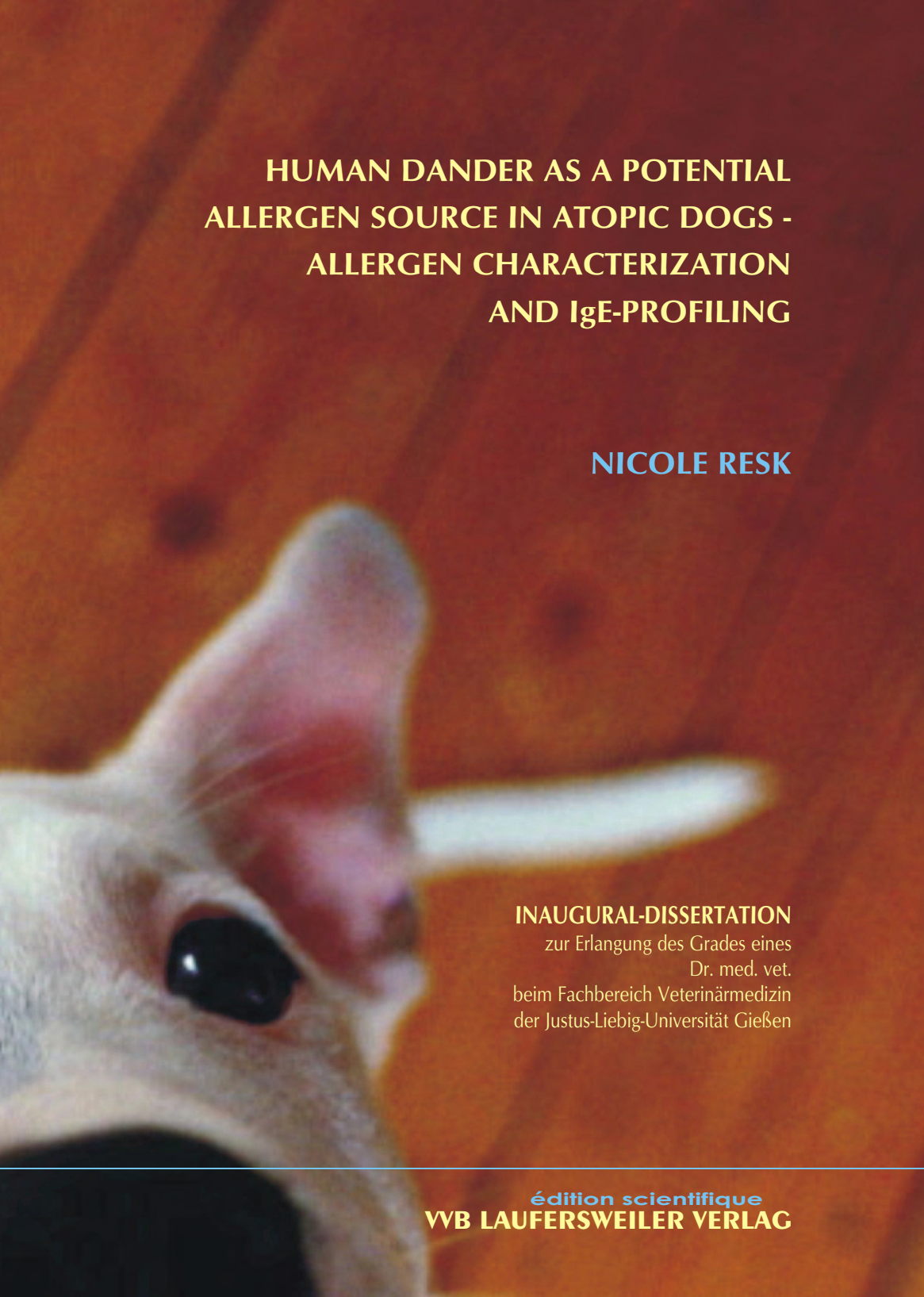

HUMAN DANDER AS A POTENTIAL ALLERGEN SOURCE IN ATOPIC DOGS - ALLERGEN CHARACTERIZATION AND IgE-PROFILING

NICOLE RESK

INAUGURAL-DISSERTATION
zur Erlangung des Grades eines
Dr. med. vet.
beim Fachbereich Veterinärmedizin
der Justus-Liebig-Universität Gießen

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Betreuer: PD Dr. A. Hoffmann**

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eingereicht von

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Philip und meiner Familie

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Abbreviations:

AD	Atopic dermatitis
ALK	Allergologisk Laboratorium Kopenhagen, Denmark (manufacturer of allergenic extracts)
Alt.a.	Alternaria alternata
AP	Alkaline phosphatase
Ara.h.	Arachis hypogaea
Art.v.	Artemisia vulgaris
Asp.f.	Aspergillus fumigatus
B	Buffer
Bet.v.	Betula verrucosa
BSA	Bovine serum albumin
Can.f.	Canis familiaris
Clad.h.	Cladosporium herbarum
Der.f.	Dermatophagoides farinae
Der f 1	Dermatophagoides farinae 1 allergen
Der.p.	Dermatophagoides pteronyssinus
Der p 1	Dermatophagoides pteronyssinus 1 allergen
DL-A	Dog leukocyte antigen
ELISA	Enzyme-linked immunosorbent assay
FcεRI	High affinity receptor for IgE
FcεRIα	Fragment of the extracellular portion of the high affinity IgE receptor alpha subunit
Fel.d.	Felis domesticus
Fel d 3	Felis domesticus 3 allergen (cystatin)
Gly.m.	Glycine max
HAL	Haarlem Allergenen Laboratorium, Amsterdam, Netherlands (manufacturer of allergenic extracts)
HD	Human dander
HDX	Human dander X (monoclonal antibody raised against human dander), Paul-Ehrlich-Institut, Langen Germany
Hom s 1	Homo sapiens 1 allergen
mP30	Monoclonal antibody Paul-Ehrlich-Institut 30 (raised against birch pollen extract), Langen, Germany

MW	Molecular weight
NC	Nitrocellulose
PEI	Paul-Ehrlich-Institut
Pen.a.	Penaeus aztecus
Pen.n.	Penicillium notatum
Phl.p.	Phleum pratense
PVDF	Polyvinylidenefluoride
RAST	Radioallergosorbent test
RBL test	Rat basophil leukemia cell release test (assay)
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
Tri.a.	Triticum aestivum

TABLE OF CONTENTS

1 INTRODUCTION

Allergic diseases, atopy in particular, have become increasingly important over the past decades. Presently, the prevalence of atopic diseases in human beings and dogs is estimated to exceed 30% and 10% respectively (OKUDAIRA, 1998; SCOTT et al., 2001). Similar to the situation in human beings, the increased prevalence in dogs is considered to be associated with environmental factors, such as the heightened exposure of pet dogs to common indoor allergens, house dust mites for example (HILLIER and GRIFFIN, 2001). Other important indoor allergens for human beings include the highly potent mammalian antigens derived from cats, dogs and other species. Conversely human dander (HD), which includes epidermals and other human proteins, is assumed to be of major clinical relevance in pet animals (SARIDOMICHELAKIS et al., 1999; STURE et al., 1995; VOLLSET, 1985; WILLEMSE and VAN DEN BROM, 1983).

1.1 Allergy: current nomenclature and historical features

Prior to dealing with the history of allergy, several common terms used in allergology will be defined. A “hypersensitivity response” is an exaggerated, often deleterious, response of the immune system to an antigen (OLIVRY et al., 2001). An “allergy” describes a disease state characterized by such a hypersensitivity response to an allergen. “Allergen” is a general term and simply refers to an antigen that favors the development of a hypersensitivity response (OLIVRY et al., 2001), though it is often used interchangeably with other terms such as “allergen molecule” and “major allergen” which further specify the allergen. “Allergen molecules” are able to bind IgE in at least one serum from a group of individuals with clinical symptoms of allergy against the specific allergen source. Most are water soluble proteins with molecular weights (MW) between 5 and 70 kilodalton (kDa) (IPSEN, 1998). “Major allergens” are allergen molecules that bind IgE from more than half the sera investigated. Half of the IgE-binding sera must bind the allergen strongly compared to the general binding pattern (IPSEN, 1998).

Allergic reactions are phenomena which have been observed in medicine since antiquity. The first studies about allergic diseases in humans were published in the beginning of the last century (COCA and COOKE, 1922; COCA and GROVE, 1925; PRAUSNITZ and KÜSTNER, 1921; STORM VAN LEEUWEN W., 1922). In veterinary medicine a case of flea allergic dermatitis was first described in 1938 (KISSILEF), followed by a report in 1941 (WITTICH) demonstrating the clinical manifestations of canine atopy in a dog with seasonal

allergic rhinitis.

Attempting a more systematic description of allergic diseases COOMBS and GELL (1975) introduced a classification of allergic reactions responsible for clinical hypersensitivity and disease into four "types".

Type I hypersensitivity (anaphylactic reaction) is the most common hypersensitivity condition to occur in the dog. Type I reactions are those of immediate humoral hypersensitivity and are associated with mast cell or basophil degranulation. Anaphylactic reactions are responsible for allergic disorders such as urticaria and atopy (REEDY et al., 2002).

Type II hypersensitivity (cytotoxic hypersensitivity) is an antibody-dependent reaction. The antibody binds to cellular antigens or complement and causes the destruction of cells by phagocytosis following the activation of complement or killer cells. Type II hypersensitivity is the cause of certain autoimmune diseases, including pemphigus and autoimmune hemolytic anemia (REEDY et al., 2002).

Type III hypersensitivity (Arthus reaction) is associated with the generation of immune complexes capable of activating the complement system, resulting in the damage of various tissues. It is also responsible for autoimmune diseases, e.g. systemic lupus erythematosus (REEDY et al., 2002).

Type IV hypersensitivity (cell-mediated immune reaction) occurs when T-helper cells respond to a foreign antigen or small molecules bound to cells to form a complete antigen. The T-helper cell elaborates a variety of cytokines, thereby inducing inflammatory lesions. It takes between 24 and 48 hours from the time of contact with the offending antigen until the reaction is clinically manifest. Allergic contact dermatitis is an example for a type IV reaction (REEDY et al., 2002).

1.2 Canine atopy

Atopy, a term first introduced in 1922 (COCA and COOKE), is a type I hypersensitivity reaction and is defined as a genetically predisposed tendency to develop IgE-mediated allergy to environmental allergens (OLIVRY et al., 2001). Atopic dermatitis (AD) is the most common manifestation of atopy in the dog. Atopic asthma, atopic rhinitis and atopic conjunctivitis are poorly defined and uncommon atopic diseases in dogs (OLIVRY et al., 2001).

1.2.1 Canine atopic dermatitis (AD)

AD is defined as an inflammatory and pruritic allergic skin disease with characteristic clinical features (OLIVRY et al., 2001) and is considered to be the most or second most common cause of canine pruritus (CARLOTTI and COSTARGENT, 1994; REEDY et al., 2002; SCOTT et al., 2001). In textbooks the prevalence is estimated to be 3-15% and 10 % respectively (REEDY et al., 2002; SCOTT et al., 2001), though these figures are not based on reliable epidemiological data (HILLIER and GRIFFIN, 2001).

1.2.1.1 Etiopathogenesis of AD

AD is a complex syndrome affecting the non specific, cellular and humoral immune system in its entirety, though the genetically controlled production of IgE to common environmental allergens and its binding to mast cells is considered to play a major role in the development of the disease (REEDY et al., 2002).

In 1921 it was shown that serum transferred from an allergic human to normal skin of a second individual by intradermal injection and subsequent intradermal injection of the allergen resulted in an anaphylactic reaction persisting at the site for over 48 hours (Prausnitz-Küstner test) (PRAUSNITZ and KÜSTNER, 1921). The biological factor responsible for these reactions was first described in 1925 and termed “reagin” (COCA and GROVE). In the 1960s and 70s “reagin” was biochemically identified as IgE and shown to be the major antibody with reaginic activity in man (BENNICH et al., 1969; ISHIZAKA and ISHIZAKA, 1967). By this discovery an important step had been taken in understanding the pathogenetic mechanisms involved in hypersensitivity reactions.

In veterinary medicine the first clinical case (WITTICH, 1941) of a dog suffering from AD also implicated a reaginic type of antibody. An anaphylactic shock was elicited when the dog underwent skin testing and serum from the same patient gave a positive Prausnitz-Küstner

test when transferred to a normal dog. In 1973 (HALLIWELL) an immunofluorescent study using rabbit sera against canine IgE demonstrated the association of IgE with mast cells. IgE-mediated degranulation of mast cells, which are regarded as the key effector cells in the pathogenesis of allergic diseases such as AD (BROSTOFF and HALL, 1993), is the initial pathologic event in the anaphylactic reaction (REEDY et al., 2002). In a predisposed individual the first contact with the offending allergen results in the excessive secretion of IgE by B-lymphocytes (REEDY et al., 2002). IgE binds to high affinity FcεR1-receptors expressed on the mast cell and basophil cell membranes. Later exposure to the same allergen leads to cross-linking of cell-bound IgE antibodies ("bridging" phenomenon) which induces a complex cascade of biochemical reactions involving G proteins, tyrosine kinases, inositol-triphosphate, protein kinase C, calcium channel activation and rearrangement of the cytoskeleton. This enables the mast cell granules to connect with the plasma membrane (HILL and MARTIN, 1998) and a host of inflammatory mediators are released (histamine, proteases, cytokines etc.) promoting the inflammatory response of the skin by participating in an interplay between the microvasculature and other inflammatory cells (HILL and OLIVRY, 2001). A time frame of 10 to 20 minutes following contact with the offending allergen until appearance of clinical symptoms is characteristic of this hypersensitivity reaction. After five hours delayed reactions can occur as a consequence of eicosanoide activity and immigration of eosinophils (PRELAUD, 2002). Though this reaction cascade is intended to be protective, it is often directed towards harmless environmental antigens and contributes to the development of allergic diseases such as AD.

Due to the fact that certain breeds are diagnosed with AD more frequently than others it is assumed that a genetic predisposition for AD exists (CARLOTTI and COSTARGENT, 1994; KOCH and PETERS, 1994; SARIDOMICHELAKIS et al., 1999; SCOTT, 1981; STURE et al., 1995; WILLEMSE and VAN DEN BROM, 1983). Many studies have focused on potential markers of AD, such as the antibody IgE, major histocompatibility haplotypes and the inheritance of the genes responsible for IgE production. VRIESENDORP and SMID-MERCX (1975) compared IgE concentrations in the serum and dog leukocyte antigen (DL-A) haplotypes in normal dogs to dogs with positive intradermal tests. Significant differences in the gene frequency of the different DL-A groups between normal and atopic dogs were not found. The combination of the DL-A haplotype 3 and R 15 was found significantly more often in atopic dogs than normal dogs, whereas the DL-A haplotype 9,4 was found significantly less frequently in atopic dogs. The authors hypothesized the former combination to increase the susceptibility to AD, whereas the latter was postulated to protect the individual

from the disease. IgE concentrations between the two groups of dogs showed no significant differences and there was no evidence of a relationship between IgE levels and DL-A haplotypes. DE WECK et al. (1995; 1997) demonstrated that repeated sensitization of selected beagle dogs within the first week of life is able to trigger high IgE levels and the authors suggested a dominant inheritance pattern for the development of high IgE levels. Therefore, the full potential of a high IgE response gene seems to be fulfilled only under certain conditions, such as early and repeated allergen exposure. This indicates that both environmental and genetic factors are of central importance for the development of high IgE levels (SOUSA and MARSELLA, 2001). ZUNIC (1998b) investigated dogs with high allergen-specific IgE levels („high responder“ dogs) regarding their reactions to intradermal testing and the development of clinical lesions of AD. The dogs showed immediate skin test reactivity but only some of them developed clinical lesions of AD after allergen exposure. The author concluded that the development of clinical disease is subject to an additional genetic control.

Although IgE indisputably plays a pivotal role in allergic asthma and rhinitis, critics cite the fact that many clinically normal individuals show a positive intradermal test as well as elevated allergen-specific serum IgE levels (HALLIWELL and DEBOER, 2001). A recent study demonstrated increased dust mite-specific IgE concentrations in the serum of clinically normal dogs (LIAN and HALLIWELL, 1998). Another study showed that IgE is also inducible in dogs via artificial sensitization, though disease is not necessarily a consequence (JACKSON et al., 1996). Many plausible explanations for this phenomenon have been suggested. In humans evidence for a functional heterogeneity of IgE antibody in terms of its ability to mediate histamine release through interaction with a histamine releasing factor was published in 1995 (MACDONALD et al.). In veterinary medicine the possibility that canine IgE might be heterogeneous received support from a study performed by PENG et al. (1997). Two biologically active forms of IgE were revealed, underlining the possibility of a relationship between different IgEs and the allergic response in the dog. A second theory suggests differences in mediator releasability of mast cells between normal and atopic dogs. Evidence for this was published in a study where histamine release from basophils of atopic dogs, from normal dogs and from dogs in which IgE antibodies to Dermatophagoides farinae (Der.f.) was induced experimentally. The leukocytes of atopic dogs had a greater tendency to release histamine than did those of normal or artificially sensitized dogs, both to allergen and anti-IgE (JACKSON et al., 1996). Other in vitro studies have also shown that the releasability of mast cells is enhanced in those isolated from atopic dogs as compared to mast cells isolated

from normal dog skin (DE MORA et al., 1996). One explanation for enhanced mast cell releasability suggests that up regulatory factors are produced in AD to prime mast cells for subsequent allergen challenge (HILL and OLIVRY, 2001). Mast cells are known to possess c-kit, the receptor for stem cell factor (LONDON et al., 1996). Experiments performed by HAMMERBERG et al. (2001) suggest that dermal stem cell factor secretion could potentize histamine release following IgE receptor cross-linking and thus, be one of the explanations for the inherent mast cell hyperexcitability observed in canine AD.

1.2.1.2 Clinical manifestations of AD

For veterinary medicine the clinical signs of canine AD were initially described in 1971 (HALLIWELL and SCHWARTZMAN). Primary symptoms are pruritus and the appearance of atopic lesions at an age of 6-12 months, often seasonally at first. Certain areas of the body are predisposed, such as the face, extremities, axillae and ventrum (GRIFFIN and DEBOER, 2001). Whether primary lesions as such exist, remains unclear. Many studies have described the appearance of primary lesions (CHAMBERLAIN, 1974; GRIFFIN, 1993; ROCKEY and SCHWARTZMAN, 1967), while other studies negate the occurrence of primary lesions (HALLIWELL and SCHWARTZMAN, 1971; SCOTT, 1981). However, dermatologists agree that if a primary lesion exists, it consists mainly of erythema. Secondary lesions resulting from chronic pruritus, automutilation, chronic inflammation and secondary bacterial infections are a common finding in atopic patients (REEDY et al., 2002; SCOTT et al., 2001; WILLEMSE and VAN DEN BROM, 1983).

1.2.1.3 Diagnosis of AD

Although history and clinical findings are suggestive of AD, symptoms in both humans and dogs are extremely variable and there is no pathognomonic feature permitting a definitive diagnosis (DEBOER and HILLIER, 2001a). The first step towards a diagnosis is the fulfillment of a minimum of clinical major criteria, i.e. pruritus, the location and appearance of lesions and minor criteria (WILLEMSE, 1988), such as the age of onset. The most recent criteria were defined by PRÉLAUD et al. (1998). In the second step, important differential diagnosis such as flea allergic dermatitis and cutaneous adverse food reactions must be ruled out (SCOTT et al., 2001). In vivo or in vitro tests are used in a third step as an adjunct to the clinical diagnosis of AD and to identify the allergens to which the animal is hypersensitive. The identification of the relevant allergens enables the clinician to employ specific allergen avoidance measures or allergen-specific immunotherapy (REEDY et al., 2002).

In vivo tests measure the mast-cell bound IgE and the resulting skin reaction produced by degranulation of the mast cells following intracutaneous application of the offending allergen. In vitro tests measure immunoreactants, particularly total serum IgE and allergen-specific IgE. Studies have shown total IgE levels between clinically healthy and atopic dogs not to be significantly different (HILL et al., 1995; OLIVRY et al., 1996). In a recent study RACINE et al. (1999) investigated total serum IgE concentrations in a beagle population and found that sex and age significantly influence IgE values. The authors hypothesized that breeds must be evaluated singly for total IgE concentration to be of diagnostic value. Furthermore, routine vaccination procedures and/or the presence of parasites may also influence total serum IgE concentrations in dogs (HILL et al., 1995).

Semiquantitative serum allergen-specific IgE assays were first reported in 1978 (HALLIWELL and KUNKLE) and have become very popular over the past few years. The semiquantitative determination of circulating allergen-specific IgE can be performed with commercially available tests (DEBOER and HILLIER, 2001b) based on enzyme-linked immunosorbent assays (ELISA), radioallergosorbent tests (RAST) or solid phase nitrocellulose (NC) -immunodot tests. These assays have several advantages compared to the intradermal test. Clipping and sedation of the patient are unnecessary and only a small amount of blood is required for testing (WASSOM and GRIEVE, 1998). Serological assays are also less time-consuming than the intradermal test and the veterinarian must not invest in the test materials that could become dated respectively expired. Furthermore the test is harmless, as there is no risk of an anaphylactic reaction, a rare complication of in vivo testing (REEDY et al., 2002).

Studies comparing the serum IgE to specific allergens in the atopic dog population (table 1) reveal highly varying results (HILL and DEBOER, 2001).

Table 1: Elevated serum IgE to selected allergenic extracts in atopic dogs in six studies (HILL and DEBOER, 2001); Figures indicate the percentage of dogs with elevated IgE levels within the designated study

Allergenic extracts	Study*					
	A	B	C	D	E	F
Dermatophagoides farinae	86	90	90	44	32	62
Dermatophagoides pteronyssinus	86	90	90	44	32	62
Human dander	-	60	0	-	-	-
Birch	54	70	0	-	-	-
Cat epithelium	-	70	0	0	-	0
Mugwort	-	10	10	-	-	-
Timothy	83	80	0	-	-	-
Penicillium	100	90	30	-	-	-
Aspergillus	81	100	20	-	-	0
Alternaria	100	100	20	2	4	0

*) Study References: A: (KLEINBECK et al., 1989); B: (BOND et al., 1994) ELISA data from test A; C: (BOND et al., 1994), ELISA data from test B; D: (HAMMERLING and DE WECK A.L., 1998) Immunodot data; E: (HAMMERLING and DE WECK A.L., 1998) ELISA data; F: (ZUNIC, 1998a) Immunodot data, "-": allergenic extracts not included in the study

At first glance it is not clear, whether the high variations in the percentage of dogs with elevated allergen-specific IgE reflect true differences in the respective sensitization patterns or whether varying sensitivities and specificities of the individual IgE-assays are responsible for this phenomenon. For example, the proportion of dogs with elevated allergen-specific serum IgE to the indoor allergen HD, vary from 0% in one ELISA to 60% in a second (BOND et al., 1994). Allergen-specific IgE levels to outdoor allergens show the same discrepancies. Elevated timothy-specific IgE levels for example, ranges from 0% to 83% (BOND et al., 1994; KLEINBECK et al., 1989). Though inconsistencies of results regarding outdoor allergens may be explained by the variable geographical regions in which the studies were performed as well as differing lifestyles, other explanations must be found for indoor allergens (HILL and DEBOER, 2001). Both test and patient factors are capable of influencing test results. Of the test factors the allergenic extract used in the assay plays a central role. In human allergology laboratories are therefore increasingly relying on biologically-standardized allergenic extracts or recombinant-source major allergens epitopes for their assays. This standard has not yet been established in veterinary medicine (DEBOER and HILLIER, 2001b). Allergenic extracts from different manufacturers are not standardized and

can contain significantly different concentrations of major allergens, consequently leading to varying assay results (MEYER et al., 1994; REEDY et al., 2002). Veterinary laboratories also lack standard quantitative reference preparations of allergen-specific IgE. Because the assays are semiquantitative, results are difficult to compare (DEBOER and HILLIER, 2001b). Furthermore mandatory or voluntary regulatory steps to maintain quality control as there are in the United States for human laboratories (DEBOER and HILLIER, 2001b) have not yet been established. One report demonstrates that results regarding the same serum sample sent to the same laboratory on different dates varied substantially (PLANT, 1994). Another major problem of allergen-specific IgE assays is the specificity of the anti-dog IgE used. Assays using monoclonal anti-IgE detection antibodies (DERER et al., 1998; HAMMERLING and DE WECK A.L., 1998) should be preferred to the less specific polyclonal anti-IgE detection reagents, which many companies and laboratories still use in their tests (BOORD, 2000; SAEVIK et al., 2003). Polyclonal anti-IgE reagents tend to cross-react with IgG which exceeds IgE concentrations in serum by far (HILL et al., 1995), therefore potentially causing false-positive results. One recently introduced highly specific assay uses a recombinant fragment of the extracellular portion of the human high affinity IgE receptor alpha subunit (FcεRIα) as a detection reagent, which does not cross-react with IgG (STEDMAN et al., 2001; WASSOM and GRIEVE, 1998). Further factors influencing test results include the reacting phase of allergen and the detection reagent employed. Though the reacting phase of allergen, mostly solid phase supports such as microtitre plates, cellulose or NC strips/discs (LIAN and HALLIWELL, 1998; ZUNIC, 1998a) probably have a negligible effect on test results, the detection reagents used may influence assay results in ELISAs. Detection reagents are mostly enzymes coupled to an antibody. MARI et al. (1999) suspect that patient serum IgE directed against common, non mammalian carbohydrate determinants may bind to carbohydrate moieties possibly available in a plant derived detection enzyme such as horse radish peroxidase, occasionally resulting in false positive reactions.

In human beings it is known that patient factors such as age, the season of year and prior immunotherapy can influence test results (OWNBY, 1998). There is only limited information available concerning these factors in the dog. Data regarding the effect of the season are variable. Some authors revealed seasonal variation in allergen-specific IgE (HALLIWELL and KUNKLE, 1978), while one study found levels of allergen-specific IgE to remain unchanged for two months following the pollen season (MILLER, JR. et al., 1992). The same study also showed that treatment with corticosteroids does not affect in vitro serum allergen-specific IgE assays if administered at moderate doses for up to two months. Therefore,

depending on what time of year the studies were performed in and patient inclusion criteria, variable test results in the studies are to be expected. Serum factors have also been reported to influence test results. The level of blocking IgG is felt to be an important cause of false negative test results (GRIFFIN, 1989; WOJDANI et al., 1985) and in humans, high total serum IgE levels are associated with more false-positive RAST results (CAPRIO et al., 1983). Total serum IgE levels in dogs are considerably higher, 190,000 ng/ml in normal dogs versus 700 ng/ml in atopic humans. Therefore the "average dog" has 20 to greater 200 times the background of IgE found in humans. The high levels of IgE in the canine are believed to be the result of their exposure to external and internal parasites. If results from humans are to be extrapolated in dogs, these high IgE levels may lead to false-positive results (GRIFFIN, 1989). Lastly, non IgG serum blockers have been hypothesized to affect test results as well (WOJDANI et al., 1985).

In a comprehensive study DeBoer and Hillier (2001b) compared the test sensitivity and specificity of serologic allergen-specific IgE assays (table 2).

Table 2: Evaluation of sensitivity and specificity of in vitro IgE assays in dogs (DEBOER and HILLIER, 2001b)

Study reference	Number of dogs	Sensitivity	Specificity
(CODNER and LESSARD, 1993)	41	100	0
(MILLER et al., 1993)	17	43-86*	33-100*
(BOND et al., 1994)	30	100	0-10
(GINEL et al., 1998)	48	72	42
(HAMMERLING and DE WECK A.L., 1998)	76	45-100*	78-92*
(WASSOM and GRIEVE, 1998)	50	64-100*	79-100*
(ZUNIC, 1998a)	48	44-78*	35-97*
(MUELLER et al., 1999)	84	90	92
(SAEVIK et al., 2003)	60	54	84

*) Where ranges are given, the reported results varied by specific allergen or allergen group, by test method, or by criteria for analysis

The apparently dominant problem of low test specificity, i.e. positive in vitro test results in conjunction with a negative intradermal test (the gold standard of diagnosis) can be the consequence of either a "technical" or a "biological" false positive result (DEBOER and HILLIER, 2001b). "Technical" false positive results may be due to nonspecific binding to proteins or carbohydrates in the extract, the measurement of other immunoglobulin isotypes or non-allergen-specific IgE. "Biological" false positive results are the consequence of

genuinely elevated allergen-specific IgE levels in clinically healthy dogs for which possible reasons have been dealt with in detail in chapter 1.2.1.1. Low test sensitivity, i.e. a negative in vitro test result in conjunction with a positive intradermal test result, can be due to either a false positive intradermal test or the interference with IgE measurement by circulating IgE immune complexes (VASSELLA et al., 1990). Even though the intradermal test was employed as the "gold standard" of diagnosis in all studies shown above it is important to remember that it does not necessarily reliably reflect the clinical status of the patient (DREBORG, 1993; PASTORELLO, 1993). For example, positive intradermal test results in normal dogs are a common finding (CODNER and TINKER, 1995; LIAN and HALLIWELL, 1998) and can be due to mast cell-bound IgE leading to degranulation during skin-testing though the animals do not exhibit clinical signs of AD ("false positive" reactions) (AUGUST, 1982; LIAN and HALLIWELL, 1998). Apart from subclinical hypersensitivity (PASTORELLO, 1993), clinically irrelevant reactions can also be due to per se irritant substances eliciting false positive reactions in non atopic dogs (AUGUST, 1982) or the necessity of other factors than mast cell-bound IgE, i.e. allergen-specific IgGd (WILLEMSE et al., 1985), to cause AD. In order to reliably calculate the test sensitivity and specificity of allergen-specific IgE assays a reliable "gold standard" of allergy diagnosis is required. Unfortunately, the only indisputably reliable "allergy test" is a provocation test, which is an extremely difficult test to perform and can cause dangerous anaphylactic reactions in the patient (PRELAUD, 2002).

1.2.2 Canine AD and cutaneous adverse food reactions

The term "adverse food reaction" refers to any clinically abnormal response attributed to the ingestion of a food or food additive. "Food allergy reactions" are considered a subgroup of adverse food reactions and are defined as an immunologically mediated adverse food reaction to food unrelated to any physiological effect of the food or food additive (ANDERSON, 1986). Food allergy in humans is an IgE-mediated hypersensitivity and a clear relationship between food allergens and AD has been established (SICHERER and SAMPSON, 1999). In veterinary medicine reports from the late 60´s and 70´s suggest that up to 30% of dogs with AD exhibit concurrent adverse food reactions (CHAMBERLAIN, 1974). In later studies less dogs with AD were reported to show concurrent adverse food reactions (CARLOTTI and COSTARGENT, 1994; SARIDOMICHELAKIS et al., 1999). In contrast 13-30% of dogs diagnosed with cutaneous adverse food reactions have been reported to exhibit concurrent AD (CARLOTTI et al., 1990; CARLOTTI and COSTARGENT, 1994; ROSSER, 1993;

WHITE, 1986). In dogs however, the pathogenesis of cutaneous adverse food reactions remains controversial. Several studies of canine models of IgE-mediated food hypersensitivity (DE WECK, 1997; TEUBER et al., 2002) have been published, as well as reports of spontaneous IgE-mediated food allergy in dogs (ISHIDA et al., 2003; JACKSON and HAMMERBERG, 2002). However, IgE-mediated hypersensitivity as the most common pathogenetic mechanism of cutaneous clinical signs of food allergy in dogs remains to be proven (HILLIER and GRIFFIN, 2001).

All foods can cause adverse reactions (REEDY et al., 2002) and lists of relevant foods differ depending on the geographic area and culinary habits (BAKER, 1990). Foods with the highest potential for causing adverse reactions are those which contain the most protein and are fed frequently. Studies have proven that the higher the intake of any one food, independent of the protein involved, the higher the probability of developing an adverse reaction (CARLOTTI et al., 1990; JEFFERS et al., 1996). Food antigens are usually protein molecules with a MW of 10 kDa or more (PRELAUD, 2002). Potentially relevant foods for dogs include most meats (REEDY et al., 2002), soy, a protein included in many low-quality pet foods (HILL et al., 2000), certain grains, such as components of wheat and barley (PRELAUD, 2002), all fruits and vegetables, haptens and food additives. In contrast to humans, peanuts as a representative of the family of legumes are a widely unrecognized potential source of cutaneous adverse food reactions in dogs. Dogs ingest peanut antigens when fed commercial pet foods containing peanut hulls added as prebiotic agents due to their oligosaccharid content (FLICKINGER and FAHEY, JR., 2002). Sea food can also cause cutaneous adverse food reactions. Fish meat itself may elicit an adverse reaction or be contaminated by large amounts of histamine due to spoiling or storage at high temperatures (REEDY et al., 2002). Crustacean proteins, common food allergens for humans (LEHRER et al., 2003) are often an ingredient of pet foods and in analogy to humans may elicit cutaneous adverse food reactions in dogs.

Clinical manifestations of cutaneous adverse food reactions involve mainly the skin and the digestive tract (BAKER, 1990). Intestinal signs may be acute or chronic and vary from mild to severe. The most important dermatological signs are urticaria and pruritus, which is generally intense and may or may not be accompanied by skin lesions (BAKER, 1990). Similar as in AD, onset of the disease is at a young age and the predilection sites are identical (PRELAUD, 2002). Otitis and secondary bacterial and Malassezia infections frequently occur (REEDY et al., 2002; SCOTT et al., 2001). Therefore it is difficult to distinguish between the two diseases on a clinical basis alone.

The diagnosis of cutaneous adverse food reactions are based on the performance of exclusion diets, serum allergen-specific IgE assays, intradermal tests, the gastroscopic food sensitivity test and colonoscopic allergen provocation (ALLENSPACH et al., 2006; BAKER, 1990; PRELAUD, 2002; REEDY et al., 2002). The exclusion diet is considered the "gold standard" method of diagnosis (SAMPSON, 1988). In the first step, the animal should receive a novel protein over a period of at least six weeks during which no other foods may be fed. In a second step, the dog is challenged with the foods it received prior to the elimination trial. If the condition worsens, an adverse food reaction can be suspected (BAKER, 1990; PRELAUD, 2002). Serum allergen-specific IgE assays for foods are controversial. In human medicine some studies report low sensitivity and low specificity (SANZ et al., 1996), while other authors claim good sensitivity, but poor specificity of such assays (SICHERER and SAMPSON, 1999). Published studies of food allergen-specific IgE assays in dogs, found them to be insensitive, nonspecific, and unreliable for the diagnosis of adverse food reactions (JEFFERS et al., 1991; MUELLER and TSOHALIS, 1998). Because it is speculated that not all adverse food reactions in dogs are IgE-mediated, and other pathogenetic mechanisms may be involved, an IgE assay would per se fail to detect some dogs with adverse food reactions (DEBOER and HILLIER, 2001b).

1.3 Allergens for dogs

The term "allergen" is a general term and is often used interchangeably with "allergenic extract" and "allergen source". An allergen is an antigen that favors the development of a hypersensitivity response (OLIVRY et al., 2001). Clinically relevant allergens include a variety of indoor and outdoor allergens, a selection of which will be discussed in the following paragraph.

1.3.1 Clinically relevant allergen sources

Many studies have identified clinically relevant allergens for dogs either by demonstration of skin test reactivity to the offending allergens and/or measurement of allergen-specific IgE or IgGd levels in serum (BOND et al., 1994; CARLOTTI and COSTARGENT, 1994; CODNER and TINKER, 1995; DEBOER, 1989; HAMANN et al., 1996; HAMMERLING and DE WECK A.L., 1998; KLEINBECK et al., 1989; KOCH and PETERS, 1994; NESBITT, 1978; NESBITT et al., 1984; SARIDOMICHELAKIS et al., 1999; SCOTT, 1981; STURE et al., 1995; VOLLSET, 1985; WILLEMSE et al., 1985; WILLEMSE and VAN DEN BROM, 1983; ZUNIC, 1998a). The spectrum of relevant allergens appears to be similar to that of

human beings including the outdoor allergens tree, weed and grass pollens and the indoor allergens mold spores, epithelial antigens and mites as components of “house dust” (IPSEN, 1998).

House dust has been recognized as a major perennial allergen for over 80 years (COCA and COOKE, 1922; KERN, 1921) and has been reviewed by a multitude of scientists (BERRENS, 1970; SUTHERLAND, 1942; WALZER, 1938; WOOD et al., 1988). House dust is a complex mixture that may contain a variety of potent allergens (WOOD et al., 1988). Apart from dust mite antigens of the Dermatophagoides species, dog, cat, human and mold antigens are highly prevalent in house dust (WOOD et al., 1988).

Der.f. in particular, appears to be among the most common allergens in Europe to elicit positive reactions in dogs with AD (HILL and DEBOER, 2001; LIAN and HALLIWELL, 1998; STURE et al., 1995). Since allergen exposure is necessary for sensitization, a recent study performed quantitation of house dust mites and house dust mite allergens in the microenvironment of dogs (RANDALL et al., 2003). It was found that Der.f. is the most common mite allergen in the household microenvironment of dogs, a finding which may explain the higher prevalence of positive reactions to Der.f. than to Dermatophagoides pteronyssinus (Der.p.) (BENSIGNOR and CARLOTTI, 2002; CARLOTTI and COSTARGENT, 1994; LIAN and HALLIWELL, 1998; STURE et al., 1995; VOLLSET, 1985). In contrast, sensitization to Der. p. is more frequent in human beings. Another reason for this phenomenon may be different major allergens recognized by man and dog (MCCALL et al., 2001; NUTTALL et al., 2001; WEBER et al., 2003).

A further very important antigenic component of house dust is constituted by epidermals and other proteins originating from humans. The term commonly used for these antigens is human dander (HD). An adult produces about 5 g of dander per week (KLIGMAN, 1964) and house dust always contains a certain percentage of HD. It has been shown that the residual allergen activity of house dust is due almost entirely to its HD content (4%) (VOORHORST, 1977). The allergenic potential of HD as an autoallergen has been known for decades. STORM VAN LEEUWEN (1922) was among the first to draw general attention to the specific human dandruff allergen as an important factor in human atopy. A study performed in 1941 (HAMPTON and COOKE) showed positive skin reactions to human dandruff extract and demonstrated skin sensitizing antibodies in the sera of these patients. SIMON, apart from various studies showing the significance of human epidermals in infantile eczema (1944), demonstrated that the allergen is particularly concentrated in the scales of the human scalp (SIMON, 1947). One study tried to partially purify the HD allergen by the method of salting

out fractionation (BERRENS and YOUNG, 1964). There have also been several attempts to isolate the relevant proteins in HD extracts via various immunochemical methods. In 1988 (YU et al.) an aqueous crude HD extract was divided into four fractions by use of high-speed gel filtration chromatography. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining demonstrated that fraction II contained two proteins with apparent MW of approximately 10 kDa and 13 kDa, respectively. Fraction I showed two major protein bands of which the apparent MW were located at 59 kDa and 52 kDa. VALENTA et al. (1998) characterized human allergen with serum IgE from patients with AD. The IgE-binding protein was shown to have an apparent MW of 55 kDa and was termed Hom s 1. Hom s 1 exhibited a broad cellular and tissue reactivity (skin, lung>>gastrointestinal tract>>muscle, brain). In veterinary medicine human epidermals and proteins are recognized as potentially important allergens in AD. WILLEMSE and VAN DEN BROM (1982) were the first to establish a skin threshold concentration for the use in intradermal tests in the canine patient. HD has been included in many studies investigating the frequency of elevated allergen-specific IgE (table 1) and positive intradermal test reactivity (table 3) in the atopic dog population. In some European studies, among house dust mite and house dust not otherwise specified, the human dandruff antigen has even been discussed to be of central importance in AD (SARIDOMICHELAKIS et al., 1999; STURE et al., 1995; VOLLSET, 1985; WILLEMSE and VAN DEN BROM, 1983). Possible reasons for the very variable results (table 1 and 3) such as the lack of standardization of allergenic extracts and false positive and false negative results in in vitro and in vivo tests have been dealt with in chapter 1.2.1.3.

Table 3: Percentage of atopic dogs reacting to HD in intradermal tests (HILL and DEBOER, 2001)

Allergen	Study*									
	A	B	C	D	E	F	G	H	I	J
HD	17	50	40	42	60	36	66	68	55	36

*) Study references: A: (NESBITT, 1978); B: (SCOTT, 1981);C: (WILLEMSE and VAN DEN BROM, 1983); D: (VOLLSET, 1985); E: (BOND et al., 1994), skin testing data; F: (CARLOTTI and COSTARGENT, 1994); G: (KOCH and PETERS, 1994); H: (STURE et al., 1995), data from Edinburgh group; I: (STURE et al., 1995), data from London group; J: (SARIDOMICHELAKIS et al., 1999)

1.3.2 Allergenic extracts

For use in the diagnosis and therapy, allergenic extracts must be prepared from clinically relevant allergen sources. “Allergenic extract” is a frequently used technological term. It refers to the intermediate product after extraction of the allergenic substances from the raw allergen source materials that patients are exposed to under normal conditions (IPSEN, 1998) and prior to the final preparation of test allergens and allergens for therapeutic purposes. Allergenic extracts are complex biological substances and are comprised of many different proteins (IPSEN, 1998). Many factors during the process of extraction and handling influence the composition, biological activity and stability of allergenic extracts. In order to ensure reliable and reproducible results in diagnosis and therapy, standardization is essential (DREBORG, 1993). A standardized allergenic extract must reflect a relevant allergen source material and be of a known composition and total allergenic potency, which is constant between batches (DREBORG, 1993).

1.3.2.1 Preparation of allergenic extracts

Allergenic extracts should have the specific ability to detect all possible IgE specificities directed against any constituent from a given allergen source (IPSEN, 1998). The preparation of an allergenic extract must neither denature the proteins or allergens nor significantly alter the ratio between the individual components. The time needed to obtain a representative extract is a compromise between denaturation and desired yield (IPSEN, 1998).

All extracts prepared in multiple dose containers must contain a substance to inhibit microbial growth and preserve the potency of the allergenic extracts in the diluents. Though phenolization can cause extracts to lose potency (NELSON, 1981), especially those with weaker dilutions at high temperatures (REEDY et al., 2002), phenole is generally included in allergenic extracts in a concentration of 0.4% to prevent contamination (PRELAUD, 2002). Glycerole is one of the most commonly used preservatives and was introduced in 1917 (CLOCK, 1917). In a concentration of 50% or more, glycerole inhibits microbial growth (ROSENAU, 1903) and in addition, has an excellent effect on the stability of allergenic extracts. Extracts with 50% glycerole can be kept at +4°C for several years (PRELAUD, 2002). Prior to intradermal use of glycerole preserved extracts a dilution to a 0,5% glycerole content is necessary, which is a concentration well tolerated by animals (PRELAUD, 2002). Higher concentrations can cause local reactions such as erythema, exudation and necrosis (SCOTT et al., 2001; VAN WINKLE, 1981).

1.3.2.2 Allergen standardization

In human medicine an increasing part of the allergenic extracts is now standardized and should meet the following requirements (IPSEN, 1998). Every batch of allergenic extract should be evaluated regarding allergen composition and content of specific allergens to ensure constant ratios. Qualitative methods include SDS-PAGE and immunoblotting, isoelectric focusing and crossed immunoelectrophoresis. The quantitative determination of specific allergens can be performed by immunoelectrophoresis and ELISA. Furthermore allergenic extracts must have a consistent total allergenic activity, measured by in vivo and in vitro methods. Recently, a new method was introduced for in vitro measurement of allergenic potency (HOFFMANN et al., 1999). The rat basophil leukemia cell mediator release assay (RBL test) is based on a rat basophil leukemia cell line which can be sensitized with murine sera or monoclonal IgE antibodies specific for the allergen. Challenge with the allergenic extract induces cross-linking of the monoclonal antibodies and degranulation of the cells. An enzyme (β-hexosaminidase) released from the cells induces a colorimetric reaction with the added substrate cocktail and is measured photometrically.

Standardized extracts are not available in veterinary medicine and those standardized for human use are of limited value in dogs, because other species may recognize a variety of different major allergens in the crude extract. Therefore false positive and negative results during intradermal testing and allergen-specific serum IgE assays using extracts for human beings are possible (HILLIER and DEBOER, 2001). So far very few major allergens including house dust mites (MCCALL et al., 2001; NUTTALL et al., 2001), cat flea (MCDERMOTT et al., 2000) and Japanese cedar (SAKAGUCHI et al., 2001) antigens have been identified for the dog by Western blotting using serum from allergic dogs as probes for the detection of relevant allergenic proteins in the crude extract. As mentioned before, apart from determining major allergens in allergenic extracts, standardization for use in intradermal tests also includes biological standardization via intradermal testing (IPSEN, 1998). Dogs show marked differences in skin reactivity depending on sex (AUGUST, 1982), coat pigmentation (AUGUST, 1982) and age (AUGUST, 1982; SCHWARTZMAN, 1984; WILLEMSE and VAN DEN BROM, 1982) as well as differences in skin thickness depending on the breed (SCHÄRER, 1997) and age (BAKER, 1967; SCHÄRER, 1997). Therefore biological standardization of allergens for use in the canine patient is an extremely difficult procedure. Because of these problems standardized extracts are not available for use in veterinary medicine (HILLIER and DEBOER, 2001).

1.4 Objective

The antigen challenge by perennial indoor allergens is most probably the main cause of atopy in dogs. In addition to mites and molds, human proteins are a major component of the antigenic materials present in house dust and are thought to play an important role in the development of canine AD. Two crucial points suggest human proteins to be of clinical importance in dogs:

Firstly, contact with human epidermals and proteins are chronic since these antigens are ubiquitous in an indoor environment. Secondly, human dandruff belongs to the family of mammalian proteins which per se harbours highly potent allergenic properties.

In order to perform further studies regarding the sensitization of dogs to human epidermals and proteins a well characterized HD extract is essential. Therefore an important issue of this study is the characterization of two available HD extracts, which complete a representative panel of allergens used to screen four typical dog populations for their allergen-specific IgE profiles. A further aim is the identification of specific IgE-binding proteins in HD extract and the elucidation of their biological nature.

2 MATERIAL AND METHODS

2.1 Dog populations

In this study the serological data of 178 dogs were analyzed. The dogs originated from four groups. In detail the first group was composed of "multi source" atopic dogs (n=88), the second group included dogs with skin diseases other than atopy (n=20) and the third and fourth group were comprised of healthy "household" (n=30) and healthy kenneled beagles (n=40), respectively.

2.1.1 "Multi-source" atopic dogs

The "multi-source" atopic dogs (group I) included a total number of 88 dogs, 55 of which were composed of a cohort of pet dogs seen by general practice veterinarians with clinical symptoms suggestive of AD. The sera of these dogs had been submitted for allergen-specific IgE screening at BIOCONTROL laboratories, Mainz, Germany. The other 33 dogs were presented at the Clinic for Small Animals, University of Berlin, Germany and were diagnosed with AD by a combination of history, clinical findings and elimination of important differential diagnosis. The intradermal test was employed as an adjunct to diagnosis. The signalment and intradermal test data were not available for this group of dogs.

2.1.2 Dogs with skin diseases other than atopy

The second group (group II) was comprised of 20 dogs presented at the Small Animal Clinic, University of Giessen, Germany. These dogs were subject to skin diseases other than AD including infestation with parasites (n=8), bacterial (n=10) and fungal skin infections (n=2). The diagnosis was made by identification of the underlying cause using skin scrapings, cytology, bacterial and fungal culture and resolution of clinical signs following specific treatment. Nine of these dogs were female and eleven were male. Various breeds were represented and the ages ranged from three months to 11 years.

2.1.3 Healthy "household" dogs

The third group (group III) was one of the control populations and was composed of 30 staff- and client-owned physically healthy dogs kept in household conditions without any present or prior history of skin problems. 12 male and 18 female dogs of various breeds were included with ages ranging from six months to 11 years. The client-owned animals were presented at the Small Animal Clinic, Internal Medicine, University of Giessen, Germany as blood donors.

2.1.4 Healthy beagle population

40 beagle dogs, maintained at HARLAN Winkelmann GmbH Borchen, Germany for breeding purposes, were the second control population (group IV). 20 of these dogs were female and 20 were male, the ages of which ranged from seven months to nine years. The dogs were kept in groups of six on a concrete floor and contact was limited to the personnel in charge of the dogs.

2.2 Serum collection

Serum samples were obtained from all dogs. 145 sera were collected between November 2000 and November 2003. 33 sera of the "multi-source" atopic dog population were collected between 1991 and 1994. Blood was drawn by jugular venipuncture or by venipuncture of the Vena cephalica or Vena saphena. The samples were left to clot at room temperature, centrifuged at 1000 rounds per minute for three minutes and stored at –20°C.

2.3 Allergenic extracts

Eleven allergenic extracts were chosen for the study (table 4). Except for shrimp, peanut, soy, birch and HD, the allergenic extracts were stock extracts from ALLERGOPHARMA, Joachim Ganzer KG, Reinbek, Germany standardized for use in human beings. Shrimp, peanut, soy and birch extract were produced in house (Paul-Ehrlich-Institute (PEI), Langen, Germany) and HD extract, which is no longer in production, was obtained as an intermediate product from the manufacturers HAL (Haarlem Allergenen Laboratorium, Amsterdam, The Netherlands) and ALK (Allergologisk Laboratorium, Kopenhagen, Denmark).

2.3.1 Processing of HD extract

Two lyophilized intermediate products of HD extracts from different manufacturers (HAL/ALK) were available for our investigations. Both extracts were resuspended 1:10 in ammonium carbonate buffer (100 mM, pH 8.1) and then disintegrated with a sonicator to increase the release of antigenic material. Subsequently, the suspensions were stirred at room temperature (RT) overnight. Sterile filtration was performed the next day using the following filters (SARTORIUS AG, Göttingen, Germany):

- Fiberglass filter (prefiltration)
- Series connected fiberglass filter and cellulose acetate filter (Pore size: 1.2 μm)

– Sterile filter (Pore size: 0.22 µm)
– The sterile suspension was prepared in 4 formulations:
  - Suspension without preservatives (native extracts)
  - Suspension with glycerole (dilution 1:1)
  - Suspension with phenole (dilution 10:1)
  - Suspension, stabilized with glycerole and phenole (dilution 5:5:1)

The extracts without added preservatives were stored at –20°C, those containing preservatives at +4°C.

Table 4: Allergenic extracts used in this study

Allergenic source	Common term	Manufacturer*	Batch number	
Indoor allergens				
Alternaria alternata (Alt.a.)	Mold	ALLERGOPHARMA	80789	30002223
Aspergillus fumigatus (Asp.f.)	Mold	ALLERGOPHARMA	82771	30004378
Canis familiaris (Can.f.)	Dog dander	ALLERGOPHARMA	60905	30003945
Cladosporium herbarum (Clad.h.)	Mold	ALLERGOPHARMA	64877	81629
Dermatophagoides farinae (Der.f.)	House dust mite	ALLERGOPHARMA	30001192	30001851
Dermatophagoides pteronyssinus (Der.p.)	House dust mite	ALLERGOPHARMA	30000247	30004090
Felis domesticus (Fel.d.)	Cat dander	ALLERGOPHARMA	30001364	30001364
Homo sapiens (HD)	Human dander	ALK	-	
Homo sapiens (HD)	Human dander	HAL	-	
Penicillium notatum (Pen.n.)	Mold	ALLERGOPHARMA	72256	30000128
Outdoor allergens				
Artemisia vulgaris (Art.v.)	Mugwort	ALLERGOPHARMA	00010744	81944
Betula verrucosa (Bet.v.)	Birch	ALLERGOPHARMA	-	
Phleum pratense (Phl.p.)	Timothy	ALLERGOPHARMA	30002169	83492
Triticum aestivum (Tri.a.)	Wheat	ALLERGOPHARMA	63298	30003753
Food allergens				
Arachis hypogaea (Ara.h.)	Peanut	PEI	-	
Glycine max (Gly.m.)	Soy	PEI	-	
Penaeus aztecus (Pen.a.)	Shrimp	PEI	-	

2.4 Determination of the protein concentration

The determination of the protein concentration of the allergenic extracts was a necessary prerequisite for the comparability of further experiments and was performed using the commercially available kit Roti®-Nanoquant (ROTH, Karlsruhe, Germany). The kit is a modification of the method described by BRADFORD (BRADFORD, 1976), who introduced the stain Coomassie Brillant Blue G 250 to reproducibly determine the protein concentration of solutions. The modified method adds Roti®-Nanoquant to the aqueous solution in question, resulting in a color change proportional to the protein concentration. This method reproducibly analyzes the protein content (>200ng) of substances.

Method:

Six doubling dilutions of the reference serum Bovine Serum Albumin (BSA) Standard (PIERCE, Rockford, U.S.A) were prepared to generate a standard curve which was included in each plate. In a second step the samples were doubly diluted with aqua bidest. 60 µl/well of blanks (aqua bidest.), reference serum and samples were pipetted into a 96-well uncoated microtitre plate (NUNC, Roskilde, Denmark). 240µl/well of Roti®-Nanoquant reagent was added and the resultant color change was measured at 590/450 nm in a programmable automated ELISA reader (Molecular Devices SPECTRAmax® 340PC, GMI, Minnesota, U.S.A.). The protein concentration was calculated by the reader software.

2.5 Determination of the biological potency of HD extracts

The RBL test (HOFFMANN et al., 1999) is a murine model of the allergic type I reaction and is based on a rat basophil leukemia cell line which can be sensitized with murine sera or mouse derived monoclonal antibodies specific for the allergen. Challenge with the allergenic extract induces cross-linking of the antibodies and degranulation of the cells. The test is used for the evaluation of the biological potency of allergenic extracts.

Method:

The rat basophil leukemia cell subline RBL-2H3 was maintained in Eagle's MEM with 10% foetal calf serum. Cells were harvested in the stationary phase and plated in 96-well tissue culture plates (NUNC) (10^5 cells/well) (18 hours at 37°C in a 5% CO_2 atmosphere). Cells were then passively sensitized by preincubation (50µl/well) with a mouse monoclonal IgE antibody raised against native ALK HD (HDX, gift from PD Dr. Hoffmann), diluted 1:5 in MEM (1 hour). Then a three cycle wash with tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4

mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10mM 2-(4-(2-Hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES) and 0.1% BSA, pH 7.4) was performed on an automated plate washer (BIO-TEK Instruments, Johannesburg, South Africa). Triggering of the RBL cells was induced by adding 100 µl/well of a serial dilution of HD extract in tyrode's buffer (one hour at 37°C in a humidified atmosphere). Each dilution was run in duplicate and following controls were included:

- Negative controls:
  - Wells without allergen to measure the spontaneous release of the RBL cells
  - Wells with cells sensitized with a nonspecific mouse monoclonal antibody raised against birch (mP30, gift from PD Dr. Hoffmann) to determine the nonspecific activation of the RBL cells
  - Wells without cells to measure the intrinsic enzymatic activity of the allergenic extract
- Positive control:
  - Total release was obtained by adding 1% Triton X-100 (SIGMA) to the tyrode buffer

To determine the enzymatic activity of β-hexosaminidase (released from the cells), 30 µl of the supernatant and 50 µl of p-nitrophenyl-N-acetyl-β-D-glucosaminide (1.3 mg/ml in 0,1 M citric acid buffer, pH 4.5) were mixed in a separate 96-well plate (1 hour at 37°C). The colorimetric reaction was terminated by adding 100µl/well of a glycine solution (0.2 M, pH 10.7) and measured at 405 nm in an automated ELISA reader. Results were expressed as percentage of total release minus spontaneous release.

2.6 Determination of allergen-specific IgE in dog sera

The development of a suitable protocol for detecting canine IgE and the semiquantitative determination of IgE levels in dog sera was performed using an NC membrane based on a solid phase assay in combination with a "grid-blot" device. The grid-blot, a modified version of a procedure developed by ALRIC et al. (ALRIC et al., 1986), is a two-piece positioning device consisting of an acrylic plate with multiple slots for applying reagent to NC and a metal plate which enables the NC to be securely (waterproof) clamped in place (see figure 1 and 3). Rotation of the NC by 90° allows the application of a second reagent perpendicular to the first resulting in a cross-wise pattern on the NC (see figure 3). The grid-blot is a reliable and simple system, frequently used for screening antibody-allergen binding on NC (AYUSO et al., 1999; LANE et al., 1989) and requires only minimum amounts of allergen and antibody. In our study we used the grid-blot device manufactured by the IDEA SCIENTIFIC

COMPANY, Minneapolis, U.S.A. (figure 1). It allows parallel testing of up to 17 allergenic extracts with 20 patient sera on a single sheet of NC.

Method:

A sheet (12,5x12,5 cm) of NC membrane (0,2 µm, SCHLEICHER&SCHUELL, Dassel, Germany), previously soaked in Tris buffered saline (TBS, pH 7.2) for 20 minutes (min), was positioned on the slotted portion of the acrylic plate. A piece of parafilm and a foam pad, followed by the metal plate, were stacked against the back side of the NC sheet and the completed grid-blot was clamped securely to immobilize and seal the NC against the acrylic plate. The allergenic extracts, diluted in TBS (pH 7.2) to a protein content of 10 µg/ml, were applied to each channel in volumes of 200µl/channel. Incubation was performed overnight at RT on a rocking platform.

Development of the detection protocol:

To establish a suitable protocol for detecting canine IgE, allergens known to be common causative agents of allergen-specific IgE in atopic dogs were chosen (see table 4 for details on the allergenic extracts):

- Native HAL HD
- Native ALK HD
- Der.p.
- Der.f.
- Phl.p.

On the next day the channels were washed with wash buffer (TBS (pH 7.2) with 0.05% Tween 20) twice (10 min) and the grid-blot was disassembled. After one more wash cycle (10 min) the NC was blocked (TBS (pH 7.2) with 0.3% Tween 20 and 1% BSA) (10 min) and cut into strips of 3-4 mm perpendicular to the run of the allergens (figure 2). Each strip now had five distinct areas, which were loaded with the allergenic extract. The strips were incubated with pooled serum from group I (diluted 1:10 in incubation buffer (TBS (pH 7.2) with 0.05% Tween 20 and 0.1% BSA)) overnight at RT. Strips were stored at –20°C and used step by step in the trials to develop and optimize the detection of canine IgE. The results are demonstrated in chapter 3.

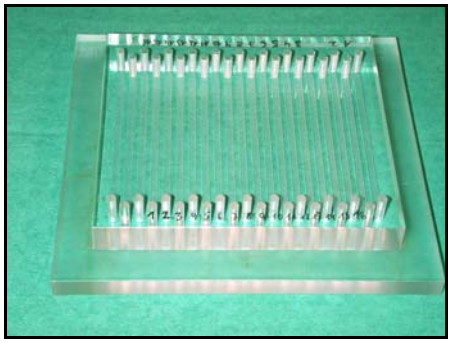

Figure 1: Acrylic plate of the grid-blot

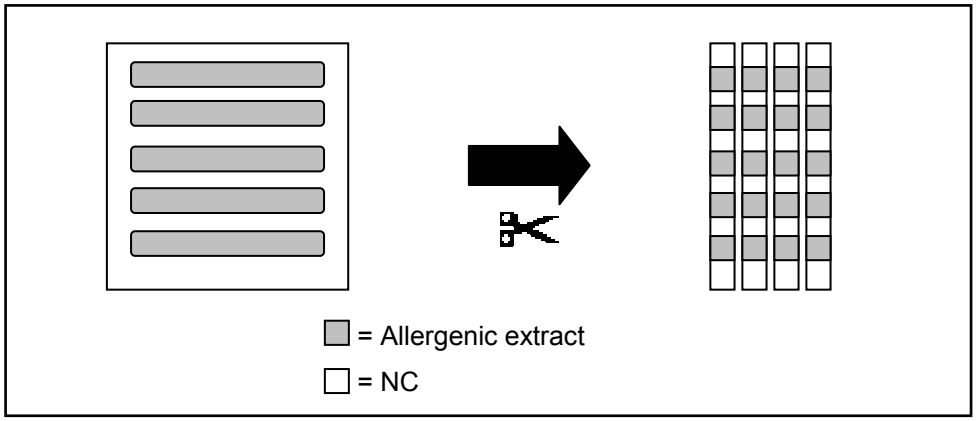

Figure 2: Preparation of NC strips

IgE-profiling:

After having established the detection protocol, individual IgE profiles to the allergenic extracts shown in table 4 were determined. The allergenic extracts were applied to the NC as explained above and incubated overnight at RT. On the next day the channels were washed with wash buffer twice (10 min) and the grid-blot was disassembled. After one more wash cycle (10 min) the NC was blocked (10 min), rotated by 90° and the grid-blot was reassembled. Channels were then perpendicular to the rows of absorbed allergenic extracts (figure 3). Each dog serum was diluted 1:20 in incubation buffer and 200µl were placed in each channel. The sera were incubated for two hours at RT. Then the channels were washed twice (10 min), the grid-blot was disassembled and one more wash cycle (10 min) was performed before continuing with immunodetection. To detect allergen-bound IgE the following protocol was used (antibodies were diluted in incubation buffer; three wash cycles, 10 min each, were performed between steps):

- Mouse α-dog IgE (SEROTEC, Raleigh, U.S.A., MCA 1895, Batch: 081100) (1:50.000, 1 hour)

- Biotinylated rabbit α-mouse IgG (SIGMA, Deisenhofen, Germany Lot: 021K4862) (1:10.000, 1 hour)
- Alkaline phosphatase (AP) conjugated streptavidin (CALTAG, Burlingame, U.S.A. Lot: 20020803) (1:4.000, 30 min)
- Visualization of antibody binding using the substrate/chromogene mixture for AP, which contains 5-bromo-4-chloro-indolyl-phosphate in aqueous dimethylformamide (DMF) and nitroblue tetrazolium in aqueous DMF solubilized in stain buffer (BIO-RAD, Hercules, U.S.A.)
- Termination of the reaction with aqua dest. and air-drying of the blots overnight

The result was a crosswise pattern (figure 3), showing each patient's IgE profile at a glance. Results were evaluated semiquantitatively using a visual classification system by comparing the reaction to a scaled scoring system (table 5). A reaction was considered grade 1 when it was stronger than the background activity.

Table 5: Scaled scoring system

Grade of reaction	0	1	2	3
Color intensity				
Evaluation	Negative reaction*	Low grade reaction	Moderate reaction	High grade reaction

*) equal to the background activity of the allergenic extract

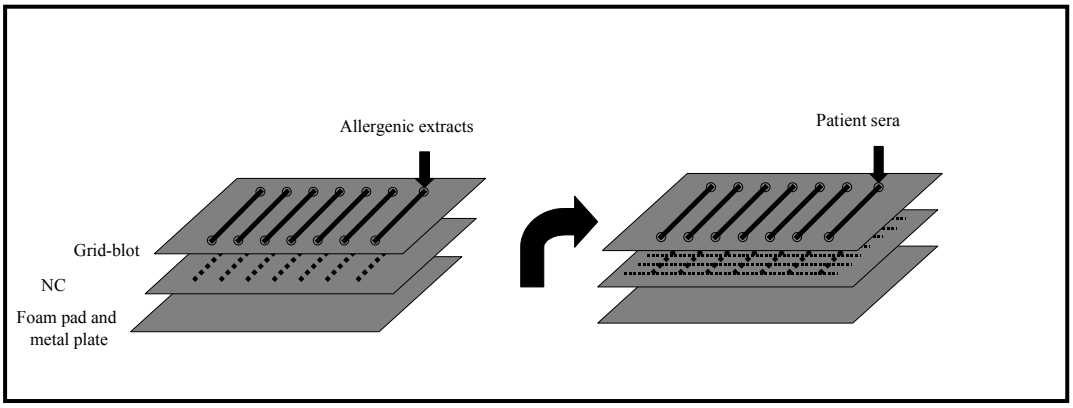

Figure 3: Schematic illustration of the grid-blot procedure

2.6.1 Statistical analysis

The Kruskal-Wallis Test was used to assess overall significant differences of allergen-specific IgE levels among the four groups of dogs. If differences were found to be significant ($P<0.05$), the Dunn´s (Bonferroni) procedure was used to perform multiple pair-wise comparisons between the four dog groups. Statistical analyses were done using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, U.S.A., www.graphpad.com.

2.7 Identification of IgE-reactive proteins for dogs

The available HD extracts were characterized by evaluating their biological potency with the rat basophil leukemia cell mediator release assay (RBL test) and determining their protein patterns using SDS-PAGE with subsequent Coomassie staining of the proteins. IgE-reactive proteins for mouse and dog sera were identified by Western blotting and N-terminal microsequencing was performed for the relevant IgE-reactive protein for dogs.

2.7.1 SDS-PAGE

PAGE separates molecules from a complex mixture according to size and charge. During electrophoresis there is an intricate interaction of samples, gel matrix buffers and electric current, resulting in separate bands of individual molecules. PAGE in the presence of SDS separates protein molecules in complex mixtures during their migration to the anode solely according to size. Depending on the expected MW of the proteins different buffer systems are used.

2.7.1.1 SDS-PAGE buffer system by Lämmli

Large molecules are separated in an SDS-PAGE buffer system according to Lämmli (LAEMMLI, 1970). Lämmli's buffer system is a discontinuous buffer system and incorporates SDS and a thiol reducing agent such as 2-mercaptoethanol in the loading buffer. In discontinuous buffer systems different buffer ions are present in the gel and electrode reservoir. By using different buffers in the gel and electrode solutions and adding a stacking to the resolving gel, which differ both in pore size and pH, samples are compressed into a thin starting band and individual proteins are finely resolved and separated. Prior to electrophoresis, proteins are denatured by heating them in the loading buffer. The resultant polypeptides take on a rodlike shape and a uniform charge to mass ratio proportional to their

MW. Proteins separate according to their MW, small proteins migrating faster through the network of pores than large ones.

Method:

For electrophoresis the Mini Protean® 3 cell system (BIO-RAD) was used. After cleaning the glass plates with alcohol, they were assembled according to the manufacturer's instructions. A 5 cm high 13% resolving gel (table 6) was poured, immediately overlaid with isobutanol to prevent oxygen contact and left to polymerize for 1 hour at RT (gel thickness: 1mm). The overlay solution was then rinsed completely with distilled water. Subsequently, a 1 cm high 5% stacking gel (table 6) containing 5% Pyronin G was poured and, depending on the experiment, appropriate combs were inserted. The gel was left to polymerize for another hour at RT. Following the manufacturer's instructions the Mini Protean® 3 cell system was assembled and Tris/Glycine/SDS running buffer (BIO-RAD) was filled into the chambers. Samples were diluted with a reducing loading buffer (Roti®Load 1, ROTH) in a ratio of 4:1 and heated at 95°C for 5 min. After removal of the combs, HD samples were loaded into the wells using a pipette (20 µg protein/well). A power of 100 V was applied until the samples reached the resolving gel as a thin starting band, then 200 V until the samples reached the bottom of the resolving gel (visualised by the additive Pyronin G). MW of resolved proteins were calculated according to the MW marker.

Table 6: Gel formulation: Lämmli buffer system

Buffers and solutions	Resolving gel 13%	Stacking gel 5%
Acrylamide/Bis (30%T/2.67%C) (Roth)	8.65 ml	1275 µl
Resolving gel buffer (1.5 M Tris, pH 8.8)	5000 µl	-
Stacking gel buffer (0.5 M Tris, pH 6.8)	-	1875 µl
SDS (10%)	200 µl	75 µl
Aqua dest.	6.050 ml	4226 µl
N,N,N´,N´-Tetramethylethylendiamin	20 µl	10 µl
Ammonium persulfate (10%)	90 µl	45 µl
Pyronin G 1%	-	5 µl

MW Markers:

- Rainbow colored protein molecular weight marker (Low molecular weight range) (AMERSHAM PHARMACIA BIOTECH)
- (2.500; 3.500; 6.500; 14.300; 20.100; 30.000; 45.000)
- Rainbow colored protein molecular weight marker (High molecular weight range) (AMERSHAM PHARMACIA BIOTECH)
- (14.300; 20.100; 30.000; 45.000; 66.000; 97.000; 220.000)
- Low molecular weight calibration kit (AMERSHAM PHARMACIA BIOTECH)
- (14.400; 20.100; 30.000; 45.000; 66.000; 97.000)
- Prestained SDS-PAGE Standards, Low Range (BIO-RAD)

2.7.1.2 SDS-PAGE buffer system by Schägger and Jagow

The resolution of peptides below 14 kDa is not sufficient in the conventional Lämmli buffer system (Tris-glycine-hydrochloric acid (HCl)). SDS-PAGE electrophoresis by Schägger and Jagow (SCHAGGER and VON JAGOW, 1987) uses Tricine as the terminating ion, which allows separation of proteins in a MW range of 1 to 100 kDa.

Method:

The Mini Protean® 3 cell system was assembled as described above (2.7.1.1.). After pouring the stacking and resolving gel (table 7) with subsequent polymerization of the gels a cathode (0.1 M Tris, 0.1 M Tricine, 0.1% SDS) and anode buffer (0.2 M Tris/HCl, pH 8.9) were filled into the respective chambers. Sample processing and electrophoresis were then performed as described in 2.6.2.1.

Table 7: Gel formulation: Schägger and Jagow buffer system

Buffers and solutions	Resolving gel 14%	Stacking gel 5%
Acrylamide/Bis (40% T/2.5 % C)	4.2 ml	700 µl
Gel buffer (3 M Tris pH 8.45, 0.3% SDS)	4.0 µl	1500 µl
Glycerole 80%	2.0 ml	-
SDS (10%)	120 µl	60 µl
Aqua dest.	1.8 ml	3760 µl
N,N,N´,N´-Tetramethylethylendiamin	6 µl	6 µl
Ammonium persulfate (10%)	60 µl	65 µl
Pyronin G 1%	-	5 µl

2.7.1.3 Coomassie brillant blue staining

Coomassie brillant blue staining is an easy way to visualise proteins separated during electrophoresis.

Method:

The gels were gently removed from the glass plates and immerged in Coomassie blue R-250 (0.1% in 50% methanol (MeOH)) on a rocking platform (10 min). After destaining with several changes of 50% MeOH, 10% acetic acid, the gels were washed in deionized water on a rocking platform (3 wash cycles, 2 min each). The DryEase® Mini-Gel drying System (INVITROGEN, GmbH, Karlsruhe, Germany) was used for drying the gels. The deionized water was decanted, the Gel-Dry™ drying solution (INVITROGEN) added and gels were equilibrated for 15-20 min. Rough edges of the gels were cut off and the gels were dried in an upright frame between two sheets of cellophane previously immerged in the Gel-Dry™ drying solution. Gels were exposed on a LumiImager and for calculations of MW the Lumianalyst software (both Roche Diagnostics, Mannheim, Germany) was used.

2.7.2 Western blotting

Western blotting is a term used for the transfer of proteins to the surface of an immobilizing membrane such as NC. The molecules adsorbed to the membrane surface are freely available for macromolecular ligands such as antibodies. Prior to specific detection, free binding sites are blocked with substrates that do not influence the ensuing reaction.

2.7.2.1 Semi-dry blotting

Semi-dry blotting is performed between two graphite plates and sheets of filter paper soaked in buffer.

Method:

First the graphite plates of the blotter were rinsed with distilled water. 14 sheets of filter paper and a piece of NC were cut to fit the gel exactly and the NC and gel were equilibrated in anode buffer II (25 mM Tris/HCl, pH 10.4, 20% MeOH) for five min. A discontinuous buffer system was used.

Assembly of the blotter:

Six sheets of filter paper (SCHLEICHER&SCHUELL) were soaked in anode buffer I (0.3 M Tris/HCl, pH 10.4, 20% MeOH) and placed on the anode graphite plate (Novablot electrodes and Multiphor II Apparatus, PHARMACIA BIOTECH, Piscataway, U.S.A.), followed by

three sheets of filter paper soaked in anode buffer II. The equilibrated NC membrane (0.2 µm, SCLEICHER&SCHUELL) and the gel were positioned on the filter papers. Five sheets of filter paper soaked in cathode buffer (40 mM 6-aminohexanacid, pH 7.6, 20% MeOH) completed the assembly. The cathode graphite plate was gently positioned and weighted with approximately one kg to allow electrolyte gas to be expelled.

Blotting:

For transfer of the proteins, 0.8 mA/cm^2 were required for 45 min. After removal of the filter papers the NC was left to dry for 30 min and was subsequently blocked (TBS (pH 7.2) with 0.3% Tween 20 and 1% BSA) twice for 15 min.

2.7.2.2 Immunodetection with dog/mice/rabbit sera

Relevant IgE-reactive proteins of allergenic extracts can be identified by probing them with sera from allergic patients. In our study immunodetection of HD extract was performed using random samples of those dog sera with grade 1 or 2 reactions to HD extract, as determined by grid-blotting as well as sera from mice and rabbits experimentally sensitized to HD.

2.7.2.2.1 Immunodetection in a Microincubation Tray

The use of a microincubation tray allows parallel incubation of NC strips from the same run with different sera and also the parallel use of different detection systems.

Method:

Using a razor blade the NC was cut into strips of three to four mm width parallel to the direction of the run and equilibrated in wash buffer (TBS (pH 7.2) with 0.05% Tween 20) (10 min) prior to immunodetection. The detection protocols are described below. Antibodies were diluted in incubation buffer (TBS (pH 7.2) with 0.05% Tween 20 and 0.1% BSA) and each step was followed by three wash cycles (10 min each) in wash buffer. All detection systems used AP as a detection enzyme and the reaction was visualized by use of the substrate/chromogene mixture for AP (BIO-RAD). Blots were exposed on a LumiImager and for calculations of MW Lumianalyst software (both Roche Diagnostics, Mannheim, Germany) was used.

Detection with dog sera:

- Step 1: patient serum (1:20, overnight)
- Step 2: mouse IgG α-dog IgE (SEROTEC MCA 1895, Batch: 081100) (1:50.000, 1 hour)
- Step 3: biotinylated rabbit IgG α-mouse IgG (SIGMA, Lot: 021K4862) (1:10.000, 1 hour)
- Step 4: streptavidin AP (CALTAG, Lot: 20020803) (1:4.000, 30 min)

Detection with mouse sera:

- Step 1: Polyclonal sera (1:10, overnight) from mice sensitized to HD (from PD Dr. Andreas Hoffmann)
- Step 2: AP marked rat IgG α-mouse IgE (SBA H021-RF29B) (1:1.000, 1 hour)

Detection with rabbit sera:

- Step 1: Polyclonal sera (1:1000, overnight) from rabbits sensitized to HD (from PD Dr. Andreas Hoffmann) (overnight)
- Step 2: AP marked rat IgG α-rabbit IgG (SIGMA A 8702 016H8938) (1:20.000, 1 hour)

2.7.2.2.2 Immunodetection with the BIO-RAD Multiscreen Device

As an alternative to the microincubation tray method the BIO-RAD Mini-Protean II Multiscreen Device (BIO-RAD) can be used. The Multiscreen Device is adapted to the size of the SDS gel separating chamber and allows the whole blot to be clamped in place. The preformed channels require only minimal amounts of serum/antibodies (<150µl) for immunodetection. For dilutions and incubation periods: see 2.7.2.2.1.

2.7.3 N-terminal microsequencing with polyvinylidenefluoride membrane

The polyvinylidenefluoride (PVDF) membrane is a Teflon® based membrane with a high binding capacity and a high mechanical stability which is used for direct protein sequencing (MATSUDAIRA, 1987).

Method:

For blotting ten sheets of filter paper and a piece of PVDF membrane (BIO-RAD) were cut to the size of the gel. The PVDF membrane was moistened with MeOH and eqilibrated in transfer buffer (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11, 10% MeOH) (5 min). Five MeOH equilibrated (5 min) filter papers (SCHLEICHER&SCHUELL) were soaked in transfer buffer and placed on the electrode, followed by the gel, PVDF membrane (with the smooth side facing the gel), five more transfer buffer soaked filter papers and the glas carbon electrode. A weight of approximately one kg was placed on top.

Transfer:

The transfer of the proteins to the PVDF membrane was performed in the Semi Dry Blotter PEGASUS (PHASE, Lübeck, Germany) with 0.8 mA/cm^2 for 45 min.

Stain:

The PVDF membrane was immerged in Coomassie blue R-250 (0.1% in 50% MeOH) on a rocking platform (10 min). Destaining was performed in several changes of 50% MeOH, followed by three wash cycles (two min) in deionized water on a rocking platform. After drying, a scalpell blade was used to cut the relevant protein band from the PVDF membrane and was submitted for microsequencing at the "Institut für Experimentelle Biologie und Medizin", Forschungsinstitut Borstel (Dr.A.Petersen).

3 RESULTS

3.1 Allergenic potency of HD extracts

The allergenic potency of HD extracts (HAL/ALK) was determined by performing the rat basophil leukemia cell mediator release assay (RBL test). Cells were passively sensitized by a specific anti-HD IgE (HDX) and a nonspecific antibody (mP30 monoclonal antibody raised against Bet.v.) as a negative control. The dose response curves indicating the mediator release induced by increasing doses of HD extract are shown in figure 4. Using a protein concentration of 15 µg/ml, the native extracts from HAL and ALK exhibited a mediator release of 60% and 30%, respectively. The nonspecific mast cell activation (using mP30) was similar for both extracts, with maximum mediator release values of slightly over 10%. When stabilizing the HD extracts with phenole and a combination of glycerole and phenole, the allergenic potency was markedly reduced. The sole use of glycerole had the least effect (figure 4).

The observed nonspecific mast cell degranulation effects of HD extracts can either be caused by nonspecific interaction with any cell-bound IgE or by IgE independent mast cell triggering. Furthermore it must be excluded that the extract itself has an inherent mediator-like activity. To differentiate between these possibilities the RBL test was performed under the same conditions with the nonspecific antibody mP30, without sensitizing antibodies and without cells. For both extracts the nonspecific (without HD-IgE) colorimetric reaction remained unchanged whether nonspecific antibodies were employed or cells remained unsensitized prior to challenge with HD extract. In the experiment without cells the ALK extract induced a colorimetric reaction whereas the extract from HAL did not (figure 5).

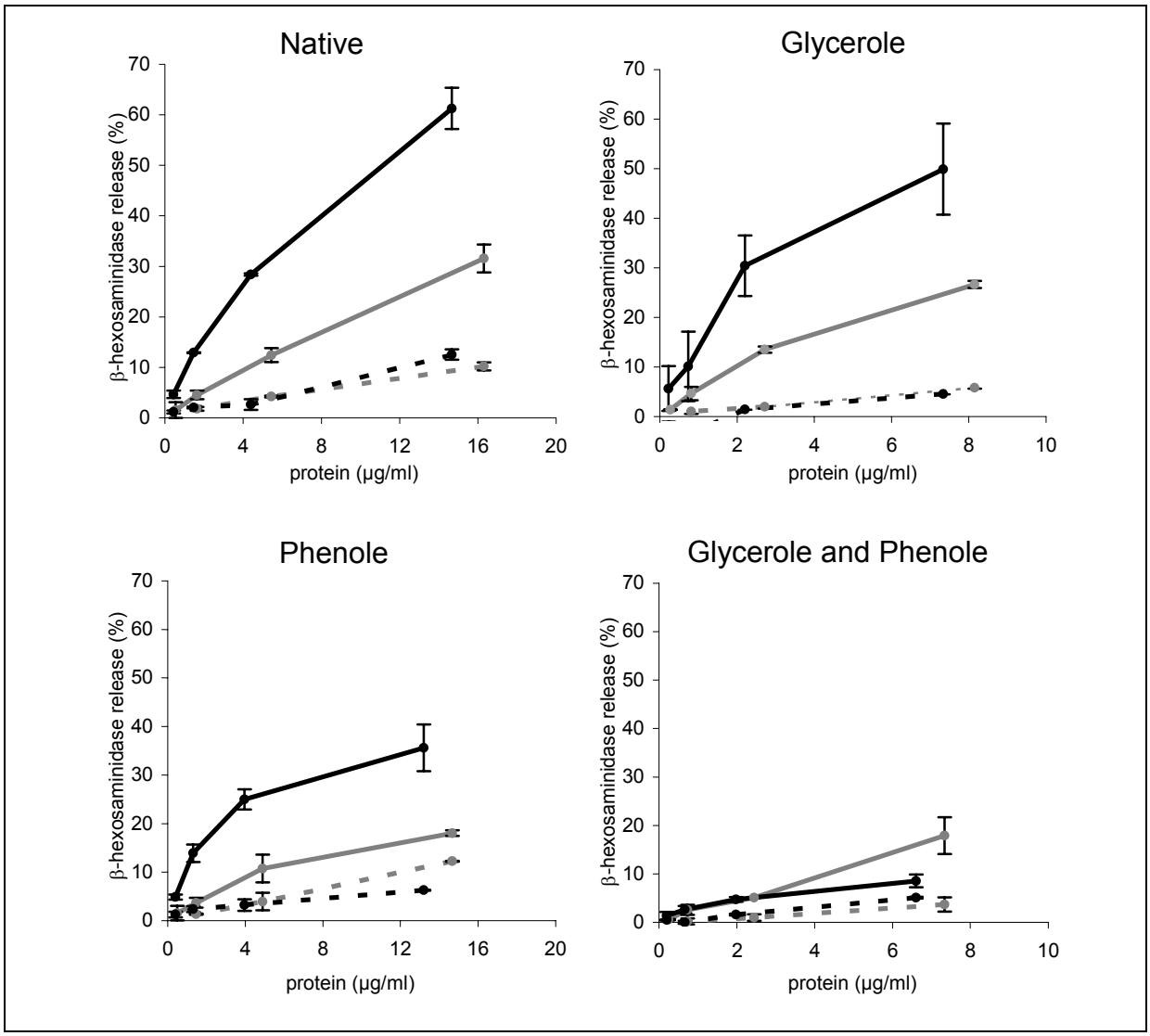

Figure 4: Comparison of native and preserved (in glycerole, phenole and glycerole/phenole) HAL (black lines) and ALK (grey lines) HD extracts in a dose response curve: β-hexosaminidase release from RBL cells plotted against HD extract concentration. Specific monoclonal antibody HDX raised against HD (continuous lines) and nonspecific monoclonal antibody mP30 raised against birch pollen extract (broken lines). Experiments were performed as duplicates. Maxima, minima and mean are shown.

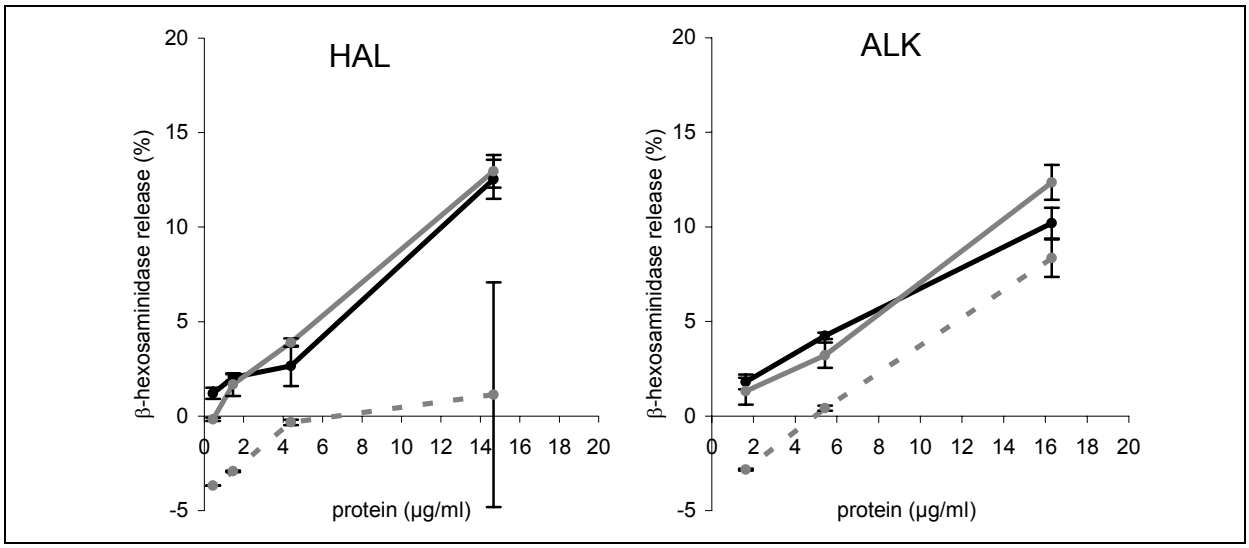

Figure 5: Comparison of nonspecific effects in native HAL and ALK extracts in a dose response curve: β-hexosaminidase release from RBL cells plotted against HD extract concentration. Nonspecific monoclonal antibody mP30 (black lines), without antibody (continuous grey lines) and without cells (broken grey lines). Experiments were performed as duplicates. Maxima, minima and mean are shown.

3.2 Determination of allergen-specific IgE in dog sera

Prior to testing the dog sera for their allergen-specific IgE profile in the grid-blot, it was necessary to develop a suitable detection protocol for canine IgE. Based on the commercial availability of recently developed anti-dog IgE antibodies, several different approaches were compared.

3.2.1 Allergen-specific IgE detection

The allergen-coated and with "putatively positive" pooled dog sera (group I: "multi-source" atopic dogs) preincubated NC strips were tested with murine or goat anti-canine IgE antibodies and a corresponding AP-labeled secondary antibody. In detail, NC strips were incubated with the primary α-canine antibodies for one hour, followed by detection with a secondary anti-species antibody coupled to the enzyme AP and subsequent visualization by use of a substrate/chromogene mixture for AP (table 8). In all direct two step detection protocols the NC strips showed no colorimetric reaction.

Table 8: Two step detection protocols

Primary detection antibody	Dilution		Secondary detection antibody	Dilution
Goat α-dog IgE	1:1.000	1:2.000	Mouse α-goat IgG AP	1:3.000
Mouse α-dog IgE	1:1.000	1:2.000	Rabbit α-mouse IgG AP	1:10.000
Mouse α-dog IgE+biotin	1:1.000	1:2.000	Streptavidin AP	1:3.000

In a further three step experiment the sensitivity was increased by introducing a biotinylated detection antibody. The formation of biotin-streptavidin-AP complexes results in a significant signal amplification due to the multiple site binding properties of biotin and steptavidin (see table 9 for details on the protocol). NC strips were incubated with each antibody for one hour. The optimal working concentration for the secondary biotinylated anti-mouse detection antibody was known and therefore further titration was not performed. This detection protocol (table 9) was shown to be sufficiently sensitive for the detection of canine IgE. For the second detection system, increasing dilutions of the secondary biotinylated anti-goat detection antibody (table 9) revealed a gradual reduction of the colorimetric reaction. In each of these three step detection protocols a nonspecific activity was noted for the native HAL extract in the negative buffer controls, which further investigations identified as a biotin-like activity of the extract.

Table 9: Enhanced detection system

Primary detection antibody	Dilution		Secondary detection antibody	Dilution			Amp-lifier	Dil-ution
mouse α-dog IgE	1:500	1:1.000	rabbit α-mouse IgG+biotin	1: 5.000			Strept-avidin AP	1:3.000
goat α-dog IgE	1:1.000		rabbit α-goat IgG+gly-cerole+biotin	1:3.000	1:10.000	1:30.000	Strept-avidin AP	1:3.000

Then serial dilutions of the dog serum and primary detection antibodies were tested in order to determine the respective optimal working concentrations. Serum was diluted 1:10 to test for the optimal dilutions of the primary detection antibody. Negative controls were run using incubation buffer instead of serum. Very high dilutions (1:128.000) of the primary detection

antibody still showed a colorimetric reaction in both detection systems, though the IgE reaction to Phl.p. became extremely weak (figure 6). The primary mouse anti-dog IgE antibody led to a stronger reaction compared to the goat system in all dilutions tested. A dilution of 1: 50.000 of mouse α dog IgE was thus selected as the optimal working concentration for the general protocol in later experiments.

Similar to the above experimental design, dog serum was also titrated to an optimal dilution. In related studies human serum (not used in this study) is generally employed in a dilution between 1:7 and 1:20. Serum IgE levels in dogs are significantly higher than in human serum and our experiments demonstrated serum dilutions of 1:20 to be sufficient to test for allergen-specific IgE in the dog.

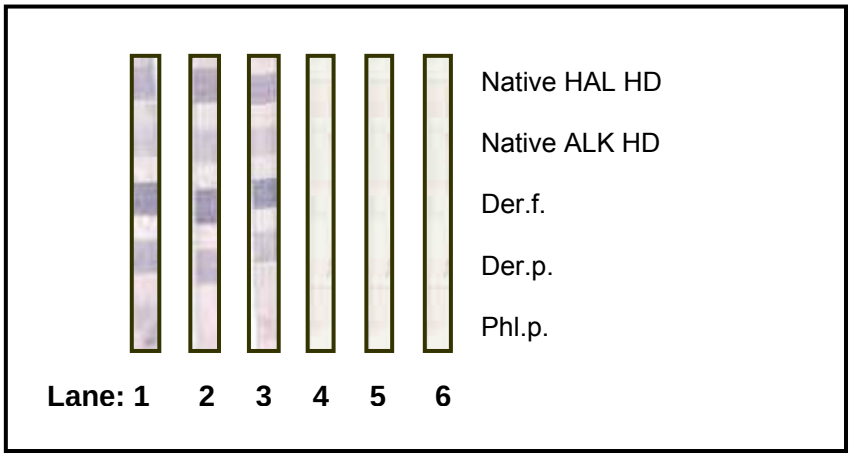

Figure 6: NC strips loaded with allergenic extracts displaying a decreasing colorimetric reaction after incubation with serial dilutions of the primary mouse IgG α-dog IgE antibody.
Lane 1: 1:32.000
Lane 2: 1:64.000
Lane 3: 1:128.000
Lane 4: 1:32.000 negative control (incubation buffer without primary antibody)
Lane 5: 1:64.000 negative control (see above)
Lane 6: 1:128.000 negative control (see above)

Immune reagents:

-Mouse IgG α-dog IgE (SEROTEC, MCA 1895 Lot: 081100)

-Mouse IgG α-dog IgE + Biotin (SEROTEC, MCA 1896B Lot: 070201)

-Goat IgG α-dog IgE (ε chain specific) (ICN, 644011 Lot: 3400E)

-Mouse IgG α-goat IgG AP (SIGMA, 12K4822)

-Rabbit IgG α-mouse IgG AP (SIGMA, 31K4823)

-Streptavidin AP (CALTAG, 19020502)

-Rabbit IgG α-mouse IgG+biotin (SIGMA, Lot: 021K4862)

-Rabbit IgG α-goat IgG+glycerole (50%)+biotin (DIANOVA, Hamburg, Germany, Lot: 35899)

3.2.2 Frequency of allergen-specific IgE in dogs

The grid-blot technique was used to determine the allergen-specific IgE levels in the sera of "multi-source" atopic dogs (group I), dogs with skin diseases other than atopy (group II), healthy "household" dogs (group III) and healthy kennelled beagles (group IV) against the allergenic extracts listed in table 4. The extracts were applied to the NC followed by incubation with the dog sera and IgE detection with the amplified mouse α-dog IgE protocol. Positive controls (P1, P2, P3) were run using dog sera demonstrating all 4 grades of reaction. Negative controls were run using incubation buffer and TBS buffer instead of serum and allergenic extracts, respectively. In addition the allergenic extract Can.f. was run as a negative control. Few allergenic extracts exhibited a high background activity (e.g. Pen.n., Asp.f. and native HAL HD), a fact taken into account when analyzing the grid-blot data. Nonspecific effects of sera, though rare, were also observed. Typical examples of grid-blots are shown in the figures 7, 8 and 9. In general, high allergen-specific IgE reactions (grade 3) to the common outdoor allergens included in the investigation were less frequent than to common indoor allergens (figure 10).

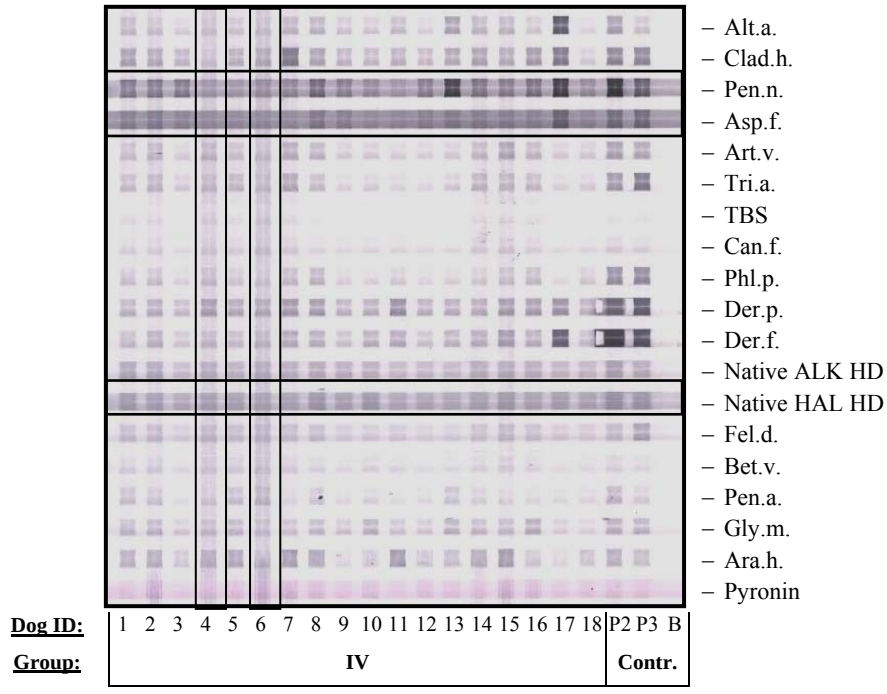

Figure 7: Grid-blot: Sera of kenneled beagle population (IV, x-axis) against selected allergenic extracts (y-axis). Sera of dogs demonstrating all 4 grades of reactions served as positive controls (P2, P3). Negative controls were performed using incubation buffer (B) instead of dog sera and TBS buffer (TBS) instead of allergenic extract. Examples of nonspecific background effects have been marked in frames.

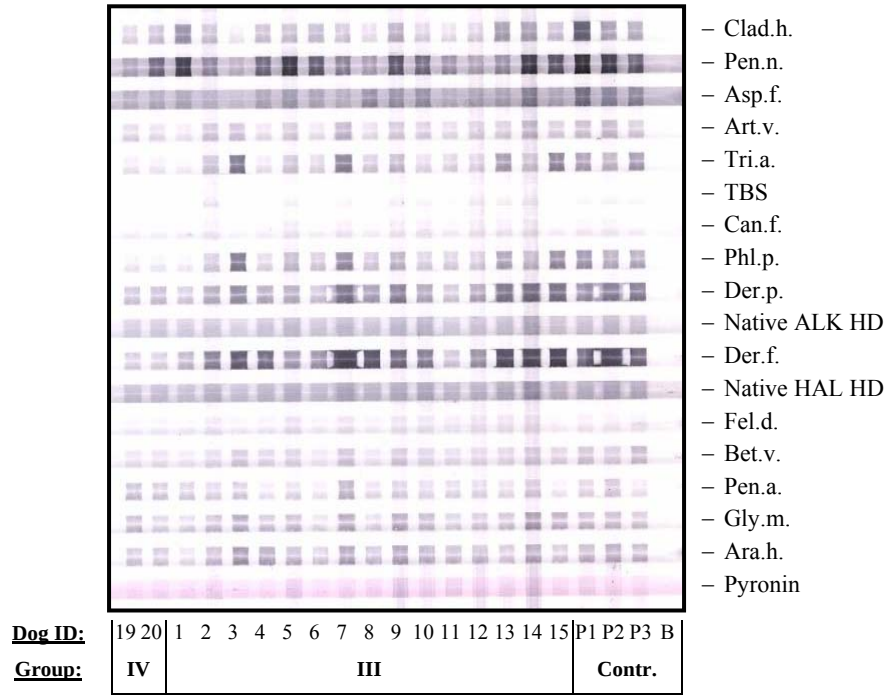

Figure 8: Grid-blot: Sera of kenneled beagle population (IV) and healthy "household" dogs (III, x-axis) against selected allergenic extracts (y-axis). Sera of dogs demonstrating all 4 grades of reactions served as positive controls (P1, P2, P3). Negative controls were performed using incubation buffer (B) instead of dog sera and TBS buffer (TBS) instead of allergenic extract.

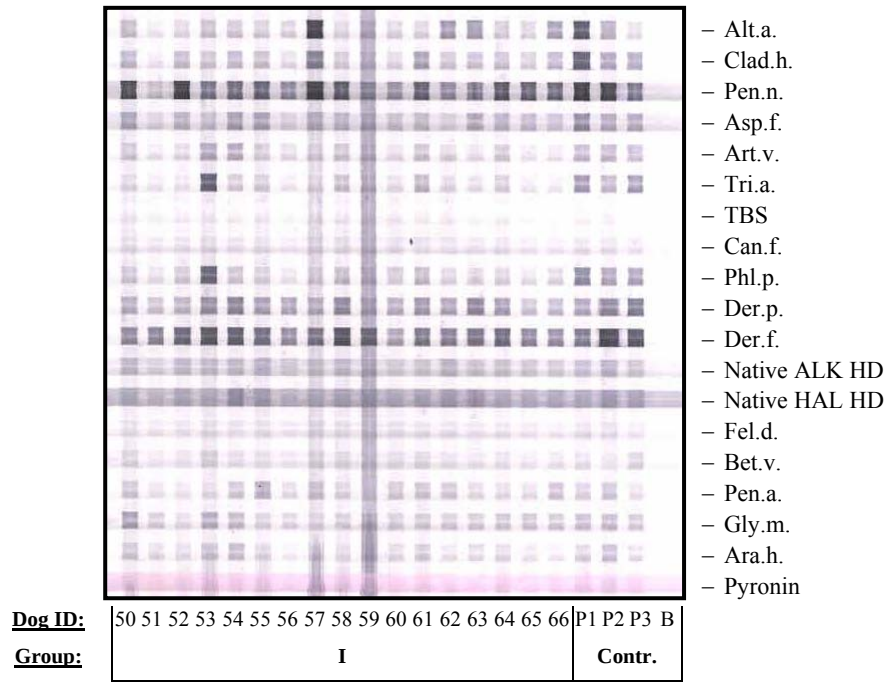

Figure 9: Grid-blot: Sera of "multi-source" atopic dogs (I, x-axis) against selected allergenic extracts. Sera of dogs demonstrating all 4 grades of reactions served as positive controls (P1, P2, P3). Negative controls were performed using incubation buffer (B) instead of dog sera and TBS buffer (TBS) instead of allergenic extract.

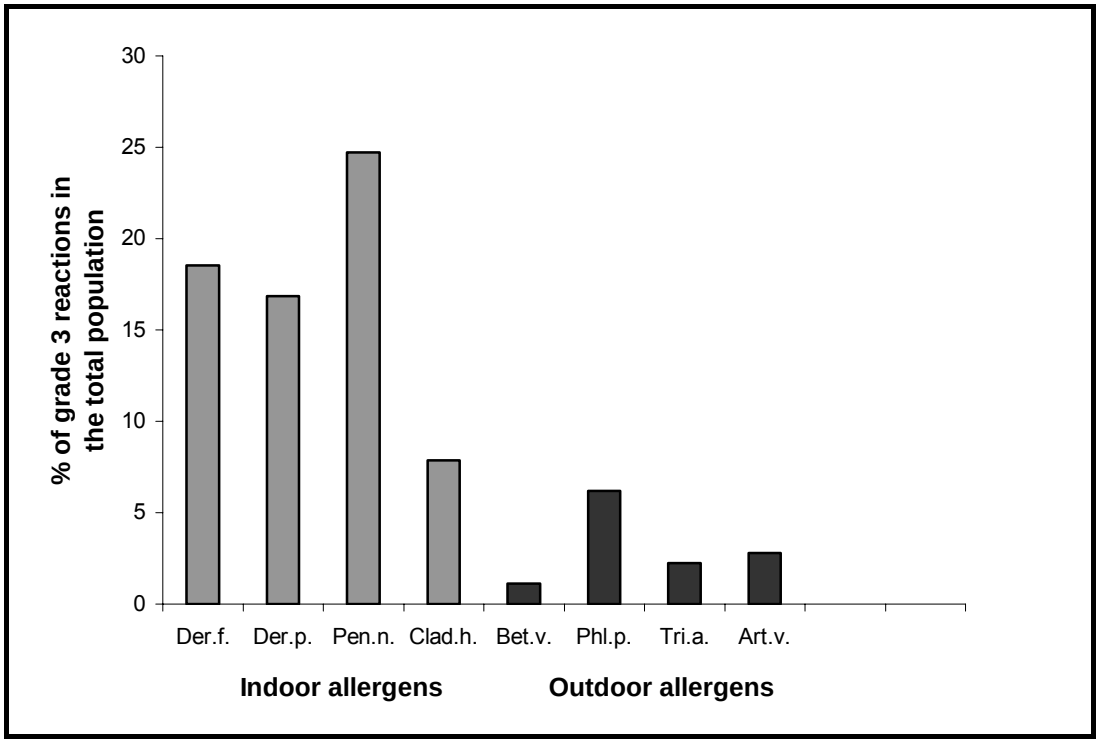

Figure 10: Dominant reactivity of common indoor allergens (grey) compared to common outdoor allergens (black)

3.2.2.1 Indoor allergens

Overall statistically significant differences were demonstrated for Der.f. and Der.p.- specific IgE levels in all four groups of dogs ($P<0.0005$ in both cases). Group IV (healthy kennelled beagles) was shown to have significantly less Der.f.-specific IgE than the three other groups of dogs (I ("multi-source atopic dogs) vs. IV: $P<0.001$, II (dogs with skin diseases other than atopy) vs. IV: $P<0.05$, III (healthy "household" dogs) vs. IV: $P<0.001$).

For Der.p. group III showed significantly more allergen-specific IgE than the other three groups of dogs (I vs. III: $P<0.01$, II vs. III: $P<0.01$, III vs IV: $P<0.01$). Comparison of the two house dust mites regarding their ability to induce IgE showed that Der.f. elicited a grade 3 allergen-specific IgE response more frequently than Der.p. in three (group I, II and III) out of four groups of dogs (table 10 and figure 11). In group IV, the allergen-specific IgE responses to Der.f. and Der.p. appeared equally represented (table 10 and figure 11). Very few dogs of all groups tested showed no IgE-reactivity to house dust mites (≤8%).

Table 10: Frequency of detectable house dust mite-specific IgE by means of grid-blot expressed as percentage (%) in dog groups

Group	I (n=88)		II (n=20)		III (n=30)		IV (n=40)	
Mite / Grade of reaction	Der.f.	Der.p.	Der.f.	Der.p.	Der.f.	Der.p.	Der.f.	Der.p.
0	6%	8%	5%	5%	0%	3%	3%	3%
1	18%	39%	30%	55%	13%	13%	65%	53%
2	39%	48%	30%	40%	30%	57%	30%	45%
3	38%	6%	35%	0%	57%	27%	3%	0%

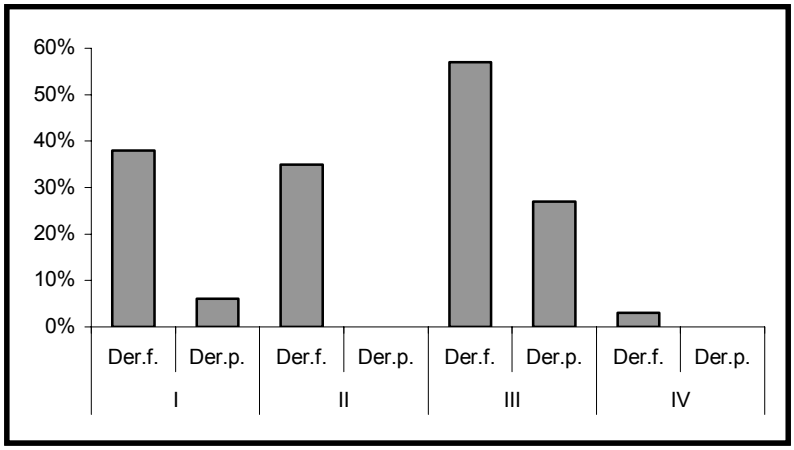

Figure 11: Comparison of the frequency of grade 3 reactions in % between Der.f. and Der.p.

Overall statistically significant differences were also revealed for the molds Pen.n. and Asp.f. ($P<0.0005$ in both cases). A statistically significant higher Pen.n.-specific IgE level was noted for group I when compared to group IV ($P<0.001$). Asp.f. only occasionally elicited a grade 3 reaction in the grid-blot (table 11 and figure 12). In the post test, group III demonstrated significantly higher Asp.f.-specific IgE levels than group II and IV and group I had a significantly higher IgE concentration than group IV (I vs IV: $P<0.01$, II vs III: $P<0.05$, III vs IV: $P<0.001$). All four groups of dogs generated very high IgE levels to Pen.n. compared to Asp.f. (table 11 and figure 12).

For both the native ALK and the native HAL HD extracts the grades of reactions were identical for each individual dog serum. Grade 1 reactions were common in all groups of dogs, whereas grade 0 and grade 2 reactions were rare. No grade 3 reactions were observed (table 12). The differences between the four groups of dogs were not statistically significant ($P=0.962$).

No statistically significant differences between the four groups of dogs were noted for the remaining indoor allergens Alt.a. (P=0.537), Clad.h. (P=0.285) and Fel.d. (P=0.796).

Table 11: Frequency of detectable Pen.n. and Asp.f.-specific IgE by means of grid-blot expressed as percentage (%) in dog groups

Group	I (n=88)		II (n=20)		III (n=30)		IV (n=40)	
Mold / Grade of reaction	Pen.n.	Asp.f.	Pen.n.	Asp.f.	Pen.n.	Asp.f.	Pen.n.	Asp.f.
0	0%	13%	0%	10%	3%	7%	0%	28%
1	10%	47%	15%	80%	20%	40%	35%	60%
2	43%	39%	70%	10%	53%	47%	60%	10%
3	47%	2%	15%	0%	23%	7%	5%	3%

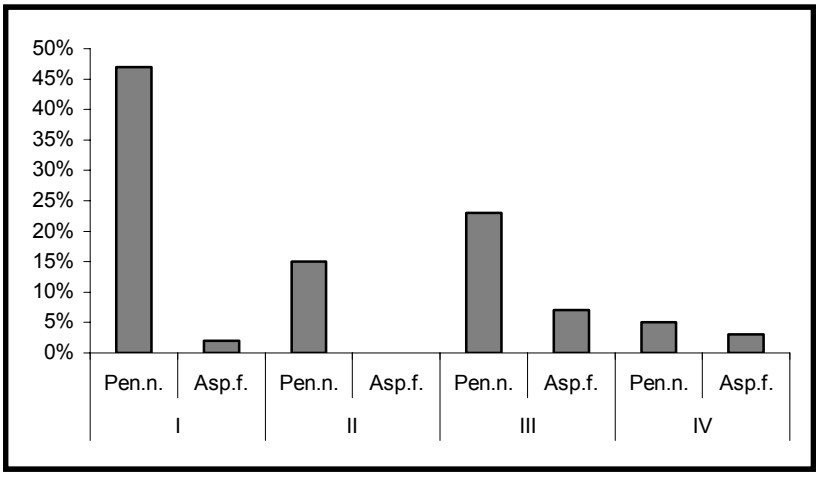

Figure 12: Comparison of the frequency in grade 3 reactions between Pen.n. and Asp.f.

Table 12: Frequency of detectable HD-specific IgE by means of grid-blot expressed as percentage (%) in dog groups

Group / Grade of reaction	I (n=88)	II (n=20)	III (n=30)	IV (n=40)
0	14%	0%	0%	5%
1	71%	100%	97%	85%
2	15%	0%	3%	10%
3	0%	0%	0%	0%

3.2.2.2 Outdoor allergens

Analysis of the results of allergen-specific IgE levels between the four dog groups for all outdoor allergens revealed statistically significant differences (Phl.p.: P<0.0005, Art.v.: P=0.001, Bet.v.: P<0.0005 and Tri.a.: P<0.0005). For Phl.p. group III demonstrated significantly higher IgE levels (I vs III: P<0.001, II vs III: P<0.001, III vs IV: P<0.01) than the three other groups of dogs. Similarly, group III also had significantly more allergen-specific IgE to Tri.a. than groups I and II (I vs III: P<0.001, II vs III: P<0.001). For Art.v. both groups III and IV had significantly more IgE than group II (II vs III: P<0.01, II vs IV: P<0.01), while for Bet.v. significantly higher IgE concentrations were noted for groups I and II vs. groups III and IV (I vs III: P<0.05, I vs IV: P<0.001, II vs III: P<0.001, II vs IV: P<0.001). The frequency of the grades of reaction to the outdoor allergens is demonstrated in figure 13.

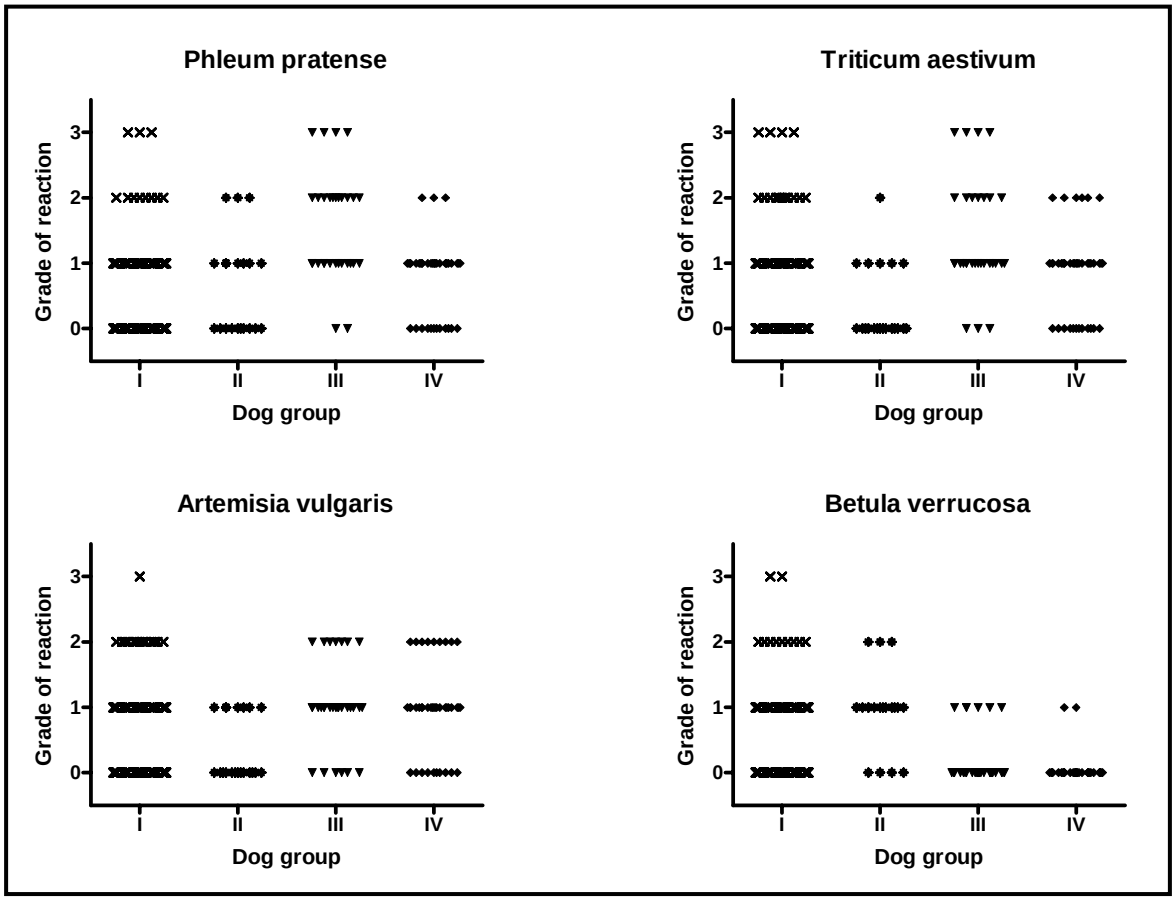

Figure 13: Scatter plots of graded IgE reactions to different outdoor allergens

3.2.2.3 Food allergens

For the food allergens an overall statistically significant difference (P<0.05) was noted for Pen.a. (P<0.0005). The Dunn´s multiple comparison test demonstrated group I to have significantly higher Pen.a.-specific IgE levels than group IV (I vs IV: P<0.001). The frequency of the grades of reaction to Pen.a. is shown in figure 14. Results for Gly.m. (P=0.182) and Ara.h. (P=0.075) were not significant.

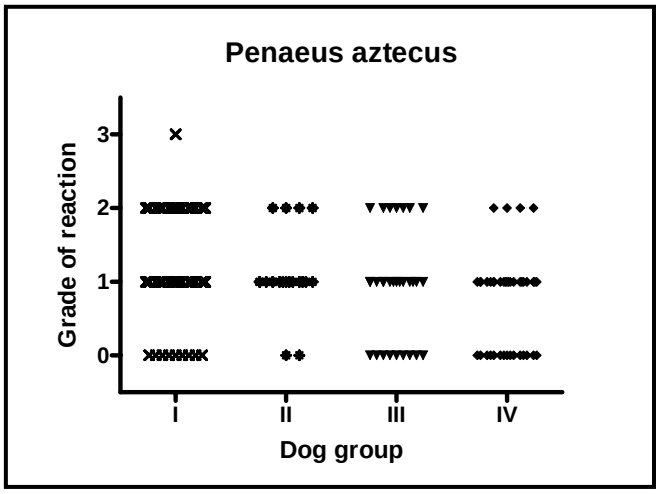

Figure 14: Scatter plot of graded IgE reactions to Penaeus aztecus

3.3 Characterization of HD extract and identification of an 11 kDa IgE-reactive protein for dogs

HD extracts were characterized by determining their allergenic potency and the proteins present in the extracts. Furthermore, the IgE-reactive proteins of HD allergen for dogs were identified by probing the extract with dog sera, followed by the identification of the relevant protein by N-terminal microsequencing.

3.3.1 SDS-PAGE analysis of native HD extract

To investigate the protein patterns of the native HAL and the native ALK HD extracts, they were separated by SDS-PAGE with a reducing agent in a Lämmli and Schägger/Jagow gel formulation.

In the Lämmli buffer system, native HD extracts were electrophoresed in 4 different protein concentrations and stained with Coomassie blue. In the lowest dilution a double band protein was visible in the low MW range (bands 3 and 4, figure 15), which was detected as a single

band protein in higher dilutions. Two other protein bands were detected in the medium MW range (bands 1 and 2, figure 15) and a further double protein band was located in the high MW range (not visible on the scanned gel) not strong enough for the imaging software to identify. Protein patterns were identical for the native HAL and the native ALK HD extracts. The MW of the protein bands in the native ALK HD extract marked with a "+" in figure 15 are shown in table 13.

A more accurate determination of the MW of the small proteins in native ALK HD extract was achieved by separation in a Schägger and Jagow buffer system (figure 16 and table 14). The protein bands in the medium MW range were calculated with approximately the same MW (band 3, 37 kDa and band 4, 29 kDa) as in the Lämmli buffer system. The protein band located in the low MW range (band 5) had an apparent MW of 9.5 kDa but a double band, as detected in the Lämmli buffer system (approximately 14/13kDa), was not visible. The two protein bands in the high MW range (band 1 and 2) were also detected and calculated to have a MW of approximately 58 and 51 kDa. The scanned blot (figure 16) does not show these bands clearly.

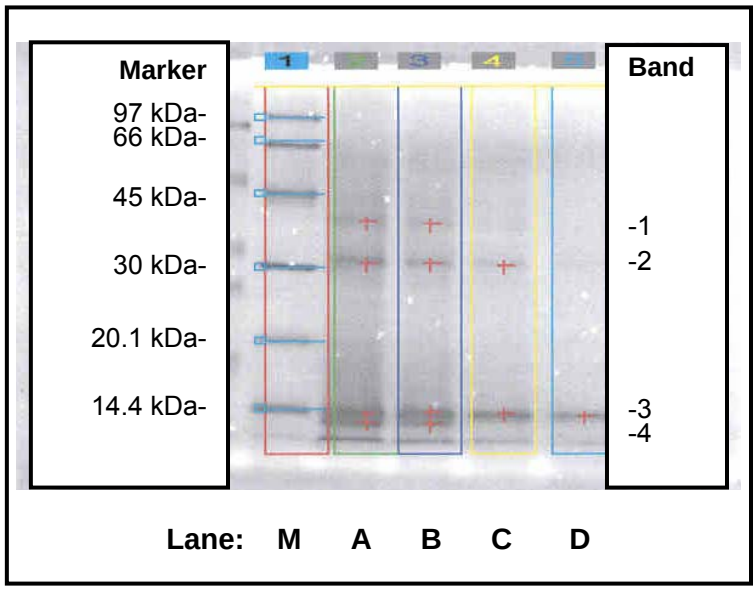

Figure 15: Separation of native ALK HD extract by Lämmli SDS-PAGE, Coomassie blue staining:
Lane M: MW standard Amersham LMW calibration kit
Lane A: 20 µg protein
Lane B: 10 µg protein
Lane C: 5 µg protein
Lane D: 2.5 µg protein

Table 13: MW of protein bands in native ALK HD extract in decreasing protein amounts separated by Lämmli SDS-PAGE

Lanes / Bands	A 20 µg protein	B 10 µg protein	C 5 µg protein	D 2.5 µg protein
	(Molecular weight in kDa)			
1	39.0	38.6	-	-
2	30.4	30.4	30	
3	13.9	14.0	13.9	13.7
4	13.2	13.0	-	-

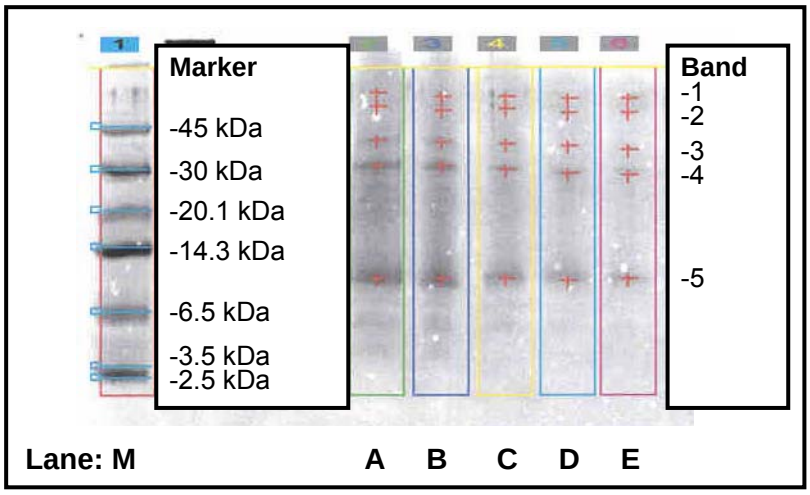

Figure 16: Separation of native ALK HD extract by Schägger/Jagow SDS-PAGE, Coomassie blue staining:
Lane M: marker MW standard Amersham Rainbow 755
Lane A: 26 µg,
Lane B: 13 µg protein,
Lane C: 7.5 µg protein,
Lane D: 3.75 µg protein,
Lane E: 1.875 µg protein

Table 14: MW of protein bands in native ALK HD extract in decreasing protein amounts separated by Schägger/Jagow SDS-PAGE

Lanes / Bands	A 26 µg protein	B 13 µg protein	C 7.5 µg protein	D 3.75 µg protein	E 1.87 µg protein
	(Molecular weight in kDa)				
1	60.4	58.3	58.3	57.2	57.2
2	53.1	51.2	52.2	50.3	50.3
3	38.8	38.1	37.4	36.7	35.4
4	30.6	30.6	29.4	28.3	27.8
5	9.5	9.5	9.5	9.3	9.5

3.3.2 IgE-reactivity of dog sera determined by Western blotting

The IgE-binding patterns of selected sera were further investigated by Western blotting using the native ALK HD extract. This extract was selected despite the higher biological activity of the native HAL HD extract demonstrated in the RBL test. This decision was based on the observation that the HAL extract demonstrated an inherent biotin-like activity in the preliminary tests (see chapter 3.2.1), raising concerns regarding the interpretation of Western blots employing the amplifier streptavidin in canine IgE detection.

Random samples of those dog sera with grade 1 or 2 reactions to HD extract, as determined by the preliminary screening method grid-blot, were chosen for Western blotting and included 19 sera from group I ("multi-source" atopic dogs). Dog sera from the three other groups of dogs were chosen as controls and included four sera from group II (dogs with skin diseases other than atopy), four from group III (healthy "household" dogs) and three from group IV (kennelled beagle dogs). The general reactivity patterns of the selected dog sera are summarized in table 17.

Native ALK HD extract was separated under reducing conditions and transferred to a NC membrane. In the Lämmli buffer system the most prominent protein band was located in the low MW range (<14.3 kDa) and was detected by six sera from group I (2 are shown in figure 17, band 5 and 6). IgE-reactivity with this protein band was not observed in the controls. Other IgE-reactive protein bands in the medium and high MW range (figure 17, band 1, 2, 3 and 4) were detected by most of the other dogs tested. The calculated MW of the proteins are shown in table 15.

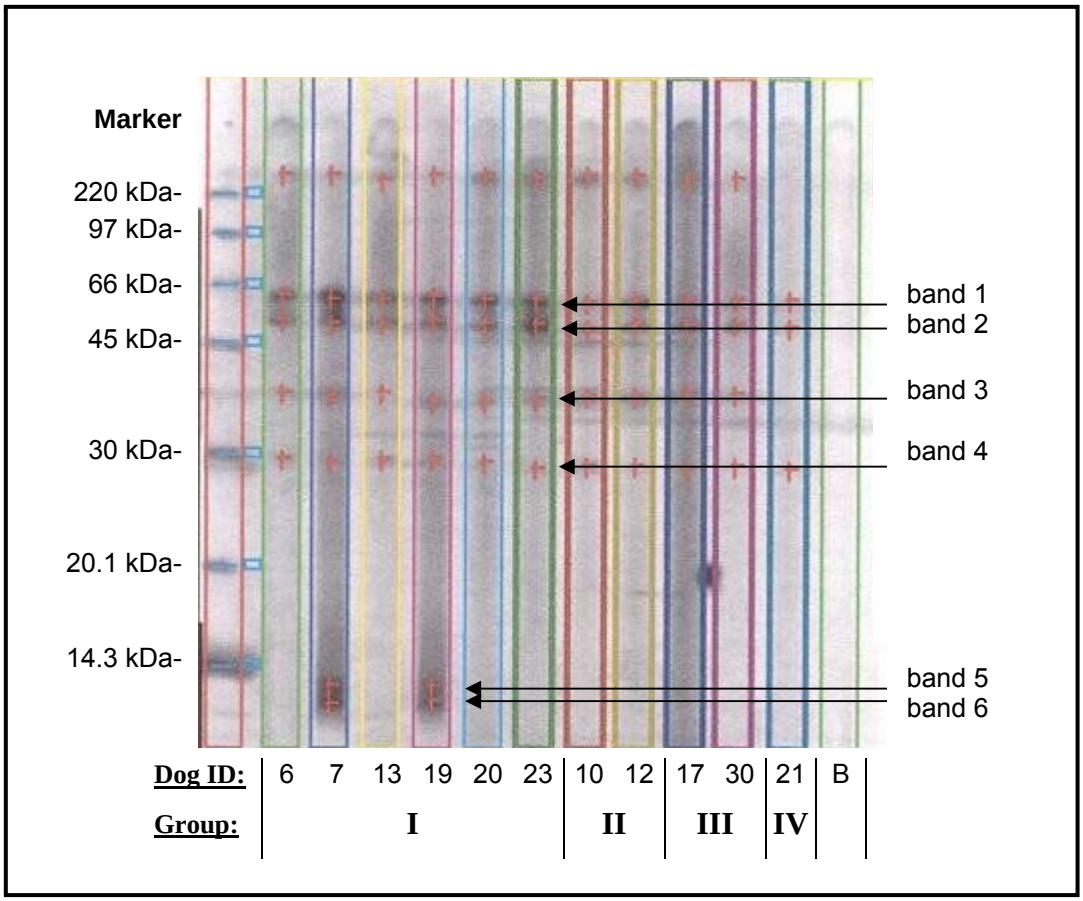

Figure 17: Western blot of native ALK HD extract (Lämmli) with canine sera:
Marker: MW standard Amersham rainbow 756
B: Buffer control
The additional "+" originate from the Lumianalyst software (Roche Diagnostics, Mannheim, Germany) used to calculate the MW

Table 15: Calculated MW of IgE-reactive proteins in native ALK HD extract (Lämmli)

Dog ID / Bands	I.6	I.7	I.13	I.19	I.20	I.23	II.10	II.12	III.17	III.30	IV.21
	(Molecular weight in kDa)										
1	61.6	60.5	60.5	61.6	60.5	60.5	59.4	60.5	60.5	60.5	60.5
2	52.7	51.6	51.6	52.7	51.6	51.6	49.4	51.6	51.6	51.6	50.5
3	38.1	37.7	38.1	36.9	36.9	37.3	37.7	37.7	38.2	38.2	-
4	29.5	29.2	29.5	29.5	29.2	28.7	28.9	28.9	28.7	28.9	28.7
5	-	13.1	-	13.1	-	-	-	-	-	-	-
6	-	12.0	-	12.2	-	-	-	-	-	-	-

In order to improve the resolution of the low MW proteins the buffer system by Schägger/Jagow was employed. In this buffer system the low MW protein bands were calculated with approximately 11 and 10 kDa (figure 18 and table 16). Because separation of low MW proteins is more accurate using the Schägger/Jagow buffer system the following paragraphs will refer to the 11 and 10 kDa protein.

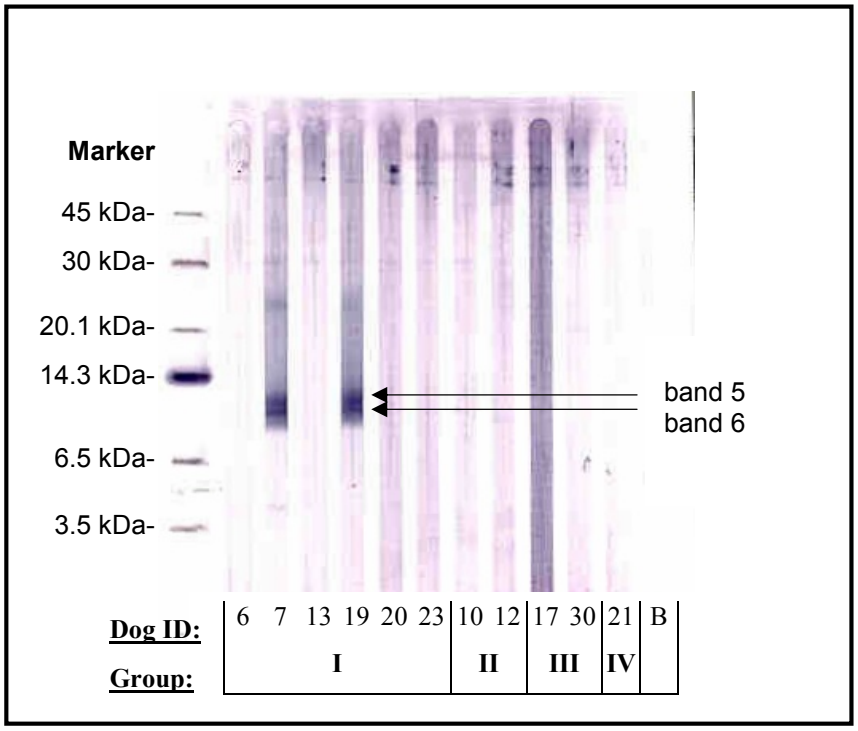

Figure 18: Western blot of native ALK HD extract (Schägger/Jagow) with canine sera:
Marker: MW standard Amersham rainbow 755
B: Buffer control
The additional "+" originate from the Lumianalyst software (Roche Diagnostics, Mannheim, Germany) used to calculate the MW

Table 16: Calculated MW of IgE-reactive proteins in native ALK HD extract (Schägger/Jagow)

Dog ID / Bands	I.6	I.7	I.13	I.19	I.20	I.23	II.10	II.12	III.17	III.30	IV.21
	(Molecular weight in kDa)										
5	-	11.1	-	11.7	-	-	-	-	-	-	-
6	-	9.7	-	9.7	-	-	-	-	-	-	-

Table 17: Reactivity patterns (grid-blot) of dog sera included in the Western blots: the allergenic extracts are shown on the x-axis and dog sera on the y-axis. Those sera with IgE-reactivity to the 11 kDa protein are marked with an arrow

		Allergens	Indoor										Outdoor				Food			
	Dogs	Alt.a.	Asp.f.	Can.f.	Clad.h.	Der.f.	Der.p.	Fel.d.	HD ALK	HD HAL	Pen.n.	Art.v.	Bet. v.	Phl.p.	Ara.h.	Gly.m.	Pen.a.	Tri.a.	
Group I	I.4	0	2	0	2	2	1	0	1	1	3	0	2	0	1	2	2	0	
	I.6	1	2	0	3	2	1	0	1	1	3	0	2	2	2	2	2	1	
	I.7	*0*	*0*	*0*	*0*	*1*	*1*	*0*	*2*	*2*	*1*	*0*	*1*	*0*	*1*	*2*	*2*	*0*	←
	I.8	0	0	0	2	2	0	0	1	1	1	0	1	0	1	1	1	0	←
	I.9	0	0	0	0	0	0	0	1	1	1	0	1	0	1	1	3	0	←
	I.11	0	1	0	1	2	1	0	1	1	2	1	1	1	2	1	2	1	
	I.13	0	1	0	1	2	2	0	1	1	2	0	3	2	3	3	2	1	
	I.19	0	1	0	1	3	1	0	2	2	2	0	0	0	1	1	1	0	←
	I.20	1	2	0	1	2	2	0	1	1	3	3	2	3	3	2	2	1	
	I.23	1	3	0	1	3	3	0	1	1	3	0	2	0	2	1	2	0	
	I.31	1	1	0	2	0	2	0	1	1	2	1	2	0	3	2	2	0	
	I.38	2	0	0	2	3	3	0	1	1	2	0	0	0	2	1	1	0	←
	I.51	0	1	0	0	2	1	0	1	1	1	0	0	0	0	1	0	0	
	I.55	2	2	0	1	3	2	0	2	2	2	1	0	1	0	1	2	1	
	I.56	1	1	0	0	2	2	0	1	1	1	1	0	0	0	1	0	0	
	I.58	1	2	0	1	3	2	0	2	2	3	1	0	2	1	1	1	2	←
	I.60	1	1	0	1	1	2	0	1	1	2	0	0	1	0	1	1	0	
	I.62	2	1	0	1	2	2	0	2	2	2	1	0	1	0	1	1	1	
	I.75	2	1	0	1	2	1	0	1	1	2	0	0	1	0	1	1	1	
Group II	II.10	1	2	0	2	2	1	0	1	1	3	1	2	2	1	1	2	1	
	II.12	1	1	0	1	3	2	0	1	1	2	1	0	1	1	1	1	0	
	II.16	1	1	0	1	3	2	0	1	1	2	1	1	2	2	1	1	1	
	II.17	1	1	0	1	2	1	0	1	1	3	1	1	2	1	1	1	2	
Group III	III.16	1	2	0	1	3	2	0	1	1	2	1	0	1	1	1	1	1	
	III.17	1	2	0	1	2	2	0	1	1	2	1	0	2	1	1	1	2	
	III.25	1	0	0	1	1	0	0	1	1	2	0	0	0	0	0	1	0	
	III.30	1	2	0	3	1	1	0	1	1	3	1	0	1	1	2	2	1	
Group IV	IV.21	1	0	0	1	1	2	0	1	1	1	2	1	2	1	1	1	1	
	IV.22	1	1	0	1	1	1	0	1	1	1	0	0	1	1	1	0	1	
	IV.23	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	

3.3.3 Identification of the 11 kDa HD IgE-reactive protein as human cystatin A

The N-terminal sequence of the 11 and 10 kDa protein was determined by resolving HD extract in a 12% SDS-PAGE gel with subsequent transfer to a PVDF membrane. After visualizing the proteins with Coomassie blue, the band was excised and submitted for N-terminal sequence analysis at the Institut für Experimentelle Biologie und Medizin, Forschungsinstitut Borstel, Germany, Laboratory of Dr. Arndt Petersson. At the N-terminus the following sequence was obtained S-P-G-G-L-S-E-A-K. Database-Alignment (SwissProt: Accession number: P01040) showed 78% consensus of the N-terminally sequenced 11 kDa

protein to the N-terminal end of human cystatin A. Further investigations (SwissProt) showed human cystatin A to be a highly conserved protein (figure 19). N-terminal sequence analysis of the 10 kDa protein was only partially possible. To a certain degree the determined amino acids agreed with the by four amino acids abbreviated cystatin A. As further proof, binding of a monoclonal antibody against human cystatin with the questionable 11 kDa protein was tested. The very clear binding of the anti-cystatin to the respective band was interpreted as a second confirmation that the 11 kDa protein in HD extracts identified by canine IgE is identical to human cystatin A (figure 20).

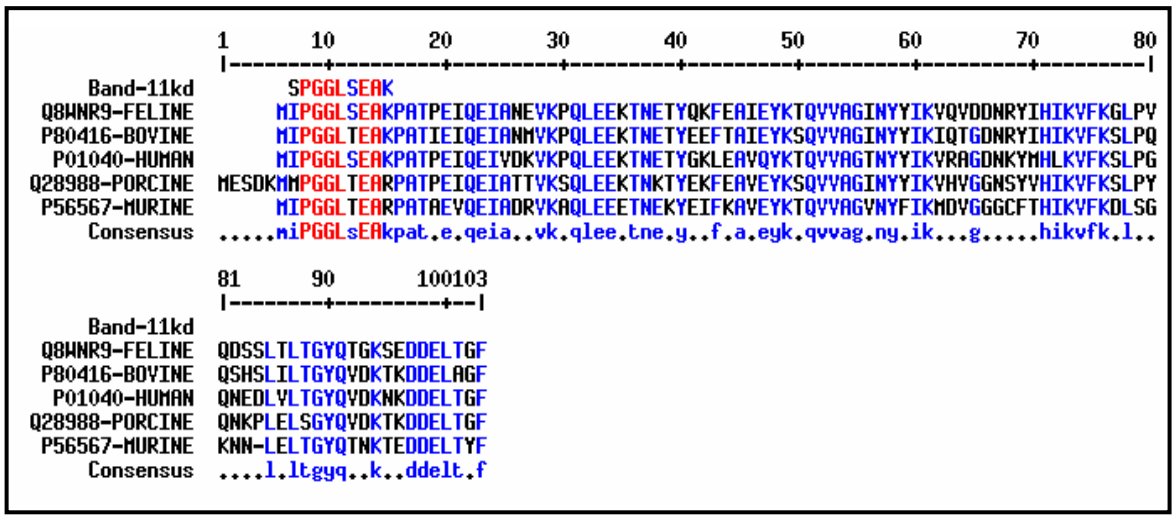

Figure 19: Alignment of mammalian cystatins in comparison to the first 9 aminoacids of the 11kDa protein determined by N-terminal sequencing (top row)

3.3.4 Dog/mouse comparison of IgE-reactivity determined by Western blotting

In many instances sera from the subjects to be investigated - dog sera in this case - are sparse, and therefore valuable, and replacing them by sera from artificially sensitized laboratory animals is of benefit. Therefore the IgE-reactive patterns to native ALK HD extract of the different mammalian species were compared in a Western blot (figure 20). The murine antibodies were raised by immunisation of BALB/c mice with HD extracts and the rabbit hyperimmune serum was also obtained by sensitizing rabbits with HD extract. All three species recognized the 11 kDa protein human cystatin A; as well as the protein in the medium MW range, at approximately 30 kDa, thus arguing for the possible use of sera from laboratory animals instead of dog sera in future studies. Serum from a non-sensitized mouse, non-sensitized rabbit and a dog from the kennelled beagle population were run as negative controls and a monoclonal antibody raised against human cystatin A was run as a positive

control. The dog serum used as a negative control recognized the 30 kDa medium MW protein as well as two other bands located in the medium MW range, though not the 11 kDa protein. A further strip of NC was run with a buffer control for each detection system.

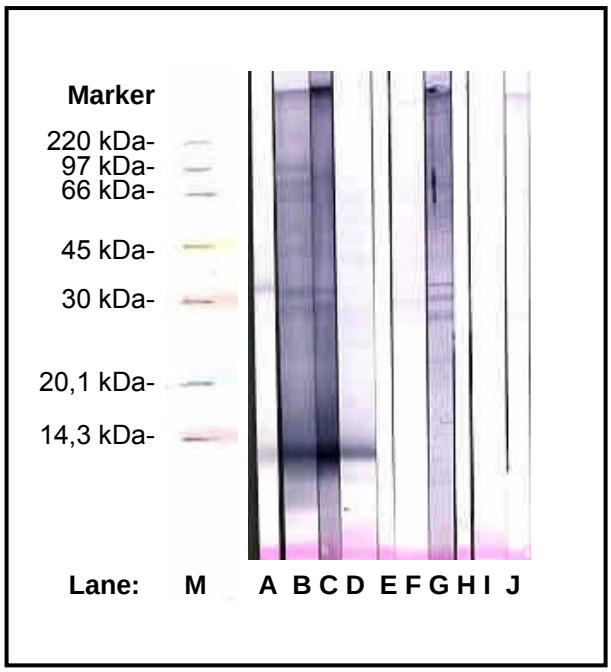

Figure 20: Western blot of native ALK HD extract (Lämmli) with canine, murine and rabbit sera;
Lane M: marker MW standard Amersham Rainbow 756
Lane A: polyclonal mouse serum sensitized against HD
Lane B: polyclonal rabbit serum sensitized against HD
Lane C: Dog serum (I.38)
Lane D: monoclonal α-cystatin antibody
Lane E: negative control: serum from a non-sensitized mouse
Lane F: negative control: serum from a non-sensitized rabbit
Lane G: Dog serum (IV.23)
Lane H: negative buffer control detection system mouse
Lane I: negative buffer control detection system rabbit
Lane J: negative buffer control detection system dog

4 DISCUSSION

At least two reasons argue for the increasing importance of indoor allergens as causative agents of allergy: Firstly, due to modern living and working habits the challenge of the individual with common indoor proteins has risen over the past decades; secondly, many patients today display allergic symptoms throughout the year. Typical indoor allergens identified by clinical experience in human allergology, are house dust mites, molds and epidermals originating from pets. These allergens have become equally or possibly even more important than outdoor allergens such as grass or birch pollens. In general dogs or other pets living mainly indoors are challenged with the same variety of indoor allergens as human occupants. However, one exception is epidermals originating from human beings themselves. It is a matter of common knowledge that mammalian proteins in particular are among the most potent allergens for human beings. Conversely human epithelial proteins, also allergens of mammalian origin, may cause allergic disease in dogs. So far, human epidermals or HD extracts have been poorly characterized as an allergen source. In order to perform further studies regarding the sensitization of dogs to human epidermals and proteins, a well characterized HD extract is essential. In this study the biological potency and protein patterns of two available HD extracts were determined by the recently developed RBL cell mediator release assay and SDS-PAGE, respectively. These characterized HD extracts were then included in a representative panel of allergens used to screen four typical dog populations for their allergen-specific IgE profiles and HD-specific IgE levels in particular. Dog sera identified as having the highest allergen-specific IgE concentrations to HD then served as probes for the identification of IgE-reactive proteins and the elucidation of their biological nature.

4.1 Characterization of HD extract: a basic requirement for the qualified diagnosis of HD-related atopy

Any qualified specific allergy treatment, i.e. both desensitization and allergen avoidance measures, requires the identification of the allergenic source responsible for evoking clinical symptoms. The application of allergenic extracts in the diagnosis and later in immunotherapy require a special degree of standardization. To date, nearly all clinically relevant allergenic extracts are available in standardized quality appropriate for application in human beings. This however, is the result of long years of effort in this field. Standardization measures include the characterization of allergenic extracts, i.e. the determination of allergenic proteins

and the evaluation of allergenic potency. The most essential tools for these procedures are sera from patients confirmedly suffering an allergy from the respective allergen source. The majority of allergenic extracts standardized for use in human beings are most likely also suitable as test allergens in dogs. The exceptions however, as mentioned above, are extracts from HD which cannot be characterized by human sera. Therefore a main focus of this study was the characterization of HD allergenic extract, a matter requiring an unusual approach for following reasons. Without HD extracts proven to be sufficiently potent or ideally, standardized test allergens, canine patients hypersensitive to human beings cannot be identified. Conversely standardized HD extracts cannot be produced without sera from patients which are confirmedly sensitive to HD. As a way out of this dilemma we employed the RBL test, a model using serum from artificially sensitized Balb/c mice to determine the biological potency of allergenic extracts. This method was originally used for the experimental biological standardization of cat, dog and horse dander extracts independent of humans (HOFFMANN, 1999). For this study, mice were artificially sensitized to HD extracts. For practical reasons however the sensitization of the RBL cells with murine sera was replaced by sensitization with monoclonal HD-specific IgE antibodies derived from spleen cells of the above mentioned mice. The RBL test allowed the thorough investigation of two intermediate products of HD extracts regarding their biological potency.

Though both native extracts demonstrated high allergenic activity in the RBL test, both also, to a lesser extent, elicited nonspecific degranulation of the RBL cells. On further investigation it was found that the nonspecific effects exhibited by the native ALK HD extract were due to a β-hexosaminidase-like activity of the extract itself. In contrast, the nonspecific effects of the native HD extract from HAL were due to nonspecific activation of the RBL cells. The phenole and phenole/glycerole preserved extracts were found to be far less potent; the sole use of glycerole had the least effect on the deterioration of the allergenic properties. Our results support the findings of a study published in 1981 (NELSON) which demonstrated phenole to be markedly inferior to glycerole.

By SDS-PAGE and Coomassie staining the protein patterns for both extracts were shown to be identical. Protein bands were detected at MW of approximately 58, 51, 37, 29 and 9.5 kDa in the Schägger and Jagow buffer system. In the Lämmli buffer system the high MW proteins were not strong enough for the imaging software to calculate. The remaining proteins had slightly different MW at approximately 39 kDa and 30 kDa and the low MW protein was demonstrated as a double protein band at approximately 14 kDa and 13 kDa. The high and low MW proteins correspond well with the MW of proteins in a HD extract calculated by a

study group in 1988 (YU et al., 1988). In this study an aqueous HD extract obtained by combing the scalp of two healthy adults, was divided into four fractions by high-speed gel filtration chromatography and subsequent investigation of the fractions by SDS-PAGE. Fraction 2 had two major protein bands with MW of 10 and 13 kDa, respectively and fraction 1 gave two major protein bands at 59 and 52 kDa. The remaining two fractions scarcely contained substances that were stainable.

SDS-PAGE and RBL test results demonstrated that both HD extracts were suitable for the planned immunological experiments, i.e. IgE-profiling and identification of IgE-reactive proteins via Western blotting.

4.2 IgE-profiling in four selected dog groups

Canine sera were screened for their IgE-reactivity with a panel of allergenic extracts in the NC-based solid phase assay "grid-blot". Using this technique a detailed investigation of the allergen-specific IgE profile for a panel of indoor, outdoor and food allergens was performed in potentially atopic dogs (group I), dogs with skin diseases other than atopy (group II) and healthy individuals, including "household" dogs (group III) and kennelled beagle dogs (group IV). Varying IgE profiles of individual dogs were successfully demonstrated with this method, suggesting the detection protocol for canine IgE to be sufficiently sensitive. However, in light of the problems encountered with the specificity of serological IgE methods and the lack of a gold standard in this study, the results obtained by the grid-blot and discussed in detail below must be interpreted with caution. In this study the serological determination of IgE levels by grid-blotting should primarily be regarded as a semiquantitative screening method for the presence of allergen-specific IgE and a prerequisite for the identification of HD-specific IgE-reactive proteins for dogs.

Using the grid-blot procedure nonspecific reactions to certain allergens, e.g. Pen.n., Asp.f. and native HAL HD, were an occasional occurrence; these phenomenona are sometimes observed in this type of assay and can be explained by a residual binding activity of biotin-labelled polyclonal rabbit α-mouse IgG antibody. Sensitization of the rabbits to other antigens in their environment other than the desired is probable and despite purification by affinity chromatography a residual binding activity to the corresponding allergenic extracts coated on the NC is possible. A further possible reason for nonspecific reactions is a biotin-like activity of allergenic extracts as was observed for the HD extract from HAL (data not shown). Nonspecific reactions of individual sera were also observed, a phenomenon caused by a high nonspecific binding capacity of the sera to the matrix used. These nonspecific reactions did

not impede the evaluation of the results as the background activity was taken into account when grading the reactions.

The statistical evaluation of the grid-blot results revealed partially significant differences between the groups of dogs for the indoor allergens house dust mites and molds.

In general both mites, though Der.f. more than Der.p., elicited very high serum IgE responses in all four groups of dogs, i.e. very few non-reactors and many grade III reactors. This finding supports the well-known fact that house dust mites and Der.f. in particular (PRELAUD, 2002) are among the most common indoor allergens to cause positive reactions in intradermal and serologic allergen-specific IgE testing in dogs (BENSIGNOR and CARLOTTI, 2002; CARLOTTI and COSTARGENT, 1994; LIAN and HALLIWELL, 1998; PRELAUD, 2002; STURE et al., 1995; VOLLSET, 1985). One explanation for the sensitization rate are the conditions in which pets are kept, moist bedding for example, resulting in a high exposure of "indoor" dogs to mite allergens (BENSIGNOR and CARLOTTI, 2002). A further possible reason for the high prevalence of IgE to house dust mites was suggested in 1995 (HEWITT et al.), where Der p 1 was shown to selectively cleave the low affinity IgE Fc receptor (CD23) from the surface of human B lymphocytes. The authors hypothesized that the loss of CD23 may promote and enhance the IgE immune response by ablating an important feedback inhibitory mechanism that normally limits IgE synthesis. Whether this mechanism is relevant for dogs remains to be proven. Dogs are more frequently sensitized to Der.f. than to Der.p., an observation which was once more confirmed in this study. This phenomenon was explained by a recent investigation performed by RANDALL et al. (2003) which quantified house dust mites and their allergens in the microenvironment of dogs. Whereas Der f 1 was detected in 100% of the homes, Der p 1 was detected in only 74% of the homes and Der.f. mites were also found more commonly and in a higher density than Der.p. mites. Group IV, kept in a virtually allergen-free environment had significantly less serum IgE to Der.f. than the three remaining dog groups, indicating the production of Der.f.-specific IgE to be primarily linked to exposition. For Der.p. group III showed significantly more IgE in serum than the other three groups of dogs. This might implicate the presence of heterogenous IgE in group III. Apart from the above mentioned statistically significant differences, surprisingly, serum IgE levels against mites in group I did not differ from the three other groups of dogs, a fact supporting the concept that alternative pathways may be responsible for clinical hypersensitivity responses, e.g. mast cell hyperexcitability.

The group of molds was comprised of four frequently found in indoor dwellings including Pen.n., Asp.f., Alt.a. and Clad.h. (OPPERMANN et al., 2001). Overall statistically significant

differences were revealed for Pen.n. and Asp.f. A comparison of Pen.n.- and Asp.f.-specific IgE levels demonstrated that high Pen.n.-IgE levels were more frequent than high Asp.f.-IgE levels. This finding is in accordance with a study performed in 1989 (KLEINBECK et al., 1989), in which serum IgE antibodies against Pen.n. were found in 100% of the atopic dog sera and Asp.f.-IgE were present in only 81% of the cases. These results suggest a higher concentration of Pen.n. than Asp.f. in homes in general. Alternatively, a higher contamination of the dog's microenvironment with Pen.n., similar to the situation for the house dust mite Der.f. (RANDALL et al., 2003) is possible. Further studies of the mold species found in the dog's microenvironment, i.e. dog beds etc. are required to confirm this theory. A statistically significant higher serum concentration of Pen.n.-specific IgE was noted for group I when compared to group IV, a finding which can be explained by a very low exposition of the kennelled dogs to molds due to housing on concrete floors and regular cleaning programs ensuring a relatively low mold contamination of the environment. The same can be stated for Asp.f. Group IV had significantly lower allergen-specific IgE levels than group III and group I.

To complete the panel of the most important indoor allergens mammalian proteins were also tested in the grid-blot. In humans, animal allergens are common causes of both acute and chronic allergic disease (CHAPMAN and WOOD, 2001). The most important animal allergens are derived from mammals, which secrete or excrete allergens into the environment. Allergens originating from cats and dogs are good examples for common causes of asthma and allergic rhinitis in man. Thus, it is only reasonable that the human being also produces allergens to which other species may develop a hypersensitivity reaction. We tested two suitable HD extracts in the grid-blot assay in order to determine the frequency of serum IgE against HD in the dog population and also to identify dogs with HD-specific IgE for further immunological experiments. Remarkably, in contrast to other studies (BOND et al., 1994; WILLEMSE et al., 1985), only few dogs had high HD-specific IgE concentrations in serum. Whereas grade 1 reactions were a common finding none of the dogs showed a grade 3 reaction and grade 2 reactions were also rarely observed.

There are several possible reasons for these conflicting results. Firstly, one might assume that our assay was not sufficiently sensitive. However, the possibility of inadequate extract potency was ruled out by evaluation in the RBL test prior to grid-blotting. The general test sensitivity was also sufficient since the other indoor and outdoor allergens, run in every grid-blot assay, served as intraassay controls of the method and the detection protocol. Conversely the in vitro assays in the previously mentioned studies perhaps may have been too sensitive,

possibly by a lack of specificity of the detecting antibodies. Early anti-IgE antibodies, especially polyclonal ones tended to detect allergen-specific IgG in addition to allergen-specific IgE, an error potentially causing false positive reactions. In studies surveying skin test reactivity nonspecific reactions of patients to the skin test antigens used may have elicited false positive test results. Despite these rather simple explanations the question appears to be more complex: If mammalian proteins are such important allergens, why do dogs show so few positive reactions? The most plausible explanation for the low sensitization rate to HD in our source of patients is a low exposure of dogs to HD in their environment. Low indoor concentrations of HD can be explained by the high hygienic standards in the industrialized countries, such as most people showering on a daily basis and changing garments regularly. This is in accordance with the high frequency of low levels of HD-specific IgE (grade 1) in dog sera, verifying that dogs are in contact with low concentrations of the human epithelial allergens. Grade 0 reactions were rare, though surprisingly, group I had 14% non-reactors. Part of these non-reactors might truly not have been sensitized to HD, though the high percentage seems improbable in the "multi-source" atopic dog population. One possible reason is the presence of cell-bound IgE, which does not circulate and therefore cannot be detected. Another possibility, which we are unable to differentiate from the above mentioned, is the involvement of a cell-mediated immune reaction. Remarkably, group IV had a high percentage of grade 2 reactors. There are several possible explanations for this finding. The environment might have been highly contaminated with HD, though higher levels of HD in a kennel environment than in a household situation seem improbable. A further possibility is cross-reactivity of HD with environmental or food allergens not included in our panel.

High allergen-specific IgE reactions (grade 3) to the common outdoor allergens included in the investigation were less frequent than to common indoor allergens. Both dogs and humans spend a greater proportion of their lives indoors and are in contact with a larger proportion of the air from the indoor environment (POPE et al., 1993), whereas contact with outdoor allergens is limited to certain seasons and is mostly restricted to a few hours a day. Because IgE production is correlated with allergen exposure, it is a logical consequence that sensitization to indoor allergens is a more common finding. A more detailed analysis of the results revealed statistically significant differences between the four groups of dogs for all outdoor allergens tested. For Phl.p. group III demonstrated significantly higher serum IgE levels than the three other groups of dogs. Similarly, group III also had significantly more allergen-specific IgE to Tri.a. than groups I and II. For Art.v. both groups III and IV had significantly more serum IgE than group II, while for Bet.v. groups I and II had significantly

more IgE than groups III and IV. The results obtained for Phl.p., Tri.a. and Art.v. implicate the presence of protective heterogenous IgE, i.e. IgE which does not lead to clinical atopy and protects the patient from developing a hypersensitivity reaction. This was not the case for Bet.v.-specific IgE, which correlated with clinical atopy and skin disease.

A third group of test allergens was comprised of the food allergens shrimp, peanut and soy bean. An overall statistically significant difference was only noted for the shrimp allergen Pen.a. Group I was shown to have significantly higher serum IgE levels than group IV. High IgE levels to Pen.a. can either be related to a "real" IgE response to shrimp allergen or possibly be the consequence of cross-reactive antibodies.

The analysis of the IgE levels to the allergens Alt.a., Clad.h., Fel.d., Gly.m. and Ara.h. revealed no statistically significant differences between the 4 groups of dogs.

Considering that IgE is thought to play the central role in atopy, this finding is surprising. However, a detailed investigation of the available literature in the recent past revealed that other studies demonstrated similar results when comparing allergen-specific IgE levels of atopic and healthy dogs (LIAN and HALLIWELL, 1998) indicating that high levels of allergen-specific IgE in serum are not solely responsible for the development of atopic disease. The importance of IgE in the pathogenesis of atopy has always been controversial and some authors suggest IgE to represent merely an epiphenomenon (HOLDEN and PARISH W.E., 1998). Though if IgE does play a major role in the pathogenesis of atopy, other explanations for the elevated allergen-specific IgE levels found in healthy individuals in this (healthy "household" and kenneled dogs) and in other studies are called for. The presence of heterogenous IgE, IgE+ and IgE-, is a theory being discussed in human allergology (MACDONALD et al., 1987). IgE+ and IgE- differ in their ability to mediate histamine release through interaction with certain co-factors, termed histamine releasing factors (HRF). In 1995 (MACDONALD et al.) a specific HRF, that is synergistic with IgE from IgE+ donors, was identified and cloned. In veterinary medicine this theory received support from two studies. Jackson et al. (1996) found that leukocytes from atopic dogs had a greater tendency to release histamine than those from normal or artificially sensitized dogs independent of total or allergen-specific serum IgE levels. Peng et al. (PENG et al., 1997) demonstrated two differing biologically active fractions of polyclonal dog IgE, which further supported the possibility of a relationship between heterogenous IgEs and the allergic response in the dog. A second theory suggests differences in mediator releasability of mast cells between atopic and healthy individuals (HALLIWELL and DEBOER, 2001). In 1996 (DE MORA et al.) a study group demonstrated enhanced mast cell releasability in atopic skin

compared to normal skin, suggesting that up-regulatory factors might be produced in canine AD to prime mast cells for subsequent allergen challenge. One of the mediators possibly responsible for the inherent mast cell hyperexcitability observed in canine AD is dermal stem cell factor, a mediator whose dermal secretion was suggested to potentiate histamine release following IgE receptor cross-linking (HAMMERBERG et al., 2001). A third concept suggests anti-IgE autoantibodies to be involved in the pathogenesis and modulation of IgE-mediated disease (DE WECK and STADLER, 1993; KOIKE et al., 1989). It is important to remember that all these concepts do not preclude one another but rather might all be intricately involved in the complex pathogenesis of IgE-mediated hypersensitivity reactions.

IgE assays are only of limited value as a specific tool in the diagnosis of atopy and until studies have examined the role of heterogenous IgE in clinical atopy, allergen-specific IgE assays clearly should be interpreted with caution. Furthermore, studies must also focus on other aspects of the pathogenesis of hypersensitivity, i.e. mast cell hyperexcitability in atopic dogs and the possible presence of heterogenous IgE, to further elucidate the complex and intricate pathogenesis of type I hypersensitivity.

4.3 Identification of cystatin A as an IgE-reactive protein of HD extract for dogs

The systematic investigation of HD extracts and the successful identification of IgE reactors in the dog population probably suffering from this allergen source allowed an approach going beyond the original goal: The identification of an IgE-reactive protein potentially of relevance for the development of clinical HD-related hypersensitivity in dogs. Sera with grade 1 and 2 reactions were used as probes for the identification of IgE-binding proteins of HD extract in Western blots. The HD extract from ALK was used for this purpose to avoid the nonspecific biotin-like effects of the HAL extract. Few dogs of the “multi-source” atopic dog population (n=6) showed a strong reaction with low MW proteins (approximately 10 and 11 kDa). In some blots this protein was only visible as a single band with an apparent MW of 11 kDa. In contrast, most of the dogs tested in Western blots throughout all four groups demonstrated less prominent IgE-reactive protein bands with apparent MW of 30, 38, 51 kDa and 60 kDa. Given the fact that most dogs displayed minor IgE-reactivity to the latter proteins the mostly low allergen-specific IgE-reactivity scores obtained during grid blotting ($\leq$1) can be explained. Because reactivity to the 51/60 and 30/38 kDa proteins were also found in a high percentage of the healthy dog population, clinical relevance seemed improbable and these proteins were not investigated further. In contrast, only individuals from the “multi-source”

atopic group demonstrated strong IgE-reactivity to the 11 and 10 kDa protein, making them more likely to be involved in clinical atopy. Therefore these two proteins were further investigated by N-terminal microsequencing. Investigation in a database (SwissProt: Accession number: P01040) showed 78% consensus of the N-terminal sequenced 11 kDa protein to the N-terminal end of human cystatin A (Stefin A), a cysteine protease inhibitor. Human cystatin A consists of 98 amino acids and has a calculated MW of 11, 006 kDa which is in agreement with the MW detected by the canine serum. The amino acids determined showed a certain agreement with the by four amino acids abbreviated cystatin A, suggesting a fragment of cystatin A. These findings indicate that fragmentation of cystatin A can occur under certain circumstances. Further confirmation was obtained by employing a monoclonal α-cystatin antibody, which bound to the low MW protein. Inhibition of the IgE-binding reactivity of dog serum by means of a monoclonal antibody against cystatin A showed a slight inhibition of the reaction (data not shown). This is to be expected because a monoclonal antibody detects only one epitope on the relevant protein and consequentially might not be able to saturate all binding regions detected by the dog serum.

Cystatins are cysteine protease inhibitors whose main physiological function is believed to be protection of the host from the harmful effects of free cysteine proteases by rapidly trapping these enzymes in tight complexes. The cystatin superfamily is divided into three subgroups, including the type I cystatins (stefins), which are mainly intracellular, type 2 cystatins, and kininogens. Type I cystatins are small acidic proteins consisting of about 100 aminoacid residues and lacking disulfide bonds. Human cystatin A functions as a cysteine protease inhibitor, forming tight complexes with papain and the cathepsins B, H and L. The primary structure of cystatin A was first determined in 1983 (MACHLEIDT et al., 1983). The protein is one of the precursor proteins of the cornified cell envelope in keratinocytes and plays a role in epidermal development and maintenance (TAKAHASHI et al., 2001).

Cysteine protease inhibitors have already been established as allergens. In 2001 (ICHIKAWA et al., 2001) cystatin as an allergenic component of cat dander was identified by screening a cat cDNA library using human IgE antibodies to cat dander and subsequent sequencing and expression of selected clones. The cat cystatin clone contained the conserved cysteine protease motif and two of three lipocalin motifs. Recombinant cystatin was produced in Escherichia coli cells and purified as an 11 kDa protein. The newly identified allergen was termed Fel d 3. Cysteine protease inhibitors are highly conserved proteins as demonstrated by the alignment in chapter 3.3.3. All species have them and bovine, porcine, murine and feline cystatin A are extremely similar to each other. Sequence homology searches of feline cystatin

revealed a high degree of identity with bovine and human cystatin A, 79% and 75%, respectively. The fact that all species have highly conserved cysteine protease inhibitors makes their allergenic properties very remarkable. This leads to a speculative question. How can an organism be allergic to a protein that it harbours itself? In the case of lipocalins, a family of extracellular proteins that most allergens of the mammalian origin belong to, it has been proposed (MANTYJARVI et al., 2000) that the immune response against the lipocalin allergens which are structurally related to endogenous lipocalins might be the pathway to allergy in genetically predisposed individuals. The same might well apply to other allergens with homologous endogenous counterparts such as the cystatins.

In summary we identified the 11 kDa molecule cystatin A, a protein capable of eliciting an IgE reaction in dogs. Further studies are necessary to determine the clinical relevance for human cystatin A in canine atopy by the use of a purified preparation of the 11 kDa protein in an intradermal test in atopic individuals and clinically healthy controls.

5 SUMMARY

Background – perennial indoor allergens, among them house dust mites and molds are known to be important inducers of IgE-mediated hypersensitivity reactions in dogs. Human dander (HD), an important constituent of house dust may also be a major cause of atopic disease in dogs. So far, HD has been poorly characterized as an allergenic source and the role HD plays in veterinary medicine is unclear.

Objective – the aims of this study were to characterize two available HD extracts, thus completing a representative panel of allergens used to screen four typical dog populations for their allergen-specific IgE profiles. A further aim of this study was the identification of specific IgE-binding proteins in HD extract and the elucidation of their biological nature.

Material and methods – the HD extracts were investigated regarding their biological potency and protein patterns using a recently developed cell mediator release assay (RBL-test) and SDS-PAGE, respectively. Both extracts were then included in a panel of common indoor (n=8), outdoor (n=4) and food (n=3) allergens to examine "multi-source" atopic dogs (n=88), dogs with skin diseases other than atopy (n=20), healthy "household" dogs (n=30) and kenneled beagle dogs (n=40) regarding their serologic allergen-specific IgE profiles using a solid-phase assay based on the "grid-blot" device. The reactions were graded semiquantitively in ascending order (0,1,2,3) according to the concentration of allergen-specific serum IgE. The identification of IgE-binding proteins in HD extract was performed by Western blot using dog sera demonstrating postitive reactions to HD (grade 1 and 2) as probes.

Results – statistically significant differences of serum IgE levels between the four groups were demonstrated for nine of the allergens tested in the grid-blot though these differences failed to follow a specific pattern. In general the sensitization rate to indoor allergens was higher than to outdoor allergens in all groups of dogs. For HD, grade 1 reactions were common in all groups of dogs, grade 3 reactions were not seen in any of the dogs tested and grade 2 reactions were also an infrequent finding. Six sera from the "multi-source" atopic dogs demonstrated IgE-binding to a protein with an apparent molecular weight of 11 kDa, which N-terminal microsequencing identified as human cystatin A. Control sera selected from the three other groups of dogs did not demonstrate IgE-reactivity with this protein.

Conclusions – semiquantitatively determined high allergen-specific IgE levels do not appear to be correlated with the development of clinical hypersensitivity in dogs and may simply reflect contact with the respective allergen source. Overall high HD-specific serum IgE levels were an infrequent finding in the canine population investigated in this study; however, in

atopic individuals, elevated HD-specific IgE can be consistent with IgE-reactivity to the cysteine protease inhibitor human cystatin A. Thus, this antigen appears to be of relevance for the development of clinical HD-related hypersensitivity in dogs.

6 ZUSAMMENFASSUNG

Hintergrund – Perenniale Allergene, wie z.B. Hausstaubmilben und Schimmelpilze gelten als verbreitete Auslöser der IgE-vermittelten Hypersensitivität beim Hund. Menschliche Hautschuppen oder Haarbälge, die meist mit Talg, Schweiß oder Speichel verunreinigt sind, und unter dem englischen Fachterminus „Human dander“ (HD) zusammengefasst werden, bilden einen proteinreichen Bestandteil im Hausstaub, welcher ebenfalls eine zentrale Rolle bei der Entwicklung atopischer Erkrankungen des Hundes spielen könnte. Bis jetzt ist HD als Allergenquelle nicht hinreichend charakterisiert worden und die Bedeutung dieses Allergens wird in der Veterinärmedizin z.Zt. widersprüchlich beurteilt.

Ziel der Studie – Ziele dieser Studie waren die Charakterisierung von HD Extrakten zweier Hersteller, sowie das Zusammenstellen von repräsentativen Allergenen zur Untersuchung vier typischer Hundepopulationen hinsichtlich ihres allergenspezifischen IgE Profils. Ein weiteres Ziel bestand in der Identifizierung IgE-reaktiver Proteine in HD Extrakten.

Material und Methoden – die HD Extrakte wurden hinsichtlich ihrer biologischen Wirksamkeit und ihres Proteinprofils mittels eines neuen Mediatorfreisetzungstests (RBL-Test) bzw. SDS-PAGE untersucht. Beide Extrakte wurden ergänzend in eine Zusammenstellung von „Indoor-“ (n=8), „Outdoor-“ (n=4) und Futtermittel- (n=3) Allergenen aufgenommen, die zur Erstellung eines serologischen IgE-Profils bei potentiell atopischen Hunden (n=88), Hunden mit Hauterkrankungen anderer Genese (n=20), gesunden im Haushalt gehaltenen Hunden (n=30) sowie Hunden (Beagle) aus Zwingerhaltung (n=40) genutzt wurde. Hierfür kam ein Festphasen IgE assay unter Verwendung einer „Grid-blot“-Apparatur zur Anwendung. Die Reaktionen wurden semiquantitativ in aufsteigender Reihenfolge (0,1,2,3) hinsichtlich ihrer IgE Konzentration im Serum ausgewertet. Die Identifizierung IgE-bindender Proteine in HD Extrakten erfolgte mittels Western Blot, unter Zuhilfenahme von Sera mit positiven Reaktionen gegen HD (Grad 1 oder Grad 2).

Ergebnisse – für neun der getesteten Allergene wurden zwischen den vier Gruppen statistisch signifikante Unterschiede nachgewiesen. Allerdings zeigten diese keine spezifische Verteilung. Im Allgemeinen war die Sensibilisierungsrate gegenüber den „Indoor“ Allergenen höher als gegenüber „Outdoor“ Allergenen. Geringe Serum-Konzentrationen an HD-spezifischem IgE (Grad 1) war in allen Gruppen ein häufiger Befund. Grad 2 Reaktionen gab es eher selten, starke Grad 3 Reaktionen wurden nicht nachgewiesen. Sechs Seren von der Gruppe der potentiell atopischen Tiere zeigten IgE-Reaktivität mit einem Protein, dessen Molekulargewicht bei 11 kDa lag. Dieses Protein wurde in der N-terminalen Mikrosequenzierung als humanes Cystatin A identifiziert. Aus den anderen drei Gruppen

ausgewählte Kontrollseren zeigten mit diesem Protein keine Reaktion.

Schlussfolgerungen – Semiquantitativ bestimmte hohe allergenspezifische IgE-Spiegel im Serum scheinen nicht mit einer klinischen Hypersensitivität korreliert zu sein und könnten lediglich den Kontakt mit der entsprechenden Allergenquelle reflektieren. Im Allgemeinen war die Sensibilisierungsrate gegen HD in der in dieser Studie untersuchten Hundepopulation gering. Bei Hunden mit erhöhten HD-spezifischen IgE Spiegeln konnte z.T. eine spezifische IgE-Reaktivität mit humanem Cystatin A nachgewiesen werden. Das humane Cystatin A scheint somit als Auslöser der HD-vermittelten Atopie beim Hund eine Rolle zu spielen.

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