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DIAGNOSIS OF DOG ALLERGY IN CHILDREN:

Molecular assessment and refined characterization

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Diagnosis of dog allergy in children: Molecular assessment and refined characterization

THESIS FOR DOCTORAL DEGREE (Ph.D)

By

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To the children who participated for the sake of the many, many children with dog allergy

It was a great pleasure to meet you all!

THANK YOU!

SAMMANFATTNING PÅ SVENSKA

Hundallergi är vanligt bland barn i skolåldern. Trots det kan diagnosen ibland vara svår att ställa. Diagnostiken baseras på barnets symptom, fysisk undersökning och ett blod- eller hudtest som detekterar allergi-antikroppar (IgE-antikroppar) mot hundextrakt. Extraktet innehåller ett antal proteiner (allergen) som kan orsaka en allergisk reaktion, men testning med hundextrakt kan inte visa vilka av dessa som patienten är allergisk mot. Dessutom kan allergitester med hundextrakt vara positiva för hundallergi, även hos barn som aldrig haft några symptom. Beskedet att man är hundallergisk och behöver undvika hundkontakter framöver kan ha stor inverkan på livskvaliteten för ett barn, och familjen kanske måste göra sig av med en älskad familjemedlem. För att läkaren ska kunna ge bästa möjliga råd och behandling är det viktigt med korrekt diagnostik.

Man kan nu analysera IgE-antikroppar i blod mot sex allergen från hund: Can f 1- Can f 6 (Can f står för *Canis familiaris*, hund på latin). Ett av dessa, Can f 5, produceras i hanhundens prostata och utsöndras endast från hanhundar. De övriga fem finns hos alla typer av hundar, i varierande nivåer. Vad det betyder att ha IgE-antikroppar mot de olika hundallergenen är ännu inte helt klarlagt.

Huvudsyftet med denna avhandling var att utvärdera om analys av IgE-antikroppar mot de olika hundallergenen kan användas för att förfina diagnostiken bland barn som har ett positivt allergitest mot hund (IgE-antikroppar mot hundextrakt). Vi undersökte också flera kompletterade metoder för diagnostik av hundallergi.

Vi tog blodprov och undersökte förekomsten av IgE-antikroppar mot de sex hundallergenen i blod hos 60 barn och ungdomar med positivt allergitest mot hundextrakt. Barnen genomgick en nasal provokation med ett extrakt som innehöll alla 6 hundallergen. Extraktet sprayades i näsan och vi observerade om barnen fick en allergisk reaktion. De genomgick dessutom lungfunktionsundersökningar och svarade på frågor om allergiska symptom.

Många av barnen visade sig ha IgE-antikroppar mot flera allergen, och ju fler allergen barnet hade antikroppar mot, desto större var risken för att reagera allergiskt vid nasalprovokationen. Barn som bara hade IgE-antikroppar mot ett allergen löpte mindre risk att reagera och risken var lägst för de som bara hade antikroppar mot hanhunds-allergenet Can f 5. Fyra av de undersökta allergenen tillhör en proteinfamilj som också förekommer hos andra pälsdjur; lipokaliner. Vi såg att barn med IgE mot något av hundens lipokaliner löpte högre risk att reagera vid nasalprovokationen än övriga. Dessutom såg vi att barn som hade höga nivåer av IgE mot lipokalinerna Can f 2, Can f 4 och Can f 6 oftare hade svår astma.

IgE-antikroppar aktiverar bland annat basofila celler i blodet. När basofila celler aktiveras frisätter de ämnen som leder till en allergisk reaktion. Genom att mäta basofil-aktivering kan man därmed mäta den biologiska aktiviteten som IgE-antikropparna orsakar. En sådan metod är CD-sens. Vi undersökte CD-sens mot hundallergenen och såg att CD-sens var högre mot lipokalinet Can 1 bland de barn som reagerade på nasalprovokationen med hundextraktet än

bland de som inte reagerade. Dessutom hade barnen med hund hemma lägre CD-sens-nivåer mot alla undersökta allergen, vilket kan tala för att dessa barn var mindre känsliga för hundallergenen.

Det finns även en annan typ av antikropp, IgG4, som kan skydda mot allergiska reaktioner. Vi undersökte om analys av IgG4-antikroppar mot hundallergenen skulle kunna användas för att se om man tål hundar, men vi såg ingen skillnad i IgG4-nivåer mellan de som reagerade och inte reagerade på nasalprovokationen med hundextrakt. Däremot hade de barn som hade hund hemma högre nivåer av IgG4 mot Can f 1 och Can f 5 än övriga barn.

Slutligen undersökte vi hur olika gener uttrycks i nässlemhinnan bland barnen med positivt allergitest med hundextrakt och jämförde med barn som hade negativt allergitest och ingen allergisk luftvägssjukdom. Flera hundra gener uttrycktes olika mellan de två grupperna och den gen vars uttryck skilde sig mest var *CST1*. Högt uttryck av *CST1* samvarierade också med inflammation och hyperreaktivitet i luftvägarna. Därmed skulle detta genuttryck kunna vara en markör för luftvägssjukdom bland barn med misstänkt hundallergi.

Sammantaget ser det inte ut som att något enskilt hundallergen kan ge hela svaret på frågan om hundallergi, men diagnostiken kan förfinas genom undersökning av alla sex hund-allergen. Risken för att ha hundallergi är högre om man har IgE-antikroppar mot flera olika hundallergen och mot just lipokaliner. Dessutom kan man ta reda på om man bara har IgE-antikroppar mot Can f 5, och då kan man kanske tåla att ha en tik utan att reagera allergiskt.

Undersökning av en patients IgE-antikroppar mot de olika hundallergenen kan få stor betydelse. Utöver förfinad diagnostik kan även behandling komma att riktas mot de molekyler som den enskilda individen visat sig reagera mot.

ABSTRACT

Dog allergy is a common cause of rhinitis and asthma in children, yet the diagnosis is a clinical challenge. Allergic sensitization, i.e. the presence of serum IgE antibodies, to dog dander affect up to 30 % of all children and adolescents, but not all sensitized children display symptoms. The most important diagnostic tool, the detection of IgE antibodies to dog dander extracts in serum does not reveal which allergen molecule in the extract that gives rise to the allergic sensitization and symptoms. Through molecular allergy diagnostics it is now possible to detect allergic sensitization to specific allergen molecules from dog, but the clinical relevance of sensitization to the different dog allergen molecules is not yet clear. When our investigations were initiated in 2014, there were six recognized dog allergen molecules, Can f 1- Can f 6, of whom Can f 1, Can f 2, Can f 4 and Can f 6 belong to the lipocalin protein family. Can f 3 is the dog serum albumin, and Can f 5 is the male dog allergen prostatic kallikrein.

The overall aim of this doctoral thesis was to improve diagnostics of dog allergy by identifying patterns of sensitization to dog allergen molecules associated with rhinitis and asthma in dog dander sensitized children and by exploring novel biomarkers and complementary diagnostic tests for dog allergy.

In paper I, we found that a positive nasal provocation test with dog dander extract was associated with an increasing number of positive sensitizations to dog allergen molecules and with sensitization to allergens from the lipocalin protein family. When investigating the impact of the different allergens, we found that sensitization to Can f 3, Can f 4 and Can f 6 conferred an increased risk for a positive *vs* a negative nasal challenge. On the contrary, monosensitization to Can f 5 was associated with a negative nasal provocation test.

In paper II, we showed that the basophil activation tests to allergen molecules, evaluated by the basophil allergen threshold sensitivity (CD-sens), were positive in a majority of the sensitized children with a positive, as well as in those with a negative nasal provocation test. However, the levels of CD-sens to dog dander and to Can f 1 were higher in children with a positive nasal provocation. The levels of IgG or IgG4 to the investigated allergens did not differ between sensitized children with a positive and a negative nasal provocation test, while sensitized children with a dog at home had higher levels of IgG4 to Can f 1 and Can f 5 and lower CD-sens to all investigated allergen molecules.

In paper III, we performed nasal transcriptomic analysis in dog dander sensitized children and healthy controls. The most over-expressed gene in dog dander sensitized children was *CST1*, coding for Cystatin 1. *CST1* expression was enhanced in a cluster of children with lower FEV1, increased bronchial hyperreactivity, pronounced eosinophilia and higher CD-sens to dog compared with other dog dander sensitized children.

Finally, in paper IV, we showed that asthma in dog dander sensitized children was associated with multisensitization to furry animal allergen molecules and to lipocalins. Children with

severe asthma had higher IgE levels to the dog lipocalins Can f 2, Can f 4 and Can f 6 than other dog dander sensitized children. Moreover, severe asthma was associated with symptoms of dog allergy evaluated by nasal provocation testing.

In conclusion, we demonstrate that a detailed assessment using molecular allergy diagnostics may help clinicians to assess the impact of allergic sensitization on dog allergy and asthma morbidity. We found that multisensitization to dog allergens and sensitization to lipocalins is associated with dog allergy and that the analysis of CD sens, IgG4 antibodies and nasal gene expression may provide further information in the diagnosis of this common disease.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications:

I. **Käck U**, Asarnoj A, Grönlund H, Borres MP, van Hage M, Lilja G, Konradsen JR. Molecular allergy diagnostics refine characterization of children sensitized to dog dander.
J Allergy Clin Immunol. 2018 Oct;142(4):1113-1120.e9. doi: 10.1016/j.jaci.2018.05.012. Epub 2018 May 29. PMID: 29852259.

II. **Käck U**, Asarnoj A, Binnmyr J, Grönlund H, Wallén C, Lilja G, van Hage M, Nopp A#, Konradsen JR#.
shared last authorship
Basophil activation testing, IgG, and IgG4 in the diagnosis of dog allergy in children with and without a dog at home. Allergy. 2020 May;75(5):1269-1272. doi: 10.1111/all.14139. Epub 2019 Dec 22. PMID: 31802499.

III. **Käck U**, Einarsdottir E, van Hage M, Asarnoj A, James A, Nopp A, Krjutskov K, Katayama S, Kere J, Söderhäll C#, Konradsen JR#
shared last authorship.
Nasal upregulation of CST1 in dog sensitized children with severe allergic airway disease. ERJ Open Research. Accepted 2021 Jan 27; in press https://doi.org/10.1183/23120541.00917-2020.

IV. **Käck U**, van Hage M, Grönlund H, Lilja G, Asarnoj A#, Konradsen JR#
shared last authorship
Allergic sensitization to lipocalins reflects asthma morbidity in dog dander sensitized children.
Manuscript submitted for publication.

CONTENTS

LIST OF ABBREVIATIONS

ACT	Asthma Control Test
AIT	Allergen-specific immunotherapy
BAMSE	Barn, Allergi, Miljö i Stockholm en Epidemiologisk studie
BAT	Basophil activation test
Can f	*Canis familiaris* (dog)
CD-sens	Basophil allergen threshold sensitivity
CI	Confidence interval
EAACI	European Academy of Allergy and Clinical Immunology
e.g.	exempli gratia (for exemple)
Equ c	*Equus caballus* (horse)
et al.	et alia (and others)
Fel d	*Felis domesticus* (cat)
FeNO	Fraction of exhaled nitric oxide
FEV1	Forced expiratory volume in one second
GINA	Global Initiative for Asthma
i.e.	id est (that is)
IgE	Immunoglobulin E antibodies
IL	Interleukin
IQR	Inter quartile range
IUIS	International Union of Immunological Societies
MADOG	Molecular assessment of dog allergy in children
MeDALL	Mechanisms of the Development of Allergy
NPT	Nasal provocation test
NPV	Negative predicitive value
OR	Odds Ratio
PD20	Dose methacholine causing a 20 % drop in spirometry FEV1
ppb	Parts per billion
PPV	Positive predictive value
RNA	Ribonucleic acid
SPT	Skin prick test
Th	T-helper cell
WHO	World Health Organization

1 INTRODUCTION:

The dog was the first domestic animal, becoming man's best friend approximately 20 000 years ago, and is today a common family member in homes all over the globe (1). Nevertheless, the human immune system does not always recognize the dog proteins as "friends but foes" and the development of dog allergies usually occur in childhood and adolescence (2).

Dog allergy is a common perennial airborne allergy among children and adolescents and is mainly characterized by rhino-conjunctivitis and asthma. Symptoms range from discomfort due to rhinitis or conjunctivitis to severe asthma with a substantial negative effect on the allergic child's quality of life (3). Thus, correct diagnosis and advice regarding dog exposure and treatment from the physician is essential.

Allergic sensitization, i.e. the occurrence of serum IgE-antibodies (IgE) to dog dander, is the most important risk factor for the development of allergic airway disease due to dog exposure. Sensitization rates above 20 % have been reported among teenagers in Nordic countries (2, 4). Whereas sensitization to dog dander has been increasing, the corresponding increase in dog allergy has been less pronounced in recent years (4).

Although dog allergy affects a considerable proportion of the population, the diagnosis is still challenging. Today, diagnosis relies mainly on the clinical history and the detection of allergic sensitization evaluated by serum IgE antibodies (IgE) or skin prick test (SPT) to dog dander extracts. However, self-reporting is known to miss-classify the allergic status in many patients (5), and the use of dog allergen extracts in the diagnosis has several limitations. There are large variations in concentrations of allergens in the extracts, which may affect the test results (6). In addition, a positive test may be the result of cross-reactivity with allergens from other furry animals and consequently of uncertain clinical significance (7). Accordingly, there is a need for improved diagnostics.

The introduction of molecular-based allergy diagnostics offers new opportunities for refined characterization (8). We are now able to investigate IgE to the allergen molecules instead of the allergen source (dog dander extract). There are today eight known dog allergen molecules, but the clinical relevance of sensitization to each of the different allergens is not fully understood. Neither the possible role of basophil activation tests, nor the occurrence of IgG and IgG4 antibodies to the dog allergen molecules in the diagnosis of dog allergy have been evaluated clinically.

The overall aim of this doctoral thesis project was to improve diagnostics of dog allergy in children by assessing the clinical relevance of sensitization to dog allergen molecules and to evaluate the usefulness of different diagnostic methods to assess severity of the disease and differentiate between dog allergy and asymptomatic dog sensitization.

2 BACKGROUND

2.1 DOG EXPOSURE

Pet- and dog keeping varies considerably between countries and regions. In Sweden, a recent nationwide register based study found that 14.2 % of pre-school children and 8.2 % of school children were exposed to dogs at home during the first year of their life (9). According to Statistics Sweden, 15.5 % of the Swedish households with children had at least one dog in 2012 (10). When comparing eleven European birth cohorts, pet ownership among the children ranged from around 60 % on Isle of Wight in the UK to 20 % in the Stockholm area, and the prevalences of dog ownership were 30 % and 6 % respectively (11).

Dog allergens are abundant in homes with dogs, but dog allergens are difficult to avoid, even for families that do not own a dog. A nation-wide US survey found that dog allergen was present in 817 of 818 investigated homes, with and without dogs (12), and a recent German study demonstrated that day care centers may reach the same levels of dog allergens as homes with a dog (13).

2.2 ALLERGIC AIRWAY DISEASE

2.2.1 Allergic rhinitis

Allergic asthma and rhinitis are among the most common chronic diseases, and the development starts early in life (14). Allergic rhinitis is defined by inflammation of the nasal mucosa lining associated with an IgE mediated immune response to an allergen. The dominant manifestations of allergic rhinitis include nasal itching, rhinorrhea, nasal blockage and sneezing. In addition the nasal symptoms are often accompanied by conjunctivitis (15).

Allergic rhinitis might be considered a mild disease, but the burden is substantial. Allergic rhinitis impairs quality of life in many affected children and adolescents (16). Furthermore, poorly controlled allergic rhinitis can affect cognitive functions and learning ability and result in absence from school (17). Allergic rhinitis is the most commonly reported symptom induced by dog exposure in individuals sensitized to dog dander and between 5 % and 12 % of Swedish school children report rhinitis due to dog exposure (2, 18).

2.2.2 Allergic asthma

The following definition of asthma has been established by the Global Initiative for Asthma (GINA) “Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation”. The asthma diagnosis should be based on clinical history and on documentation of variable expiratory airflow limitation (19). Severe asthma in childhood is characterized by deficient asthma control despite medication with high doses of

corticosteroids and complementary asthma control medication (20). Children with severe asthma are sensitized to a larger extent to aeroallergens, display higher FeNO-levels and increased bronchial hyperresponsiveness (21).

The relationship between allergic sensitization to furry animals and allergic asthma is well established (22). Allergic asthma often debuts in childhood and is generally associated with other allergic manifestations such as allergic rhino-conjunctivitis or atopic dermatitis (23). Allergic sensitization to aeroallergens early in life is a major predictor of asthma in school children (24). Furthermore, allergic asthma that starts in childhood is often associated with severe asthma in adulthood (25). Allergen-specific immunotherapy to airway allergens has shown to improve symptom control, medication use and airway hyperresponsiveness (26). However, in the treatment of dog allergy, allergen-specific immunotherapy has shown conflicting results, which has been attributed to the quality of dog dander extracts and to complex sensitization profiles to dog allergen molecules in the patients (27).

Allergic asthma triggered by dog exposure is somewhat less common than allergic rhinitis, between 3 % and 4.5 % of Swedish school children report asthma due to dog exposure (2, 18).

2.2.3 The united airways

The relationship between asthma and allergic rhinitis is strong. In patients with allergic rhinitis 15 % to 38 % have asthma. In patients with asthma, between 6 % and 85 % show nasal symptoms (28). Patients with rhinitis are at increased risk for developing asthma (29, 30) and allergic rhinitis among pre-school children is associated with bronchial hyperreactivity at the age of seven (31). Moreover, severe rhinitis can predict a less favorable evolution of asthma (30). Appropriate treatment of allergic rhinitis can have a beneficial effect on asthma symptoms and therefore these two conditions should be assessed and treated concomitantly (15). It has also been shown that allergen-specific immunotherapy in patients with allergic rhinitis not only improves rhinitis symptoms, but also prevent the development of allergic asthma (32). Taken together, these associations between rhinitis and asthma are referred to as different manifestations of an united airways disease (30).

2.3 ALLERGIC SENSITIZATION

2.3.1 Prevalence

The prevalence of allergic sensitization to dog is increasing during childhood and adolescence. In a large Swedish birth cohort study, sensitization rates to dog dander increased from 4.8 % to 22.6 % between 4 and 16 years of age. (2, 33). A recent follow up showed that IgE-sensitization rates remained relatively unchanged from late adolescence up to age 24 years, and that male sex was associated with airborne and dog dander sensitization (34). In another Swedish pediatric population based cohort sensitization rates among 11 and 12 year old children reached 31.5 % (18). A lower sensitization rate, around 10 %, has been reported

in a German birth cohort (35), and there are large variations between different geographic areas (36). The increase in prevalence of sensitization over time is pronounced in countries with previously moderate rates. In Brazil, sensitization to dog among allergic, as well as non-allergic children, increased sharply between 2004 and 2016. In 2016, 28 % of the investigated non-allergic children showed IgE reactivity to dog (37).

Accordingly, allergic sensitization does not always induce allergic symptoms, some IgE-sensitized individuals do not display any allergic reactions. In a 16 year follow up of the Swedish population based BAMSE cohort, 23 % of the adolescents with IgE directed to different allergens had not developed allergic symptoms (38).

2.3.2 The process of allergic sensitization to an airborne allergen

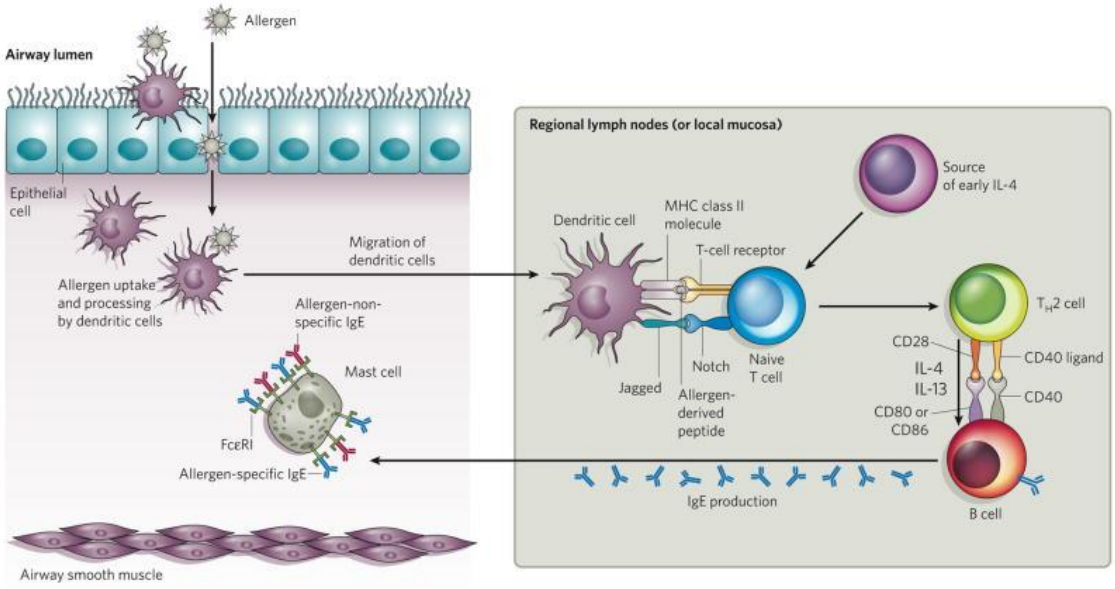


Figure 1: The process of allergic sensitization in the airways. With permission from the publisher. Galli et al. Nature 2008 (39).

Allergic sensitization is the underlying mechanism of an allergic disease. For an airway allergy, the development of allergic sensitization begins when an inhaled antigen (allergen) penetrates the airway mucosa. The allergen is recognized as foreign and taken up and processed by dendritic cells. The peptide-derived antigens are then presented to naïve T cells through MHCII molecules on the dendritic cell surface. Under the influence of IL-4 (interleukin 4), the naïve T cells will develop into effector T helper 2 cells (Th2) and T follicular helper cells (Tfh) and are stimulated to produce IL4 and IL13. These cytokines stimulate in turn B lymphocytes to switch to IgE-producing plasma cells, and to produce large amounts of specific IgE directed to the initially presented antigen (39).

2.3.3 The IgE mediated allergic reaction in the airway mucosa

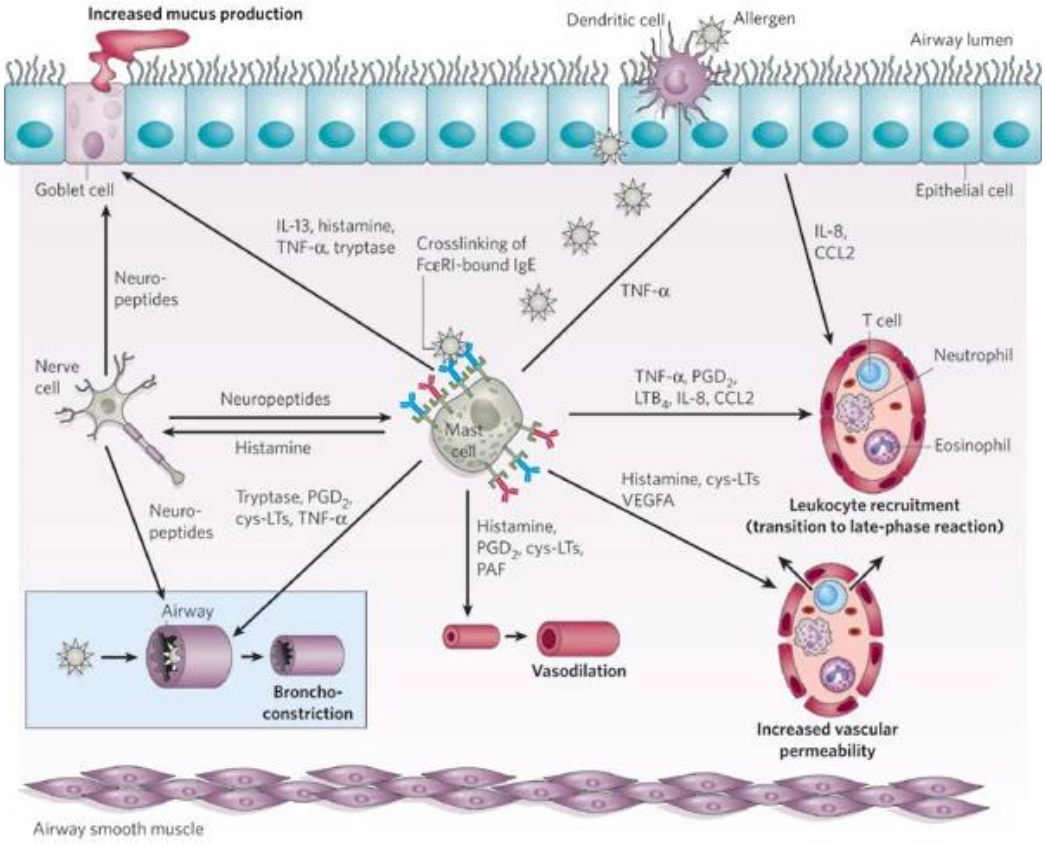


Figure 2: Early phase of the airway inflammation induced by an allergen. With permission from the publisher. Galli et al. Nature 2008 (39).

IgE antibodies produced by plasma cells in a sensitized individual bind to high-affinity FcƐ receptors expressed on mast cells and basophils. Re-exposure to the allergen induces an acute-phase response by cross-linking of FcƐ-bound IgE on the mast cell surface. This leads to degranulation and secretion of e.g. histamine, tryptase and subsequently leukotrienes and prostaglandins. The mediator release causes increased mucus production, vasodilatation, broncho-constriction and increased vascular permeability with acute onset of allergic symptoms: rhinitis in upper airways and asthma symptoms from the lower airways. The mediator release further initiates the recruitment and migration of inflammatory cells, including T cells, eosinophils and neutrophil granulocytes, which subsequently will lead to the late phase allergic reaction. The late phase reaction occurs hours after the early phase. Eosinophils and neutrophils cause tissue damage through release of proteases and the T cells may exacerbate the allergic reaction by further release of cytokines.

Why certain individuals produce IgE to normally harmless proteins is still largely a question to be resolved. The explanation to these events is thought to be due to the cytokines produced by the Th1 cells and Th2 cells, with an excess of Th2 cell cytokines. The etiology of the imbalance leading to allergic sensitization is multifactorial, including host factors, e.g. genetic and epigenetic factors, the microbiome and environmental exposures as important

determinants (40). Decreased Th1- and increased Th2-associated chemokine levels during childhood has been associated with allergic symptoms and sensitization in children possibly influenced by the maternal immunity during pregnancy (41).

Hereditary predisposition is a well-known and important contributing factor to allergic sensitization (42). This link seems particularly strong in airway allergy. A recent prospective population based study could demonstrate that parental history of atopy (allergy, eczema and asthma) was associated with increased risk of physician-diagnosed inhalant allergy, but not with food allergy in children at age 10 (43).

Early exposure to micro-organisms has been suggested to protect against allergies since the hygiene hypothesis was presented by Strachan in 1989 (44). A recent Swedish nation-wide cohort study could demonstrate that early exposure to farm animals was associated with a decreased risk for asthma in both pre-school and school children (9). Furthermore, there is increasing evidence that living with a cat or a dog during the first years of life is associated with a decreased risk for future allergy (45, 46).

2.3.4 Allergens

Allergens are antigens with the ability to cross-link IgE, and subsequently activate mast cells and basophils. Allergens are, with a few exceptions, proteins that share some important features, such as several binding sites for IgE (epitopes) and low molecular weight. Several epitopes are needed for the ability to cross-link IgE (47). Lately, adjuvant properties of the allergens and interaction with the airway epithelium have come into focus (48). Some allergens, i.e. several pollens, have the ability to impinge the epithelial barrier through protease activity (49).

Dog dander extract is an allergen *source* consisting of several allergens. All allergen molecules are recorded and named using the systematic nomenclature by the World Health Organization and the International Union of Immunological Societies (WHO/IUIS) (50). The three first letters of the Latin/linnean name are followed by the first letter of the species name and finally a number indicating the chronology of allergen purification, i.e. the first recognized dog (*Canis familiaris*) allergen is Can f 1. Allergen molecules eliciting an IgE response in more than 50 % of the population sensitized to an allergen source are generally regarded as “major allergens” (51).

2.3.5 Cross-reactivity

Some allergens are thought to be specific for the allergen source, whereas others cross-reactive with several allergens from other furry animals (52, 53). Cross-reactions occur between allergens with similar binding sites or epitopes: IgE antibodies produced in response to one allergen recognizes similar binding sites/epitopes on another allergen and can bind to these sites. This results in a positive IgE response to both allergens and can, in some cases, initiate an allergic reaction to both allergens from different allergen sources. Generally cross-

reactivity requires high peptide sequence identity (> 50 %) and/or similar tertiary protein structure (53). Accordingly, cross-reactions mainly occur between allergens from the same protein families, for example serum albumins from different furred animals. A primarily horse or cat allergic individual may thus have a positive IgE response to dog due to serum albumin sensitization. It has also been shown that serum albumin peptides from horse inhibit IgE to dog and cat as well as horse (54). Serum albumins have been estimated to account for the cross-reactivity observed in around one-third of patients sensitized to cat, dog and horse (55).

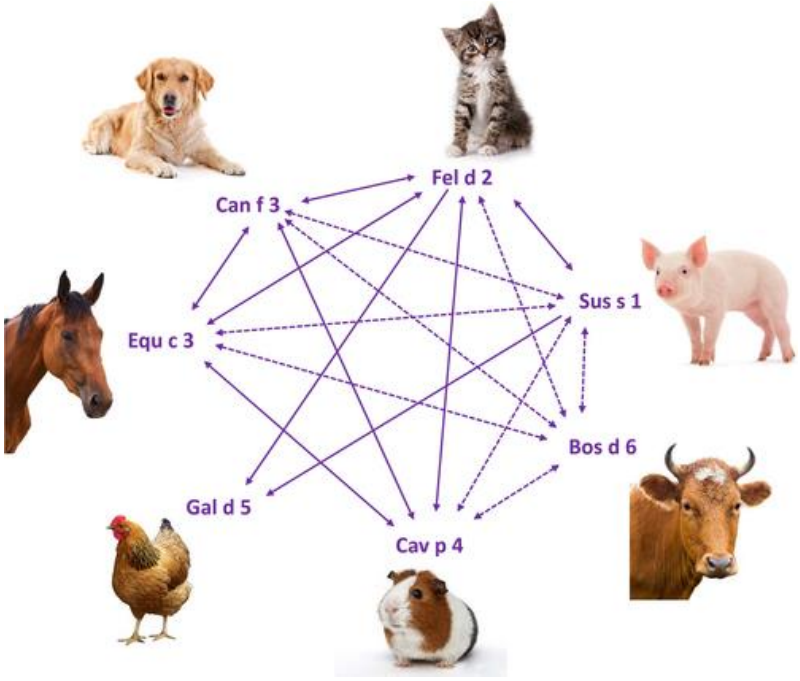


Figure 3: IgE cross-reactivity between serum albumins. The lines represent documented cross reactivity and the dotted lines represent possible cross-reactivity due to high peptide sequence-identity. With permission from the publisher. Matricardi et al. Pediatric Allergy and Immunology 2016 (52).

The detection of cross-reactivity has emerged as an important diagnostic tool in food allergy, for example peanut allergy, to differentiate between severe, sometimes life threatening reactions, and the itching and swelling in oral allergy syndrome (56). However, when investigating allergy to furry animals, it has been challenging to elucidate the clinical significance of cross-reactivity and more research is needed (57).

2.3.6 Molecular spreading and poly-sensitization

The concept of "molecular spreading" refers to the timely development of multiple sensitizations to distinct non cross-reacting allergens from the same allergen source. This process generally starts with an "initiator molecule" (58). In 2012, Hatzler et al could demonstrate a typical progression of IgE sensitization to timothy (*Phleum pratense*, Phl p) in children over time, starting with sensitization to Phl p 1, followed by Phl p 4, Phl p 5 and subsequently several other timothy allergens. The initial sensitization and the beginning of the molecular spreading often preceded symptoms of grass pollen related rhinitis (59).

Similar patterns of evolution of IgE sensitization to mite (*Dermatophagoides pteronyssinus*-Der p) have been demonstrated, and early onset was associated with stronger molecular spreading, which in turn predicted allergic rhinitis related to mite exposure and asthma (60). These patterns have been proposed to be useful for predicting severe symptoms and to advocate for early allergen-specific immune therapy (58). Moreover, early age sensitization to a number of "risk-allergen molecules" from different allergen sources have been shown to identify children with a high risk of developing allergic rhinitis and asthma comorbidity at the age of 16 (61).

The molecular evolution of IgE responses to allergens from dog have been demonstrated by Asarnoj et al. The prevalence of children with sensitization to any of five investigated allergen molecules increased from 3.6 % at age 4 through 8.2 % at age 8 to 14.8 % at the age of 16. Early polysensitization to allergen molecules from dog could predict allergy at age 16 significantly better than IgE to dog extract (2). Furthermore, sensitization to more than three allergen molecules from the lipocalin, prostatic kallikrein and secretoglobin protein families has been associated with severe asthma (62). The pan-European research network MeDALL (Mechanisms of the Development of Allergy) has recently introduced the concept that mono- and polysensitized individuals represent different phenotypes. They demonstrate that polysensitization is associated with multiple manifestations of allergic disease and with more severe disease (63).

Taken together, these findings from different cohorts demonstrate different appearances of allergic sensitization in relation to clinical presentation and highlight the need for in-depth knowledge regarding the role of specific allergens in allergic disease.

2.4 THE DOG ALLERGENS

When our investigations started in 2014, there were 6 recognized dog allergens in the WHO/IUIS Allergen Nomenclature Sub-committee database, Can f 1- Can f 6. The list has since then expanded with two more allergens, Can f 7 and Can f 8, and currently eight dog allergens are registered (64).

Table 1: Dog allergens currently recognized by the WHO/IUIS Allergen Nomenclature Sub-committee, their molecular weight and the prevalence of sensitization among dog dander sensitized.

Dog allergen molecules	Protein family	Molecular weight	Prevalence of sensitization
Can f 1	Lipocalin	23-25 kDa	50-75 %
Can f 2	Lipocalin	19 (27) kDa	20-33 %
Can f 3	Serum albumin	69 kDa	35 %
Can f 4	Lipocalin	16-18 kDa	35-81 %
Can f 5	Prostatic kallikrein	28 kDa	70 %
Can f 6	Lipocalin	27 and 29 kDa	23-61 %
Can f 7	NPC2	16 kDa	10-20 %
Can f 8	Cystatin	14 kDa	13 %

2.4.1 Dog lipocalins

A majority of the mammalian allergens are lipocalins (57, 65). There are four known dog-derived lipocalins: Can f 1, Can f 2, Can f 4 and Can f 6 (52). The lipocalins are small molecules (150-200 amino-acids) found in dog dander, saliva and urine. They are carried by relatively small particles and become easily airborne and can be found in homes as well as in schools and other public areas (13, 66). Lipocalins were initially thought to be species specific due to relatively low amino acid sequence homology but have subsequently been shown to cross-react with lipocalins from other mammalian species (57, 67). It has also recently been shown that sensitization to furry animal allergens from the lipocalin family, are independently associated with asthma and rhinitis in children (68).

Can f 1 was the first recognized dog allergen (69) and is generally considered a major allergen with sensitization rates between 50-75 % among dog dander sensitized individuals (57). Can f 1 is secreted from the dog's sebaceous gland and found in fur and saliva (70, 71). Due to the small size of the carrier molecules, Can f 1 can be inhaled more easily into the lower airways than larger particles, such as pollen grains, and initiate an asthma attack (72).

IgE to Can f 1 has been found to be associated with persistent rhinitis in patients with allergy to furry animals (73). Sensitization to Can f 1 in childhood has also been shown to predict dog allergy at age 16 better than sensitization to dog dander (2). Nevertheless, IgE to Can f 1 is insufficient to diagnose dog allergy (74). Can f 1 has been regarded as a species specific allergen for dog, but has extensive sequence homology and cross-reacts *in vitro* with the cat lipocalin, Fel d 7, which make clinically significant cross-reactions plausible (75).

Can f 2 was detected as "dog allergen 2" by de Groot et al. in 1991, and the authors stated that Can f 2 was a less important allergen with a sensitization rate of 23 % among dog allergic patients (76). Can f 2 is a salivary protein produced by tongue and parotid glands (77). In a recent study of dog allergen content in dog dander extract, Can f 2 was found in low levels in fur as well as in skin prick test extracts (71). IgE to the lipocalin Can f 2 occurs mainly as concomitant sensitization with Can f 1 (74), and 20-33 % of dog dander sensitized individuals have eventually been estimated to be sensitized to Can f 2 (7). Despite findings indicating that Can f 2 is of less importance for dog allergy, IgE reactivity to Can f 2 was more common in children with severe asthma than in children with controlled asthma (3). Furthermore, in an adult population, IgE to Can f 2 has been shown to be associated with asthma diagnosis (73). Despite important structural similarities with the horse lipocalin Equ c 1, no IgE cross-reactivity was detected between these allergens. However, Can f 2 has shown patient-dependent cross-reactivity with the cat lipocalin Fel d 4, despite a low sequence homology, but the clinical relevance has not yet been established (78).

Can f 4 is abundant in dog fur and in dog saliva (71). Can f 4 was purified by Mattsson et al. and cross-reacts *in vitro* with a protein from bovine dander, but not with any known allergen from cat or dog. IgE to Can f 4 is present in between 35 % and 81 % of dog allergic subjects (79, 80). This large variation in sensitization rates is thought to be due to the denaturation of

the protein which affect the IgE binding capacity (80). The detection of IgE to Can f 4 in patients has not been available for clinical settings until recently, consequently little has been reported regarding the clinical significance and utility of Can f 4 as a marker for allergic disease.

In the search of a dog lipocalin protein that had shown extensive sequence homology with Fel d 4, **Can f 6** was purified by Hilger et al. (81). Can f 6 shows high peptide sequence identity to cat Fel d 4 (67 %) and to horse Equ c 1 (57 %) and cross-reacts with these allergens with an uncertain clinical impact (82). Sensitization rates to Can f 6 are estimated between 23 % and 61 % among dog dander sensitized individuals (81, 82). Since clinical settings have not had the possibility to investigate sensitization to Can f 4 and/or Can f 6, reports on the clinical relevance of these two dog allergens are scarce.

2.4.2 Dog serum albumin

Serum albumins are abundant in saliva and dander. They display extensive cross-reactivity between serum albumins from different mammal species and are generally considered minor allergens with around 35 % sensitization rates among dog allergic individuals (55, 83, 84). The dog serum albumin **Can f 3** has been considered to be a less important allergen and rather a marker for cross-reactivity (85), but the results from clinical studies are somewhat contradictory. Among patients attending an allergy clinic, a strong association between sensitization to Can f 3 and severe respiratory symptoms has been reported (73). However, in a pediatric population based cohort, sensitization to Can f 3 was reported to be uncommon and no association with asthma was seen (18)**.**

2.4.3 Dog prostatic kallikrein

Dog prostatic kallikrein was identified in 2009 by Mattsson et al and was labeled **Can f 5**. The authors reported that around 70 % of a dog allergic population was sensitized to this allergen. Can f 5 is produced in the male dog's prostate, secreted in the urine and present both in urine and dander (86). Can f 5 has not been found to disperse in society in the same way as lipocalins and direct exposure to male dogs is thought to be the main source of sensitization (87). Exposure to male dogs has recently been described as a risk factor for exclusive sensitization to Can f 5 (88).

A considerable proportion of dog dander sensitized individuals seem to be monosensitized to Can f 5 (sensitized to Can f 5, but no other dog allergens) and accordingly, these individuals might have an exclusive male dog allergy. In a Swedish pediatric population-based study 56 % of all dog sensitized 16 year's old were monosensitized to Can f 5, and the proportions have been rather high in Spanish (37 %) and Italian (58 %) disease specific cohorts (2, 89, 90). However, the concept of "monosensitization" has in most studies been based on the sensitization to Can f 1, Can f 2, Can f 3 and Can f 5 and no previous studies have taken all known dog allergens into account.

A case report could confirm that a woman, who was exclusively sensitized to Can f 5 had a positive conjunctival provocation test with male dog dander extract, but not with female dog dander extract (91). This finding was recently verified in a group of Can f 5 monosensitized children (92). Even though monosensitization to Can f 5 has been investigated in several populations, the prevalence of exclusive male dog allergy is not yet known. Asarnoj et al. found that monosensitization to Can f 5 was common among sensitized, but dog-asymptomatic children (2). Despite this finding regarding monosensitization, Can f 5 seems to play a role in airborne allergy, especially in concomitant sensitization with other dog allergens: Uriarte et al. found a strong association between the presence of IgE to Can f 5 and reported severe persistent rhinitis (73). Moreover, a strong relationship between sensitization to Can f 5 and asthma has been reported (93). Fall et al, could show that children who grew up with female dogs had a lower prevalence of asthma at age 6, compared to children who grew up with male dogs (94), which raises the hypothesis that excretion of Can f 5 from male dogs and subsequent Can f 5 sensitization in the children could explain this difference.

There are no known cross-reactions between Can f 5 and any other mammalian allergen, but Can f 5 shows 60 % sequence identity and cross-reacts with human prostate-specific antigen (95). Consequently, sensitization to Can f 5 in women might lead to allergic reactions to human seminal fluid at intercourse. There are now several clinical reports of Can f 5 involvement in human seminal plasma allergy (96-98).

2.4.4 More recently discovered dog allergens

Can f 7, the dog NPC2 protein, was recently characterized. Can f 7 was previously known as a dog epididymal protein and a structural homologue to the human epididymis protein HE1, but not as an allergen. Sensitization rates to this dog allergen has been estimated to 10-20 % among dog allergic individuals. (99, 100). A Cystatin allergen **Can f 8**, with a 13 % sensitization rate among dog dander sensitized, was recently added to the WHO/IUIS database of recognized allergens (64).

2.5 CAT- AND HORSE ALLERGENS

There are currently eight registered cat allergens, of whom Fel d 1, the cat uteroglobin is dominant. Around 95 % of all cat allergic subjects display IgE reactivity to Fel d 1 (101), making the molecular diagnosis for cat allergy more straightforward than for dog allergy. There are two known cat lipocalins, Fel d 4 and Fel d 7. The cat serum albumin is Fel d 2.

Five horse allergens are registered, of whom two are lipocalins: Equ c 1 and Equ c 2. Equ c 3 is the horse serum albumin (64). Up to 76 % of patients with horse allergy are sensitized to Equ c 1 (67), and sensitization has been associated with severe asthma in children (3).

2.6 DOG ALLERGY: THE DIAGNOSTIC APPROACH

A detailed structured allergy history and physical examination is the basis for allergy diagnostics. Which organs are affected? Are the symptoms perennial? Are the symptoms progressing? Which allergen source is thought to cause symptoms? Are there any plausible differential diagnoses? Skin prick test or IgE to dog dander extract can confirm dog dander sensitization in an individual with suspected dog allergy. In cases where the clinical history and the sensitization test are concordant, this evaluation may be sufficient for the dog allergy diagnosis. However, if the diagnosis is still uncertain, The European Academy of Allergy and Clinical Immunology (EAACI) Molecular Allergology User's Guide proposes that molecular based allergy diagnostics can be useful in differentiating between primary and cross-sensitization, and to detect risk molecules. Nasal provocation test with the suspected allergen source (e.g. dog dander extract) should be considered in uncertain cases (52).

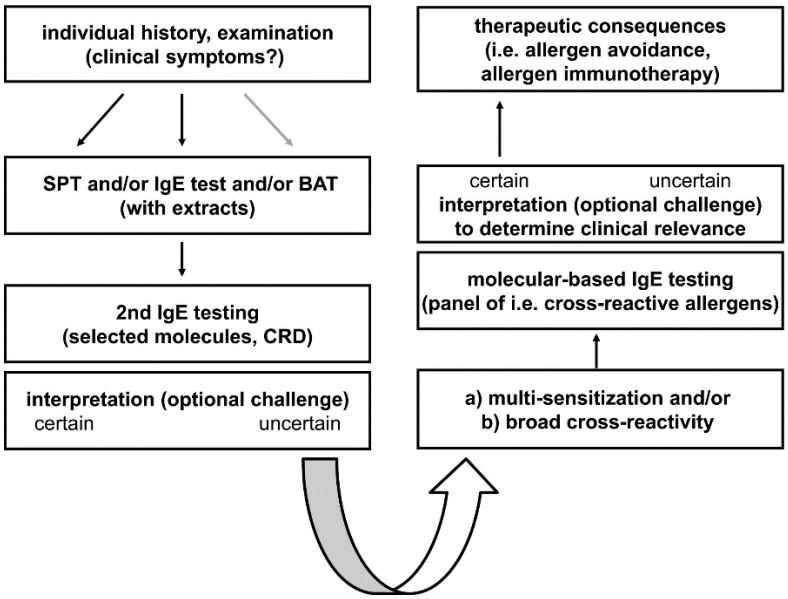


Figure 4: Standard diagnostic approach completed with broad molecular based IgE testing proposed by EAACI. This "U-shaped" approach, has been proposed for complex cases. (CRD; component resolved diagnostics; molecular diagnostics.). With permission from the publisher. Matricardi et al. Pediatric Allergy and Immunology 2016 (58).

Further EAACI has proposed a "U-shaped" approach for complex cases with molecular diagnostics detecting multisensitization and broad cross-reactivity and assess these patterns for further targeted molecular based testing in relation to the clinical symptoms. However, there are still several questions regarding the relevance of dog allergen sensitization (102).

2.6.1 Skin prick tests (SPT)

The SPT is a test of cutaneous reactivity as a marker for allergic sensitization. A droplet of dog dander extract is placed on the patient's forearm. The skin is then superficially punctured with a lancet. The allergen causes a local reaction due to mast cell degranulation after IgE

cross-binding by the tested allergen in a sensitized individual. A wheal size ≥ 3 mm is considered positive (103). The GA(2)LEN skin test study found a positive SPT with dog dander extract to be clinically relevant in 60.3 % of the sensitized cases attending European allergy clinics (104). A later evaluation showed that the positive predictive value (PPV) of a positive SPT (wheal ≥ 3 mm) was 57 % for reported clinical symptoms, and to obtain a 80 % PPV a wheals size of 10 mm was required, which is larger than for most inhalant allergens (105). There are still some obvious advantages: the test provides an immediate response, it is cheap and considered safe (103). Important disadvantages are that dog dander extracts have shown marked variations in content of major and minor allergens, salivary allergens tend to be underrepresented, and they do not reveal which allergen is responsible for the reaction (6, 71, 106).

2.6.2 Serum IgE assays

The serum IgE assay provide direct proof of allergic sensitization to dog dander extract from a blood sample. The most extensively studied assay is the Immuno-CAP System (Thermo Fisher, Uppsala, Sweden), where 1 International Unit (IU) is equal to 2.42 ng of serum-IgE (107). The allergen extract is coupled to a solid phase, and the patient's serum is added. Serum IgE directed against the allergen will bind to the allergen. Fluorescent anti-IgE is then added and the allergen bound IgE can thus be quantified.

Diagnostic testing with serum IgE detection and SPT to aeroallergens has, according to previous studies, showed similar performance in terms of sensitivity and specificity, but the serum IgE assay has shown better predictive values for future rhino-conjunctivitis in children (108).

A major advantage with the serum IgE assays is that they quantify the IgE levels (109). Serum IgE assays can be performed in patients when the SPT is not feasible, for example in patients who have extensive allergic skin disease, or who are taking antihistamines that can interfere with the SPT result (110). The disadvantages with serum IgE assays to dog dander are mainly the same as for SPT, since testing with allergen extracts do not reveal the sensitizing allergen. In addition, commonly used cut-off values for a positive test are determined on the basis of detection limits rather than clinical significance (111). Thus the IgE test or the SPT to dog dander can be regarded as a screening test and if the result do not lead to a satisfactory diagnostic conclusion, molecular allergy diagnostics can be performed (52).

2.6.3 Molecular allergy diagnostics

It is now possible to detect IgE to purified natural or recombinant allergen molecules instead of allergen extracts. Sensitization to allergen molecules can be detected using the same methodology as with serum IgE to dog dander extract (singleplex ImmunoCAP) or multiplex ImmunoCAP Immuno Solid-phase Allergen Chip (ISAC) assays detecting IgE to a large number of allergen molecules from different allergen sources (109). Analysis of serum IgE to

allergen molecules can not only detect the allergen responsible for the allergic reaction, but also reveal more complex patterns of sensitization, such as multisensitization and cross-sensitization. Sensitization to several allergens from the same species has shown to be a risk marker for pet allergy (2, 18). By investigating the patterns of sensitization to different allergen molecules from an allergen source, the diagnostic precision may be improved (112). In dog allergy diagnostics, however, there is still a need for more knowledge regarding the impact of sensitization to the specific allergens on rhinitis and asthma and on the severity of the disease.

2.6.4 Nasal provocation testing (NPT)

Nasal provocation testing (NPT) reproduces the allergic reaction of the nose under standardized and controlled conditions (113). NPT's are considered gold standard in the diagnosis of allergic rhinitis as they provide direct proof of symptoms and have shown good repeatability (114, 115). Nasal challenges are also important in clinical research and provide the possibility to evaluate treatment effects. Despite a broad area of applications there have, until recently, been no international consensus guidelines for nasal provocation testing (116). Criteria for positivity, methodologies and allergen preparations utilized in challenges have not been uniform (117-119), which have resulted in divergences that make international comparisons difficult. Recently the European Academy of Allergy and Clinical Immunology (EAACI) presented a position paper on the standardization of nasal allergen challenges (115).

The main recommendations include a bilateral nasal provocation test with a standardized allergen solution, using a spray device offering 0.1 mL per nostril. Positivity criteria can be based on symptom scoring or a combination of symptom scoring and objective measurement of nasal patency, for instance peak nasal inspiratory flow (120). Assessing symptoms is the most relevant outcome parameter in nasal allergen provocation test (117, 120), and there are several accepted symptom scores containing the key symptoms: sneezing, nasal pruritus, rhinorrhea, nasal obstruction, and ocular symptoms (121, 122).

As with SPT and serum IgE performed with whole extract, the result may be hampered by poorly standardized allergen extracts with too low allergen concentration of the relevant allergen, and lead to a false negative result (115). The results from nasal provocation testing with dog dander extract have never been investigated in relation to sensitization to the dog allergen molecules.

2.7 COMPLEMENTARY DIAGNOSTIC METHODS AND BIOMARKERS

2.7.1 Basophil activation test

Basophilic granulocytes share important features with mast cells. They originate from the same precursor cell in the bone marrow and bind IgE to the cell surface. The cells are activated through cross-binding of allergens to IgE and histamine-containing granulae are released. While mast cells primarily are tissue resident, basophils are accessible for analysis

through a blood sample (123). After anaphylactic degranulation, basophils express CD63 from the inside of the histamine-containing granulae on the cell surface, which can be measured by flow cytometry. The basophil activation test (BAT) is a measure of allergic activity. While serum IgE determination can only confirm the presence of IgE, BAT measures the biological function: the result of IgE cross-linking by an allergen which leads to basophil activation and degranulation (124). The basophil activation test may thus be a possible *in-vitro* alternative to *in-vivo* provocation tests.

There are two common measures of basophil activation, basophil reactivity and basophil sensitivity. Basophil reactivity measures the basophil response at a given concentration of allergen and provides a positive or negative result. Basophil sensitivity can be assessed by stimulating basophils with increasing concentrations of allergen (125). The basophil response (upregulation of CD63) to the allergen is plotted on a curve of reactivity *vs* allergen concentration and provide a measure of sensitivity.

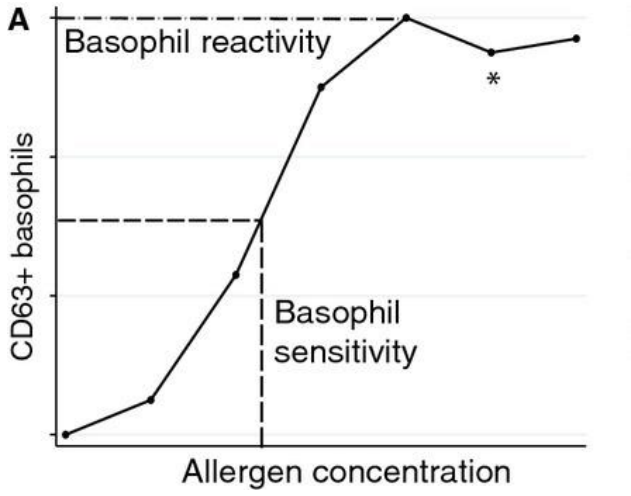


Figure 5: Basopil reactivity and baspophil sensitivity. The maximum basophil response represents the basophil reactivity, and the allergen concentration leading to 50% of the maximum basophil response (EC50) represent the basophil sensitivity. *The basophil response may be suppressed at high allergen concentrations. With permission from the publishers. Hoffman et al. Allergy 2015, adapted from Patil et al. CEA 2012 (124, 126).

In our studies we used the basophil allergen threshold sensitivity (CD-sens) as a measure of basophil sensitivity. The allergen concentration giving 50 % (EC50) of the maximum CD63 upregulation is calculated, and CD-sens is defined as the inverted value of EC50 multiplied by 100 (127). Thus, activation of basophils at low concentrations corresponds to high allergen sensitivity.

CD-sens has shown to correlate with *in-vivo* allergen provocations both in the upper and lower airways (128, 129), and also to correlate with peak nasal inspiratory flow and reported nasal symptoms in grass pollen allergic subjects (128). However, CD-sens has not yet been evaluated in relation to sensitization to dog allergen molecules or as a diagnostic tool for dog allergy. Nor has CD-sens to dog been evaluated in relation to the severity of the allergic disease.

2.7.2 IgG and IgG4 as possible markers for tolerance

Whether exposure to furry animals induces tolerance or allergy is a question that has been debated (130). Multiple studies do now report a possible protective effect of pet ownership on allergic airway disease, but the mechanisms of this protective effect are still not known (11, 94, 130-133). A suggested mechanism of tolerance at exposure is the induction of IgG and IgG4 which has been classified as a "modified Th2 immune response" (134). IgG is the most common immunoglobulin in humans, and there are four subclasses. IgG4 is the least abundant of the IgG-antibodies, and the appearance of IgG4 is usually associated with continuous exposure to an allergen and sometimes a decrease in allergic symptoms (135). Allergen-specific IgG4 antibodies are thought to protect from allergic reactions by blocking binding sites for IgE on basophil and mast cells (135, 136).

Clinical studies of IgE and IgG antibodies to cat show that IgG4 covariates with exposure, with divergent results regarding the clinical protective effect. Perzanowski et al. have shown lower prevalence of IgE and higher prevalences of IgG and IgG4 antibodies to the major cat allergen Fel d 1 in children and adolescents with a cat at home. On the other hand, the occurrence of IgG4 could not predict symptoms (93, 133). However, in cat sensitized individuals, decreased exposure to cat has also shown to lead to a decreased titer of IgG and IgG4 to Fel d 1, and in some cases the recurrence of clinical symptoms upon cat exposure (137). Investigations of microarrayed dog, cat and horse allergen molecules have shown weak correlations between allergen-specific IgE and IgG responses, which suggest a non-sequential class switch and that IgG and IgE to furry animals may be directed towards different binding sites of the allergen (138).

Few studies have yet evaluated the clinical significance of allergen-specific IgG- and IgG4 responses to dog allergens and whether IgG antibodies mainly reflect exposure or tolerance. However, Burnett et al. showed that teenagers symptomatic after dog exposure had higher Can f 1 serum IgE levels and lower serum IgG4/IgE, but similar levels of IgG4 compared with asymptomatic participants (139).

2.7.3 Gene expression in dog dander sensitized children

Genetic mechanisms do, as previously mentioned, play an important role in the individual's development of allergic disease. However, gene expression, the production of m-RNA, differs between body tissues (140), as well as between individuals in different physiological conditions (141). Microarray based gene expression analysis of bronchial airway epithelial brushings in adults with asthma has revealed a number of genes with dysregulated expression in the bronchial airways (142). Furthermore, patterns of Th2-driven inflammation that was characterized by the expression of several IL-13 inducible genes was seen in a sub-group of the asthmatic subjects. These gene expression patterns correlated with higher IgE levels, response to inhaled corticosteroids and higher peripheral blood eosinophil counts (143). Since gene expression patterns seem to reflect the phenotypic heterogeneity in asthmatic patients,

gene expression profiles may be valuable in the diagnosis of allergic asthma and in monitoring the disease.

However, to access the bronchial epithelium, a bronchoscopy is required, which is unreasonably invasive in routine practice, especially in children. The unified airway hypothesis proposes that disease mechanisms and airway remodeling detected in the lower airways are also reflected in the upper airway epithelium (144, 145). Recently, investigations of correlations between gene expression in nasal and bronchial epithelium in asthmatic children could show that the bronchial differential expression was strongly correlated with the nasal differential expression (146). Moreover, gene expression profiles were altered in the nasal brushings of asthmatic children versus those of healthy control children (147). Finally, children experiencing asthma exacerbations exhibited altered gene expression in the nasal airways compared with children whose asthma was stable (148).

Differential gene expression patterns in dog sensitized individuals compared to non-sensitized have not yet been investigated and could provide biomarkers for allergy to furry animals and future targets for therapy.

3 RESEARCH AIMS

Overall aims

The overall aim of this doctoral thesis project is to improve diagnostics of dog allergy in children by identifying patterns of sensitization to dog allergens associated with rhino-conjunctivitis and asthma and by exploring novel biomarkers and complementary diagnostic tests for dog allergy.

Specific aims

To investigate the prevalence of sensitization to dog allergen molecules in children and adolescents sensitized to dog and describe the patterns of IgE reactivity associated with dog allergy, evaluated by NPT and clinical history (Paper I).

To investigate how the results from basophil activation testing (CD-sens) and analysis of IgG antibodies to dog allergens relate to dog allergy, evaluated by NPT and to dog exposure at home (Paper II).

To investigate nasal gene expression in children sensitized to dog dander compared to non-sensitized control children and relate these gene expression patterns to clinical symptoms and biomarkers of allergy (Paper III).

To investigate sensitization to dog allergens in relation to clinical manifestations of asthma through evaluation of symptom scoring, lung function (spirometry), airway inflammation (exhaled NO) and airway responsiveness (methacholine provocation) (Paper IV).

4 MATERIALS AND METHODS

This thesis is based on the MADOG study (Molecular assessment of dog allergy in children), which is an observational explorative study of dog dander sensitized children.

4.1 STUDY POPULATION

Children and adolescents between 10 and 18 years of age participated. All patients were recruited from pediatric outpatient clinics in the Stockholm region they were attending due to suspected or confirmed airway allergy. The primary inclusion criterion was positive IgE (≥ 0.1 kU_A/l) or positive skin prick test (wheal size > 3 mm) to dog dander. Patients with known impaired lung function due to other causes than asthma and patients with ongoing or completed immunotherapy to furry animals were excluded. Patients were invited to participate regardless of symptoms of dog allergy, as the relation between patterns of IgE sensitization and symptoms was a main focus of this research project.

Twenty age matched healthy controls were recruited from the same geographic area through advertising. Healthy controls were included if they reported no symptom of rhinitis or asthma and had a negative serum IgE to dog dander (IgE < 0.1 kU_A/l).

4.2 STUDY DESIGN

Included dog dander sensitized patients made two visits at Barnforskningscentrum and the healthy controls one visit:

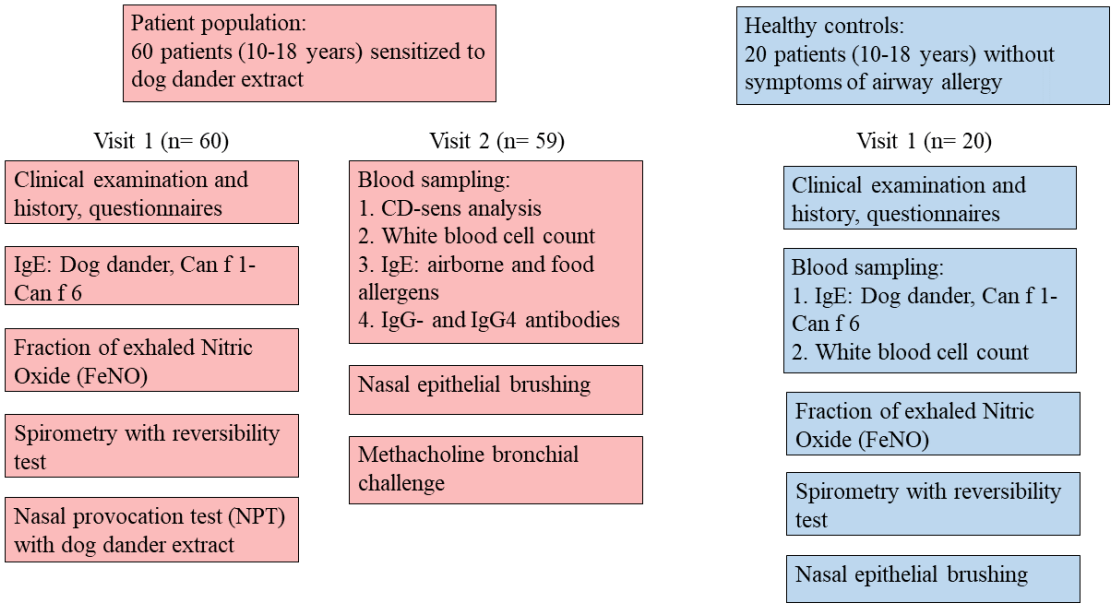
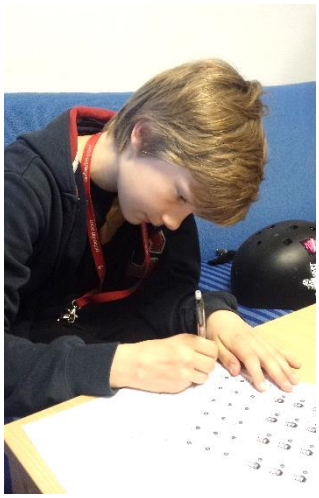


Figure 6: Schematic overview of the MADOG procedures. All but one dog sensitized child completed the two visits; one only participated in visit 1 and could only be included in Paper I. Among the healthy controls 3/20 had IgE to dog dander ≥ 0.1 kU_A/L and were excluded.

4.3 STUDY PROCEDURES

All procedures, except from analysis of the nasal brushings (RNA extraction and transcriptome library preparation and sequencing), were performed at Barnforskningscentrum, Södersjukhuset, Stockholm, Sweden. I conducted the interviews and investigated all patients in collaboration with two research nurses throughout the studies.

Interviews (Paper I-IV): All children and their parents were interviewed according to a standardized questionnaire which was a modified version of the questionnaire used in the Environmental and Childhood Asthma Study (149). The interview included questions regarding demographic data; family and patient history of rhinitis and asthma, other atopic manifestations, exposure to pets as well as symptom triggers, symptoms and medication for asthma and rhinitis.



Asthma Control Test (Paper III and IV): Asthma control was assessed according to the Pediatric Asthma Control Test among children 10-11 years of age (maximum score 27) and Asthma Control Test for individuals above the age of 12 (maximum score 25). A score below 20 indicates deficient asthma control for both tests (150, 151).

Physical examination: Prior to the nasal provocation test a physical examination was conducted including lung and heart auscultation, inspection of the oral cavity and the skin. Height and weight were recorded.

Analysis in blood and serum (Paper I-IV): Blood samples were collected on two separate occasions in dog dander sensitized patients and on one occasion in healthy controls after application of local anesthesia.

IgE to dog dander and IgE to the dog allergen molecules Can f 1- Can f 6 were analyzed. Further, IgE against other airborne allergens (cat- and horse dander, timothy, birch, mugwort, *Dermatophagoides pteronyssinus, Dermatophagoides farinae* and *Cladosporium herbarum*) and the food mix Fx5 (egg white, peanut, cow's milk, wheat, soy bean and codfish) were analyzed. Sera that scored positive (IgE $\geq$ 0.10 kU_A/l) for cat and horse extracts were further analyzed for IgE against cat allergens (Fel d 1, Fel d 2, Fel d 4) and horse allergen (Equ c 1). Sera showing an IgE $\geq$ 0.35 kU_A/l for Fx5 were analyzed for the single allergens included in the mix. All IgE determinations were performed using the ImmunoCAP System (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's instructions. The results are presented as kU_A/L and the cut-off level for single allergens was $\geq$ 0.10 kU_A/L.

IgG and IgG4 antibodies to dog dander and to the dog allergen molecules Can f 1- Can f 6 were analyzed using the ImmunoCAP system. The results are presented as mg/L and the cut-off for allergen-specific IgG was $\geq$ 2 mg/L and for IgG4 $\geq$ 0.05 mg/L.

Blood cell counts were analyzed at the Department of Clinical Chemistry, Karolinska University Hospital.

Basophil activation test (Paper II and III): Basophil activation test was performed to dog dander and to the two dog allergen molecules eliciting the highest IgE-levels in each individual. To obtain dose-response curves for CD-sens analysis, basophils were stimulated with increasing concentrations of dog dander extract (Aquagen, ALK-Abello, Copenhagen, Denmark, final concentration: 0.5-5000 SQ-E/ml) (127, 152) and the allergen molecules Can f 1- Can f 6 (final concentration: 0.05-500 ng/ml). Anti-FcƐRI (Bühlmann Laboratories AG, Schönenbuch, Switzerland) was used as positive control and RPMI (cell culture media developed at Roswell Park Memorial Institute) as negative control. To differentiate the basophils from the leukocyte population they were stained for CD203c. To detect activated basophils, the cells were stained for CD63 (Immunotech, Marseille, France) followed by analysis in a Navios flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). Patients, whose basophils after stimulation with the positive control (anti-FcƐRI) responded with less than 5 % CD63 upregulation, were regarded as non-responders. Individuals with a response to the positive control between 5 % and 16 % were classified as low responders. The cut-off of 16 % was calculated (mean 76 % – 3 SD) from the positive controls of an in-house reference material of 264 allergic children and adults (152). Cut-off determining a positive test was set to 5 % of CD63-positive basophils in response to the tested allergen.

CD-sens (Paper II and III): To determine the basophil allergen threshold sensitivity, CD-sens, the eliciting allergen concentration resulting in 50 % (EC50) of maximum CD63 % upregulation of the dose–response curve was calculated. CD-sens is defined as the inverted value for EC50 multiplied by 100 (127). When basophils only react at the highest allergen concentration, a CD-sens value cannot be calculated, nor can the test be ruled out as negative. These test results were regarded as positive, but they were not included in the analysis of CD-sens levels.

Nasal provocation test (Paper I-IV): Nasal provocation test (NPT) was performed with a commercially available dog dander extract; Aquagen 100 000 SQ-E/ml (ALK-Abello, Copenhagen, Denmark). The extract was analyzed for the content of the investigated dog allergen molecules by competitive inhibition ELISA to ascertain representative concentrations.

Table 2: Content of allergens in the dog dander extract used for NPT.

Specific component	Content of specific allergen in dog dander extract (ng/ml)
Can f 1	256 ng/ml
Can f 2	10 ng/ml
Can f 3	923 ng/ml
Can f 4	282 ng/ml
Can f 5	255 ng/ml
Can f 6	8 ng/ml

The NPT was performed in a two-step manner, with two different concentrations of dog dander extract. One spray-dose, 0.1 ml, of the lower concentration (10 000 SQ-U/ml) was deposited in each nostril. Symptoms during NPT were scored according to a modified Lebel scoring scale, before and 5, 15 and 30 minutes after administration (121). Children with a negative test at the first step proceeded to the second step; One spray-dose, 0.1 ml, of the higher concentration (100 000 SQ-U/ml) in each nostril and the scoring was repeated 5, 15 and 30 min after administration. The scoring system identifies the three cardinal symptoms of rhinitis: sneezing, rhinorrhea, and nose-blockage. In addition, nasal pruritus, ear pruritus and eye symptoms were registered. The maximum score was 12.

Table 3: Symptom scoring according to Lebel (121).

LEBEL SYMPTOM SCORE

Symptom	Score
Sneezing, 3-4 times	1 p
≥ 5 times	3 p
Rhinnorea:	0-3 p
Nose blockage:	0-3 p
Pruritus, nose	1 p
Pruritus, palate or ear	1 p
Conjunctivitis	1 p
MAX SCORE	12 p

A score of ≥ 5 at any scoring occasion was considered positive and a score ≤ 2 was considered negative. Nasal steroids and oral antihistamines were withheld for 14 /3 days prior to the investigation. Children with seasonal rhinitis due to pollen were investigated outside the pollen season.

Spirometry with reversibility test (Paper III and IV): Dynamic spirometry with reversibility test (Salbutamol 0.2 mg x 2) was performed using a Vitalograph® 2120 (Vitalograph®, Ennis, Ireland), in accordance with recommendations from the European Respiratory Society using the reference values reported by Polgar (153). An increase in FEV1 >12 % was considered a positive reversibility test.

Methacholine bronchial provocation (Paper III and IV): Bronchial hyperresponsiveness to a challenge with methacholine was assessed utilizing a Spira nebulizer (Spira Respiratory Care Centre, Hämeenlinna, Finland). The dose-response slope (DRS) and the dose methacholine (µmol) leading to a 20 % drop in FEV1 (PD20) were calculated (154).

Exhaled Nitric Oxide (Paper III and IV): A NIOXTM analyzer (Aerocrine AB, Solna, Sweden) was used to measure the fraction of nitric oxide in exhaled air (FeNO) in accordance with international guidelines (155). A FeNO level above 20 ppb was considered elevated and above 35 ppb was considered high (156).

Nasal epithelial brushings (Paper III): Nasal epithelial brushings were performed in patients and healthy controls. Among cases, nasal provocation tests and nasal epithelial brushings were performed at different occasions, at least five days apart. Nasal epithelial cells were collected from behind the inferior nasal turbinate using a cervical cytology brush (Bastos Viegas, Penafiel, Portugal). Cells were immediately stored in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA), initially at 4°C overnight, followed by long term storage at - 80°C until RNA extraction.

RNA extraction (Paper III): Total RNA was extracted from nasal epithelial brushings using Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA quality and quantity were assessed using NanoDrop 8000, Qubit Fluorometric Quantitation (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), and an RNA integrity number ≥ 8 was used as cut-off for inclusion.

Transcriptome library preparation and sequencing (Paper III): A modified version of the Single-cell Tagged Reverse Transcription (STRT) method (157) was used to prepare two 48-plex Illumina-compatible sequencing libraries from 20 ng of each epithelial RNA. The libraries were sequenced on four Illumina HiSeq2000 (Illumina, San Diego, CA, USA) lanes each, using the Illumina TruSeq v3 60-bp single-read protocol. Sequencing was performed at the Bioinformatics and Expression Analysis core facility at Karolinska Institutet, Sweden. Sequence data were converted to fastq files using Casava 1.8.2 (Illumina), and quality control performed using the STRTprep pipeline (158).

4.4 STATISTICAL ANALYSIS

Categorical data are presented as numbers (n) and proportions (%). Values are presented as means (SD) for normally distributed data and as medians and inter-quartile ranges (IQR) or ranges for non-normally distributed data.

Categorical data were compared using the Chi-Squared test or Fisher’s exact test when subgroups were small.

The Student’s t-test was used for group comparisons of normally distributed continuous variables and log-transformed values of IgE levels. Wilcoxon rank sum test (Mann-Whitney U) was used for group comparisons of non-normally distributed continuous variables and for ordinal variables (e.g. number of sensitizing allergens). In study II, Wilcoxon rank sum test was used for group comparisons of all continuous variables.

Logistic regression was used to calculate odds ratios (OR) with 95 % confidence intervals (CI). Adjustments for concomitant sensitization were performed to determine possible independent markers for a positive NPT result among the analyzed components.

ORs for a positive NPT result in relation to the number of sensitizing dog allergens were estimated by using logistic regression models and 95 % CIs. Fitted predicted probability estimates were plotted according to the number of IgE-reactive (≥0.1 kU_A/L) dog allergen molecules by using results from logistic regression.

The diagnostic performance of IgE measurements to different allergen molecules and dog dander extract was compared by using receiver operating characteristic (ROC) curve analysis.

Spearman rank-order correlation test was performed to investigate correlations between variables since compared data were not normally distributed.

Positive and negative predictive values (PPV and NPV) and likelihood ratios (LR) for a positive basophil activation test as a marker for a positive *vs* a negative nasal allergen challenge were calculated.

All statistical analyzes above were performed with Stata statistical software (release 14.2, Stata Corp, Texas, USA). A p-value < 0.05 was considered significant.

In study III, differential gene expression and the statistical significance were tested in R, using the SAMstrt package (159). When comparing sample groups q < 0.05 was considered as significantly variable expression and genes with a q-value < 0.05 and Fold change (FC) > 1.2 or FC < 0.5 were considered to be significantly differentially expressed.

4.5 ETHICAL CONSIDERATIONS

All studies were approved by the Swedish ethical review authority (Dnr 2014/1453-31/4 and supplement 2015/194-32-5/2).

Children and adolescents should be regarded as a vulnerable group in clinical research; partly because they may not understand the full meaning of participation, partly because they can have difficulties making their voices heard when being or feeling improperly treated. Further, a child is represented by a legal guardian, whose role is to see to the child's best, but who may also have separate interests. Research on a vulnerable group should only be conducted if the research cannot be carried out in a non-vulnerable group. Further, the research may only be conducted if the importance of the purpose outweighs the risks and burdens to the research patients. Possible risk also has to be minimized and monitored.

There is a lack of knowledge regarding the clinical relevance of sensitization to dog allergens in children. As allergic sensitization to furry animals is developing during childhood and adolescence, and patterns of sensitization differ between adults and children, more knowledge in this field is of special interest for dog dander sensitized children. Results from our studies may for instance improve advice regarding pet exposure and future choice of profession.

In this thesis, several investigations were performed; e.g. blood sampling on two occasions, and nasal brushing. We also conducted provocation tests; a nasal provocation test with dog dander extract which is expected to give rise to symptoms of rhino-conjunctivitis in a large proportion of dog-sensitized subjects and methacholine challenge provoking asthma symptoms, especially in individuals with bronchial hyperreactivity. All these investigations may entail discomfort and risks if not conducted in a responsible way and if the child is not fully informed and motivated. These risks should be compared to the benefits of increased knowledge regarding dog sensitization, not only for a general population but also for the participating patient.

All patients were thoroughly informed regarding the study. Local anesthesia was applied before blood sampling. We also ensured that the children were healthy at the time of investigation. Immediately after the nasal provocation test, antihistamine was given. After the methacholine challenge, a bronchodilator was administered. Equipment for the hazard of a more serious adverse reaction was always available. For several children there was also a direct interest to participate as they saw this experience as a possible help in decisions regarding pet keeping. Some of the tests performed in the study are not used in clinical routine, but may give advice regarding dog allergy. Most patient were also very interested in the nasal provocation test as they had the opportunity to experience their own reaction in a controlled setting.

In clinical research, children are under-represented, perhaps because of ethical principles regarding vulnerable groups, but it is important to conduct ethically well-founded research also in children.

5 MAIN RESULTS

The main findings included in this thesis are presented in this section. For complete results, please see the published papers and manuscripts.

5.1 CLINICAL CHARACTERISTICS (PAPER I-IV)

Dog dander sensitized children were included regardless of symptoms of dog allergy. However, manifestations of airway allergy were common, 85 % had an asthma diagnosis and 50 % reported dog exposure as a trigger for asthma. Further, 97 % reported allergic rhinitis and 68 % reported rhinitis triggered by dog exposure. A curious finding is that despite the fact that all children were sensitized to dog and the majority reported allergic disease, 25 % had a dog at home, which is slightly higher than in the general Swedish population (10). The age matched non-sensitized healthy controls did not report any allergic airway symptoms.

Table 4. Clinical characteristics of dog dander sensitized children and healthy controls.

Parameter	**Dog dander sensitized children n= 60**	**Non sensitized healthy children n= 17**
Mean age (s.d)	13.1 (2.3)	13.3 (3.0)
Female gender, n (%)	21 (35)	9 (53)
At least one parent with dog allergy, n (%)	19 (32)	4 (24)
Exposure, n (%)		
Dog at home	15 (25)	0 (0)
Cat at home	3 (5)	2 (12)
Exposure to horse	12 (20)	4 (24)
Asthma, n (%)		
Asthma diagnosis	51 (85)	0 (0)
Asthma triggered by dog exposure	30 (50)	0 (0)
Asthma triggered by cat exposure	14 (23)	0 (0)
Rhinitis, n (%)		
Rhinitis	58 (97)	0 (0)
Rhinitis triggered by dog exposure	41 (68)	0 (0)
Rhinitis triggered by cat exposure	37 (62)	0 (0)

5.2 IGE REACTIVITY (PAPER I-IV)

5.2.1 Sensitization to dog

IgE levels to dog dander among dog dander sensitized children ranged from 0.19-219 kU_A/L. IgE reactivity to the dog allergen molecules Can f 1 and Can f 5 was most common, followed by IgE to Can f 4, Can f 2 and Can f 6 whereas sensitization to Can f 3 was least common (Figure 7). We also found that a large proportion of the investigated children had IgE reactivity to several dog allergens; 67 % were sensitized to two or more allergens. Sensitization to allergen molecules from the three different protein families; lipocalins, serum albumin and prostatic kallikrein was likewise common. Fifty-two percent were sensitized to allergen molecules from more than one protein family, and 23 % were sensitized to all three.

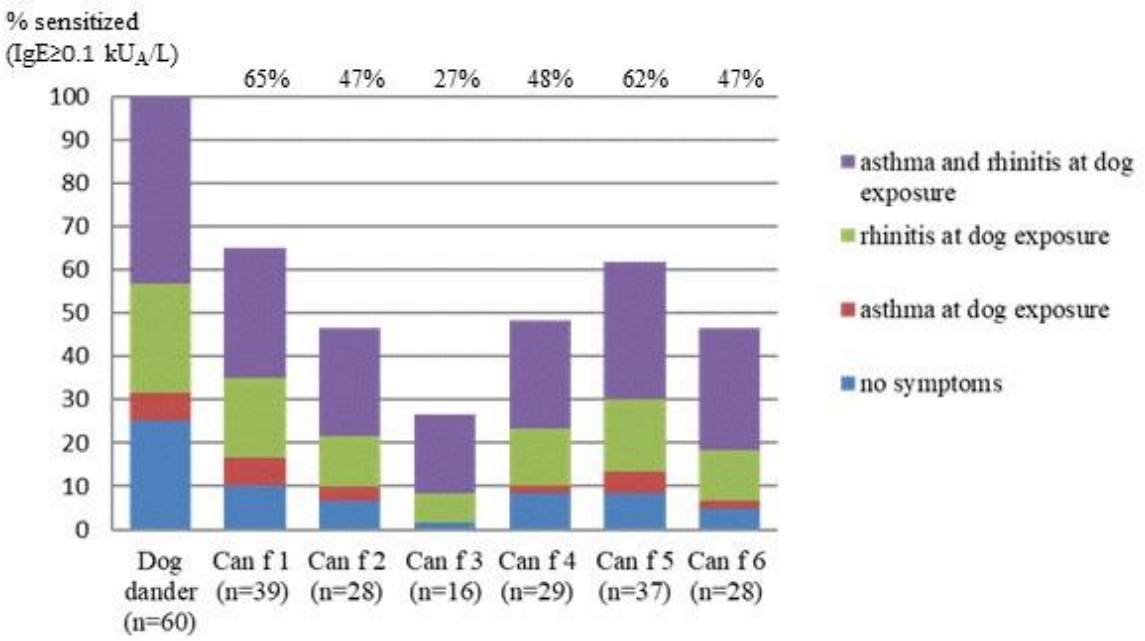
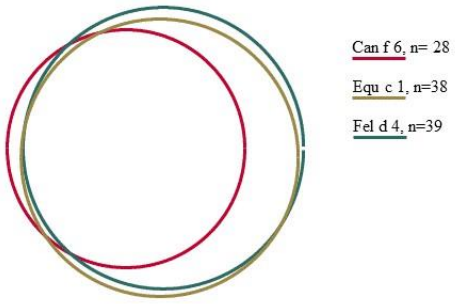


Figure 7: Frequencies of IgE reactivity to dog dander and dog allergen molecules in relation to reported symptoms of rhinitis and asthma triggered by dog exposure.

There were large variations in patterns of positive IgE reactivities among the 60 dog dander sensitized children. Sensitization to all six investigated allergens was most common (n= 9), followed by monosensitization to Can f 5 (n= 7), sensitization to dog dander but none of the investigated allergens (n= 6) and sensitization to Can f 1, 2, 4, 5, 6 but not to Can f 3 (n= 6).

The 20 healthy controls in paper III were included based on the lack of allergic airway symptoms. Their sensitization status was not known prior to the investigation. Fifteen percent (3/20) exhibited low IgE levels to dog dander (0.11, 0.37 and 0.39 kU_A/L) without any history of airborne allergy and were excluded.

5.2.2 Sensitization to cat and horse



Among the dog sensitized children, we further analyzed IgE reactivity to cat and horse and found that they were to a large extent sensitized to cat (97 %) and horse (80 %) as well as to the investigated allergen molecules from cat, Fel d 1 (81 %), Fel d 2 (25 %), Fel d 4 (66 %), and horse, Equ c 1 (64 %). There was a considerable overlap between sensitization to the cross-reactive serum albumins as well as to the lipocalins.

Figure 8: Numbers of individuals with overlapping sensitization to the cross-reacting lipocalins Can 6 (dog), Equ c 1 (horse) and Fel d 4 (cat).

5.3 DOG ALLERGY EVALUATED BY NASAL PROVOCATION (PAPER I)

All dog dander sensitized children underwent nasal provocation testing with dog dander extract. Twenty-five children had a positive NPT result. Twenty-one children had a negative response to the nasal provocation, with a symptom score of 2 or less. Fourteen children scored 3 to 4 but were still not clearly unaffected by the NPT, and their results were considered inconclusive.

5.3.1 Positive *vs* negative NPT and sensitization to dog allergens

In the unadjusted analysis, IgE reactivity to the lipocalins Can f 4 and Can f 6 as well as to the serum albumin Can f 3 was associated with a positive nasal provocation test with dog dander (Figure 9).

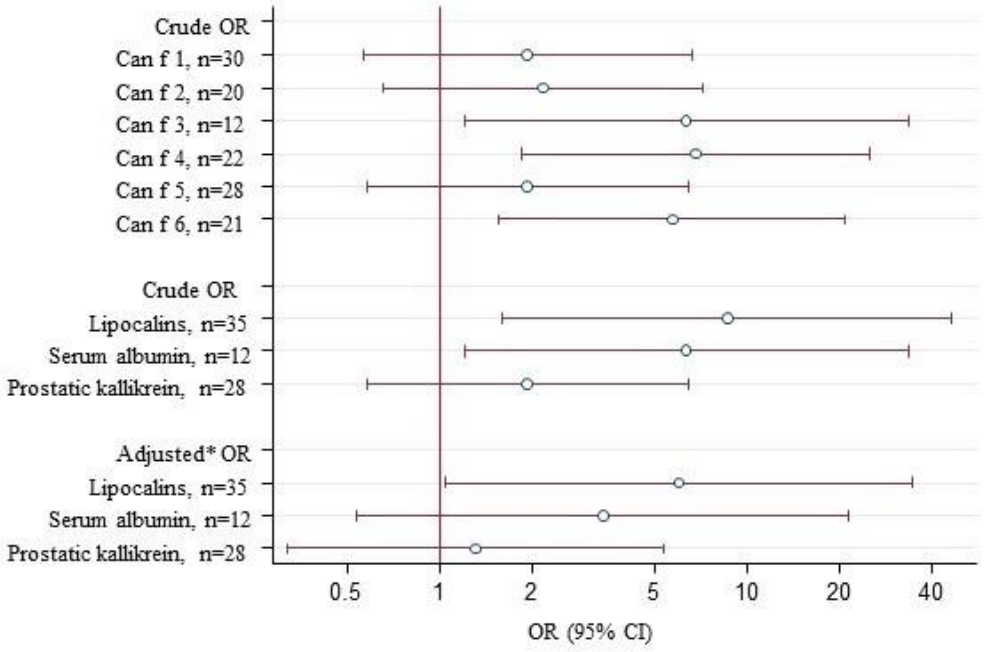


Figure 9: Crude and adjusted OR for a positive *vs* a negative NPT result in relation to IgE reactivity to individual dog allergen molecules and allergens from different protein families. *Adjusted for concomitant sensitization to allergens from other protein families.

In the analysis of a positive *vs* a negative NPT that was adjusted for sensitization to allergens from the investigated protein families, sensitization to lipocalins remained associated with a positive NPT.

5.3.2 Positive *vs* negative NPT and sensitization patterns

We investigated associations between sensitization to different combinations of allergen families and a positive *vs* a negative NPT. The highest odds ratio for a positive NPT were found in individuals with IgE reactivity to allergens from all three protein families, OR 5.34

(95 % CI: 1.01-28.4), the lowest odds ratio in individuals sensitized to prostatic kallikrein only, though not significant, OR 0.13 (95 % CI: 0.01-1.25).

Further, we analyzed the relationship between the number of positive IgE reactivities and NPT-results. We could demonstrate that sensitization to an increasing number of dog allergen molecules entailed a higher likelihood for a positive NPT (Figure 10).

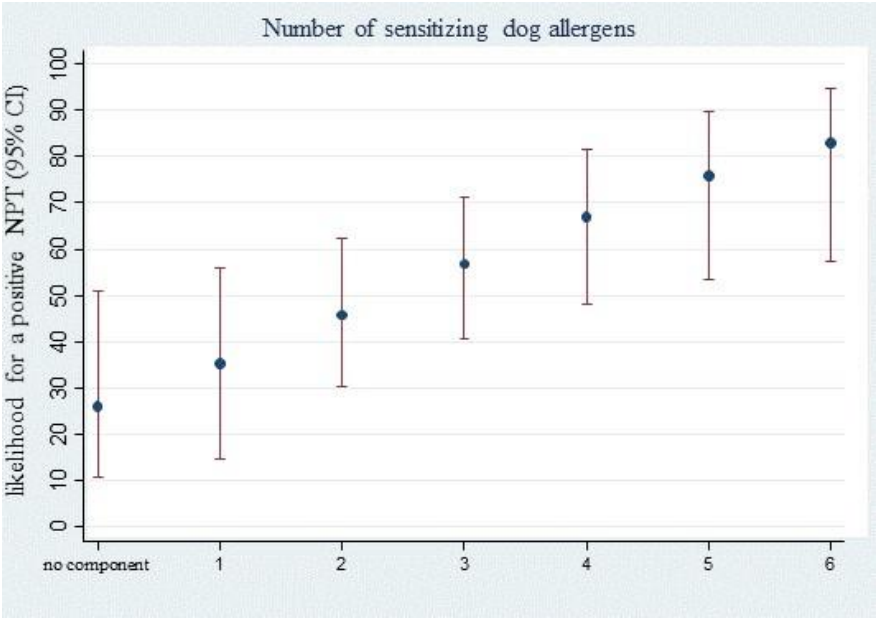
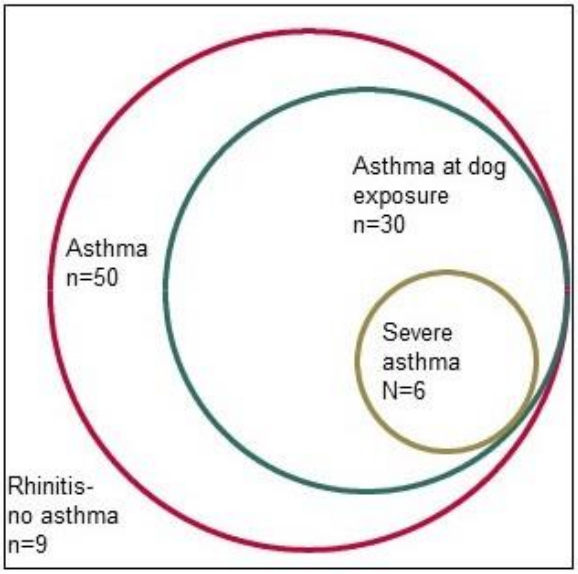


Figure 10: Likelihood for a positive NPT in relation to IgE reactivity to an increasing number of dog allergen molecules (0-6), p= 0.01.

5.3.3 Negative NPT and sensitization

To evaluate whether there were any patterns of sensitization that suggests dog tolerance despite dog dander sensitization, we analyzed relationships between a negative NPT and IgE. We found an association between monosensitization to Can f 5 and a negative nasal challenge (OR 5.78, 95 % CI 1.01-33.0). Neither sensitization nor monosensitization to any other specific allergens or investigated combinations of allergens from different protein families or even sensitization to no allergen molecule (dog dander only, n= 6) could be associated with a negative nasal challenge.

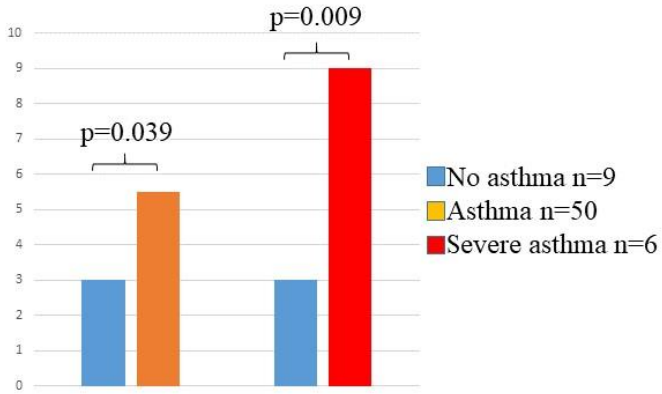
5.4 ASTHMA AND IGE-REACTIVITY (PAPER IV)



A large proportion of the dog dander sensitized children had an asthma diagnosis. We investigated the relationships between asthma diagnosis and sensitization to dog allergens, as well as to important allergen molecules from cat and horse among 59 dog dander sensitized children in the MADOG study. In addition, we investigated associations between sensitization and asthma control, airway inflammation and bronchial hyperreactivity.

Figure 11: Numbers of individuals with asthma (red), severe asthma (yellow) reported asthma triggered by dog exposure (green) and rhinitis only (outside circle).

5.4.1 Asthma diagnosis and sensitization



In line with the results from the nasal provocation testing, multi-sensitization to furry animal allergens was more common in asthmatics than in non-asthmatic dog sensitized children. A median of 5.5 positive sensitizations was observed for asthmatics, 9 for severe asthmatics vs 3 positive sensitizations for non-asthmatic dog dander sensitized children.

Figure 12: Numbers of positive IgE reactivities to furry animal allergen molecules (y-axis 0-10 sensitizing allergen molecules) in children with no asthma, asthma and severe asthma.

Children with **asthma diagnosis** were more frequently sensitized to the dog lipocalin Can f 6 (54 % *vs* 11 %, p= 0.03) and showed a tendency towards more frequent sensitization to the horse- and cat lipocalins Equ c 1 and Fel d 4 compared to dog sensitized children without asthma. There were no differences in IgE levels to dog allergen molecules, but IgE to the horse allergen Equ c 1 was elevated in asthmatic children compared to dog sensitized children without asthma (median 7.61 *vs* 0.17, p= 0.02).

Severe asthma, defined by the combination of Asthma Control Test (ACT) < 20 p, high FeNO (> 35 ppb) and pronounced bronchial hyperreactivity (PD20 < 2µmol) was associated with increased levels of IgE to the lipocalins Can f 2 (44 *vs* 2.9 kU_A/l, p= 0.014), Can f 4 (5.8

vs 0.83 kU_A/l, p= 0.016) and Can f 6 (1.3 *vs* 0.69 kU_A/l, p= 0.039) in comparison with dog sensitized children without severe asthma. We also found that dog allergy, evaluated by nasal provocation test with dog dander, was more common among children with severe asthma compared to dog sensitized children without severe asthma (83 % *vs* 35 %, p= 0.034).

Asthma triggered by dog exposure*:* Fifty-one percent (30/59) of the dog dander sensitized children reported symptoms of asthma at dog exposure and sensitization to the lipocalin Can f 6 was more common among these children compared to dog sensitized children not reporting dog exposure as a trigger for asthma (60 % *vs* 34 %, p= 0.05). Children reporting dog exposure as a trigger for asthma symptoms had higher IgE levels to the dog specific prostatic kallikrein Can f 5 compared to children who did not report asthma symptoms upon exposure to dog (median 5.8 *vs* 1.3 kU_A/L, p= 0.02).

5.4.2 IgE reactivity and asthma manifestations

IgE reactivity and asthma control: Mean score on the Asthma Control Test among dog dander sensitized children with asthma was 20.6 (SD 3.4). Thirty-five percent (n= 17) of the investigated children with asthma showed insufficient asthma control with a score below 20. No differences in sensitization rates to furry animal allergen molecules were seen between the asthmatic children with ACT < 20 and asthmatic children with ACT ≥ 20. However, IgE levels to the dog lipocalins Can f 2 and Can f 4 were increased among asthmatics with ACT < 20 compared to asthmatics with ACT ≥ 20. (32 *vs* 2.9 kU_A/L, p= 0.005 and 3.4 vs 0.9 kU_A/L, p= 0.03 respectively).

IgE reactivity and airway inflammation*:* The median FeNO level was 33 ppb (IQR 20-69) among the dog dander sensitized children. FeNO above 20 was seen in 42 (71 %) and 28 (47 %) had high FeNO (> 35 ppb). Children with high FeNO displayed higher IgE levels towards dog dander and the dog lipocalins Can f 1 and Can f 4 than children with FeNO < 35 ppb (19 *vs* 2.6 kU_A/L, p < 0.001 and 2.2 *vs* 0.58 kU_A/L, p= 0.01).

IgE reactivity and bronchial hyperreactivity: A majority, 69 % (37/54), of the investigated dog dander sensitized children had a positive bronchial methacholine challenge (PD20 < 8 µmol methacholine), while 46 % (25/54) showed pronounced bronchial hyperreactivity (PD20 < 2 µmol methacholine). No significant associations between sensitization rates or IgE levels to the investigated allergens and bronchial hyperreactivity were observed.

5.5 IN VITRO ALLERGEN CHALLENGE (PAPER II)

The performance of the basophil activation (BAT) test as an *"in vitro"* challenge was investigated in 58 of the 60 children in the MADOG study. One child was a low responder and one patient did not consent with blood sampling for BAT and they were excluded.

Allergen	BAT performed (n)
Dog dander	**58**
Can f 1	**34**
Can f 2	**20**
Can f 3	5
Can f 4	8
Can f 5	**23**
Can f 6	5

Table 5: Titrated BAT with increasing concentrations of allergen was performed to dog dander and to the two dog allergen molecules eliciting the highest IgE levels in each individual. Few children were tested regarding Can f 3, Can f 4 and Can f 6 due to generally lower IgE levels, and therefore BAT to these allergens were not further analyzed.

5.5.1 BAT and nasal provocation

A vast majority of the children sensitized to dog dander as well as to the investigated allergens displayed basophil activation upon stimulation with the corresponding allergen. All Can f 1-sensitized children with a positive NPT result and 60 % with a negative NPT, had positive basophil activation test to Can f 1 (p= 0.01), figure 13. None of the four Can f 1 basophil negative children had a positive NPT. For BAT to dog dander, Can f 2 and Can f 5 there were no significant associations between the basophil activation test and the nasal provocation test.

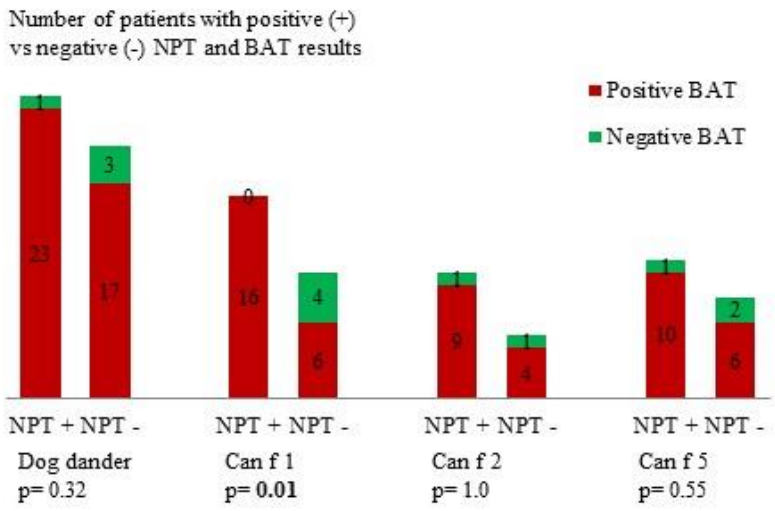


Figure 13: Numbers of patients with positive vs negative NPT results and BAT results to dog dander and the investigated allergen molecules. Individuals with inconclusive NPT results are not shown.

5.5.2 CD-sens and nasal provocation

We obtained basophil allergen threshold sensitivity (CD-sens) levels to dog dander in 51 children, to Can f 1 in 26, to Can f 2 in 19, and to Can f 5 in 20 children.

Children with a negative nasal provocation test had lower CD-sens to dog dander (0.10 vs. 0.67, p= 0.04) and to Can f 1 (2.35 vs. 34.8, p= 0.025) compared to children with a positive test, suggesting lower biological basophil allergen sensitivity among the NPT negative children. We found no significant differences in CD-sens levels to Can f 2 or Can f 5 between children with a positive or a negative NPT.

The CD-sens levels showed significant correlations with IgE-levels to dog dander and to the investigated allergens: dog dander r_S= 0.66, (p < 0.001), Can f 1 r_S= 0.52 (p= 0.006), Can f 2 r_S= 0.71 (p < 0.001) and Can f 5 r_S=0.81 (p < 0.001).

5.6 TOLERANCE TO DOG (PAPER II)

Among the dog sensitized children living with a dog at home, only one out of 15 had a positive NPT, which is a significantly lower proportion than among those without a dog. Despite this difference in NPT results, IgE levels to dog dander did not differ significantly between the groups. However, the median CD-sens level to dog dander was lower for children with a dog at home (Table 6).

Table 6: Levels of IgE, IgG, IgG4 and CD-sens among sensitized with and without a dog at home.

	Dog at home (n= 15) median(IQR)	**No dog at home (n=43) median(IQR)**	**P-value**
Positive NPT- dog dander	1	23	p= **0.002**
Dog dander (n= 58)			
IgE	11 (4-41)	16 (2.8-47)	p= 0.67
IgG	13 (9.8-25)	9.9 (8.4-14)	p= **0.019**
IgG4	2.3 (1.4-4.9)	1.8 (0.81-2.7)	p= 0.16
Ratio IgG4/IgE	97 (38-279)	44 (17-160)	p= 0.19
Level CD-sens (n= 51)	0.11 (0.07- 0.44)	0.50 (0.13-2.0)	p= **0.038**
Can f 1 (n= 38)			
IgE	2.1 (0.68-5.9)	13 (2.6-33)	p= **0.006**
IgG	3.2 (2.1- 6.4)	2.0 (0– 2.9)	p= 0.07
IgG4	0.5 (0.2- 1.6)	0.18 (0.07-0.3)	p= **0.039**
Ratio IgG4/IgE	141 (36-315)	5.7(1.9-12)	p= **0.006**
Level CD-sens (n= 26)	1.0 (0-8.8)	23 (4.1-50)	p= **0.026**
Can f 2 (n= 28)			
IgE	1.6 (0.42-14)	10 (1.1-34)	p= 0.22
IgG	2.2 (0- 4.3)	0 (0- 2.2)	p= 0.06
IgG4	0.2 (0- 0.4)	0 (0- 0.1)	p= 0.10
Ratio IgG4/IgE	13 (0-34)	0 (0-2.5)	p= 0.11
Level CD-sens (n= 19)	20 (0-41)	224 (70-271)	p= **0.018**
Can f 5 (n= 37)			
IgE	0.61 (0.25-1.5)	5.8 (2.1-16)	p< **0.001**
IgG	1.1 (0- 5.0)	2.1 (0- 3.1)	p= 0.62
IgG4	0.13 (0- 0.31)	0 (0- 0.09)	p= **0.025**
Ratio IgG4/IgE Can f 5	34 (0-247)	0 (0- 4.7)	p= **0.01**
Level CD-sens (n= 20)	0 (0-0.24)	2.5 (1.3-3.6)	p= **0.005**

Levels of IgE are shown in kU_A/L and levels of IgG and IgG4 are shown in mg/L.

The median CD-sens levels to all investigated dog allergen molecules were lower among children with a dog at home, as were IgE to Can f 1 and Can f 5. The IgG4 levels to Can f 1 and Can f 5 in sensitized children were inversely higher in children with dog exposure at home compared to children without a dog at home (Table 6).

Additionally, we compared the levels of IgE, IgG and IgG4 to dog dander and Can f 1, Can f 2 and Can f 5 among sensitized children with a positive and a negative NPT. The median IgE levels to dog dander and to Can f 1 were higher in children with a positive NPT than among children with a negative NPT. However, no significant differences in levels of IgG or IgG4 to dog dander, Can f 1, Can f 2 or Can f 5 were seen between sensitized children with positive and negative nasal provocation test result (data not shown).

5.7 NASAL GENE EXPRESSION (PAPER III)

We performed whole genome transcriptomic profiling (RNA) from the nasal mucosa in 49 dog dander sensitized children and 17 healthy controls to investigate associations between nasal gene expression, allergic sensitization to dog and clinical manifestations of airway allergy.

We found that 321 genes were significantly differently expressed among dog dander sensitized children compared to non-sensitized controls. The most over-expressed gene in dog dander sensitized children was *CST1*, with a median fold change of 21 compared with the controls. The second most over expressed gene was *CCL 26*, with a median fold change of 4.5 compared with the controls (Figure 14).

Further, unsupervised clustering of the nasal brushing samples based on the ten most up- and downregulated genes revealed a distinct cluster of ten dog dander sensitized children. The clearly most over-expressed gene in this cluster was *CST1* with a median fold change of 47 compared with other cases and > 500 compared with the controls.

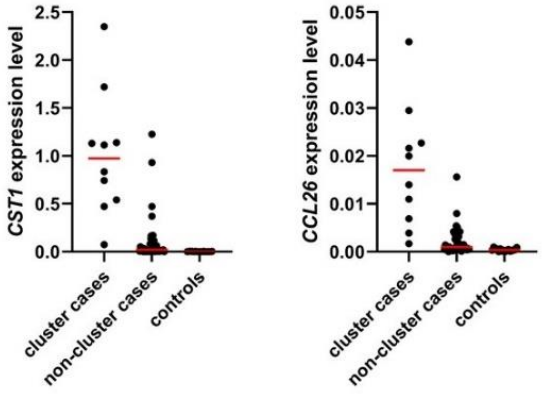


Figure 14: The two most over-expressed genes, *CST1* and *CCL26*, in dog dander sensitized children and healthy control children.

5.7.1 Clinical characteristics of *CST1*-high cluster cases

The *CST1*-high cluster-cases differed clinically from the rest of the dog sensitized cases through lower FEV1 and pronounced bronchial hyperresponsiveness (low methacholine PD20), figure 15, but they did not report asthma or rhinitis to a larger extent. Furthermore, the *CST1*-high cluster cases showed higher blood eosinophil count (median 0.65 $x10^9$/l *vs* 0.3 x 10^9/l, p= 0.02) and higher CD-sens levels to dog dander (median 1.8 *vs* 0.20, p= 0.01) compared with the rest of the dog dander sensitized study population.

Despite the differences in CD-sens levels to dog dander, the *CST1*-high cluster cases did not display significantly higher IgE levels to dog dander than other dog sensitized cases. When investigating sensitization rates to dog allergen molecules, we found higher sensitization rates to the dog lipocalins Can f 2 (80 % *vs* 42 %, p= 0.04) and Can f 6 (80 % *vs* 36 %, p= 0.03) among *CST1*-high cluster cases compared to other dog sensitized children.

The *CST1*-high cluster cases were in a greater extent multisensitized to lipocalins to furry animals than other dog dander sensitized cases, median 6 *vs* 3 positive sensitizations (p= 0.03). No differences in sensitization rates or IgE levels to cat or horse allergens were found. We further investigated sensitization rates to food allergens but found little differences between the *CST1*-high cluster and other dog sensitized children.

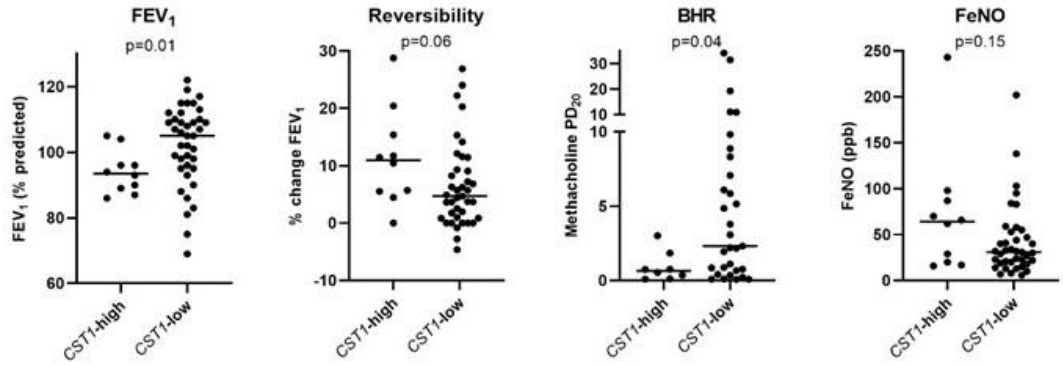


Figure 15: FEV1, spirometry reversibility, bronchial hyperresponsiveness and exhaled FeNO in *CST1* high cluster cases compared to other dog dander sensitized children (*CST1*-low).

6 DISCUSSION

The main objective of this doctoral thesis project was to improve diagnostics of dog allergy in children by identifying patterns of sensitization to dog allergen molecules associated with rhino-conjunctivitis and asthma and by exploring complementary diagnostic tests for dog allergy and novel biomarkers.

The relations between sensitization to dog and allergic airway disease have never been investigated in such detail as in the current project. We found that multisensitization to dog allergen molecules and sensitization to lipocalins are associated with symptoms of dog allergy and asthma severity. The BAT does not seem to be able to replace a provocation test, but CD-sens might be useful in monitoring the biological allergen sensitivity. IgG4 antibodies to dog allergen molecules seem to reflect exposure to dog, but could not be used to indicate tolerance. Finally, we explored nasal gene expression in dog dander sensitized children and found that *CST1* may be a marker for allergic airway disease. Our results refine the interpretation of sensitization to dog allergen molecules and will improve the pediatrician's advice to the dog dander sensitized patient.

6.1 STUDY DESIGN: STRENGTHS AND WEAKNESSES

This is an observational explorative investigation of dog dander sensitized children. Few studies regarding sensitization to dog allergen molecules and their relation to airway allergy have been conducted in pediatric patient populations. Our study population was chosen to reproduce the clinical situation where children with sensitization to dog are attending a pediatric outpatient clinic due to suspected or confirmed airway allergy. Accordingly, we think that our results are valid for pediatricians in the clinical evaluation of dog dander sensitized children.

To our knowledge, this is the first study to investigate sensitization to all clinically available dog allergen molecules and to perform nasal provocation tests. Most studies regarding dog allergy rely on the clinical history. Furthermore, we performed bronchial provocations, investigated lung function and airway inflammation and all patients were thoroughly investigated through interviews, questionnaires, and blood and nasal samplings. Another strength is that all investigations were performed by two research nurses in the field of pediatric allergology and myself at one site (Barnforskningscentrum, Södersjukhuset), to assure a uniform procedure.

The detailed procedure was, on the other hand, limiting the number of participants. Some sub-groups were small, which is illustrated by wide confidence intervals for certain results. The selection of patients from pediatric clinics entails a high risk for selection bias. Further, the choice of patient population limits the external validity, thus our results cannot be generalized on a population basis.

6.2 CLINICAL HISTORY AND NPT

All but one child with a positive NPT reported rhinitis triggered by dog exposure. There was a highly significant association between reported symptoms and a positive NPT, the gold standard in the diagnosis of allergic rhinitis (160). However, 38 % of the individuals with a negative response to the nasal provocation test reported rhinitis at dog exposure and 29 % reported asthma at dog exposure. The relatively high numbers of reported rhinitis and asthma due to dog exposure compared to those with positive NPT may have several explanations. In our study, IgE-sensitization to dog dander was a primary inclusion criterion, which means that all subjects were aware of being sensitized to dog. In some cases, confirmed dog dander sensitization may have been interpreted by the patient as meaning clinical allergy and we believe that there is a risk for over-reporting symptoms from this highly atopic cohort. However, our results are in concordance with a previous study where a structured allergy history alone resulted in a 27 % false-positive rate for dog allergy compared with a combined allergy assessment of clinical history and skin prick test (5). The discrepancy between reported symptoms and a positive NPT highlights the challenge of diagnosing dog allergies.

6.3 SENSITIZATION TO DOG ALLERGEN MOLECULES

The diversity in sensitization patterns to dog allergen molecules was striking. We found a large number of different patterns of sensitization to the dog allergen molecules among whom sensitization to all 6 allergens was most common and monosensitization to Can f 5 was second most common. To our knowledge, this is the first study to evaluate sensitization rates to all six clinically available dog allergen molecules and there is no single study to compare the complete results. However, the proportions of IgE reactivity in the investigated population mostly agree with previous data from dog dander sensitized subjects (7).

We found that sensitization to two or more dog allergen molecules occurred in 67 % of the patients, which contrasts with population based cohorts. Sensitization patterns to furry animal allergens were recently described among adults by Suzuki et al. The most common sensitization pattern to dog was monosensitization and the most common sensitizing dog allergen was Can f 5. Mono-sensitization was seen in 5.6 %, double sensitization in 1.5 % and multi-sensitization in 2.1 % on a population basis (161), however Can f 4 and Can f 6 were not investigated.

We found monosensitization to Can f 5 in 12 %, which is lower than in previous observations among dog dander sensitized. The lower prevalence of Can f 5 monosensitization may depend on the detection of IgE to Can f 4 and Can f 6 in our study, and perhaps also reflects the age of the study population, since Can f 5 sensitization has shown to increase in prevalence during adolescence (2).

The observed sensitization rates to cat and horse, as well as the overlap between sensitization to Can f 6, Equ c 1 and Fel d 4 underlines that clinically relevant sensitization to dog rarely occur without simultaneous sensitization to cat and/or horse. Likewise, it was expected to

find that 15 % of the initially recruited healthy controls exhibited IgE reactivity to dog dander, without any history of airborne allergy (38).

This heterogeneity in sensitization to dog allergen molecules is a challenge in diagnosis as well as in treatments. When treating a dog allergic patient with allergen-specific immunotherapy it should be ascertained that the treatment is performed with an extract containing the allergens to which the patient actually reacts.

6.3.1 Cut-off for a positive IgE

IgE levels can be evaluated with either quantitative or semi-quantitative methods. We used a quantitative method for all IgE determinations (ImmunoCAP). Several previous studies have been using IgE ≥ 0.35 kU_A/L as cut-off for allergic sensitization, but since a few years ≥ 0.1 kU_A/L, has been considered as a positive reaction (18, 73, 93). These levels are generally not based on proof of significance or manifest allergy, but rather on the detection limit of the assay at the time of investigation (111, 162). However, IgE levels to dog below the previous cut-off ≥ 0.35 kU_A/L have shown to indicate clinically relevant sensitization (162). We used the cut-off ≥ 0.1 kU_A/L because this is the current cut-off level used in most clinical practices. This lower detection level will result in higher sensitization rates than for some previous studies.

6.4 ALLERGIC SENSITIZATION AND RHINITIS

6.4.1 Lipocalins and serum albumin

A positive NPT result was associated with IgE to the lipocalins Can f 4 and Can f 6 and to the serum albumin Can f 3 in the unadjusted analysis. Moreover, a positive NPT was associated with IgE to lipocalin allergen molecules in the analysis that was adjusted for co-sensitization with serum albumin and prostatic kallikrein. Positive IgE to Can f 1, generally regarded as the major dog allergen could not be associated with a positive NPT, but the IgE levels to Can f 1 were higher among children with a positive NPT compared to children with a negative NPT.

Since IgE reactivity to Can f 3, Can f 4 and Can f 6 was less common than to Can f 1 and Can f 5, the association between sensitization to these allergens and a positive NPT may reflect a higher degree of multisensitization. IgE to Can f 3 has previously been suggested to be a marker for allergic airway disease in multisensitized, highly atopic individuals (73), but not specific for dog allergy. We show that lipocalins are important markers for dog allergy, however no single allergen molecule can provide a response that is more discriminative to the diagnosis than IgE to dog dander extract. The added value of molecular allergology among dog dander sensitized children is achieved by combining the information obtained after analyzing IgE antibodies to all the available dog allergen molecules as the likelihood for a positive NPT increased with the number of test positive molecules.

6.4.2 Prostatic kallikrein

Sensitization to Can f 5 did neither entail a significantly increased OR for a positive NPT, nor were IgE levels to Can f 5 higher among children with a positive *vs* a negative NPT. Can f 5 has been considered to account for allergic reactions specific to dog exposure since there are no cross-reactions with known allergens from furry animals. In a previous population based study among school children in the north of Sweden, 41 % of the dog sensitized children with IgE reactivity exclusively to Can f 5 reported rhino-conjunctivitis at dog exposure while 69 % of the children with sensitization to both Can f 5 and Can f 1 and/or Can f 2 reported rhino-conjunctivitis at dog exposure. Sensitization to Can f 3, Can f 4 or Can f 6 was not reported (18). Similarly, we observed a higher OR for a positive NPT among children co-sensitized to lipocalins and prostatic kallikrein compared to those sensitized only to allergens from one of the two protein families.

Interestingly, we found that monosensitization to Can f 5 was associated with a negative NPT among dog dander sensitized. This result might depend on low concentration of Can f 5 in the extract used for NPT (102). However, the concentration of Can f 5 in the extract used for our investigations was 255 ng/ml, which was higher than the mean concentrations of Can f 5 in fur extracts sampled from the groin of male dogs (71). Accordingly, low concentration of Can f 5 in the extract is an unlikely explanation.

Can f 5 monosensitization can be identified by investigating sensitization to all available allergen molecules, and female dog ownership might be an option for Can f 5 monosensitized individuals. However, it is important to bear in mind that allergic sensitization is a dynamic process, and an individual monosensitized to Can f 5 may be at risk for developing sensitization towards other allergens. Moreover, we may not yet have detected all relevant dog allergens and consequently, the clinical history has to be thoroughly reviewed before the decision of getting a female dog.

6.4.3 The nasal provocation test

A nasal challenge with a standardized extract may give the clinician and the patient accurate guidance for diagnosis and management. We did not experience any severe adverse event during the provocations, on the contrary most patients took a great interest in the investigations. Still, nasal challenges are often described as time consuming and burdensome for the patient and are rarely performed in clinical practice.

One drawback with nasal provocation tests has been the lack of standardization regarding i.e. the quality of the allergen extract, technique for allergen application and assessment of symptoms. However, EAACI published a consensus document on the standardization of NPT's in 2018 (115), stating that clinical symptoms are the most relevant outcome. Several recognized symptom scores are available. We chose the Lebel symptom score for several reasons: The Lebel score was developed based on verified correlations between threshold release of inflammatory mediators and the cut off for a positive challenge (121). Moreover,

evaluating NPT by the Lebel symptom score correlates closely with evaluation by combined rhinomanometry and symptom scoring (163).

6.5 ALLERGIC SENSITIZATION AND ASTHMA

We investigated the associations between asthma diagnosis, asthma manifestations and sensitization to dog as well as to important cat and horse allergen molecules. Multisensitization was common among asthmatic children and even more pronounced among children with manifestations of severe asthma. Sensitization to Can f 6 was more common among children with asthma and interestingly, all children but one, who were sensitized to Can f 6 were also sensitized to the cross-reactive lipocalins Fel d 4 and Equ c 1 from cat and horse. The clinical impact of Can f 6 has, prior to our investigations, only been studied scarcely. A clinical case report suggests that the cross-reactivity between Can f 6 and Equ c1 may lead to clinically relevant symptoms to dog, even if the primary sensitizing source is horse (164).

Our observations regarding minor and cross-reactive allergens are in line with observations made among cat sensitized young asthmatics. Tsolakis et al. have shown that sensitization to the cat lipocalin Fel d 4 is associated with increased blood eosinophil count and that the cat serum albumin Fel d 2 is associated with increased FeNO in young asthmatics (165). The authors suggest that evaluation of sensitization to these minor cat allergens can be useful in the assessment of asthma severity among cat allergic patients.

6.5.1 Severe asthma

Severe asthma affects around five percent of all children with asthma, and only a few children in our investigations met the criteria of high dose steroids and ACT < 20. However, we had access to lung function measures allowing us to identify a sub-group of children with manifestations of severe asthma such as reduced asthma control, increased airway inflammation and increased bronchial hyperresponsiveness (21).

We could show that IgE levels to the dog lipocalins Can f 2, Can f 4 and Can f 6 were higher in children with severe asthma manifestations. Thus, sensitization to minor lipocalins seems to play an important role as markers for asthma severity. It has previously been demonstrated that children with severe asthma had more complex spreading of IgE to furry animal allergen molecules than asthmatic children with controlled asthma. They were to a larger extent sensitized to Can f 2 and Equ c 1, and those sensitized to multiple lipocalin molecules were at a greater risk of having severe asthma (3).

6.5.2 Dog exposure as a trigger for asthma

Children reporting dog exposure as a trigger for asthma symptoms had higher levels of IgE to Can f 5 than other dog dander sensitized children. Hence, high IgE levels to Can f 5, particularly in double- or poly-sensitized children, seem to be a marker for asthma triggered

by dog exposure. Our results are supported by Bjerg at al. who found that the prevalence of asthma symptoms related to dog exposure was low among children with IgE reactivity to either Can f 5 (5 %) or Can f 1/f 2 (13 %), compared to 37 % among children with IgE reactivity to both Can f 5 and Can f 1/f 2 on a population basis (18).

6.5.3 The united airways

We could confirm the associations between allergic rhinitis and asthma through the association between a positive NPT and manifestations of severe asthma in the study population. We also found that sensitization patterns among children with a positive NPT and asthma share traits; sensitization to minor lipocalins was more common and multisensitization was associated with both conditions. A recent population based study demonstrated that mono and double sensitization to furry animal allergen molecules increased the risk for rhinitis, while polysensitization increased the risk for asthma (166). The explanation to this difference probably lies in the selection of the patient population, those with very mild symptoms may not seek healthcare. Further, the number of investigated allergens to furry animals was higher in the MADOG study.

6.6 BASOPHIL ACTIVATION TEST AND CD-SENS

Performing a BAT has been suggested when there is a discordance between the clinical history and serological testing and as an alternative method to provocation tests (167). The performance of BAT to dog dander, Can f 1, Can f 2 and Can f 5 in our sensitized children was investigated in relation to the results from the NPTs. The basophil activation tests were positive in a majority of the children with a positive, as well as a negative NPT, which seems to limit the utility of the BAT as a diagnostic tool in dog dander sensitized children. However, a negative BAT to Can f 1 in Can f 1 sensitized children was associated with a negative NPT, but the total number of investigated individuals were low and the results has to be confirmed. The reason for positive BAT results in an asymptomatic, but generally atopic individual has previously been suggested to be due to non-specific hyperreactivity of basophils in atopic individuals (168).

The basophil allergen threshold sensitivity, CD-sens, was used to investigate the allergen sensitivity in children with positive and negative NPTs. We found that basophils in children with a positive NPT were significantly more sensitive to dog dander and to Can f 1 than in children with a negative NPT. A high CD-sens demonstrate that lower allergen concentrations can activate the individual’s basophil cells, which may be reflected by the positive NPT response. Conversely, we found that the children with a dog at home had lower CD-sens levels to dog and to all investigated allergen molecules. The design of our study did not permit us to reveal whether these children were desensitized by the dog exposure at home or if they had a lower basophil allergen sensitivity from the start, allowing them to tolerate a dog at home.

Nevertheless, we provide the first investigation of basophil allergen sensitivity to dog allergen molecules among dog exposed dog dander sensitized children. CD-sens has previously been shown to be useful for monitoring patients treated with allergen-specific immunotherapy (AIT) as the basophil allergen sensitivity decreases during the early up-dosing of the allergen (169). Since the effect of AIT to dog is uncertain (27), the CD-sens response to dog allergen molecules might be useful to identify for which individuals AIT is an appropriate treatment, and to monitor the AIT response in patients treated with AIT for dog allergy.

Another interesting finding regarding CD-sens to dog was made in paper III. Based on the expression of the 10 most upregulated and 10 most downregulated genes, we identified a distinct cluster of ten sensitized children who displayed lower mean FEV1, more pronounced bronchial airway responsiveness and higher blood eosinophil counts than other dog dander sensitized children. They did not display higher median IgE levels to dog dander, but they had higher median CD-sens to dog than the rest of the dog dander sensitized population. This is in line with an investigation of CD-sens to cat in severe asthmatic children (170). Children with severe asthma had higher CD-sens levels but not higher IgE levels to cat than children with controlled asthma. They also showed lower ACT score, reduced FEV1 and higher blood eosinophils. Thus, basophil allergen threshold sensitivity to cat, as well as to dog, seems to reflect morbidity and the allergic inflammation in severe asthma.

A concern regarding our investigations of BAT and CD-sens was that a relatively high number of children had basophils that only reacted to the highest concentration of the allergen. In these cases, CD-sens, which requires a dose-response curve, could not be calculated. Further it has to be elucidated whether this low grade of activation is clinically relevant and if these test results should be regarded as positive, or rather as an effect of generally hyperreactive basophil cells in atopic individuals.

6.7 TOLERANCE

A significant proportion of children with IgE directed towards dog dander are known to be tolerant. It has been reported that prolonged exposure to high doses of cat allergen (exposure in the home) results in tolerance due the deviation of the immune system towards a “modified Th2 response”(134). One outcome of this response is IgG4, which can function as a blocking antibody, preventing cross-linking of IgE (171). Can f l-specific IgG4 antibodies have in several previous studies shown to increase during immuno-therapy (172).

We found no significant differences in IgG4 antibody levels to any of the investigated dog allergen molecules in sensitized children with a positive *vs* a negative NPT. Our results agree with findings by Burnett et al. who demonstrated that isolated IgG4 to Can f 1 could not distinguish tolerant children from dog allergic children (139). According to our findings, this also seem to be the case for Can f 2 and Can f 5, thus, the clinical utility of IgG4 to dog allergen molecules in the diagnosis of dog allergy appears to be limited. However, we found higher IgG4 levels to Can f 1 and Can f 5 among children with a dog at home. Thus, IgG4 to

allergen molecules seem to reflect exposure. Accordingly, the use of IgG4 to dog allergen molecules might be useful to confirm efficient exposure during allergen-specific immunotherapy.

6.8 NASAL GENE EXPRESSION

Previous findings demonstrating that nasal gene expression patterns reflect bronchial gene expression, make investigations of the nasal transcriptome in dog dander sensitized children particularly interesting.

We found that *CST1* was the most upregulated gene among dog dander sensitized children. Several recent investigations demonstrate an upregulation of *CST1* in airway allergy. *CST1* was the most differentially expressed mRNA in nasal epithelial brushings from children with allergic rhinitis (173) and dust mite allergy (174). Our study adds the information that nasal overexpression of *CST1* is associated with several clinical and biochemical markers for airway allergy among dog dander sensitized children.

The protein product of *CST1*, Cystatin 1 (also named Cystatin SN), has shown to be upregulated in individuals with eosinophilic chronic rhino-sinusitis with nasal polyps, and specifically in those with asthma. Among these subjects, Cystatin 1 enhanced eosinophil recruitment and activation in the nasal mucosa (175).

Accordingly, *CST1* is a possible target for future therapies and a potential marker for severity of the allergic airway disease in dog dander sensitized children.

7 CONCLUSIONS

Through the MADOG project we show that molecular assessment can refine the diagnosis of dog allergy in dog dander sensitized children. Based on the presented results, we conclude the following:

Sensitization to an increasing number of dog allergen molecules as well as sensitization to lipocalins, is associated with dog allergy. Moreover, high levels of IgE to the lipocalin Can f 1 is associated with rhinitis symptoms at dog exposure. By investigating all known dog allergen molecules the physician may identify individuals who are monosensitized to Can f 5, and may actually tolerate female dogs. Accordingly, when the clinical history and investigations are not conclusive, molecular allergy diagnostics can provide valuable information in the diagnostic work-up of children with suspected dog allergy (paper I).

Asthma in dog dander sensitized children is associated with multiple sensitizations to furry animal allergen molecules and lipocalins. Children with severe asthma have higher IgE levels to dog lipocalins than other dog dander sensitized children. In particular, we show that IgE levels to the previously scarcely investigated lipocalins Can f 4 and Can f 6 seem clinically relevant. Thus, a detailed assessment using molecular allergy diagnostics may help the clinicians to assess the impact of allergic sensitization on asthma morbidity (paper IV).

Basophil activation test cannot replace in vivo allergen challenges in dog dander sensitized children. However, the basophil allergen threshold sensitivity (CD-sens) to dog dander and to Can f 1 is higher in symptomatic than in asymptomatic sensitized children and a negative test to Can f 1 in Can f 1-sensitized children is associated with a negative NPT. The presence of IgG4 antibodies to dog allergen molecules can reflect dog exposure but do not seem to be markers of tolerance (paper II).

The most over-expressed gene in dog dander sensitized children compared to healthy controls was *CST1*. Enhanced expression was seen in a cluster of children with increased bronchial hyperreactivity, higher blood eosinophil count and basophil allergen threshold sensitivity towards dog dander, suggesting that *CST1* may be important as a biomarker and a mediator of allergic disease (paper III).

8 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The investigation of allergen molecules is successively being implemented in clinical practice. Analyze of dog allergen molecules is still a complement to current extract based investigations (176), and can be performed in individuals where the initial investigations, including the clinical history, physical examination and serum IgE testing with extracts, are not conclusive.

Our results underline that sensitization patterns, rather than sensitization to individual allergen molecules should to be evaluated. A possible approach is to investigate all six available dog allergen molecules. Multiple sensitizations to dog allergen molecules are associated with a high likelihood for dog allergy and allergic airway disease. In the case of monosensitization to Can f 5 the option of having a female dog may be considered. Sensitization patterns can further provide information on disease severity, as severe asthma is associated with increased IgE levels to minor lipocalins among dog dander sensitized children.

The following diagnostic approach was suggested in an editorial comment on our findings regarding sensitization and NPT results (177):

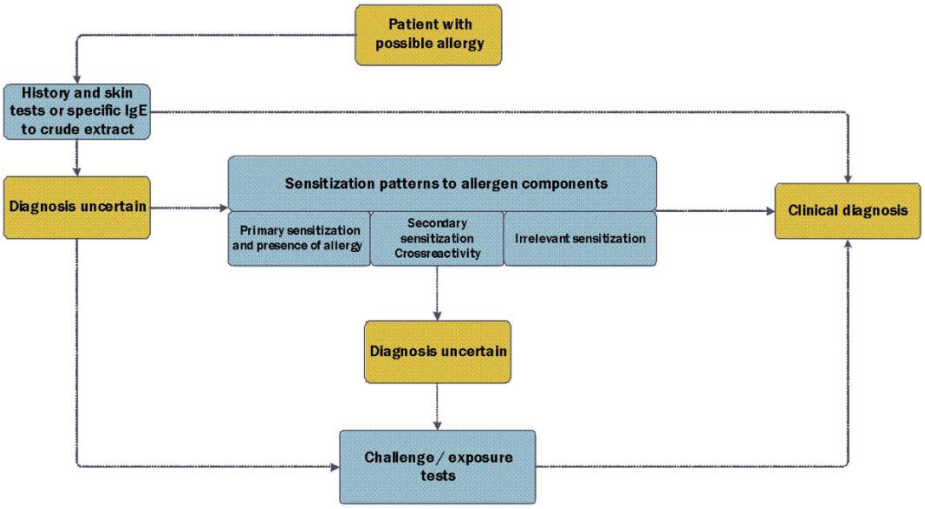


Figure 16: Proposed diagnostic approach for patients sensitized to furry animal allergens. With permission from the publisher. van Wijk, Journal of Allergy and Clinical Immunology (177).

We show that sensitization to minor and cross reactive lipocalins may serve as markers for dog allergy and allergic airway disease, but further investigations are needed to elucidate the role of cross-reactive allergens in the pathogenesis of the allergic disease.

New allergen molecules are continuously being discovered. Since the beginning of this project, two more dog derived proteins have been added to the list of dog allergens, Can f 7, the dog NPC2 protein and Can f 8, a dog cystatin. Sensitization rates of 10-20 % and 13 %

respectively have been reported, but very little is yet known regarding the clinical impact of these proteins (64).

The increasing number of recognized allergens and detectable sensitizations generate complex sensitization patterns. Several recent studies have investigated allergic airway disease and disease severity based on patterns of sensitization, rather than specific IgE to individual allergen molecules. Machine learning techniques have been used to identify co-occurring sensitizations and their relations to different phenotypes of asthma and allergy (178). Differences in patterns of IgE sensitization have been demonstrated between severe and mild to moderate asthma and strong connections between IgE to furry animal allergens were seen in severe asthma (179). Computerized analysis including recently added dog allergen molecules might be useful in the future assessment of dog allergy.

The divergences in sensitization profiles to dog, without one clearly dominant sensitizing allergen, has been used as an explanation for poor and conflicting results on the efficacy of allergen-specific immunotherapy to dog (27). A molecular approach might be used to identify individuals where dog allergen-specific immunotherapy is suitable. However, further investigations are needed to clarify the usefulness of molecular allergology in allergen-specific immunotherapy to dog.

Molecular allergology is a key to individually tailored advice and treatment and can path the way towards a precision medicine-oriented management of dog allergy.

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