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„Do hypoallergenic cats exist? - Secretion of Fel d 1 in the Neva Masquarade versus domestic cat breeds.“

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Zusammenfassung

Allergien gegen das eigene Haustier sind keine Seltenheit mehr. Besonders in Europa werden, neben Hunden und Nagetieren immer mehr Katzen als Haustiere gehalten. Die Fälle von Katzenallergien steigen leider rapide an und erzeugen für beide Seiten, Katzenbesitzer und Tier, eine stressvolle Situation. Leider funktioniert die Allergenimmuntherapie gegen Katzenallergien zumeist ungenügend. Bis jetzt war daher die einzige Lösung, um einer Allergie gegen die eigene Katze zu begegnen, die Abschaffuind des Tieres. Doch selbst Jahre nach Entfernung der Katze können deren Allergene noch immer im Haushalt vorhanden sein und Symptome auslösen. Die Katzenallergene werden nach ihrem lateinischen Namen Felis domesticus mit dem Kürzel Fel d und einer Nummer gekennzeichnet. Das Problem der Katzenallergei, aber auch der damit zusammenhängende Markt wurde von Katzenzüchtern erkannt und aufgegriffen, indem hypoallergene Katzen gezüchtet und angeboten werden.

Das Ziel meiner Diplomarbeit war es, den wissenschaftlichen Hintergrund der möglichen „Hypoallergenität“ der Katzenrasse Neva Masquarade zu erarbeiten, welche von vielen Züchtern als geringer allergen angepriesen wird.

Hierfür wurden Proben von 8 normalen und 8 hypoallergenen Katzen aus unterschiedlichen Bereichen ihres Körpers, wie Gesicht, Brust, Speichel und auch von ihren Schlafplätzen gesammelt. Die aufbereiteten Proben wurden auf ihren Gesamtproteingehalt und speziell auf die des Hauptallergens Fel d 1 untersucht. Weiters wurden die Proben via SDS-PAGE nach ihrer Molekularmasse getrennt und die Proteine analysiert. Ebenso wichtig war es, diese Proteine nach ihrer IgE Reaktivität zu untersuchen, indem Seren von Katzenallergikern darauf im Immunoblot getestet wurden. Die Ergebnisse des Gesamtproteingehalts im und BCA Proteinbestimmungstestes und der Fel d 1 Konzentration im Enzym-Immuno Sorbent Assay (ELISA) wiesen darauf hin, dass die Neva Masquarade im Vergleich mit normalen Katzen weniger Protein, darunter weniger Fel d 1 produzieren und in ihrem Umfeld verteilen.

Die Silber-gefärbten SDS-Polyacrylamid-Gel-Elektrophorese (SDS-PAGE) zeigten, dass hypoallergene Katzen weniger Protein auf der Höhe des Fel d 1 Heterodimers (18kDa) als auch auf der Höhe der quaternären Form (35kDa) zeigten. Im Immunoblot erkanntes IgE von Katzenallergikern in Extrakten von 6/6 normalen Katzen Banden bei 18 und 35kDa, also auf der Höhe des Fel d 1 Heterodimers, bzw. des kompletten quaternären Fel d 1 Moleküls. Im Gegensatz dazu wurde in Extrakten von hypoallergenen Katzen in 6/6 Fällen das 18kDa Allergen, jedoch nur in 4/8 Fällen das 35kDa Allergen über IgE erkannt.

Die erhobenen unterstützen, dass Neva Masquarade als eine hypoallergene Katzenrasse bezeichnet werden kann. Die Hypoallergenität begründet sich in einer geringeren Fel d 1 Produktion und daher auch geringeren Verteilung auf dem Fellklleid.

Abstract

Allergies against the own pet isn't a curiosity. Especially in European countries the number of cats in the households is exploding besides rodents and dogs. To avoid allergies against cats, the only solution is to get rid of the cat, however even years after of the removal of the cat, their allergens can be still present in the household or can induce continuously the symptoms. For both sides, cat owner and animal, these disease can induce very stressful situations without a real solution. Because of the rapid increase of the allergy against the major cat allergen Fel d 1, also the number of hypoallergenic cat breeders rises.

The aim of this diploma thesis was to find out, what is the scientific background, that the cat breed Neva Masquarade, which is promoted of many breeders as one of the hypoallergenic cat breeds, induces less or no symptoms by cat allergic patients.

Therefore I collected samples from 8 normal and 8 hypoallergenic cats from various areas like face, chest, saliva and the cats sleeping places. All prepared samples were tested for their protein content and for their Fel d 1 amount. I also separated the proteins of my samples according to their molecular weight on SDS-PAGE and analysed in detailed for the protein content. Equally important was to detect the IgE reactivity of allergic patients sera with the samples and analyse for Fel d 1 IgE binding.

The results of the total protein content and Fel d 1 concentration measured by ELISA showed, that the samples of Neva Masquarade imply less proteins and Fel d 1 and also distribute less allergen in their environment. Protein separation on SDS-PAGE supported our findings, that the hypoallergenic cats produce less or even no special allergen Fel d 1 in comparison with normal cats. Analysing the IgE binding proteins of our samples with cat allergic patients sera, hypoallergenic cats showed a single band at the molecular weight of monomer Fel d 1, while the normal cats more intense signal by the monomer and another band by the dimer. Determination of binding proteins with anti Fel d 1 antibody verified the hypothesis, that the major difference between normal and hypoallergenic cats is the binding capacity of Fel d 1.

From our data we conclude, that that hypoallergenic cat breeds exist with less production of Fel d 1 and therefore induce less allergic reactions in cat allergic patients as normal cats.

1. INTRODUCTION

1.1. Allergy

The term allergy originates from the old Greek words “àllos” and “èrgon”, which mean foreign and reaction. Allergies were first described in 1906 by Clemens von Pirquet, a Viennese pediatrician. He described an allergy as the ability of the human body to react against foreign substances [1]. In an experiment, Prausnitz and Küstner, showed in 1921, that allergen-specific sensitivity could be transferred by a soluble factor termed reagtion to a non-allergic person by injection of serum. The major component of allergic reaction, IgE was finally discovered in 1966 by two independent groups. [2] Due to these achievements the allergy research experienced a high upturn. The reasons for allergic disease can result from different exogenous or endogenous factors, such as air pollution or genetic background of an individual. **For instance it has been shown that people who experience long-term exposure to traffic-related pollutants have a higher risk of getting allergic bronchial asthma** [3]**.** Fossil fuels are of these outdoor air pollutants and lead to an increased production of antigen-specific IgE and histamine, higher susceptibility for allergic diseases. Besides outdoor air pollutants, also indoor pollutants, such as tobacco smoke and other chemical substances present in furniture, paint, plastics, hygienic materials like **toothpastes** and **deodorants** play an important role [4]. Also natural components are affecting allergic people, for example house dust mites and grass pollen are the most common allergens responsible for IgE-mediated allergies in European countries [5]. According to the “Erster österreichischer Allergiebericht” in 2006 the main allergens affecting the European population and leading to allergic rhinitis are 63.2% grass pollen, 43.6% cats, 35.9% birch pollen and 35% house dust mites [6]. Hence cat dander is among the most important allergen sources. The prevalence of allergic aptients in Austria is given in Figure 1.

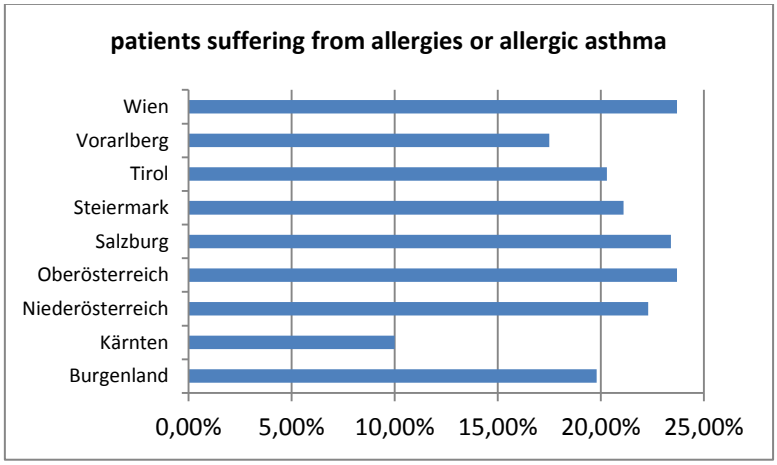


Figure 1: Percentage of patients suffering from an allergy or allergic asthma in Austria (self-made) **[7]**.

Not only respiratory allergens can cause allergy. About 4% of children suffer from food allergy against eggs, peanuts, tree nuts, fish or wheat. Studies have shown that early contact with food can induce specific tolerance [8].

Another interesting topic is the so-called “hygiene hypothesis”, described by David P. Strachan in 1989 [9]. The worldwide higher risk of getting affected by an allergy was correlated to increasingly smaller family sizes, increased cleanliness and decreased exposure to micro-bacteria. Exposure to farm environment appears to protect from both non-atopic and atopic asthma [10].

All these environmental exposures are now affecting all parts of population worldwide [11]. A questionnaire from the International Study of Asthma and Allergies in Childhood (ISAAC) was conducted in Korea and showed, that families with a history of atopic diseases, such as allergic conjunctivitis, food allergy or additionally raising pets were positively associated with atopic dermatitis in children [12].

In spite of the high and rising number of people suffering from allergies, only part of them seek help by a physician. In Germany only 28% of allergy-affected patients received sublingual immune therapy, 58% were practicing self-medication and only 21% were ever treated with anti-allergic drugs [13].

1.1.1. Hypersensitivity reactions in human

Hypersensitivity reactions, are excessive, undesirable reactions of the immune system, towards exogenous antigens like in allergies, or to self antigens like in autoimmune diseases. Hypersensitivity requires primary sensitisation of the body followed by effort phases which were according to the different immune mechanisms classified in 4 groups by Coombs and Gell in 1963 (Table 1).

Type I – hypersensitivity reaction

Immediate anaphylactic allergic reactions caused by IgE against innocuous allergens are termed type I reactions. The reactions may involve different organs like skin (dermatitis, food allergy), eyes (conjunctivitis), nasopharynx and lung (rhinitis, asthma) or the gastrointestinal tract (food allergy). The detailed description of the mechanism is presented in the next section.

Type II – hypersensitivity

The Type II hypersensitivity reaction is also called cytotoxic reaction. The reaction is primarily medited by IgM, IgG antibodies in context with complement. The antigens of this type of hypersensitivity are in general endogenous, but may also be exogenous, such as chemicals. When attached to a body cell membrane these haptens may achieve the status of a complete antigen. Macrophases or dendritic cells recognize and present the antigen to T-helper cells which then support the B-cells in antibody production, in this case prominently IgM or IgG. These antibodies again bind to the antigen, forming immune complexes and complement activation. The subsequent cell destruction may be caused either by complement-dependent cytotoxicity (CDC), or via antibody-dependent cell-mediated cytotoxicity (ADCC). The reaction takes hours to a day. Diseases, which belong to this category are for example Goodpasture`s nephritis or pemphigus.

Type III – hypersensitivity

In contrast to the above, it is soluble antigens that form immune complexes with IgG, IgM or IgA antibodies in Type III hypersensitivity reactions. The resultin immune complexes may precipitate in tissues such as joints, kidney or vasculature. Their pathogenic potential is determined by the ratio of antigen to antibody, by their size and by the amount, affinity and

isotype of the involved antibody. In a setting with more antibody that antigen, systemic type III hypersensitivity reactions may arise. This typically was associated with intravenous injection of sera or antibodies from foreign species into human patients for passive immunotherapy (protection against infections, cancer, snake bites) and hence, called serum sickness. These immune complexes can be deposited in different tissues and can cause inflammation due to complement activation followed by inflammation, with platelets and neutrophils being involved. In contrast, when a foreign antigen is injected locally into a hypersensitive individual, the localized form of type III hypersensitivity reaction may occur, termed Arthus reaction. In this case the antigen is precipitated immediately at the entry site by preformed antibodies of the IgG or IgM class. The resulting immune complexes bind to the IgG receptor FcγRIIIa. [14]

Type IV - hypersensitivity

In contrast to types I-III, the type IV hypersensitivity reaction is a T-cell mediated and thus delayed-type of hypersensitivity. In this case sensitization occurs to a hapten such as Nickel ions. The antigen-presenting cells, macrophages secrete IL-12, which stimulated the proliferation of CD4+ T-cells. Specific CD4+ T helper cells recognize the antigen in complex with MHC class 2, molecules on an antigen presenting cell. When the antigen is presented by MHC class I. molecules, it may stimulate CD8+ cytotoxic T-cells (CTL). Also these cells have been recognized to participate in skin hypersensitivity and inflammation. The prototypic delayed-type, or tuberculin type IV. Reaction is for instance used to determine if a patient has previously been infected with M. tuberculosis. The eczema form of type IV reactions is caused by direct skin contact with antigens and is mediated by antigen-specific TH1 cells as well as by TH2 cells. [14]

	Type I	Type II	Type III	Type IV
Immune reactant	IgE	IgG/IgM	IgG/IgM	TH1/TH2 CTL
Antigen	Soluble, via body surfaces or mucosa	Exogenous or endogenous cell-surface bound antigen	Soluble, injected	Hapten
Effector mechanism	Activation of effector cells that express high affinity IgE receptor	Antibody and complement-mediated cytotoxicity	Immune complex mediated complement activation	Macrophage activation, IgE production
Hypersensitivity reaction	Allergic asthma, a. rhinitis, part of atopic eczema mechanism, systemic anaphylaxis	Drug allergies and autoimmune diseases	Serum sickness, Arthus reaction	Allergic contact dermatitis, tuberculin reaction, aspect of atopic eczema mechanism

Table 1: Classification of the 4 types of hypersensitivity reactions according to Coombs and Gell.

1.1.3. Mechanism of type 1 hypersensitivity reaction - allergy

In the pathomechanism of type I allergic reactions, allergen-specific immunoglobulin E (IgE) plays a key role. Compared to IgG concentration in human serum (10 mg/ml), serum IgE concentrations are around 150 ng/ml in healthy individuals, but this concentration can be elevated significantly during allergic reaction. The type 1 hypersensitivity reaction is subdivided into two phases, the sensitisation phase and effector phase, the latter occurring immediately (typically within seconds or minutes to an hour) or late (after 6-8 hours). Allergens pass the natural barriers of the human body, such as skin, mucosa or respiratory system and get in contact with all over existing antigen presenting cells (APC). These APC can be dendritic cells (DC) including Langerhans-cells of the skin, myelotic DC, interdigital DC, inflammatory dendritic epidermal cells (IDEC) and follicular DC, but also macrophages. These APCs have the ability to capture an entering antigen, which causes the activation and maturation of these cells. The mature APC presents the antigen by MHC II class molecules to the T-cells (signal 1). TH2 cells release a number of interleukins (IL), such as IL-4, IL-5 and IL-13. Interleukin 4 and 13 are very important cytokines because of their ability to induce

the production of IgE by B-lymphocytes. In the meantime also so called memory B-cells are stimulated by the intact antigen, taken up and presented by the B-cells themselves to T-lymphocytes. The cytokines from the Th-cells stimulate antibody production and isotype switch, e.g. IL-4 and IL-13 stimulate switch to IgE production. The formed IgE antibodies only shortly circulate in the blood, because they quickly bind to the IgE-receptors on the surface of mast cells and other IgE effector cells. Mast cells arise from progenitor cells in the bone marrow, but their maturation occurs in the peripheral tissue under the influence of different cytokines, such as IL-3, IL-4, IL-6 and IL-9. They carry a variety of receptors on their surface, including the high affinity IgE receptor FcεRI. Mast cells can not only be activated via IgE, or IgG1, IgG2a and IgG2b, but degranulation also can be induced by crosslinking of immunoglobin free light chains. [15] However, after second contact with the specific antigen in the human body, memory B-cells produce IgE, which are catched by FCεRI on the mast cells and basophils and binds to the low affinity receptor FCεRII (CD23). Mast cells release different vasoactive amines, lipid mediators, chemokines or other cytokines, such as histamine, which all can lead to acute local responses such as oedema formation, tissue swelling, or bronchoconstriction. Mast cells also play an important role on the late phase responses of an allergic response by the release of chemotactic and pro-inflammatory mediators. (2) Late phase responses occur usually after 2 till 24 hours after the first reaction and after the migration of other leukocytes, such as eosinophils and macrophages to the initial site. [16] These reactions are caused by release of mediators from eosinophils and also are dependent on TH2 cell activity. [17]

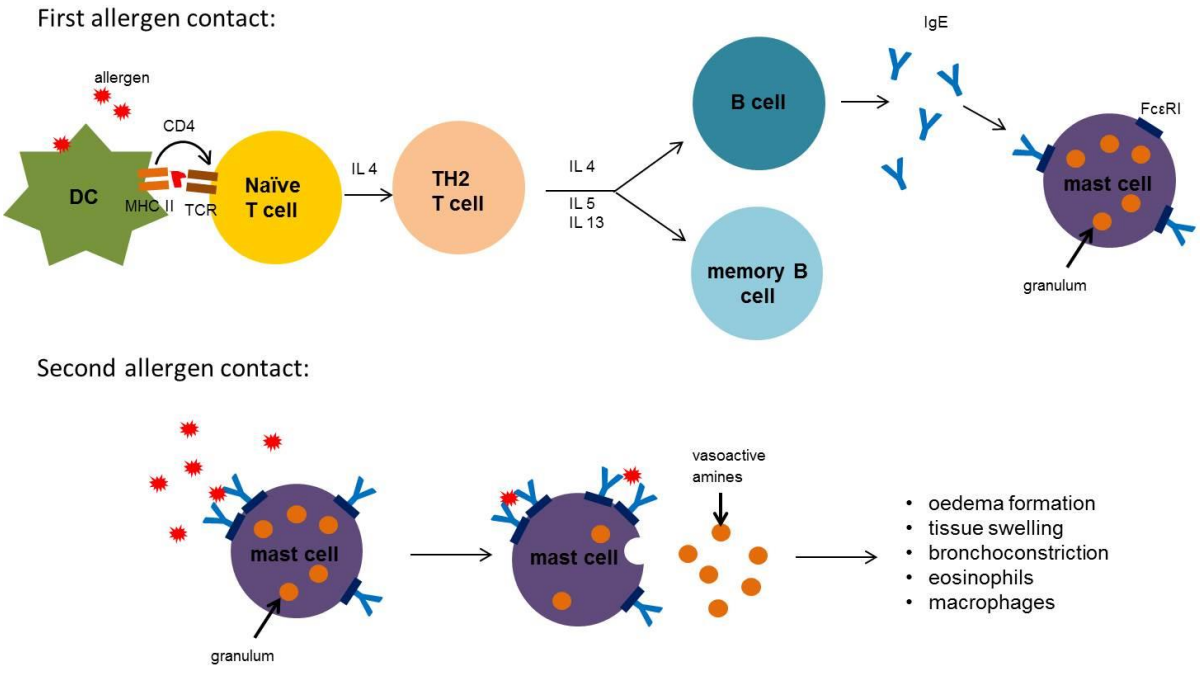


Figure 2: Mechanism of Type I hypersensitivity reaction, adapted from []. (self-made).

1.2. Allergy to cats

It is known, that cats (Felis domesticus) are the source of one of the most common aeroallergens, causing mild to severe acute and chronic respiratory reactions [18]. The raising pet ownership in many industrialized countries simultaneously increased the level of exposure to pet allergens. It has been shown, that cat allergens also are present in households without cats, which can be explained by its distribution via clothes of cat owners into cat-free environments. Recent studies have shown, that allergic sensitization to cats is more likely to develop in children exposed to moderate levels of this allergen than in children exposed to high amounts of the major cat allergen Fel d 1 [19]. Cat allergic patients suffer from itchy eyes and nose, coughing and wheezing, red eyes, sneezing, runny and stuffy nose due to inhalation of the allergen, or redness of the skin where a cat has bitten, licked or scratched. These symptoms may develop within a few minutes, up till hours and continuous exposure may result in chronic rhinitis or asthma.

1.2.1. Cat allergens

Fel d 1

Of all known cat allergens, *Felis domesticus* allergen 1 (Fel d 1) is the most potent and best studied. About 95% of cat allergic patients show IgE antibodies to Fel d 1 in their sera [20]. Fel d 1 was already described three decades ago [21] and since then the biochemical and immunological nature of Fel d 1 has been revealed. The allergen is produced by salivary and sebaceous gland, secreted onto the animal skin and fur, but is also found in large amount in anal sacs [22]. It is a 35kDa tetrameric glycoprotein formed by noncovalent interaction of two heterodimers of each 18kDa-19kDa. Each heterodimer again is formed by one 70 amino acid residue peptide of 4kDa (chain1, α-chain) and one 85, 90 or 92 amino acid residue peptide of 14kDa (chain 2, β-chain) [23]. However, samples from various parts of a cat's body may be composed by different forms of Fel d 1 with variable molecular weights. For example, Bienboire-Frosini et. al. verified that the tetrameric Fel d 1 in the chest area of cats has a molecular mass of 30kDa and from anal sacs 40kDa [22]. The tetrameric form arises from a non-covalent interaction, but the two chains of the heterodimers are linked by three disulfide bonds [24]. Studies have achieved in vitro expression conditions in Escherichia coli for the appropriate folding of recombinant Fel d 1 using a direct linkage of chain 1 to chain 2, called

Fel d 1 (1+2) or, Fel d 1 (2+1) [25]. A schematic view of the Fel d 1 tetrameric complex is shown in Figure 3.

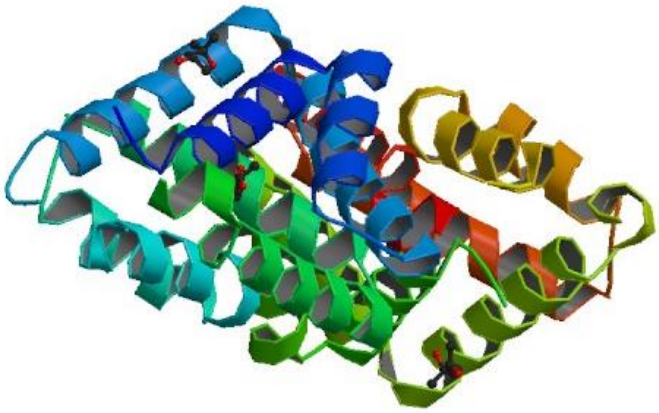


Figure 3: Molecular model based on crystal structure of Fel d 1. PDB: 1PUO **[26]**

Experiments with cat allergic patients have verified that the immunological activities of Fel d 1 (1+2) and (2+1) are equivalent to natural Fel d 1 and that the crystal structure of the recombinant Fel d 1 (2+1) is similar to the structure of uteroglobin, which is a steroid-inducible cytokine-like molecule. Uteroglobin is a member of the secretoglobin superfamily and has potent anti-inflammatory and immunmodulatory properties. More interestingly, this secretoglobin superfamily also includes the human protein Clara Cell 16 (CC16), which already has been shown to provide a protective effect in the lungs against pathogens and also plays an important role in the control of inflammation [27].

Fel d 2

Fel d 2 belongs to the family of albumins with an size of 69kDa. Cat serum albumin cDNA was procured from cat liver. The prevalence of Fel d 2 recognition lies between 14 and 23% in cat allergic patients, meaning that almost a quarter of tested cat allergic patients showed IgE binding to Fel d 2 in immunoblots. Albumins are a family of globular proteins. They have a water soluble and crystallisable character and it is the most common protein of human blood plasma. [28] [29]

Fel d 3

The Fel d 3 allergen originates from the family of stefins, the family 1 cystatins type and has a size of 11kDa. Stefins are small, acidic proteins consisting 100 amino acid residues, which lack disulphide bonds. The cat stefin Fel d 3 has 98 amino acid residues, without cysteine

and shows 80% sequence identity to bovine and human cystatin A. The model for Fel d 3 is similar to the 3D-strucure of stefin B. It has been shown, that about 60-90% of sera from cat-allergic patients contained IgE antibody to this protein. [30]

Fel d 4

Fel d 4 is a lipocalin, with an size of 22kDa. The main function of lipocalins is the transport of small hydrophobic molecules, for example lipids and steroids. This allergen also binds IgE at relatively high frequency in allergic patients. It also has a high amino acid sequence identity with many other mammalian lipocalins, such as Equ c 1 the major horse allergen or boar salivary lipocalin Sal 1. This similarity suggests the possibility of the presence of cross-reactive IgE epitopes. About 62,96% of all cat-allergic patients having Fel d 4-specific IgE. Fel d 4 has the typical folding pattern of lipocalins. A cDNA clone of the allergen isolated from the submandibular salivary gland of the cat encoded a 19,7kDa lipocalin. [31]

Fel d 5 – Fel d 8

The available information on the allergens Fel d 5 (400kDa), 6 (800-1000kDa), 7 and 8 is so far restricted. Fel d 5 is the cat Immunoglobin A, Fel d 6 is cat IgM and Fel d 7 is the so-called Von Ebner's gland protein. The Von Ebner's glands are exocrine glands inside the mouth and are named after the Austrian histologist Gilbert Victor von Ebner, Ritter von Rosenstein. The salivary Von Ebner's gland protein is a cysteine proteinase inhibitor. One study has shown that Fel d 7 has the size of 17.5kDa and Fel d 8 the size of 24kDa. The frequency of IgE binding of cat allergic patients to Fel d 7 and 8 was 39.6 and 19.3%, respectively. [32] [33] [34] [35] [36] [37]

1.3. Hypoallergenic cat breeds

There exist many myths about hypoallergenic cats. In theory, cats which do not induce allergic symptoms would offer a possibility for cat allergic persons to hold a cat pet. Many cat breeders sell cats as "hypoallergenic" without any scientific proof. They often argue with reports from their customers. The seven breeds, which are described to be hypoallergenic are Balinese, Oriental Shorthair, Oriental Semilonghair/Javanese, Devon Rex, Cornish Rex, Sphynx and Siberian. Specifically for the Siberian cat, previous investigations by a company specialöized for measuring the level of indoor allergen contaminations (Indoor Biotechnology,

Charlottesville, US) suggested that Siberian cats harbour less major allergen than other cats, a statement which was later corrected [38].

The World Cat Federation (WCF) is an internationally operating federation and has more than 370 single organizations worldwide. Besides other assignments, one area of their responsibility is the definition of the standardization of the various cat breeds. The following summarized descriptions in table 2 of the 7 different hypoallergenic cat breeds are determined by the WCF and can be looked-up for more detailed information on http://www.wcf-online.de. [39]

Cat breed	Coat	Colour varieties
Balinese	medium length silky texture no undercoat furnished tail, forming a plume	all colours without white Siamese points
Cornish Rex	short length wavy, soft, plush and elastic less guard hair	all colours
Devon Rex	very short length wavy, soft and fine less guard hair	all colours
Oriental Semilonghair / Javanese	medium length glossy and silky texture no undercoat furnished tail, forming a plume	all colours without white
Oriental Shorthair	very short glossy and silky texture no undercoat close lying to the body	all colours without white and without points
Siberian Cat	medium length very dense, soft and fine undercoat firm and glossy top coat	all colours without any combination of chocolate, cinnamon, lilac and fawn pointed variety with Siamese points is

	top coat is water repellent under party of body ad back on hind legs only with undercoat long hair on the neck, chest, trousers and tail	called Neva Masquerade
Sphynx	only covered by slight down skin has appearance and texture of human skin and has same functions	any colours

Table 2: Summary of coat and colour verities of the 7 considered hypoallergenic cats from the homepage of WCF [37].

For illustration phenotypic differences between hypoallergenic cat breeds, the 3 winners of the “WCF Worldchampion Worldpremior” are shown in figure 4.

A  B  C 

Figure 4: Winners of the “WCF Worldchampion Worldpremior” award A) Sphynx B) Oriental Shorthair C) Siberian **[40]**

All hypoallergenic cats used for sampling in this study are from the “Animalfarm” of the emerging breeder Markus Kronberger in Upper Austria. The farm is located at the sea level of 770m in the Upper Austrian region Salzkammergut. Besides Neva Masquerade cat breed, a subclass of the Siberian Forest Cat, breeding of other **chastity’s**, such as Iceland horses breed, red deer breed and poultry breed takes place at the isolated farm. The cats have the possibility to move around the whole huge area of his 13-hectare large estate. Regardless of this extraordinary breeding characteristic, the breeder is aware of the strict reproduction only between his two breeding cats and no other cat, which were also tested due my diploma thesis. The breeding tomcat Sven, abbreviated HC2, is a Siberian Forest Cat and the breeding cat Ronja, abbreviated HC1 is a beautiful Neva Masquerade both shown in figure 5 and 6. [41]

Figure 5: Breeding cat HC1 being a Neva Masquarade. **[41]**

Figure 6: Breeding tomcat HC2 is a Siberian Forest Cat. **[41]**

2. AIM OF THE STUDY

The aim of my diploma study was to examine the possible cat hypoallergenicity specifically of the Siberian “Neva Masquerade” breed because of their extraordinary phenotype. The major allergen Fel d 1 was selected as a marker molecule of prognostic value for asthma due to cat allergy.

There was another reason for my strong interest in performing the diploma work on specifically this topic: Many family members of mine, including myself, are allergic to cats and suffer from asthma, which makes it very difficult for us to have one in our household. Meeting a breeder of hypoallergenic cats and reading about them contributed to the decision to workn on this subject. Hopefully the results of the diploma work will help cat especially asthmatic patients.

3. MATERIALS AND METHODS

3.1. Instruments

- DUSTREAM® Collector Kit (Indoor Biotechnologies; Charlottesville, Virginia, USA)
- ELISA Reader TECAN infinite M200 PRO (Tecan Group Ltd., Männerdorf, Germany)
- Lyophilisator CHRIST Alpha 2-4 LSC (CHRIST GmbH, Osterodl am Harz, Germany)
- Film Processor AGFA CP 1000 (Agfa-Gevaert N.V.; Belgien, Mortsel)
- Centrifuge Sigma 4K15C (Qiagen; Venlo, Netherlands)

3.2. Materials

- 96-well flat-bottom plates (Nunc Immunoplate; Roskilde, Denmark)
- Protease Inhibitor Cocktail (Sigma-Aldrich; Vienna, Austria)
- SnakeSkin® Dialysis Tubing 10K MWCO 33mm dry I.D. 35 feet (Thermo Fischer Scientific; Vienna, Austria)
- Fel d 1 – ELISA Kit (Indoor Biotechnologies; Charlottesville, Virginia, USA)
- BSA – Bovine serum albumin (Sigma-Aldrich; Vienna, Austria)
- Ready-SET-Go Avidin HRP 250x 0.5ml (eBioscience; San Diego, USA)
- ABTS – 2,2‘-azino-bis3-ethylbenzthiazoline-6-sulphonic acid (Sigma-Aldrich; Vienna, Austria)
- Pierce® BSA Protein Assay Kit (Thermo Fischer Scientific; Vienna, Austria)
- Tween®20 (Sigma-Aldrich; Vienna, Austria)
- APS – Ammonium Persulfate (Thermo Fischer Scientific; Vienna, Austria)
- TEMED - N,N,N’,N’-Tetramethylethylendiamin (Thermo Fischer Scientific; Vienna, Austria)
- PageRuler™Plus Prestained Protein Ladder (Thermo Fischer Scientific; Vienna, Austria)
- X-Ray Film (GE HealthCare Life Sciences; Buckinghamshire, Great Britain)
- Super Signal West Pico Chemiluminescent Subrate (Thermo Fischer Scientific; Vienna, Austria)

3.2. Buffer compositions

- 10x PBS
    - 2g KCl + 2g KH_2PO_4 + 80g NaCl + 11.5g $Na_2HPO_4*H_2O$ + 1000ml distilled water, pH 7.2-7.4
- 0.05% PBST
    - 500µl Tween20 + 1000ml 1x PBS

Allergen sample collection

- Washing buffer
    - 0.5ml protease inhibitor cocktail + 100ml distilled water

ELISA

- Coating buffer
    - 1.96g Na_2CO_3 + 2.64g $NaHCO_3$ + 500ml distilled water; pH 9.6
- 1% BSA-PBST
    - 2mg BSA + 200ml PBST 0.05% Tween20
- Streptavidin HRP buffer
    - 1% BSA-PBST + Streptavidin HRP (dilution 250x)
- Citrate acid buffer
    - 7.35g citrate monohydrate + 8.9g Na_2HPO_4 + 500ml distilled water; pH 4
- ABTS buffer
    - 10ml citrate acid buffer + 10µg 2,2'-azino-bis3-ethylbenzthiazoline-6-sulphonic acid + 10µl 30%H_2O_2

SDS-PAGE

- 1x15% Separation gel
    - 3.65ml distilled water + 2.5ml running buffer + 100µl 10% SDS + 3.75ml 40% acrylamid + 50µl 10% APS + 10µl TEMED
- 2x Stacking gels
    - 2.7ml distilled water + 1.25ml stacking buffer + 50µl 10% SDS + 1ml 40% acrylamid + 25µl 10% APS + 10µl TEMED
- 10x Electrophoresis buffer
    - 60.6g TRIS + 292g glycin + 20g SDS + 2000ml distilled water; pH 8.3
- Reducing SDS-PAGE buffer
    - 378mg TRIS + 1.15g SDS + 5ml 87% Glycerol + 1.25ml Bromphenol blue of 1% solution in ethanol + 2.5ml Mercaptoethanol + 50ml distilled water; pH 6.8

- Non-reducing SDS-PAGE buffer
    - 378mg TRIS + 1.15g SDS + 5ml 87% Glycerol + 1.25ml Bromphenol blue of 1% solution in ethanol + 50ml distilled water; pH 6.8

Silver staining

- Gel fixing solution
    - 800ml methanol + 200ml acetic acid + 1000ml distilled water
- Washing solution
    - 150ml ethanol + 500ml distilled water
- Thiosulfate reagent
    - 200mg sodium thiosulfate + 1000ml distilled water
- Silver nitrate reagent
    - 400mg silver nitrate + 400µl 3.7% formaldehyde + 200ml distilled water
- Developer
    - 15g Na_2CO_3 + 2.5ml 3.7% formaldehyde + 12.5ml thiosulfate reagent + 500ml distilled water
- Stop solution
    - 2.5g glycine + 500ml distilled water

Immunoblot

- Gold buffer
    - 30g Na_2HPO_4 x $12H_2O$ + 2.5g NaH_2PO_4 x $2H_2O$ + 1g NaN_3 + 10ml Tween20 + 2000ml distilled water
- Blocking buffer
    - Gold buffer + 3% (w/v) BSA (50ml Gold buffer + 1.5g BSA)
- Dilution buffer
    - Gold buffer + 0.5% (w/v) BSA (5ml blocking buffer + 25 ml Gold buffer)

3.3. Cats

All hypoallergenic (HC) and normal cats (NC) were visited in their households by the experimenter. Samples were collected in their field and questionnaire about the cats were filled out by the owners.

Questionnaire:

Name of the pet: _____

Breed: _____

Gender: male ☐
female ☐

Castrated: yes ☐
no ☐

Dewormed: yes ☐
no ☐

Age: _____

Fur (colour, etc.):_____

Living place/Environment: _____

Did the pet recently suffer from any disease?
Yes ☐
No ☐
If yes, what kind of: _____

Anomaly? (birth control pill, operations, drugs, chronic illness, etc.)

The collected data about the cat features are summarized in Table 3. From all cats pictures were taken after sample collection, three cats each from both groups are presented in figure 7.

Cat	Age	Gender	Breed	Fur	Castrated	Dewormed	Environment
NC1	6 years	Male	Felis silvestris	White-grey-black striped, brindled	Yes	Yes	Free roaming
NC2	6 years	Female	Felis silvestris	black	Yes	Yes	Free roaming
NC3	16 years	Male	Felis silvestris	Red-white brindled	Yes	Yes	Domestic
NC4	7 years	Male	Felis silvestris	White-grey-black striped, brindled	Yes	No	Free roaming
NC5	6 years	Male	Felis silvestris	White-grey-black striped, brindled	Yes	Yes	Free roaming
NC6	7 years	Male	Chantilly Tiffany Cat	black	Yes	Yes	Free roaming
NC7	4 years	Female	Felis silvestris	White-grey-black striped, brindled	Yes	Yes	Free roaming
NC8	8 years	Male	Felis silvestris	White-grey-black striped, brindled	Yes	Yes	Free roaming
HC1	5 years	Female	Neva Masquarade	Sealpoint	No	Yes	Free roaming
HC2	1,5 years	Male	Siberian Forest Cat	Blue-tabbypoint /white	No	Yes	Free roaming
HC3	5 months	Female	Neva Masquarade	Sealpoint	No	Yes	Free roaming
HC4	2 years	Male	Neva Masquarade	Sealpoint	No	Yes	Free roaming
HC5	1,5 years	Female	Neva Masquarade	Sealpoint	No	Yes	Free roaming
HC6	1,5 years	Male	Neva Masquarade	Sealpoint	Yes	Yes	Free roaming
HC7	5 months	Male	Neva Masquarade	Blue-tabbypoint /white	No	Yes	Free roaming
HC8	5 months	Female	Neva Masquarade	Blue-tabbypoint /white	No	Yes	Free roaming

Table 3: Individual informations from normal and hypoallergenic cats based on the questionnaires.

Figure 7: Representative pictures from normal cats NC2, NC3 and NC7 and from hypoallergenic cats HC5, HC6, HC7 (self-made).

3.4. Human patients

Fel d 1 represents one of the major allergens from cat, inducing allergen-specific IgE antibodies and therefore causing symptoms by affected patients. Human patients were selected based on their Fel d 1-specific IgE antibody titer, tested by Immuno Solid-phase Allergen Chip (ISAC) in the outpatient allergy diagnosis center AllergyCare®, Vienna, Austria, listed out in table 4. The human study was performed with the permission of the Austrian Ministry of Science 2002/2012.

Cat allergic patients		Non-allergic patients	
Patient number	Fel d 1 (AU)	Patient number	Fel d 1 (AU)
1/9	5360	1/3	0
2/1	2319	1/6	0
2/11	15862	1/7	0
3/19	2003	1/18	0
5/6	1462	2/3	29
6/1	5204	2/18	0
6/5	2118	3/8	0
6/7	13547	3/10	0
6/8	4653	3/13	0

Table 4: List of cat allergic and non-allergic patients.

3.5. Methods

3.5.1. Preparation of allergen samples

Step 1 – Fel d 1 sample collection

Fel d 1 samples from hypoallergenic and from normal cats were collected by washing the respective surface area of cat. Cotton pads were moistened with washing buffer containing protease inhibitor to hinder the enzymatic activity of some enzymes in the dust samples. 4x4cm area from the face and 8x8cm from the chest were gently rubbed with these cotton pads and put into 50ml falcon tubes with 20ml washing buffer and 0.05% NaN_3 and stored at -20°C till further use. The chest sample collected from cat NC1 is presented in figure 8A. For the collection of dust from the sleeping places, Dustream® Collector from Indoor Biotechnologies was used. The components of the kit are shown in figure 8B. For the usage, a filter and adaptor are stuck together and put on the vacuum cleaner. The most favourite places of the cats were vacuum-cleaned till the filter was at least one quarter full. After dissembling the adaptor the filter was opened and put in a 50ml falcon tube including 20ml washing buffer. When for the collection of saliva samples a dry Q-tip was gently stuck into

the cats' mouth cats started immediately licking it. The cotton-covered end was cut and put in a 20ml falcon tube with 5ml washing buffer, including NaN_3. In the case of longer storage, all samples were frozen at -20°C.

A  B 

Figure 8: A) Self-made picture from chest sample collection by NC1. B) Components of Dustream Collector Kit. **[42]**

Step 2 – Lyophilisation

After collection samples were filtered into 50ml falcon tubes to purify them from bigger dust particles (Fig. 9A). For lyophilisation the top of frozen tubes was perforated with a needle, and samples subjected lyophilissation overnight (ON) (Fig. 9B).

A  B 

Figure 9: A) sample filtration with paper filter and cone into a falcon tube; B) Lyophilisator (CHRIST Alpha 2-4 LSC).

Step 3: Dialysis

To further clean the samples and exchange the buffer, lyophilised samples were dissolved in 1ml distilled water and filled into perforated Eppendorf tubes, covered with a dialysis membrane (10K MWCO, molecular weight cutoffs, Thermo Fischer) and fixed into a Styrofoam-plate. Tubes were placed bottom-up in distilled water and dialyzed at 4°C, ON under constant agitation on a rocker table and finally transferred into new 1.5ml Eppendorf tubes. Perforated Eppendorf tubes without and with dialysis membrane are pictured in figure 10 A and B.

A  B 

Figure 10: A) Perforated Eppendorf tubes without and B) with dialysis membrane (self-made).

3.5.2. BCA Protein Assay Kit

Principle of the test

The protein contents in the samples were determined to compare between the total protein and the specific allergen. The Pierce BCA Protein Assay is based on a detergent-compatible formulation of BiCinchoninic Acid (BCA), which is used in colorimetric detection. In this assay the of Cu^{+2} to Cu^{+1} by protein in alkaline medium is combined with the highly sensitive and selective colorimetric detection of the cuprous cation, using Reagent A, containing bicinchoninic acid. As a result is a purple coloured reaction occurs due to the chelation of two molecules of BCA with one cuprous ion detectable at an absorbance at 590nm. A colouration occurs only when four particular amino acids are present in the samples: cysteine, cystine, tryotophan or tyrosine. The macromolecular structure of the protein and the number of peptide bonds contribute to the colouration process in BCA.

Process

To determine the protein concentration collected samples were diluted 1:2 and 1:5 in distilled water, to a final volume of 15µl/well in 96-well ELISA plates. For calculation of a reference, curve (standard) BSA (bovine serum albumin) was diluted to the following concentrations: 2000µg/ml, 1500µg/ml, 1000µg/ml, 750µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 50µg/ml, 25µg/ml. The reagents A and B from BCA-Kit were diluted 50:1 (in final 200µl/well) and were added to the samples and control wells. As a negative control, wells were left empty (blank) and were incubated just with reagents A and B. The plates were incubated at 37°C for 30 minutes. Then plates were cooled down to room temperature and read in ELISA Reader at 562nm. All samples and controls were set up in duplicates, and the mean value calculated. Dependent on the protein amount in sample, the colouration turns from green (low protein amount) to purple (higher content). An example of a final 96-well ELISA plate with detected and colourised samples is shown below in figure 11.

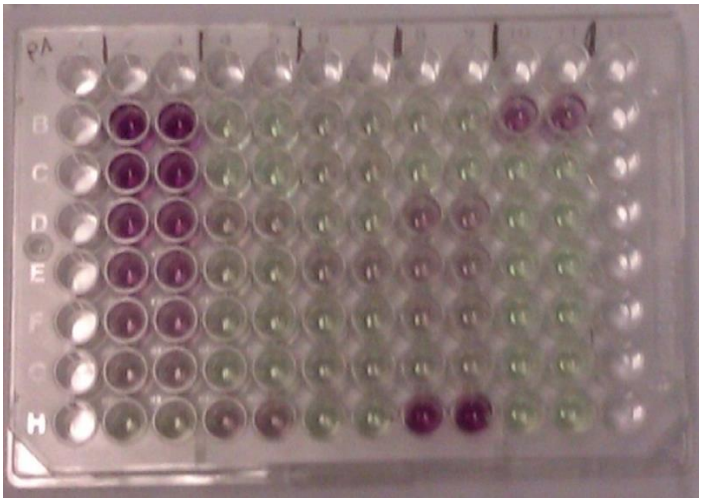


Figure 11: Determination of the protein concentration with BSA standard by BCA Kit on a 96-well ELISA plate (self-made).

3.5.3. Enzyme-Linked ImmunoSorbent Assay (ELISA)

Principle of the test

The enzyme-linked immunosorbent assay is an enzymatic assay, which is based on an antibody-mediated detection of an antigen made visible by enzymatic colouring reaction. The ELISA method is applied to detect different proteins, viruses, hormones or toxins in samples. First a target molecule is immobilised on an absorbing 96-well flat-bottom plate, followed by incubation with a specific antibody, which may detect the absorbed antigen, but which is still invisible. For the detection of this antibody, subclass specific anti-antibodies linked to an enzyme are added. When a specific substrate is added, colouration occurs. In this study the

anti-antibodies where attached to biotin, which binds to streptavidin or avidin with an extremely high affinity, our specific substrate, and makes the protein of interest visible. The reaction can be measured at specific emission/extinction wavelength by a spectrophotometer usually called ELISA reader. This technique is routinely applied in medical diagnostes and plant pathology, and is also intensively used in quality control in the industry (Fig. 12).

Another type of ELISA called catching ELISA is slightly different from the above descibed method. In this case catching antibody, specific to the antigen/allergen will be coated, followed by the incubation of the sample of interest. For detection, specific and chemically marked antibody will be used. In my diploma thesis we used this type of ELISA for Fel d 1 detection.

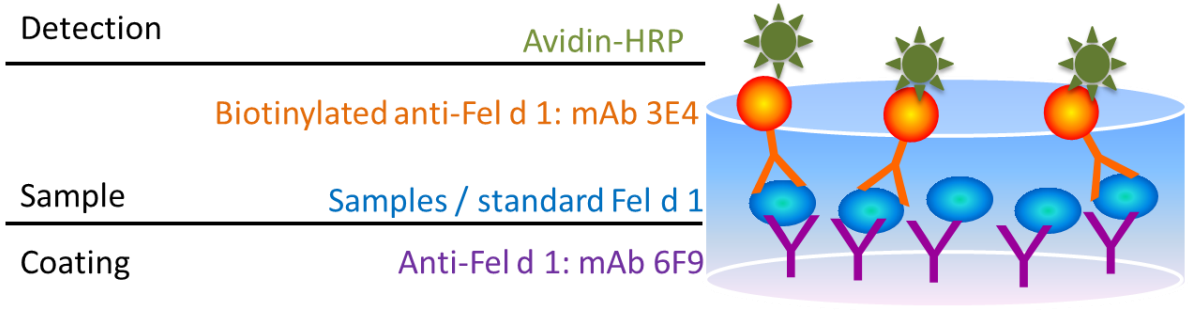


Figure 12: Scheme of the Fel d 1 catching ELISA (self-made).

Process

To measure the cat-specific allergen Fel d 1 concentration in the different cat samples, the catching monoclonal anti-Fel d 1 antibody (clone: 6F9) was diluted 1:1000 in coating buffer and incubated 100µl/well ON at 4°C in 96-well flat-bottom plates. Wells were washed 3 times (200µl/well) with 0.05% PBST. After blocking for 30 minutes with 100µl/well 1% BSA-PBST, samples and the standard were diluted in 1% BSA-PBST: face samples 1:10, chest samples and Dustream samples 1:2000, saliva samples were not diluted. After sample incubation for 1h at room temperature (RT), wells were repeatedly washed (200µl/well) with 0.05% PBST. For detection, biotinylated monoclonal Fel d 1-specific antibody (clone: 3E4) was used 1:1000 in 1% BSA-PBST and incubated 100µl/well for 60 minutes at RT. Wells were washed again and streptavidin HRP was added 1:250 in 1% BSA-PBST (100µl/well) for 30 minutes at RT. After final washing, specific binding was developed with ABTS solution. The reaction was detected in an ELISA microplate reader (Tecan Infinite M200) at an optical density of 405nm and at 490nm. (Fig. 13A and B)

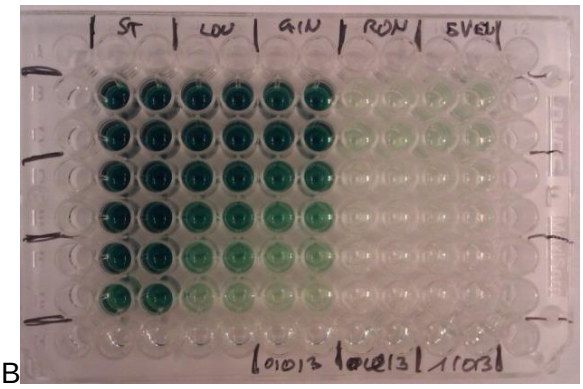
A  B 

Figure 13: A) The Tecan Infinite M200 ELISA Reader working station. B) 96-well flat-bottom plate with detected Fel d 1 samples next to the standard (self-made).

3.5.4. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Principle of the test

Sodium **d**odecyl **s**ulphate **p**oly**a**crylamide **g**el **e**lectrophoresis (SDS-PAGE) is a method to separate proteins according to their mass (kilo Dalton - kDa) and compare them with standard proteins (marker), w.g. PageRuler™ Plus Prestained Protein Ladder (Thermo Fischer Scientific) (Fig. 14). Acrylamide gels are prepared which act as protein mashes due to polymerisation of acrylamide monomers, initiated by APS (ammonium persulphate) and TEMED (tetramethylethylendiamin). By charging the protein negatively with SDS (sodiumdodecylsulfate; 1.4g SDS/g protein;) electrophoretic mobility in the gel independent of molecular charges is guaranteed. Also the protein to be separated is incubated in a buffer with the detergent SDS to confer negative charges to it. Hence, the negatively loaded protein will migrate to the positive pole during the electrophoresis in the acrylamide gel (Fig. 15).

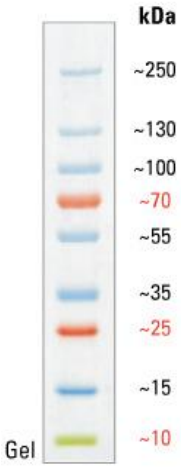


Figure 14: SDS-PAGE band profile of PageRuler™ Plus (Thermo Fischer Scientific) **[43]**

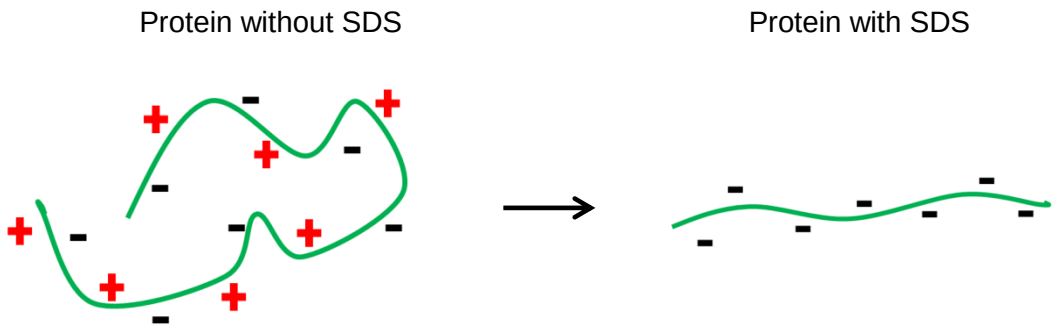


Figure 15: The effect of SDS on protein structure. When a protein is incubated with SDS, SDS charges the protein negatively (self-made).

Bisulphite bond fix secondary structures, but can be reduced by thiol compounds. Based on their molecular mass, the new charged proteins run from cathode to anode. In this context the gel functions as a filter mash, where smaller proteins pass through faster than bigger ones. SDS-PAGE can be used for analytical of preparative samples. By an analytical gel, many different samples can be applied at the same time to compare the protein contents and compositions. In a preparative gel, a bigger amount of one sample is loaded, mostly for transfer to a membrane or further analysis.

Process

For separation of Fel d 1 allergen in the collected samples, a 15% SDS polyacrylamide gel was prepared using the following ingredients, as listed in Table 5:

Ingredient	1 separation gel 15%	2 stacking gels
Distilled water	3.65ml	2.7ml
Running buffer	2.5ml	1.25ml
10% SDS	100µl	50µl
40% Acrylamid	3.75ml	1ml
10% APS	50µl	25µl
TEMED	10µl	10µl

Table 5: Recipe for a 15% SDS-PAGE gel.

First the separation gel was poured between two glass plates. When completely polymerized a stacking gel was poured on top (Fig. 16). Depending on the purpose of an analytical or preparative gel, an analytical or preparative comb was immediately placed into the stacking gel, forming either 10 or one big chamber. After polymerization the gel between the glass plates was inserted into the electrophoresis gadget. The protein samples of interest were mixed with the reducing or non-reducing loading buffer in the ratio of 1:4 (5µl buffer + 15µl sample). For the reducing and also non-reducing condition, samples in the reducing/non-reducing buffer were heated for 5 minutes at 95°C. Prepared samples and the not heated marker (PageRuler™Plus Prestained Protein Ladder, Thermo Scientific, Fig. 17 A, B, C) were loaded into the polyacrylamide gel pockets and subjected electrophoresis at 200V and 60mA in electrophoresis buffer till the protein front arrived 5mm before the lower end of the glass plates (Fig. 18).

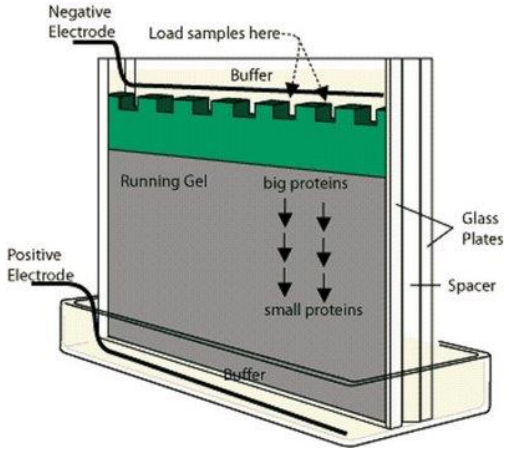


Figure 16: Principle of an SDS-PAGE gel apparatus **[44]**.

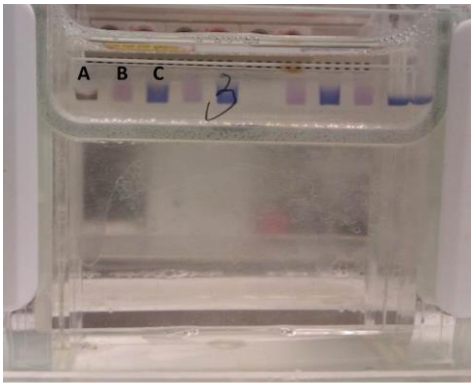


Figure 17: Gel Elektrophoresis A) marker; B) sample with reducing buffer; C) sample with non-reducing buffer front view (self-made).

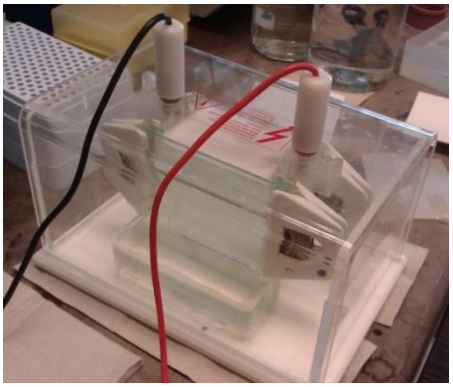


Figure 18: Gel Elektrophoresis apparatus filled with gels between glass plates and electrophoresis buffer, top view (self-made).

3.5.5. Silver staining

Principle of the test

Silver staining is a widely used technique to detect the appearance of proteins in polyacrylamide gels. It is based on the reduction of silver nitrate when subjected to a reducing agent, which causes formation to black elementary silver.

Process

SDS-PAGE gels with separated cat protein samples fixed with fixing solution for at least 60min and washed 3 times for 20 minutes in washing solution under gently agitation at RT. Gels were incubated with thiosulfate reagent for 1 minute and washed 3 times for 20 seconds with distilled water. After washing the gels, they were incubated with silver nitrate reagent for 20 minutes at RT under gently agitation and washed again for 5 seconds with distilled water. Finally the gels were developed with developer solution for an appropriate time (5-15 minutes) and blocked with stop solution. Note: All procedure carried out with gloves to avoid contamination with exogenous proteins.

3.5.6. Western blot

Principle of the test

The Western blot, also called immunoblot is an analytical method to identify specific proteins of interest within a mixed protein sample containing different proteins. On the other hand, also serum antibody reactivity can be determined by the method (serology). For this, the samples first have to be separated in an SDS-PAGE. Protein transfer from the gel onto a nitrocellulose membrane can be achieved in an electrical files (electrophoresis method). Proteins transferred from the gel are then covalently bound to a solid phase, such as a nitrocellulose membrane. Proteins immobilized on such a membrane can then be incubated and detected by specific antibodies.

Transfer - Process

The Western-blot-sandwich was prepared with a grill gadget: i) a sponge was moistened well with distilled water and placed on the first grill-plate; ii) two layers of filter paper were wetted and laid on the sponge; iii) the nitrocellulose membrane was placed next, followed by iv) the SDS-PAGE-gel with the separated proteins. Finally, on the gel, the same layers were placed again before closing the grill-gadget. Note: Any air-bubbles must be carefully avoided and gloves worn during the whole process. The complete gadget is then placed into a blot-chamber filled with transfer-buffer and placed in a bowl filled with ice. The transfer is then carried out 200mA for 100 minutes. The gadget composition is illustrated in Figure 19.

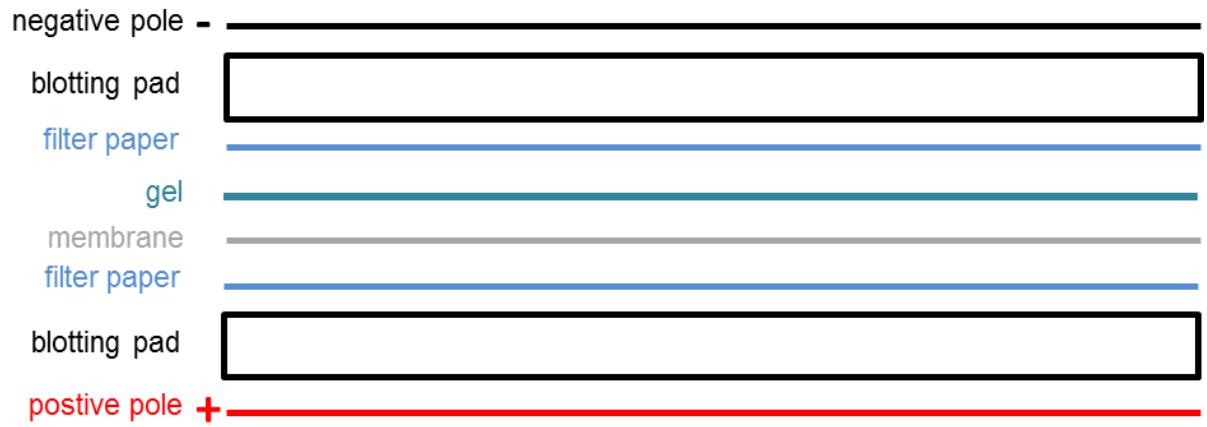


Figure 19: Scheme illustrating the layers of the Wet-Blot sandwich (self-made).

Indian Ink - Staining

Principle of the test

It is important to evaluate the whole protein contents transferred during electroblot to a nitrocellulose membrane. This coloration is based on the static attachment of colloidal particles to loaded amino acids of proteins. Indian Ink is a colloid of dispersers carbon in aqueous solution.

Procedure

One strip of the immunoblot membrane is cut and stained with Indian Ink to visualize the transferred proteins on the membrane. The strip is then washed 3 times for 10 minutes in BSA-PBST blocking buffer with gently agitation and incubated with Indian Ink for 30 minutes at RT. Finally, the strips with colored bands are scanned for archivation.

Immunoblot with I^{125}-labeled anti-human IgE antibody

Procedure

The nitrocellulose membrane was cut into strips, which were first blocked with blocking buffer for 30 minutes at room temperature. After washing with Gold buffer 3 times for 10 minutes, incubation with diluted (1:10 in Gold-buffer) cat allergic and control patients sera followed, 1ml/strips for ON at 4°C. On the next day, sera were recovered and strips were washed with Gold buffer as before. For detection, strips were incubated with I^{125}-labeled anti-human IgE antibody (1ml/strip, diluted 1:10 in Gold buffer) and incubated ON at RT.

On the third day, the radioactive labeled antibody was discharged carefully into tanks for radioactive waste and all stripes were intensively washed. The strips were dried, wrapped in saran wrap and X-ray film (GE HealthCare Life Sciences) was marked and placed in the developing cassette. For detection, cassette was stored at -80°C 3 and 7days and development was carried out with CP 1000 (Agfa-Gevaert). All procedures were carried out with gloves in a special radioactive room. All used materials, such as pipettes etc. were measured with a hald-held Geiger scanner to monitor contamination.

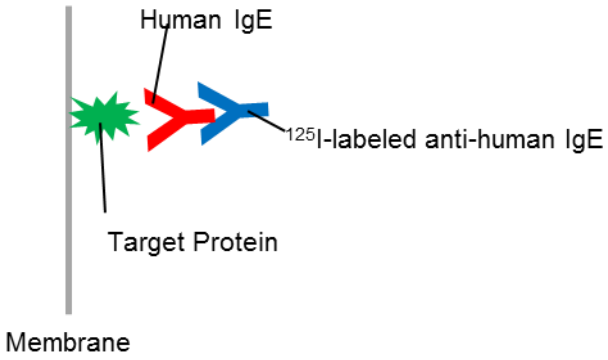


Figure 20: Schematic representation of the principle of an anti IgE-immunoblot detection (self-made).

Immunoblot with biotinylated anti - Fel d 1 antibody

Procedure

Nitrocellulose strips were first blocked with blocking buffer for 120 minutes at room temperature and washed with Gold buffer 3 times for 10 minutes afterwards. After washing, strips were incubated with diluted (1:1000 in Gold buffer) biotinylated monoclonal antibody 3E4 for ON at 4°C. Next day, antibody was discarded and strips thoroughly washed with Gold buffer 3 times for 20 minutes, the strips were then incubated with diluted Streptavidin-HRP (1:1000 in Gold buffer) for 60 minutes at room temperature. Streptavidin-HRP was

discarded and strips washed with Gold buffer as before. For development, strips were marked with a pencil, and developed with chemiluminescent substrate solution (1:1). Strips were then fixed on paper and placed in the developing cassette onto a film (Amersham Hyperfilm ECL, GE Healtcare) for 5 seconds. The development was carried out with CP 1000 (Agfa-Gevaert).

4. RESULTS

4.1. Determination of total protein concentration

During sample collection, standardization was attempted by taking samples from same square areas of the animals as described in the M&M section. Hence, the whole protein content in an area might be different and typical for normal or hypoallergenic cats (“non-adjusted” samples) and reveal interesting information. When total protein levels were determined by BCA Protein Assay in collected samples from normal (NC) and hypoallergenic (HC) cats, there was no significant difference between normal and hypoallergenic cats in face, chest and saliva samples (Fig. 21). In the face samples the proteins ranged between 261-499µg/ml in normal and 112-579µg/ml in hypoallergenic cats. In contrast, the chest samples of both groups showed less proteins with 26-300µg/ml. The saliva samples had a similar protein content with a stable mean value of 259µg/ml in NC and 245µg/ml in HC. The highest variability was detected in the dust samples, from 403µg/ml to 7773µg/ml (Fig. 21).

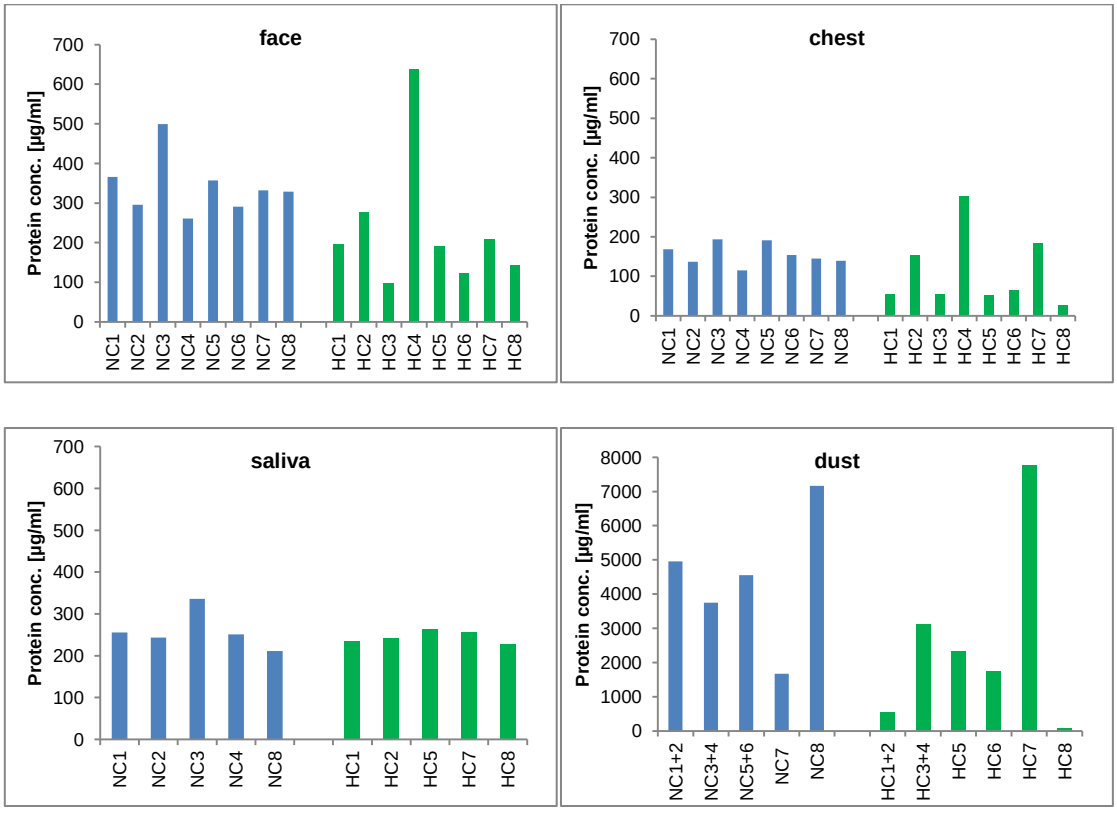


Figure 21: ELISA: Protein levels in face, chest, saliva of normal and hypoallergenic cats, and from dust samples of their households.

4.2. Evaluation of Fel d 1 concentration

Even though the total protein amounts appeared to be comparable in standardized areas from normal and hypoallergenic cats, still, single compounds might differ. Thus the next aim was to measure Fel d 1 in “non-protein adjusted” face and chest samples using a Fel d 1-specific ELISA Kit. In these non-adjusted samples, the 8 hypoallergenic cats showed significantly less Fel d 1 in face samples compared to normal cats, and this difference was even more pronounced in the chest samples (Fig. 22 A, B). After adjusting the samples to the same protein concentration, no differences could be detected any more indicating the similar Fel d 1 concentrations (Fig. 22 C, D) on the face and chest area.

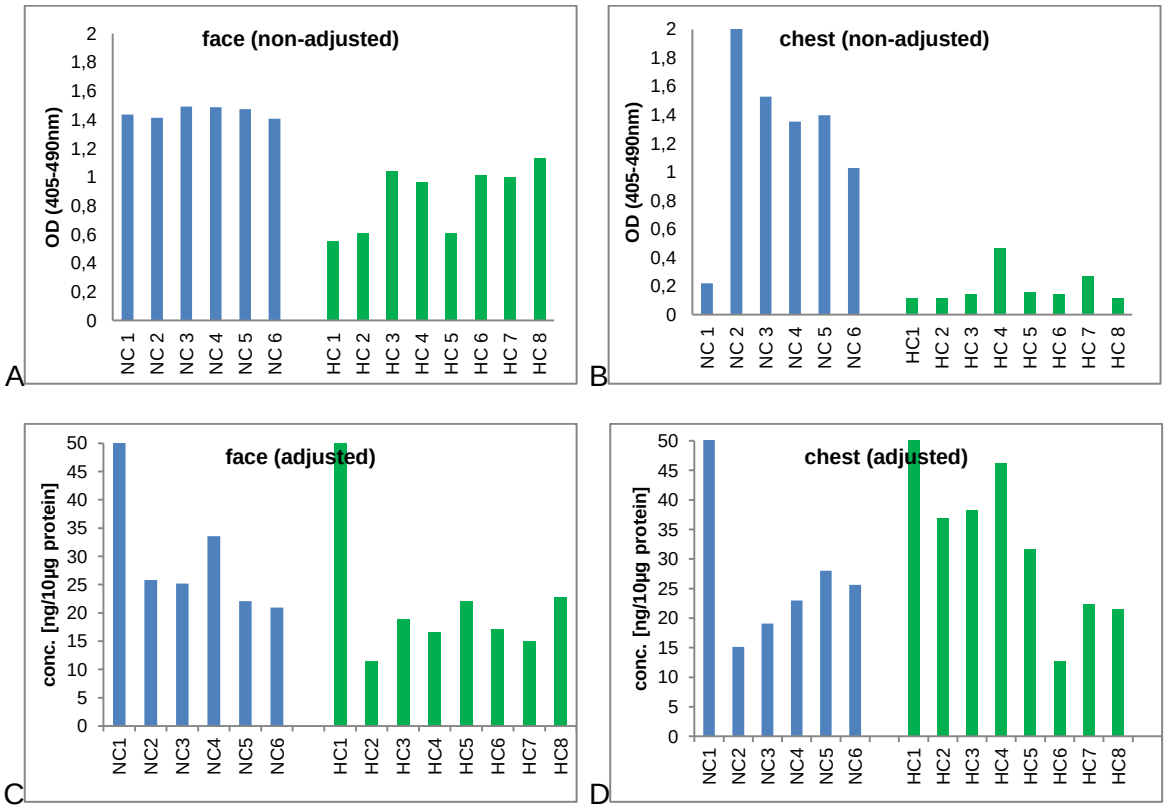


Figure 22: Fel d 1 levels in A) non-adjusted face, B) non-adjusted chest, C) adjusted face, D) adjusted chest;

For Fel d 1 detection in the saliva and dust samples we used the samples without adjusting the protein concentration were tested. The saliva samples of HC cats showed significantly lower Fel d 1 compared to the NC group (Fig. 23A). In the dust samples, in the case of normal cats, the concentration was higher, while in the hypoallergenic cats, two of the cats show very low level of Fel d 1, but and four others show more intense signal (Fig. 23B).

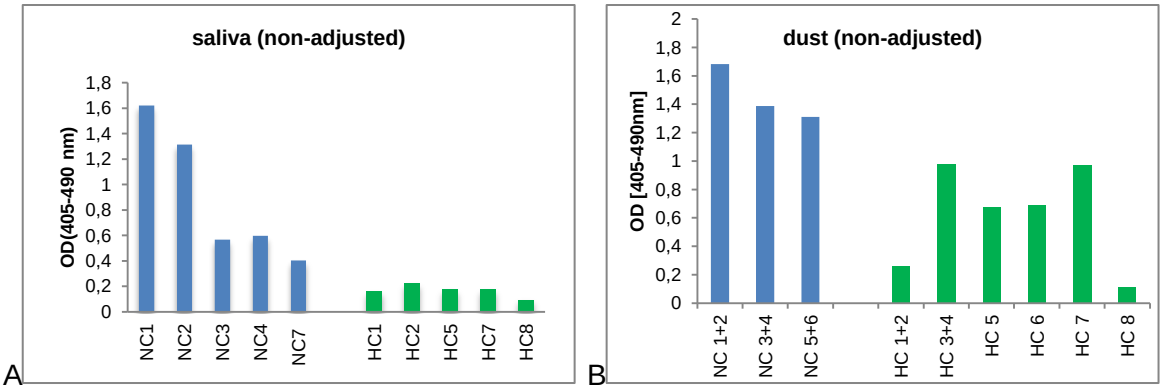


Figure 23: Detected Fel d 1 levels in A) saliva, B) dust samples.

4.3. Detection of protein bands on SDS-PAGE

To analyse in more detail the allergen and protein composition of the samples, normal and hypoallergenic face and chest samples were pooled and separated by 15% SDS-PAGE under reducing (+) and non-reducing (-) conditions, and subjected silver staining (Fig. 24A, B). Based on the composition of the natural Fel d 1 in non-reduced conditions the quaternary complex formed of two heterodimers should be seen at 35kDa and the single heterodimer at 18kDa. Upon denaturation by cooking and reduction the heterodimer should be split into a 4kDa and 14kDa chain 1 and 2.

However, as whole cat extracts was analysed the resulting picture was to be more complex. Indeed the reduced samples of normal cats showed protein bands above ~70kDa, at ~30-20kDa, ~15kDa and ~10kDa, and weaker bands at ~55kDa, ~35kDa, 14~kDa, ~11kDa. In the non-reduced samples of normal cats intense bands could be observed at ~70kDa, but also at ~55kDa, ~35kDa, ~30-20kDa and 18-15kDa.

In the hypoallergenic cat samples the reduction resulted in intense signals at above ~70kDa, at ~25-20kDa, ~18-15kDa and ~10kDa and weaker at ~20kDa and ~13kDa. It can clearly be seen that the 18kDa protein (putative Fel d 1 heterodimer) is reduced to chain 1 of 4kDa and chain 2 at 14kDa upon reduction. The non-reduced samples of hypoallergenic cats showed strong signals at ~55kDa and ~25-20kDa and weaker bands at ~70kDa, ~20kDa and ~13kDa. Taken together, in the HC samples the 35kDa band (putative Fel d 1 quaternary allergen) and lower molecular weight cat proteins were expressed to a less amount, in favour of higher molecular weight allergens within the total protein load.

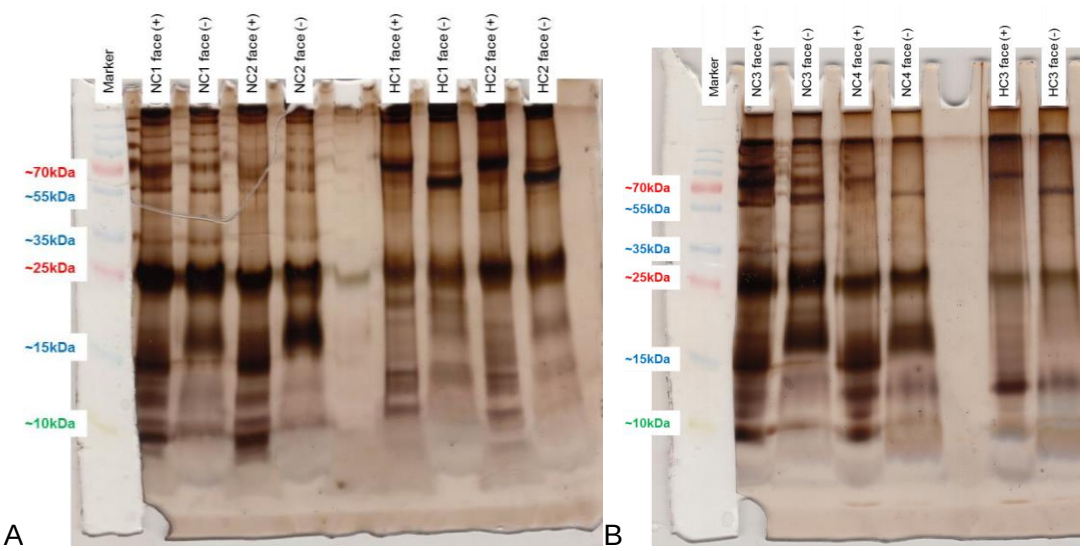


Figure 24: SDS-PAGE colored with Silver Stain with A) single cat samples NC1, NC2, HC1, HC2, B) single cat samples NC3, NC4, HC3 with reducing (+) and non-reducing (-) buffer.

For improved analysis the proteins starting at a protein concentration of 150µg/ml were serially diluted with distilled water 1:2, 1:5, 1:10, 1:50, 1:100, 1:200 and 1:500 (Fig. 29A, B). The results verified the results above in both cases, in hypoallergenic cats and normal cats. Representive examples are presented in figure 25.

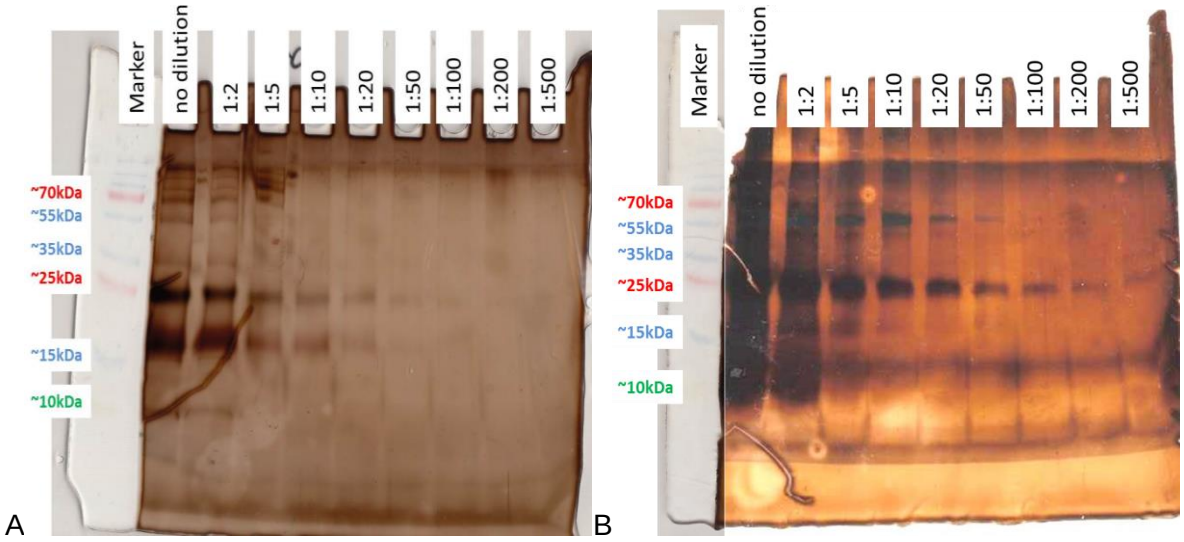


Figure 25: Serial dilution of non-reduced cat protein samples separated by 15% SDS-PAGE silver stained A) normal cat (NC2), B) hypoallergenic cat (HC2).

4.4. Identification of IgE binding proteins

To identify the IgE binding proteins/allergens in samples from normal (NC) versus hypoallergenic cats (HC), pooled samples of face were adjusted to the same protein concentration of 150µg/ml. Separated on SDS-PAGE under reducing (+) and also under non-reducing (-) conditions and transferred to a nitrocellulose membrane. For allergen-specific IgE detection, serum pool of cat allergic patient's sera was added and non-allergic patient's sera were used as control. When loading the same total protein concentration in samples NC and HC, IgE binding was detectable just under non-reducing, but not under denaturing and reducing conditions (Fig. 26). A strong single band was visible at 18kDa and a weaker band around 35kDa in NC and HC and a weaker also single band around 35kDa just in NC. The non-allergic patient's sera, as well as the buffer control, did not show any binding activity.

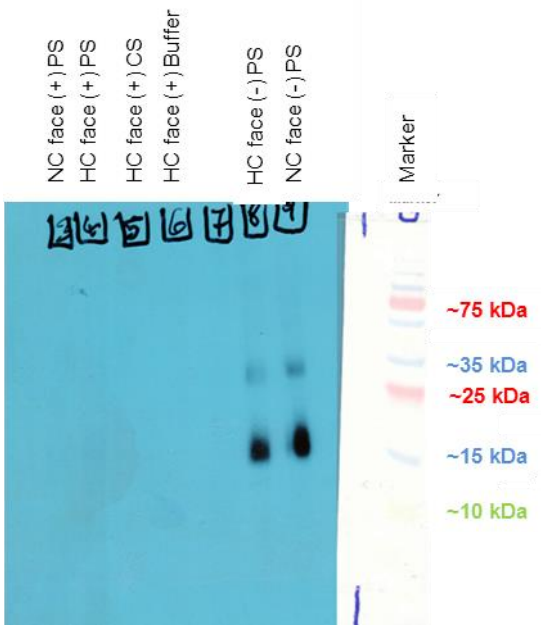


Figure 26: Fel d 1 detection in pooled face samples with pooled cat allergic patients sera.

Based on these IgE immunoblot results the consecutive blots were run under non-reducing conditions and using single cat allergic patients' sera. The results presented in Figure 27 again revealed two IgE-binding bands at 18 and 35kDa. IgE binding activity was higher in samples of face than chest at same protein concentration. Moreover, the bands on hypoallergenic samples seemed to be slightly less intense than in normal cat samples when testing with single patient sera, although same protein amounts were loaded.

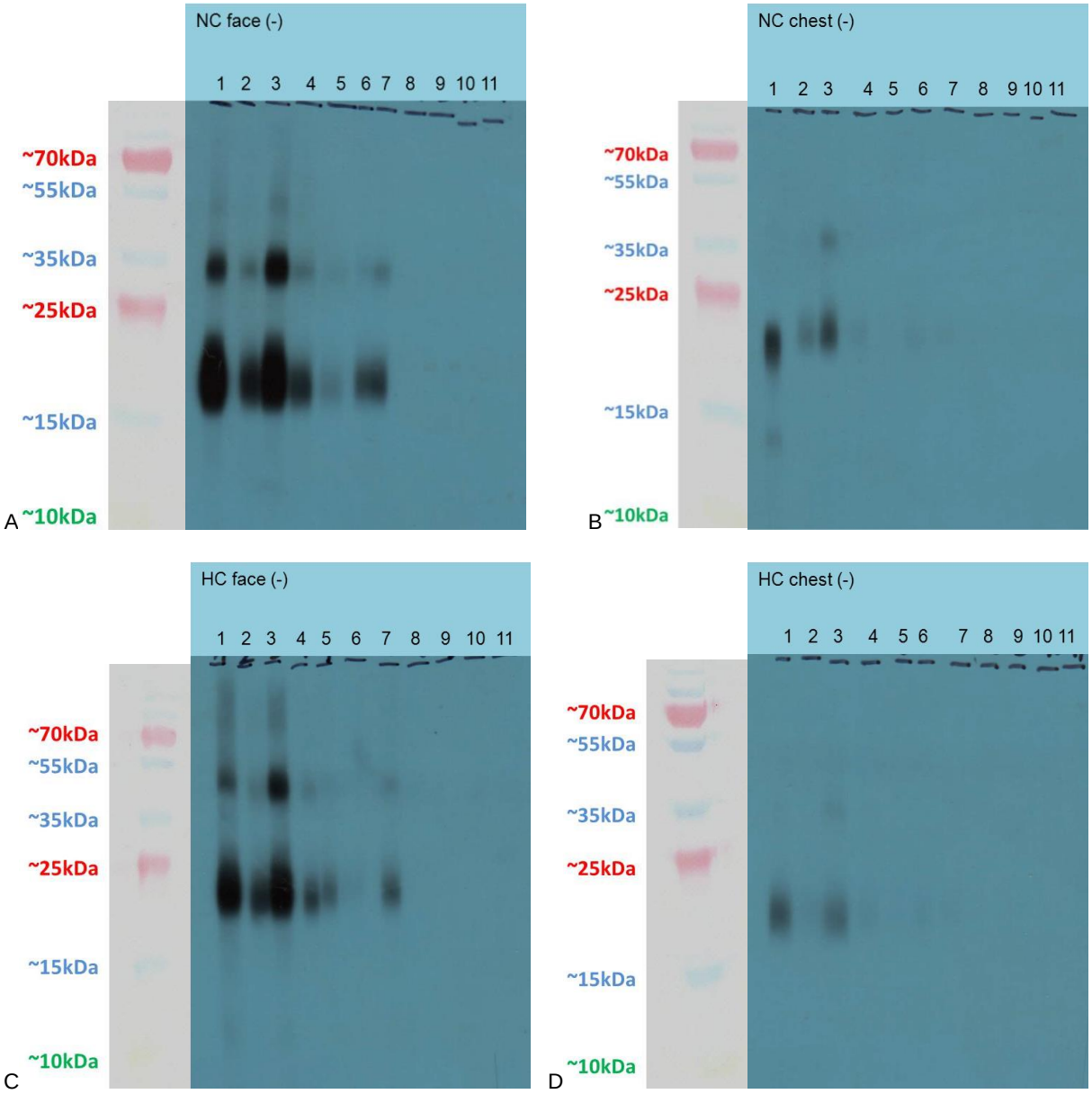


Figure 27: Immunoblot of pooled non-reduced samples from normal (NC) or hypoallergenic cats (HC) tested for IgE binding with single patient's sera A) NC face B) NC chest C) HC face and D) HC chest.

Vice versae we aimed to determine the IgE-binding to samples from individual cats. Therefore, single cat samples were immunoblotted and incubated with pooled sera of allergic patients. In 6/6 tested extracts IgE-binding reactivity to predominant bands at 18kDa and 5/6 to a 35kDa band were seen when testing extracts from normal cats (NC). Testing hypoallergenic cats (HC) extracts, in 6/6 cases IgE binding to a 18kDa allergen could be seen, but only in 3/8 cases to the 35kDa allergen (Fig. 27 A,B).

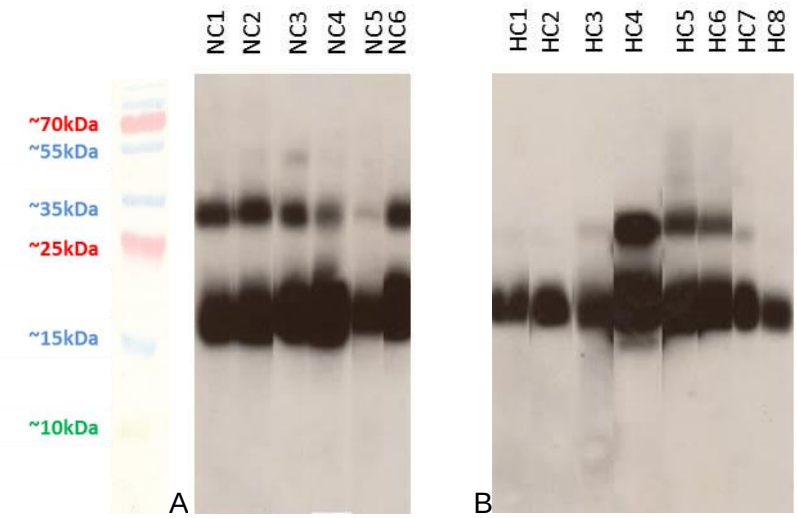


Figure 28: IgE-immunoblot with pooled patient's sera and non-reduced, but protein adjusted single cat samples of A) normal and B) hypoallergenic cats.

To verify the identity of the IgE binding bands, the immunoblot experiment was carried out with the anti-Fel d 1 monoclonal antibody 3E4. Face samples of single cats (NC2 and HC2) were adjusted to the same protein concentration separated on SDS-PAGE and transferred to a nitrocellulose membrane. The blot strips were incubated with biotinylated monoclonal antibody 3E4, followed by both bands at ~18kDa and ~35kDa in the selected normal and hypoallergenic cats represent Fel d 1 molecules, possibly representing the single heterodimer and the complete quaternary form. Further, in the selected hypoallergic cat HC2 showed only a 35kDa band, likely a single heterodimeric unit (Fig. 29). The dilution experiment indicated that the 35kDa band appears at higher protein concentrations only. This again proved that Fel d 1 is expressed at less concentration in the investigated cat breed Neva Masquarade.

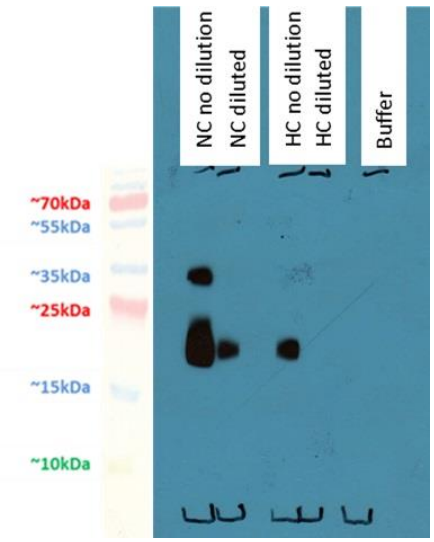


Figure 29: Detection of Fel d 1 in blotted face samples of hypoallergenic (HC2) and normal (NC2) cats with monoclonal anti – Fel d 1 antibody.

5. DISCUSSION

Cat allergens are among the major inducers of symptoms like rhinitis, conjunctivitis and asthma in allergic patients. However cats are also very important pets and family members in many households around the world. In settings where pet owners are at allergic the allergic symptoms also mental stress arises. Since till now, no satisfying therapy is available for cat allergic patients. The only solution is to despair cats from the homes which represents an emotional problem. Hypoallergenic cat breed may open up a new perspective for these patients.

All owners of hypoallergenic cats participating in this diploma study reported that none of their cat allergic relatives shows symptoms when exposed to the cats in their home even during long time. Cat breeder Markus Kronberger reported 9 out of 10 potential cat buyers visiting his cat farm were interested in his cats due to their own or a family member's allergy and because they had heard about the "hypoallergenic" Neva Masquarade breed. He further reported that the feedback from his customers is very positive with respect to symptoms which could be avoided or at least much reduced with the Neva Masquarade cats.

There is a previous web-based report by Indoor Biotechnologies on the examination of Siberian cats for allergen contents. Based on the examination of 4 cats for the content of Fel d 1 major allergen they state that the Fel d 1 levels in Siberian samples are significant (200-2000µg/g/. they proposed that more studies are needed before being able to give clear-cut recommendations to cat allergic interested in hypoallergenic breeds. Therefore, the aim of this diploma thesis was to investigate in more detail the possible hypoallergenicity of the Siberian cat species, with special focus on the Siberian subtype Neva Masquarade.

Cats are sources at least of 8 different allergens, with Fel d 1 and Fel d 4 being the most important ones, called major allergens. In our study we focused on Fel d 1 and collected samples for our analyses of 8 domestic ("normal") and 8 hypoallergenic (Neva Masquarade) cats. We used for the determination sampling devices and immune detection kits by Indoor Biotechnologies.

For the sample collection, we classified four potential allergen sources: face, chest, saliva and dust from the favourite places of the cats in their homes. In the analysed dust samples similar levels of Fel d 1 were detected, possibly due to the fact that in all cases Neva Masquarade cats were held together with normal cats, sharing also the sleeping places. When the face, chest and saliva samples were analysed for their total protein contents, they showed slightly lower protein concentrations in hypoallergenic than normal cats (Fig 21.). Accordingly, Neva Masquarade cats secrete and distribute also less Fel d 1 via their saliva

onto their fur coat than normal cats (Fig. 22 A, B). Therefore, the Neva Masquarade cas might emit less allergen in their environment than normal cats and thus may be classified as “hypoallergenic”.

For a more thorough analysis single cat samples were separated by SDS-PAGE and silver-stained. Further, samples were tested und reduced and non-reduced conditions. Reduction is a method to destroy intramolecular and interchain disulphide bridges and thus to separate and linearize peptides or proteins. Often, conformational epitopes are thereby destroyed and IgE binding may be lost. The Fel d 1 heterodimer is held together by 3 disulphide bridges. The silver-stained SDS-PAGE analysis indicated that reduction of normal cat samples resulted in diminishment of a 18kDa band in favour of 4 and 14kDa peptides, putative chain 1 and 2 of the heterodimer Fel d 1. The protein pattern in hypoallergenic cat extracts was different: The 35kDa band and lower molecular weight bands were stained much less. Instead, the >70kDa protein was expressed more intense than in normal cats. The 25kDa bands showed approximately the same intensity in both cat types.

Denaturation by reduction and cooking destroyed the IgE-epitopes completely on blotted extracts from normal and hypoallergenic cats. The immunoblot and non-reduced extract from normal cats exhibited IgE-binding to 18 and 35kDa bands that by use of a monoclonal anti-Fel d 1 antibody could be identified as Fel d 1. Apparently, hypoallergenic cat extracts contained less of the 35kDa entity and showed IgE-binding mostly to the 18kDa allergen only. We hypothesized that the quaternary form might assemble at higher concentrations, In fact, dilution experiments indicated that also in normal cat samples the complete 35kDa Fel d 1 complex disappeared in higher dilutions.

Principally extracts from cats may contain single heterodimeric or a double heterodimeric allergen (quaternary state). The assembly of molecules to symmetric complexes facilitates the crosslinking IgE when bound to FceRI on mast cells and other effector cells (Schöll. J. Immuik. 2004). Hence the Neva Masquarade by secreting less protein and less Fel d 1 allergen, may favour the single heterodimeric form of the major cat allergen Fel d 1 (Fig. 24) with likely lower crosslinking-capacity and this lower allergenicity.

Out data propose that the cat breed Neva Masquarade produces less Fel d 1 allergen in their saliva, distributes less allergen on their fur and may thus be termed “hypoallergenic”. Taken together, our study gives an optimistic view for cat allergic and especially asthmatic patients and is worth continuing.

Limitation of the study

Clearly, more individual cats should be investigated, as well as their age and sex status include, to improve the significance of our observations. Even though Fel d 1 is a highly important asthma marker, also cat allergens other than Fel d 1 should be considered when investigating hypoallergenicity of cats. It will be also of interest to evaluate whether the single heterodimeric Fel d 1 form indeed prevents IgE crosslinking in cat allergic patients. However, this study provides the solid base for a systematic approach.

6. ABBREVATION

%	percent
(-)	non-reducing
(+)	reducing
(w/v)	weight per volume
°C	degree Celsius
µl	microliter
ABTS	2,2‘-azino-bis3-ethylbenzthiazoline-6-sulphonic acid
ADCC	antibody-dependent cell-mediated cytotoxitiy
APC	antigen presenting cells
APS	Ammonium Persulfate
BCA	bicinchoninic acid
BSA	bovine serum albumin
CC16	Clara Cell 16
CD 4+	cluster of differentiation 4+ T cells
CDC	complement-dependent cytotoxicity
cDNA	complementary deosyribonucleic acid
cm	centimetre
conc.	Concentration
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
FcγRIIIa	type III gamma receptor a
FcεRI	type I Fc epsilon receptor
Fel d 1	Felis domesticus 1 (allergen)
Fig.	figure

g	gram
HC	hypoallergenic cat
HRP	horseradish peroxidase
I^{125}	Iodine-125
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
ISAAC	International Study of Asthma and Allergies in Childhood
ISAC	Immuno Solid-phase Allergen Chip
kDa	kilo Dalton
m	meters
mA	milliampere
mAb	monoclonal antibody
mg	milligram
MHC II	major histocompatibility complex 2
Min	minutes
MWCO	molecular weight cutoffs
ml	milliliters
NC	normal cat
ng	nanogram
nm	nanometres
ON	overnight
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween-20

pH	power of hydrogen
RT	room temperature
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel elektrophoresis
sec	seconds
T cell	thymus cell
TEMED	N,N,N',N'-Tetramethylethylendiamin
TH cell	thymus helper cell
V	volt
WCF	World Cat Federation

7. REFERENCES

[1]. **Benedikt, H.** 100 Jahre Allergie: Clemens von Pirquet - sein Allergiebegriff und das ihm zugrunde liegende Krankheitsverständnis. Teil 1: Leben und Werk. *Wiener Klinische Wochenschrift - The Middle European Journal of Medicine.* 2006, pp. 573-579.

[2]. **Ishizaka, K., Ishizaka, T. and Hornbrook, MM.** Physico-chemical properties of human reaginic antibody. IV. Presence of a unique immunoglobulin as a carrier of reaginic activity. *Journal of Immunology.* July 1966, pp. 75-85.

[3]. **Carlsten, C. and Melèn, E.** Air pollution, genetics, and allergy: an update. *Current opinion in allergy and clinical immunology.* October 2012, 5, pp. 455-60.

[4]. **Lee, SY., Chang, YS. and Cho, SH.** Allergic diseases and air pollution. *Asia Pacific allergy.* 2013, 3, pp. 145-154.

[5]. **Worm, M., Lee, HH. and Kostev, K.** Prevalence and treatment profile of patients with grass pollen and house dust mite allergy. *Journal der Deutschen Dermatologischen Gesellschaft.* July 2013, Vol. 11, 7, pp. 653-661.

[6]. **Maucher, J.** Einschätzung und Realität. *Österreichische Ärztezeitung.* 10 Juni 2006, pp. 56-57.

[7]. Stallergenes. [Online] [Cited: 1 Jänner 2014.] www.stallergenes.at/fileadmin/images/filiales/at/folder1/AllergieFakten_Bundeslaender.pdf.

[8]. **Longo, G., et al.** IgE-mediated food allergy in children. *Lancet.* 8 July 2013.

[9]. **Strachan, DP.** Hay fever, hygiene, and household size. *British medical journal.* 18 November 1989.

[10]. **C. Brooks, N. Pearce, J. Douwes.** The hygiene hypothesis in allergy and asthma: an update. *Current opinion in allergy and clinical immunology.* February 2013.

[11]. **Kim, KH. and SA. Jahan, E. Kabir.** A review on human health perspective of air pollution with respect to allergies and asthma. *Environment international.* 11 Juni 2013, pp. 41-52.

[12]. **Baek, JO., et al.** Analysis of the Prevalence of and Risk Factors for Atopic Dermatitis Using an ISAAC Questionnaire in 8,750 Korean Children. *International archives of allergy and immunology.* 27 Juni 2013, pp. 79-85.

[13]. **Augustin, M., et al.** Allergies in Germany -- prevalence and perception by the public. *Journal of the German Society of Dermatology.* 11 Juni 2013.

[14]. **Murphy, Kenneth P.** *Janeway's immunobiology.* United States of America : Garland Science, Taylor & Francis Group, 2012.

[15]. **Nauta, A. J., et al.** Mechanism of allergy and asthma. *European Journal of Pharmacology.* 2008, pp. 354-360.

[16]. **Grimbaldeston, M. A., et al.** Effector and potential immunoregulatory roles of mast cells in IgE-associated acquired immune responses. *Current Opinion in Immunology .* 2016, pp. 751-760.

[17]. **Holt, P. G. and Sly, P. D.** Th2 cytokines in the asthma late-phase response. *The Lancet.* 20-26 October 2007, Vol. 370, 9596, pp. 1396-1398.

[18]. **Bush, RK.** Indoor allergens, environmental avoidance, and allergic respiratory disease. *Allergy and asthma proceedings : the official journal of regional and state allergy societies.* November-Dezember 2008.

[19]. **Liccardi, G., et al.** Focus on cat allergen (Fel d 1): immunological and aerodynamic characteristics, modality of airway sensitization and avoidance strategies. *International archives of allergy and immunology.* September 2003.

[20]. **Kaiser, L., et al.** Production, crystallization and preliminary crystallographic study of the major cat allergen Fel d 1. *Acta crystallographica. Section D, Biological crystallography.* June 2003, Vol. 59, 6, pp. 1103-1105.

[21]. **Ohman, JL., Lowell, FC and Bloch, KJ.** Allergens of mammalian origin. |||. Properties of a major feline allergen. *Journal of immunology.* December 1974, Vol. 113, 6, pp. 1668-1677.

[22]. **Bienboire-Frosini, C., et al.** Variable Content of Fel d 1 Variants in House Dust and Cat Extracts May Have an Impact on Allergen Measurement. *Journal of Investigational Allergology & Clinical Immunology.* 2012, Vol. 22, 4, pp. 270-279.

[23]. **Duffort, OA., et al.** Studies on the biochemical structure of the major cat allergen Felis domesticus I. . *Molecular immunology.* April-May 1991, Vol. 28, pp. 301-309.

[24]. **Kristensen, AK., Schou, C. and Roepstorff, P.** Determination of isoforms, N-linked glycan structure and disulfide bond linkages of major cat allergen Fel d 1 by a mass spectrometric approach. August 1997, Vol. 378, 8, pp. 899-908.

[25]. **Grönlund, H., et al.** Formation of disulfide bonds and homodimers of the major cat allergen Fel d 1 equivalent to the natural allergen by expression in Escherichia coli. *The Journal of biological chemistry.* October 2003, Vol. 278, 41, pp. 40144-40151.

[26]. **Kaiser, L., et al.** RCSB PDB - Protein Data Bank. [Online] [Cited: 9 September 2013.] http://www.pdb.org/pdb/explore/explore.do?structureId=1PUO.

[27]. **Kaiser, L., et al.** Structural Characterization of the Tetrameric form of the Major Cat Allergen Fel d 1. *Journal of molecular biology.* 2007, Vol. 370, pp. 714-727.

[28]. Allergen Nomenclature IUIS Allergen Nomenclature Sub-Committee. [Online] [Cited: 29 August 2013.] http://www.allergen.org/viewallergen.php?aid=324.

[29]. **Hilger, C., et al.** Allergic cross-reactions between cat and pig serum albumin. Study at the protein and DNA levels. *European journal of allergy and clinical immunology.* February 1997, pp. 179-187.

[30]. **Ichikawa, K., et al.** Molecular cloning, expression and modelling of cat allergen, cystatin (Fel d 3), a cysteine protease inhibitor. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* August 2001.

[31]. **Smith, W., et al.** Fel d 4, a cat lipocalin allergen. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* November 2004.

[32]. Allergen Nomenclature IUIS Allergen Nomenclature Sub-Commitee. [Online] [Cited: 29 August 2013.] http://www.allergen.org/viewallergen.php?aid=320.

[33]. Wikipedia The Free Encyclopedia. [Online] [Cited: 29 August 2013.] http://en.wikipedia.org/wiki/Von_Ebner%27s_gland.

[34]. Allergen Nomenclature IUIS Allergen Nomenclature Sub-Committee. [Online] [Cited: 29 August 2013.] http://www.allergen.org/viewallergen.php?aid=323.

[35]. Allergen Nomenclature IUIS Allergen Nomenclature Sub-Committee. [Online] [Cited: 29 August 2013.] http://www.allergen.org/viewallergen.php?aid=665.

[36]. **Hof, W. van't, et al.** The Salivary Lipocalin Von Ebner's Gland Protein Is a Cysteine Proteinsase Inhibitor. *The Journal of biological chemistry.* 17 January 1997.

[37]. **Smith, W., et al.** Two newly identified cat allergens: the von Ebner gland protein Fel d 7 and the latherin-like protein Fel d 8. *International archives of allergy and immunology.* 17 May 2011.

[38]. Siestadream. [Online] 7 Jänner 2014. www.siestadream.com/aler.htm.

[39]. World Cat Federation. [Online] [Cited: 29 August 2013.] http://www.wcf-online.de/.

[40]. World Cat Federation - Hall of Fame. [Online] [Cited: 7 Jänner 2014.] http://www.wcf-awards.com/eingang2.htm.

[41]. Animal Farm Bäckerberg. [Online] [Cited: 29 August 2013.] http://www.bäckerberg.at/animal-farm.

[42]. Indoor Biotechnologies. [Online] [Cited: 29 August 2013.] http://www.inbio.co.kr/image/dustream_lg.jpg.

[43]. Thermo Scientific Pierce Protein Biology Products. [Online] [Cited: 29 August 2013.] http://www.piercenet.com/browse.cfm?fldID=717F6181-FACE-A3DF-CA2B-80430903A433.

[44]. Blogspot. [Online] [Cited: 29 August 2013.] http://2.bp.blogspot.com/_Tytr3wMN6t0/SKobCgG3GQI/AAAAAAAAAjw/UXcpZivHgk0/s400.

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1. Szalai K., **Satorina J.,** Lukschal A., Willensdorfer A., Mothes-Luksch N., Jensen-Jarolim E. Monitoring Fel d 1 concentration in normal *versus* hypoallergenic cat breeds.

Posters

1. Szalai K., **Satorina J.**, Willensdorfer A., Jensen-Jarolim E. Do hypoallergenic cats exist? – Fel d 1 concentration in normal versus hypoallergenic cats. Annual Meeting “Veterninärmedizinische Arbeitskreis” of the German Society for Immunology, Vienna, Austria, 5-6 April, 2013.

2. **Satorina J.,** Lukschal A., Willensdorfer A., Jensen-Jarolim E., Szalai K. Validation of hypoallergenic cat breeding by determination of the major allergen Fel d 1. PhD Symposium of Medical University of Vienna, Vienna, Austria, 19-20 June, 2013.

3. Szalai K., **Satorina J.,** Lukschal A., Willensdorfer A., Mothes-Luksch N., Jensen-Jarolim E. Why cat allergic patients react less toward some cats: Determination of Fel d 1 levels in normal versus hypoallergenic cats. 2nd meeting of Middle-European Societies for Immunology and Allergology. Opatija, Croatia, 10-13 Oct. 2013.

4. **Satorina J.**, Szalai K., Lukschal A., Willensdorfer A., Mothes-Luksch N., Jensen-Jarolim E. Different production of major cat allergen Fel d 1 in normal versus hypoallergenic cat breeds? 4th Retreat of Center for Pathophysiology, Infectiology and Immunology, Vienna, Austria, 17&19 Sept. 2013.