)	154–164	
20	1272.606	1272.606	(K)/GTYYPLLGMDK/(A)	165–175	Oxidized methionine
21	1287.674	1287.674	(K)/EMQDGIIELIK/(M)	341–351	
22	1303.669	1303.669	(K)/EMQDGIIELIK/(M)	341–351	Oxidized methionine
23	1418.777	1418.777	(K)/VKGQLATFEGELK/(G)	152–164	Missed cleavage
24	1425.703	1425.704	(K)/LQNAQDCHSLLK/(K)	17–28	Acetylation
25	1657.781	1657.780	(K)/GYPFNPMLTEAQYK/(E)	133–146	
26	1673.776	1673.775	(K)/GYPFNPMLTEAQYK/(E)	133–146	Oxidized methionine
27	1688.798	1688.798	(K)/TFLMWVNEEDHLR/(I)	217–229	
28	1704.793	1704.793	(K)/TFLMWVNEEDHLR/(I)	217–229	Oxidized methionine
29	1720.788	1720.788	(K)/TFLMWVNEEDHLR/(I)	217–229	Oxidized methionine, Oxidized tryptophan
30	1764.872	1764.872	(K)/TFAALFDPIIDDYHK/(G)	77–91	
31	1792.903	1792.894	MVDQATLSKLEAGFQK/(L)	1–16	Formylation. missed cleavage
32	1807.894	1807.886	(R)/FLQAANACRYWPVGR/(G)	194–208	Acetylation. missed cleavage
33	1825.940	1825.939	(R)/LGYLTFCPTNLGTTIR/(A)	265–280	Acetylation
34	1933.902	1933.901	(R)/GTAGEHTESVGGIYDISNK/(R)	310–328	
35	2092.997	2092.996	(K)/HPQTDFGNIEHFVNVDPK/(N)	99–116	
36	2212.095	2212.094	(R)/DDRLGYLTFCPTNLGTTIR/(A)	262–280	Acetylation. missed cleavage
37	2308.039	2308.039	(K)/GYPFNPMLTEAQYKEMETK/(V)	133–151	Oxidized methionine (2). Missed cleavage
38	2440.223	2440.222	(R)/MGLTEYQAVKEMQDGIIELI K/(M)	331–351	Oxidized methionine (2). Missed cleavage
39	2866.404	2866.404	(K)/GFKPTDKHPQTDFGNIEHFV NVDPK/(N)	92–116	Missed cleavage
40	3442.645	3442.646	(K)/TDMGATLLDVIQSGVENLDS GVGIYAPDAQSYK/(T)	44–76	Oxidized methionine
41	3554.745	3554.746	(K)/KTDMGATLLDVIQSGVENLD SGVGIYAPDAQSYK/(T)	43–76	Missed cleavage

Appendix 14: Results of peptide mass fingerprinting for identity verification of recombinant proteins

Allergen	Expressed Sequence	Coverage (%)
Der p allergens		
Der p 2	**DQVDVKDCANHEIKKVLVPGCHGSEPCIIHRGKPFQLEAVFEANQNTK**TAK**IEIKA SIDGLEVDVPGIDPNACHYMKCPLVKGQQQYDIKYTWNVPKIAPKSENVVVTVKV MGDDGVLACAIATHAK**IRDLEHHHHHH	90
Der p 5	**EDKKHDYQNEFDFLLMERIHEQIKKGELALFYLQEQINHFEEKPTKEMKDKIVAE MDTIIAMIDGVRGVLDRLMQRKDLDIFEQYNLEMAKKSGDILERDLKKEEARVK** KIEVLEHHHHH	91
Der p 7	**MDPIHYDKITEEINKAVDEAVAAIEKSETFDPMKVPDHSDKFERHIGIIDLKGELD MRNIQVRGLKQMKRVGDANVKSEDGVVKAHLLVGVHDDVVSMEYDLAYKLGD LHPNTHVISDIQDFVVELSLEVSEEGNMTLTSFEVR**QFANVVNHIGGLSILDPIFAVLS DVLTAIFQDTVRAEMTK**VLAPAFKKELERNNQLEHHHHHH**	81
Der p 10	**MEAIK**NK**MQAMKLEKDNAIDRAEIAEQKARDANLRAEKSEEEVR**ALQK**KIQQIEN ELDQVQEQLSAANTKLEEKEKALQTAEGDVAALNRRIQLIEEDLERSEER**LK**IATA KLEEASQSADESER**MRK**MLEHRSITDEERMEGLENQLK**EAR**MMAEDADRKYDEV ARKLAMVEADLERAEER**AETGESK**IVELEEELRVVGNNLKSLEVSEEKAQQREEA HEQQIRIMTTKLKEAEARAEFAERS**VQKLQK**EVGRLEDELVHEKEK**YKSISDELDQ TFAELTGYLEHHHHHH	83
Der p 13	**ASIEGKYKLEKSEKFDEFLDKLGVGFMVK**TAAK**TLKPTFEVAKENDQYVFRSLST FKNTEIKFKLGEEFEEDRADGK**RVKTVINK**DGDNKFVQTQFGDKEVKIVREFNGD EVVVTASCDGVTSVR**TYKRILEHHHHHH	82
Der p 20	**MVDPATLSKLEAGFQKLQNAQDCHSLLKKYLTRDVFDQLKNKK**TDMGATLLDVI QSGVENLDSGVGIYAPDAQSYK**TFAALFDPIIDDYHKGFKPTDKHPKTDFGNIENFV NVDPKNEYVLSTR**VR**CGRSLNGYPFNPMLTEAQYKEMETKVKGQLATFEGELKG TYYPLLGMDKATQQQLIDDHFLFKEGDRFLQAANACRYWPVGRGIFHNDKKTFL MWVNEEDHLRIISMQKGGDLKEVYGRLVKAVKHIEQKIPFSRDDRLGFLTFCPTN LGTTIRASVHIKLPKLAADRKKLEEVAGRYNLQVRGTAGEHTESVGGIYDISNKR RMGLTEYQAVKEMQDGILELIKMEK**SMHHHHHH	88
Der p 21	**MFIVGDKK**EDEWR**MAFDRLMMEELETKIDQVEKGLLHLSEQYK**ELEKTKSK**ELK EQILRELTIGENFMK**GALK**FFEMEAKRTDLNMFERYNYEFALESIKLLIKKLDELA KK**VKAVNPDEYYLEHHHHHH	73
Der p 23	**MANDNDDDPTTTVHPTTTEQPDDKFECPSRFGYFADPKDPHKFYICSNWEAVHKD CPGNTR**WNEDEETCTELHHHHHH	77
Der f allergens		
Der f 2	**MDQVDVKDCANNEIKKVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTK**TAK**IEI KASLDGLEIDVPGIDTNACHFMKCPLVKGQQQYDIKYTWNVPK**IAPK**SENVVVTVK LIGDNGVLACAIATHGKIRDHHHHHH**	94
Der f 5	MEPK**KHDYQNEFDFLLMQRIHEQMRKGEEALLHLQHQINTFEENPTKEMKEQIL GEMDTIIALIDGVRGVLNR**LMK**RTDLDIFERYNVEIALKSNEILER**DLKKEEQRVK**K IEVHHHHHH**	85
Der f 20	**MVDQATLSKLEAGFQKLQNAQDCHSLLK**KYLTR**DVLDQLK**TK**KTDMGATLLDVI QSGVENLDSGVGIYAPDAQSYKTFAALFDPIIDDYHKGFKPTDKHPQTDFGNIEHF VNVDPKNEYVISTR**VRCGRSLK**GYPFNPMLTEAQYKEMETKVKGQLATFEGELK GTYYPLLGMDK**ATQQK**LIDDHFLFK**EGDR**FLQAANACRYWPVGRGIFHNDKKTF LMWVNEEDHLRIISMQKGGDLKEVFGR**LVKAVKHIEQKIPFSR**DDRLGYLTFCPTN LGTTIR**ASVHIK**LPK**LAADRK**KLEEVAARYNLQVRGTAGEHTESVGGIYDISNK**RR MGLTEYQAVKEMQDGIIELIK**MEKSLHHHHHH	82
Der f 21	**MFIVDVDTEDKWRNAFDHMLMEEFEEKMDQIEHGLLMLSEQYKELEK**TKSKELK EQILRELTIAENYLR**GALK**FMQQEAKRTDLNMFERYNFETAVSTIEILVKDLAELA KKVKAVKSDDHHHHHH**	91

Amino acids covered by matching peptides are indicated in bold and blue.

Appendix 15: Characterization of study subgroups used for molecular sensitization profiling

	SG1	SG2	SG3	SG4	SG5	P
Characteristics	Rhinitis-only	Asthma	non-AD [a]	AD high sIgE [b]	AD low sIgE [b]	
Phenotype	AR+ AA- AD+/-	AR+/- AA+ AD+/-	AR+/- AA+/- AD-	AR+/- AA+/- AD+		
N	85	91	110	98	87	
Sex (m / w)	34 / 51	33 / 58	43 / 67	71 / 27	40 / 47	< 0.0001
Age [years] mean (min-max)	41 (19–87)	44 (18–80)	47 (18-87)	44 (23–85)	42 (23–86)	0.3349
Total serum IgE mean, IU/ml (95% CI)	368.7 (± 115.9)	700.5 (± 355.9)	288.5 (± 77.4)	5639.7 (± 2242.8)	1520.1 (± 385.3)	< 0.0001
Total serum IgE median, IU/ml (IQR)	190.6 (88.1–461.8)	231.1 (120.5–719.3)	200.8 (88.3–389.9)	3993.5 (2236.8–10400)	865.0 (212–2573.5)	
Specific IgE concentrations [kU/l]						
sIgE Der p extract median, kU/l (IQR)	7.3 (3.4–39.0)	11.6 (3.0–39.0)	7.0 (2.0–20.0)	344.8 (203.5–822.5)	11.9 (2.3–25.3)	< 0.0001
sIgE Der f extract median, kU/l (IQR)	7.4 (2.8–29.9)	14.1 (4.7–41.4)	8.2 (2.3–25.8)	N/A	N/A	0.1931
Allergic diseases						
Rhino-conjunctivitis Yes (%)	85 (100%)	72 (79%)	86 (78%)	21 (57%)	11 (31%)	< 0.0001
Allergic asthma Yes (%)	0 (0%)	91 (100%)	48 (44%)	25 (68%)	12 (34%)	< 0.0001
Atopic dermatitis Yes (%)	21 (25%)	36 (40%)	0 (0%)	37 (100%)	35 (100%)	< 0.0001
Hay fever Yes (%)	51 (60%)	72 (79%)	67 (61%)	32 (86%)	30 (86%)	0.0003
Animal dander allergy Yes (%)	33 (39%)	47 (52%)	37 (66%)	23 (62%)	17 (49%)	0.0106
Food allergy Yes (%)	42 (49%)	~~44~~ (48%)	45 (41%)	26 (70%)	20 (57%)	0.0328
Crustaceae allergy Yes (%)	4 (5%)	8 (9%)	7 (6%)	7 (19%)	3 (9%)	0.1054

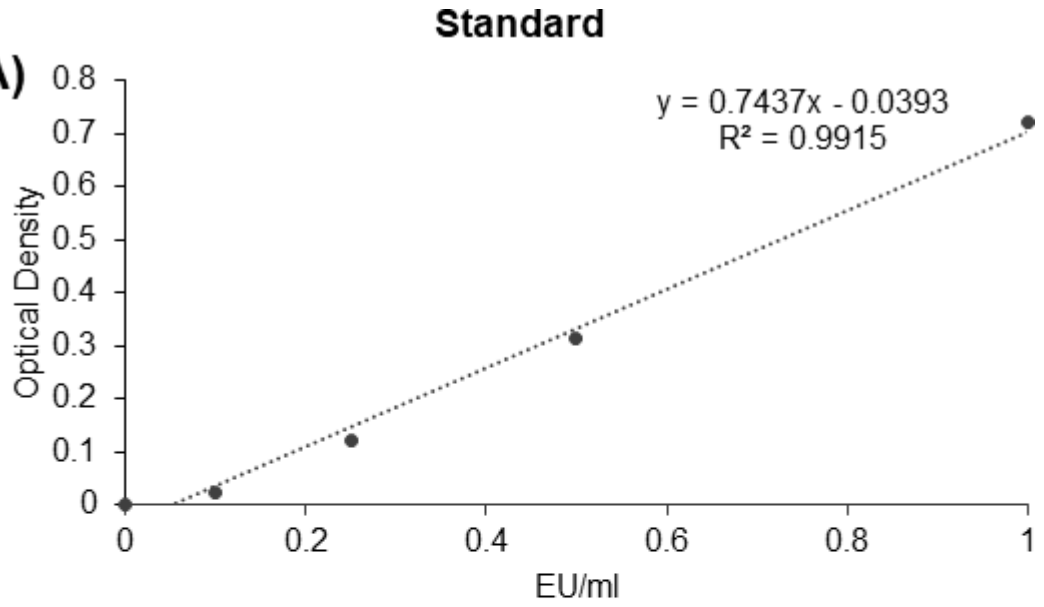
Comparisons between predominant airway and predominant AD patients by Chi-square (prevalences) or Kruskal-Wallis (metric variables) tests. IQR = interquartile range

[a] SG1–SG3 are derived from patients with predominant airway symptoms (SG1 + SG2). Patients in SG3 had no medical history of AD and stated in the questionnaire that they had never suffered from AD.

[b] SG4–5 are derived from patients with predominant atopic dermatitis. Only 37 (SG4) / 35 (SG5) patients completed the *post hoc* questionnaire. All patients suffered from AD as assessed by trained dermatologists.

Appendix 16: Results of Limulus amebocyte lysate assay for endotoxin quantification

(A)



(B)

Allergen	Endotoxin content @ 0.1mg/ml
Der p 5	1.3 EU/ml
Der p 10	9.1 EU/ml
Der p 20	0.4 EU/ml[a]
Der p 21	1.1 EU/ml

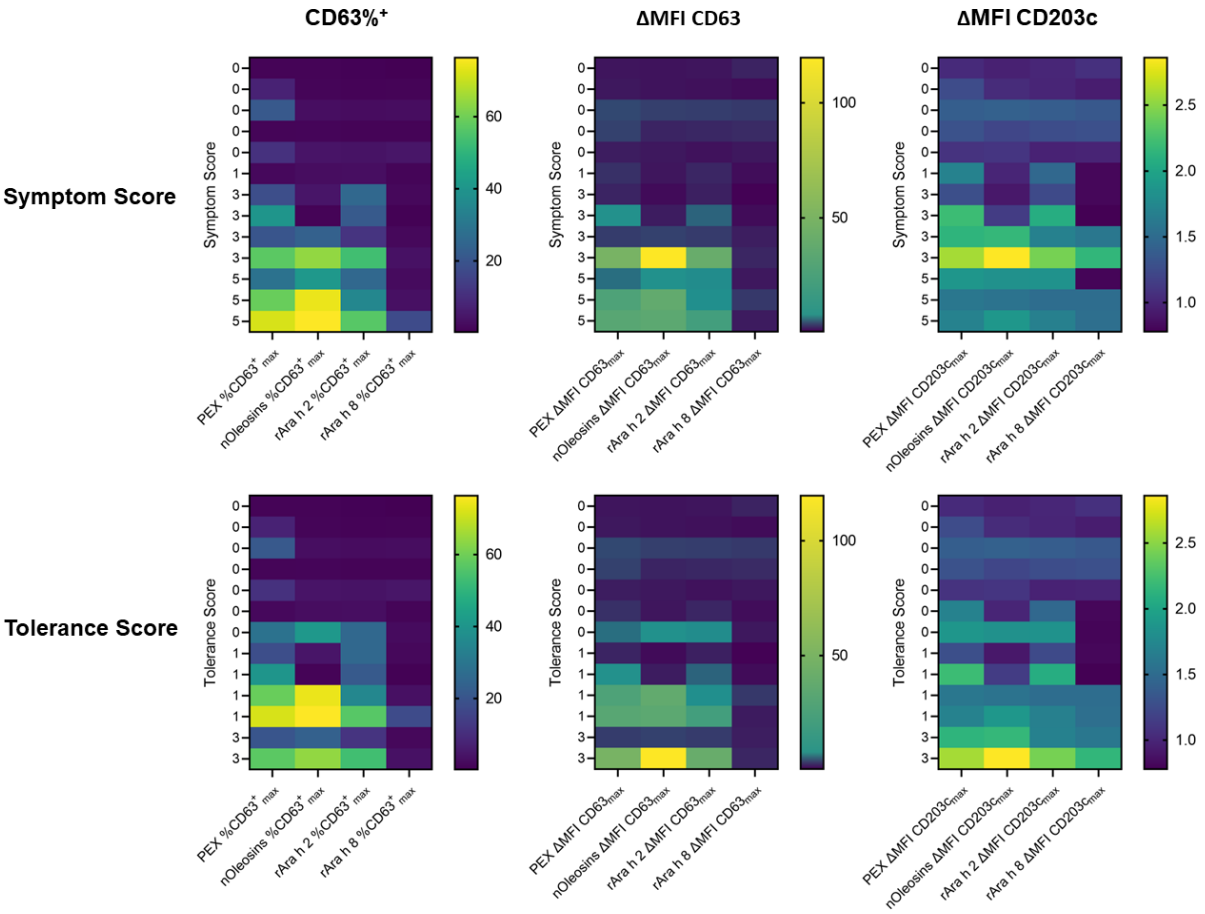
[a] Determined externally at MHH

Detailed results of LAL assay used for determining endotoxin content in the allergen samples for cell culture experiments.

A: Generated endotoxin concentration standard curve of the chromogenic assay measured at 405 nm

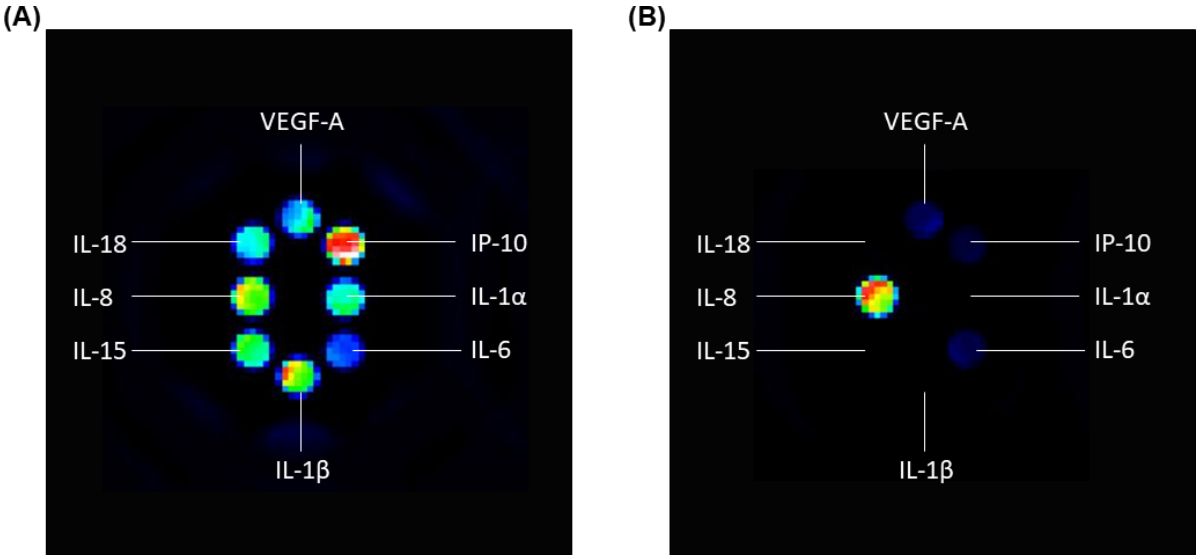
B: Calculated endotoxin contents of the allergens used for stimulation experiments at a concentration of 0.1 mg/ml. The endotoxin content of Der p 20 was determined externally at MHH.

Appendix 17: Heat map of BAT parameters and isolated symptom and tolerance scores



Maximum percentage of activated basophils observed during BAT for each stimulus and study participant or maximum fold change of CD63 or CD203c MFI compared to PBS for each stimulus and study participant using isolated symptom score and tolerance score each.

Appendix 18: Signals of electrochemiluminescence-based 8-plex detection assay after stimulation of human bronchial epithelial cells.

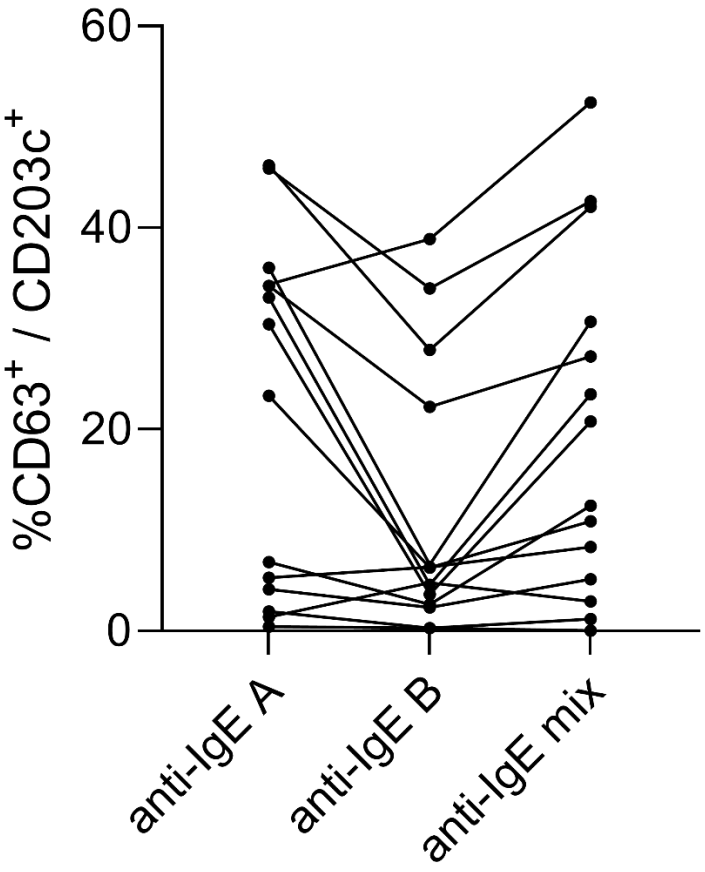


Recorded image by the MSD Quickplex SQ 120 for the 8-plex assay used to measure the concentrations of IL-1α, IL-1β, IL-6, IL-8, IL-15, IL-18, IP-10 and VEGF-A simultaneously after stimulating human bronchial epithelial cells. Clear division of the spots demonstrated that no overexposure occurred for IL-6 and IL-8 despite the high concentrations measured in the assay.

A: Highest standard concentration

B: Stimulation with 200 µg/ml of Der p 21

Appendix 19: Basophil activation by different anti-IgE antibodies as positive controls.



Comparison of individual basophil activation against 2 different anti-human IgE antibodies and a mix thereof, all at a final concentration of 0.001 mg/ml. Both antibodies are directed against the ε-chain of IgE according to the manufacturers.

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