Evaluation of Veterinary Allergen Extract Content and Resultant Canine Intradermal
Threshold Concentrations

Thesis

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Abstract

Allergen-specific immunotherapy (ASIT) is the preferred treatment for allergic diseases in humans and animals, as it can modify the immune system, improve clinical signs and halt disease progression with fewer side effects than traditional pharmaceuticals. Treatment of atopic dermatitis (AD) with ASIT in animals relies on identifying offending allergens. Although numerous methods of allergy testing exist, intradermal testing (IDT) remains the gold standard test in veterinary medicine. IDT is performed with unstandardized allergen extracts at threshold concentrations (TCs) that result in positive reactions in $\leq 10\%$ of a normal canine population. Limited information is available on the optimal TCs for IDT in dogs. Additionally, the lack of standardization of allergen extracts may lead to variable protein compositions and cause false negative and positive results on IDT. The objectives of this study were to survey the protein heterogeneity of tree, grass, weed, and mite allergen extract lots between manufacturers, and to determine IDT allergen extract TCs for healthy dogs using allergens from two veterinary allergy manufacturers. We hypothesized that IDT TCs and protein composition would vary according to allergen extract and manufacturer.

Eleven allergens which included giant/short ragweed mix, lamb's quarter, English plantain, American elm, black walnut, box elder, red cedar, white oak, Johnson grass, timothy grass and *Dermatophagoides farinae*, were obtained from ALK-Abelló and

Stallergenes Greer. The protein concentrations of the allergen extracts were evaluated using a Bradford-style assay and the electrophoretic patterns were determined by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). IDT was performed in 25 privately owned, clinically healthy dogs and 10 laboratory beagles with the 11 allergens from each company. IDT was performed in two groups: group 1 (n=22) and group 2 (n=13). Intradermal testing dilutions were adjusted for the IDT to be used for group 2 when the percentage of dogs with significantly positive reactions ($\geq 2+$) to any allergen tested was $\leq 10\%$ in group 1. Both groups 1 & 2 were tested with 96 allergen dilutions, 11 allergens used at four different concentrations from each manufacturer and *D. farinae* tested using four additional weight per volume (w/v) concentrations from each manufacturer. Subjective measurements were scored on a scale of 0 to 4+, whereas objective measurements were obtained by averaging the vertical and horizontal diameter of each reaction at 15 minutes. Threshold concentrations were defined as the highest concentration of an allergen where $\leq 10\%$ of dogs had a positive reaction ($\geq 2+$) at 15 minutes. Subjective and objective measurement concordance was determined from the combined data from both companies and from each allergy manufacturer separately with receiver operating characteristic (ROC) curves. Exact threshold concentrations were determined using generalized estimating equations.

Allergen extract protein quantity varied within and between manufacturers despite sharing the same PNU/mL values. ALK-Abelló extracts labelled as 40,000 PNU had protein amounts that ranged from 210.5 $\mu\text{g/mL}$ (American elm) to 1117.2 $\mu\text{g/mL}$ (timothy grass). Greer[®] extracts labelled as 40,000 PNU had protein amounts that ranged from 231.0

µg/mL (white oak) to 864.29 µg/mL (black walnut). Between manufacturers the protein concentration of identical allergens designated to have 40,000 PNU also differed, with the largest disparity identified in American elm with a 1.4-fold difference (210.5-513.1 µg/mL). The qualitative protein composition showed heterogeneity of electrophoretic patterns between ALK-Abelló and Greer[®] extracts. Using the TC cut-off of ≤10% positivity for the Group 2 dogs, the generalized estimating equations produced TCs for nine PNU/mL allergen extracts from ALK-Abelló: lamb's quarter 1420 ± 286 , American elm 786 ± 836 , black walnut 890 ± 450 , box elder 576 ± 108 , red cedar 613 ± 321 , white oak $3930 \pm 33,300$ Johnson grass 92 ± 19.2 , timothy grass 146 and *Dermatophagoides farinae* 24 ± 8.72 ; and eight PNU/mL allergen extracts from Greer[®]: ragweed 1760 ± 9450 , lamb's quarter 846 ± 244 , English plantain 303 ± 269 , American elm 216 ± 400 , black walnut 605 ± 2310 , box elder 583 ± 3380 , Johnson grass 220 ± 427 and timothy grass 177 ± 66.4 . For two allergens (ragweed and English plantain) from ALK and three allergens (red cedar, white oak and *Dermatophagoides farinae*) from Greer[®], the TCs could not be determined. When evaluating *Dermatophagoides farinae* extract as w/v concentrations, the TCs could not be determined for either manufacturer. The percent concordance of a subjective positive reaction having a large objective measurement was 75.5% when data from both companies was combined and 77.3% for ALK-Abelló and 75% for Greer[®] allergens.

Veterinary allergen extracts labeled with the same genus and species as well as with the same PNU/mL varied in potency and composition between lots and manufacturer. In parallel, the determined TCs also differed between manufacturers. More

clinical studies are needed to establish if these differences influence clinical outcomes of skin test reactivity expected from an IDT and thereby clinical response to ASIT.

Dedicated to Van, Dad, Mom and Laura

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Fields of Study

Major Field: Comparative and Veterinary Medicine

Studies in Dermatology

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Chapter 1. Introduction

The inception of allergy began in the nineteenth century with the description and naming of the immune system. Initially the immune system was thought to only be protective, however further studies and observations revealed that the immune response could also be hypersensitive, harmful, and produce signs of illness. The term allergy now encompasses any antibody or cell-mediated hypersensitivity.¹ Atopy, characterized by immunoglobulin E (IgE)-mediated hypersensitivities to environmental allergens, includes allergic disorders of asthma, allergic rhinitis and atopic dermatitis (AD). Humans and animals are affected by AD. Canine AD has received increased attention as a spontaneous model for the study of AD, due to the increasing prevalence of human AD in industrialized countries.^{2,3} Atopic dermatitis currently affects 15-30% of children and 2-10% of adults.⁴

The pathogenesis of AD is multifactorial and has been intensely studied in humans. Although the precise pathomechanisms remain unknown, development of AD involves a genetic predisposition, exposure to environmental factors, microbiota dysbiosis, skin barrier dysfunction and a skewed immune response.^{4,5} Specifically, genetic impairments in epidermal function, as well as, an increased number of T-helper-2 (Th2) cells, characterizes the progressive pattern of cutaneous inflammation and allows enhanced colonization of bacteria.⁶ Atopic dermatitis frequently begins in infancy and

may cause life-long skin problems and predispose to food allergy, allergic rhinitis and allergic asthma.⁴ This exact pattern, known as the “atopic march”, is rarely observed in dogs and asthma has never been documented.^{2,7,8} However, humans and dogs do share the cutaneous clinical hallmarks of AD of intense pruritus, dry skin and inflammatory skin lesions consisting of erythematous macules and patches.³

Aside from the clinical characteristics, dogs also share more features of AD with humans including a genetic predisposition, early age of onset, epidermal barrier defects and altered cutaneous bacterial colonization.⁸ Numerous breeds have been identified with having an increased risk of developing AD and include the beagle, bichon frise, boxer, English bulldog, bull mastiff, bull terrier, boxer, cairn terrier, chow chow, Dalmatian, fox terrier, French bulldog, great Dane, golden retriever, Labrador retriever, Lhasa apso, Newfoundland, pug, shar-pei, schnauzer, springer spaniel, Staffordshire terriers and West Highland white terriers.^{2,9} The age of onset is commonly between 6 months and 3 years, but has been reported in dogs as young as 4 months and as old as 7 years.^{10,11} Skin barrier impairments consist of increased transepidermal water loss, decreased filaggrin expression and changes in lipid composition.^{3,11,12} Skin immunity is further influenced by the alteration in microbiota diversity. Lesional skin of dogs with AD have a lower microbiota diversity. This allows overpopulation of certain bacterial populations such as *Staphylococcus pseudintermedius* that cause recurrent superficial pyodermas and contribute to the development of chronic skin lesions.^{3,8,13} However, AD does not have pathognomonic signs and diagnosis is based on excluding other pruritic skin conditions with similar clinical presentations.¹⁴

Diagnosis of AD relies exclusively on patient history and clinical features as no specific laboratory findings or tests have been established. The disease pattern may be seasonal or nonseasonal. Therefore, differential diagnoses of ectoparasites and cutaneous adverse food reactions must be ruled out.¹⁴ Sets of diagnostic criteria have also been developed to assist in diagnostic confirmation, however, they have variable sensitivity and specificity.^{3,11,14,15} Documenting elevated allergen-specific IgE is only a minor criterion and not used for diagnostic purposes.^{15,16} Once a clinical diagnosis of AD has been made, allergy testing is performed to identify allergens to include in allergen-specific immunotherapy (ASIT).¹¹

Allergen sensitization is assessed with intradermal testing (IDT) or serum allergy testing (SAT). Intradermal and serology testing employ different methods of measuring IgE reactivity. Intradermal testing evaluates IgE indirectly through mast cell reactivity, whereas serology assays detect allergen specific IgE antibodies.¹⁴ False negative and positive results can occur with either test. In IDT, false negative results may be due to drug interference, using expired allergens, using allergens at inappropriate dilutions, poor technique and seasonal variability.^{11,17} Reasons for false positive reactions include irritant reactions from tested allergens, subclinical sensitivity, poor technique, irritable skin, and dermatographism.^{11,17} Limited information is available on the effects of extrinsic factors on serology testing in dogs. Although IDT requires clinical training and is more invasive for the patient, it remains the preferred method of allergen selection for ASIT.¹¹

In human medicine, 19 allergen extracts have been standardized to ensure uniformity and consistency in skin testing and immunotherapy.¹⁸ As allergens are

biological products, quality can be influenced by geographical location of source material and by extraction and manufacturing protocols. The FDA develops and maintains U.S. reference standards by mandating testing of each manufactured lot for potency and stability.¹⁹ Overall allergenicity assessment is based on IDT and examining the erythema and size of a reaction. This method is termed IntraDermal dilution for 50 mm sum of Erythema and determines the bioequivalent ALlergy units (ID₅₀EAL) and allows comparison of extract allergenicity regardless of source. For a few extracts, such as short ragweed and cat, the bioequivalent allergy unit (BAU) was based on their major allergen content.¹⁸ In veterinary medicine, identification of major allergens for dogs is in its infancy. Furthermore, extracts have not been standardized, allowing for potential variability in total allergenic activity.¹¹ Non-standardized extracts are labeled by weight per volume (w/v) or protein nitrogen units (PNU).^{17,19} Neither measurement directly measures the allergen content in an extract, as neither the weight or protein directly correlate to potency.¹⁷ Therefore, extracts may vary by allergen manufacturer as well as by lot.

Establishment of optimal extract concentrations for IDT remains a challenge given this inherent variation in allergen potency and lack of standardization. In accordance with the purpose of performing IDT to identify IgE-mediated reactions in allergic dogs, allergen extracts should be tested at the maximum concentration that does not result in an irritant, or false positive reaction. The threshold concentration (TC) of an allergen has been defined as the highest concentration that induces a positive reaction in $\leq 10\%$ of a normal canine population.²⁰ Limited studies in veterinary medicine have

examined TCs for IDT and even fewer have compared findings from different allergen manufacturers and from several lots. In veterinary practice, most pollens are tested at concentrations of 1000 PNU/mL and mites at 250 PNU/mL.^{11,20} However, the findings in newer studies have conflicted with these concentrations, indicating the need to further characterize TCs.²¹⁻²³

As there is no standardization for veterinary allergen extracts, a significant need exists to establish TCs for use in IDT. Additionally, a deficiency exists in comparing allergen extracts from multiple manufacturers. Beginning by establishing TCs in non-allergic, clinically normal dogs with allergens from two manufacturers will allow the results of future allergy studies to be compared. We hypothesized that allergen extracts produced by two different companies would have different IDT TCs when evaluated in a population of apparently healthy dogs. The aims of the present study were as follows: (i) to survey the protein heterogeneity and concentration of tree, grass, weed, and mite allergen extract lots between and within manufacturers, and (ii) to determine IDT allergen extract TCs for healthy dogs using extracts from two different veterinary allergen manufacturers.

Chapter 2. Literature Review

2.1 History of Allergy

Observations of human cutaneous allergy initially occurred in 1798, when Edward Jenner witnessed localized injection site reactions to the cowpox vaccine, yet decreased sensitivity to development of disease when the same individuals were exposed to smallpox and cowpox.²⁴ Inhalant allergy was then described in 1828 by John Bostock who reported his own symptoms to *catarrhus aestivus*, now commonly known as hay fever.^{25,26} Additional reactions to inhalant allergens were then demonstrated by Charles Blackley in the 1870s who studied grass pollen in the United Kingdom. He showed that application of pollens to the mucous membranes of the nose produced the symptoms of hay fever, while inhalation produced asthma.²⁷ The oral tolerance method was discovered in the first century B.C. by Mithradates VI when he continuously exposed himself to incrementally larger amounts of toadstool toxins. His later goal of ingesting a large amount of this same toxin for an attempted suicide failed, demonstrating the concept of desensitization.²⁸ Then in 1906, Clemens von Pirquet proposed that serum sickness was caused by antibodies against foreign serum. He developed the term allergy (from the Greek *allos*, meaning other or different' and *ergia*, meaning 'energy or action') to express the transformation in immune reactivity to an organic poison.^{1,29} The first report of allergic sensitization occurred in 1912 in industrial workers that developed a dermatitis

due to 2,4-dinitro-1-chlorobenzene.³⁰ In 1921, Prausnitz and Kustner revealed that serum of a sensitized individual when transferred to a non-sensitized individual could elicit an urticarial reaction in response to the trigger.^{31,32} Ishizaka and Ishizaka later found that immunoglobulin epsilon (IgE) antibodies mediated skin sensitivity in the Prausnitz and Kustner reaction and has since been employed in serological assays for the identification of unknown allergens in allergic patients.^{33,34} Coca and Cooke suggested further classification of hypersensitivities by categorizing reactions as normal (serum sickness and dermatitis venenata) and abnormal (anaphylaxis, atopy, and hypersensitivity of infection) according to the type of reaction and the species involved. By this definition, animals only experienced the abnormal type of hypersensitivity.^{1,31}

Wittich questioned Coca and Cooke's division of hypersensitivities, when he reported seasonal conjunctivitis and rhinitis and subsequent hyposensitization in a dog in 1941. He proposed that both humans and animals experience the same allergic reactions.³⁵ Eventually, in 1999, the American College of Veterinary Dermatology (ACVD) developed the Task Force on Canine Atopic Dermatitis. The task force standardized terminology and defined allergy as, "A disease state characterized by hypersensitivity responses to allergens and oftentimes mediated by IgE reagenic antibodies".³⁶ This committee has since evolved into the International Committee on Allergic Diseases of Animals (ICADA) and continues to make recommendations on diagnosis and treatment of canine allergies.^{14,37}

2.2 Hypersensitivity Reactions in Humans

The goal of the immune system is to fight infections by recognizing foreign molecules, antigens, and mounting a response. However, the immune system can also be activated or overreact in response to benign antigens. The World Allergy Organization (WAO) defined the term hypersensitivity to describe “objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons”.³⁸ In 1963, Philip Gell and Robin Coombs classified types of hypersensitivity diseases. The classification considered these reactions damaging and divided them into types I to IV based on the initiating immune mechanisms: type I immediate IgE-mediated, type II antibody-mediated cytolytic, type III immune complex, and type IV delayed hypersensitivity reactions.³⁹⁻⁴¹ Sell proposed a more complex classification scheme based on seven immunologic mechanisms with both protective and destructive functions: 1) cytotoxic or cytolytic antibody reactions; 2) immune complex reactions; 3) delayed hypersensitivity reactions; 4) inactivation or activation antibody reactions; 5) T-cell cytotoxic reactions; 6) granulomatous reactions and; 7) allergic reactions.^{41,42} The Gell and Coombs classification remains relevant for disorders such as allergy.

2.2.1 Type I Immediate IgE-Mediated Hypersensitivity

Type I hypersensitivity is an immediate reaction to an antigen.⁴⁰ Classic examples of type I hypersensitivity reactions include AD, asthma and allergic rhinitis. Other examples of type I hypersensitivity reactions include anaphylaxis, food allergy, drug reactions, urticaria, angioedema and reactions to insect bites or stings. IgE classically

mediates a type I hypersensitivity.³⁹ A type I hypersensitivity can also occur in the absence of detectable allergen-specific IgE. Mouse models have demonstrated that IgG can also induce anaphylaxis.⁴³

Allergens processed by antigen presenting cells stimulate Th2 cells in regional lymphoid tissues. The Th2 cell promotes a humoral response with expansion of antigen-specific B cells that switch toward IgE production.⁴⁴ This generates high concentrations of antigen-specific IgE which bind to high affinity fragment crystallizable (Fc) receptors (FcεRI) on the surface of circulating mast cells and basophils, to complete the first phase of sensitization.⁴⁴ The second phase is initiated upon re-exposure of the IgE-coated mast cell to the sensitized antigen.⁴⁴ Upon contact, the antigen is bound by two or more IgE molecules, which cross-links these antibodies and initiates a signaling pathway within the mast cell via Lyn tyrosine kinase, located on the cytoplasmic tail of the FcεRI molecule.^{44,45} Lyn tyrosine kinase activates and phosphorylates other proteins in the signaling cascade such as mitogen-activated protein (MAP) kinase cascade and phospholipase Cγ.⁴⁵ Phospholipase Cγ catalyzes a sequence of cellular events that ultimately yields calcium.⁴⁵ Calcium promotes mast cell cytokine gene transcription, cytoplasmic granule exocytosis and lipid mediator production.⁴⁵

In response to FcεRI cross-linking, transcription factors stimulate the production and secretion of cytokines including TNF-α, MIP-1α, IL-3, IL-4, IL-5, IL-6, IL-13 and GM-CSF.⁴⁵⁻⁴⁷ IL-3, TNF and MIP-1α induce mast cell proliferation. IL-4 and IL-13 stimulate IgE production and mucus secretion.⁴⁵ IL-5 is chemotactic for eosinophils.⁴⁵ Mast cells may also release preformed or newly synthesized inflammatory mediators.

Following FcεRI cross-linking, the activation of protein kinases allows SNARE proteins to mediate the fusion of mast cell cytoplasmic granules with the plasma membrane.⁴⁵ After membrane fusion, the cytoplasmic granules are released and preformed mediators are secreted.⁴⁵ Preformed mediators consist of histamine, heparin, serotonin, kininogenase, adenosine, proteases (tryptase and chymase), lysosomal enzymes, acid hydrolases, cathepsin G and carboxypeptidase.^{40,44-47} The release of histamine causes vasodilation, vascular leakage and smooth muscle spasm. The preformed enzymes degrade microbial structures and are involved in tissue damage and remodeling.^{44,45} Lipid mediator production is dependent on the cytosolic enzyme phospholipase A2 (PLA₂). Elevated calcium and MAP kinase activates PLA₂, which then hydrolyzes membrane phospholipids to release arachidonic acid. Modification by cyclooxygenase produces thromboxanes and prostaglandins, while conversion by lipoxygenase leads to leukotrienes.⁴⁵ Platelet-activating factor (PAF) is also synthesized and is a derivative of membrane phospholipids.⁴⁵ They trigger vasodilation, bronchoconstriction, leukocyte chemotaxis and adhesion, mucous secretion, increased vascular permeability, and oxidative burst.⁴⁵

Similar to mast cells, basophils express FcεRI and can be activated in the same way by IgE binding and crosslinking of IgE-labelled antigen.⁴⁵ Basophils are therefore recruited to tissues sites with antigen. They also release a variety of mediators including peptides, amines, lipids and cytokines. Histamine is the major preformed mediator stored in basophils and leukotrienes is the major lipid produced on activation. Basophils produce large amounts of IL-4, which in turn induces eosinophil migration and

infiltration. Other cytokines and chemokines produced by basophils include IL-6, IL-13, TNF- α , CCL3 (MIP-1 α) and CCL4 (MIP-1 β).⁴⁸

Chemokines released by basophils and mast cells such as IL-3, IL-5 and GM-CSF recruit eosinophils to the sites of type I hypersensitivity reactions.⁴⁵ Infiltration into tissues is dependent on CCL11 produced by epithelial cells at the sites of allergic reactions.⁴⁵ Eosinophils contribute to the late phase immune response by releasing their cytoplasmic granules, synthesizing lipid mediators and producing cytokines. The granule contents of eosinophils include major basic protein and eosinophilic cationic protein. Both cationic polypeptides are noxious to parasites, bacteria and host tissue. Lipid mediators synthesized by activated eosinophils include PAF, leukotrienes and lipoxin.^{45,47}

2.2.2 Type II Antibody-Mediated Hypersensitivity

Type II hypersensitivity reactions consist of antigen and antibody interactions, leading to activation of the complement system and recruitment of polymorphonuclear leukocytes. Usually IgG or IgM and less so IgA is directed against an altered self-protein or a foreign antigen bound to a tissue or cell.⁴¹ Exogenous antigens may consist of drugs or microbial proteins. The tissue or cell is then destroyed by complement-mediated lysis, antibody-dependent cell-mediated cytotoxicity (ADCC), or altered cellular function.⁴⁷ Cytotoxicity can be mediated by activation of the complement system, which is an enzymatic cascade resulting in cytolysis through the formation of the membrane attack complex (MAC) or by C3b opsonization facilitating phagocytosis.^{41,47} Complement activation also results in the generation of anaphylatoxins C3a and C5a. These molecules

mediate vasodilation, are chemotactic for neutrophils and macrophages and can cause mast cell degranulation.⁴⁹ Antibodies can also opsonize cells marking them for destruction by cells with FcR or cause cell lysis by cellular toxicity. Lastly, antibodies may act against surface receptors and modify cell function.⁴⁷ Examples of type II hypersensitivity include vasculitis, hemolytic anemia, neonatal isoerythrolysis, transfusion reactions, drug reactions, myasthenia gravis and bullous pemphigoid.^{39,47}

2.2.3 Type III Immune Complex Hypersensitivity

Type III immune complex hypersensitivity is due to the formation of insoluble antibody-antigen complexes, which activate the complement system. The complexes are formed locally at the site of antigen deposition or within blood vessels. Depending on the quantity of antigen-antibody complexes in the wall of vessels, signs may range from mild edema to vascular necrosis. Immune complexes can also circulate and then become lodged in joints or organs. An inflammatory reaction also occurs at the times of immune complex deposition.⁴⁷ The complement system, neutrophils and macrophages are activated by FcR on the surface of the complexes. Chemokines and cytokines are released secondary to complement activation and by neutrophils and macrophages. Examples of type III immune-complex hypersensitivity reactions include allergic pneumonitis, systemic lupus erythematosus, glomerulonephritis, serum sickness and Arthus reaction.⁴⁷

2.2.4 Type IV Delayed Hypersensitivity

Type IV delayed hypersensitivity is the result of activation of sensitized T lymphocytes to a specific antigen.⁴⁷ The reaction involves a sensitization and effector phase.⁴⁷ During the sensitization phase, antigen-specific memory T lymphocytes (CD4+) are formed.^{47,50} CD8+ lymphocytes may also be activated during the sensitization phase. They are cytotoxic to cells showing antigen-specific TCRs. Death is induced by apoptosis through the delivery of cytotoxic proteins and by the interaction of membrane-bound Fas ligand.⁴⁷ Once CD4+ T lymphocytes are activated in response to antigenic peptide presentation they can develop into Th1 lymphocytes initiating the effector phase. The Th1 response is also augmented by the production of IL-2, IL-3, IFN- γ and TNF- β by CD4+ lymphocytes. IL-2 supports the proliferation and survival of T lymphocytes. IL-3 induces the growth and differentiation of Th1 lymphocytes and NK cells.⁴⁷ IFN- γ activates macrophages. Macrophages are the principle effector cell and activated macrophages have enhanced phagocytic and killing mechanisms.⁵⁰ They also express increased levels of class II MHC molecules and cell adhesion molecules and can therefore function more effectively as antigen-presenting cells.⁵⁰ IFN- γ along with TNF- β facilitate monocyte extravasation by acting on endothelial cells.⁵⁰ Examples of Type IV delayed hypersensitivity reactions include contact dermatitis, transplant rejection, tuberculosis and chronic allergic disease.⁴⁷

2.3 Atopic Disease in Humans

“Atopy” is the familial tendency to become sensitized and produce IgE antibodies after normal exposure to allergens and display clinical signs of asthma, rhinoconjunctivitis or eczema.⁵¹⁻⁵⁴ In 1941, Rackemann and Mallory divided asthma based on the presence (“extrinsic”) or absence (“intrinsic”) of allergy and this concept has been applied to AD to also encompass individuals that do not display elevated serum IgE, but have AD.⁵² Extrinsic AD is associated with an increased total serum IgE.^{5,52} Intrinsic AD is defined by low levels of serum IgE and occurs in up to 45% of patients with AD.⁵⁵ Humans with intrinsic AD are also absent of specific sensitization to aeroallergens or foods.^{5,51,56} Intrinsic and extrinsic AD cannot be differentiated based on clinical presentation.⁵¹

2.3.1 Immunology of Atopic Disease

The immunology of AD involves a type 1 hypersensitivity reaction, initially driven by T helper (Th) 2 cytokines with progression to a Th1 response with chronicity. Acutely, Th2 and Th22 signals dominate and chronic lesions exhibit a substantial Th1 response.^{57,58} Mechanical injury, allergens such as pollens, house dust mites and certain foods and microbes trigger the skin’s innate immune system and promotes a Th2-skewed immune response.^{5,58} Dendritic cells express a high affinity receptor for IgE enabling them to uptake IgE labelled antigens.⁵⁹ Langerhans cells, a subset of dendritic cells, perform antigen surveillance by penetrating tight junctions with their dendrites to sense outside antigen.^{5,59} After antigens are processed, Langerhans cells and dendritic

epidermal cells travel to the regional lymphoid tissue and preferentially signal the antigen-specific Th0 cell to differentiate to a Th2 cell.^{45,58} Langerhans cells and dendritic epidermal cells also produce chemokines CCL17, CCL18, and CCL22 to attract and amplify the Th2 response.⁵⁸ Barrier defects are closely related to the Th2 and Th22 polarization.⁵ Cytokines produced by Th2 cells, such as IL-4, IL-5 and IL-13 downregulate tight junction proteins, allowing penetration of more antigens.⁵⁸ IL-22 produced by Th22 cells inhibits epidermal differentiation and promotes epidermal hyperplasia.⁶⁰ Other cytokines such as IL-5 and granulocyte-monocyte colony-stimulating factor (GM-CSF) activate and enhance survival of eosinophils and macrophages.⁶¹ IL-4 promotes B lymphocyte isotype switching into the IgE production pathway.⁶¹ IL-13 also increases the differentiation of mononuclear cells into fibrocytes which promotes tissue remodeling.⁶²

Chronic Th1 differentiation is induced by Interferon (IFN)- γ and IL-12.⁶³ The continued Th1 inflammatory cascade is characterized by the influx of IFN- γ , IL-12 and GM-CSF.⁵⁸ IFN- γ promotes increased cutaneous inflammation and keratinocyte apoptosis.⁵⁸ The loss of skin cells due to apoptosis may allow gaps in the skin barrier and allow increased antigen penetration.⁵⁷ The IFN- γ receptor is expressed on mast cells. IL-12 amplifies the inflammatory process, triggering the proliferation of additional IFN- γ , T cells and NK cells.⁵⁸ Although upregulated acutely, the continual stimulation of GM-CSF causes persistent inflammation.⁶⁰

2.3.2 Atopic Dermatitis

Atopic dermatitis in humans, also called eczema or atopic eczema, is a chronic, pruritic, recurrent inflammatory disease, with initial onset early in childhood or in adulthood.^{3,51,59} The course of the disease is relapsing and may fade before puberty or persist into adulthood.⁵¹ Prevalence in children is estimated at 10% to 30% and in adults ranges from 0.3% to 14.3%.^{51,60} Acute skin lesions consist of erythematous papules and skin associated with excoriations, erosions and serous exudate.⁶⁴ Chronically, there is development of lichenification and fibrotic papules.⁶⁴ Patients with AD have a reduced threshold for pruritus and it can be exacerbated by allergens, stress, reduced humidity, excessive sweating, wool, acrylic soaps and detergents.^{64,65} Food allergens are the major trigger in children with AD, while inhalant allergens are the main trigger in adults.⁶⁰

2.3.3 Pathogenesis of Atopic Dermatitis

The pathogenesis of AD is multifactorial and includes a genetic predisposition and environmental factors.^{58,66} Genetics strongly influences atopic diseases.⁶⁷ A study evaluating twins showed a proband concordance rate of 86% of AD in monozygous twins, while dizygous twins only showed a concordance of 21%.^{67,68} Children with one atopic parent have a 19.8% risk of developing atopic disease, compared to a 42.9% risk for children with two atopic parents and a 72.2% risk when both parents had identical the same type of atopic disease, respiratory or skin.^{67,69} Numerous genes are involved in the development of various mild to severe AD phenotypes, which supports a polygenic mode of inheritance in AD.^{67,69} Gene mutations affecting epidermal barrier proteins, innate

immunity, adaptive immunity, pattern-recognition receptors (PRRs) and cytokines contribute to the complex pathogenesis of AD.⁶⁶ For example, a gene discovered on chromosome 5q31.1 was identified to have a polymorphism that encodes IL-4.^{70,71} Additionally, a point mutation on chromosome 17q11.2-q12 was found to increase production of RANTES, a chemokine that promotes mobilization of memory T cells.^{70,72} Other chromosomes believed to be linked to the development of AD include 3q21, 1q21 and 17q25.⁷³⁻⁷⁵ Genetic effects are also influenced by exposure to environmental factors such as pollution and tobacco smoke.^{66,67}

2.3.3.1 Barrier Dysfunction

The skin is the largest organ of the body and has many functions including acting as a physical, chemical and biological barrier and immune organ that prevents transepidermal water loss and colonization of pathogenic microbes.^{57,67} The epidermis, the most superficial structure of the skin is composed of five layers (stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, stratum basale) which largely includes different stages of keratinocytes with lesser intertwining dendritic cells and sensory cells.⁶⁷ The stratum corneum, the outermost layer of the epidermis, is chiefly responsible for the barrier function of the skin.⁷⁶ As keratinocytes mature they flatten and lose their nucleus and are termed corneocytes.^{67,76} Beneath the cell membrane of corneocytes, a barrier structure called the cornified envelope is formed.⁷⁶ The extracellular space between the corneocytes is filled with lipids including cholesterol, ceramides, and free fatty acids.^{67,76,77} These hydrophobic lipids inhibit water loss.⁷⁷ Lipid

precursors including glucosylceramides, phospholipids and cholesterol sulfate are stored in an organelle called lamellar bodies within the keratinocytes of the stratum granulosum.⁷⁶⁻⁷⁸ Lamellar bodies also deliver enzymes such as β -glucocerebrosidase, acidic sphingomyelinase and cholesterol sulfate that produce the ceramides and free fatty acids.^{77,78} Proteases and antiproteases that regulate shedding of corneocytes, desquamation as well as antimicrobial peptides are also supplied by lamellar bodies.^{77,78}

Defects in the formation of the cornified envelope or intercellular lipids or any of the components within the lamellar bodies contribute to the development of AD.⁷⁶

Corneocyte adhesion is dependent on intercellular connections called desmosomes. A homozygous mutation in an important desmosomal protein, desmoglein 1, results in severe dermatitis, multiple allergies and metabolic wasting (SAM syndrome).^{76,79}

Tmem79 is a five-transmembrane protein localized to lamellar bodies and a missense mutation in the TMEM79/MATT gene has a significant association with AD.^{76,80}

Additionally, homeostasis of the stratum corneum is maintained by desquamation of corneocytes which is influenced by the inherent acidic pH of the skin.⁵⁷ Desquamation is regulated by a proteolytic cascade of kallikrein related peptidases.⁷⁶ Barrier abnormalities in patients with AD leads to an increase of skin pH which over activates kallikrein.⁷⁸ This negatively affects the stratum corneum barrier by promoting corneocyte desquamation, decreasing processing of lamellar bodies and increasing generation of inflammatory cytokines such as IL-1.⁷⁶ An autosomal recessive disorder, Netherson syndrome, caused by loss-of-function mutations in SPINK5, the gene encoding the serine protease inhibitor lymphoepithelial Kazal-type trypsin inhibitor type 1, is characterized by severe AD-like

dermatosis, mucosal atopy and anaphylactic reactions to food antigens due to unrestricted kallikrein activity.^{7,66,78} Other Th2 cytokines such as IL-4 and IL-13 downregulate the production of components of the cornified envelope, desmosomal proteins, and ceramides.⁷⁶ Therefore, defects in barrier function increase skin pH, allow foreign antigens to penetrate into the epidermis, activate inflammatory cytokines and compromise antimicrobial defenses.

2.3.3.2 Filaggrin Mutations

Mutations in the filaggrin gene are important in the pathogenesis of AD and have been associated with an early-onset of disease.^{7,81} Approximately 50% of moderate to severe cases of AD and 15% of mild to moderate cases of AD can be attributed to filaggrin mutations.^{66,82} Filaggrin is expressed as the precursor protein profilaggrin, a cationic phosphoprotein composed of hydrophobic amino acids.^{7,57} Profilaggrin is stored as keratohyalin granules in keratinocytes in the stratum granulosum.⁸³ During keratinocyte maturation profilaggrin is dephosphorylated and proteolytically processed to filaggrin.^{77,83} Filaggrin aggregates and organizes keratin filaments which contributes to the mechanical strength of cells.^{7,83} At the upper stratum corneum, filaggrin is further proteolyzed into amino acids that are deaminated into polycarboxylic acids known as natural moisturizing factors.^{5,57,77}

The filaggrin gene is located in the epidermal-differentiation complex on chromosome 1q21, specifically exon 3 encodes almost the entire profilaggrin protein.^{66,82,83} The epidermal differentiation complex also encodes other epidermal

protein genes including involucrin, loricrin, small proline-rich proteins, trichohyalin and several S100A proteins.⁸⁴ Numerous mutations of the profilaggrin molecule have been identified, however loss-of-function mutations within exon 3 result in complete absence of filaggrin.⁶⁶

Decreased filaggrin expression can result in decreased keratohyalin granules causing disruption of the stratum granulosum and normal differentiation of the stratum corneum.⁵⁷ Filaggrin deficiency also causes dysregulation of skin acidification further enhancing kallikrein and increased desquamation.^{5,83} Filaggrin deficiencies also promote barrier dysfunction by decreasing stratum corneum hydration and promoting increased transepidermal water loss.^{5,77} The loss of natural moisturizing factors also affects the skin biome and allows aggregation of *Staphylococcus aureus*.⁵⁷ Similarly, mutations in tight junctions proteins that control the permeability of the epidermis, such as claudin 1 have been identified. Claudin 1 deficient mice had increased transepidermal water loss and loss of function of the skin barrier.⁸⁵ These changes due to filaggrin mutations and tight junction proteins, may explain and contribute to the dryness, increased penetration of allergens and irritants as well as greater skin colonization of pathogenic organisms seen in humans with AD.⁸²

2.3.3.3 Role of Keratinocytes

Keratinocytes are strongly influential in coordinating the innate immune response of the skin.^{86,87} They express several pattern recognition receptors including, Toll-like receptors (TLRs), protease-activated receptors (PARs), Nod-like receptors (NLRs), RIG-

I-like receptors and C-type lectin receptors which recognize invading pathogens.^{86,87}

Activation of TLR receptors results in a Th1-skewed immune response and production of type-1 interferons.⁸⁶ TLR2 is the primary receptor that recognizes staphylococcal ligands and mutations in this receptor correlate to increased skin infection with *Staphylococcus aureus* and are associated with a more severe phenotype of AD.⁸⁶ In response to invading microorganisms or tissue injury, keratinocytes produce inflammatory cytokines and antimicrobial peptides such as IL-1 α , IL-1 β , IL-6, IL-36, TNF- α and GM-CSF.⁸⁶⁻⁸⁹ IL-1 α and IL-1 β have similar biological activities and stimulate acute-phase proteins, cellular adhesion, chemotaxis and T and B cell proliferation.^{87,90} IL-6 activates lymphocytes and is a potent inducer of Th17 differentiation from naïve T cells.⁸⁷ IL-36 activates dendritic cells and promotes the production of pro-inflammatory mediators IL-17A, IL-22, IFN- γ , TNF- α , IL-6 and IL-8.⁹¹ TNF- α mediates inflammation by recruiting macrophages, epidermal Langerhans cells and increasing expression of adhesion molecules E-selectin, ICAM-1 and VCAM-1.^{90,92} GM-CSF is a growth factor for myeloid progenitors and has potent effects on macrophages and dendritic cells. Keratinocytes also produce thymic stromal lymphopoietin (TSLP), which activates dendritic cells to prime naïve T cells to produce IL-4 and IL-13 and thereby promote Th2 cell differentiation.⁸⁸ Keratinocytes can also recruit T cells and Langerhans cell precursors to the skin by expressing CCL17, CXCL9, CXCL10 and CXCL11.^{86,87}

2.3.3.4 Role of Dendritic Cells

Dendritic cells (DCs), professional antigen-presenting cells, like keratinocytes also mediate T cell differentiation.^{93,94} In the skin, DCs inhabit the epidermis and dermis and constantly scan for foreign antigens, acting as a link between innate and adaptive immunity.^{93,94} Three main DC subtypes exist: Langerhans cells (LCs), classical myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs). LCs are the DC present in the epidermis and are localized to the basal and suprabasal layers.⁹³ All DCs express CD45 and major histocompatibility complex (MHC) class II molecules.⁹³ LCs also express CD1a, which facilitates presentation of microbial lipid antigens to specific T cells.⁹³ LCs make up about 2-8% of all epidermal cells and are the initial cell to encounter skin and mucosal pathogens.⁹³ LCs tolerate commensal bacterial through the production of regulatory cytokines such as IL-10.⁹³ LCs are also modulated in an autocrine fashion by TGF- β , which has been shown in a murine model to inhibit LC maturation and migration.^{93,95} The skin of humans with AD has been reported to have increased numbers of LCs and upregulated IgE receptors, Fc ϵ R1 and Fc ϵ R2.⁹⁴ Upon protein antigen exposure, TSLP receptor is upregulated on LCs and can trigger migration to draining lymph nodes.^{5,96} LC migration out of the epidermis is mediated by TNF- α through its downregulation of the cell adhesion molecule E-cadherin.⁹³ Once in the lymph node, mature LCs display antigenic peptides in the context of MHC class II resulting in activation, stimulation and proliferation of naïve CD4⁺ T cell.⁹⁴ TSLP also drives naïve T cells to Th2 phenotypes.^{5,97} The lesional skin of patients with AD also contain a second mDC called inflammatory dendritic epidermal cells (IDECs).^{5,93,98}

Unlike LCs, IDECs lack Birbeck granules and Langerin expression and instead express the mannose receptor CD206.⁹³ IDECs have a stronger expression of FcεR1 compared to LCs, and when cross-linked by IgE, release the proinflammatory cytokines IL-8 and TNF-α as well as IL-12 and IL-18 promoting development of IFN-γ producing T cells *in vitro*.^{5,93} IDECs are integral in the switching of Th2 to Th1 in chronic AD.⁵ pDCs circulate in peripheral blood and are low to absent in normal and atopic skin.⁹⁸ pDCs produce type 1 interferons in response to viral DNA or RNA, which may explain why patients with AD develop lesions associated with herpes simplex virus (eczema herpeticum).^{98,99}

2.3.3.5 Skin Microbiota

Human skin microbiota is developed at birth and continues to evolve based on body site, age, gender, hygiene, geographical, interpersonal and temporal interactions.^{13,100} Protection of the skin from invasion by pathogenic microorganisms is also accomplished by the presence of commensal microorganisms and a diverse microbiome.^{13,101} Three genera *Staphylococcus*, *Corynebacterium*, *Propionibacterium* comprise about 60% of the bacterial load on the skin.^{13,100,102} *Staphylococcus epidermidis* inhibits biofilm formation by invading pathogens through the secretion of Esp, a serine protease.^{100,103} Staphylococci also enhance production of β-defensin 2, an antimicrobial peptide, through activation of TLR2 on human keratinocytes.^{100,104} They can also produce peptide antibiotics that are bactericidal against *S. aureus*.^{101,105} In a model with germ free mice known to be deficient in IL-17 production, colonization with *S. epidermidis*,

rescued production of this cytokine through IL-1 induction, suggesting that resident commensals maintain optimal cutaneous immunity.¹⁰⁶

Dysbiosis of this commensal bacterial population can result in excessive inflammation.¹⁰⁰ The predominant bacteria causing skin infection in patients with AD is *S. aureus*.¹⁰⁷ *S. aureus* express fibronectin-binding proteins that adhere to fibronectin on corneocytes.^{101,108} Increased levels of fibronectin are observed in the stratum corneum of AD skin compared to normal skin.¹⁰⁸ *S. aureus* has a density 100 to 1000 times higher in AD skin in comparison to healthy skin.¹⁰⁹ In children with AD, disease severity correlated with lower skin bacterial diversity and increased levels of *S. aureus*.^{13,110} *S. aureus* has many virulence factors that promote colonization and pathogenesis.^{101,102} Peptides involved in virulence and inflammation are phenol-soluble modulins (PSMs). PSMs trigger the immune system by promoting neutrophil chemotaxis, are cytotoxic and contribute to biofilm development.^{102,111} Specifically, δ -toxin induces mast cell degranulation.^{102,112} *S. aureus* also express superantigens such as toxic shock syndrome toxin-1 and the staphylococcal enterotoxin serotypes A through U (SEA-SEU).^{101,113} These exotoxins bind to MHC class II on APCs resulting in the production of T cell cytokines and IgE antibodies.^{101,113} Mast cell degranulation is then triggered through binding of IgE to Fc ϵ RI.¹⁰¹ The presence of IgE antibodies to *S. aureus* and its' superantigens correlates with more severe AD symptoms.^{101,102,114} Although *S. aureus* significantly contributes to the pathogenesis of AD, the restoration of microbiome diversity with antimicrobial and anti-inflammatory medications can improve lesional skin and disease severity.^{101,110,115}

2.3.3.5.1 Antimicrobial Peptides

Production of antimicrobial peptides (AMPs) by epidermal keratinocytes, sebocytes, phagocytes, T cells and mast cells helps maintain the immunologic barrier of the skin.^{102,116} AMPs contribute to both the innate and adaptive immune response.¹¹⁶⁻¹¹⁸ AMPs can be bacteriostatic or bactericidal and can modulate inflammatory cell migration, cytokine and chemokine production and promote angiogenesis.^{116,117,119,120} The major types of AMPs include defensins, cathelicidins, S100 proteins, ribonucleases and dermcidin.¹¹⁶ Human defensins are cationic peptides and human β -defensins (hBDs) 1-4 are principally located in epithelia of skin.^{116,117,119} hBD-1 is constitutively expressed by epithelial cells and hBD-2 and hBD-3 are present in low levels in keratinocytes and are inducible in the presence of TNF- α , IL-1 β , IL-17, IL-22, bacteria and yeast.^{119,121} hBD2- is stored within lamellar bodies in the stratum spinosum and released into the intercellular space during formation of the cornified envelope. hBD-2 attracts dendritic cells and memory T cells by the expression of CCR6.¹²¹ Humans only have one cathelicidin, hCAP18 (human cationic antibacterial protein of 18 kDa).^{116,121} hCAP18 is a precursor of LL-37, which can be upregulated in keratinocytes following infection, injury or inflammation.^{116,120,121} LL-37 can act as a pro-inflammatory mediator by downregulating IL-10 and upregulating IL-1 β , IL-12p40 and IL-18 and inducing type 1 interferons in pDCs and keratinocytes.¹¹⁶ Anti-inflammatory properties of LL-37 consist of suppressing IFN- γ , IL-4 and IL-12.¹¹⁶ Additionally, LL-37 can promote angiogenesis by an increase in endothelial cell proliferation and vessel formation.¹²¹ Of the S100 proteins, S100A7 is

one of the primary AMPs found in normal skin and is predominantly expressed in low levels in keratinocytes and neutrophils.^{116,121} S100A7 acts as a chemokine for CD4⁺ T lymphocytes and neutrophils.^{116,121} RNase 7 is the most highly expressed ribonuclease in keratinocytes and promotes DC maturation and chemotaxis.¹¹⁶ Dermcidins are predominantly expressed in eccrine sweat glands and active against *S. aureus* in an acidic environment.^{116,121,122}

Decreased expression of AMPs impairs the skin barrier function and predisposes to increased skin infections.¹¹⁶ hBD-3, LL-37 and S100A7 improve tight junction barrier function and lower levels of hBDs and LL-37 are found in the skin of AD patients.^{116,117,119,121} Th2 cytokines such as IL-4 and IL-13 produced during AD suppress keratinocyte expression of hBD-2 and block the mobilization of hBD-3 on the surface of *S. aureus*.^{117,119} The sweat of patients with AD also contains lower amounts of dermcidins.¹¹⁶ However some patients with AD have overexpression of AMPs, which can also prove to be detrimental, as hBDs and LL-37 stimulate keratinocytes to release IL-6, IL-10, monocyte chemoattractant protein-1 and macrophage inflammatory protein-3a.^{116,121}

2.3.3.6 Thymic Stromal Lymphopoietin

Thymic stromal lymphopoietin (TSLP) is a cytokine that is primarily expressed by epithelial cells at barrier surfaces such as the skin and stimulates the maturation of T-cells through the activation of DCs and macrophages.^{59,99,123,124} TSLP binds a heterodimeric receptor complex composed of the IL-7 receptor α -chain and the TSLP

receptor chain, which is expressed on DCs, macrophages, CD4⁺ T cells, CD8⁺ T cells and Treg cells, NK cells, monocytes, basophils, mast cells and eosinophils.^{7,123,124} TSLP is undetectable in healthy skin and non-lesional skin in atopic humans.¹²⁴ However, high levels of TSLP are found in the lesional skin of AD patients.¹²⁴⁻¹²⁶ Cytokines upregulated during AD such as TNF- α , IL-1 β , IL-4 and IL-13 are known to induce TSLP expression by keratinocytes.^{125,126} The expression of TSLP can be further augmented through exposure to viral, bacterial peptidoglycan, parasitic pathogens and aeroallergens by the activation of TLRs, PAR-2 and the transient receptor potential vanilloid type 1 (TRPV1).^{7,124} In mouse models, overexpression of TSLP causes elevated serum IgE, mediates Th2 skewing and increases eosinophilic infiltration of the skin.¹²⁷ In humans, TSLP can directly stimulate naïve CD4⁺ T cells and CD8⁺ T cells to develop into Th2 cells by inducing IL-4 gene transcription.¹²⁴ TSLP also stimulates mast cells, NK cells, basophils and eosinophils to induce Th2 cytokines, particularly IL-4, IL-5 and IL-13.^{7,124} *In vitro*, TSLP can also induce eosinophil inflammation in humans by delaying eosinophil apoptosis through the regulation of adhesion molecule CD18 and ICAM-1.¹²⁴ TSLP also contributes to the chronic fibrotic remodeling that occurs in AD by enhancing fibrocyte differentiation and collagen production by directly binding to the TSLP receptor on fibrocytes.^{62,124} TSLP has also been noted as an endogenous mediator for the induction of chronic pruritus in patients with AD.^{99,128} TSLP induces pruritus by activation of transient receptor potential ankyrin 1 (TRPA1) in cutaneous sensory neurons.^{99,128}

2.3.3.7 Vitamin D Pathway

Vitamin D is a fat-soluble vitamin that is produced by plants as ergocalciferol (vitamin D₂) and derived from animal-based foods as cholecalciferol (vitamin D₃).¹²⁹ The major source of Vitamin D in humans is by the cutaneous synthesis of sunlight.¹²⁹ Keratinocytes synthesize vitamin D₃ from its precursor 7-dehydrocholesterol (7-DHC) under UVB light.¹²⁹⁻¹³² Regardless of the source, vitamin D₃ is hydroxylated twice, initially in the liver by vitamin D 25-hydroxylase (CYP2R1) enzyme to form 25-hydroxyvitamin D₃ (calcidiol) and then in the kidney by 1 α -hydroxylase (CYP27B1) to form an active metabolite, 1,25-dihydroxyvitamin D₃ (calcitriol).^{129-131,133} Vitamin D levels in the body are regulated by feedback mechanisms of calcium, phosphorus, parathyroid hormone, fibroblast growth factor and vitamin D.¹²⁹ Aside from calcium homeostasis, Vitamin D also plays a role in cell proliferation, differentiation, apoptosis and immune modulation.^{129,130}

Vitamin D has a dose dependent effect on keratinocyte proliferation and differentiation because keratinocytes express the nuclear vitamin D receptor (VDR).¹³⁰ Low concentrations of vitamin D promote keratinocyte proliferation, while high concentrations are inhibitory.¹²⁹ The antiproliferative effects of vitamin D are induced by the decreased expression of c-myc and cyclin D and the increased expression of the cell cycle inhibitors p21^{cip} and p27^{kip}.¹²⁹ Calcitriol can also promote keratinocyte differentiation through the upregulation of involucrin, transglutaminase, loricrin, filaggrin, ceramides and increased intracellular calcium levels.^{129,130} By increasing synthesis of components of the cornified envelope, calcitriol also improves the barrier

function of the skin.¹²⁹ Similar to the effects of vitamin D on cellular proliferation, at high levels it triggers keratinocyte apoptosis.¹²⁹ Vitamin D is also involved in the innate and adaptive immune system through promotion of inflammatory cells and antimicrobial peptides.¹³³ VDR is also expressed on T cells, B cells, neutrophils, macrophages and dendritic cells.^{131,133} Calcitriol decreases the production of IL-2 by inhibiting the nuclear factor of activated T cells, which leads to decreased CD4⁺ T cell proliferation.^{131,132} Cathelicidins and β -defensins are also directly induced by binding of calcitriol to the VDR.¹²⁹ Calcitriol can also affect the production of AMPs in the skin by regulating the synthesis of serine proteases KLK5 and KLK7.¹²⁹ Vitamin D can decrease inflammation by suppressing TLRs production by monocytes, enhancing mast cell production of IL-10, inducing Treg cells and inhibiting B lymphocyte function and IgE secretion.¹³³

Vitamin D levels have been shown to effect the prevalence and severity of AD.¹²⁹ An increased prevalence of AD has been demonstrated in populations with less sun exposure.¹²⁹ Vitamin D levels are also lower in children and adults with AD and correlated with disease severity.^{129,133-135} Low serum vitamin D₃ levels also correlated with low serum LL-37 in AD patients.¹²⁹ Increased levels of *S. aureus* virulence factors were associated with lower calcidiol levels.¹³³ Adults in Turkey with the VDR single nucleotide polymorphism in BsmI were at an increased risk for the development of AD.¹³⁶

2.3.3.8 Nerve Growth Factors

Neurons and neurosensory structures are targets of the inflammatory process, but also participate in the regulation of the immune response by releasing soluble mediators.¹³⁷ The main products of activated sensory neurons are a family of protein growth factors called neurotrophins (NTs).¹³⁷ Four members of this family have been identified and include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5).^{137,138} NTs are involved in neuronal development, survival and function.¹³⁷⁻¹³⁹ However, their biological roles have been further elucidated to also include regulating the allergic response.¹³⁷ NTs contribute to cutaneous inflammation by promoting vascular permeability, plasma extravasation, release of proinflammatory cytokines, nerve fiber sprouting and recruitment of keratinocytes and mast cells that secrete more neurotrophins.¹³⁷ Elevated serum NT levels have been identified in patients with AD and correlate with disease severity.^{137,140}

In the skin, keratinocytes primarily synthesize and secrete NGF, with lower release of BDNF, NT-3 and NT-4/5.¹³⁷⁻¹³⁹ In normal skin, other cells that express NGF include mast cells, endothelial cells and macrophages.¹³⁸ High affinity receptors for NT's belong to the tyrosinase kinase family (Trk; TrkA, TrkB, and TrkC).^{138,139} In skin of AD patients, TrkA expression is observed in all layers of the epidermis.¹³⁹ Binding of NGF to TrkA and NT-3 to TrkC induces keratinocyte proliferation.^{138,139} NGF expression is enhanced in AD and its' production is promoted by TNF- α and IL-1.^{139,141,142} The Th2 cytokine IL-4, upregulates TrkA expression in normal human epidermal keratinocytes.¹³⁹ Eosinophils express cell TrkB and the low affinity receptor p75NTR. Binding of these

receptors by BDNF promotes eosinophil survival through antiapoptotic signaling.^{137,138,143} Additionally, through neuronal expression of ICAM-1, VCAM-1 and Eotaxin-1, eosinophils are recruited to the nerves and when they degranulate cause increased nerve branching.^{137,141} NGF promotes mast cell degranulation and acts as a cofactor with stem cell factor to prevent mast cell apoptosis.¹³⁸ This increases histamine release from mast cells, upregulates neuropeptides such as substance P and contributes to the “itch-scratch cycle” encountered in patients with AD.¹³⁷ Substance P also enhances NK cell activity, proliferation of CD4⁺ T cells and induces IFN- γ and TNF- α production.¹⁴¹ NGF can stimulate nerve fiber elongation into the basement membrane or within the dermis by upregulating matrix metalloproteinases.¹⁴² NGF also promotes DC differentiation and the release of proinflammatory cytokines IL-1, IL-6, IL-12, TNF- α and TSLP.¹³⁷

2.3.3.9 JAK-STAT

The Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway contributes to hematopoiesis and immune development.¹⁴⁴ The human JAK family consists of four JAKs: JAK1, JAK2, JAK3 and TYK2.¹⁴⁴⁻¹⁴⁶ JAKs are a type of tyrosine kinases that are bound to the cytoplasmic regions of type I and II cytokine receptors.¹⁴⁵ The STAT family contains seven STATs: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6.¹⁴⁴⁻¹⁴⁶ The JAK-STAT pathway transfers signals from cell-membrane receptors to the nucleus.¹⁴⁵ Activation of the receptors associated with JAKs by cytokine attachment, initiates dimerization and JAK transphosphorylation.¹⁴⁶

The activated JAKs create docking sites for the recruitment of cytoplasmic transcription factors, STATs. Once phosphorylated, STATs dimerize through SH2 domains and translocate to the nucleus and bind to specific DNA sequences.^{145,146} Inhibitors of the JAK-STAT pathway include suppressors of cytokine signaling (SOCS) protein family, protein inhibitors of activated STAT (PIAS) and protein tyrosine phosphatases (PTPs). The SOCS proteins inhibit signaling by binding and inhibiting JAKs, by binding to docking sites and blocking STAT recruitment and by promoting ubiquitination and degradation of the JAK-receptor complex.^{145,146} Members of PIAS are constitutively expressed and inhibit STAT DNA binding and gene transcription.¹⁴⁵ PTPs negatively regulate JAK-STAT pathway functions by binding to JAKs and by removing phosphorus and preventing phosphorylation.¹⁴⁵

Th2 skewing of immune cells plays an integral role in the pathogenesis of AD. STAT6, STAT3, JAK1 and JAK3 are important in Th2 polarization. The Th2 specific transcription factor is GATA3. STAT6 and IL-2 upregulates GATA3 and promotes expression of the IL-4 receptor.^{145,146} STAT6-deficient B cells are unable to undergo class switching and produce IgE.^{146,147} Increased production of IgE by B cells secondary to strong JAK3 phosphorylation was demonstrated in murine models.¹⁴⁴ STAT3 contributes to Th2 cell differentiation by binding the Th2 cell-associated gene loci.¹⁴⁸ Another promoter of Th2 differentiation, TSLP, has been shown to activate STAT1, STAT3, STAT5, JAK1 and JAK2.^{147,148} A loss-of-function mutation in TYK2 results in enhanced Th2 differentiation and in humans, caused AD-like skin lesions.¹⁴⁷ Other polymorphisms in STAT3 and STAT6 are linked to hyper-IgE syndrome and an

increased risk of AD.^{147,148} JAK1, JAK2 and STAT3 are also activated by IL-31, a pruritogenic cytokine.¹⁴⁹

2.3.3.10 Cytokines in Atopic Dermatitis

The inflammatory infiltrate encountered in the skin of AD patients reveals a predominant expression of Th2 cytokines in the earlier phases of disease and Th1 cytokines chronically.^{57,150,151} When evaluating skin of patients with AD, acute skin lesions had significantly greater numbers of IL-4, IL-5 and IL-13 compared to normal and unaffected skin.¹⁵⁰⁻¹⁵⁴ IL-4 and IL-13 promote initial tissue inflammation and mediate B cell isotype switching to IgE synthesis, while IL-5 fosters eosinophil survival.^{155,156} Chronically, greater increases in IFN- γ , IL-6, IL-12, TGF- β and GM-CSF are observed in lesional skin compared to IL-4.^{150,155} IFN- γ acts as a stimulant for tissue remodeling by regulation of plasminogen activator inhibitor type 1 and tissue-type plasminogen activator.¹⁵⁷ IL-12 downregulates IL-4 in the chronic phase.¹⁵⁷ A shift from Th2 cells to Th0/Th1 cells was also noted as skin lesions became more chronic, paralleling the changes in dominant cytokines.^{150,155}

2.3.3.10.1 Newer Cytokines

Apart from the previously describe Th1 and Th2 cytokines, IL-17, IL-19, IL-22, IL-25, IL-31 and IL-33 are also key participants in allergic disease. A Th-cell lineage, Th17 cell, produces IL-17.¹⁵⁸ Th17 cells are negatively regulated by IFN- γ and IL-4.¹⁵⁸ Cellular producers of IL-17 include $\gamma\delta$ T cells, NK cells, neutrophils, and

monocytes.^{158,159} IL-17 triggers the differentiation of IL-4 producing Th2 cells.¹⁵⁸ IL-17 can also induce production of TSLP and stimulates keratinocytes to generate GM-CSF, TNF- α , IL-8, CXCL10 and VEGF.¹⁵⁸ IL-17 also promotes IgE production by B cells and contributes to an impaired barrier function by downregulating filaggrin.^{95,156,160} IL-4 and IL-17 can induce IL-19 expression by human keratinocytes and IL-19 has been reported to be upregulated in the lesional skin of AD.¹⁶¹ IL-19 can increase Th2 cytokine expression and specifically upregulate IL-4.¹⁶² IL-22 and IL-25 has also been found at increased levels in lesional AD skin.^{7,151} IL-22 has been indicated as a mediator of epidermal hyperplasia.¹⁵⁶ IL-25 can inhibit filaggrin expression.^{7,163} IL-31 is a cytokine associated with pruritus and is increased in acute lesions and correlated with disease severity.¹⁵⁶ IL-4 induces release of IL-31 from Th2 cells.¹⁴⁹ Other cells that secrete IL-31 include dendritic cells, basophils, mast cells and eosinophils.¹⁴⁹ The IL-31 receptor is expressed on macrophages, DCs, basophils, cutaneous neurons, keratinocytes and neurons in the dorsal root ganglia.¹⁴⁹ Studies in mice, monkeys, dogs and humans have demonstrated that IL-31 directly induces pruritus.^{149,164-166} In murine models, IL-31 was also shown to increase epidermal thickness and promote transepidermal water loss.^{164,167} IL-33 is secreted by macrophages, dendritic cells, fibroblasts, adipocytes, smooth muscle cells, endothelial cells, bronchial, osteoblast and intestinal epithelial cells. IL-33 binds to TLR/IL1R super family (ST2). The ST2 receptors are expressed by innate lymphoid group 2 cells (ILC2s), mast cells, basophils, DCs, NK cells and Th2 lymphocytes.¹⁵⁸ Increased expression of IL-33 and ST2 were found in AD skin after allergen exposure.¹⁶⁸ IL-33 triggered ILC2s to produce IL-5 and IL-13 in allergic conditions and have found to

be increased during the onset of AD and expand in number after allergen exposure .¹⁵⁸

IL-33 also potentiates IL-4- induced IL-31 release.¹⁴⁹ Crosslinking of IgE on murine mast cells also enhances production of IL-33 mRNA.¹⁶⁸

2.3.3.11 Epigenetic Alterations

Given the increased prevalence of AD, considerable research has examined how genetic and epigenetic factors predispose to disease development.^{66,84,169} Epigenetics can be defined as heritable changes and biological processes that result in altered gene expression, without affecting the underlying DNA sequence.^{84,170,171} Examples of these mechanisms include DNA methylation, histone modification and regulation through non-coding RNAs.^{84,171-173} Environmental risk factors suggested to cause epigenetic alterations include increased stress, nutrition, obesity, sedentary lifestyle, urbanization, pollution, low vitamin D levels or the overuse of antibiotics.^{66,169,171} In a meta-analysis of candidate genes associated with AD, the most common mutations were found in filaggrin and genes involved in skin barrier function as well as the Th2 and vitamin D signaling pathways.¹⁶⁹ Prenatal maternal stress has been associated with an increased risk of AD in the offspring, secondary to activation of the hypothalamic pituitary axis, which has been suggested to drive a Th2 cytokine profile.^{65,174,175} Air pollutants are thought to predispose to AD through the production of reactive oxygen species (ROS) that lead to the damage of proteins, lipids and DNA.¹⁷⁶ Tobacco smoke has been demonstrated to cause hypomethylation of the TSLP gene (CGI) and lead to the development of AD.^{84,176-178} Atopic monocytes were also found to be hypomethylated at the *FCER1G* locus causing overexpression of FcεR1.¹⁷⁹ Additionally, expression of the non-coding RNA molecule

microRNA-155 was highly upregulated in CD4⁺T cells from AD skin and serum specimens and correlated with an increase in IL-17 and decreased cytotoxic T lymphocyte-associated antigen (CTLA-4) levels, an inhibitory molecule of T-cell activation.^{84,180,181} The exact epigenetic link between environmental risk factors and the development of AD has not been identified in every scenario. A high body mass index (BMI), obesity and low physical activity in children is significantly associated with the development of AD.^{182,183} Use of broad spectrum antibiotics in the first and second year of life for the treatment of upper respiratory and urinary tract infections was also associated with the subsequent development of atopic disorders.¹⁸⁴

2.3.3.12 Pathogenesis of Pruritus in Atopic Dermatitis

The skin is densely innervated by somatosensory neuron terminals, primarily C fibers, as free nerve endings that terminate in the stratum spinosum.¹⁸⁵ The itch impulse begins in cell bodies located in the trigeminal ganglia and dorsal root ganglia (DRG) at the spinal cord, crosses to the contralateral spinothalamic tract, ascends to the thalamus and then stimulates the brain.¹⁸⁵⁻¹⁸⁷ Pruriceptive itch originates from the activation of pruriceptive receptors by itch ligands (pruritogens) that causes an influx of calcium and activates intracellular cell signaling pathways.¹⁸⁶ Many ligands have been reported and examples of some pruritogens include histamine, proteases (trypsin, tryptase, cathepsins and kallekreins [KLK]), opioids, substance P, calcitonin gene-related peptide (CGRP), neurotrophins, endocannabinoids and acetylcholine.¹⁸⁶ Histamine specifically induces itch through histamine receptors (H₁ and H₄).^{185,186} Other pruritogens activate voltage gated ion channels by exciting TRPV1 (histaminergic) or TRPA1 (non-histaminergic).¹⁸⁸

Pruriceptor neurons express multiple receptors including TLRs and formyl peptide receptors (FPRs) and can also activate transient receptor potential (TRP) channels.^{187,189} Protease-activated receptor-2 (PAR2), activated by pruritogenic proteases, has increased expression at nerve endings in the skin and is significantly increased in AD.¹⁸⁶ In addition, keratinocytes have voltage-gated adenosine triphosphate channels and adenosine receptor ligands, similar to C-nerve fibers that are also involved in the generation and transduction of itch.¹⁹⁰

Pruritogens are also increased in canine AD. Specifically, KLKs are upregulated in epidermal nerves and keratinocytes in AD skin.¹⁹⁰ KLKs also contribute to an impaired skin barrier through increased desquamation of the stratum corneum and can therefore mediate a positive feedback loop between keratinocytes, barrier disruption and itch.¹⁸⁵ Endothelin-1 (ET-1) also results in the sensation of itch by directly activating neurons in the DRG.^{185,186} Patients with AD have a significantly increased concentration of β -endorphin (a μ -opioid receptor agonist) and these levels are correlated with both itch intensity and severity.¹⁹⁰ Substance P induces the release of IL-1, IL-8 and VCAM-1. CGPR increases expression of vascular endothelial growth factor (VEGF) in cultured keratinocytes.¹⁸⁵ Keratinocytes also mediate itch by the release of NGF which sensitizes somatosensory neurons and can induce neurite outgrowth and degranulate mast cells.¹⁸⁵ NGF expression is increased in keratinocytes and mast cells in AD.¹⁹⁰ Additionally, activation of keratinocytes by damage to the epidermal barrier or by pruritogens causes the release of proinflammatory cytokines IL-1, IL-6 and IL-8 and pruritogenic cytokines such as IL-31 and TSLP.^{185,190} IL-31 is overexpressed in the skin of atopic patients and

causes severe pruritus and nerve elongation and branching.^{186,188,191} TSLP expression is increased in keratinocytes from AD patients and directly activates sensory neurons through the TSLP receptor.¹⁸⁵ The increase in nerve density, pruritogens and pruritoceptors in patients with chronic AD, may cause the increased itch sensation encountered with this disease and lead to the “itch-scratch cycle”.¹⁸⁸

2.4 Canine Allergic Disease

2.4.1 Canine Atopic Dermatitis

Canine AD is a spontaneous model for human AD.^{2,3,192,193} Therefore, extensive research has revolved around the incidence and predisposition of AD in dogs. The prevalence of AD in the canine population is estimated to be between 10-15%.^{194,195} Similar to human AD, a genetic predisposition, immune system dysregulation, cutaneous barrier impairment, environmental allergen exposure and bacterial dysbiosis contributes to the development of AD in dogs.^{3,192} Clinically, canine AD is similar to human AD, as dogs develop lesions on the face, flexural surfaces and distal extremities.^{14,192}

2.4.1.1 Pathogenesis

AD is classified as a genetic predisposition to develop a type 1 immediate hypersensitivity most commonly associated with IgE antibodies in response to environmental allergens.^{194,196,197} House dust, house dust mites, pollens, molds and wool are the most common antigens associated with canine AD.^{196,198,199} Antigens may enter through the respiratory tract or by percutaneous absorption.^{192,194} IgE bound antigen

cross-links FcεR1 on mast cells and basophils leading to chemical and enzymatic reactions that causes their degranulation and release of inflammatory mediators.^{36,200} The cutaneous inflammatory cell infiltrate in canine AD is similar to what has been identified in humans. Mast cells, eosinophils, LCs and CD4⁺ T cells are significantly increased in canine AD skin compared to normal canine skin.^{201,202} Furthermore the cytokine profile in canine AD also mimics human AD, with a predominance of the Th2 cytokines such as IL-4, IL-5 and IL-13 and fewer Th1 cytokines like IFN-γ.²⁰³⁻²⁰⁷ Lower expression of TGF-β was also reported in canine AD, suggesting a breakdown of immune tolerance.²⁰⁵

In a model of epicutaneous allergen challenge in house dust mite (HDM)-sensitive beagles, significant increases in IL-6, IL-13, IL-12p35, IL-18 and TARC mRNA expression was also detected upon allergen challenge.²⁰⁸ IL-6 was the only cytokine to significantly increase compared to baseline.²⁰⁸ IL-6 is a proinflammatory cytokine and co-stimulator of T-lymphocyte activation. TSLP also plays an integral role in the differentiation of Th2 cells and the development of allergic inflammation by activating DCs.²⁰⁹ Similar to humans, increase expression of TSLP occurs in the skin of atopic dogs.^{3,209} IL-31 is also produced by Th2 cells. An *in vitro* study demonstrated HDM specific peripheral blood mononuclear cells produced IL-31 upon exposure to HDM and *Staphylococcus* enterotoxin B.²¹⁰ IL-31 has been shown to induce pruritus in experimentally injected dogs and was also identified in 57% of dogs with naturally occurring AD, but not in the normal dogs.²¹¹ Recently, keratinocytes in chronic lesional skin of canine AD were shown to express IL-33, while expression was not recognized in normal skin of healthy dogs.²¹² IL-33 levels were significantly higher in lesional skin

with excoriations, than those without, suggesting an upregulation in response to scratching behaviors.²¹²

Although Th2 cytokines such as IL-4 and IL-13 trigger production of allergen specific IgE, total serum levels of IgE in dogs does not necessarily correlate with the presence or absence of AD.^{16,196,213} Numerous studies have evaluated total serum concentrations of IgE in normal and atopic dogs and found no significant difference between both populations. In addition, both dogs with AD and normal dogs can have detectable levels of allergen-specific IgE that does not correlate with total serum IgE concentrations.^{201,214-216} However, a higher percentage of IgE positive cells have been identified in lesional and non-lesional atopic skin compared to normal controls.²¹⁶ Interestingly, allergen-specific IgE may decrease in response to hyposensitization with immunotherapy.^{16,217} Rarely, dogs that are clinically diagnosed with AD have no demonstrable allergen-specific IgE.²¹⁸ Studies evaluating other antibodies such as IgG have demonstrated an elevated allergen-specific IgG_d in 89% of atopic dogs compared to 0% of healthy non-allergic dogs.²¹⁹ No difference was found in the measurement of serum IgA in normal versus atopic dogs.²²⁰

Determining the role of skin barrier abnormalities in the pathogenesis of canine AD is in its infancy. Studies have reported alterations in filaggrin expression, decreased levels of ceramides, and increased transepidermal water loss in dogs with AD.³ Filaggrin mRNA expression was significantly decreased in lesional atopic skin of West Highland white terriers compared to non-lesional non-atopic skin.²²¹ Examination by electron microscopy of the stratum corneum of atopic skin showed an abnormal structure of lipid

lamellae deposition compared to normal canine skin.²²² Ceramides and free fatty acids have also been found to be lower in the skin of dogs with AD.²²³ Specifically, ceramides 1 and 9 were shown to be significantly lower in dogs with AD than in control subjects.²²⁴ Sphingosine-1-phosphate (S1P) another structural lipid and signaling molecule was significantly lower in homogenized lesional canine AD skin compared to non-lesional AD and healthy skin.²²⁵ Decreased levels of ceramides are thought to accelerate transepidermal water loss and a negative correlation was identified between transepidermal water loss and the amounts of ceramide in both lesional and non-lesional skin of dogs with AD.²²⁶

Barrier dysfunction also contributes to the increased incidence of superficial bacterial infections in canine AD. On healthy canine skin, the predominant bacteria belong to *Porphyromonas*, *Staphylococcus*, *Streptococcus*, *Propionibacterium* and *Corynebacterium* species.²²⁷ During AD flares, dogs have decreased diversity of the microbiota on their skin and this correlates with an increase of *Staphylococcus spp.*^{227,228} Attachment of microorganisms to epithelial cells is an initial step in the pathogenesis of infection.²²⁹ An older study by McEwan, demonstrated a statistically significant greater adherence of *S. intermedius* to keratinocytes in AD dogs with pyoderma compared to keratinocytes in normal dogs.²³⁰ Adherence of *S. intermedius* to corneocytes was shown to be significantly greater in dogs with high pruritus scores.²³¹ Additionally, alopecia, lichenification and lesion score is inversely correlated with microbial diversity.²²⁷ The presence of pathogenic bacteria such as *S. pseudintermedius* and *S. schleferi* can induce and exacerbate the clinical signs of AD and *S. pseudintermedius* has been shown to be

more prevalent in AD skin, which suggests that decreased microbial diversity is associated with lesion severity.^{227,228} Exposure to beneficial bacteria, such as the probiotic *Lactobacillus rhamnosus*, has been demonstrated to reduce the clinical severity of AD and decrease IL-10 levels.²³² These results support the influential role of bacteria in modulating the immune response in canine AD.

The increase prevalence of cutaneous infections in canine AD may also be due to decreased production or production of nonfunctional AMPs, however no clear correlation has been identified.^{233,234} AMPs aid in the management of pathogenic bacteria. Discordant results have been demonstrated in the expression of cathelicidins in canine AD skin.²³³ Multiple studies have shown increased mRNA expression of cBD1, cBD1-like, cBD3, cBD3-like and cCath in lesional and nonlesional skin of dogs with AD compared to healthy controls.^{233,235,236} While another study revealed a lower mRNA expression of cBD1-like in lesional canine AD skin compared to nonlesional AD skin.²³³ Furthermore, no significant difference in cCath secretion between healthy and atopic keratinocytes has also been observed.²³⁶

2.4.1.2 Clinical Signs

Pruritus is the primary feature of canine AD.^{194,196,200} Classically, the onset of pruritus occurs between one to three years of age, however; dogs as young as six months of age may develop clinical signs.^{10,194,196} Relocation to a new environment can result in the development of AD in an older dog.¹⁹⁴ Pruritus is exhibited as rubbing, scratching, licking and chewing and may be localized or generalized and include the muzzle,

periocular areas, neck, chest, axilla, inguinal area, extremities, antebrachial areas, paws, trunk or ears.^{3,10,194,200,237} Primary lesions consist of rarely seen papules and erythema.¹⁹⁶ A minority of dogs may develop conjunctivitis and may rub their noses or sneeze.¹⁹⁶ Ocular signs of allergic conjunctivitis consist of conjunctival hyperemia, pruritus, chemosis, ocular discharge and epiphora. Conjunctival provocation tests with *D. farinae* and *D. pteronyssinus* have demonstrated a causal relationship with these allergens and ocular signs in mite sensitized atopic dogs.²³⁸ with Asthmatic symptoms such as increased or difficulty breathing rarely occurs.²⁰⁰ More commonly, as a consequence of pruritus acute secondary lesions such as excoriations and alopecia are noted.¹⁹⁶ Chronically, salivary staining, lichenification, hyperpigmentation, scales, crusts and alopecia can become more prominent.^{10,194,196,200} Pruritus, impaired epidermal barrier function and microbiota dysbiosis predispose dogs with AD to developing a superficial pyoderma or folliculitis as well as otitis externa.^{3,194,200} Clinical signs may be seasonal or nonseasonal depending on the particular hypersensitivity.^{3,195,196} Breeds reported to be predisposed to AD include West Highland white terrier, Labrador retriever, German shepherd, golden retriever, boxer, French and English bulldogs, Scottish terriers, Cairn terriers, wirehaired terriers, Dalmatians, Irish setters, Lhasa apsos, miniature schnauzers, poodles, beagles, cocker spaniels, dachshunds and German shorthaired pointers.^{194,200,239}

2.4.2 Cutaneous Adverse Food Reactions

Cutaneous adverse food reaction (CAFR) can be described as an immunologic reaction caused by the ingestion of food.²⁴⁰ Due to a lack of precise etiology, many terms

have been used to describe this disorder including food allergy, food hypersensitivity, food intolerance and food sensitivity.²⁴⁰ The incidence of CAFR is low and is estimated to occur in about 1 to 2% of the canine population and overlaps with a diagnosis of AD in 9 to 40% of dogs.^{241,242} Diagnosis of CAFR is based on a food trial with a novel source of protein and carbohydrate or a hydrolyzed diet for 5 to 8 weeks.²⁴³⁻²⁴⁵ Identifying a diet with novel ingredients is challenging due to the demonstration of IgE cross-reactivity between unrelated foods.²⁴⁶ Studies evaluating the ingredients in prescription and over the counter commercial novel or limited ingredient and hydrolyzed diets discovered discrepancies between the labelled ingredients and proteins found through ELISA or PCR testing.^{243,247-249} Additionally, the use of extensively hydrolyzed diets rather than partially hydrolyzed diets is preferred due to the chance of higher molecular weight peptides inducing an IgE-mediated hypersensitivity.^{250,251} After the designated food trial time, a food provocation or rechallenge with the initial or “old” diet is required to confirm CAFR. A diagnosis of CAFR is established based on the recurrence of clinical signs during the rechallenge period.²⁴⁴

2.4.2.1 Pathogenesis

The immunologic mechanisms of CAFR is thought to involve both an immediate hypersensitivity involving types I and III as well as a delayed hypersensitivity involving type IV.^{241,252} This reaction differs from a food intolerance which occurs from a lack of a digestive or proteolytic enzyme or when the food itself has a compound that induces the release of inflammatory mediators.²⁴¹ Ingested food is reduced to oligopeptides by

digestive enzymes in the gastrointestinal tract.²⁵³ Antigen may enter through M cells in Peyer's patches, epithelium covering the lamina propria or through lymph draining to the mesenteric lymph nodes.^{244,253} Antigens are then internalized by DCs, macrophages and B cells for presentation via MHC class II molecules to T cells.^{244,253} A natural state of tolerance is established in the gastrointestinal tract due to the presence of regulatory T cells (T-regs) and their secretion of IL-10 and TGF- β .²⁴⁴ In a mouse model it was determined that CD4⁺ CD25⁺ T-regs create oral tolerance to food to control the IgE-mediated food hypersensitivity response.²⁴⁴ A role for lymphocytes in the pathogenesis of CAFR has been demonstrated, as 82% of confirmed CAFR cases were positive for a lymphocyte blastogenic response using a radioisotope during the provocation period.²⁵⁴ Additionally, a proliferation test evaluating CD4⁺ CD25^{low} lymphocytes showed a significant difference in lymphocyte proliferation between the dogs with CAFR compared to healthy controls.²⁵⁵ Bacterial components, specifically *Staphylococcal* enterotoxin B can also modulate the development of CAFR by causing upregulation of T-cell immunoglobulin-domain and mucin-domain (TIM) proteins on DCs. Sensitization to food allergens may also occur percutaneously, as epithelial exposure can trigger a Th2 response resulting in gastrointestinal sensitization.²⁴⁴ Any food can invoke an allergic response, although proteins are frequently the cause of dietary allergy in the dog.^{241,256} Typically, glycoproteins with molecular weights between 10 and 70 kD incite the immune response by cross-linking two or more IgE molecules bound by Fc ϵ R1.^{244,252,257} In humans, peptides composed of only 3 amino acids can induce proliferation of CD4⁺ T

cells.²⁵⁸ The most common foods to elicit reactions include beef, chicken, dairy, wheat, lamb, corn, eggs, pork, fish and rice.^{244,252,259}

2.4.2.2 Clinical Signs

Clinically CAFR can present as non-seasonal pruritus, recurrent superficial pyodermas or otitis externa.²⁵² Primary lesions can include erythematous wheals, papules, macules and plaques. Secondary to pruritus and trauma, dogs can develop excoriations, ulcerations, alopecia, lichenification and hyperpigmentation.^{244,252} Cutaneous signs are indistinguishable from other allergic dermatosis such as canine AD.²⁵² However, the pattern of otic and perianal pruritus, “ears and rears” is attributed to CAFR.²⁵² Uncommonly, food-induced vasculitis and erythema multiforme have been observed.^{252,260,261} Gastrointestinal signs such as vomiting, diarrhea, increased bowel movements, flatulence, fecal mucus and blood have also been reported.^{240,244} The onset of pruritus can occur in a bimodal distribution with young dogs (<1 year) and old dogs (>7 years) primarily showing signs, although CAFR can occur at any age.^{244,252}

2.5 Diagnosis of Canine Atopic Dermatitis

Canine AD has no pathognomonic clinical signs or definitive diagnostic tests.¹⁴ Guidelines were developed by the International Committee for Allergic Diseases in Animals (ICADA) to offer an outline to assist in canine AD diagnosis and include: 1) Exclusion of disease with clinical signs that can mimic AD such as ectoparasites, microbial skin infections, flea allergy, CAFR and contact dermatitis; 2) Application of

Favrot's criteria to historical and clinical features; and 3) Allergy testing with intradermal testing (IDT) or serum allergy testing (SAT).¹⁴ Favrot's criteria consists of two sets of clinical features designed to be used in clinical studies or to evaluate the probability of the diagnosis of AD after exclusion of similar diseases with overlapping signs of AD.^{14,239} Importantly, demonstrating allergen-specific IgE is not a criterion of AD diagnosis. Allergy testing is most beneficial in identifying allergens to include in allergen-specific immunotherapy, after a clinical diagnosis of AD has been established.³

2.5.1 Intradermal Allergy Testing

In veterinary medicine, intradermal and serum allergy tests are the most common diagnostics used to identify IgE-dependent hypersensitivity in dogs.¹⁴ In human medicine, skin prick testing, IDT and SAT are used to identify offending allergens in an immediate-type hypersensitivity. Whereas, patch testing is used in humans with suspected contact dermatitis.²⁶² Patch testing in canine patients has reproduced the immunologic sequence seen with spontaneous AD, however; studies supporting its use in allergen identification are few.^{263,264} Similarly, skin prick testing has not yet been validated as a tool for allergen identification in canine AD.²⁶⁵ Although controversial, IDT is still generally preferred over SAT as it measures the ability of mast cells to degranulate upon exposure to allergens, which better mimics natural sensitization.²⁶⁶

IDT is performed most commonly on the lateral thorax in sedated dogs to decrease endogenous steroid release.^{11,14,17} Prior to testing, the thorax is shaved and the injection sites are marked.^{14,20} A small volume of allergenic extracts, typically 0.05-0.1

mls, are injected intradermally.^{11,14,20} In clinical practice, reactions are read subjectively on a score from 0 to 4+ at 15 minutes. Histamine phosphate at a concentration of 1:100,000 weight per volume (w/v) serves as the positive control and is given a grade of 4+, while phenolated saline is the negative control and graded as 0. Subjective reactions are compared to the positive and negative controls and in general, reactions graded as $\geq 2+$ are considered significant, however a minority of veterinary practices deem reactions $\geq 3+$ as significant.^{14,20,23} Positive reactions are represented as wheals and they are evaluated subjectively by visual inspection and palpation for size, erythema and turgidity.^{11,14} Positive reactions can also be evaluated objectively as having wheal diameters that are equal to or greater than the average diameter measurement of the positive and negative controls.¹¹ As subjective scoring is not standardized, interobserver variability can occur due to individual experience. Conversely, as objective scoring only considers one aspect of subjective scoring, size, this method may exclude truly positive IDT reactions. In one study, a moderate level of correlation was identified when subjective and objective IDT scores were compared.²⁶⁷

IDT results can be influenced by numerous factors. Drugs that can inhibit the release of histamine and other inflammatory mediators can cause false negative IDT results.¹⁴ The influence of oral antihistamines on IDT results have been evaluated in three studies. The antihistamines evaluated were hydroxyzine and cetirizine. Two of the studies showed inhibition of histamine reactivity which returned to normal between 36 hours and 14 days.²⁶⁸⁻²⁷¹ Given the short duration of antihistamine administration, the effect of longer treatment times and use of other antihistamines on IDT reactivity is not known and

therefore a seven to 14 day withdrawal time is recommended.^{11,14} Based on the route of administration, corticosteroids have difference recommended withdrawal times. The recommended withdrawal time for topical glucocorticoids is 14 days, for oral short acting glucocorticoids is 14 to 21 days, and for long acting injectable glucocorticoids a minimum of 28 days.^{11,14,268} Anesthesia and sedatives can alter IDT results and opioids, ketamine/diazepam, propofol and acepromazine should be avoided.^{11,14} Other drugs that may interfere with IDT results include progestational compounds, β_2 adrenergic agonists, bronchodilators, tricyclic antidepressants and essential fatty acids.¹⁴ Alpha 2 agonists, cyclosporine, tacrolimus, oclacitinib, pentoxifylline and ketoconazole appear to have no effect on skin test reactivity.^{11,14,268}

An additional cause of inaccurate IDT results include seasonal variability in environmental allergen load.¹¹ To avoid false negative results, the suggested optimal time to perform IDT is at the end or within 2 months of the peak allergy season. This timing is thought to avoid low IgE levels and minimize mast cell exhaustion.^{11,17} Similarly, young dogs that are still developing hypersensitivities may only show positive results to a narrow range of allergens.¹⁷ False negative IDT reactions can occur due to low allergen extract concentration, inhibitory medications, improper technique, estrus, pseudopregnancy and severe stress.^{11,196} A reaction that resembles a positive erythematous wheal, but is not IgE-mediated is considered a false positive result and may occur secondary to irritant test allergens, improper technique, irritable skin, contaminated test allergens or dermatographism.^{11,17} Therefore, all positive reactions should be interpreted in light of the patient's historical clinical signs.

Although highly suspected, a concrete role for IgE in the development of canine AD has not been established, therefore, IDT should not be used as a screening test.^{218,266} While most dogs with AD have positive results on allergy testing, rarely a negative skin test can also occur in small subset of dogs, where other causes of pruritus have been excluded and IDT was performed according to the previously listed guidelines. As an IgE-mediated reactivity cannot be documented, this group of dogs is given the condition of atopic-like dermatitis.^{16,272} Clinically normal dogs can also have positive reactions. Lian and Halliwell observed 91.7% of normal dogs react positively to at least one allergen on IDT.²¹⁴ These positive reactions in normal dogs may represent a subclinical hypersensitivity. As a result, IDT should be used for the identification of offending allergens to be included in allergen-specific immunotherapy, when a clinical diagnosis of AD has already been reached.

2.5.1.1 Threshold Concentrations

In theory, the concentration of allergens used in canine IDT should optimize true positive results, while minimizing the occurrence of false positive and negative results. Therefore, the optimal extract concentration used in IDT should be the highest concentration that does not cause an irritant or false positive reaction.^{11,17} The TC has been arbitrarily defined as the allergen concentration that results in positive reactions in $\leq 10\%$ of normal dogs.²⁰ The ideal population for determination of TCs would be a pathogen free animal with no previous exposure to environmental allergens, to eliminate positive reactions due to a subclinical hypersensitivity. However, more commonly IDT is

performed in client-owned or laboratory dogs. To establish TCs allergens are serially diluted and then adjusted based on the $\leq 10\%$ cut off. The recommended IDT concentration in dogs for pollens is 1000 PNU/mL, 250 PNU/mL for dust and storage mites and 1000 PNU/mL for insects.¹⁷ The few canine studies evaluating TCs suggest that these concentrations may no longer be appropriate.²¹⁻²³

Wellington *et al.* evaluated a house dust mite allergen extract mix with equal parts *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* in laboratory and privately owned dogs. Privately-owned dogs had significantly more reactions than laboratory dogs, supporting the use of animals with minimal environmental allergen exposure. A concentration of 31.25 PNU/mL was determined as the 0% TC.²⁷³ The optimal histamine concentration and TC for 48 commercial allergen extracts was evaluated by Hensel *et al.* in non-allergic privately-owned dogs. A histamine concentration of 1:10,000 w/v (0.1 mg/mL) was deemed preferable due to the size, turgidity and color of the resultant wheal. When Wellington *et al.* evaluated TCs based on a histamine concentration of 1:100,000 w/v (0.01 mg/mL) all tested grasses (except Johnson), weeds, trees, molds and insects (except moth and flea) had a TC of ≥ 1750 PNU/mL. Specific TCs were determined for Johnson grass (1500 PNU/mL), *Dermatophagoides pteronyssinus* (100 PNU/mL), human epithelia (300 PNU/mL) and moth (1000 PNU/mL). The TCs could not be determined for *Dermatophagoides farinae* and *Tyrophagus putrescentiae* due to excess reactivity at the lowest concentration or for sheep epithelia, duck feathers, goose feathers and flea due to lack of reactivity.²¹ Bauer *et al.* evaluated 23 allergen extracts and determined the 0% and $\geq 10\%$ irritant TC (ITC) in

31 privately-owned dogs. Thirteen ITCs were determined for the 0% cut-off and were as follows: Johnson grass 1000 PNU/mL; Bermuda grass, lamb's quarter, ash red/green 2000 PNU/mL; bahia grass, rye/perennial grass, pigweed, Virginia oak tree 3000 PNU/mL; marsh elder, maple red 4000 PNU/mL; sorrel/sheep weed 5000 PNU/mL; and cocklebur and willow black 7000 PNU/mL. The $\geq 10\%$ cut-off ITC was determined in Bahia grass (5000 PNU/mL), Johnson grass (2500 PNU/mL), marsh elder (6000 PNU/mL), pigweed (6000 PNU/mL), box elder (3000 PNU/mL) and black willow (8000 PNU/mL). For all other tested allergens, the ITC was either greater than 8000 PNU/mL or less than 1000 PNU/mL.²² The first study to primarily evaluate IDT reaction differences in relation to allergen extract manufacturer was performed by Koebrich *et al.* IDT was performed with four mite allergens (*Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, *Acarus siro*, *Tyrophagus putrescentiae*) from two suppliers at a concentration of 250 PNU/mL in 17 healthy laboratory beagles. Seven dogs reacted to at least one mite allergen and there was significant correlation between extract manufacturers.²⁷⁴ Similarly, Foust-Wheatcraft *et al.* evaluated nine allergens from two allergen extract suppliers in 20 privately-owned clinically non-allergic dogs, but only determined the TC for one allergen, cat dander. The TCs differed between manufacturers and was 750 PNU/mL for the ALK extract and 2000 PNU/mL for the Greer[®] extract.²³ In totality, these studies suggest that current TCs are inadequate for most allergens used in IDT. Additionally, there is a lack of studies comparing allergen extracts supplied by different manufacturers.

2.5.2 Allergen-Specific Serum IgE Testing

Serum allergy tests (SAT) detect the presence of allergen-specific IgE and are routinely used in laboratory research and clinical practice.^{11,275,276} Similar to IDT, positive results are not diagnostic for AD, but are used to identify allergens for inclusion in allergen-specific immunotherapy.^{11,275} The benefits of SAT include the ease of performance and convenience, less drug interference and ability to perform in dogs with skin lesions.^{11,20,277} The most commonly used *in vitro* tests include radioallergosorbent test (RAST), enzyme-linked immunosorbent assays (ELISA) and liquid-phase immune-enzymatic assay.¹¹ RAST involves an initial extraction of allergen-specific IgE and IgG antibodies bound to activated solid-phase polysaccharides, usually adhered to a paper disc.^{278,279} Unbound antibodies are removed through buffer washes.²⁷⁹ The solid-phase allergen-antibody complex reacts with a radionuclide or enzyme.²⁷⁹ After washing for a second time, the bound radioactivity is measured and is proportional to the quantity of IgE antibodies produced in the first step.²⁷⁸

In veterinary medicine, ELISA, rather than RAST testing is now widely used. ELISA is based on the measurement of allergen-specific IgE using monoclonal or polyclonal antibodies or the Fc- ϵ receptor technique.²⁸⁰ ELISA and RAST have similar mechanisms. With ELISA, the patient's serum is added to wells containing antigens. The plates are allowed to incubate and then are washed to remove the serum. Enzyme-linked monoclonal or polyclonal antibodies are added to the wells and allowed to incubate for a second time. After washing, chemicals are added to the wells that cause a color change.

The color change correlates with the concentration of allergen-specific antibody in the patient's serum.²⁰

Initial studies of RAST performed by Halliwell and Kunkle compared the tests ability to detect canine pollen hypersensitivities to IDT results.^{20,276,281} They showed that the agreement between positive RAST and positive IDT ranged from 82% for ragweed to 12.5% for dandelion. The concordance of negative RAST and IDT was 100%.^{20,281} Willemse *et al.* used ELISA for the detection of allergen-specific IgG_d antibodies in dogs. Antibodies were identified in 89% of atopic dogs, 55% of clinically atopic dogs with a negative IDT and 0% of normal healthy dogs. The agreement between positive ELISA and IDT also varied based on allergen.^{20,219} ELISA assays were performed with polyclonal anti-IgE with questionable results in specificity due to cross reactivity with IgG.²⁸² To improve on this, De Boer produced a mouse monoclonal anti-dog IgE.²⁸³ Derer *et al.* showed that the use of a combination of monoclonal anti-IgE antibodies in the CMG IMMUNODOT which uses a nitrocellulose strip test, enhanced test sensitivity.²⁸² Hammerling compared two serological tests that use the nitrocellulose strip tests (CMG IMMUNUDOT and CMG DOG TOPSCREEN) to an ELISA plate test using the anti-IgE monoclonal antibody described by De Boer and IDT. The percentage of dogs with positive results was similar. A correlation of 82% was noted between the IMMUNODOT and TOPSCREEN test for indoor allergens (house dust mites, storage mites, flea, human and cat dander). In comparison to the nitrocellulose strip tests for indoor allergens to IDT results, ELISA had poorer sensitivity and almost equal specificity. For all three serological tests concordant positive results for house dust mite

sensitivity occurred in 29% of cases and concordant negative results in 36% of cases.²⁸⁴ Rather than using a monoclonal or polyclonal antibody, a commercial assay was developed based on the human high-affinity mast cell receptor for IgE, FcεR1α, due to its selectivity for only allergen-specific IgE in canines as no binding to canine IgG was reported.²⁸⁵ Biotinylated FcεR1α and monoclonal ELISA assays showed high correlation in their abilities to detect anti-*D. farinae* IgE.²⁸⁵ Furthermore, in a study evaluating a FcεR1α based assay the sensitivity was 86% with a specificity of 92% compared to IDT.²⁸⁶ More laboratories are now using an FcεR1α based assay. When intra- and interlaboratory variability of this assay was examined, the overall disparity within laboratories was 3.14% and the between laboratories differences was also considered low at 4.76%.

Differences in SAT results can occur due to a variety of test specific and patient factors. Similar to IDT, laboratories use allergen extracts from a range of suppliers and differences in protein concentration and content can occur.²⁷⁶ For example, a human study comparing allergen extract potency from four manufacturers, found extensive heterogeneity in protein and carbohydrate composition. Specifically, the results of RAST inhibition of *Aspergillus fumigatus* revealed that the extract supplied from one company was approximately 35 times more potent compared to the other three suppliers.²⁸⁷ As previously discussed, depending on the type of IgE-specific detection, false positives can occur due to IgG binding. Additionally, diverse signaling molecules are used to identify allergen-specific IgE and no standardized IgE reference values have been developed across laboratories. Therefore, no system calibration is alike and no quality control

measures on are in place.²⁷⁶ Canine risk factors associated with elevated serum levels of allergen-specific IgE was evaluated in a cross-sectional Norwegian study. Samples of serum from dogs with suspected AD were analyzed and a greater proportion of female and older dogs had positive test results as well as samples taken during summer and autumn. Geographic location and IgE were statistically significant and Boxer dogs had a significantly higher proportion of positive test results compared to the overall average across breeds. These results suggest the existence of variation based on gender, season, location and breed.²⁸⁸

2.6 Treatment

2.6.1 Allergen-Specific Immunotherapy

Allergen-specific immunotherapy (ASIT) remains the only treatment suggested to target the cause of AD rather than the symptoms. The process of ASIT includes exposing patients to increasing doses of causative allergens to decrease the intensity of the allergic response and achieve tolerance.^{289,290} The history of immunotherapy dates back to 1900 when Curtis relieved the clinical signs of asthmatic patients with the immunization of aqueous pollen extracts.^{289,291} However, excessive doses resulted in anaphylaxis which caused a short-term hiatus in this approach.^{289,291} Then in 1907, Besredka and Steinhardt identified that sensitized guinea pigs were protected from anaphylaxis by the repeated administration of tolerable doses of antigen.^{289,291} In 1910, Noon designed dosages of allergen extract based on pollen derived weight (Noon unit) and started immunizing humans.^{289,291} Noon's colleague, Freeman, continued his work and in 1914 treated 84

patients with grass pollen extract and reported immunity lasting for 1 year after treatment.^{289,291} Currently ASIT is used to treat allergic rhinitis, asthma and venom hypersensitivity in humans.²⁹² Traditionally, ASIT is not recommended as a general treatment option for humans with AD, however successes have been reported in house dust mite sensitized patients.^{293,294} Based on the European Academy of Dermatology and Venereology 2015 position paper, ASIT may now be considered as an option for treatment of severe AD with a positive atopy patch test to house dust mite, birch or pollen allergens.²⁹⁴ Typically, patients start receiving a low dose of allergen administered either by a subcutaneous or sublingual route, that increases until a maintenance dose is reached and then continued for three to five years.²⁹⁵ SLIT in a tablet form has also been approved in humans by the FDA for ragweed, grass and house dust mite.²⁹⁶ Several immune alterations are thought to occur during immunotherapy.^{289,295} The mechanisms believed to modify the immune system include the increased development of IgG allergen-specific antibodies, decreased synthesis of IgE allergen-specific antibodies, reduction in T-cell and eosinophil recruitment, and production of suppressor cells and cytokines.^{289,295}

2.6.1.1 Mechanism of Action in Humans

ASIT is thought to act on both humoral and cellular immune mechanisms involved in allergic inflammation.²⁹⁷ B cells shift from producing allergen-specific IgE to IgG subset.²⁹⁸ Allergen-specific IgG antibodies, particularly IgG4, increase and may compete with IgE for allergen binding to mast cells and basophils.^{298,299} IgG4 can block

allergen-induced IgE-dependent histamine release by basophils and inhibits binding of IgE-complexes to antigen presenting cells.^{299,300} A murine model revealed that IgG induces expression of the inhibitory receptor FcγRIIb, which competes with the high affinity IgE receptor FcεRI and can inhibit mast cell activation.^{299,301} ASIT also results in tolerant DCs that skew naïve T-cells towards Treg cells, leading to a more robust Th1 response and shifting the ratio of Th2 to Th1 cells.^{298,302} A decrease in Th2 cytokines IL-4 and IL-13 follows.^{295,299} This was demonstrated with *in vitro* activation of mononuclear cells from patients suffering from rhinoconjunctivitis and asthma and receiving ASIT. Compared to the placebo group, humans receiving ASIT had decreased release of IL-4 and IL-13.³⁰³ After four years of ASIT, significant increases in the Th1 cytokine, IL-12, was observed, which is consistent with the transition toward a Th1 response.^{293,304} When patients with allergic rhinitis and asthma were administered the major allergens in *Dermatophagoides pteroyrnissinus* (Der p 1) and birch pollen (Bet v 1), suppression of the Th1 cytokines IFN-γ and the Th2 cytokines IL-5 and IL-13 occurred. An increased secretion of IL-10 and TGF-β was also noted.³⁰⁵ Treg cells play a critical role in inducing tolerance during ASIT. Inducible Tregs produce the regulatory cytokines IL-10, TGF-β and IL-35.²⁹⁸ Treg cells inhibit the activation and proliferation of effector T cells and inhibit Th2 cytokine expression and proliferation in response to allergens.³⁰⁶ Furthermore, IL-10 and TGF-β induce peripheral T-cell tolerance, skew the allergen-specific antibody isotype towards IgG4 and IgA and directly suppress IgE production.^{293,306,307} IL-10 also suppresses antigen presentation by APCs and production of IL-5 by Th₀ and Th₂ cells.^{298,308}

2.6.1.2 Mechanism of Action in Canine Patients

Less research has been focused on the mechanisms of action of ASIT in dogs, but the results do show similarities to humans.^{290,309} In 20 atopic dogs receiving ASIT for at least 6 months, allergen-specific IgG levels were higher compared to both atopic dogs not receiving ASIT and non-atopic dogs.^{302,310} Adding to this finding, concentrations of IgG₁ were determined to be significantly increased in seven atopic dogs with a good clinical response to ASIT.^{302,311} In parallel during ASIT, AD dogs sensitized to *Dermatophagoides farinae* showed an increase in total IgG upon *in vitro* mite exposure.³¹² After 10 dogs with AD were treated successfully with ASIT, the cytokine balance shifted from Th2 to mainly Th1. The ratio of IFN- γ to IL-4 was significantly higher after ASIT due to increases in IFN- γ expression.^{302,313} Finally, *in vitro* analysis of serum from AD dogs receiving ASIT revealed an increase in Treg cells and IL-10 compared to healthy dogs and a significant decrease in IgE in AD dogs with a clinical response to ASIT compared to non-responders.^{302,314}

2.6.1.3 Patient Selection

Based on guidelines designed by WHO for immunotherapy in humans, a set of criteria has been proposed for dogs.³¹⁵ ASIT is indicated when the following occurs: 1) The dog has clinically relevant, positive results on IDT or SAT; 2) The specific sensitized allergens cannot be avoided and can also be included in ASIT; 3) The patient fails to respond to other antipruritic drugs or is experiencing severe side effects secondary to the

therapeutic management; and, 4) The client or owner has requested ASIT and agrees to the cost, understand the risk of side-effects, efficacy and administration.^{290,302,315,316} In general, factors that do not appear to influence the clinical efficacy of ASIT in dogs include age of disease onset, time of immunotherapy initiation and length of disease before treatment commenced.^{11,302,317-320} Inclusion of offending allergens based on IDT or SAT also does not appear to change response to treatment.^{11,316,318,319} Studies show conflicting results on whether seasonality of clinical signs, dog breed, sex, and the number and type of allergen included ASIT influence treatment outcome.^{290,302,318-320}

2.6.1.4 Allergen Types

An allergen is defined as an antigen primarily composed of protein that stimulates and interacts with IgE and may result in allergic diseases.^{291,321} IgE can also be produced in response to polysaccharides, glycoproteins and lipoproteins and is usually formed in reaction to more than one source.^{291,321} The majority of clinically relevant aeroallergens are derived from pollens, mites, insects, fungal spores and animal danders.^{20,321} Over 700 allergens have been identified and are catalogued in multiple databases based on nucleotide sequence, protein sequence and function and protein structure.³²¹ Allergens recognized by more than 50% of allergic patients are named “major” allergens, in contrast to “minor” allergens in which <50% of allergic patients recognize.^{291,321} The International Union of Immunological Societies (IUIS) and WHO has developed a nomenclature scheme for allergenic molecules.³²¹ They are named by the three or four letters of the genus, followed by the first one or two letters of the species name and a

number indicating order of allergen isolation or clinical importance.^{291,321} To describe structurally related allergens with similar molecular weights from different species, within the same genus or closely related genera the word “group” is used.²⁹¹

Grass pollens are mainly present during the spring and summer months and cause between 10 to 30% of all human and dog IgE mediated allergies.^{291,321-323} Pollens produced by grasses range in size from 5 to greater than 200 μm . In humans, a concentration of 20 to 100 pollen grains/ m^3 can cause allergic signs.³²¹ The most recognized grass allergens were extracted from rye-grass (Lol), Kentucky bluegrass (Poa) and timothy grass (Phl).³²³ Commonly grasses contain Group 1, 2 and 3 allergens which are β -expansins, that are involved in cell-wall loosening required for growth.^{291,321} The grass Group 1 allergens are acidic glycoproteins with molecular weights between 27-35 kDa and are identified in most grasses except for Bermuda. The Group 2 allergens are acidic proteins and found in rye, fescue, orchard and velvet grasses.^{20,291} The Group 3 allergens are basic proteins.²⁹¹ Group 7 and 12 contain pan-allergens that are responsible for 10-15% of cross-reactivity between grass, tree and other weed pollens.^{291,323}

Weed pollination occurs during the late summer and throughout the fall.²⁹¹ Within the Compositae family, Asteraceae species are considered clinically important and include ragweed, mugwort, sunflower and feverfew.³²¹ Ragweed has received the most attention because it is the major cause of hay fever in humans.^{290,291} Numerous ragweed species exist in North America, but six species including short and giant ragweed are the most commonly encountered.²⁹⁰ The major allergens in humans from short ragweed pollen is pectate lyase, Amb a 1 (38 kDa).^{291,321} Mugwort's major allergen Art v 6 is also

a pectate lyase and cross-reactivity and cosensitization with ragweed frequently occurs.³²⁴ Paralleling human major allergens, 81% of serum from atopic dogs showed IgE binding to proteins at 38 kDa, with smaller percentages recognizing proteins at 45 kDa and 27-30 kDa.³²⁵

Tree's pollinate during the spring, but tend to be a less significant allergen compared to grasses and weeds.²⁰ The most significant class of tress includes gymnosperm and angiosperm. Gymnosperms consist of conifers such as pines, spruce, cedars, hemlocks and golden larch which release large grain pollens and the cypress family which produce smaller spherical pollen grains.²⁹⁰ The gymnosperm, Japanese cedar, is a predominant allergen source in America and Europe.²⁹¹ Numerous major human allergens have been identified from Japanese cedar such as Cry j 1, Cry j 2 and Cry j 3.³²¹ Similarly, two studies in Japan have shown that 100% of atopic dogs demonstrated specific anti-Cry j 1 IgE and 73% of dogs also showed anti-Cry j 3 IgE.^{326,327} Angiosperm are flowering trees.²⁹⁰ The major angiosperm allergens consist of Bet v 1, the major birch allergen, part of Fagales group 1, which is the primary cause of human allergic rhinoconjunctivitis and asthma.²⁹¹ Bet v 1 acts as a plant steroid carrier and demonstrates relation to pathogenesis related proteins (PR-10).³²¹ Other trees within the order Fagales share homologous allergens to Bet v 1 such as alder (Aln g 1), hazel (Cor a 1), hornbeam (Car b 1), white oak (Que a 1) and chestnut (Cas s 1).²⁹¹ Humans sensitized to Bet v 1 have developed secondary food allergies due to cross reaction with homologous PR-10 family proteins in hazelnut, apple, bell pepper, celery and jackfruit.³²⁸⁻³³⁰

Domestic mites include house dust mites (Pyroglyphidae family) and storage mites (Acaridae, Glycyphagidae and Chortoglyphidae families) and live in human and canine habitats. They eat protein rich cereals, cheese, dried fruit, molds and skin scales.³³¹ The most important species of house dust mites are *D. pteronyssinus* and *D. farinae* and storage mites are, *Acarus siro*, *Tyrophagus putrescentiae*, *Lepidoglyphus destructor*.^{291,331} *Blomia tropicalis*, most prevalent in the tropics, has been recognized as both an important house dust and storage mite in those regions.³³² A study evaluating clinically relevant aeroallergens of atopic children in Poland, found that the most frequent allergic reactions on SPT were *D. pteronyssinus* and *D. farinae*.³³³ Similarly, in Korea *D. farinae* and *D. pteronyssinus* caused a reaction in 40% and 43.6% of atopic dogs, respectively.¹⁹⁸ The major allergens in mites are derived from digestive enzymes, actin-associated proteins, ligand-binding proteins or proteins of unknown function.³²¹ Approximately 31 *D. farinae* and 21 *D. pteronyssinus* allergens are listed in the official database by the WHO and IUIS (www.allergen.org).³²¹ In humans with respiratory allergies, the most commonly recognized major allergens from *D. pteronyssinus* are associated with fecal particles and have low molecular weights.³³⁴ They include Group 1 cysteine proteases (Der p 1, 25 kDa), Group 2 MD-2-related protein (Der p 2, 14 kDa), and Group 23 peritrophin-like protein (Der p 23, 8 kDa).³³⁴⁻³³⁶ In contrast, humans with AD produce IgE antibodies to high molecular weight allergens associated with mite bodies such as Group 11 paramyosin (Der p 11, 103 kDa) and chitin-binding protein (Der p 18, 60 kDa).^{334,337,338} Interestingly, potential major allergens identified in dogs with AD include the high

molecular weight allergens in *D. farinae* Der f 15 (98/109 kDa), Der f 18 (60 kDa) and *D. farinae* mite body-derived Zen-1 (150-250 kDa).^{334,335,339,340}

2.6.1.4.1 Allergen Sources

The quality and composition of allergen extracts used in the diagnosis and treatment of allergic disease is influenced by the extract source material.³⁴¹ The characteristics of the raw materials vary according to geography and climate.³⁴¹ The amounts of environmental contaminants such as fungal spores, bacteria, algae, insects, other pollens and pollution also vary by location and may contribute to immunogenicity.³⁴² Individual plant growth is dependent on local sunlight, rain fall and soil composition.^{341,343} The timing and duration of pollination also fluctuates by year.²³⁷ Ragweed pollen extract collected from the same location over 15 years showed a 10-fold variation in Amb a 1 depending on the year of collection.³⁴⁴ Additionally, when six different varieties of olive trees were analyzed over four years, variations in the most prevalent allergen Ole e 1 content were noted and total allergenicity positively correlated with rainfall.³⁴⁵ Mites are typically grown as pure cultures, but similar differences can be detected between lots of extract and by manufacturer.³⁴⁴ The composition of the extract can dictate the amount of major allergens that are present. Higher mite fecal material can cause increased levels of Group 1 allergen content.³⁴⁴ Standardized *D. farinae* extract from four manufacturers revealed varying levels of Der f 1 and Der f 2 content.³⁴⁴ Aside from extract variability due to location, allergen collection, purification and storage conditions can also influence the inherent purity, potency and composition.^{341,342} Pollen

collection can occur in a greenhouse through water setting, on the farm by vacuum or in the laboratory by cutting, drying and sieving flower heads.^{237,341,346} The water-set method involves placing the plants on a tray of water just prior to pollination.²³⁷ The vacuum method is used for most grasses and weeds and is performed during pollination.²³⁷ The cut/dry/sieve method is typically used for mature flower heads collected before pollination.^{237,341} After collection, delipidation occurs with solvents and then extraction is performed.³⁴⁶ Extraction is done carefully as to not denature heat-labile or protease sensitive allergens.²³⁷ Additives such as phenol or glycerin may be combined with extracts to prevent microbial and fungal growth.^{237,346} The extract then passes through systems for clarification and purification and finally strained through a sterilizing filter.^{237,346} Storage conditions such as temperature can also affect extract quality and potency.³⁴⁴ Given the inherent variability of natural allergen sources and the multitude of stages in collection, extraction and storage causes, lots from different years and locations should be combined for a more consistent product.

2.6.1.4.2 Allergen Extracts

Allergen extracts can be prepared in multiple ways; however, the most common formulations are aqueous, glycerin, lyophilized and alum-precipitated.³⁴⁶ Aqueous allergens are water based and typically formulated in a buffered saline solution with a phenol preservative. They are most commonly used in North America for immunotherapy and IDT. Aqueous allergens are the easiest to process and higher concentrated extracts can be obtained compared to glycerinated extracts.³⁴⁶ Oil can also be added to aqueous

allergen to form an emulsion, but have limited use in humans.³⁴⁷ Glycerin can be added to extracts during the extraction process at a 50% concentration. Glycerin is an effective protein stabilizer, but can be irritating and sting during intradermal injection, Therefore, glycerin is more commonly used in SPT and sublingual immunotherapy.³⁴⁶ Lyophilized extracts are produced by extracting the soluble components of an allergen in water with a variable excipient. The solution is frozen and the water is vacuum separated. The product must then be reconstituted prior to use.³⁴⁶ Alum-precipitated extracts are formulated from aqueous extracts. The addition of aluminum potassium sulfate at a neutral pH causes a gel to form in the extract that includes the protein components of the allergen extract. Nonadsorbed extract is washed and the resulting precipitate is added to a saline solution. Alum-precipitated extracts may cause injection site reactions, and newer studies have begun to question the long term effects of aluminum accumulation in the body.³⁴⁶⁻³⁴⁸ Other adjuvants that can be combined with allergenic extracts to induce and improve the immune response include immunostimulating complexes, monophosphoryl lipid A, polymeric nanoparticles, polyactides, polyesters and vitamin D.³⁴⁷

2.6.1.4.3 Allergen Stability

The stability of allergen extracts is influenced by the preservative, labelled concentration, temperature, storage container and time.^{20,237} Glycerin is the most stable extract solution and has a longer storage life compared to aqueous solutions.²³⁷ The FDA has mandated that bulk standardized glycerin extracts can be stored for 3 years following extraction and then an additional 3 years after shipping.³⁴⁹ Extracts not containing this

level of glycerin, expire in half the time.³⁴⁹ Allergens should be stored in a refrigerator, as phenol extracts kept at 35°C lose approximately 50% of their antigenicity within seven days.^{20,237} The surface of plastic and glass may absorb active components in extracts. Allergens stored in plastic syringes lose potency faster compared to allergens kept in glass syringes.^{20,237} Additionally, diluted are more affected than concentrated extracts.²³⁷ In general, allergen extract dilutions formulated for IDT should be replaced every 30 to 60 days.²⁰ Due to the inherent proteases within extracts, degradation of protein content occurs over time which may translate to a decrease in potency. Anecdotally, an increase in false-positive results have also occurred, suggesting that degraded extracts can also act as irritants.²⁰ Even the mixing of fresh extracts can lead to degradation, due to the presence of proteases notably in mold and insect allergens.²³⁷ Although glycerin has been noted to stabilize protease activity and allows for the mixing of all extracts, penicillium was shown to compromise German cockroach potencies at 25% and 50% glycerin when stored together after seven to 12 months.^{237,350} Human serum albumin has also been evaluated as an alternative preservative to glycerin.^{349,351,352} The addition of human serum albumin at 300 µm/mL in saline-phenol to allergen extracts has shown improved stability of major allergens compared to 10% and 50% glycerin.^{349,352} Human serum albumin may reduce degradation by interacting as an alternative protein source with allergen enzymes.³⁵²

2.6.1.4.4 Allergen Standardization

Allergen manufacturers within the USA must follow the Food and Drug Administration (FDA) and Center for Biologics Evaluations and Research (CBER) regulations for pollen extracts.^{237,343} Both agencies maintain 19 US reference standards for potency: six Hymenoptera venoms, two house dust mites, two cat dander extracts, one short ragweed pollen, and eight grass pollens.³⁵³ Standardized pollens have mandated limits for relative pollen potency or major allergen concentrations.³⁴¹ Grass and mite allergen extracts are regulated by comparing overall allergenicity.^{237,353} Cat and ragweed allergen extracts are standardized by evaluating single allergen determinations.^{237,353} Hymenoptera venoms and cat pelt allergenicity are determined by assessing two allergens for each lot.^{237,353} For assessment of overall allergenicity, CBER developed measurements based on serial intradermal testing of highly allergic patients, called intradermal dilution for 50 mm sum of erythema determines the bioequivalent allergy units (ID₅₀EAL).²³⁷ In ID₅₀EAL, the mean diameters of erythema for at least 15 patients determines the D₅₀ and extracts with similar D₅₀ responses are considered bioequivalent.²³⁷ The bioequivalent allergen unit (BAU) is commonly used in standardized extracts.³⁵³ When this measurement was developed, extracts with a mean D₅₀ of 14 were arbitrarily assigned the value of 100,000 BAU/mL.^{237,353} However, most allergen extracts available in the US are nonstandardized and therefore lack regulations.³⁴² Nonstandardized allergen extracts are commonly labelled according to protein nitrogen unit (PNU) or weight of source material extracted with a given volume of extracting fluid (w/v).²³⁷ As neither w/v or PNU directly relate to major allergen

content within allergens, differences in potency and protein content are common within batches from the same allergen suppliers and between different manufacturers. In Europe, the committee for Medicinal Products for Human Use (CHMP) has suggested guidelines for allergen extract standardization, however they can be modified as needed by manufacturers.²³⁷ Curin *et al.* evaluated dog allergen extracts from five European manufacturers and revealed a 20-fold variation in total protein content as well as heterogeneity in the major and minor allergens Can f 1, Can f 2 and Can f 3.³⁵⁴ Similarly, Focke *et al.* showed high variability in protein and major allergen content in four commercial timothy grass allergen extracts.³⁵⁵

2.6.1.5 Formulation

Of the allergic diseases, ASIT is primarily considered as a treatment for humans suffering from allergic rhinoconjunctivitis and/or asthma that demonstrate evidence of IgE-sensitization to one or more clinically relevant allergens.^{342,356,357} In dogs, ASIT is used as therapy for AD. Primarily the selection of allergens is based on IDT and SAT results in combination with symptom seasonality and history.^{290,358} Although, response rate does not seem to correlate to the type of allergy test that was performed.^{11,302} Given the variability in results and agreement between IDT and SAT, the validity in this method of allergen selection has been questioned.^{302,359-361} Therefore, a method of allergen selection purely based on regional aerobiology, regionally-specific immunotherapy, (RESPIT), has also been developed for the use in animals.³⁵⁹

ASIT protocols for dogs are widely variable and there is no conventional standard for optimal dose, number of included extracts and mixing of extracts.^{302,358,362} Response rates of dogs to low or high dose immunotherapy are conflicting.^{358,363,364} In humans, both monosensitized and polysensitized benefitted equally from single allergen immunotherapy.³⁶⁵ Conversely, administration of a *D. farinae* restricted immunotherapy solution was insufficient in managing dogs with multiple hypersensitivities.³⁶⁶ Studies evaluating if certain aeroallergens are more effective than others to include ASIT are conflicting.^{290,302} As pollens cross-react, including all offending allergens may not be necessary and mixing of allergens may cause increased degradation and ineffectiveness.^{11,290,302} Dogs receiving mold allergens combined with pollens had less successful treatment outcomes compared to dogs solely receiving pollen or dust mite allergens.^{11,302} However, when mold allergens were placed in separate vaccines, improvement was comparable.^{11,302,318}

2.6.1.6 ASIT Administration

ASIT protocols are not standardized.²⁹⁰ The frequency, volume and concentration of ASIT increases until a maintenance level or “maximum tolerated dose” is achieved.^{290,362,367} Typical doses of maintenance vials are 10,000 to 20,000 PNU/mL.³⁵⁸ ASIT protocols start with a loading dose twice daily to once every three days and that may increase every seven to thirty days.^{11,290} The response of the individual dog to ASIT can vary. Treatment schedules can be altered based on the absence, presence or worsening of pruritus.¹¹ The majority of dogs will respond with three to 12 months of

treatment.^{192,358,367,368} When effective, dogs require lifelong treatment with ASIT.³⁵⁸

Prolonged improvement has been noted in 4 to 35% of dogs after discontinuation of ASIT.³⁵⁸

2.6.1.7 Types of Immunotherapy

Routes for administration of immunotherapy include subcutaneous and sublingual.³ In subcutaneous immunotherapy (SCIT) aqueous, saline-phenol preserved extracts are used.³⁶² Intradermal injections are advantageous because less frequent administration is required. Sublingual immunotherapy (SLIT) uses glycerinated extracts, but requires daily dosing. SLIT can be considered in dogs that have experienced side effects with injections or for owners that feel uncomfortable giving injections.³ To reduce the induction phase, rush immunotherapy has been evaluated as an alternative to the conventional ASIT loading phase. Rush immunotherapy involves reducing the loading phase of ASIT to more quickly reach maintenance.^{11,358} Currently no studies have proven that rush immunotherapy has more value compared to traditional ASIT.³⁶⁸ Evidence from human studies suggests that intralymphatic immunotherapy (ILIT) may provide a safer and faster method of ASIT.³⁶⁹ ILIT with alum-precipitated allergens was performed in 20 dogs and 60% improved.³⁷⁰ A long-term study evaluating the efficacy and safety of ILIT also found that of 22 dogs that completed the study all had significant prolonged improvement in pruritus.³⁷¹ Due to a lack of standardized extracts in veterinary medicine, one trial investigated the use of a recombinant *D. farinae* major allergen in ASIT. Dog were sensitized to Der f 2 and then treated with SCIT consisting of recombinant Der f 2

adjuvanted with a matotriose polymer pullulan. No adverse reactions occurred and clinical reactivity to Der f 2 challenge was absent in 83% of dogs after four monthly injections.³⁷²

2.6.1.8 Adverse Effects

Acute side effects of ASIT are reported as rare, however anaphylaxis may occur.³⁵⁸ Therefore, after administering ASIT, the dog should be monitored for 30 to 60 minutes for the development of hives, facial swelling, angioedema, vomiting, diarrhea, weakness and collapse.^{11,358,373} Serious reactions have been reported to happen in less than 1% to 1.25% of dogs.³⁷⁴ Milder reactions may also occur such as pruritus, decreased appetite, lethargy and anxiousness.^{358,373} Injection site reactions such as pain and swelling infrequently occur secondary to SCIT and usually resolve rapidly.³⁶⁶ Other adverse reactions include salivation, coughing, panting, hyperactivity, increased bowel sounds, changes in urinary habits and frequent swallowing.^{11,366} To alleviate or reduce the frequency of side effects, antihistamines may be given prior to ASIT or the dose can be decreased.

2.6.1.9 Use in Canines

ASIT is used in dogs for the long-term management of AD.¹⁹² In canine AD, few blinded placebo-controlled studies have been conducted to investigate the effectiveness of ASIT.³⁶⁷ However, results of open uncontrolled and retrospective studies have shown successful results for ASIT in the treatment of canine AD.^{317,358,368,375} Treatment with

ASIT is estimated to improve clinical signs in 36% to 100% of dogs with AD.^{317,367,368,373,375} ASIT has many advantages over other therapeutics, given its low side effect profile and the ability to alter the immune system.^{313,367} Disadvantages of ASIT include expense and delayed clinical improvement^{317,358,367}

2.6.2 Alternative Therapy in Canine Allergic Disease

Management of pruritus in dogs can be accomplished with both topical, oral and injectable medications. Options for topical therapy include glucocorticoids, tacrolimus and essential fatty acids.^{37,192,376} Additionally, microbial infections are commonly the reason for an acute flare and therefore regular bathing or application of antimicrobial sprays, mousses and wipes may also both decrease pruritus and the frequency and severity of bacterial and yeast infections.^{37,192} Topical or oral essential fatty acids have been shown to increase and restore lipid abnormalities in the epidermis.^{37,367} Oral medications that have been demonstrated to have high efficacy in acute flares in dogs include glucocorticoids and oclacitinib.^{367,376,377} Both medications have comparable speed of actions.³⁷⁸ The subcutaneous injection of the lokivetmab, a caninized anti-IL-31 monoclonal antibody, has a fast onset of action in one to three days and lasts for at least one month.³⁷⁹ Antihistamines provide minimal benefit and relief in acute flares of canine AD, but may be included in multimodal therapy as a steroid sparing agent.^{37,317,367} Given the late onset of effect of cyclosporine, this medication is commonly reserved for long-term management of AD.^{37,367} The efficacy of cyclosporine is comparable to both

prednisolone, methylprednisolone, oclacitinib and lokivetmab.^{316,376,380-383} However, glucocorticoids and oclacitinib may also be used for long-term therapy.^{3,37,367,384}

Chapter 3. Evaluation of Veterinary Allergen Extract Content and Resultant Canine Intradermal Threshold Concentrations

3.1 Abstract

Background – Limited information is available on threshold concentrations (TCs) and protein composition for common allergens from different allergen extract suppliers.

Objectives – To characterize the protein heterogeneity of tree, grass, weed, and mite allergen extract lots between and within two manufacturers, and to determine intradermal allergen extract TCs for healthy dogs using allergens from two veterinary allergen extract manufacturers.

Animals – Twenty-five privately owned, clinically healthy dogs and ten purpose-bred beagles were used in this study.

Methods – Protein concentration and heterogeneity of 11 allergens from two manufacturers were evaluated using a Bradford-style assay and SDS-PAGE. Intradermal testing was performed with 11 allergens from each company at six dilutions. Immediate reactions were subjectively scored (0 to 4+), and objectively measured (mm) and their percent concordance was evaluated. TCs were determined by a positive reaction ($\geq 2+$) at 15 min and then by fitting generalized estimating equations.

Results – Allergen extract protein quantity and composition varied within and between manufacturers despite sharing the same PNU/mL values. TCs of one weed, five trees, two grasses and a house dust mite were determined for ALK extracts, whereas TCs for three weeds, three trees and two grasses were identified for Greer extracts. Receiver operating

characteristic curve analyses determined a percent concordance of the objective and subjective values of 77.3% for ALK and 75% for Greer allergens.

Conclusions – Veterinary allergen extracts labeled as the same species and PNU/mL are nonstandardized, therefore, can show variability in composition and potency within and between manufacturers. Variability in extracts between manufacturers will require different testing concentrations for use in canine IDT.

3.2 Introduction

Identifying potential environmental triggers in atopic patients is accomplished using allergy testing. The intradermal test (IDT) measures levels of tissue-bound immunoglobulin E (IgE) whereas serum allergy tests SAT identify circulating serum IgE antibodies using enzyme-linked immunosorbent, radioallergosorbent or molecularly defined allergens distributed on microarray or multiplex nanotechnology panels.^{17,266,276,385,386} Selection of the identified allergens for inclusion in allergen-specific immunotherapy (ASIT) is based on the presence of regional sensitizers, relative allergenicity, clinical relevance, compatibility of extracts on mixing and cross-reactivity with other allergens.³⁰² Effective ASIT reduces clinical signs and concurrent pharmacotherapy while improving quality of life.

Allergen extracts are aqueous extractions produced from natural source materials that contain various amounts of major, minor, and isoallergens, as well as other non-allergenic compounds.³⁸⁷ The quality of ASIT depends exclusively on the content of allergen preparations and manufacturers are challenged to produce allergen extracts with

consistent compositions. Human IDTs and skin prick tests (SPT) use a combination of standardized and non-standardized extracts. As of 2016, the United States has 19 allergen extracts that are standardized for potency and are currently used for skin testing and formulation of ASIT. The reference standards are maintained by the US Food and Drug Administration Center for Biologics Evaluation and Research.¹⁸ Comparing standardized allergen extracts to reference standards ensures relative equivalent potencies. This makes skin testing and ASIT with standardized extracts less variable and facilitates the comparison of studies using standardized extracts. Importantly, standardization ensures safety and efficacy of ASIT by minimizing the risks of variations in allergen dosing when switching from one lot of manufactured extract to another, and by providing an objective measure of stability of each lot of allergen extract over time. Standardized allergens are extracted in glycerinated saline, and the utility of these extracts for IDT in veterinary medicine have not had been validated. One of the challenges in veterinary aeroallergy is the interpretation of IDT reactions due to the variability of the aqueous extracts used for IDT. Currently, there are no standardized allergen extracts for use in the IDT in veterinary medicine.

Non-standardized allergens do not have reference standards established, thereby allowing for greater variations in quality, (potency and immune reactivity) both among lots and between suppliers. Inconsistencies in protein content and the presence of major allergens between manufacturers has been demonstrated in human medicine. Initial studies evaluating protein nitrogen unit (PNU) values and Group 1 antigens in six species of grass from five manufacturers showed that actual PNU values varied as much

as 10-fold from the labeled value.³⁸⁸ Furthermore, Group 1 antigen content did not necessarily correlate with allergen extract concentration, but did strongly correlate with IDT reactivity.³⁸⁸ Similar disparities have been identified in fungal and tree extracts. When two lots each of *Aspergillus* extracts from eight manufacturers were compared, a 100-fold variation was found in the content of the major allergen ASP f 1, suggesting potential differences in manufacturing methods.³⁸⁹ In tree extracts, the protein content and concentrations of the major allergen Bet v 1 in birch pollen from five European allergen manufacturers varied more than 10-fold. Additionally, the size of the wheal reaction produced by SPT in 17 birch sensitized patients corresponded with the concentration of Bet v 1.³⁹⁰ Differences in both protein and major allergen content may pose a challenge in determining appropriate extract concentrations to diagnose hypersensitivity in allergic patients.

Optimal allergen extract threshold concentrations (TCs) for IDT has been defined as the highest concentration of an allergen that results in positive reactivity in $\leq 10\%$ of a normal population.³⁹¹ This is used to minimize false positive and negative reactions in clinically diagnosed dogs with atopic dermatitis (AD).³⁹¹ Historical, TCs routinely used in veterinary medicine for pollens is 1000 PNU/mL and for dust mites is 250 PNU/mL.^{17,20} However, an earlier study revealed that house dust mite dilutions greater than 31.25 PNU/mL, caused false positive reactions.²⁷³ When a greater proportion of clinically non-affected animals react to an injected allergen at a given concentration, false positive reactions to those specific allergens are also more likely to occur in dogs with AD. False positive reactions resemble an erythematous wheal of an IgE-mediated

reaction to an allergen, and can represent a clinically irrelevant sensitization, an irritant reaction not mediated by IgE, contaminated test allergens, poor injection technique, irritable skin, dermatographism and mitogenic allergens.^{17,392}

The first veterinary study to evaluate IDT reaction differences in relation to allergen extract manufacturer was performed in Europe by Koebrich *et al.*²⁷⁴ Intradermal testing was performed with four mite allergen extracts from two suppliers in non-atopic purpose-bred beagle dogs. The percent correlation of the subjective positive results between manufacturers ranged from 57-78%.²⁷⁴ Mite allergen extracts were evaluated at a single concentration and variability in TCs between the different suppliers was not addressed. Recently, Foust-Wheatcraft *et al.*²³ evaluated irritant TCs (ITC) for nine allergens from two different manufacturers in non-allergic dogs. Differences in the ITC between the two manufacturers for one allergen was reported, however, the ITC for the remaining eight allergens could not be determined.

As there is no standardization for veterinary allergen extracts, a significant need exists to establish TCs for use in IDT. Additionally, a deficiency exists in comparing allergen extracts from multiple manufacturers. Establishing TCs in non-allergic, clinically normal dogs with allergens from both manufacturers should aid with interpretation of both IDT reactions and future allergy studies. We hypothesized that allergen extracts produced by two different companies would have different IDT TCs when evaluated in a population of apparently healthy dogs. The aims of the present study were as follows: (i) to survey the protein heterogeneity and concentration of tree, grass, weed, and mite allergen extract lots between and within manufacturers, and (ii) to

determine IDT allergen extract TCs for healthy dogs using extracts from two different veterinary allergen manufacturers.

3.3 Materials and Methods

3.3.1 Study design

This was a prospective study. The study protocol and owner consent form were approved by Institutional Animal Care and Use and the Clinical Research Committees.

3.3.2 Allergens

Eleven commercially available aqueous allergen extracts were used for protein analysis and for IDT. The allergen extracts included three weeds (giant/short ragweed mix, lamb's quarter, English plantain), five trees (American elm, black walnut, box elder, red cedar, white oak), two grasses (Johnson grass, timothy) and one house dust mite (*Dermatophagoides farinae* [*D. farinae*]). Allergens were obtained from Stallergenes Greer® Laboratories Inc. (Lenoir, NC, USA) and ALK-Abelló (Round Rock, TX, USA). All allergen extracts were used within their expiration date.

Mixing of lots was done to replicate the variation in lots that would occur when dermatologists reorder allergens. At least two lots were used for each mix with some mixes containing up to three lots. Mixes were made by the primary investigator by combining aliquots from each allergen extract lot to equal the lowest PNU/mL or weight/volume (w/v) concentration of any lot (Table 1).

3.3.3 Protein analysis

3.3.3.1 Total protein quantification

Allergen extracts were obtained from ALK-Abelló (Round Rock, TX, USA) or Greer® (Lenoir, NC, USA) (Table 1) and total protein concentration ($\mu\text{g/mL}$) was determined by a Bradford-style assay (#23236, Pierce™ Coomassie Plus [Bradford] Assay Kit, ThermoFisher Scientific, Waltham, MA, USA). Bradford-style assays were performed in three to four replicates for each sample via the microplate method, using bovine serum albumin as a protein standard.³⁹³ Protein concentrations for each allergen are represented as the mean ($\pm$ SD) of the three to four replicates.

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

The protein composition of each allergen extract lot and mi ALK-Abelló mixes were analyzed using equal PNU/mL concentrations and volume (30 $\mu\text{L}/\text{lane}$) by SDS-PAGE. Extracts were diluted to the same PNU concentration and treated with the reducing agent, dithiothreitol (DTT), and loaded in buffer (#1610737 2x Laemmli Sample Buffer, Bio-Rad Laboratories, Inc., Hercules, CA, USA), then heated to $>95^{\circ}\text{C}$ for 5 min before loading into the wells of pre-cast 10-20% tris-glycine gels (#XP10200BOX, Novex™ WedgeWell, Life Science, Invitrogen Waltham, MA, USA). Electrophoresis was conducted 100 V/cm for 1 h to separate the proteins in 1X tris-glycine SDS running buffer (#LC2675, Novex™ Tris-Glycine SDS Running Buffer

[10X], Invitrogen, Waltham, MA, USA) and subsequently stained with coomassie stain (#LC6060, SimplyBlue™ SafeStain, Invitrogen, Waltham, MA, USA) to visualize linear bands. The size of the separated proteins were identified by molecular weight using a pre-stained protein standard (#161-0375, Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standard, Bio-Rad Laboratories, Hercules, CA, USA).

3.3.4 Animals

Twenty-five privately owned, clinically healthy dogs and ten purpose-bred beagles were used in this study. Privately owned dogs were a minimum age of two years old and lived in their current owner's homes for at least one year. A questionnaire (Figure 1) was used to ensure that none of the privately owned dogs had any history of pruritus, dermatologic and/or otic diseases. If an owner answered "yes" to any question regarding pruritus, cutaneous or otic lesions, history of antibiotic use for skin or ears, ocular or nasal discharge or vomiting and diarrhea, the dog was excluded from the study. A complete blood count and biochemistry panel were performed at the time of enrollment. Prior to the IDT, all privately owned dogs were fed a standardized diet (BLUE Basics® Grain-Free Turkey Potato Recipe, Blue Buffalo Co., Ltd.), with an omega 6:3 ratio of 7.8, and treats (BLUE Basics® Turkey & Potato Biscuits, Blue Buffalo Co., Ltd.) for a minimum of two months.

Purpose-bred beagles were a minimum of one year old at time of enrollment. They were kept in kennels that were sprayed down with water repeatedly throughout the day and cleaned thoroughly with a disinfectant scrub each morning. The dogs were kept on a rubber coated metal floor with plastic bowls for food and water, had interactive indestructible food toys filled with their diet, but had no blankets. All of the dog's medical records were evaluated

to ensure they did not have a history of dermatologic and otic disease. Only dogs without abnormalities on general physical, dermatological and otoscopic examinations were included. A complete blood count and biochemistry profile were not required for enrollment. All purpose-bred beagles were fed a standardized diet (Laboratory Canine Diet 5006*, LabDiet®), with an omega 6:3 ratio of 4.5, for a minimum of two months before enrollment.

Dogs could have never received antipruritic medications including topical, oral or injectable glucocorticoids, antihistamines, Staphage Lysate, pentoxifylline and tacrolimus. Dogs could have previously received nonsteroidal anti-inflammatories, essential fatty acid supplementation, gabapentin, monoamine oxidase inhibitors for reasons other than pruritus. All dogs had been without any medication that could influence intradermal testing for at least 8 weeks, including the aforementioned supplements and medications. All dogs were receiving monthly flea, tick and heartworm preventative.

3.3.5 Intradermal testing

All allergens and dilutions of allergens for IDT were stored in glass vials at 4°C as recommended by the manufacturer. The allergens were removed 10 min before the IDT was performed. Each of the 11 allergens from both companies were diluted with 0.4% phenolated saline (ALK-Abelló, Round Rock, TX, USA) to obtain four different test concentrations in PNU/mL and w/v (Table 2). Dilutions were re-made every four weeks as needed from their respective stock vials. All individual allergen dilutions contained at least two and up to three different lots of that specific allergen from the same company. The mixed lot allergens were used in all the dogs.

Dogs were sedated with dexmedetomidine hydrochloride (Dexdomitor[®]; Zoetis, Kalamazoo, MI, USA) at 8-12 µg/kg intravenously. The skin over the left or right ventro-lateral thorax wall was carefully clipped using a number 40 clipper blade (Oster[®] PowerPro[®] Ultra Cordless Clipper Kit, McMinnville, TN, USA). A waterproof permanent marker (Sharpie[®] Newell Rubbermaid Inc, Hoboken, NJ, USA) was used to create a testing template, which consisted of three horizontal rows of 20 dots. Intradermal injections of control solutions and allergens were made above and below the dots. Syringes (1 mL 27G; BD, Franklin Lakes, NJ, USA) with a permanently attached needle were preloaded with 0.25 mL of the saline diluted allergens to avoid under- or over injection. Each dog was then injected intradermally with 0.25 mL of 1:100,000 w/v histamine (Greer[®]) solution as the positive control^{20,394}, a negative control solution, 0.4% phenolated saline (ALK-Abelló) and 96 allergen dilutions using 1 mL syringes and 27 gauge (3/8 inch intradermal bevel) needles. New syringes preloaded with 0.25 mL of allergens were used for each dog.

Each skin test site was evaluated 15 min post-injection for evidence of a reaction. Reactions were evaluated and scored by the same investigator. Subjective scores used a scale of 0 (negative) to 4+ (maximum positive) based on wheal size, erythema and turgidity by comparison to the positive and negative controls.^{17,20} Allergen concentration values used for the statistical model that predicts TCs were derived from the highest concentration of an allergen used in the IDT where $\leq 10\%$ of dogs had a subjective reaction of $\geq 2+$ at 15 min. Concentrations of allergens that caused $>10\%$ of dogs to have positive subjective 15 min reactions were considered probable irritant reactions. An objective measurement of the

vertical and horizontal diameter of each reaction was also performed at 15 min following injection using digital calipers.

Intradermal testing of dogs was performed in two groups: group 1 (n=22) and group 2 (n=13). The concentrations of allergens used in group 1 were based on a pilot study that tested eight serial dilutions for each allergen in eight purpose-bred beagles and outbred hounds (results from the pilot study are not included). Intradermal testing dilutions were adjusted for group 2 when the percentage of dogs with significantly positive reactions ($\geq 2+$) to any allergen tested was $>10\%$ in group 1. Both groups 1 & 2 were tested with 96 allergen dilutions, 11 allergens used at four different concentrations from each manufacturer and *D. farinae* tested using four additional weight per volume (w/v) concentrations from each manufacturer. Due to expiration of five lots before completion of the study, all dogs in group 2 had three allergens (*D. farinae*, English plantain and mixed ragweed) with different lot numbers than were used for IDT in group 1, which were tested with the initial lots obtained at the start of the study.

Following IDT, a 1% hydrocortisone spray (MalAcetic[®] Ultra Spray Conditioner, Dechra, Overland Park, KS, USA) was applied to the test site and then an ice pack wrapped in a towel was applied for 10 min. Once testing was completed, sedation was reversed with atipamezole hydrochloride (Antisedan[®]; Zoetis, Parsippany, NJ, USA) at 5000 $\mu\text{g}/\text{m}^2$ intramuscularly in the epaxial muscles.

3.3.6 Statistical analyses

Descriptive statistics were performed to describe protein quantification and composition of each allergen extract lot. The TC was defined as the allergen dose which resulted in a positive reaction ($\geq 2+$) on $\leq 10\%$ of the dogs. Probit regression models were fit separately to the allergens from each company, using generalized estimating equations to account for the repeated measures on each dog. Subjective data results from both group 1 and group 2 were used to fit each model, with separate dose-response curves estimated for each group. The TC was estimated from each model for each group using the equation $(\Phi^{-1}(0.1) - \beta_0)/\beta_1$ where β_0 and β_1 are the intercept and slope coefficients from the logistic regression for the corresponding group and Φ^{-1} is the inverse of the standard normal cumulative distribution function. Standard errors of the TCs were reported along with the estimate. Concordance between a subjectively positive IDT reaction ($\geq 2+$) at 15 min with the objective measurement was determined using receiver operating characteristic (ROC) curves along with the corresponding area underneath the curve (AUC). The AUC, or concordance index (C-index), gives the probability that a randomly chosen dog with a subjective positive reaction will have a larger objective measurement than a randomly chosen dog with a subjective negative reaction. All analyses were carried out using R version 3.4.1 (R Foundation, Vienna, Austria) or SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

3.4 Results

3.4.1 Extract protein quantification and composition

Differences in the protein concentration of allergen extracts were observed within and between manufacturers despite equivalent PNU/mL labeling (Table 1). When evaluating extracts labeled as 40,000 PNU, the protein amount varied from as low as 210.5 µg/mL for American elm to 1117.2 µg/mL for timothy grass from ALK-Abelló allergens. Whereas for Greer® allergens labeled as 40,000 PNU protein concentration was from 231.0 µg/mL for white oak to 864.29 µg/mL for black walnut. The largest difference in protein concentrations between the same allergen extract lots was for ALK-Abelló. ALK-Abelló American elm extracts had a 1.1-fold difference (210.5-452.1 µg/mL). Most extracts from ALK-Abelló had small differences in protein concentrations between lots, as shown for timothy grass with a 0.4-fold difference (820.5-1117.2 µg/mL), black walnut with a 0.3-fold difference (925.54-1201.4 µg/mL), box elder with a 0.2-fold difference (279.46-341.8 µg/mL) and ragweed mix with a 0.1-fold difference (581.8-613.4 µg/mL). White oak had the most consistent protein concentrations with only a 0.03-fold difference (340.98-349.8 µg/mL) between lots.

The largest protein quantification differences within Greer® allergen extracts of 40,000 PNU was with black walnut lots demonstrating a 0.4-fold difference (638.8- 864.29 µg/mL). Similar to ALK-Abelló allergen extracts, most Greer® allergen extracts had comparable protein concentrations between lots as shown for English plantain with a 0.2-fold difference (368-434.9 µg/mL), ragweed mix with a 0.2-fold difference (639.88-789.8 µg/mL) and

timothy grass with a 0.05-fold difference (756.0-797.11 µg/mL). Box elder extract had nearly identical protein concentrations (373.1-373.7 µg/mL).

Between manufacturers, the protein concentration of the identical allergens designated to have 40,000 PNU also differed. The largest difference in protein concentration was identified in American elm with a 1.4-fold difference (210.5-513.1 µg/mL). Johnson grass had a 1-fold difference (262.79-522.3 µg/mL). Black walnut had a 0.9-fold difference (638.8-1201.4 µg/mL) whereas white oak (231-349.8 µg/mL) and timothy (756-1117.2 µg/mL) both had a 0.5-fold difference. Ragweed mix had a 0.4-fold difference (581.8-789.8 µg/mL). Box elder had a 0.3-fold difference (279.46-373.6 µg/mL). Lamb's quarter had the least fold difference at 0.1 (402.1-459.2 µg/mL).

The ratio of µg/1000 PNU for all allergen extracts ranged from 5 µg/1000 PNU to 41 µg/1000 PNU. When comparing ALK-Abelló extracts with at least two lots of the same allergen, the largest difference was detected in American elm with a 1.2-fold difference (5-11 µg/1000 PNU). Within Greer® extracts, the largest difference in the ratio of µg/1000 PNU between two lots was for black walnut extracts with a 0.4-fold difference (16-22 µg/1000 PNU). Between manufacturers, the largest difference in µg/1000 PNU ratio was identified in black walnut with a 0.9-fold difference (16-30 µg/1000 PNU).

The mean of ALK-Abelló to Greer® µg/1000 PNU ratio for each allergen extract ranged from 0.7 to 1.5. Black walnut had the largest mean µg/1000 PNU ratio difference with 1.5, whereas *D. farinae*, English plantain and Johnson grass had equal ratios. All other allergen extracts fell between 0.7 to 1.2 (Table 1).

The qualitative protein composition of all 11 extracts was compared by SDS-PAGE and subsequent coomassie staining. *Phleum pratense* (timothy grass) extracts (Figure 2) from both manufacturers had a comparable intensity in protein bands between ~ 31-34 kDa; however, the Greer® extract in lane 2 had an additional small band of ~ 30 kDa that was not present in the other extracts. Heterogeneity of protein bands between ALK-Abelló and Greer® extracts were identified ~50-70 kDa (Figure 2a). Greer® extracts (lanes 2-3) had lower molecular weight bands at 50 kDa that were absent in ALK-Abelló extracts (lanes 4-6). However, there was an additional band in the ALK- Abelló extracts between 50-70 kDa that was not present in the Greer® extracts. Despite loading equal amounts of protein by PNU, differences in intensity of bands can be seen between extracts from the same company, as demonstrated by the bands at ~68 kDa of lanes 2, 3, 5 and 6.

Overall, the bands for *Sorghum halepense* (Johnson grass) extracts (Figure 2b) from ALK-Abelló (lanes 4-7) were stronger in intensity compared to Greer® extracts (lanes 2-3). Notably, protein bands at ~30 kDa were nearly absent to faint in the Greer® extracts (lanes 2-3) whereas they were prominent in ALK-Abelló extracts (lanes 4-6), but less robust in the ALK extract shown in lane 7. Likewise, ALK-Abelló extracts in lanes 4-6 had a band at 50 kDa, but this was absent in Greer® extracts (lanes 2-3) and the ALK-Abelló extract in lane 7.

The extracts with the most similar protein compositions between manufacturers were lamb's quarter, ragweed mix and white oak (Figure 3f,g,i, respectively). The protein electrophoresis of the extracts American elm, black walnut, box elder, *D. farinae*, English

plantain, and red cedar showed heterogeneity in intensities for specific protein components within and between manufacturers (Figure 3a-e and h). The heterogeneity in protein extract components is highlighted by *D. farinae* extracts (Figure 3d). Distinct bands at ~14-15 kDa were noted for ALK-Abelló extracts (lanes 3-5), but not in the Greer[®] extract (lane 2). Proteins of 14 kDa correspond with the Group 2 allergen, MD-2-related protein. All *D. farinae* extracts show a range of very faint to strong protein bands between 150-250 kDa, a molecular weight range where the allergen, Zen-1 would be expected to fall within.

3.4.2 Animals

The privately owned dogs included 13 neutered males and 12 spayed females. Their ages ranged from 2 to 10 years of age (mean age 5.8 years). Breeds included American pit bull terrier (1), collie (1), German shepherd dog (3), German shorthair pointer (1), golden retriever (2), greyhound (3), mixed breed (5), pit bull mix (7), presa canario (1) and standard poodle (1). Complete blood counts and biochemical profiles performed at study inclusion had no significant abnormalities. The purpose-bred beagles included six intact males and four intact females with an age range of 1 to 3 years old (mean age 1.5 years). Group 1 included 22 client-owned dogs. Group 2 contained three client-owned dogs and 10 purpose-bred beagles. No further dogs were enrolled in Group 2, due to expiration of allergen extracts. At the time of enrollment and IDT, all dogs were systemically healthy with no cutaneous or mucosal abnormalities.

3.4.3 Fifteen-minute allergen extract threshold concentrations

The allergen extract dilutions used for Group 2 dogs provided the greater number of evaluable TCs compared to the dilutions used in Group 1 dogs (Table 2). Using the TC cut-off of $\leq 10\%$ positivity for the Group 2 dogs, the generalized estimating equations produced TCs for nine PNU/mL allergen extracts from ALK-Abelló and eight PNU/mL allergen extracts from Greer[®] (Table 3, Figure 4). The TCs ranged from 24-3930 PNU/mL for ALK-Abelló allergens and from 177-1760 PNU/mL for Greer[®] allergens. The standard error for the TCs for ALK-Abelló allergens ranged from 8.72 PNU/mL for *D. farinae* (24 ± 8.72 PNU/mL) to 33,000 PNU/mL for white oak ($3930 \pm 33,300$ PNU/mL). The standard error for the TCs for Greer[®] allergens ranged from 66.4 PNU/mL for timothy grass (177 ± 66.4 PNU/mL) to 9450 PNU/mL for ragweed mix (1760 ± 9450 PNU/mL). TCs could not be determined for the ALK-Abelló allergens of ragweed mix and English plantain and for the Greer[®] allergens of red cedar, white oak and *D. farinae*. When evaluating *D. farinae* extract as w/v concentrations, the TCs could not be determined for either manufacturer.

3.4.4 Comparison of subjective and objective IDT measurements

Receiver operating characteristic (ROC) curves were performed to evaluate the concordance of a subjectively positive IDT reaction ($\geq 2+$) at 15 min with the objective measurement. The area under the curve (AUC), the measure of test accuracy, was 0.7555 when the data from both companies was combined (Figure 5a). A percent concordance of 75.5% was interpreted to mean that a randomly selected dog that had a subjective positive reaction had a larger objective measurement than that of a randomly chosen dog from the

negative group 75.5% of the time. When the data from both companies was separately analyzed, the percent concordance was 77.3% for ALK-Abelló and 75% for Greer® (Figure 5b).

3.5 Discussion

Repeatable and clinically interpretable results obtained by performing an IDT are accomplished by using allergen extracts with consistent protein concentrations at appropriate testing concentrations. In this study, differences in protein concentration of allergen extracts manufactured by two different veterinary allergen suppliers were found in extracts labeled as having contents with the same genus and species of allergen and PNU/mL values within and between manufacturers. Based on this initial survey, lots from the same and different raw source materials should be analyzed on a larger scale for more accurate characterization of allergen extract protein composition. If protein concentrations are truly higher or lower than the purported extract value, this may negate IDT results through the creation of false negative and positive test reactions.

Traditional expression of allergen extract potency has been measured by PNU or w/v. However, each measure differently assesses the potency of an extract. Protein nitrogen values are measures of protein nitrogen in the extract, which is converted to units to give a PNU, whereas the ratio of w/v reflects the grams of the source material to volume of extraction solution.²⁰ A disadvantage of PNU is that extracts are only available in specific PNU concentrations (e.g. 10,000-40,000 PNU/mL) and this requires that extract be diluted from the strength obtained during the extraction process. Therefore, the

most potent PNU extract available might be weaker than the most concentrated w/v measure for any given allergen extract. Additionally, PNU determination may not be performed in the same manner by each allergen manufacturer, which could attribute to the difference in protein concentration observed in this study. A disadvantage of both PNU and w/v is that neither measurement directly assesses the total protein concentration in an extract. Both PNU/mL and w/v potency measures do not consider the contribution of major and minor allergens. Extract potency and therefore patient sensitivity on IDT are affected by the most prevalent major and minor allergens. Mass spectrometry represents a novel method to measure allergen extract potency. Mass spectrometry qualitatively and quantitatively identifies major and minor allergens and their isoforms in an extract by measuring individual peptide concentrations. This can be advantageous when standardizing allergen extracts as the potency of allergen extracts used for ASIT should be expressed in units describing clinical efficacy because there is no relationship between therapeutic dose and skin test potency.³⁹⁵ In dogs, there is a lack of homogenous placebo-controlled, randomized-prospective clinical trials comparing allergen dose, immunotherapy protocols and resultant efficacy.³¹⁶ In humans, an improved response to ASIT is observed with exposure to higher doses of allergen and when the optimal maintenance dose of the major allergen is 5-20 µg, which is a useful measure of quantification.³⁹⁶ Conversely, exposure to high dose allergens may also predispose to an increased occurrence of adverse reactions.^{358,396}

Factors to consider as causes of variation in skin test reactivity are not only protein concentration of the allergen extract, but also the heterogeneity of the protein

content due to allergen source material and manufacturing processes, and the stability of the protein in the extract. In human medicine, discrepancies in potency as documented by skin test reactions between manufacturers still exist. A European study evaluated, SPTs performed in humans with house dust mite allergy using equal concentrations of *Dermatophagoides pteronyssinus* produced by nine manufacturers. Like veterinary extracts, European mite extracts are not standardized and revealed inconsistent positive reactions and false-negative results. Of the mite allergic patients, 30% had a negative SPT with at least one of the extracts tested.³⁹⁷ Our study also found heterogeneity of non-standardized mite extracts. *D. farinae* protein content varied between allergen manufacturer. The TCs for *D. farinae* w/v extracts could not be determined, but the TC was found for PNU/mL extracts by ALK-Abelló. This suggests that the w/v TCs for mites are much different from the PNU TCs in normal dogs. Studies in dogs evaluating IDT using aqueous allergens from multiple manufacturers have also had inconsistent results. One study reported significant agreement and correlation in positive test results to four mite allergens in normal dogs when comparing allergen batches from different suppliers.²⁷⁴ A more recent study reported significant differences in subjective and objective measurements for three other allergens, *Alternaria*, cat dander and timothy grass, produced by two manufacturers.²³ Differences due to protein concentration and/or heterogeneity in each extract were not examined in either study.

Standardization of the multistep process of producing allergen extracts would regulate manufacturers and may provide a measure to help ensure more uniformity in IDT results and immunotherapy efficacy. The structures and properties of extract

components can be influenced by a large number of external factors including allergen source (phylogenetic, geographic), collection procedures (vacuum, cutting, drying, grinding), extraction conditions (time, temperature, pH, extraction fluid composition, degree of wetting, mixing), post-extraction processing steps (clarification, purification, concentration, sterilization), and storage conditions.³⁴⁶ Extracts manufactured by a single supplier vary from lot to lot, due to the nature of the source material being utilized and uncontrollable circumstances influencing natural products. We observed mild variability in protein composition between the same pollen and mite extracts made by the two manufacturers. However, given the small number of analyzed allergen lots, the identified variability in protein complexity may or may not make a difference in extract performance based on the inclusion or exclusion of immunodominant allergens. The translation of these differences into clinical outcomes of skin test reactivity expected from an IDT and clinical response to ASIT is unknown. European standards for human allergen production allows the total protein content of standardized allergens to range from 50 to 150% of the stated amount to account for variability in the source materials.¹⁹ Similar variation in standardized extracts is allowed in the US, as the range of the major allergen Fel d 1 content in cat extracts can contain 10-19.9 U/mL.²⁹¹

An IgE-mediated response in an allergic dog is caused by an allergenic protein. Intradermal testing in canines using extracts that contain the whole protein of the pollen, mold or mite has been considered the gold standard to identify IgE antibodies to environmental allergens. In humans, an allergen is classified as a "major allergen" if it is recognized by more than 50% of allergic individuals. Major allergens have been

determined for many grass, tree, weed, fungi, arthropod and dander allergens. It had been suggested that using major allergens to diagnose hypersensitivity, would reduce positive reactions due to cross-reactivity and allow formulation of immunotherapy based on individual proteins rather than total protein of an allergen.³⁹⁸ In dogs, identification of major allergens is in the early stages. Zen-1, an 188 kDa protein, has been suggested as a major allergen in atopic dogs sensitized to *D. farinae*.³³⁵ All *D. farinae* extracts in this study demonstrated a band between 150-250 kDa that falls within the size range inclusive of Zen-1 and may represent this protein. However, the recognized major allergen in humans and dogs for *D. farinae*, Der f2 MD-2-related protein, a protein of 14 kDa, was absent in the Greer[®] extract lot used for the electrophoresis analysis, but was present in the ALK extract lot.³⁹⁹ By using extracts that lack a major allergen, false negative results may occur during IDT. Conclusive absence of Der f2 is made by directly measuring Der f2 in the Greer[®] extracts by Western blot analysis, but this was not evaluated by this study.

Two populations of clinically healthy dogs were used to determine the 15 min TCs of 11 environmental allergens. Client-owned dogs were considered non-atopic based on the pre-enrollment owner questionnaire, physical and dermatologic examination findings and complete bloodwork. Purpose-bred beagles were deemed non-atopic based on their medical history physical exam and origin of derivation from well-characterized purpose-bred stock. Although all client-owned dogs were greater than 2 years of age, it is impossible to definitely say that none of the enrolled dogs would not exhibit allergic signs in the future. The ideal study would evaluate TCs in purpose-bred pathogen-free dogs. In other studies evaluating TCs in clinically normal dogs, exact TCs could not be

determined or were much higher than what was found in this study.^{21,22,274} In the only study evaluating TCs in extracts manufactured by ALK-Abelló, exact TCs could not be determined for lamb's quarter or Timothy grass.²³ Potentially, by combining both populations of animals, a more precise evaluation of TC has been accomplished.

Similar to allergen extracts, there is no standard method of scoring IDT. Both objective measurements and subjective scoring (0-4+) are used in grading IDT. A subjective score is based on the perceived size, erythema and turgidity of the wheal.¹⁷ A previous study revealed poor interobserver agreement of subjective scoring alone.²³ Combining subjective and objective measurements theoretically should lessen variability of IDT grading. Using ROC analyses, we observed at least a 75% agreement between a positive subjective reaction and objective measurement in normal dogs. Our results paralleled the findings in a study which found a moderate level of correlation between the subjective and objective scores in atopic dogs.²⁶⁷ When considering the results of our ROC curves in normal dogs and the previous study in atopic dogs, these would imply that combining subjective and objective scores and equally weighting size, erythema and turgidity, that the incidence of perceived false-positive and false-negative results may be reduced.

There are recognized limitations of this study. The first limitation is that the sample size contributed to the high standard errors of the calculated TCs. The standard error depends on the standard deviation, a measure of variability of the values obtained and the sample size.⁴⁰⁰ With a larger sample size, the standard error could have been reduced as the chance of variation is minimized. The different total protein

concentrations and heterogeneity of allergen extracts could have contributed to the larger standard errors as well. Other limitations were associated with the recruitment length, the eight-week controlled diet enrollment criteria and allergen lot expiration. Five allergen lots expired between the time the IDTs were performed in group 1 and the time they were performed in group 2. As we found differences in protein concentration and composition between lots of the allergen extracts from the same manufacturer, this may have been reflected in the IDT results for group 2 and the overall TCs. Allergen extracts also have different proteolytic activity, which can lead to heightened or decrease degradation, which may ultimately affect IDT reactivity over time. A final limitation is that PNU/mL were used as the predominate unit of measure for the IDT concentrations. The amount of PNU per allergen is influenced by the extraction procedure and evaluation of PNU content in each extract varies according to the method of measurement. Therefore, using only allergens with standardized reference standards would be ideal for use in determining TCs. However, since PNU and w/v labeled non-standardized extracts are used in clinical practice, this study applies to a practical setting.

In conclusion, this study showed that protein concentration and composition varied between lots of allergen extracts and by manufacturer in the limited number of analyzed allergen extracts. This study also determined TCs for allergens using a novel statistical model from an existing and new veterinary allergy manufacturer. We recognize that basing TCs purely on results from clinically normal outbred and inbred dogs poses an inherent risk of miscalculating the “irritant” TC in a hypersensitive patient, as these may deviate from the TCs identified in normal animals. Therefore, studies are needed in

atopic dogs with defined hypersensitivities to the allergens used in this study to identify the allergy detection TCs. This concept would be similar to the "IntraDermal dilution for 50 mm sum of Erythema determines the bioequivalent ALlegry units" (ID₅₀EAL) testing method used to designate a bioequivalent allergy unit (BAU/mL) of biological potency of an allergen. The BAU measures allergenicity of vaccines regardless of source. Crude non- standardized- aqueous allergen extracts are inherently biologically complex mixtures that differ in potency and content between companies. The specialty of veterinary dermatology would benefit from future studies that determine if these qualities affect IDT results and subsequent response to ASIT.

Table 1. Allergen extracts and total protein content.

Extract	w/v formulation	Manufacturer	Lot number	Vial volume	Expiration date	PNU value	Total protein concentration (µg/mL)	Standard deviation	Ratio µg/1000 PNU	Average ALK-Abelló to Greer® µg/1000 PNU ratio
American elm		Greer®	292825	50 mL	7/13/17	40,000	513.1	28.8	13	
		Greer®	290642	10 mL	8/15/17	20,000	NE			
		ALK-Abelló	Mix			40,000	575.39	23.7	14	0.8
	1:10	ALK-Abelló	1914827	10 mL	6/14/17	76,000	827.7	39.5	11	
		ALK-Abelló	1769694	10 mL	6/14/17	40,000	210.5	10.2	5	
Black walnut		ALK-Abelló	1622000	50 mL	5/30/17	40,000	452.1	72.5	11	
		Greer®	272840	30 mL	7/13/17	40,000	864.29	188.6	22	
		Greer®	279332	10 mL	11/1/17	40,000	638.8	96.2	16	
		ALK-Abelló	Mix			40,000	1542.0	160.2	39	1.5
		ALK-Abelló	1855732	10 mL	6/10/17	40,000	925.54	77.5	23	
		ALK-Abelló	1760608	10 mL	6/14/17	70,000	1352.6	207.7	19	
		ALK-Abelló	1754045	50 mL	5/30/17	40,000	1201.4	172.6	30	
Box elder		Greer®	284729	10 mL	5/17/17	40,000	373.7	46.7	9	
		Greer®	285621	30 mL	5/17/17	40,000	373.1	38.6	9	
		ALK-Abelló	Mix			40,000	308.7	22.7	8	0.9
		ALK-Abelló	1660444	50 mL	5/30/17	40,000	291.9	14.8	7	
		ALK-Abelló	1855733	50 mL	5/30/17	40,000	279.46	22.8	7	
		ALK-Abelló	1855734	50 mL	5/30/17	40,000	341.8	11.2	9	
		ALK-Abelló	1855734	50 mL	5/30/17	40,000	341.8	11.2	9	
<i>D. farinae</i>	1:100	Greer®	284410	30 mL	7/13/17	12,000	372.69	39.0	19	
	1:100	Greer®	289275	10 mL	8/11/17	12,000	NE			
		ALK-Abelló	Mix				392.9	57.2	18	
	1:50	ALK-Abelló	1153655	50 mL	12/18/16	22,000	382.4	36.0	17	1.0
	1:50	ALK-Abelló	1459535	50 mL	5/30/17	25,000	521.8	103.5	21	
	1:50	ALK-Abelló	2293574	10 mL	8/6/18	20,000	NE			
		ALK-Abelló	2293574	10 mL	8/6/18	20,000	NE			
English plantain		Greer®	288179	30 mL	7/13/17	40,000	368.4	36.1	9	
		Greer®	280811	10 mL	12/13/17	40,000	434.9	46.6	11	
		ALK-Abelló	Mix			20,000	360.66	20.1	18	1.0
	1:10	ALK-Abelló	1863072	10 mL	6/14/17	61,000	493.0	65.4	8	
		ALK-Abelló	1459581	50 mL	5/30/17	40,000	323.2	14.9	8	
		ALK-Abelló	1643775	50 mL	1/7/17	20,000	122.5	16.9	6	
	1:10	ALK-Abelló	2320391	10 mL	8/6/18	53,000	NE			
Johnson grass		Greer®	277588	30 mL	7/13/17	40,000	522.3	43.8	13	
		Greer®	287540	10 mL	2/2/18	20,000	232.73	25.6	12	
		ALK-Abelló	Mix			40,000	817.0	24.0	20	1.0
	1:10	ALK-Abelló	1475181	10 mL	6/9/17	126,000	1573.5	204.9	12	
	1:10	ALK-Abelló	1815590	10 mL	6/14/17	117,000	1174.01	222.4	10	
		ALK-Abelló	1512897	50 mL	5/30/17	40,000	262.79	31.7	7	

Continued

Table 1 continued

Lamb's quarter		Greer®	287494	10 mL	2/18/18	20,000	204.6	15.5	10	
		Greer®	293948	30 mL	7/13/17	40,000	459.2	58.0	11	
		ALK-Abelló	Mix			40,000	538.9	39.8	13	1.2
	1:10	ALK-Abelló	1745060	10 mL	6/14/17	59,000	922.4	93.7	16	
		ALK-Abelló	1655743	50 mL	5/30/17	40,000	402.1	10.4	10	
Mixed ragweed		Greer®	279390	30 mL	7/13/17	40,000	789.8	43.1	20	
		Greer®	286862	10 mL	2/7/18	40,000	639.88	63.4	16	
		ALK-Abelló	Mix			40,000	1019.64	80.6	26	1.1
	1:10	ALK-Abelló	1810076	10 mL	12/5/16	117,000	2606.4	200.6	22	
		ALK-Abelló	1802842	50 mL	11/30/16	40,000	613.4	26.6	15	
		ALK-Abelló	1810066	50 mL	10/30/16	40,000	581.8	20.7	15	
	1:10	ALK-Abelló	2339860	10 mL	12/22/17	129,000	NE			
	1:10	ALK-Abelló	2563885	10 mL	2/6/18	128,000	NE			
Red cedar	1:10	Greer®	285427	10 mL	3/28/18	8,600	326.9	49.9	38	
	1:10	Greer®	289096	30 mL	7/13/17	9,300	350.2	54.6	41	
		ALK-Abelló	Mix			7,000	214.8	26.0	31	0.7
		ALK-Abelló	1741124	50 mL	5/30/17	7,000	170.5	13.2	24	
		ALK-Abelló	1802869	50 mL	5/30/17	8,000	226.2	23.3	28	
Timothy grass		Greer®	278062	30 mL	7/13/17	40,000	756.0	38.9	19	
		Greer®	288475	10 mL	7/4/18	40,000	797.11	146.1	20	
		ALK-Abelló	Mix			40,000	794.8	19.8	20	1.2
		ALK-Abelló	1701311	50 mL	5/30/17	40,000	820.5	106.2	21	
		ALK-Abelló	1817751	50 mL	5/30/17	40,000	1117.2	85.4	28	
White oak		Greer®	278633	50 mL	7/27/17	20,000	402.6	79.6	12	
		Greer®	294247	30 mL	7/13/17	40,000	231.0	12.3	10	
		ALK-Abelló	Mix			40,000	466.7	49.6	12	0.9
	1:10	ALK-Abelló	1576733	10 mL	6/14/17	76,000	870.1	5.7	11	
		ALK-Abelló	1601290	50 mL	5/30/17	40,000	340.98	72.6	9	
		ALK-Abelló	184079	50 mL	5/30/17	40,000	349.8	33.6	9	

Comparison of total protein concentrations of allergen extract lots from ALK-Abelló and Greer. Several allergens were labelled with both w/v and PNU/mL values. Mixed allergens were composed of at least two allergen extracts of different lots from the respective manufacturer. Allergen extracts with NE were obtained after protein analysis by Bradford style assay was performed. The average ALK-Abelló to Greer® µg/1000 PNU ratio was calculated by dividing the average ratio of µg/1000:PNU of each allergen for ALK-Abelló by the same value for Greer®. Concentrations are expressed in weight/volume (w/v) and protein nitrogen units/mL (PNU/mL). NE, not evaluated.

Figure 1. Owner Questionnaire

1. Has your dog lived in central Ohio for at least 1 year?
2. Does your dog itch, scratch, lick, chew, bite at his/her skin?
3. Does your dog stop eating to scratch?
4. Does your dog stop playing to scratch?
5. Does your dog lick or chew the paws?
6. Does your dog have unusual hair loss?
7. Does your dog have red skin?
8. Does your dog itch his/her ears?
9. Does your dog rub his/her face or ears on furniture or carpets?
10. Has your dog ever received ear cleaners or medications?
11. Does your dog shake its head daily?
12. Has your dog's skin or ears ever been evaluated by a veterinarian?
13. Has your dog ever been on antibiotics for a skin infection?
14. Does your dog have runny eyes?
15. Does your dog sneeze?
16. Does your dog have vomiting or diarrhea?
17. Does your dog scoot?
18. Does your dog lick her/his genital region?
19. Has your dog ever had a hot spot?
20. Is your dog on flea control?
21. Have you ever seen a rash on your pet?
22. Have you ever seen ear discharge from your dog?
23. Does your dog chew its claws?

Table 2. Intradermal test allergen extract concentrations

	IDT CONCENTRATION RANGES			
	Group 1		Group 2	
Allergen	ALK	Greer	ALK	Greer
American elm	1250-2000 PNU/mL	400-1000 PNU/mL	312.5-1500 PNU/mL	100-600 PNU/mL
Black walnut	750-1500 PNU/mL	400-1000 PNU/mL	187.5-1000 PNU/mL	100-600 PNU/mL
Box elder	600-1200 PNU/mL	400-1000 PNU/mL	150-800 PNU/mL	100-600 PNU/mL
<i>D. farinae</i>	20-50 PNU/mL	20-50 PNU/mL	5-30 PNU/mL	5-30 PNU/mL
<i>D. farinae</i>	1:10,000 - 1:100,000 w/v	1:10,000 - 1:100,000 w/v	1:25,000 - 1:200,000 w/v	1:25,000 - 1:200,000 w/v
English plantain	5000-8000 PNU/mL	200-800 PNU/mL	1250-6000 PNU/mL	50-400 PNU/mL
Johnson grass	100-250 PNU/mL	100-250 PNU/mL	25-150 PNU/mL	25-200 PNU/mL
Lamb's quarter	1000-4000 PNU/mL	1000-4000 PNU/mL	250-2000 PNU/mL	250-2000 PNU/mL
Ragweed mix	200-1000 PNU/mL	1500-2250 PNU/mL	50-400 PNU/mL	375-1750 PNU/mL
Red cedar	750-1500 PNU/mL	750-1500 PNU/mL	187.5-1000 PNU/mL	187.5-1000 PNU/mL
Timothy grass	600-1200 PNU/mL	250-400 PNU/mL	150-800 PNU/mL	62.5-300 PNU/mL
White oak	750-1500 PNU/mL	200-800 PNU/mL	187.5-1000 PNU/mL	50-400 PNU/mL

Each allergen was diluted with 0.4% phenolated saline to four concentrations within the listed range. PNU, protein nitrogen unit; w/v, weight per volume.

Figure 2. SDS-PAGE analysis of *Phleum pretense* (timothy grass) (a) and *Sorghum halepense* (Johnson grass) (b).

SDS-PAGE analysis of *Phleum pretense* (timothy grass) (a) and *Sorghum halepense* (Johnson grass) (b). Protein concentration of *Phleum pretense* extracts were 10,000 PNU per lane and the protein content of *Sorghum halepense* extracts were evaluated at 20,000 PNU per lane. (a) Lanes: 1) Ladder. 2) Greer[®] timothy grass Lot #278062. 3) Greer[®] timothy grass Lot #288475. 4) ALK-Abelló timothy grass mix. 5) ALK-Abelló timothy grass Lot #1701311. 6) timothy grass Lot #1817751. (b) Lanes: 1) Ladder. 2) Greer[®] Johnson grass, Lot #277588. 3) Greer[®] Johnson grass, Lot #287540. 4) ALK-Abelló Johnson grass mix. 5) ALK-Abelló Johnson Lot #1475181. 6) ALK-Abelló Johnson Lot #1815590. 7) ALK-Abelló Johnson Lot #1512897. Mixed allergens were composed of at least two allergen extracts of different lots from the respective manufacturer.

Figure 3a-i. SDS-PAGE analysis of allergen extracts

(a) SDS-PAGE analysis of *Ulmus Americana* (American elm). Protein concentration of *Ulmus Americana* extracts were 20,000 PNU per lane. Lanes: 1) Ladder. 2) Greer® American elm Lot #292825. 3) ALK-Abelló American elm mix. 4) ALK-Abelló American elm Lot #1914827. 5) ALK-Abelló American elm Lot #1769694. 6) ALK-Abelló American elm Lot #1622000. Mixed allergens were composed of at least two allergen extracts of different lots from the respective manufacturer.

continued

Figure 3 continued

(b) SDS-PAGE analysis of *Juglans nigra* (black walnut). Protein concentration of *Juglans nigra* extracts were 20,000 PNU per lane. Lanes: 1) Ladder. 2) Greer® black walnut Lot #272840. 3) Greer® black walnut Lot #279332. 4) ALK-Abelló black walnut mix. 5) ALK-Abelló black walnut Lot #1855732. 6) ALK-Abelló black walnut Lot #1760608. 7) ALK-Abelló black walnut Lot #1754045. Mixed allergens were composed of at least two allergen extracts of different lots from the respective manufacturer.

continued

Figure 3 continued

(c) SDS-PAGE analysis of *Acer negundo* (box elder). Protein concentration of *Acer negundo* extracts were 20,000 PNU per lane. Lanes: 1) Ladder. 2) Greer[®] box elder Lot #284729. 3) Greer[®] box elder Lot #285621. 4) ALK-Abelló box elder mix. 5) ALK-Abelló box elder Lot #1660444. 6) ALK-Abelló box elder Lot #1855733. 7) ALK-Abelló box elder Lot #1855734. Mixed allergens were composed of at least two allergen extracts of different lots from the respective manufacturer.

continued

Figure 3 continued

(d) SDS-PAGE analysis of *Dermatophagoides farinae*. Protein concentration of *D. farinae* extracts were 10,000 PNU per lane. Lanes: 1) Ladder. 2) Greer® *D. farinae* Lot #284410. 3) ALK-Abelló *D. farinae* mix. 4) ALK-Abelló *D. farinae* Lot #1153655. 5) ALK-Abelló *D. farinae* Lot #1459535. 6) Ladder. Mixed allergens were composed of at least two allergen extracts of different lots from the respective manufacturer.

continued

Figure 3 continued

(e) SDS-PAGE analysis of *Plantago lanceolata* (English plantain). Protein concentration of *Plantago lanceolata* extracts were 10,000 PNU per lane. Lanes: 1) Ladder. 2) Greer® English plantain Lot #288179. 3) Greer® English plantain Lot #280811. 4) ALK-Abelló English plantain mix. 5) ALK-Abelló English plantain Lot #11863072. 6) ALK-Abelló English plantain Lot #1459581. 7) ALK-Abelló English plantain Lot #1643775. Mixed allergens were composed of at least two allergen extracts of different lots from the respective manufacturer.

continued

Figure 3 continued

(f) SDS-PAGE analysis of *Chenopodium album* (lamb's quarter). Protein concentration of *Chenopodium album* extracts were 10,000 PNU per lane. Lanes: 1) Ladder. 2) Greer[®] lamb's quarter Lot #287494. 3) Greer[®] lamb's quarter Lot #293948. 4) ALK-Abelló lamb's quarter mix. 5) ALK-Abelló lamb's quarter Lot #1745060. 6) ALK-Abelló lamb's quarter Lot #1655743. Mixed allergens were composed of at least two allergen extracts of different lots from the respective manufacturer.

continued

Figure 3 continued

(g) SDS-PAGE analysis of *Ambrosia* spp. (mixed ragweed). Protein concentration of *Ambrosia* spp. extracts were 20,000 PNU per lane. Lanes: 1) Ladder. 2) Greer® mixed ragweed Lot #279390. 3) Greer® mixed ragweed Lot #286862. 4) ALK-Abelló mixed ragweed mix. 5) ALK-Abelló mixed ragweed Lot #1810076. 6) ALK-Abelló mixed ragweed Lot #1802842. 7) ALK-Abelló mixed ragweed Lot #1810066. Mixed allergens were composed of at least two allergen extracts of different lots from the respective manufacturer.

continued

Figure 3 continued

(h) SDS-PAGE analysis of *Juniperus virginiana* (red cedar). Protein concentration of *Juniperus virginiana* extracts were 3,500 PNU per lane. Lanes: 1) Ladder. 2) Greer® red cedar Lot #285427. 3) Greer® red cedar Lot #289096. 4) ALK-Abelló red cedar mix. 5) ALK-Abelló red cedar Lot #1741124. 6) ALK-Abelló red cedar Lot #1802869. Mixed allergens were composed of at least two allergen extracts of different lots from the respective manufacturer.

continued

Figure 3 continued

(i) SDS-PAGE analysis of *Quercus alba*. (white oak). Protein concentration of *Quercus alba* extracts were 10,000 PNU per lane. Lanes: 1) Ladder. 2) Greer® white oak Lot #278633. 3) Greer® white oak Lot #294247. 4) ALK-Abelló white oak mix. 5) ALK-Abelló white oak Lot #1576733. 6) ALK-Abelló white oak Lot #1601290. 7) ALK-Abelló white oak Lot #1840799. Mixed allergens were composed of at least two allergen extracts of different lots from the respective manufacturer.

Table 3. Comparison of statistically derived allergen extract threshold concentrations from two manufacturers.

Allergen Extract	ALLERGEN THRESHOLD CONCENTRATIONS	
	ALK-Abelló (PNU/mL $\pm$ SE)	Greer® (PNU/mL $\pm$ SE)
Ragweed, mixed	†	1760 ($\pm$ 9450)
Lamb's quarter	1420 ($\pm$ 286)	846 ($\pm$ 244)
English plantain	†	303 ($\pm$ 269)
American elm	786 ($\pm$ 836)	216 ($\pm$ 400)
Black walnut	890 ($\pm$ 450)	605 ($\pm$ 2310)
Box elder	576 ($\pm$ 108)	583 ($\pm$ 3380)
Red cedar	613 ($\pm$ 321)	†
White oak	3930 ($\pm$ 33,300)	†
Johnson grass	92 ($\pm$ 19.2)	220 ($\pm$ 427)
Timothy grass	146	177 ($\pm$ 66.4)
<i>D. farinae</i>	24 ($\pm$ 8.72)	†

Concentrations expressed as protein nitrogen unit/milliliter (PNU/mL); $\pm$ SE. standard error; †, not determined as fitted values from the estimating equations did not reach the 10% response threshold.

Figure 4. Plots of fitted lines based on generalized estimating equations for each group of dogs.

Plots of observed proportions (solid circles) and fitted lines for each allergen. Fitted lines were based on probit models using generalized estimating equations fitted to the proportions for each group of dogs (group 1: n = 22 dogs; group 2: n = 13 dogs). Protein nitrogen unit (PNU), Ragweed mix (Ragweed), Lamb's quarter (Lamb's quart) and English plantain (English plan).

continued

Figure 4 continued

Plots of observed proportions (solid circles) and fitted lines for each allergen. Fitted lines were based on probit models using generalized estimating equations fitted to the proportions for each group of dogs (group 1: n = 22 dogs; group 2: n = 13 dogs). Protein nitrogen unit (PNU).

continued

Figure 4 continued

Plots of observed proportions (solid circles) and fitted lines for each allergen. Fitted lines were based on probit models using generalized estimating equations fitted to the proportions for each group of dogs (group 1: n = 22 dogs; group 2: n = 13 dogs). Protein nitrogen unit (PNU), Johnson grass (Johnson grass).

continued

Figure 4 continued

Plots of observed proportions (solid circles) and fitted lines for each allergen. Fitted lines were based on probit models using generalized estimating equations fitted to the proportions for each group of dogs (group 1: n = 22 dogs; group 2: n = 13 dogs). Protein nitrogen unit (PNU), weight per volume (w/v), *Dermatophagoides farinae* (D. farinae)

Figure 5. Receiver operating curves (ROC) for percent concordance of IDT subjectively positive reactions ($\geq 2+$) and objective measurements (mm).

Receiver operating curves (ROC) for percent concordance of IDT subjectively positive reactions ($\geq 2+$) and objective measurements (mm). (a) The area under the curve (AUC) when combining results from both manufacturers. (b) The AUC for ALK-Abelló (blue) and the AUC for Greer® (red). The black diagonal line (random) extending from 0,0 to 1,1 represents the benchmark for matching a subjective result with a larger objective measurement based on random chance alone.

Chapter 4. Conclusion and Future Direction

The present study evaluated allergen extracts from two manufactures and found differences in protein concentration and composition by a Bradford-style assay and SDS-PAGE. Major limitations of natural allergen extracts are the potential for variable composition and allergen content. Quality of the allergen extracts has the potential to affect allergen sensitization identification and treatment outcomes in ASIT. The other finding of this study was that TCs were also different between manufacturers. Interestingly, American elm had the largest difference in protein concentration between manufacturers, with a 1.4-fold difference and the greatest variation in TC, with a 2.6-fold difference between manufacturers. However, American elm allergen extracts showed similar protein compositions regardless of manufacturer or lot. Suggesting, this variability may have occurred due to various allergen extract potency, major allergens and patient differences.

Many factors intrinsic and extrinsic factors contribute to allergen extract potency such as the source of raw materials, extraction procedures, extract manipulation and length of storage of allergen extracts within veterinary clinics. The allergen extracts were measured at the beginning of the study; however, IDT was performed throughout a year-long period. Allergen extracts can lose potency due to inherent proteases and by adsorption to the holding container. As the allergen extract potency was not measured at

the end of the study, the amount of degradation is not known. It is possible that the extracts from the different companies have varying levels of proteases and definitely protein content, thereby potentially affecting the allergen potency over time.

Both PNU and w/v extracts were analyzed and compared in this study. However, these measurements may also not be appropriate in reflecting potency, protein content and composition of allergen extracts. We demonstrated that extracts labelled with the same PNU and w/v had very different protein contents and composition. Furthermore, neither PNU or w/v consider the content of major and minor allergens. Newer studies should focus on determining the important major and minor allergens in dogs, as they have been shown to differ from humans. This then may allow for the standardization of veterinary allergens and thereby reduce variation.

The current method of determining TCs of allergenic extracts may be inherently flawed due to the variability demonstrated in allergen extracts. Currently TCs are established by performing IDT in normal healthy dogs with arbitrary serial dilutions of allergen extracts. At best, this method can provide a range for the optimal TC. We investigated TCs with a mathematic model to provide exact values. TCs were not found for every allergen, likely due to the small number of dogs used in this study. However, the TCs that were obtained, differ from the concentrations used in clinical practice.

Additional studies with more lots of allergen extracts would be needed to confirm the heterogeneity in protein concentration and composition that was observed in this study. To develop a better understanding of the rate of allergen extract protein degradation and the potential influence on TCs, analysis of protein concentration,

composition and IDT should be performed at two time points. In conclusion, future studies with larger numbers of dogs, should compare IDT reactivity between both privately owned, purpose-bred and atopic dogs to determine the best model to study TC and potentially aid in the standardization of veterinary allergen extracts.

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