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MOLECULAR APPROACHES FOR CHARACTERIZATION, DIAGNOSIS AND THERAPY OF ALLERGY TO PETS

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Molecular approaches for characterization, diagnosis and therapy of allergy to pets

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To all my fur babies, past, present and future

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'

Isaac Asimov

ABSTRACT

Allergy to furred animals is a common affliction, where up to 20% of the population in affluent countries are sensitized to one or more pet allergens. Several causative allergens from an array of species have been identified, but there are still gaps in our knowledge. This complicates diagnosis and treatment of allergy to pets. Conventional methods rely on naturally derived allergen extracts, with poor characterization and lack of broadly accepted standards. Due to the nature of the extracts, some allergens could be missing or present in clinically non-significant concentrations. The aim of the thesis is to improve diagnostics by characterizing extracts and evaluating component based microarrays to conventional methods. Further, to identify new allergens as well as describe previously only partially characterized allergens, their distribution and properties.

Paper I compares two multiplex microarray assays in allergy diagnostics to the standard methods ImmunoCAP and skin prick test with doctor's diagnosis in a cohort of children with persistent asthma (n=71). The results showed that 75% of all children were sensitized to at least one allergen and that nearly half was multisensitized to three or more allergens. The accuracy of the methods was comparable, they all had a pair-wise concordance of 90% and above. However, the microarray assays contributed with positive IgE responses to new allergens not described in the diagnosis in almost half the patients (47%). The added value of microarray analysis in multisensitized patients is not to belittle.
In **paper II**, an analysis of the content and composition of dog dander extracts for skin prick test is conducted. Results from five commercial manufacturers display a large variation, not only between the different suppliers, but also between batches albeit to a lesser extent. Anatomical location of the source material also differs, hair, dander and epithelia was analyzed. Two of the allergens were detected only at low amounts and patient sera sensitized to these failed to induce an allergic response to the extracts in a basophil activation test. Moreover, a population of dogs was investigated in regard to their allergen profile, also resulting in a substantial divergence between individuals. The heterogeneity of the extracts calls for better content characterization and standardization. Substandard quality could render flawed diagnosis and jeopardize patient safety.
Paper III characterizes sensitization to horse allergens, where several allergens are only described partially, or analysis of IgE binding performed in a small cohort. We present sensitization data for four horse allergens including a novel protein, designated Equ c 7, with a prevalence of 38%. Equ c 7 is a homologue to the cat allergen Fel d 1, suggesting possible cross-reactivity. Further, we describe two forms of full-length Equ c 2 and the potential roles of isoforms. Here we contribute to the panel of horse allergens. Allergy to horse is less investigated than to other pets and likely more allergens await discovery.
Aerodynamic properties of allergens are investigated in **paper IV**, where three dog allergens were sampled, however, only two could be detected. Particles associated with airborne allergen differed in size. Can f 1 was only detected on particles larger than 2.8 µm, diversely from Can f 4 which was found on particles of all sampled sizes >8.3-<0.34 µm. Smaller particles reach the small airways and are causative of inflammation and airway responsiveness. Different sampling methods also yielded different proportions of the allergens, indicating that they possess different airborne properties.

To summarize, furred animals produce a wide array of allergens and there is a large individual variation in expression levels/profiles. This is mirrored in the extracts used for diagnosing allergy, where poor quality counteracts correct diagnosis. Characterization of novel allergens and production of recombinant equivalents could improve the accuracy of diagnostics and therapy.

LIST OF SCIENTIFIC PAPERS

I. Annica Önell*, Anna Whiteman*, Björn Nordlund, Francesca Baldracchini, Giorgio Mazzoleni, Gunilla Hedlin, Hans Grönlund & Jon R. Konradsen
Allergy testing in children with persistent asthma: comparison of four diagnostic methods
Allergy, 2017; 72: 590–597
*Shared first authorship
Note: Anna Wintersand changed name from Whiteman

II. Anna Wintersand, Klara Asplund, Jonas Binnmyr, Erik Holmgren, Ola B. Nilsson, Guro Gafvelin och Hans Grönlund
Allergens in dog extracts: Implication for diagnosis and treatment
Allergy, 2019;74:1472–1479

III. Jonas Binnmyr*, Anna Wintersand*, Erik Holmgren, Ola B. Nilsson, Mattias Bronge, Guro Gafvelin och Hans Grönlund
IgE profiling using a panel of horse allergens, including the novel allergen Equ c 7 and full length Equ c 2
Manuscript
*Shared first authorship

IV. Anna Wintersand, Malin Alsved, Jonas Jakobsson, Sasan Sadrizadeh, Hans Grönlund, Jakob Löndahl och Guro Gafvelin
Airborne allergens from dogs - quantitation and particle size
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AIT	Allergen specific immunotherapy
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cell
BCR	B cell receptor
CD	Cluster of differentiation
DAMPs	Damage-associated molecular patterns
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen binding
Fc	Fragment crystallizable
HDE	Horse dander extract
HDM	House dust mite
HLA	Human leukocyte antigen
IEC	Ion exchange chromatography
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILIT	Intralymphatic immunotherapy
ILC2	Innate lymphoid cells type 2
IMAC	Metal chelate affinity chromatography
MHC	Major histocompatibility complex
NK cell	Natural killer cell
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cells
PSA	Prostate-specific antigen
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SLIT	Sublingual immunotherapy
SCIT	Subcutaneous immunotherapy
TCR	T cell receptor
T_H	T helper cell

1 INTRODUCTION

As living beings, we are constantly under attack from pathogens, which require our physiology to reproduce and thrive. The downside for us is that our bodies are damaged in that process, sometimes unrepairable or worse even fatally. Thus a war is constantly being waged between us and invasive microorganisms. To that end, the immune system has evolved in an arms race, to help us gain the upper hand in survival. Unfortunately, sometimes the immune system mistakes a harmless object for a pathogen and responds accordingly. This may result in causing harm instead of protecting the body, manifesting diseases like autoimmunity and allergy. This thesis contributes with new insights regarding the allergens causing allergic disease to pets, their variability, properties and distribution, to improve diagnosis and treatment of allergy.

1.1 THE IMMUNE SYSTEM

1.1.1 The innate immune system

The immune system constitutes two major parts, the innate and the adaptive immune system. The innate immune system, which is present at birth, includes the first line of defense with physical outer barriers such as the skin, mucosa and epithelial layers. Their role is to prevent entry into the body, but if damaged or compromised pathogens might penetrate. When that occurs, antimicrobial proteins, the complement system and innate immune cells are circulating to identify and neutralize the threat. The innate immune system is non-specific but responds quickly to pathogens or cell damage using pathogen-associated molecular patterns respectively damage-associated molecular patterns (PAMPs/DAMPs) (1). These receptors have a limited diversity, about 100 receptors have the ability to recognize around 1000 antigens (2). But the antigens identified represent commonly distributed preserved essential features of pathogens, such as lipopolysaccharide of bacterial cell walls or double stranded RNA, frequent in viruses but foreign in humans. The PAMPs and DAMPs are found on cells associated with the innate immune response such as neutrophils, macrophages or NK-cells, but also on epithelial cells in the barriers. When a pathogen is identified, these cells try to eliminate the microorganism by phagocytosis, opsonization or cytotoxicity also causing inflammation (3). Secreting inflammatory mediators as chemokines and cytokines also helps signal to attract more immune cells to the site of infection and to appropriately activate them. Specialized antigen presenting cells (APCs) also express PAMP/DAMP receptors. In this way they constitute a bridge between the innate and the adaptive arm of the immune system, and dendritic cells for instance, have the ability to activate the adaptive immune system to get a tailored, more persistent response to the pathogen.

1.1.2 The adaptive immune system

The adaptive immunity takes longer to engage, but carries substantially more specificity. It also harbors a memory which increases the efficiency towards the same antigen following

repeating encounters (4). However, more recent research has shown an advanced interplay between the innate and the adaptive systems, where some cells perform functions or share characteristics attributed to both systems (5-8). The adaptive immune system requires multiple activation signals, including co-receptors and cytokines to upregulate and mature into effector cells. Innate immune cells engage and activate the adaptive cells, namely lymphocytes during an infection. Lymphocytes comprises of T and B cells, these cells have a much broader repertoire of antigen recognition through the T or B cell receptors (TCR/BCR), compared to the innate cells. However, each individual cell carries only one specificity, determined through hypervariable regions and somatic gene rearrangement. These features allow a vast array of possible combinations and specificities (9).

1.1.3 T cells

T cells are lymphocytes that are specialized for specific antigen recognition through the TCR and responsible for cell mediated immunity. All T cells display the surface marker CD3 (cluster of differentiation), a co-receptor in conjunction with the TCR and ζ-chain forming the TCR complex. T cells are classified in two major groups depending on the presence of a second surface co-receptor, CD4 or CD8, giving them different capabilities and function. The CD8+ cells, or cytotoxic T cells, recognize major histocompatibility complex (MHC) class I molecules presented on all of the own body's cells when infected with an intracellular pathogen. The MHC complexes are encoded in the human leukocyte antigen (HLA) genes, and are like the TCR/BCR, highly diverse. Together they represent most polymorphic genes in the genome, to provide a broad repertoire of specificity. When activated the CD8+ T cell kills off the infected cell, mainly through cell lysing mechanisms by releasing their granules containing perforin and granzymes.

CD4+ T cells are called T helper cells due to their ability to activate and facilitate differentiation of other immune cells. The CD4+ T cell recognizes antigens presented on the MHC class II molecule of antigen presenting cells, which are extracellular foreign antigens digested and displayed as peptides on the surface of the APC. The activated CD4+ cell differentiate into an effector cell which produces cytokines to recruit and activate different target cells depending on the present microbe (2). To that end different subpopulations of CD4+ cells have evolved. T_H1 type cells are characteristic of combating viruses and intracellular pathogens, macrophages have been shown to induce this pathway (10) as well as dendritic cells (11) through interleukin (IL)-12 and interferon gamma (IFNγ). The T_H1 cells in turn produce more IFNγ and express the CD-40 ligand to enhance macrophages' ability to kill ingested microorganisms. The T_H2 pathway is initiated by IL-4 from mast cells or epithelial cells, in response to extracellular parasites (12). The typical cytokines produced by T_H2 are IL-4 to continue the T_H2 differentiation, IL-5 to recruit eosinophils for killing parasites and IL-13 to increase mucus secretion and contractions of smooth muscle (13). These cytokines also promote the alternative pathway of macrophages, to produce growth factors and stimulate fibrosis (14). T_H17 cells differentiation occurs in response to bacterial or fungal infections, where dendritic cells secrete IL-1, IL-6, IL-23 and TGF-β (15). These cells

produce IL-17 and IL-22 (16) recruiting neutrophils and driving inflammation as well as repairing epithelia. Recent research has also linked them with the defense against intracellular bacteria (17). Further, additional subsets have been identified, e.g. T_H22, which is a pro-inflammatory lineage of T cells infiltrating the epidermis, producing IL-22 and TNF-α (18) and shown to assist in cutaneous immunity (19). T_H9 is another subset of T helper cells, which have been implicated in contributing to allergic responses by secretion of IL-9. The cytokine, together with IL-4 stimulate B cells to produce IgE (20) and are necessary for mast cell amassment during inflammation (21).

1.1.4 B cells

B cells belong to the second class of lymphocytes, responsible for the humoral branch of the adaptive immune system, the major effector function being antibody production. In contrast to T cells, the antigen receptor immunoglobulin (Ig) can exist either as a membrane receptor (BCR) or in soluble form as an antibody. Both types share the same specificity on an individual cell. Another distinction between T and B cell receptors is that while the TCR recognizes peptides and linear epitopes, the immunoglobulin bind to the whole antigen and thus conformational epitopes (9). Two membrane bound Ig classes are expressed on naïve B-cells, IgM and IgD. These receptors recognize and bind antigen, but need a second stimulus to cause activation. The mode of activation can either be T cell dependent if the antigen in question is a protein, or T cell independent during polysaccharide or lipid encounters where the repetitive antigens have the ability to cross-link adjacent Ig molecules or bind innate toll-like receptors on the B-cell. The activated B cell undergoes clonal expansion and differentiation into either antibody producing plasma cells or memory B cells. The T cell dependent pathway also have the ability to induce class switching and affinity maturation to further tailor the response (22). The remaining three Ig classes are a result of isotype switching depending on the type of T cell help and the milieu.

1.1.5 Antibodies

Antibodies, the secreted form of immunoglobulin, consist of two variable regions, (fragment antigen binding, Fab) and a constant one (fragment crystallizable, Fc). The Fab portion of the antibody is highly diverse and can bind a vast array of antigens. Due to the rearrangement of the V(D)J domains of the genome this polymorphism is possible. Further specialization occurs by class-switch recombination and somatic hypermutation, where AID plays a vital role. AID is a single stranded DNA that causes mismatched DNA by deamination. When DNA repair systems removes the mismatch, mutations arises causing further diversity (23). The Fc region determines the isotype of the antibody and thus the function. The region bind receptors on cell surfaces eliciting a cellular response causing either up- or downregulation. Hence, Fc receptor signaling determines the immune response from the affected cells (24).

The Ig isotype IgM is either expressed as a surface bound monomer or secreted as a pentamer during infections where it opsonizes pathogens (25). IgD can also be secreted as a soluble

form in blood serum. It's role in immune responses is still unclear, but it is suggested to be involved in maintaining homeostasis (26). IgG can trigger up-regulation and activation of myeloid cells and some endothelial cells, facilitating phagocytosis, endocytosis and antibody-dependent cellular cytotoxicity (ADCC). IgG can also opsonize pathogens to be recognized by NK-cells and destroyed. However, IgG can also bind to the inhibitory receptor FcγRIIb expressed on mast cells, basophils and monocytes to initiate a down-regulation of the cell (27). IgE is engaged in the defense against helminth infections and are pathologically associated with allergic diseases and asthma (28). IgE binds to the high affinity receptor FcεRI, present on mast cells, basophils, eosinophils and DC. Mast cells, the main expresser of FcεRI, become coated with antibodies when sensitized. Upon antigen interaction the cell degranulates releasing mediators toxic to parasites, causing inflammation. The antibody is also involved in IgE dependent phagocytosis as well as B-cell maturation and survival (29). Lastly, IgA is found in serum, but predominantly on mucosal surfaces and in secretions. IgA can act both pro-inflammatory, when binding an antigen forming an immune complex causing cytokine release, cytotoxicity and phagocytosis, and anti-inflammatory (30). The IgA immune complex can also pass though membranes via the polymeric transmembrane receptor pIgR, eliminating the pathogen from circulation and function as a way of clearing infections (31, 32).

1.1.6 Immune dysregulation

The immune system is highly effective in eradicating pathogens and keeping us healthy. The aptitude however comes at a price. Sometimes the immune system recognizes harmless molecules as threats or worse, the own self as foreign. Hereditary defects in the genome or mutations can also cause immune deficiencies. For example, defective T cell development can cause severe combined immunodeficiency (SCID), the individuals lacking functional T cells and generally having a low lymphocyte population. The condition causes critical infections and predisposes to early death (33). Loss of CD40 or CD40L due to mutations resulting in an inability of B-cells to undergo class-switch, causes hyper-IgM syndrome as well as a shortage of the other antibody isotypes. This manifests as recurrent bacterial infections, autoimmunity and malignancy (34). Hyper-IgE syndrome (HIES) patients have a defect in the transcription factor STAT3, critical for differentiation of T cells into T_H17 cells and activation of innate lymphoid cells type 3 (ILC3). Absence of these cells causes a multitude of bacterial and fungal skin and pulmonary infections including eczema. The reason for the elevated IgE levels is not fully understood, however these patients lack a pronounced T_H2 phenotype and the IgE is not allergen-specific, resulting in less allergic disease compared to atopic patients (35). Immune deficiencies can also be acquired from pathogen exposure. The retrovirus HIV causes acquired immune deficiency syndrome (AIDS), characterized by a depletion of CD4+ cells, eventually causing recurrent infections and malignancies, resulting in death if untreated (36).

During lymphocyte development in the thymus and bone marrow, the cells undergo several selection steps to ensure that the naïve lymphocytes have the ability to identify foreign

antigens meanwhile discriminating self-antigens, thus inducing self-tolerance. When the immune system fails to distinguish one's own antigens from those foreign, the result can lead to severe tissue damage manifesting as autoimmunity (37). T cell mediated autoimmunity includes for example diabetes type I where T_H1 cells react towards pancreatic islet antigens on β-cells, destroying these resulting in lack of insulin production. Multiple sclerosis is another severe form of autoimmunity, where the autologous T cells attack the myelin sheaths of the central nervous system, creating plaques causing the myelin to dissolve and the nerve signals to diminish. Common symptoms are muscle weakness, ataxia, blindness and ultimately paralysis. Several autoantigens have been suggested as targets, myelin oligodendrocyte glycoprotein (MOG) being one of the first identified, but questioned more recently. However, advances are being made in detecting novel autoantigens and more sensitive methods are under development (38).

Allergic disease is also a consequence of a dysfunctional immune response, in this instance hypersensitivity. The immune system mistakes a harmless molecule, for instance pollen or food, for a pathogenic one and responds accordingly causing harm to the body in the process.

1.2 ALLERGY

1.2.1 Hypersensitivity

Clemens von Pirquet first defined allergy as the odd disposition of some people to respond with hypersensitive symptoms to specific substances in 1906 (39). Gell and Coombs, two British immunologists suggested in 1963 that there were four types of allergic reactions that was the result of hypersensitivity (40). They proposed a classification, despite lack of knowledge on immune mechanisms like T cell functions and cytokine repertoire at the time drafted, which was adopted for decades. The classical classification was defined as:

Type I – Acute type, IgE mediated. When the antigen is recognized and bound by IgE antibodies on mast cells, IgE crosslinks causing degranulation and release of mediators instantaneously.

Type II – Semi-delayed, antibody mediated. Autoreactive IgG recognizing host cell and opsonizes it. Cell is lysed by complement or antibody-dependent cellular cytotoxicity by NK cells.

Type III – CIC mediated. Immune complexes are formed and deposited in tissues from abundant antigen bound to antibodies and complement. Causing inflammation and tissue damage.

Type IV – Delayed-type, cell mediated. The antigen is presented by an APC to a T cell, activation occurs and cytokines are released.

However, the validity for all types of hypersensitivity reactions have been debated as well as the lack of a more complex accurate description (41). A new revised classification was

proposed by Johansson et al, accepted by EEACI in 2001 (42) and by the World Allergy Organization in 2003 (43). The consensus of the current hypersensitivity classification is as follows:

Non-allergic hypersensitivity

Allergic hypersensitivity
- IgE-mediated
  - Atopic
  - Non-atopic, e.g. insect sting, helminth infection, pharmaceutical,
- Non IgE-mediated
  - T cell-mediated, e.g. celiac disease
  - Eosinophilic, e.g. eosinophilic esophagitis
  - IgG-mediated, e.g. allergic alveolitis
  - Other

1.2.2 IgE-mediated allergy

We are constantly exposed to potential allergens from the environment. People with IgE-mediated allergy respond to these allergens by producing IgE antibodies, referred to as sensitization. However, some people develop allergic disease and other do not. Hereditary predisposition to develop allergies is described as atopy (from Greek atopos, meaning out of place) (44). Having atopic parents increases the likelihood of the child to develop allergic disease (45, 46). Further, the gender of the child may influence the parental contribution to atopy. A higher risk of girls developing allergy has been shown to depend of maternal allergy and the same has been shown for boys and paternal allergy (47). Certain alleles, like a polymorphism of the 17q21 locus, have also been linked with high risk of developing asthma, which could be an explanation for the diverging results of the hygiene hypothesis and pet keeping (48). Mutations in filaggrin functionality also have an effect on risk of sensitization, but the outcome differs depending on the allergen and age of exposure (49). However, several factors influence the risk of developing allergy, besides the genes, and the cause is likely multifactorial (50).

The hygiene hypothesis has long been a favored explanation for increased risk of sensitization. It was coined by Strachan in 1989, who observed that children growing up in larger families had a lower incidence of hay fever and eczema compared to children in smaller families (51). He postulated that the unhygienic contact with siblings or infected mother would explain the difference. Thus early exposure to microorganisms would provide protection against development of allergies and overly clean environments would be disadvantageous. Some later studies have supported the hypothesis (52), while others have shown more conflicting results depending on the pathogen exposure (53), where e.g. RSV infection in infancy seems to increase the risk for allergy (54, 55). However, growing up on a farm decreases the risk of developing allergies according to several studies (56-59). Living with cats and dogs during the first year of life also provide a protective effect against allergic

disease (60-62). The composition and diversity of the gut flora during infancy has proven to impact the risk of sensitization and asthma (63, 64), where T-regulatory cells are important for keeping homeostasis (65). Further, even later in life altered microbiota have been linked with inflammatory disease e.g. rheumatoid arthritis (66). The same is true for the airways, where an abundance of certain strains of Proteobacteria is linked with incidence of asthma (67) and *Moraxella* or *Haemophilus* infiltration associated with neutrophilia (68).

1.2.3 Sensitization

The first time an allergen is encountered in an atopic individual, sensitization occurs without symptoms. The allergen breaches the mucosal layers in the airways, gastrointestinal tract or penetrates disrupted epithelia in the skin, see figure 1. House dust mite (HDM) allergen even have the ability to disrupt tight junctions using protease activity (69). Dendritic cells in the tissue capture and process the allergen, causing the DC to mature and migrate to the regional lymph node. In the lymph node the DC presents the processed allergen peptides to naïve T cells using the MHC class II molecule. If recognized, depending on the milieu and presence of IL-4 from mast cells or epithelial cells, the naïve T cell will differentiate into an effector T_H2 cell. The T_H2 cells start to produce more IL-4 and IL-13, and activate B cells and phagocytes by engaging their CD40 receptor using the CD40 ligand and CD80/86 with CD28 (70). The B cell undergoes class switch and starts producing IgE, and with help of resident T follicular helper cells increases the affinity of the antibodies, making them more potent (71). The T follicular helper cells are also thought to drive inflammation by producing IL-4 (72). The secreted IgE is spread by the blood systemically, and further into tissues where it encounters mast cells, to which it binds using the high affinity receptor FcεRI. The mast cells are now sensitized and ready to combat the allergen during subsequent exposure.

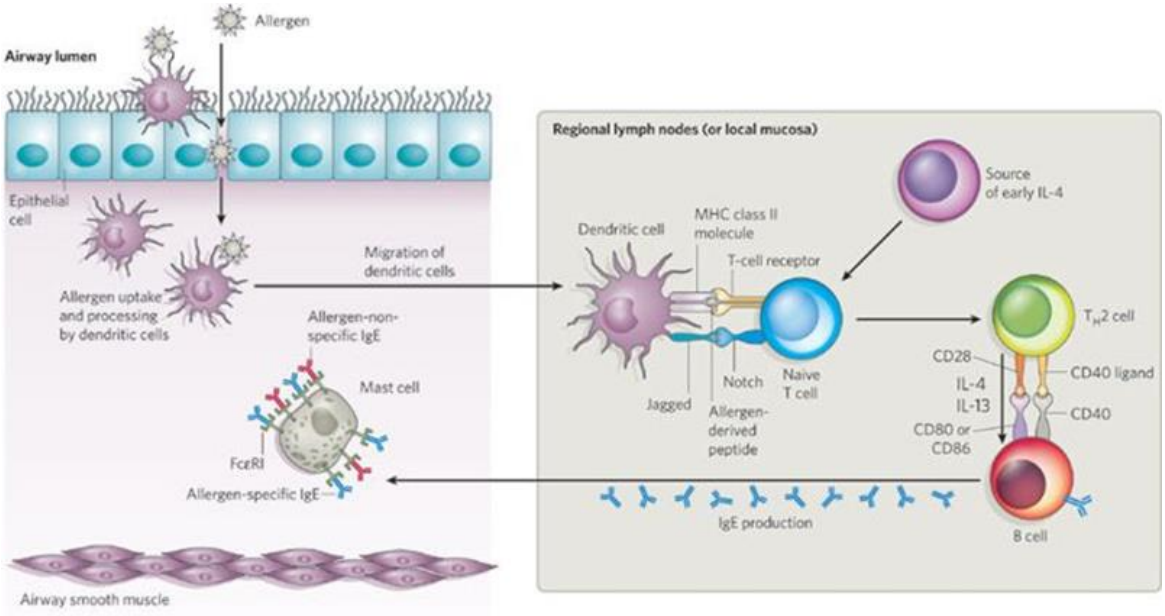

Figure 1: Sensitization to allergens in the airway. Galli, S., Tsai, M. & Piliponsky, A. Nature 454, 445–454 (2008).

1.2.4 Effector response

The following time the allergen is encountered an allergic response initiates. The effector phase can be described as three stages, early, late and chronic phase. A few minutes after the allergen exposure, the early phase occurs when IgE-coated mast cells degranulate upon recognition of the allergen, cross-linking of FcεRI and releasing mediators causing inflammation, see figure 2. Symptoms occur such as asthma due to bronchoconstriction, swelling and redness because of vasodilation and rhinitis from mucus production (73).

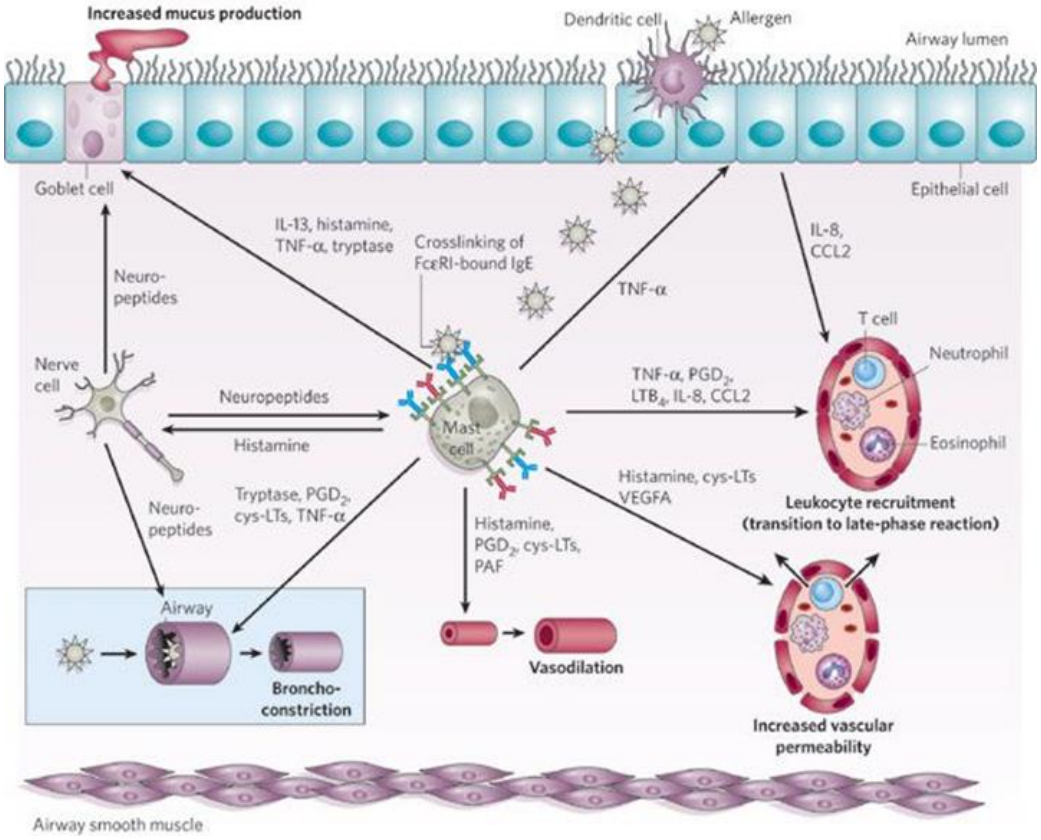

Figure 2: Early phase of allergen-induced airway inflammation. Galli, S., Tsai, M. & Piliponsky, A. Nature 454, 445–454 (2008).

The late phase response occurs hours after the allergen exposure, and is a result of the innate and adaptive immune cells recruited by the chemokines, cytokines and mediators present at the site of inflammation. The innate cells cause damage to the epithelia, neutrophils release elastase causing collagen degradation (74) and eosinophils their major basic protein (MBP) destroying cells (75), see figure 3.

If the inflammation is persistent, long-term damage to the airways through remodeling can occur. The smooth muscle cells in the lungs increase both in numbers and size, decreasing the lumen size, while mucus producing goblet cells become more abundant (76). With constant infiltration of immune cells, cytokines are further injuring the tissue (77). In non-allergic individuals, T regulatory cells contribute to keeping the balance of the immune system and produce IL-10 to inhibit activation of the T_H2 pathway (78).

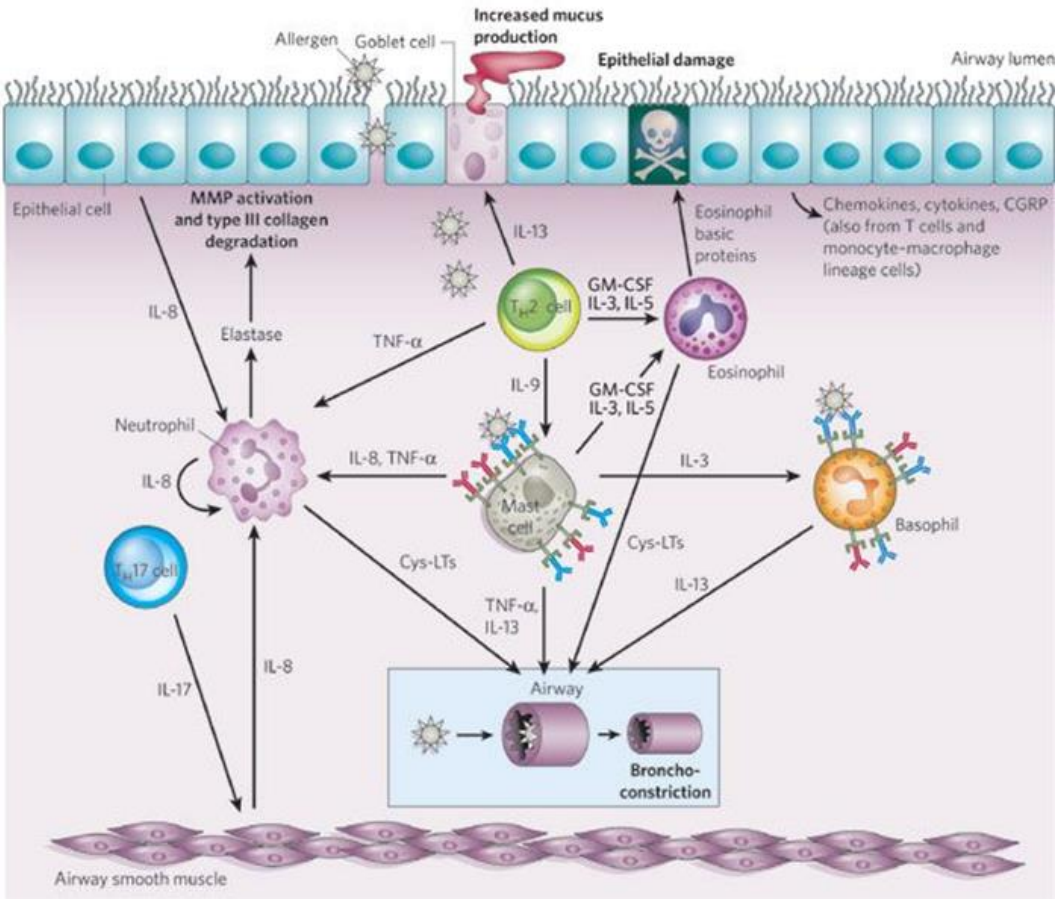

Figure 3: Late phase of allergen-induced airway inflammation. Galli, S., Tsai, M. & Piliponsky, A. Nature 454, 445–454 (2008).

1.2.5 The mucosa in allergy

The mucosal layers on the inside of the body are the surfaces that are directly exposed to pathogens and if breached, the normal entry point for microbes. These layers consist of a mucus secreting epithelium that covers the entire gastrointestinal tract, the respiratory tract, the urogenital tract and the middle ear, forming the largest part of the immune tissues (9). However, these tissues are permeable to allow substances such as nutrients or oxygen to pass, making defense an important feature. In an allergy context, when the body tries to fight off a perceived threat in the form of an allergen, the cells driving inflammation also causes damage in the bronchial tract. Lymphocytes in patients susceptible to allergic disease differentiate into T_H2 cells, that together with innate lymphoid cells type 2 (ILC2) activate and start producing cytokines (79), figure 4. Secretion of IL-13 causes airway remodeling and airway obstruction, features of allergic asthma (80). Recruitment of eosinophils via the cytokine IL-5 further drives inflammation and degranulation causing epithelial damage (81). Production of IL-4 induces naïve T cells to differentiate into T_H2 cells creating a positive feedback loop and more recently has been shown to synergize with IL-3 to further enhance T_H2 promotion (82). In addition, IL-4 has been shown to polarize macrophages into the alternatively activated M2 macrophages through the inflammasome NLRP3 (83), potentially causing fibroblast accumulation and tissue remodeling through TGF-β secretion (84).

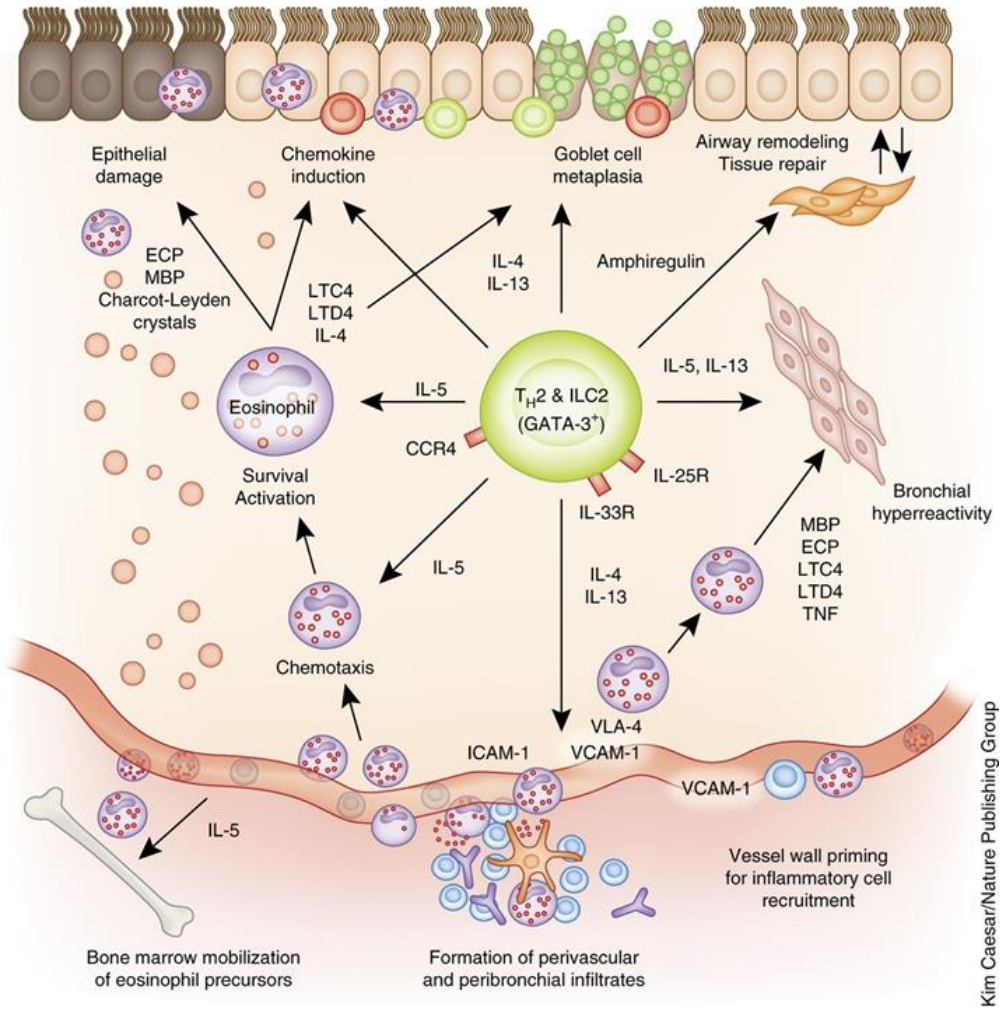

Figure 4. Overview of functions of TH2 cells and ILC2 cells in asthma. (Lambrecht B.N. & Hammad H., Nature Immunology 16, 45–56(2015))

The gut also plays a major role in the development of allergies, studies have shown that diversity of microbiota during infancy affects allergy and asthma outcome (85, 86). Further, impaired IgA ability to bind fecal bacteria predisposes children to develop allergies (87) and consuming breast milk with lower diversity of microbiota could facilitate this development (88). Richness of bacterial flora as well as composition in the saliva also impacts the probability to acquire allergic disease and discussions are ongoing regarding efficacy of probiotic prophylaxis (89).

1.2.6 Mast cells and basophils

Mast cells are found in all tissue vascularized in the body, but more abundantly close to epithelia, airways and the gastrointestinal tract. Diversely, basophils are encountered in circulation and only migrate into tissue during an immunological event (90). However, both cells express the high affinity receptor for IgE, FcεRI. When sensitized, allergen-specific IgE is bound to the receptor coating the cell. Upon allergen encounter, two or more IgE molecules bind the allergen, crosslinking the receptors leading to an aggregation of FcεRI (91). The aggregation activates a downstream process where the protein tyrosine kinases LYN and

FYN initiate a cascade resulting in degranulation, synthesis of pro-inflammatory lipid mediators and activation of transcription factors encoding cytokines, chemokines and growth factors (92). When degranulation occurs, histamine and lipid mediators, among others prostaglandins and leukotrienes, which cause vasodilation and swelling, are released. Further chemokines which induce migration of leukocytes to the site of inflammation, as well as TNF-α causing bronchoconstriction and pro-inflammatory cytokines such as IL-4, IL-5 and IL-13 are secreted (93). FcεRII or CD23 is the low affinity equivalent and can exist both membrane bound or soluble. It has the ability to inhibit IgE production when engaged on B cells (94) and when in soluble form has cytokine-like activity, promoting proliferation and sustaining cell growth (95). FcεRII is expressed on among others T and B cells (96), granulocytes (97) and monocytes (98). A new study has found a cross-talk between mast cells and basophils, where IL-33 produced by mast cells induce basophils to drive allergic inflammation (99).

1.3 ALLERGENS

1.3.1 Structure

Allergens are generally small hydrophilic proteins or glycoproteins derived from the animal, plant or fungi kingdoms. Allergens contain conformational epitopes or in rare cases carbohydrate residues recognized by immunoglobulins (100). Galactose-α-1,3-galactose (α-Gal), a carbohydrate residue with the capacity to induce IgE antibodies are associated to red meat allergy, but sensitization has been linked to tick bites (101). However, the mechanism is still not fully determined. Further, an allergen must have the ability to cross-link IgE on mast cell and basophil surfaces, which requires at least two IgE epitopes. Consequently, many allergens naturally exist as dimers or oligomers to facilitate the immune cell activation capacity (102).

Another inherent ability of allergens is the stability of the protein, especially in regard to food allergens that have to withstand the gastrointestinal tract and proteases (103). The major peanut protein Ara h 1 and the hazelnut allergen Cor a 14 for instance, are able to survive digestion and to elicit severe allergic reactions (104, 105). The capacity to maintain conformation could also serve to slow down digestion by lysosomes after phagocytosis and contribute to a prolonged MHC class II presentation (106, 107). Lately, focus has been shifted to the adherent adjuvant properties of some allergens, such as their function as proteases and thus the ability to affect epithelial cells directly and drive T_H2 inflammation (108).

A large portion of the pet allergens belong to the lipocalin protein family (109), and the similarities within the group could explain the common pattern of multi-sensitization across species (110). The other three major protein families connected with pet sensitization are kallikrein, serum albumins and secretoglobin (111). Most animal allergens studied belong to pet mammals like cat and dog, or those in our proximity e.g. house dust mite. Less is known

about uncommon pets like reptiles or birds, however a toxin has been described for spiders (112).

Allergen nomenclature is derived from the host species name. The Latin name of the Linnean system for dog is *Canis familiaris* and the allergens are thus named Can f and allocated a number in sequence, for instance Can f 1, Can f 2 and so forth. *Equus caballus* is Latin for domestic horse and the nomenclature for these allergens Equ c. The World Health Organization and the International Union of Immunological Societies Allergen Nomenclature Sub-Committee are responsible for recording and classifying characterized allergens (113).

1.3.2 Sensitization to pets

Sensitization to pet allergens increases the risk of developing allergy and asthma (114) and over 50% of studied children admitted to hospital for asthma exacerbations were shown to be sensitized to pet allergens, signifying the connection (115). The prevalence of allergy however differs between geographic locations and populations, where the westernized countries are more prone to disease development (116-118). Further, exposure to urban environments during childhood was connected with a higher risk of sensitization as an adult compared to rural settlement (119). During the last decades the sensitization rate in childhood to inhalant allergens has increased dramatically (120), although in older children in high prevalence areas, asthma development increase seems to come to a halt (121). Studies suggest a sensitization rate of 20% to dogs and 18% to cat in Sweden, but only 6% and 10% respectively in Palermo (116). In the US, a prevalence of 12% has been recorded for both dogs and cats (122). Sensitization to horse has been reported to 5% in one study in Italy (123) and 3% in another (124).

1.3.3 Dog allergens

Regarding dogs, eight allergens have been described to date, Can f 1-8 (Table I). Can f 1 is a major lipocalin allergen 23-25 kDa, found in dander and saliva (125). Approximately 70% of dog allergic individuals are sensitized to Can f 1 (126). Recently, a study revealed that the N and C-terminus of Can f 1 both contained a conformational IgE epitiope, possibly a target for future therapeutics (127). Can f 2 is a minor lipocalin, detectable in saliva, with an IgE reactivity of 23% and a size of 19 kDa (128). Can f 3, is serum albumin, abundant in blood, but can also be found in saliva and skin, possibly leaking through from damaged epithelia or gums. It is a larger protein, 69 kDa and cross-reactive with serum albumins from other mammals (129). Due to this, Can f 3 is less immunogenic with a prevalence of 35% sensitization (130). Can f 4, an 18 kD lipcocalin, was first thought to have a sensitization rate of 35% (131). It was in a later study displaying positive titers of up to 81%, suggesting the classification as a major allergen (132), possibly due conformational stability. Can f 4 also has the ability to form dimers, possibly affecting the allergenicity (133). Can f 5, prostatic kallikrein of 28 k Da, is also a major allergen with prevalence up to 76% among dog sensitized patients (134). Being a homologue to human prostate-specific antigen (PSA), it is

only expressed in male dogs, making avoidance easier (135). Case studies have also confirmed the link between Can f 5 sensitization and allergy to human seminal fluid (136, 137). Another cross-reactive allergen of 27-29 kDa denoted Can f 6, has also been described in both fur and saliva with a sensitization of 38% (138). Can f 7 or Niemann Pick type C2 protein, has been characterized as a 16 kDa protein with an IgE positivity of 10-20% (139). Recently, a novel allergen has been added to the IUIS Allergen database, a 14 kDa cystatin with a prevalence of 13% named Can f 8 (113).

Mentions of hypoallergenic dogs have been frequent in media and popularity of cross-breeds with these claimed traits has risen. However, no scientific data supports the statement regarding hypoallergenic dog breeds. On the contrary, one study describes higher concentrations of Can f 1 in the acclaimed hypoallergenic breeds compared to other breeds (140). Others report no difference in allergen concentration between homes of so-called hypoallergenic dogs compared to control dogs (141). Further, a higher incidence of allergy in children growing up with these claimed hypoallergenic breeds was found. Conversely, a reduced risk of developing allergy was instead correlated with female dogs and keeping two dogs or more (142).

Table I. List of currently known dog and horse allergens, their molecular weight, prevalence of sensitization and biochemical name.

Dog allergens	**Size**	**Prevalence**	**Biochemical name**
Can f 1	23-25 kDa	70%	Lipocalin
Can f 2	19 kDa	23%	Lipocalin
Can f 3	69 kDa	35%	Serum albumin
Can f 4	18 kDa	35-81%	Lipocalin
Can f 5	28 kDa	76%	Kallikrein
Can f 6	27-29 kDa	38%	Lipocalin
Can f 7	16 kDa	10-20%	NPC2
Can f 8	14 kDa	13%	Cystatin
Horse allergens	**Size**	**Prevalence**	**Biochemical name**
Equ c 1	25 kDa	51-70%	Lipocalin
Equ c 2	17 kDa	50%	Lipocalin
Equ c 3	67 kDa	40%	Serum albumin
Equ c 4	17 kDa	77%	Latherin
Equ c 5	"	"	"
Equ c 6	15 kDa	Not reported	Lysozyme

1.3.4 Horse allergens

Six horse allergens have been registered in the IUIS Allergen database (Table I). Equ c 1, the major horse allergen, is a 25 kDa lipocalin protein (143, 144) with a reported IgE prevalence of 51-76% (145, 146). Equ c 2 has previously only been described as two incomplete isoforms of a 17 kDa lipocalin protein. IgE recognition in immunoblot was detected in about 50% of patient samples, however no distinction was made between the different isoforms and only 23 patients were analyzed (147). Equ c 3 is serum albumin and holds a molecular weight

close to other albumins, 67 kDa (148). Sensitization rate reported is approximately 40% (149). Equ c 4, diversely is a latherin of 17k Da, with surfactant properties found in sweat and saliva (150). Equ c 5 was later described as an independent allergen, but found to be identical to Equ c 4 and removed from the IUIS database. Prevalence was only reported in the Equ c 5 paper, 77% of 22 patients (151). Lastly Equ c 6, a lysozyme, was described in a case study and classified both as a contact allergen causing urticaria and a food allergen after ingestion (152).

Similarly to dogs, hypoallergenic horses has been a hot topic. However, in concordance with the results for dogs, no evidence for the hypothesis has been found. Curly horses are classified as a hypoallergenic breed, but had significantly higher levels of horse dander antigens, Equ c 1 and Equ c 4, compared to several other breeds (153). Equ c 4 has a high variation both within and between ten different breeds, where only stallions had significant consistently higher concentration compared to mares and geldings (154).

1.3.5 Cross-reactivity

Due to pet allergens generally belonging to a select few protein families, cross-reactivity is common. Clinically, pet allergic patients often are multi-sensitized, making diagnosis more complicated, with mono-sensitization being as low as 5% (149). To elucidate the primary allergen source in poly-sensitized patients, molecular based allergy diagnostics is a useful tool (155). Two super-families of pet allergens are similar in structure and reported as cross-reactive, lipocalins and serum albumin (156, 157). Lipocalin Can f 6, shares similar tertiary structure and sequence with the cat allergen Fel d 4 (158). Fel d 4 together with horse allergen Equ c1, also inhibit IgE binding to Can f 6 *in vitro*, implying cross-reactivity between the three allergens (138). However, in spite of a low sequence identity, Fel d 4 also displayed cross-reactivity with Can f 2 (159). Dog allergen Can f 4 also cross-reacts with an odorant binding cow allergen (131). Further, Fel d 7, a cat allergen that 46% of cat sensitized individuals react to, was shown to moderately correlate to Can f 1 and inhibit IgE-binding in dog sensitized patients (160). Three peptides from horse serum inhibit IgE and IgG from horse allergic patients to not only horse, but also dog and cat albumin (161). One third of patients sensitized to dog, cat and horse displayed cross-reactivity to all the serum albumins (162). Taken together, patients sensitized to above mentioned protein families should be further investigated, because the assumed primary sensitizing allergen might only be a cross-reactivity, and another allergen actually the principal cause of allergic symptoms.

1.3.6 Distribution

Pet allergens are abundant in homes with resident pets, but can also be found in public places like schools and hospitals, making avoidance measures difficult for allergic individuals (163, 164). A recent study even detected a higher concentration of allergen in day care centers compared to homes (165). Allergen reduction tools have proven some decrease of allergen

concentration and allergic symptoms, including air cleaners and recurrent washing of the animal (166-169), but avoidance if sensitized is recommended.

The aerodynamic properties of allergens varies, where house dust mite allergens group I are carried mainly on particles averaging 20 µm (170). Cat and dog allergen associated particles however, belong to a broader range, where 20% of particles were smaller than 5µm (171). Smaller particles have the capacity to reach further down the airways, increasing the probability of uptake and encountering of mast cells, and sensitization to these have been correlated to inflammation and asthma (172). Comparison of allergens in settled dust in relation to the airborne compartment, showed correlation for homes with dogs, but not in cat households, suggesting these particles have further unknown properties (173). Airborne levels of horse allergen has been shown to disperse quickly, even in the vicinity of stables (174), where concentration was 500-fold lower just outside compared to inside the stable increasing to a 3000-fold difference 12 meters away (175).

1.4 ALLERGY DIAGNOSTICS

1.4.1 Skin prick test

The golden standard of allergy diagnostics is still skin prick test (SPT) and the most common practiced (176). The method is usually performed using naturally derived aqueous extracts acquired from the allergen source (177). The standard procedure is conducted as described (178) by placing a grid on the patient's forearm and adding a small drop of each allergen to be analyzed, as well as a negative (diluent) and a positive control (histamine solution). The skin is then pierced using a lancet. The grid is removed and excess solutions wiped off. After 15 minutes the results is recorded by measuring the diameter of the wheal formed by the local immune reaction. A wheal diameter ≥3 is considered positive. The advantage of using the skin prick test is the cheap cost, it is quick to perform and well proven (179). To consider when using the skin prick test is that the positive control can influence the negative control if in close proximity (180). The physicians interpreting the result differently can also impact diagnosis (181). Further, because the nature of the extracts and lack of a consensus standardization (182), false negative responses can occur if an allergen is lacking from the analytic preparation or not present in adequate amounts to produce a reaction (183).

1.4.2 Serology

Detection of specific IgE antibodies in serum is another well-established method of diagnosing allergy (184). It was introduced in 1967 as a radioallergosorbent test (RAST) by Johansson and Wide (185). Several different methods are in practice today, where the single-plex assays ImmunoCAP and Immulite 2000 are most commonly used (186, 187). The analyzed allergen can either be in an extract or a component, coupled to a solid phase. Sera is added and if allergen-specific IgE is present, it binds to the allergen. Detection is done by a fluorescent anti-human IgE, in proportion to the patient sIgE (188). The advantage of these

assays is that they quantify the sIgE in units per liter (kU/L), the sensitivity and precision is high, it is reproducible and IgG does not interfere with the measurement (189). The downside of the method is again the extracts and their content, or lack thereof. Especially in regard to food allergy where different molecules of the allergen source are connected with severity of symptoms (190). Lack of allergens in the extracts can, as in the skin prick test produce false negative results (191). Recently, multiplex analyses have been developed. These assays test for a large panel of common allergens simultaneously and use lesser sera per allergen tested (192). Another advantage is that they are often component resolved, which improves the accuracy (193). Further, in multi-sensitized individuals, deducing cross-reactivities and detecting the primary sensitization profile is less complicated (194).

1.4.3 Extracts and components

As mentioned, the extract based assays struggle with sensitivity, due to the varying composition of extracts (195). Preparation of the raw material into aqueous extracts also causes inconsistencies. Some methods destroy conformational epitopes and, additionally, selection of the raw material can impact the composition of allergens (177). Even different batches from the same manufacturer differ in patient SPT responses (196). The varying protein content and composition of SPT solutions have been demonstrated for dog dander and the possible impact on the accuracy of diagnosing allergy to dogs (197). Further, due to the allergen variability of dogs and lack of low molecular weight allergens in extracts, inconsistencies cause difficulties in diagnosis (198). The same phenomenon has been shown for fish extracts, where different manufacturers varied 10-fold in content, (199) and in extracts for cockroach allergy diagnostics (200). Molecular components provides a solution to the inconsistencies of extracts, by having a consistent allergen content, thus making standardization possible (201). Further, the issue with unknown molecules and their properties are non-existent. In cockroach extracts for example, glycinin is not unlikely present, due to contamination from their food source. Patients sensitized to soybean can respond positively although they are negative for cockroach, providing a false diagnosis (202).

1.5 TREATMENT OF ALLERGY

1.5.1 Allergen specific immunotherapy

The only curative treatment currently available for allergies is allergen specific immunotherapy (AIT), where desensitization is achieved by administrating the relevant allergen in low doses over a long period of time gradually increasing the dose (203). However, no standardized protocol exist, physicians worldwide practices different doses and frequency of treatment, complicating understanding of efficacy (204). As with the skin prick test, AIT relies on allergen extracts. Due to the absence of a common standardization and wide range of administered doses, knowledge on effective doses are lacking (205). The mechanism of AIT encompasses shifting the T_H2 immune response into a T_H1 pathway

including blocking IgG antibodies and induction of IL-10 secreting T regulatory cells (206). IgG_1 and IgG_4 in response to AIT, can compete with IgE-allergen complexes for binding the low affinity FcγRIIb on B cells, thus inhibit the antigen presentation to T cells promoting tolerance (207).

Two methods are in practice in the clinics today, subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT). SLIT is less invasive, where tablets are orally administered compared to SCIT, where the allergen is injected under the skin, but suggested as less effective (208). However, from a safety perspective SLIT is advantageous, less adverse systemic reactions are reported but local responses are more frequent. Although uncommon, SCIT can cause potentially lethal anaphylaxis, which should be taken into account when initiating treatment (209). Studies on treatment of allergy to pets display divergent results. For dog AIT, a review of clinical trials show inadequate results after therapy attributed to inferior extracts and the numerous dog allergens in relation to patients' divergent sensitization profiles (210). A case study report states that AIT to dog can decrease allergic responses to other furry animals, likely due to cross-reactivity (211). Not many studies have been conducted on AIT on horse allergic patients, but one study found that rhinitis lessened for 93% of the patients and asthma symptoms reduced for 90% (212).

1.5.2 Immunomodulation

A large concern regarding immunotherapy is safety, anaphylaxis and severe systemic reactions poses danger to patients undergoing treatment (213). To combat the issue, as well as increasing the efficacy of the treatment, different strategies has been applied to modify the therapeutic allergens. For example, allergoids are chemically modified allergens, by polymerization with aldehydes conformational IgE-epitopes can be destroyed while sequential T cell epitopes are retained, thereby reducing allergenicity while maintaining immunogenicity (214). A cat dander allergoid induced a T_H1 and T regulatory response *in vitro* and lesser IgE interaction (215). Further, a HDE allergoid has proven well-tolerated in several clinical trials (216). Another strategy involves DNA containing unmethylated CpG oligodeoxynucleotide (ODN), a feature of bacterial and viral genomes. It is recognized by toll-like receptor 9, which has the capability of switching a T_H2 response into a T_H1 milieu (217). CpG ODN has been utilized as an adjuvant in treatment of peanut allergy (218, 219). Traditionally, adjuvants has been added to vaccines for boosting the efficacy, where aluminum hydroxide has been frequent choice. However, the mechanisms are still not fully understood, but several advances have been made (220).

2 THESIS AIM

The aim of this thesis is to increase the understanding of the molecular aspects of pet allergens, specifically those belonging to dog and horse, and their implication in diagnosis of allergy to pets. By identifying causative allergens, characterize their properties and distribution and thoroughly examine allergen profiles and patient sensitization patterns the goal is to improve molecular diagnostic methods and subsequent treatment.

Specific aims:

Paper I: To compare two microarray multiplex assays to conventional diagnostic methods, skin-prick test and singleplex serology, in terms of specificity and sensitivity.

Paper II: To analyze commercial skin prick test extract used for diagnosing allergy, determining their content and levels of allergen components. Further, to examine allergen profiles of a dog population.

Paper III: To determine IgE profiles in horse sensitized patients and to characterize novel and previously poorly described horse allergens.

Paper IV: To detect dog allergens in the air utilizing different sampling methods and to elucidate the aerodynamic properties of individual allergens.

3 MATERIAL AND METHODS

Below follows a recount of the patient material analyzed in the papers and a description of the major methods experimentally applied. Further details are accessible in the material and methods section of each respective paper.

3.1 MATERIAL

3.1.1 Patient material

In **paper I**, children (n=71) were recruited from a nationwide study of severe asthma. The children underwent an extensive doctor's examination and were well characterized before receiving the diagnosis severe problematic asthma (221). Written informed consent was obtained from all participants. Sera from dog allergic patients were obtained from voluntary donors for **paper II**, written informed consent was given and the material stored in our biobank in accordance with GDPR. Horse allergic patient sera (n=180) were provided by Clinical immunology at Karolinska University Hospital, Stockholm, Sweden and control sera (n=25) from Thermo Fisher, Uppsala, Sweden for **paper III**. All studies were approved by the regional board of ethics.

3.1.2 Animal material

In **paper IV**, four dogs and their owners were recruited. No ethical approval was obtained, as it is not needed for privately owned pets according to Swedish Board of Agriculture statutes, provided that certain conditions are fulfilled during the experiment. Thus the set requirements were met, i.e. owners informed consent, the animal reside in its normal environment (not separated from the owner), no invasive actions, no fixed positioning of the animal, no activity causing suffering or distress. Polyclonal rabbit antibodies were purchased from Agrisera AB, which hold a permission from Umeå animal ethics board for antibody production. BALB/c mice were immunized with recombinant allergens to produce polyclonal anti-Can f 4 and anti-Can f 6 for **papers II and IV**. The immunizations were approved by the Stockholm animal ethics board.

3.2 METHODS

Aerosol particle collection (Paper IV)	Particle collection both by PTFE filters at flowrate 20 L/min or 40 L/min operated by an air pump and liquid cyclone sampling into PBS at flowrate 200 L/min.
Airborne particle detection (Paper IV)	Sampling of air at 60 L/min using a cascade impactor with eight size fractions range

	<0.34->8.13 µm.
Antibody production (Paper II & IV)	Immunization of BALB/c mice or rabbits with recombinant allergens subcutaneously to induce an allergen specific immune response.
Basophil activation test (BAT) (Paper III)	Determination of basophil degranulation upon stimulation of whole blood with allergens by flow cytometry after staining for the basophil marker CD203c and the degranulation marker CD63.
Inhibition ELISA (Paper II & IV)	Allergen measurement by ELISA, where sample competes with biotinylated recombinant allergen for allergen specific antibody binding. Detection with secondary enzyme labelled antibody.
ImmunoCAP (Paper I & III)	Quantification of allergen-specific serum IgE by singleplex assay. The test vial contains a solid cellulose phase coupled with allergen extracts or components. Detection by secondary fluorescent antibody.
ImmunoCAP ISAC (Paper I)	Quantification of allergen-specific serum IgE by multiplex assay with biochip technology. 112 allergen components are immobilized in a microarray. Staining with secondary antibody after serum incubation.
Microtest allergy system (Paper I)	Quantification of allergen-specific serum IgE by a multiplex assay with 35 allergen extracts and components from 22 sources immobilized on a glass slide. Detection by secondary fluorescent antibody.
Protein identification (Paper III)	Candidate allergens were identified by immunoblot. Corresponding protein bands excised and digested by trypsin. Peptides were discerned by tandem mass spectrometry and pBLAST search for full length sequence.

Protein quantification (Paper II)	Protein concentration determination by bicinchoninic acid assay, BCA. Detection by colorimetric measurement.
Purification of antibodies (Paper II & IV)	Immunized sera from mice or rabbits are purified by affinity chromatography using the allergen of interest as the immobilized ligand.
Production of allergens from natural source (Paper II, III & IV)	Purification of allergens from natural source, animal dander or urine, by size exclusion chromatography (SEC) and ion exchange chromatography (IEC).
Production of recombinant allergens (Paper II, III & IV)	Synthetic genes of interest are cloned into expression vectors for protein expression in *E. coli* or HEK293 cells (Can f 5, Paper II). The expressed recombinant proteins, containing histidine tags, are purified by immobilized metal chelate affinity chromatography (IMAC), size exclusion chromatography (SEC) and ion exchange (IEC) chromatography.
Quantitative ELISA (Paper II & III)	Allergen specific serum IgE is quantified using recombinant or natural allergens. Detection with secondary enzyme labelled antibody.
Skin prick test (SPT) (Paper I & II)	Skin allergen challenge with naturally derived extracts. Sensitization is measured by diameter of wheal in response to challenge and compared to positive and negative controls.
Protein analysis (Paper II)	Size and purity analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separation of proteins according to size under reducing conditions.
Statistical methods	Paper I: Spearman rank correlation coefficient was used for the concordance of

(Paper I-IV)	methods. Sensitivity, specificity and the positive and negative predictive values were calculated as well as the positive and negative likelihood ratios. Paper II: To compare gender, Mann-Whitney U test was used, to compare breeds, Sidak's multiple comparisons, and to compare Can f 4 to the other allergens Dunnett's multiple comparisons test was utilized. Paper III: Linear regression was used to assess correlation between the two Equ c 2 forms. Paper IV: No statistics applied due to it being a pilot study with only four participants.

3.3 ETHICAL STATEMENT

All experiments on human and animal material were approved by the regional ethics committee and conducted in accordance with Swedish regulation and animal welfare laws. Written consent was obtained from all blood donors providing sera for serology and basophil activation test. For the human material, no major invasive procedures occurred, only a small puncture wound for venous blood sampling. The children in paper I were further subjected to skin prick test, which has no severe patient risks, only local irritation and swelling. In regard to this, safety issues is not a major cause for concern. However, the collection of patient data and storage of personal information needs to be taken into consideration. This could jeopardize patient integrity if misplaced and should be handled with care. We keep all our records coded on a secure server in accordance with general data protection regulation (GDPR). In regard to this, all serum samples, marked with their study code, are stored in our biobank in accordance with the Swedish biobank act. Patients give their consent to store samples in a biobank, are informed that their samples will only be used for the purpose they consent to and that they have the right to request at any time to have their stored samples destroyed.

The animal experiments imply the use of other species without their consent and for human benefit, which is a general ethical dilemma. The immunizations are minimally invasive and likely the handling causes more stress than the injection. Drawing of blood causes slightly more distress and tends to render the animals exhausted. At end point the animals are euthanized, which again raises ethical concerns. To minimize damage and suffering when using animal experiments, we implement the three Rs, reduce, replace and refine. Further, all personnel handling the animals have the appropriate education where ethics and animal welfare are central.

4 RESULTS AND DISCUSSION

4.1 ALLERGY TESTING IN CHILDREN WITH PERSISTENT ASTHMA: COMPARISON OF FOUR DIAGNOSTIC METHODS [I]

Routine diagnostics of suspected allergy is conducted by skin prick test and/or serology (222, 223). However, these methods mostly rely on naturally derived extracts with poor characterization of composition for analysis (224). Multi-sensitization is common for pet allergic patients, where determining the primary sensitization source can be challenging (225). To add complexity, many of those sensitized are children or adolescents (226, 227). Further, to understand cross-reactive patterns and predictions of severity of allergic reactions, knowledge of component sensitization is essential (228). Multiplex assays containing an array of allergen molecules could provide more detailed information and improve the accuracy of diagnosis (229). In this paper we compared two multiplex assays, ImmunoCAP ISAC 112 and the Microtest allergy system, to the conventional methods skin prick test and the singleplex ImmunoCAP, and with doctor's diagnosis on children with persistent asthma from a defined cohort (221). To account for the two standard methods limit in number on analyses practicable, ten allergens were selected for comparison, despite the actual number tested by the multiplex assays. Of seventy-one participating children, 27% were sensitized to 1-2 of the 10 selected allergens and 48% were multi-sensitized to 3-8 of the identical allergens, see figure 5. This indicates that allergies are a common affliction in persistent asthma and children with the condition should be investigated for sensitization.

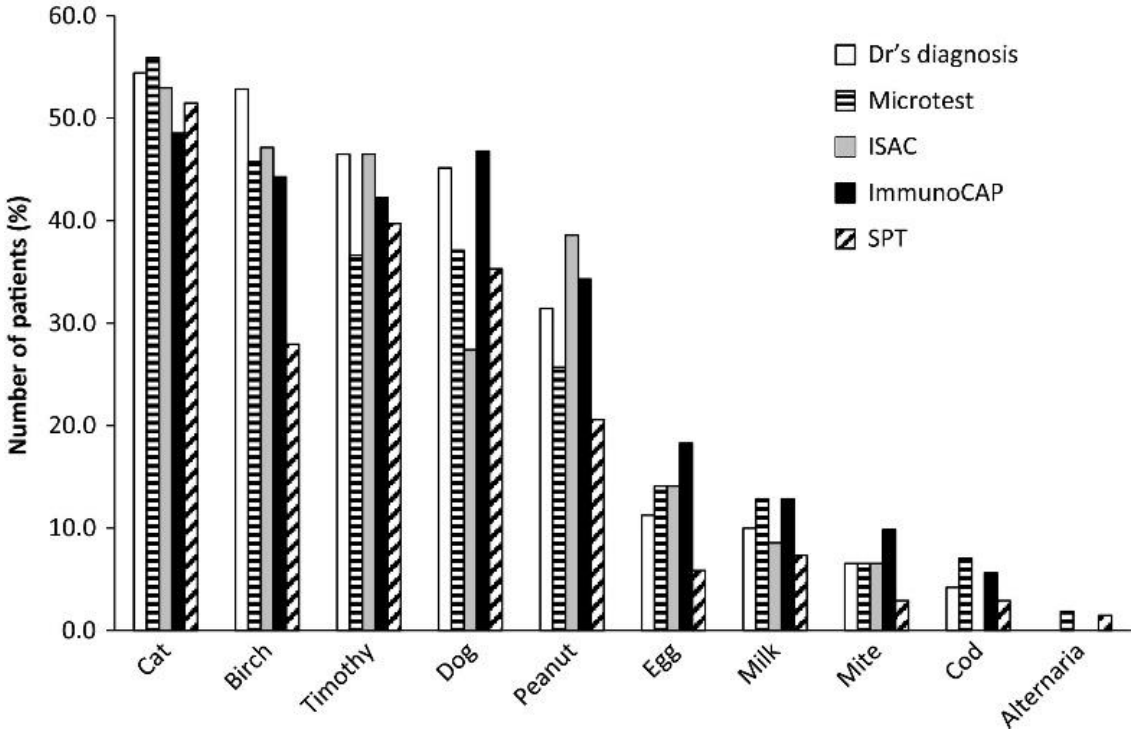

Figure 5. Prevalence of allergy/IgE sensitization according to doctor's diagnosis, Microtest, ISAC, ImmunoCAP and SPT.

Analyzing the results for concordance of methods, comparing them two and two yielding 3964 pairs, gave 90-92% concomitance regardless of method. However, at low sensitization levels all chosen allergens and irrespective of method showed divergence, possibly due to the allergen extracts or technological limitations of the assays. The issues with extracts were further demonstrated by a negative SPT to birch in 18 highly sensitized patients according to all three *in vitro* methods and doctor's diagnosis. This was later explained by the manufacturer as a faulty batch, but highlights the problems with naturally derived extracts.

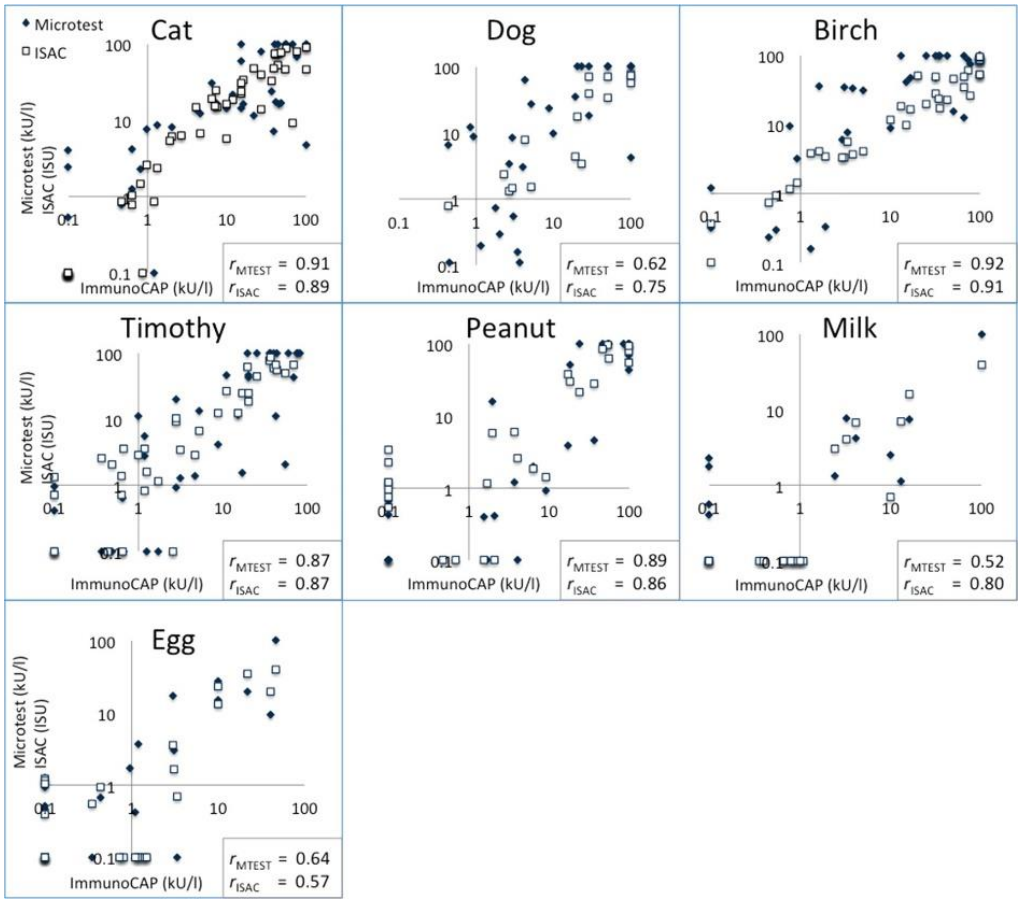

Figure 6. Quantitative comparison with ImmunoCAP IgE concentrations on the x-axis vs ISAC and Microtest sIgE concentrations on the y-axis for different allergen sources and the Spearman correlation coefficient (*r*). Each patient's results are represented by two dots: filled diamond represent ImmunoCAP vs Microtest and unfilled squares represent ImmunoCAP vs ISAC.

Further, the accuracy of the methods was compared by evaluating the ability to detect specific IgE towards an allergen extract. Of 710 results, 593 observations were in negative or positive agreement across all four methods. No significance in accuracy was discovered between the two microarray methods compared to the standard procedures, see figure 6. Interestingly, detection of dog sensitization on ISAC failed to recognize 13 of 28 patients with positive IgE on ImmunoCAP and doctor's diagnosis. This could be due to ISAC lacking several dog allergen components and the patients being sensitized to those, underscore the importance of identification of new allergens and the importance of a full panel of allergens in diagnostics.

The same 710 results were moreover compared with doctor's diagnosis, where the positive divergent outcomes ranged from six to fifteen, where SPT had the fewest discrepancies and ImmunoCAP the most. The negative inconsistent observations were more abundant, from 21 (ImmunoCAP) to 41 (SPT). Overall, sensitivity was calculated to 0.77–0.88, and the specificity to 0.97–0.99 of the four methods compared to doctor's diagnosis. Interestingly, both standard methods had the most dissonant results.

To assess the added value of multiplex assays and the increased information received, allergen components were analyzed. Six children were sensitized to Ara h 8, a peanut allergen which exhibit cross-reactivity with birch (230). Distinguishing sensitization to Ara h 8 and Ara h 2, another peanut allergen is also of great importance, as Ara h 2 is associated to systemic reactions (231). Multi-sensitization to animal lipocalins of several species were also found in eight patients. Seven children were also found to have positive IgE to molds and mites, this is uncommon in Sweden, and could forego detection during conventional diagnosis. However, the information regarding sensitization is valuable to minimize exposure. Comparison of sensitization profiles made possible to distinguish species specific IgE reactivity from cross-reactivities in nine children. In total, 47% of the patients received additional specific IgE information when analyzing components. Microarray analysis are more costly, but in multi-sensitized patients they offer added value, high-resolution and likely a more accurate diagnosis. Further, in children the benefit of lesser sera used per analysis is also advantageous.

4.2 ALLERGENS IN DOG EXTRACTS: IMPLICATION FOR DIAGNOSIS AND TREATMENT [II]

Allergy to dogs is a common disease, where 5-10% of the population is affected and regionally up to 20% (120). Skin prick test is one of the common methods of diagnosing allergy. The test is performed using aqueous extracts produced from the natural allergen source (232). Unfortunately, there is a lack of standardization of extracts and content is poorly defined (179). Further, the source differs between manufacturers, where hair, epithelia and dander are used. Eight dog allergens have been described to this day, Can f 1-8 (113). In this paper we have cloned and produced or purified six of these allergens to characterize five commercial skin prick test extracts. The results display a large variety of both concentration and composition. The total protein content differed from 1.53 to 3.07 mg/mL, where the defined allergen content detected only reached a fraction of the protein amount. As little as 0.26% from one manufacturer up to 10% as the maximum portion. This raises the question of what the rest of the content consists of. It has been shown that some manufacturers add albumin as a buffer solution, however albumin of different species is cross-reactive which could affect the results of the skin prick test (197). Due to the largely uncharacterized protein content, positive reactivity could be towards other potential allergens. It could also be partially dissolved proteins, different extraction methods can affect the protein structure and could denature some proteins independently of others. Resulting in rendering conformational epitopes nonfunctional for some allergens, while possibly leaving others intact.

The six allergens analyzed were detected in all of the extracts, however the amount differed considerably. Can f 3 showed the most diversity in proportions, from 19% to 98% of the total allergen content, see figure 7. We also tested the extracts for human serum albumin, but found only low levels, not enough to explain the large proportion of albumin. Possibly it could belong to another species, which could recognize our Can f 3 antibody, due to the homology of serum albumins. Can f 1, the major allergen was only found in minute concentrations (15ng/mL) in one of the extracts, compared to the others ranging 1200-9100 ng/mL. This could have implications for diagnosis, when 70% of dog allergic patients are sensitized to Can f 1. Overall, Can f 2 and Can f 6 were also detected at low concentrations. It is questionable if the amounts are clinically significant and there is risk of false negative diagnosis when applying these extracts. Can f 6, with a proportion of down to 100 fold lower than Can f 1, still is a consequential allergen due to the cross-reactivity to cat and horse allergens. Even between batches the relation between allergens differed, albeit not to the extent as between manufacturers. A problem is that the manufacturers have not means of analyzing allergen components. The only assay available commercially is for detecting Can f 1, which some companies refer to as calibration. However, since the extracts are composed from naturally derived sources, these can differ not only in concentration but in proportion between allergens.

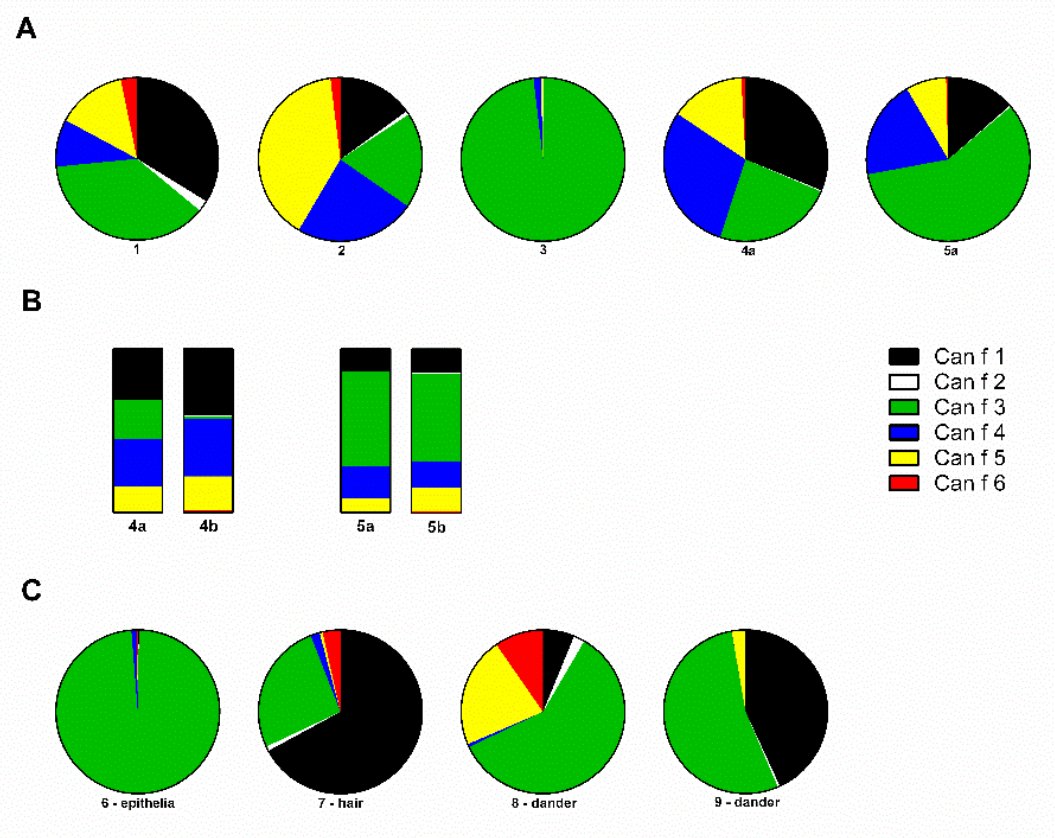

Figure 7. Distribution of six dog allergens, Can f 1-6, in ready-to-use SPT solutions (1-5) from five suppliers (A), two batches (a and b) of skin prick test (SPT) solutions from two manufacturers (B) and in allergen source material extracts (6-8) from one vendor and (9) from another supplier (C).

Lastly, raw material from three different sources, epithelia, hair and dander, was analyzed and found to heterogeneous. Epithelia and dander contained mostly Can f 3, while in hair, Can f 1 was more dominant. Highlighting the importance of carefully selecting source material when manufacturing extracts. Choosing a more diverse source will likely produce a more balanced and well comprised extract. An alternative could be to mix material, including additional sources such as saliva or urine, that could contain site specific allergens.

The lack of sensitivity was also demonstrated in a basophil activation test, specifically the CD-sens method was applied. Three dog allergic patients with different sensitization profiles donated blood for testing the extracts capability of inducing an allergic response. One of the patients was monosensitized to Can f 6 and only reacted to two of the extracts. The second patient with low IgE towards three of the dog allergen components, only responded to the highest concentration of two of the extracts, not sufficient to produce a CD-sens value. The last patient, sensitized to a large extent towards all allergens displayed positivity to all extracts except one, where again only the highest concentration was able to evoke reactivity. The extract used in Sweden's routine allergy diagnostics would fail to detect the allergic response from two of these patients, hence those might not receive a correct diagnosis.

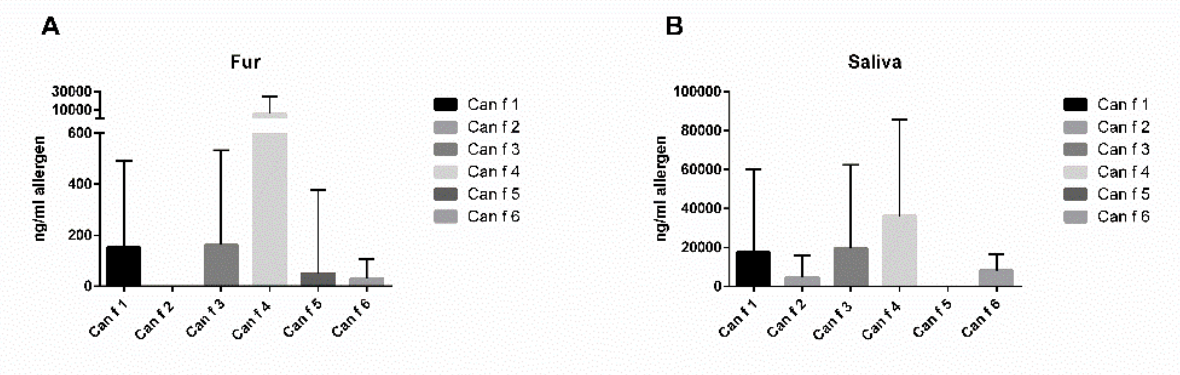

Figure 8. Concentration (ng/mL, y-axis) of six allergens (x-axis) analyzed in extracts of fur sampled from the neck (A) and of saliva (B) collected from 120 dogs. Mean with standard deviation shown. Can f 2 is not detectable in fur. Saliva concentration is approximately 100-fold higher compared to fur.

Lastly, to increase understanding of the nature of the extracts, a dog population (n=120) was tested for levels of allergen components in fur and saliva, see figure 8. In general, higher concentrations of allergen were accounted for in saliva, which could cause difficulty for allergic people despite the dog being washed regularly. The most abundant allergen in both fur and saliva was Can f 4. A recent study has implicated Can f 4 as a major allergen, with sensitization rates up to 81% (132). Thus, it is possible to have as great an impact on dog allergic patients as Can f 1, where most studies on sensitization and prevalence have focused. Moreover, the dominance of Can f 4 on dogs did not reflect the composition of the extracts. Sensitization can thus be underestimated, due to the extracts' imbalanced content. Can f 2 was only detected in saliva, not in fur. The extracts are made from hair and dander, where Can f 2 was detected only in low amounts accordingly. This again could have implications

for monosensitized individuals whom could remain undetected and wrongly diagnosed. The same but opposite was true for Can f 5, the PSA homologue found only in fur, likely due to contamination from urine. Can f 5 was further also detected only in intact male dogs, which was expected, none the less not proven previously. Trace amounts were found in fur samples from a few female dogs, this could be due to contamination from the household having several dogs. Not only did the variation of allergen profiles vary greatly in the population, individuals from the same breed exhibited as large diversity within the breed as inter breeds. Thus, the idea of hypoallergenic dogs are false, in concordance with previously published results (140).

4.3 IGE PROFILING OF HORSE SENSITIZED SUBJECTS USING A PANEL OF HORSE ALLERGENS, INCLUDING THE NOVEL ALLERGEN EQU C 7 AND FULL LENGTH EQU C 2 [III]

Allergy to horse is dependent on sensitization to horse allergen components, where knowledge is incomplete and still to be described allergens likely to contribute (145). Unfortunately, not many studies on horse allergy and treatment have been conducted, despite horses being utilized by mankind for thousands of years (233). Currently, five horse allergens, one duplicate, have been described, denoted Equ c 1-6 (113). In this article, we aimed to establish the sensitization rates to allergen components in a horse sensitized patient cohort. For this purpose, we produced recombinant Equ c 1, the major horse allergen, as a reference allergen. Sixty-one percent displayed positive IgE binding to the allergen, in range of previously reported 51-76% (145, 146).

Moreover, we produced two forms of Equ c 2, previously only partially characterized (147). Using pBLAST search, a protein originating from Przewalski's horse, a feral descendant of domesticated horse (234) aligned with the Equ c 2 N-terminal sequence. Further search using Przwalski's Equ c 2 as template yielded a homologue from modern horse. The two Equ c 2 forms displayed a 67.1% sequence identity and 90.6% similarity, rendering speculation regarding the two forms being isoforms justified, due to the relatively low identity. However, analyzing patient sensitization patterns, the forms correlate strongly and the vast majority sensitized to Equ c 2 responded to both forms. Interestingly, Przewalski's Equ c 2 induced higher IgE titers, compared to the modern horse form, indicating more allergenic properties see figure 9. Many isoforms of allergens have different capability of inducing an allergic response, and even hypoallergenic isoforms exist (235, 236). Further, sensitization to the two Equ c 2 forms was quantified to 15 and 16% of horse sensitized patients, compared to the previously reported incidence of 50% (147). Nevertheless, that prevalence was deduced using immunoblot applying not fully characterized allergen extracts, possibly including several isoforms. A possible additive effect from the allergenicity of different isoforms could contribute to a higher reactivity observed. Another implication of Equ c 2 sensitization is due to it being a lipocalin, because the protein family often cross-reacts with other members and determining the primary sensitization allergen can prove cumbersome (237).

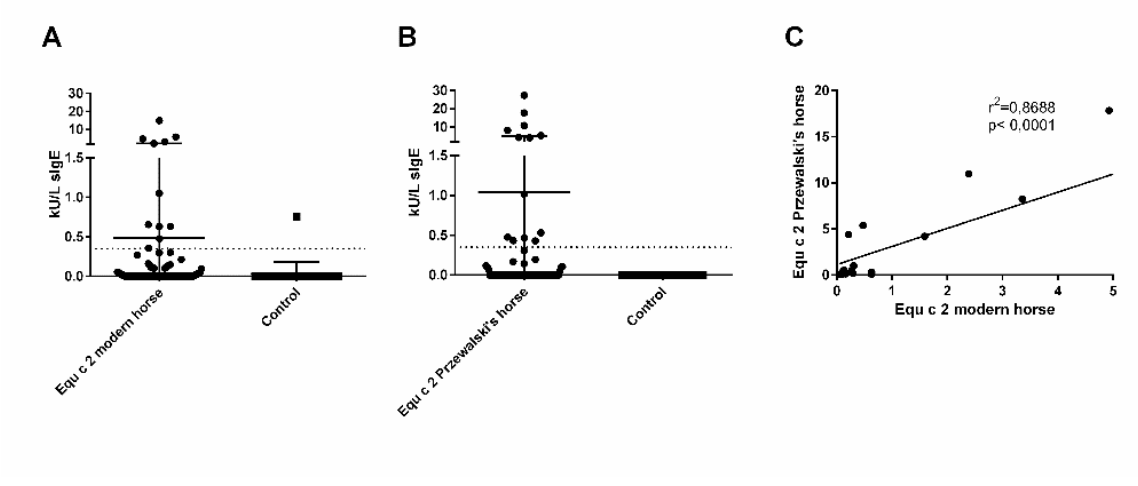

Figure 9. Quantitative analysis of IgE to two forms of Equ c 2 by ELISA.

A. IgE titers (kU/L) to modern horse Equ c 2 among 80 HDE positive sera. B. IgE titers (kU/L) to Przewalski's Equ c 2 among 80 HDE positive sera. Included also IgE-values of 25 negative control sera to each component. The dotted line indicates positive cut off (0.35 kU/L). Bars indicate mean value of the full population and one SD. C. Correlation between the two Equ c 2 variants.

Additionally, we discovered a novel horse allergen, Equ c 7, by analyzing SDS-PAGE and immunoblot of horse dander extract. Sequence alignment identified a homologue in cat, the major allergen Fel d 1, to which 90% of cat allergic patients respond to (238). Protein sequence identity was confirmed to 55.7% with 82.0% similarity. The IgE reactivity was also confirmed with 38% of horse allergic patients responding to the allergen, see figure 10. Due to the similarities, cross-reactivity is likely to manifest and some of these patient might be primarily sensitized to Fel d 1. Further investigation of the allergen and its cross-reactive nature is warranted.

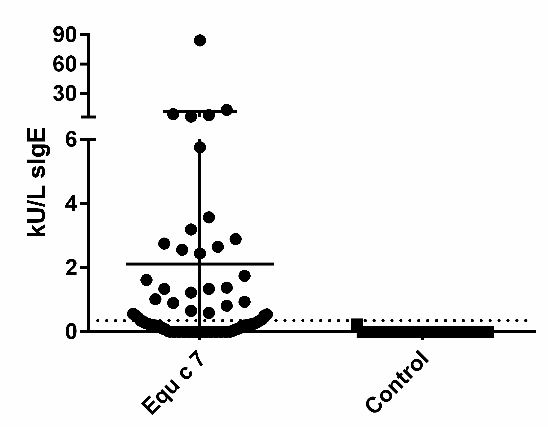

Figure 10. Quantitative analysis of IgE to the novel allergen Equ c 7 by ELISA

IgE titers (kU/L) to Equ c 7 among 80 HDE positive sera. Included also 25 control sera to each component. The dotted line indicates positive cut off (0.35 kU/L). Bars indicate mean value of the full population and one SD.

Lastly, to increase the understanding of the allergenicity of Equ c 4, latherin was purified from horse dander extract. Previous sensitization has been reported to 77%, indicating the importance as a major allergen. However, the study was performed on what was then though to be a novel allergen and not regarded as Equ c 4, further, only 22 horse sensitized patients were analyzed in the same study (151). Latherin is a surfactant protein and present in horse sweat, thus hard to avoid in proximity to horses. Moreover, stallions have a higher secretion of Equ c 4 compared to mares and geldings, which could contribute to the severity of allergic response (154). Examining IgE response of 100 consecutive horse sensitized patients revealed a prevalence of 59%, still significant as a major allergen and should be taken into consideration when diagnosing allergic individuals. The panel of horse allergens are still not fully characterized and likely undescribed allergens contribute to sensitization. As we have shown in dogs, certain allergens are restricted to specific anatomical sites of the animal. Horse AIT are not conducted in Sweden currently, due to low efficacy. This could be a result of allergen not present in the extracts. Further, a large heterogeneity exist in commercially available extracts, as shown for dogs and fish (197, 199). Indeed, it is a common feature among allergen extracts to exhibit a large variety in content. Action is urgent to characterize allergens and producing them recombinantly, spiking extracts with these could improve diagnostics drastically and possibly therapy.

4.4 AIRBORNE ALLERGENS FROM DOGS - QUANTITATION AND PARTICLE SIZE [IV]

Exposure to animal allergens occur in our everyday life and the molecules they shed disperse and deposit in most public places (239, 240). Knowledge about allergen structure, secretion, aerodynamic properties and dispersion pattern are crucial to prevent unnecessary subjection if allergic. Data collected from earlier studies have only focused on Can f 1, showing that the allergen when airborne, is connected with mostly larger particles (> 9µm) but can also be found on particles as small as < 4.7µm (171). Smaller particles reaches further down the airways when inhaled, thus have the ability to deposit in the alveoli and have been associated with inflammation and asthma (241). To investigate the properties of airborne dog allergens Can f 1, Can f 3 and Can f 4, we utilized a $27m^3$ steel chamber with controlled air flow rate. The advantage of the chamber is the enclosed environment and reduction of contamination found in other studies partaking in people's homes. Four dogs spent 2 hours each inside with their owner keeping the dog active. Fur and saliva samples were also collected for comparison with airborne particles. The saliva had generally higher allergen levels, for Can f 1 24000-2200 ng/mL and >1600-256 ng/mL for Can f 4. In fur the range was 9.2-95 ng/mL for Can f 1 and <2.5-3.6 ng/mL for Can f 4. Can f 3 was not detected in any fur sample and only in saliva from dog #4. Can f 3 being serum albumin, therefore it might not be found in fur unless the dog has a wound or fractured skin where blood can leak out. The inability to detect Can f 3 in the samples is thus not surprising. The absence of Can f 3 in the dogs' fur mirrored the airborne results, where Can f 3 was not detected regardless of method.

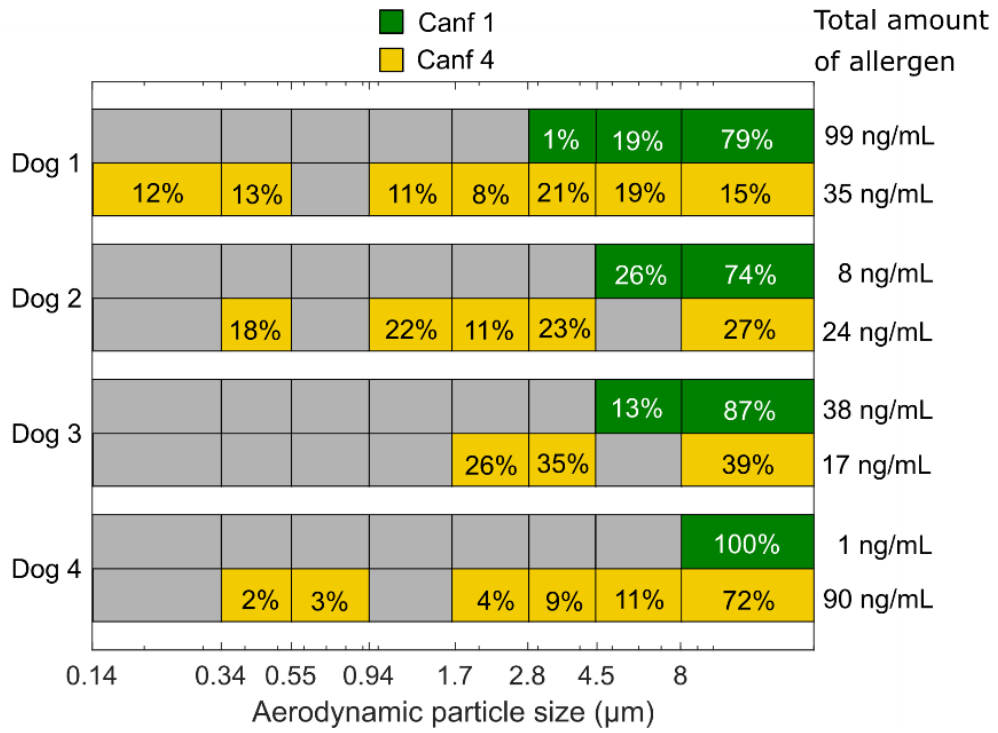

Figure 11. Amount of dog allergen detected in fractionated airborne particles. Particle size in fractions as follows from left to right <0.34 µm, 0.34 – 0.56 µm, 0.56 – 0.95 µm, 0.95 – 1.67 µm, 1.67 – 2.85 µm, 2.85 – 4.51 µm, 4.51 – 8.13 µm and >8.13 µm. Can f 1 was found in the three largest size fractions, no detection in the other fractions. Can f 4 was detected in all fractions.

To detect and separate particles of different sizes a cascade impactor with eight compartments was used. For all four dogs, Can f 1 was only detected in the three largest fractions, belonging to particles with sizes over 2.85 µm. Diversely, Can f 4 was found in all fractions, connecting the allergen with particles of many different sizes, see figure 11. The capability to attach to smaller particles could also influence the allergenicity of Can f 4, where lesser particles can reach the small airways and thus increase the likelihood to encounter mast cells and macrophages to initiate an allergic response (242). Allergens connected with particles less than 5 µm, have in sensitized patients been associated with asthma and inflammation (172). As recent studies has implicated Can f 4 as a major allergen with a sensitization rate up to 81% (132), the impact of Can 4 exposure can contribute to eliciting allergic responses to large degree. Of note, as shown in Paper II, Can f 4 is the most abundant allergen in dog fur, further underlining its importance. However, in the dogs examined in the present study Can f 1 was more abundant than Can f 4. This apparently contradictory result can be explained by the high individual variation in allergen levels between dogs.

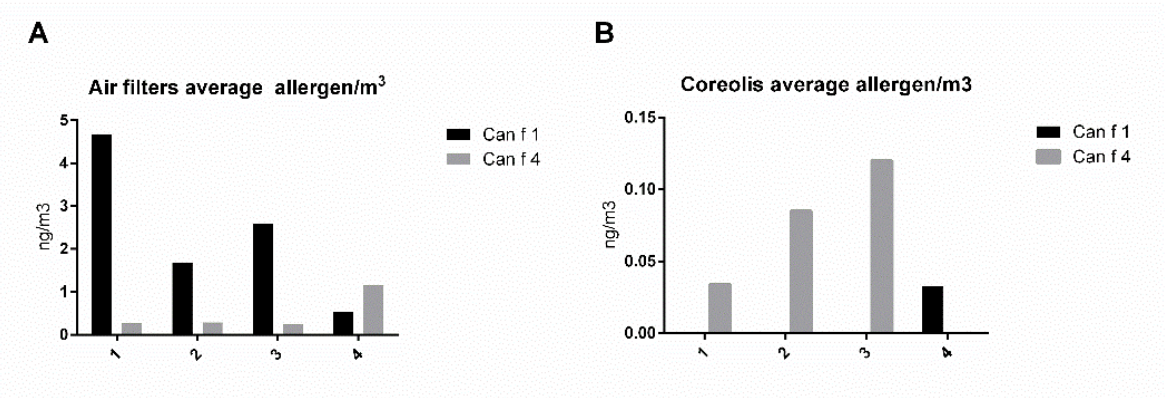

Figure 12. Allergens collected by air pump and air cyclone. A. Amount allergen per m3 collected from air filters (average of 20 lph and 40 lph) from each dog (1-4). B. Amount allergen per m3 in the liquid cyclone samples, 10 minutes collection per sample, average of two samples per dog (2x10 minutes). Low levels (<1.8-3.6ng/ml) of Can f 4 was detected from dog #1-3.

The chamber had two PTFE filters, with an air flow rate of 20/40 liters per hours installed. Can f 1 and Can f 4 was detected in all air filters, however in samples from dog #1-3 Can f 1 was the dominant allergen, while higher levels of Can f 4 compared to Can f 1 were detected from dog #4, see figure 12. Samples were also collected using a liquid air cyclone, 2x10 minutes per dog. Interestingly, the results were opposite to those observed from PTFE filters. In samples from dog #1-3, only Can f 4 could be detected and from dog #4 Can f 1 was exclusive. The overall higher proportion of Can f 4 in the cyclone samples could be a consequence of different aerodynamic properties. As shown with the cascade impactor, Can f 4 was connected with particles of all sizes, not only larger ones. This could enable them to prolonged time airborne or reach further distances. Also, the collection time between the filters and the cyclone differed, 2 hours compared to 2x10 minutes, which could account for the discrepancy of composition. Further studies are needed to investigate the properties of airborne particles and to find out how collection method impacts the recovery of allergens and implications thereof.

5 CONCLUSIONS

Paper I: We compared two microarray assays to standard diagnostic methods, skin prick test and ImmunoCAP with doctor's diagnosis. Concordance of accuracy was similar regardless of method, suggesting no diagnostic drawbacks of microarrays. The advantage of Microtest is to provide both extracts and components, decreasing the likelihood of missing a relevant component not present on the chip. ISAC however, is vastly superior in component diversity with 112 allergens, but could fall short in rare sensitization profiles. The downside of microarrays is the cost compared to the conventional methods and in monosensitized patients they provide no extra benefit. The additional specific IgE information achieved in half the sensitized children from microarray analysis provides physicians with improved tools aiding diagnosis. In multi-sensitized individuals this could be valuable, distinguishing primary sensitization from cross-reactivities. Certain allergen components are more clinically relevant and thus important to identify. As we show in paper IV, allergens have different aerodynamic properties and some are localized on different parts of the animal. Understanding of the sensitizing component's characteristics could help in managing the allergen, for example avoiding male dogs or saliva.

Paper II: The study analyzed commercially available skin prick test extracts for allergen concentration and composition. The results showed a large variation of content, where some allergens were only present in minute concentrations. The allergens present at very low concentrations may appear as not clinically relevant. The physician to diagnose the patient will have a difficult dilemma, where the test is negative but the patient's anamnesis say differently. There is further a large portion of uncharacterized dog proteins, proteins, which may be allergenic or non-allergenic, but possibly also from other sources than dog. Because the extract is naturally derived, there is a possibility of cross-contamination with for example pollen or fungi. This could result in an opposite dilemma, rendering a false positive response. In our study, the dominating allergen found in the extracts differed to that of a population of dogs. This could be due to extraction methods favoring certain allergens while dissolving others, the implication is, nonetheless, that the extracts are capable of only detecting sensitization to select allergens. Utilizing poorly defined extracts for diagnosis could jeopardize patient safety, as insufficient amounts of certain allergens of clinical relevance and inconsistencies between batches could result in an incorrect diagnosis. Moreover, extracts for allergen specific immunotherapy are derived from the same source material, possibly rendering a costly and lengthy treatment ineffective.

Manufacturers need to urgently improve the quality of extracts, to ensure consistent content and sufficient concentrations. They should consider the possibility of adding recombinant allergens to guarantee satisfactory amounts and representation of all relevant allergens, or mixing different source material for a better allergen representation. A unified standardization and full content declaration should be a minimum.

Paper III: Sensitization to horse allergen components is less investigated compared to dogs and cats and there is a lack in knowledge about horse allergens. Here we analyze sensitization rates to four horse allergens, describe a novel allergen and further characterize full-length Equ c 2. We produced two forms of Equ c 2. Although sensitization prevalence to these was low, the allergen still is clinically relevant being a lipocalin. The protein family share many similarities and cross-reactivity to other lipocalins is not unlikely. Deducing primary sensitization source will aid patients in assessing severity of symptoms and avoidance measurements. We also identified a new horse allergen, Equ c 7. It shared homology with the major cat allergen Fel d 1, which has a sensitization rate over 90%. Likely, Equ c 7 will cause an immune response in many cat allergic individuals. In this paper we have further characterized a new horse allergen and for the first time described full-length Equ c 2. This contributes to a better understanding of horse allergens and could improve diagnostics and treatment of allergy to horse. However, probably several horse allergens remain uncharacterized and better understanding of the known ones is required. Determining cross-reactivities and location of allergens on the horse is imperative. Most allergens are discovered in horse dander extract, but studies on other species implicates both saliva and urine for harboring allergens. Further, as seen in paper IV, the aerodynamic properties of allergens differ and could result in altered allergenicity or capability of spreading. Further studies about horse allergens and their characteristics are warranted.

Paper IV: We have quantified two airborne dog allergens using three different sampling methods in a controlled manner, reducing contaminants and defining space and volume. Detecting particles associated with allergens, revealed that distinct allergens are connected to different particle sizes. This can have implications for sensitized individuals, because the size of the associated particles can impact the allergenicity of the allergen. Lesser particles can travel into the small airways where they can cause a more severe systemic allergic response, compared to larger particles. Smaller sized particles have indeed been linked with asthma. The study also revealed that different sampling methods displayed opposite accumulation of two analyzed allergens. This may reflect their ability to associate to particles with diverse characteristics. It is likely that smaller particles have a different dispersion pattern, possibly remaining airborne over greater time and space. This is the first study showing that allergen molecules have a unique particle size association pattern, which could impact on their clinical relevance. Different collection methods yield dominance of certain allergens, showing the importance of carefully choosing sampling technique. Distinct allergens hold separate aerodynamic properties which should be taken into account when sampling environments and when considering avoidance strategies. Further studies should be performed on other allergens to determine their airborne characteristics and increasing the cohort for statistical soundness.

6 FUTURE PERSPECTIVES

This thesis has contributed to the knowledge of dog and horse allergens, their distribution and properties. Further it describes a novel allergen and provides refined characterization of others. Importantly, it has highlighted the issues with current diagnostic methods and substandard allergen extracts. Yet, there is still much to be done. Improving accuracy of allergy diagnosis and treatment success could improve quality of life for the large proportion of allergic individuals (243). It could also benefit society as a whole, for example by reducing instances of pollen allergic people taking sick leave due to severe symptoms in spring or reduce the cost of asthma medication if the underlying allergy is treated.

In **paper I**, we explore new diagnostic tools, two microarrays with the capacity to analyze an assemblage of allergens compared to standard methods ImmunoCAP and skin prick test. They all performed equally, but the microarray methods were able to provide additional sensitization information in nearly half of the patients. It is clear that component resolved diagnostics improve specificity and have the ability to distinguish between allergen components with different clinical implications from the same source. To further refine the analysis, more allergen components needs to be identified. Likely, there is an array of unknown allergens from the common sources, pets, food, pollen, mold and insect venom. From my perspective, pet allergens should be further explored. Cat and dog allergens have been thoroughly studied, but less is known about horse allergens. Further studies should be conducted on the equine family. If less is known on horse allergen, rare and exotic pets are an unmapped domain. Ferrets are kept as pets, and single allergens have been described (244), but their allergen profile remain veiled. The same condition applies to small rodents; mice, rats and guinea pigs have been described as causing occupational allergies (245, 246), but other species from the same order are less studied. Identifying new allergens and producing them recombinantly for the purpose of improving allergy diagnostics is an important project. As mentioned before, many allergen are similar in structure and most belong to a few allergen families. Studies regarding cross-reactivities of these allergens would be the next logical step, to deduce primary sensitization targets and potential risk sources. Improving production of recombinant allergens can also hopefully reduce the cost, which is another factor for the clinician when deciding diagnostic method.

Paper II highlights the lack of standardization and content cohesion in extracts intended for diagnosis by skin prick test. The results clearly shows the discrepancies, not only between manufacturers, but also from batch to batch and display how source material affect the composition. The extracts needs to improve in quality and content declaration. Spiking the extracts with recombinant allergens is one possibility, at least one which would ensure enough allergen material for clinical relevance. A problem would still be the uncertainty of the remaining uncharacterized content in the extract. Our study showed that the allergen proportion in regard to the entire protein concentration was at best a tenth. It would be interesting to identify the remaining proteins and to determine if they are relevant from an allergen perspective. If these extracts are produced from naturally derived source, it is

possible they can contain bacterial, fungal or even other allergenic material from other species residing in the fur, such as pollen during season or house dust mites, fleas and lice. It could also be just dissolved proteins and peptides or uncharacterized allergens, but the answer is still unknown. Performing proteomic analysis and examining the extracts by mass spectrometry would be compelling. Another possibility is to produce purely recombinant allergen preparations, the risk being the opposite. There might be novel allergens or various numbers of isoforms with different allergenic properties, as Equ c 2 displayed in paper III, in the extracts and those would be missing in the recombinant mixture. Monosensitized patients to those would receive a flawed diagnosis. Identifying new allergens would decrease the risk of applying defined recombinant allergens for diagnosis and that is always urgent. A new dog allergen was recently described, so clearly the panel is not complete (113). There is more to be done in the field of describing allergens. A study similar to ours but on AIT extracts could give incentive to improve therapy. Unsuccessful treatment is a common problem and proving poor quality of AIT extracts could pave way to a molecular approach to therapy.

Further, a dog population was analyzed yielding their allergen profiles for six dog allergens. The result showed a wide variety of allergen composition and concentration. No correlation between breeds or genders was found, except for Can f 5 which is exclusive for male dogs being a prostatic protein. Analyzing the two newly described allergens not included in our study would be interesting. They have not been detected on dogs, so proving that they indeed are dog allergens and present on dogs would render them more valid. Dog saliva has been shown to contain a wide variety of allergens including potential novel ones (247), yet new allergens are normally detected using immunoblot in dog fur extracts. Our study also show a higher concentration of allergens in saliva compared to fur. The prospective of discovering new allergens using saliva or urine should be considered. Our study measured allergen profiles from a single collection time. Knowledge on whether allergen profiles are stable over time is lacking. To address this, dog populations need to be sampled over time to discern variation due to seasons, if female dogs are affected by being in heat, differences from puppy to adolescent to adult and if ageing induce changes. These are unknown factors and would contribute to avoidance advice and possibly in selecting source material for the extracts.

The identification of a novel allergen, Equ c 7, in **paper III** is exciting. This opens up a lot of possibilities regarding further exploration. The allergen is a homologue to Fel d 1, the major cat allergen. A study on cross-reactivity would be interesting including inhibition experiments to identify the level of conformational similarities. Further, determining the primary sensitization source could aid future diagnosis. Confirming that Equ c 7 is present in horse extract or horse source material is also a priority. The horse extract in Sweden has been removed from allergen specific immunotherapy, due to unsatisfactory results indicating the poor quality of the extracts. This could also affect diagnosis of horse allergy, since the same source material can be used to produce both SPT and AIT extracts. A study investigating the extracts used for skin prick test for allergen concentration and composition would likely be an eye opener. The solution of recombinant allergens are relevant here too. Since horse is less

studied compared to dogs and cats, the panel of horse allergens is likely incomplete and efforts should be made to identify new allergens. Can f 5 is a major allergen in dogs, possibly a homologue exist in horses. The prostatic kallikrein is a conserved protein and humans have a homologue (136), investigating stallions could prove prosperous. Horse saliva is another allergen candidate, immunoblot should be performed to identify allergen candidates. Cellular responses would also be of interest, stimulation of peripheral blood mononuclear cells (PBMC) using horse allergen components is a possibility. Our group have developed a fluorospot assay to detect activation of rare antigen-specific T-cells by cytokine release in MS and cancer. The analysis could be adjusted for application in allergy, correlating cytokine profiles from a well characterized patient cohort to an allergen panel. Additionally, the assay can be used to monitor a recombinant vaccine experiment *in vivo*, to compare pro-inflammatory cytokines to those inducing tolerance.

The field of aerodynamic properties of allergens is largely unexplored. Previous studies have solely analyzed Can f 1. In **paper IV** we investigate three dog allergens in aerosol samples. The logical continuation would be to analyze the remaining dog allergens. Can f 2 is a saliva exclusive protein, but could become airborne in exhaled air or in slobber. Due to the fact of only having one participating male, Can f 5 was excluded, but in a larger cohort it would be interesting to analyze. Can f 6 is present in low amount in fur, but as seen with the other allergens, detection was made despite low concentrations. This being a pilot study, with only four participating dogs, a larger study would be warranted including more dogs and all dog allergens. Identifying the particles associated with the allergens would increase the understanding of their properties. Speculation regarding the allergenicity divergence due to the size of associated particles is also relevant. An allergen challenge could be performed in an animal study using the particle bound allergens. Analysis of bronchoalveolar lavage (BAL) and airway responsiveness using a Flexevent (small animal ventilator) would provide information whether the different particles affect the development of asthma (248-250).

The field of allergy is still filled with gaps in knowledge and there is much to be done. Especially in improving diagnosis and treatment, which is of critical clinical importance. Recombinant allergens is a promising field, with many routes to explore. Our group has previously created a recombinant multimer allergen comprising of Can f 1, 2, 4 and 6 (251). This has been applied in our inhibition ELISA assays to detect allergen in natural samples. It is a promising candidate for a vaccine, and has the ability to induce an IgG response *in vivo*. Further refining this molecule for therapeutics would be interesting. The advantage of the multimer being the known concentration and relation of all inherent allergens. However, adverse events are problematic when treating allergy. Due to the hypersensitivity, allergen exposure can cause severe reactions and worse case anaphylaxis. A solution to minimize the risks of AIT is to explore immunomodulatory substances or techniques. For instance, by introducing mutations into the recombinant allergen molecules, IgE binding capacity can be reduced while maintaining the capability to induce an IgG response (252). Another approach is peptide based hypoallergenic vaccines, where the linear T cell epitopes remain intact, while

conformational B cell epitopes lose their ability to cross-link IgE (253). Another strategy to modulate the allergic immune response is to produce allergen preparations including immunomodulatory substances, for example non-digestible oligosaccharides which has been shown to modulated the T_H2 into a T regulatory response (254). These are all viable routs to further explore our vaccine candidate.

Route of administration is another field to consider. Traditionally AIT is performed either as SCIT or SLIT, but efficacy and number of adverse events are inconsistent between studies (255). Further the lengthy and frequent treatment discourages patients and compliance can be low (256). An option we have considered is intra-lymphatic injections, where the allergen is administered straight into the lymph node, thereby bypassing mast cell activation and directly introducing the allergen to T cell presentation (257). A further advantage of intra-lymphatic injections is the shorter duration and fewer treatment occasions in combination with lower allergen dose, increasing the likelihood of patient compliance (258). In summary, continuing to explore new allergens and their properties to complete the panel of known proteins capable of inducing an immune response feels urgent. Further, utilizing that knowledge will support the creation of molecular based diagnostic tools and refine treatment by the aid of immunomodulatory techniques and molecules.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Allergi är en av våra stora folksjukdomar, där upp till 30% av befolkningen i Sverige är drabbad. Symptomen innefattar hösnuva, klåda samt i vissa fall även astma. De mest allvarliga fallen kan utlösa livshotande tillstånd. Allergi beror i de flesta fall på ett överkänsligt immunförsvar, där celler som ska skydda oss från sjukdomsframkallande ämnen inte kan skilja på dessa och harmlösa ämnen och startar en immunreaktion. Dessa ofarliga ämnen som har förmågan att initiera allergiska symptom kallas allergen. Allergen är proteinmolekyler som förekommer i många kontexter, till exempel hos pälsdjur, i födoämnen, hos växtpollen eller i insektsgift. Dessa allergen finns ofta i flertal hos en källa, till exempel hund har åtta kända allergen och de flesta hundallergiker reagerar på en eller ett par av dessa allergen. Det är därför allergiker kan reagera på en individ av en art, men förbli opåverkad av en annan. Vilket allergen man reagerar på är individuellt. Oftast utgörs allergenen av de proteiner som har en förmåga att ta sig in i kroppen genom slemhinnan i lungorna eller mag-tarmkanalen, där de påträffas av immunceller. Hygienhypotesen är en förklaringsmodell för uppkomsten av allergi, där en minskad exponering av bakterier i barndomen på grund av ökad renlighet leder till ett felprogrammerat immunsystem. Miljöer där man tidigt i livet utsätts för bakterier och bakterieprodukter, såsom på bondgårdar eller husdjur i hemmet, har påvisat skyddande effekter. Även andra orsaker är kopplade till benägenhet att utveckla allergi, såsom gener och ärftlighet, bakteriefloran i tarmen hos nyfödda och RS-virus infektion i tidiga år.

Pälsdjursallergi är en av de mest förekommande typer av allergisk sjukdom. 15-20% av svenska invånare reagerar mot hund och runt 5% mot häst. Svårigheten med pälsdjursallergi är liksom pollenallergi, den begränsade möjligheten att undvika exponering av allergenen. Studier har påvisat hundallergen både i skolor, på bussar och sjukhus. Pälsdjursallergen är ofta lika varandra i uppbyggnad, vilket gör att reaktiviteten ofta visar sig i överkänslighet mot flera olika arter. Diagnos ställs av läkare med hjälp av kliniska tester. Den vanligaste metoden är pricktest, där en liten droppe extrakt från källan för den misstänkt allergin läggs på patientens hud. Därefter punkteras huden och irritationen som uppkommer lokalt mäts i diameter som mått på överkänslighet. Den andra rutinmässigt förekommande analysen är mätning av en speciell typ av patientens antikroppar i serum från blodet, IgE, som reagerar mot allergenet. Vanligtvis sker detta med analyssystemet ImmunoCAP. Finns det en ökad mängd IgE antikroppar som är specifikt riktade mot allergenet kallas det sensibilisering. Dock är allergi svårdiagnostiserat och det krävs en läkares utlåtande för att konstatera sjukdom. Metoden båda dessa analyser grundar sig på, innefattar extrakt producerat av naturligt material från allergenkällan, där alla allergen från arten bör ingå. För hund innebär det att extraktet är gjort på en stor mängd insamlat hundhår eller mjäll och hudceller, från en blandad population hundar, där allergenen utvunnits genom kemiska metoder. Extraktionsmetoderna kan dock skada allergenen och omfördela balansen inbördes mellan dessa. Vissa allergen finns utöver det bara i saliv eller urin, vilket gör sannorliheten liten för att dessa finns representerade i extrakten. Det innebär att tillförlitligheten hos metoderna

varierar beroende på kvaliteten på extrakten och att beroende på vilket allergen du är allergisk mot, kan testet vara felvisade. Dessa extrakt används även för behandling av allergi med allergivaccination, s.k. allergen specifik immunoterapi. Genom att långsamt vänja kroppen vid låga doser allergen och sedemera öka dessa kan tolerans uppnås. Denna behandling är utdragen, minst 2-3 års behandlingstid samt mellan 80-100 injektioner med allergenextakt, därutöver kostsam. På grund av extraktens varierande kvalitet och patientens egen överkänslighetsprofil, är behandlingen inte alltid lyckosam och förfinade metoder efterfrågas.

I **delarbete I** jämförs två nyare diagnostikinstrument med de tidigare nämnda rutinmetoderna pricktest och ImmunoCAP, samt mot läkarens diagnos. De två nya metoderna har en panel av allergen där man simultant kan testa sensibilisering mot en mängd olika allergen och allergenextrakt. Fördelen är att det krävs mycket mindre serum samt att man kan analysera fler allergen jämfört med konventionella metoder. Detta är av särskild vikt när patienten reagerar mot allergen som är strukturmässigt lika mellan olika arter. Är patienten sensibiliserad mot ett hundallergen som är närbesläktat med ett hästallergen kan denne få allergiska symptom av både hund och häst. Sådan information är viktig vid behandling och för förebyggande åtgärder. I denna studie deltog 71 barn med ihållande astma, bland vilka allergi mot 10 vanliga allergenkällor undersöktes. Totalt 75% av barnen reagerade mot något av de 10 utvalda allergenen och nästan hälften av alla vara överkänsliga mot tre eller fler allergenkällor. Resultaten visade att alla fyra metoderna var likvärdiga i noggrannhet och samstämmiga i 90-92% av alla analyser. Utöver det erhölls ny information om ytterligare allergier i 47% av alla fallen vid paneltesterna. Panelerna bidrar således med mer nogrannhet och har inte sämre träffsäkerhet eller känslighet jämfört med nuvarande metoder. Majoriten av barnen med astma hade underliggande allergi, vilket föranleder att barn av denna kategori bör utredas för allergi rutinmässigt.

Delarbete II undersökte innehållet i hundextrakt som används i rutinvården för pricktest vid allergidiagnostik. Extrakten består av naturligt material och deras innehåll är undermåligt beskrivna. Vi har tagit fram analysmetoder för att mäta sex olika hundallergen i extrakten och från hundars päls och saliv. Resltaten visade att de fem olika testade extrakten varierade mycket, både innehållsmässigt och avseende allergenens inbördes proportioner. Vissa allergen detekterades endast i mycket små mängder, där det är tveksamt ifall dessa skulle ge utslag i ett pricktest. Vi jämförde också extrakt från olika källor, och de skiljde sig markant mellan hår, hudceller och mjäll. För att undersöka de kliniska aspekterna, testade vi immuncellers reaktionsförmåga mot dessa extrakt från tre hundallergiker. Flertalet extrakt lyckades inte skapa en immunologisk reaktion, trots att patienterna hade allergiska symtom vid kontakt med hundar. Detta är oroande, då patientsäkerheten kan hotas p.g.a. den skiftande kvaliteten på extrakten . Trots detta används de rutinmässigt vid allergidiagnostik. Då extrakten baseras på naturliga råvaror analyserades också etthundratjugo hundar för allergenförekomst. Dessa varierade också ansenligt, där skillnaden på individnivå var större än mellan rastillhörighet. Saliven hade en högre mängd allergen jämfört med päls och intressant nog återfanns allergenet Can f 4 i störst kvantitet. I extrakten var i motsats Can f 3

högst i koncentration. Detta kan bero på hur extrakten produceras, där vissa allergen kanske delvis sönderfaller. Dock återspeglar extrakten inte den naturliga fördelningen av allergen, vilket även kan påverka diagnostiken. Risken är att patienter får en felaktig diagnos och att sjukdomstillstånd inte upptäcks. Samma typ av extrakt används även vid behandling av allergi, där effekten riskerar att utebli och patienten genomgår en utdragen ineffektiv terapi.

Delarbete III fokuserade på hästallergen och hur många allergiker som reagerar på dessa. Allergi mot häst är betydligt mindre studerat än allergi mot katt och hund, trots att hästar och hästsport är en vanlig fritidssysselsättning i vårt samhälle. Fem hästallergen finns idag identifierade, dessvärre är vissa inte så väl undersökta. Vi har med DNA-teknik producerat allergenet Equ c 2, som tidigare inte varit komplett beskrivet. Två former av detta allergen togs fram, dels från urhästen Przewalskis häst samt från nutida häst. Dessa två former är identiska till 2/3, men skiljer sig åt tillräckligt för att ifrågasätta om de två studerade formerna kan sägas vara samma allergen. Vi undersökte sera från misstänkt allergiska individer och 15-16% av serana reagerade mot Equ c 2 och den stora majoriteten av dessa svarade på båda formerna, vilket indikerar stor likhet dem emellan. Utöver detta upptäcktes ett nytt allergen, kallat Equ c 7. Detta allergen uppvisade samstämmighet med ett av kattens allergen Fel d 1, sannorlikt orsakar det symptom hos både häst- och kattallergiker. Mer än en tredjedel av de undersökta serana reagerade mot detta allergen, vilket antyder att det kan ha en viktig roll inom diagnosik och behandling. Huvudallegenet mot häst, Equ c 1, analyserades också och påvisade att en stor andel är känsliga mot detta allergen, dessutom att en övervägande andel är sensibliserade mot Equ c 4, ett allergen som har tvättmedelslika egenskaper (löddrar samt tar bort ytspänningen) och återfinns i hästars svett. Den här artikeln bidrar med en utökad kunskap om hästars allergen och hur personer som är överkänsliga mot häst reagerar mot de enskilda allergenen. Denna kunskap kan förhoppningsvis bidra till en nogrannare diagnosik och mer träffsäker behandling.

Luftburna allergens egenskaper studerades i **delarbete IV**. Hundens allergen Can f 1 är det enda allergen som undersökts tidigare för aerodynamiska attribut, d.v.s. hur allergenet sprids på luftburna partiklar. I denna studie analyserades tre allergen för första gången, Can f 1, Can f 3 och Can f 4. Can f 3 detekteras tyvärr inte i luftproverna, däremot de övriga två. Ett av analysinstrumenten detekterade storleken på partiklar som allergen sammankopplas med. Can f 1 återfanns på större partiklar inom spektrat, medan Can f 4 hittades på partiklar utav alla storlekar. Informationen är vikteg eftersom mindre partiklar kan tränga längre ner i lungorna och på så sätt ha större möjligheter att initiera en inflammation. Mindre partiklar associeras också med astma i större utstäckning. Prover togs även från luftpumpar, där Can f 1 detekteras i högre utstäckning än Can f 4. Tvärtom var det i insamlat material från en luftcentrifug där proportionen Can f 4 var större. Sammantaget visar detta att olika insamlingsmetoder har varierande förmåga att detektera lufrburet allergen i materialet. Därför bör tonviken vid val av metod, läggas på frågeställningen. Olika allegen har särskilda aerodynamiska egenskaper och detta bör vägas in i hur hälsovådliga dessa är för allergiker, hur man exponeras för dessa och riskerna med att utsättas för dem.

Sammanfattningsvis lämnar diagnostiken av allergi mycket att önska. Extrakten som testerna vanligtvis baseras på är undermåliga och med dessa som underlag riskerar fel diagnos att ställas. Därför är nyare analysinstrument intressanta då de kan detektera många fler allergen simultant och mer specifikt. Även mer kunskap om okända allergen och allergens luftburna egenskaper är viktiga för att kunna förbättra diagnos, behandling och prevention.

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