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New insights into the pathogenesis and therapy of adverse food reactions in dogs

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“To be successful, the first thing to do is fall in love with your work”

Sister Mary Lauretta

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List of abbreviations

7-AAD	7-aminoactinomycin D
AA	Arachidonic acid
AD	Atopic dermatitis
AFR	Adverse food reaction
AG	Specific antigen
ALP	Alkaline phosphatase
ALT	Alanine transferase
APCs	Antigen presenting cells
Ara h 1	Arachis hypogaea 1
ASIT	Allergen-specific immunotherapy
AST	Aspartate transferase
B-cell	B lymphocytes
Ber e 1	Bertholletia excelsa 1
Bos d 6	Bos domesticus 6
Bos d 7	Bos domesticus 7
BSA	Bovin serum albumin
BW	Body weight
C. diet	Commercial diet
C+H diet	Commercial and home-made diet
CAD	Canine atopic dermatitis
CADESI	Canine Atopic Dermatitis Extent and Severity Index
CAFR	Canine adverse food reactions
CCD	Cross-reactive carbohydrate determinants
CD	Cluster of Differentiation
CD4+ CD25+ cells	CD4+ CD25+ regulatory T lymphocytes
CD4+ cells	CD4+ helper T lymphocytes
CD8+ cells	CD8+ cytotoxic T lymphocytes

CLA	Cutaneous lymphocyte antigen
CM	Cow milk
CO_2	Carbon dioxide
Con A	Concanavalin A
Cor a 1	Corylus avellana 1
Cor a 8	Corylus avellana 8
DBPC	Double-blind, placebo controlled
DCs	Dendritic cells
DN T cells	Double negative T cells (CD4-,CD8-)
DP T cells	Double positive T cells (CD4+,CD8+)
EFAs	Essential fatty acids
ELISA	Enzyme-Linked Immunosorbent Assay
EPIT	Epicutaneous immunotherapy
FA-SLIT	Food allergen-specific sublingual immunotherapy
FA	Food allergy
FAD	Flea allergy dermatitis
FAS system	Cell-surface FAS receptor /APO-1,CD95 and FAS ligand
FCS	Faecal consistency scores
FCS	Fetal calf serum
FcεRII	low affinity receptor for IgE
FcεRI	Cellular Fc receptor
FIAD	Food induced atopic dermatitis
Foxp3	Forkhead box P3
FPIES	Food protein induced enterocolitis syndrome
FRE	Food responsive enteropathy
Gal d 1	Gallus domesticus 1
Gal d 2	Gallus domesticus 2
GALT	Gut Associated Lymphoid Tissue
GBSS-1	Granule-bound starch synthase-1

GI	Gastrointestinal
Gly m 5	B-conglycinin α chain
Gly m 6	Glycin
GLUB	Glutelin type B
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gr	Grams
H. diet	Home-made diet
HTY	Healthy
ICAM-1	Intercellular adhesion molecule-1
IDTv	Intradermal test
IELs	Intraepithelial lymphocytes
IFAs	Interfollicular areas
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgAs	Secretory immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG4	Immunoglobulin G subclass 4
IgM	Immunoglobulin M
IL	Interleukin
ITT	Intention-to-treat
JAK1	Janus kinase 1
Jug 2	Juglans regia 2
kDa	Kilodalton
kg	Kilograms
LA	Linoleic acid
LCT	Long chain triglycerides
LPS	Lipopolysaccharides
LPT	Lymphocyte proliferation test
M cells	Microfold cells

mAb	Monoclonal antibody
mg	Milligrams
MHC	Major histocompatibility complex
MIICs	Major histocompatibility complex class II-enriched compartments
MLNs	Mesenteric lymph nodes
mRNA	
n	Number of dogs
NFIAD	Non-food induced atopic dermatitis
NFICAD	Non-food-induced canine atopic dermatitis
OD	Optical density
OFC	Oral food challenge
OIT	Oral immunotherapy
OVA	Ovalbumin
P group	Placebo group
P/S	Penicillin/streptomycin
PAMP	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
pH	Potential of hydrogen
PNU	Protein nitrogen units
PP	Peyer's patches
PUFA	Polyunsaturated fatty acid
PUV	Pulsed ultraviolet
PVAS	Pruritus Visual Analogue Scale
QoL	Quality of life
RBC	Red blood cells

RCT	Randomized controlled trial
SBP	Sucrose-binding protein
SCD	Successfully consumed dose
SCIT	Subcutaneous immunotherapy
SD	Standard deviation
SLIT	Sublingual immunotherapy
SOCS-3	Suppressor of cytokine signaling 3
Sol t 2	*Solano tuberous* aspartic protease inhibitor
SSRI	Selective serotonin reuptake inhibitor
T cells	T lymphocytes
T group	Treatment group (FA-SLIT)
T-AT	Pollen toxin-antitoxin
tbs	Table spoons
TGF- β	Transforming growth factor beta
TGF	Transforming growth factor
Th1	T helper 1 lymphocytes
Th2	T helper 2 lymphocytes
Th3	T helper 3 lymphocytes
TLR	Toll-like receptors
Tr1	Type 1 regulatory T lymphocytes
Treg	Regulatory T cells
TSB	Tryptone Soya Broth
γδTCR	γδ T cell receptor
μg	Micrograms
μL:	Microliters

Chapter I:
Literature review

1. Adverse food reactions

1.1. Introduction

In this literature review, levels of evidence (LoE) are assigned to studies based on the methodological quality of their design and validity (Table 1.1).

Table 1.1: Levels of evidence (Adapted from Sackett et al. 2000)

LoEI	Individual randomized controlled studies (RCTs) Systematic reviews of RCTs
LoEII	Individual cohort studies and low quality RCTs Systematic reviews of cohort studies
LoEIII	Systematic reviews of case-controlled studies Individual case-controlled studies
LoEIV	Case series and poor quality cohort and case-control studies
LoEV	Expert opinion based on clinical experience

Hypersensitivity skin diseases in dogs fall into three main categories: canine atopic dermatitis (CAD), adverse food reaction and flea allergy dermatitis (FAD). This distinction is based on the causative agent. The former is an inflammatory reaction against environmental allergens, AFR against food and the latter against fleas. Since food driven skin disease may look indistinguishable from the one triggered by environmental allergens, the traditional separation between AFR and CAD may be no longer an appropriate one. Thus, we should not refer to CAD as a disease but rather as a clinical dermatological manifestation of an allergy, therefore including all those cases with environmental allergy, flea allergy and food adverse reactions that present characteristic cutaneous signs (Marsella and De Benedetto 2017) (LoEII). However, for an easier reading of this thesis, we will use the term CAD only to refer to pruritic allergic skin disease triggered by pollens and other environmental allergens.

The term “adverse food reaction” (AFR) refers to any abnormal clinical reaction due to ingestion of food or food additives. AFR can be divided into toxic and non-toxic reactions to food (Figure 1.1) (Bruijnzeel-Koomen et al. 1995; Cortinovis et al. 2016). The former reactions can occur in everybody, are dose-dependent and are provoked by substances that are natural constituents of food (e.g. sulfoxide in fresh garlic), that are present after food preparation or contamination (e.g. aflatoxins in corn). Non-toxic

AFR depend on an individual's susceptibility to develop AFR upon ingesting food. These are either non immune-mediated reactions, called food intolerances, or immune-mediated reactions, called food allergies (Figure 1.1). Food intolerances, which account for the majority of adverse food reactions, include enzymatic reactions (e.g. lactose intolerance due to lactase deficiency) and those resulting from the pharmacological properties of the food (e.g. the caffeine in coffee that causes nervousness or anaphylactic shock secondary to the presence of vasoactive amine in food) (Bruijnzeel-Koomen et al. 1995; Hillier et al. 2001).

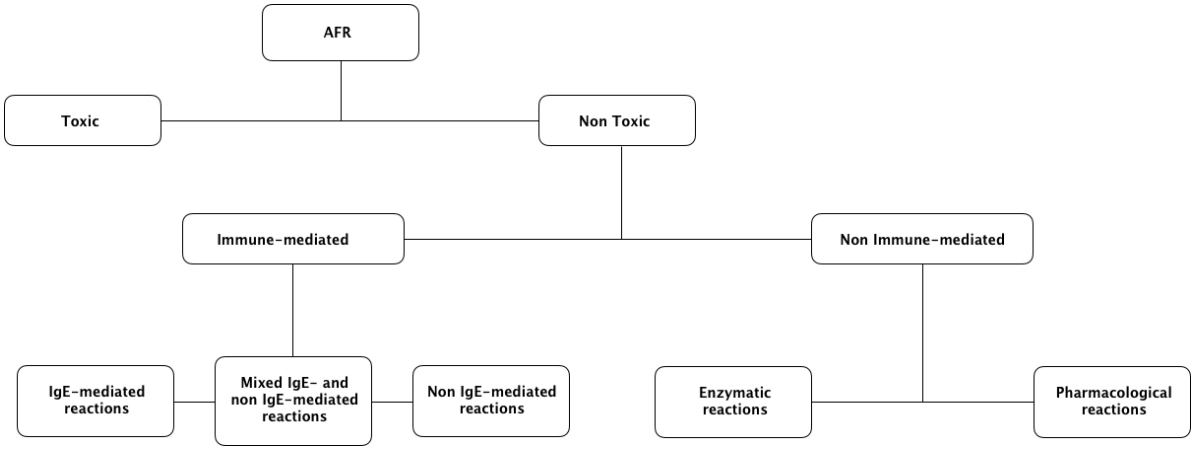

Figure 1.1: Classification of adverse reactions to food. AFR can be classified as toxic or non-toxic reactions. Non-toxic reactions are categorized further as immune-mediated or non immune-mediated. Immune-mediated AFR are then additionally divided in those associated with the formation of IgE against food allergens, in mixed IgE- and non-IgE-mediated reactions and in non-IgE-mediated AFR where other mechanisms, e.g. cell-mediated, are involved. Non immune-mediated reactions are further divided in enzymatic and pharmacological reactions.

Food allergies are abnormal, specific and reproducible immunologic responses to ingested food (Boyce et al. 2010). These responses may be immunoglobulin (Ig) E-mediated, mixed IgE- and non-IgE-mediated, or non-IgE-mediated hypersensitivity responses. IgE-mediated responses are the most studied and best defined in human literature. They include urticaria/angioedema, rhinoconjunctivitis/asthma, pollen food allergy syndrome, gastrointestinal symptoms, anaphylaxis and food dependent exercise-induced anaphylaxis (Sicherer and Sampson 2010). Mixed IgE and cell-mediated food-induced allergic disorders gather atopic eczema/dermatitis and eosinophilic gastrointestinal disorders. In human, the group of non-IgE-mediated hypersensitivity responses includes food protein induced enterocolitis syndrome (FPIE) and food protein-induced proctitis/proctocolitis. In dogs, it is more difficult to

make this differentiation because pathogenetic mechanisms are poorly investigated and clinical manifestations are scarce and often overlapping. But, most importantly, there is no accurate assay for their diagnosis and distinction. For these reasons, the term AFR is preferred even to refer to allergies.

1.2. Prevalence

AFR is the 3rd most common canine skin allergy (after flea allergy dermatitis (FAD) and canine atopic dermatitis (CAD)). Their precise prevalence is unknown and it is difficult to gauge due to the heterogeneity in clinical signs, the type of allergens, and diagnostic criteria. A recent review reported that prevalence of AFR in dogs was found to vary depending upon the type of diagnosis made: 1-2% of any diagnosis; 0-24% among skin diseases; 9-40% of dogs with pruritus; 8-62% of dogs with any skin allergic condition; and between 9 and 50% of dogs with skin lesions suggestive of atopic dermatitis (Olivry et al. 2016) (LoEII). The large difference among studies may be due to the different assays used, the diagnostic workout and differences in the geographical populations studied (Olivry et al. 2016) (LoEV). Although the presence of a genetic component has not been proven, some breeds, such as West Highland White terrier, Cocker spaniel, Labrador retriever, Boxer, Rhodesia ridgeback, German Shepherd Dogs, and Pug, show a higher prevalence of AFR (Picco et al 2008; Proverbio et. 2010) (LoEIII). In addition, AFR prevalence is higher in young (< 1 year) and old dogs (> 6 years) (Picco et al 2008; Proverbio et. 2010) (LoEIII).

1.3. Pathogenesis

Food allergy is considered to result from a failure in oral tolerance even though many factors are involved in the onset of food sensitization, including host and food factors (Pabst and Mowat 2012). Before introducing the concept of oral tolerance, the following sections will illustrate the gut anatomy and its immune system to better understand their physiology.

1.3.1. Gastro-intestinal tract

The gastro-intestinal tract is a very long and winding tube that starts at the mouth, extends into the oropharynx, through the oesophagus, stomach, small intestine and

large intestine and ends at the anus. The histological composition of the digestive canal from the inside to outside includes the following structures as shown in figure 1.2 (Abraham and Kierszenbaum 2002).

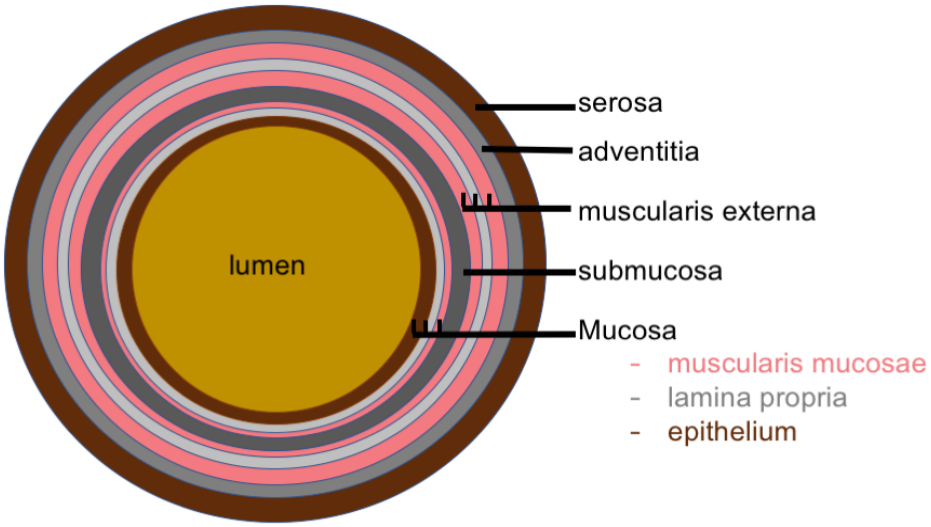

Figure 1.2: histological composition of the alimentary canal.

The lumen is lined by an epithelium, which rests on a vascular connective tissue, the lamina propria. The lamina propria is in turn surrounded by a narrow band of smooth muscle (muscularis mucosae). These three tissues are collectively referred to as the mucosa of the alimentary canal.

Beneath the mucosa we find the submucosa which consists of loose connective tissue and contains vessels and a nerve plexus (submucosal plexus or Meissner's plexus). The submucosa is surrounded by smooth muscle, which is typically divided into two differently oriented layers: an inner circular and an outer longitudinal layer. These muscle layers are together referred to as the muscularis externa. Between the two muscle layers we find the second nerve plexus innervating the alimentary canal (myenteric plexus or Auerbach's plexus) (Gabella 1979). The alimentary canal is separated from other tissues by a layer of loose connective tissue, the adventitia. In the case of the intraperitoneal parts of the alimentary canal, a simple squamous epithelium, the serosa, delimits the adventitia from the peritoneal cavity. Glands may be present in some parts of the wall of the alimentary canal (Ross and Pawlina 2015). These glands are called mucosal glands if they are located luminal (or superficial) to the muscularis mucosae. If the glands extend into the submucosa they are called submucosal glands. Of particular interest for this thesis, the mucosa of the small

intestine, has various structural features which considerably increase the luminal surface area supporting the absorption of the degraded components of the food. The entire intestinal mucosa forms intestinal villi (about one mm long), which increase the surface area by a factor of ten (Figure 1.3). The surface of the villi is formed by a simple columnar epithelium of absorptive cells called enterocytes. Each enterocyte of the epithelium forms numerous microvilli (1 µm long and about 0.1 µm wide). Microvilli increase the surface area by a factor of 20 (Figure 1.3) (Mooseker 1985). Among enterocytes there are goblet cells, which secrete mucin, for lubrication of the intestinal contents and protection of the epithelium.

Between the intestinal villi there are the openings of simple tubular glands, the crypts of Lieberkühn (Figure 1.3) (Ross et al. 2003). They extend through the lamina propria down to the muscularis mucosa and contain epithelial cells, undifferentiated cells to regenerate the epithelium, scattered mucus-secreting goblet cells, and endocrine cells. At the bottom of the crypts there are also the Paneth cells with an anti-bacterial function (Ross and Pawlina 2015).

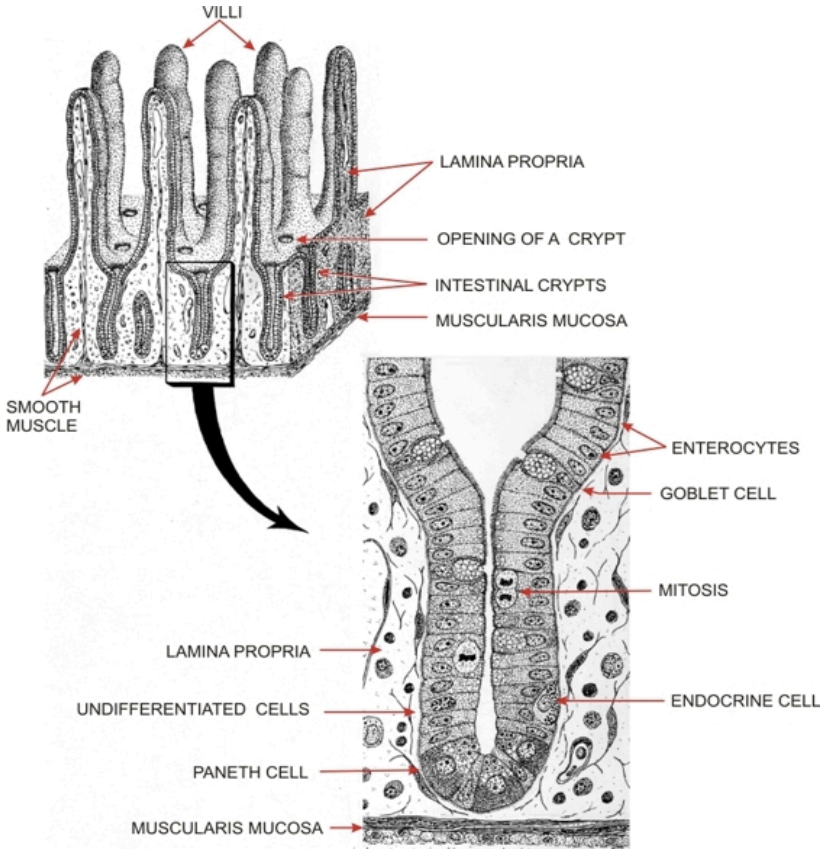

Figure 1.3: Diagram of the mucosa of the small intestine showing a three-dimensional representation of the mucosa (top left) and the components of an intestinal crypt of Lieberkühn (Clermont et al. 2013).

The lamina propria contains inflammatory cells which usually consists of plasma cells and lymphocytes. Lymphocytes often invade the epithelium (Intraepithelial lymphocytes) or form solitary lymphoid nodules in the lamina propria. Lymph nodules may form longitudinal aggregations of 30-50 nodules in the lamina propria of the ileum. These large aggregations are called Peyer's patches.

The functions of the digestive system can be divided into four main categories: digestion, absorption of nutrients, motility (movement through the digestive tract), and elimination of feces. The gastro-intestinal tract is also designed to protect the body against bacteria, toxins and other harmfull ingested antigens (Ross and Pawlina 2015).

1.3.2. Barrier functions

The gastro-intestinal tract is continuously exposed to foreign antigens derived from food, the microbiota or pathogens. Some of these antigens are harmless, while others are dangerous and have to be removed. The first structure that prevents the entry of harmful antigens is the GI mucosal barrier, which consists of non-immune and immune components (Turner 2009). The former includes the intestinal peristalsis, the mucus layer, glycocalyx and microvilli, which prevent antigen penetration to the epithelium. The epithelium maintains a selective barrier function through the formation of complex protein-protein networks, called tight junctions, that mechanically link adjacent cells and seal the intercellular space. Furthermore, gastric acid, pepsins, pancreatic and intestinal enzymes degrade the ingested antigens destroying their conformational epitopes. This results in a loss of their immunogenic epitopes, resulting in immunologic ignorance. The immunological components include antigen-specific secretory IgA (SIgA) (Figure 1.4). Luminal SIgA is believed to interfere with pathogen adherence to mucosal epithelial cells, a process called immune exclusion.

1.3.1. Uptake of antigens in the intestine

Proteins that are not digested and processed in the lumen of the gut will contact the epithelium and mucosal immune system beneath it in various manners. In the gut, dendritic cells can sample antigens by extending processes through the epithelium and into the lumen. Specialized epithelial cells, called microfold cells or M cells, that

overlie Peyer patches can take up, via active transport, particulate antigens and deliver them to subepithelial dendritic cells (Chehade et al. 2005) (Figure 1.4 and 1.5).

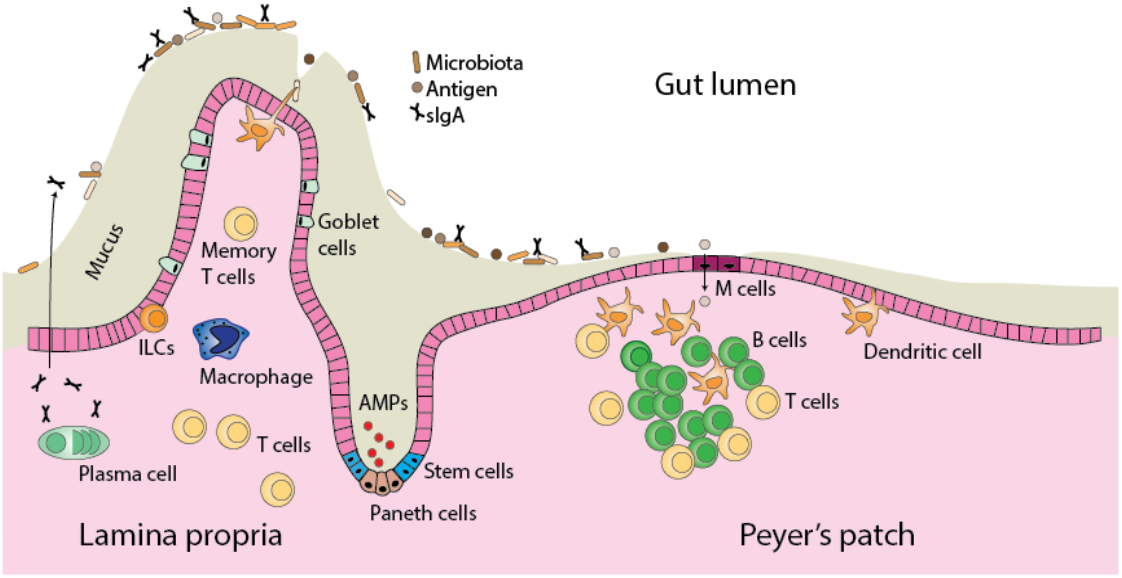

Figure 1.4: Immunological components of the intestine. ILCs: innate lymphoid cells, AMPs: anti-microbial peptides (From website: http://theibdimmunologist.com/learning/the-immune-system-in-the-healthy-gut (consultation date 31st May, 2018).

Upon delivery to local dendritic cells (DCs), these antigens might be presented directly to T cells in the Peyer's patches or the antigen-loaded DCs might move across lymphatic vessels to the mesenteric lymph nodes (MLNs). Free antigens can also move directly to the lymph nodes. Alternatively, antigens smaller than 10 kDa might gain direct access using the paracellular transport: antigens diffuse between cells to the lamina propria passing across the epithelium through pores in the tight junctions between the epithelial cells (Figure 1.5). Conversely, antigens bigger than 600 Da might penetrate into the gut tissues by transcellular transport involving endocytosis/exocytosis (Figure 1.5). Antigens that are taken up at the apical side of the enterocytes can be released at the basolateral side as immunogenic peptides (40%), fully degraded into amino acids (50%) or in their intact form (10%). Once antigens are released into the lamina propria, they might move directly into blood stream or be taken up by local antigen-presenting cells. Transcytosis of antigens can be mediated by membrane receptors binding antibody-antigen complexes (IgA, IgE and also IgG). These immune complexes are transported intact across the intestinal epithelium and are released on the basolateral side. This is what happens in celiac

disease, where gliadin is transported upon binding to IgA via the transferrin receptor CD71, which shows an ectopic expression at the apical membrane of the epithelial cells (Matysiak-Budnik et al. 2008). The same mechanism applies to IgE-mediated transport. In humans, mice and rats the low affinity receptor for IgE (FcεRII, CD23) (Bevilacqua et al. 2004; Kaiserlian et al. 1993 Yang et al. 2000) is overexpressed at the apical surface of epithelial cells in patients with food allergies compared to healthy individuals, resulting in an increased transport of IgE-antigen immune complexes from the lumen to the lamina propria.

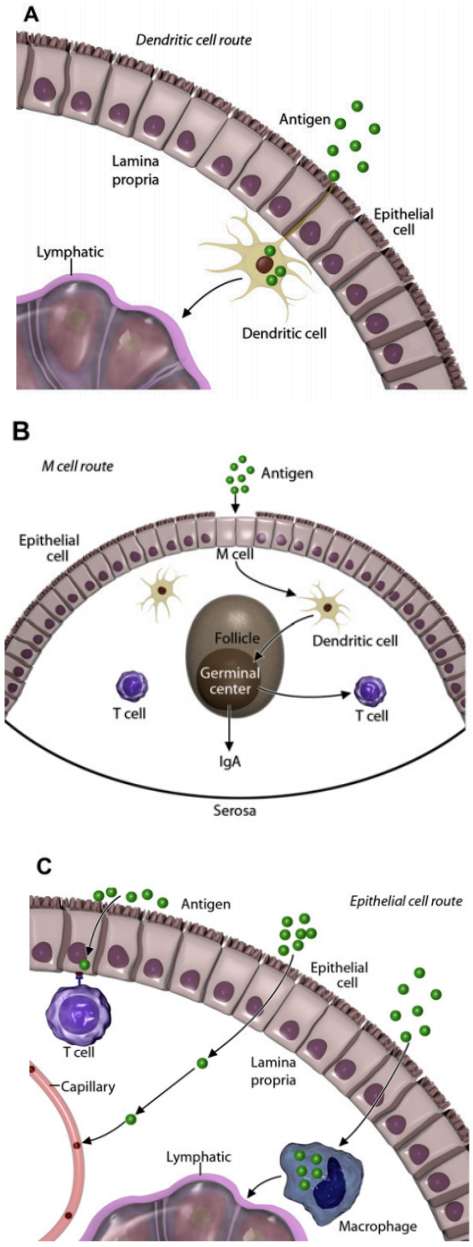

Figure 1.5: Antigen sampling in the gut. A, Dendritic cells extend processes through the epithelium and into the lumen. B, M cells overlying Peyer's patches take up particulate antigens and deliver them to subepithelial dendritic cells. C, Soluble antigens possibly cross the epithelium through transcellular or paracellular routes to encounter T cells or macrophages in the lamina propria. (Burk et al. 2008)

This causes IgE-mediated FcεRI cross-linking on mast cells. Monocytes and dendritic cells can sample protein directly from the gut lumen using their pseudopodia. Tight junctions are opened and after sampling new tight junctional complexes are formed. CD103+ DCs can also capture luminal antigens by extending processes through goblet cells (McDole et al. 2015) which function as a conduit between the intestinal lumen and the mucosa. In addition, they can sample antigens that are transported across the intestinal epithelium by paracellular or transcellular pathways or sampling by M cells (Berin et al. 2013) and carry it into the lamina propria. Goblet cells can directly transport small peptides (<10 kDa) and particles across the intestinal wall (McDole et al. 2015), however their role in uptake of food proteins has not yet been documented in humans nor in dogs. Dietary proteins that escape proteolysis in the gut can be taken up by intestinal epithelial cells. The epithelial cells can act as nonprofessional APCs given that they constitutively express MHC class II molecules on their basolateral membranes and can present antigen to primed T cells.

Even though the intestinal route seems to be the most important site where the absorbed antigen can contribute to oral tolerance induction, primary sensitization to food allergens may result from exposure to protein allergens on other body parts, such as the skin. Indeed, epicutaneous exposure to environmental allergens may induce both the sensitization and the perpetuation of atopic dermatitis in dogs, since animals topically exposed developed signs of atopic dermatitis and CD1c+ dermal dendritic cells contained allergens (Pucheu-Haston et al. 2008). In humans and dogs with atopic dermatitis there is evidence that skin barrier defects due to abnormal lipid and protein production can modify the skin barrier favoring antigen penetration. Moreover, the epidermal barrier blocks the entry of allergens in atopic dogs as removal of the stratum corneum facilitated sensitization to these allergens. This mechanism could also play a role in food allergy. In human and mice, topical application of peanut through a disrupted skin barrier is related to peanut allergy (Lack et al. 2003; Strid et al. 2005). This topical exposure, after stratum corneum removal, interferes with the induction of oral tolerance, since IL-4 and allergen-specific IgE production were increased in epicutaneously exposed animals when the stratum corneum was previously removed (Strid et al. 2005). These studies also showed that disrupting the epidermal barrier is

necessary for allergens to pass (Strid et al. 2005). In addition, local inflammation of the skin could be the cause of sensitization to allergens (Lack et al. 2003). As in human, dogs can be sensitized to peanut by epicutaneous application of a peanut cream, although this does not require the removal of the stratum corneum (Marsella 2015). Since dogs with food allergy have the same signs as atopic dogs two diseases could share the same skin abnormalities and gene mutations and therefore the same mechanism of allergen entry with an overlapping sensitization.

It is worth mentioning that dogs with food allergy only showing skin signs did not differ in cellular composition or mRNA expression of Th1-, Th2- and Treg-related genes into the duodenum before and after allergens exposition (Veenhof et al. 2010). This implies that the gut is not the first site for sensitization to food allergy. This hypothesis is supported by another study that found a significantly increased number of CD8+γδTCR+ T cells and an increased expression of IL-4, IL-13, SOCS-3 and Foxp3 genes in the lesional skin of dogs with food allergy compared with healthy dogs during the provocation diet even if no change in cell phenotype was found after the elimination diet (Veenhof et al. 2011). Inversely, children with also an intestinal manifestation of food allergy had a switch from a Th1 to Th2 cell population (Beyer et al. 2002) and a decreased TGF-beta expression in duodenal lymphocytes in the epithelium and lamina propria (Perez-Machado et al. 2003). A hypothesis could be that in humans, allergens gain access via the gut, while in dogs, allergens can penetrate through the gut in those animals showing intestinal signs or cross the skin in those dogs with skin disorder or through both resulting in skin and intestinal disorders.

1.3.2. Induction of oral tolerance to harmless antigens

The mechanism which inhibits immune responses to harmless antigens and still allows a strong response to pathogens is called oral tolerance. Chase was the first to characterize the concept of tolerance in a murine study in 1946 (Chase 1946) and since then several studies have confirmed this concept. However, the exact mechanism of oral tolerance is still incompletely understood. There are two primary effector mechanisms for inducing oral tolerance: active suppression by regulatory T cells or clonal anergy or deletion (Figure 1.6), where the dose of antigen is one of the prime determinants (Friedman et al. 1994).

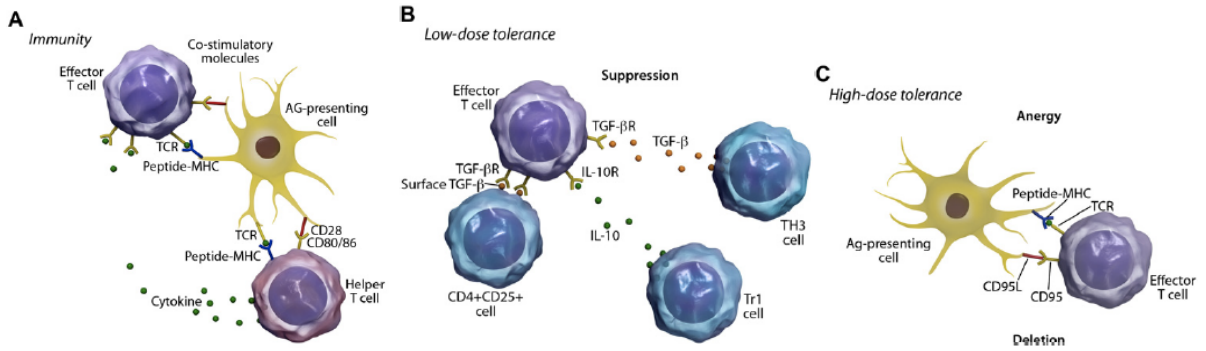

Figure 1.6: Mechanisms of oral tolerance. A, Immune responses require T-cell receptor ligation with peptide-MHC complexes in the presence of appropriate costimulatory molecules (CD80 and CD86) and cytokines. B, Low doses of antigen promote tolerance driven by regulatory cells, which suppress immune responses through soluble or cell surface-associated downregulatory cytokines, such as IL-4, IL-10, and TGF-β. C, High-dose tolerance is mediated by lymphocyte anergy or clonal deletion. Anergy can occur through T-cell receptor ligation in the absence of costimulatory signals. Clonal deletion occurs by means of FAS-mediated apoptosis (CD95). TCR, T-cell receptor; Ag, antigen. (Burk et al. 2008)

Repeated low-dose exposure to antigen favours the induction of regulatory T-cell (Tregs), whereas higher doses favour the induction of T cell anergy or deletion. Tregs are classified as naturally occurring, thymic selected FoxP3+CD4+CD25+ regulatory T cells and inducible regulatory T cells, which, in principle, have the same surface markers. Other cells with a regulatory function are generated in the periphery (Figure 1.7).

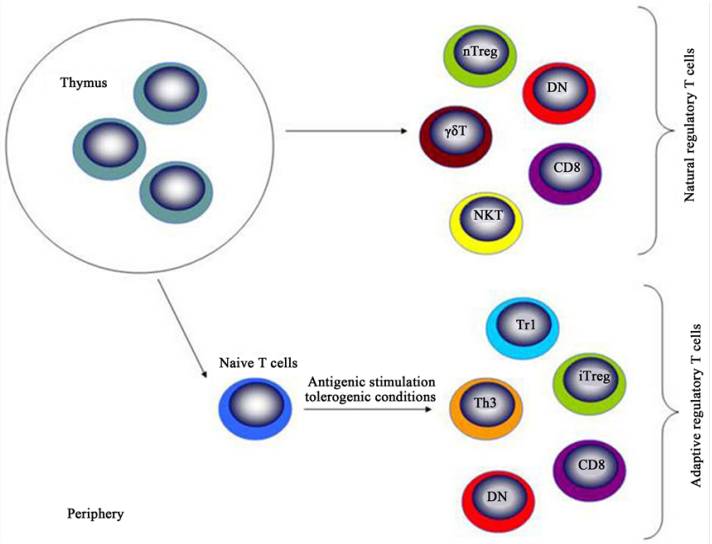

Figure 1.7: Natural and adaptive regulatory T cells. Natural regulatory T cells produced by thymus and adaptative regulatory T cells are induced in the periphery upon allergenic stimulation of naïve T cells under tolerogenic conditions. Abbreviations: nTreg, naturally occurring CD4+ CD25+ Foxp3+ Treg cells; iTreg, induced CD4+ CD25+ Foxp3+ Treg cells; NKT, natural killer T cells; DN, double negative Treg cells; Th3, T helper type 3; Tr1, type 1 regulatory T cells (Adapted from Zhu 2015).

Antigens are collected in the lamina propria and in the Peyer's patches by CD103+ dendritic cells which promote the differentiation of induced regulatory T cells (iTregs), by a mechanism depending on retinoic acid (RA), TGF-β and the expression of indoleamine-2,3-dioxygenase (IDO). iTreg cells work through soluble or cell surface-associated downregulatory cytokines, such as IL-4, IL-10, and TGF- β which suppress systemic immune reactions and antigen-specific sensitization.

Antigen-specific regulatory cells migrate to lymphoid organs, where they inhibit the generation of effector cells, as well as to target organs, where they release non–antigen-specific cytokines (Faria et al. 2005). The intake of food allergens can also promote natural Tregs and type 1 regulatory T cells (Tr1). The latter secretes IL-10 and TGF-β, resulting in allergen tolerance. Tr1, conversely to the previous, do not express FoxP3 nor CD25 (Palomares et al. 2010; Akdis et al. 2007). Uptake of food allergens promotes the induction of a specific CD4+ T cells, called Th3, expressing on their surface the latency associated peptide (LAP) (Weiner 2001). These cells do not express CD25 and Foxp3 because their mechanism is TGF-β dependent. T helper 3 cells (Th3) have also a suppressive function being able to produce TGF-β, and varying amounts of IL-4 and IL-10. Beside IL-10 and TGF-β, Tregs also secrete several novel suppressive cytokines such as IL-35. This interleukin is constitutively secreted by FoxP3+ Tregs and induces IL-10 production from CD4+CD25+ Tregs increasing their regulatory capacity and IFN-γ production in CD4+CD25- effector T cells (Collison et al. 2007; Niedbala et al. 2007). IL-35 is also secreted by a novel regulatory T cell subset, which mediate suppression without secreting IL-10 nor TGF-β (Collison et al. 2010). Furthermore, CD8+ CD28- T cells prevent upregulation of the B7 molecule induced by T helper cells on APC. This suppressive activity is important for the development of oral tolerance (Keet al. 1996). TCRαβ+ CD4-CD8- double negative regulatory T cells suppress immune responses mediated by CD4+ T cells and by CD8+ T cells. γδ T cells can produce an array of different cytokines with both proinflammatory and immunoregulatory functions, via the production of Th1-, Th2-, and Th17-associated cytokines. Lahn et al. (2002) showed that γδ T cell-deficient mice when exposed to ovalbumin developed increased airway responsiveness, suggesting that γδ T cells exert a suppressive role in the Th2 response to allergen challenge (Lahn et al. 2002). In addition, natural killer cells and certain subset of invariant natural killer

cells have suppressive function against specific T cell responses (Deniz et al. 2008; Fujita et al. 2009; Ishii et al. 2008).

High-dose tolerance is mediated by lymphocyte anergy or clonal deletion (Figure 1.6). Anergy occurs when the immune system does not react due to the absence of costimulatory signals (Appleman et al. 2003). Clonal deletion occurs by means of FAS-mediated apoptosis (Chen et al. 1995). Macrophages and DCs clean up the apoptotic cells and exhibit up-regulation of TGF-β and down regulation of inflammatory cytokines. Apoptotic cells can also secrete TGF-β, which is critical for inducing and maintaining iTreg. Interestingly, it has been suggested that low- and high-dose tolerance might not be mutually exclusive and might have overlapping functionality (Faria et al. 2005).

The small intestine is considered as the most important site for tolerance induction. However, there is evidence that the liver can also lead to tolerance of those allergens that, after passage through intestinal epithelial cells, are absorbed into capillaries that drain into the portal vein and then reach the liver. This phenomenon is called portal tolerance. Indeed, portacaval shunts in rats led to abrogation of tolerance to a soluble protein antigen (Callery et al. 1989). Portal tolerance seems to be mediated by liver lymphocytes carrying the NK1.1 marker (NK1.1 T cells), as demonstrated in a study in mice, where depletion of these T cells blocked oral tolerance induction (Trop et al. 1999). Beside murine experiments, it has been shown that dogs without a portacaval shunt had reduced antibody responses to oral antigens as compared to control dogs (Cantor et al. 1967) (LoEIII). Furthermore, a number of case series described the development of food allergy in children undergoing liver transplantation. Thus, the liver may have an important role for peripheral immune tolerance induction.

In addition to the small intestine and the liver, the oral mucosa could also play a role in the development of oral tolerance. Although its function in triggering oral tolerance is not completely understood, it seems that dendritic cells residing in the oral mucosal epithelium and the release of SIgA by plasma cells present into the salivary glands might play an important role in oral tolerance induction (Novak et al. 2008).

1.3.3. Factors influencing the development or leading to tolerance breakdown

Although these mechanisms are very efficient in most of the population, individuals might be sensitized against food because of a deficient induction of oral tolerance or

a breakdown of established oral tolerance (Chelade and Mayer 2005). The inciting events leading to these anomalous responses are poorly understood but it is likely that multiple pathways are involved. A deficient induction of oral tolerance might be caused for instance by a genetic increased susceptibility to mount IgE responses or to environmental factors such as dietary composition, age at which food antigen is introduced or bypassing of oral tolerance induction sites (Chelade and Mayer 2005; Holgate et al. 1999; Lee and Burks 2006). Nevertheless, even though a proper oral tolerance is established this can still be compromised later. Indeed, bacterial infections may damage barrier function by disturbing the intestinal mucus layer, or altering the epithelial tight junctions increasing the intestinal permeability and allowing antigens invading the mucosa, permitting normally tolerogenic responses to most luminal antigens to be modified into immunogenic responses (Figura et al. 1999). Defects in barrier function together with immune activation, and subsequent persistent inflammation would further damage the epithelium and drive the vicious cycle which is responsible for the breakdown of the gastrointestinal homeostasis (Egawa and Kabashima 2018).

Host factors

The genetic background most likely is an important factor in humans predisposing for AFR because the incidence of food allergy was higher in monozygotic twins than in dizygotic twins (Sicherer et al. 2000). Also in dogs, genetics are likely predisposing for AFR since a higher risk is reported for some breeds (Chesney 2002; Loeffler et al. 2004; Loeffler et al. 2006) (LoEIII).

Age is also an important factor. In humans, the prevalence of food allergy is higher in children as compared to adults and also dogs often show the first symptoms of food allergy at a young age (< one year old) (Denis et al. 1994; Harvey 1993; Rona et al. 2007; Rosser 1993) (LoEIII). A higher prevalence of AFR in children and puppies might be related to the increased permeability at birth for most nutrients, which may facilitate priming of humoral and cell-mediated immune responses in predisposed subjects (Buddington et al. 2003; Fergusson et al. 1990; Sampson 1999; Rona et al. 2007). Indeed, ingesting dietary proteins before four months of age has been associated with a 2.9% higher risk to develop adverse food reactions in children. It is worth noting that even a delayed food introduction has been reported as a predisposing factor for

developing AFR. Recent guidelines recommend solid-food introduction not be delayed past 4-6 months of life defining a window of intervention in which allergens can be introduced safely leading to tolerance induction (Togias et al. 2017). The protective effect of the early introduction of solid foods in dogs has not been investigated yet. It is not clear when a puppy has the maturity to develop oral tolerance, but it is estimated that they must be older than six weeks. If a new food is consumed before that age, oral tolerance could not develop resulting in food allergy (Strombeck 1999) (LoEIV). Beside an immature intestine, in dogs, the passage of allergens can be increased when the epithelial barrier is damaged as a result of malnutrition or acute gastrointestinal disorders (Sanderson et al. 1993) (LoEIII). In detail, malnutrition and gastrointestinal disorders induce loss of diversity of the microbiota, decreasing commensal bacteria with an enrichment in proinflammatory species (Lupp et al. 2007). The consequent dysbacteriosis promotes inflammation which may damage the intestinal epithelium (Kane et al, 2015; Round and Mazmanian 2009). Once damaged, macromolecules can easily pass across the intestinal barrier and set up an immune response (Heyman et al. 1984). Furthermore, loss of commensal bacteria, important to support Treg stability and functionality, together with the increased inflammation, lead the unstable iTreg to become effectory T cells (Charbonnier et al. 2015; Komatsu et al. 2014; Smith et al. 2013). In addition, the inflammation and microbial stimuli activate cells to release danger signals, such as TSLP, which promote Th2 differentiation and proliferation (Ito et al. 2005; Reche et al. 2001; Soumelis et al. 2002). Finally, gastrointestinal upset may impede a proper food digestion resulting in the presence of large polypeptides, which are more difficult to ignore by the immune-system.

In addition to a well-developed intact intestinal barrier, it is important that the pH of the gastrointestinal tract and its bacterial flora are preserved. Indeed, a study in humans showed that anti-acid treatment induced food sensitization in 25% of the patients (Untersmayr et al. 2005). Further studies in mice demonstrated that anti-ulcer drugs inhibiting or neutralizing gastric acid allowed the persistence of intact food and enhanced the capacity to sensitize and elicit allergic reactions. Malnutrition, gastric disorders and any changes that increase the pH of the stomach will perturb the bacterial microbiota of the gastrointestinal tract interfering with a correct tolerance development (Bashir et al. 2004). Indeed, in rodents it has been demonstrated that commensal bacteria such as *Bifidobacterium*, *Bacterioides* and certain *Clostridia*

strains, exert protective effects for food allergy by modulating type 2 immunity, maintaining a regulatory tone of the mucosal immune system, regulating basophil homeostasis and promoting the function of the intestinal barrier (Cahenzli et al. 2013; Faith et al. 2014; Geuking et al. 2011; Herbst et al. 2011; Hill et al. 2012; Round et al. 2010; Stefka et al. 2014). Administration of probiotics, such as *Lactobacillus GG*, can also improve the barrier function in humans (Berni et al. 2012; Berni et al. 2017; Majamaa et al.1997; Tang et al. 2015). Unfortunately, there are no data about the role of probiotics in alleviating canine food allergy. However, a study on dogs with intestinal bowel syndrome (IBD) suggested that the use of probiotics may be beneficial by increasing the number of mucosal FoxP3+ regulatory T cells (Rossi et al. 2014) (LoEIII). An increase in pH might also result in incomplete digestion which has been reported to elicit food allergy in dogs (Roudebush et al. 2000) (LoEIII).

A different entrance route of an antigen can result in loss of tolerance by bypassing the gastrointestinal tract. This has been demonstrated in the respiratory tract but also in the skin, where food allergy was elicited in humans, mice and dogs by applying a cream containing peanut oil (Borghesan et al. 2008; Lack et al. 2003; Leser et al. 2001; Marsella 2015; Strid et al. 2005.) (LoEIII). Further, there are several studies demonstrating that parenteral injection of proteins may cause sensitization in dogs (Guilford et al. 1991; Ermel et al. 1997; Teuber et al. 2002) (LoEIII).

Adverse food reactions have been associated with an immune response clearly biased toward a type 2 cytokine-associated phenotype due to a defect in one or multiple components of the immune system, such as phagocytic innate immune cells, tolerogenic antigen presenting cells (APCs) and regulatory cells of the adaptive immune system.

In murine models of food allergy, it has been demonstrated that intestinal epithelial cells express IL-33, which increases mucosal permeability and induces OX40 ligand expression on CD103+ intestinal DCs. OX40L, in turn, promotes Th2 skewing by interacting with OX40 on T cells (Chu et al. 2013). IL-33 promotes the expansion and activation of group 2 innate lymphoid cells (ILC2), which respond by producing large amounts of IL-4. In addition, IL-33 contributes to acute reactions to food by acting directly on mast cells and enhancing IgE-mediated activation (Galand et al. 2016).

In genetically allergy-prone subjects, a dysregulated IgE mast cells activation further increases the production of IL-4, which was shown to suppress the generation of Treg

cells in the skin, lung, and small intestine and to amplify Th2 responses (Hammad et al. 2015; Noval Rivas et al. 2016).

IL-9, a growth factor for mast cells, is another key cytokine, which is overrepresented in allergic subjects and has been associated with allergic responses to food in human and mice (Chen et al. 2015; Tordesillas et al. 2017).

Cutaneous application of food antigen after stratum corneum removal in mouse or human skin, induces keratinocytes to express IL-33, IL-25, and thymic stromal lymphopoietin (Tordesillas et al. 2017). Of particular interest, TSLP expression has been shown to be elevated in skin of human and dogs with atopic dermatitis and in lungs of asthmatic patients (Klukowska-Rötzler et al. 2013; Soumelis et al. 2002; Ying et al. 2005) (LoEIII). This cytokine induces DC maturation, a proces which is indispensable to trigger the activation of effectory T cells and thus impeding normal tolerance induction. TSLP, conversely to common pathogens, does not act through IL-12, therefore it leads to Th2 polarisation with production of IL-4, IL-13 and TNF-α. Moreover, intestinal T cells can also upregulate, in response to IL-4, low affinity IgE receptor CD23 to bind specific igE and therefore facilitate antigen passage through the epithelium.

At the level of antigen presentation, binding of OX40 ligand to OX40, TIM4 to TIM1, and jagged to notch on DCs and naive T cells, respectively, can regulate T cell differentiation from Tregs toward Th2, as previously reviewed (Berin and Shreffler 2008). Environmental interactions may drive this differentiation. For example, staphylococcal enterotoxin B (SEB) can break tolerance and promote food allergy, and it was shown mechanistically to induce TIM4 expression on DCs that is necessary for Th2 skewing (Ganeshan et al. 2009; Yang et al. 2007).

Last but not least, the induction of regulatory T cells is decreased or their function impaired in allergy-predisposed individuals. It has been demonstrated that atopic patients have decreased capacity to suppress effectory CD4+ T cells and to decrease Th2 cytokine secretion. The above mentioned dysregulated activation IgE-mediated of mast cells, the increased ILC2 activity, and the increased production of IL-13 lead to an increase in IL-4. This increases the intracellular GATA-3 levels in Tregs, a FoxP3 inhibitor (Mantel et al. 2007). Since Fox3P acts as a master switch transcription factor for Treg development and function (Fontenot et al. 2003), once inhibited, Treg function is suppressed. Finally, a proallergic inflammatory environment characterized by chronic inflammation may reprogram allergen-specific Tregs from a regulatory to an

effector Th2 phenotype, hence perpetuating and aggravating the disease. Similarly, chronic inflammation can modify the intestinal microbiota from tolerogenic to pathogenic bacteria. Indeed, it has been demonstrated that commensal bacteria produce short-chain fatty acids, such as butyrate, that increase FxoP3 protein acetylation, thereby stabilizing and strengthening the Treg response (Arpaia et al. 2013).

As a consequence of the factors mentioned above, allergy-prone individuals, develop a CD4+ T helper 2 (Th2) biased immune response to orally delivered food antigens, resulting in a B cell class switch towards IgE responses (Akdis et al. 2009; Akdis et al. 2013). More specifically, in an allergic individual, luminal antigens are captured by dendritic cells and presented to naïve T cells to further induce allergen-specific CD4+ Th2 (Figure 1.8). Once generated, effector Th2 cells undergo clonal expansion and produce IL-4, IL-5 and IL-13, which lead to the development and recruitment of eosinophils and class switching to the ε immunoglobulin heavy chain in B cells with production of food allergen-specific IgE antibodies. Allergen-specific IgE binds to the high affinity receptor FcεRI, on the surface of mast cells and basophils in all examined species, which in turn allows for an increased uptake of allergens (Akdis et al. 2011). In humans and dogs, expression has also been demonstrated on monocytes and dendritic cells (LoEIII).

The engagement of IgE on effector cells leads to the sensitization of patients to a specific allergen (Akdis et al. 2011 b.). The effector phase is initiated when the patient is re-exposed to the sensitized allergen. Upon re-exposure receptor-bound IgE molecules are crosslinked, which in turn results in the activation and release of mediators that cause the development of type I hypersensitivity reactions (Akdis M et al. 2011 b. and c.). During the development of allergic diseases, effector Th2 cells not only produce traditional Th2 cytokines, such as IL-4, IL-5, IL-9 and IL-13, but also novel cytokines with proinflammatory functions, such as IL-25, IL-31 and IL-33 (Bousquet et al. 2011; Deniz et al. 2011; Gaschen et al. 2011; Jones et al. 2009; Kostadinova et al. 2013; Nowak-Wegrzyn et al. 2011; van Wijk et al. 2007; Zuidmeer-Jongejan et al. 2012). These cytokines induce allergen-specific IgE, eosinophilia, mucus production and the recruitment of inflammatory cells to inflamed tissues.

Predominance of Th2 cells might be caused by an increased tendency to activation-induced cell death of high IFN- γ -producing Th1 cells as it is commonly observed in

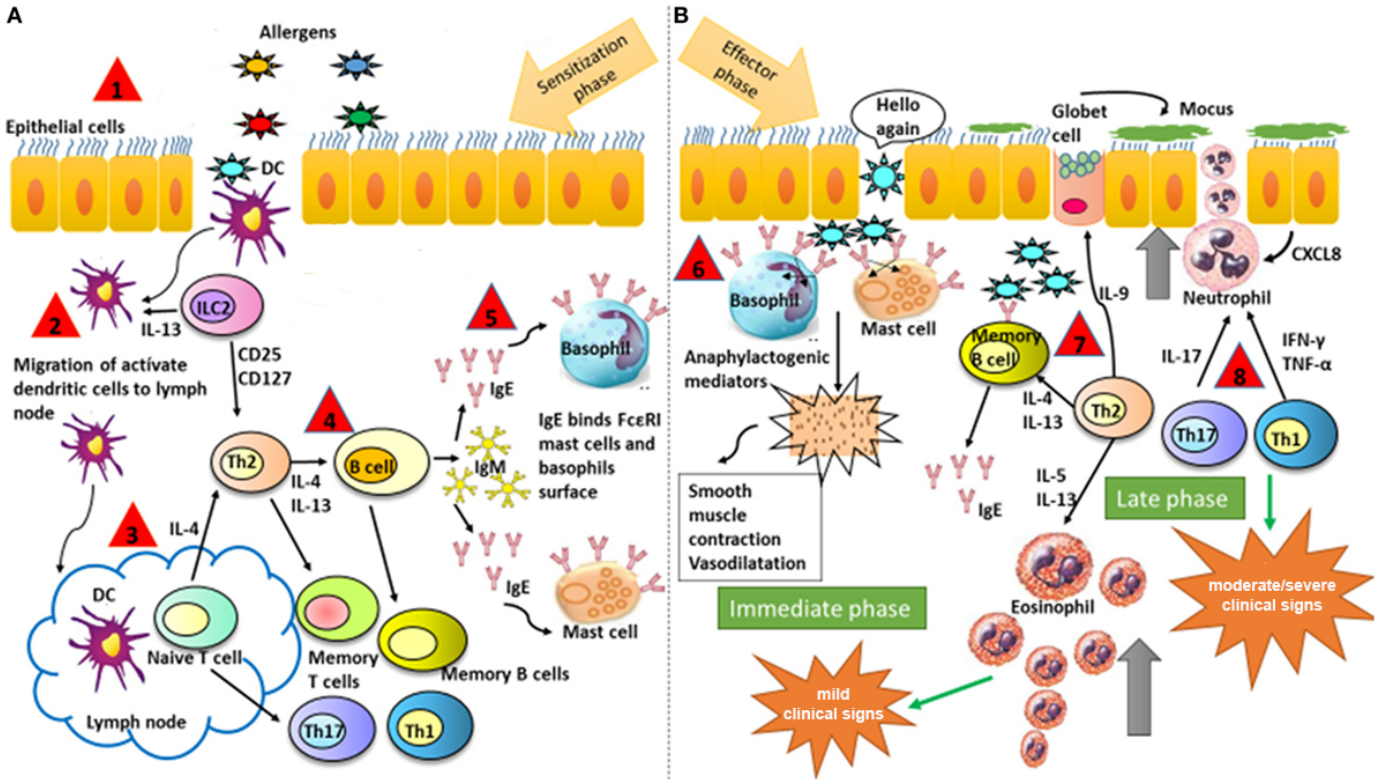

Figure 1.8: Schematic illustration representing major mechanism during the development of allergy: (A) sensitization phase: the sensitization phase is initiated when the allergen is introduced in the organism for the first time and it is captured by dendritic cells (1). Activated DC s migrate from the lamina propria to the lymph node (2) where antigen is presented to the naïve T cell (3) with further differentiation to Th T cells (4). Following Th2 T cells maturation, clonal expansion and Th2 cytokine production, B cells are stimulated to produce IgE (5). IgE binds to FcεRI on mast cells and basophils. During the effector phase (B), re-esposure to the same allergen triggers an immediate reaction due to the binding of the allergen to the FcεRI on on mast cells and basophils (6) with a consecutive release of inflammatory mediators. Further responses are mediated by memory B-cells and Th2 cytokines with recruitment and activation of eosinophils, neutrophils (7). In the late phase, other T helper subsets are involved such as Th 1 and Th17 (8). (Adapted from: Martin-Orozco et al. 2017)

patients with atopic disorders (O'Mahony et al. 2011). In addition, CD4+ naïve cells also differentiate into distinct T cell subsets, such as Th9-, Th17- and Th22-type memory and effector cells depending on the status of the cells and existing cytokines in the microenvironment (Akdis et al 2011 b.).

Immune regulatory mechanisms normally operative to maintain tolerance to allergens break down for reason that are still unclear. However, evidence points to a reduced frequency of Tregs as a possible explanation (Llyod et al 2009; Mamessier et al. 2008). Indeed, allergen-specific IL-10 secreting Tregs were shown to be decreased in blood obtained from patients with persistent allergic rhinitis (Vale-Pereira et al. 2011). Furthermore, pulmonary CD4+ $CD25^{hi}$ Tregs in bronchoalveolar lavage fluids were significantly decreased in untreated asthmatic children and, interestinghly, the treatment restored the Treg compartment in the blood and in the bronchoalveolar lavage fluid (Hartl et al 2007). Moreover, infiltration of CD4+ CD25+ FoxP3+ Tregs has been reported to be absent in atopic dermatitis lesions and atopic patients with active disease have lower levels of circulating CD4+ FoxP3+ T cells compared to asymptomatic control subject (Orihara et al. 2007; Verhagen et al. 2006). Altogether, these data indicate that an impaired allergen-specific suppressive function of Tregs is a predisposing factor to develop allergic diseases.

Recent evidence supports that also a reduction in number of regulatory B cells (Bregs) exacerbates symptoms of allergic conditions (Noh et al. 2011). Indeed, it has been shown that B cells also can have a regulatory function, besides their key role in humoral immune responses. These Bregs exhibit anti-inflammatory and suppressive functions. Through secretion of IL-10 they modulate the development, proliferation and maintenance of CD4+ effector, memory and regulatory T cells. TGF-β has also been identified as a Breg-associated suppressor molecule. This cytokine supports the conversion of naïve CD4+ T cells to Tregs (Akdis et al. 2016). Beside this, Bregs can also indirectly suppress the effector T cells through inhibition of dendritic cell maturation and production of IgG4 antibodies (Habener et al. 2017; van de Veen et al. 2013). There are many subsets of IL-10 producing regulatory B cells. In humans, the major phenotypes are: $CD27^{+}CD24^{hi}CD148^{hi}CD48^{hi}$B10/pro-B10 cells, $CD24^{hi}CD38^{hi}$ immature B cells, $CD73^{-}CD25^{+}CD71^{+}$ type 1 regulatory B (Br1) cells, and $CD27^{int}$ $CD38^{+/hi}$ plasmablasts (Blair et al.201; Iwata et al. 2011; Matsumoto et al. 2014;

Shalapour et al. 2015; van de Veen et al. 2016). Regulatory B cells have not been described yet in dogs with allergic conditions.

Food allergen factors

Food is defined as any substance, raw, semi-cooked or cooked that is intended for consumption and includes milk, treats and dietary supplements. Food allergens are those components of food that are recognized by allergen-specific immune cells.
The most important allergen sources are proteins, but carbohydrates and probably also additives can induce allergy. There are several studies reporting culprit allergens from dietary provocation in dogs, but they differ depending on the case numbers, the type of study and for which allergen was tested (Carlotti 1990; Chesney 2002; Denis et al. 1994; Harvey 1993; Ishida et al. 2004; Jackson et al. 2002; Jeffers et al. 1991; Jeffers et al. 1996; Kunkle et al. 1992; Mueller et al. 1998; Paterson 1995; Walton 1967). This lack of standardization leads to unreliable outcomes. Nevertheless, beef, chicken, dairy products, lamb and wheat are the most commonly recognized food allergens in dogs, while soy, corn, eggs, pork, fish and rice are of lesser importance (Mueller et al. 2016) (LoEII). Other food stuffs, such as peanut and walnut, have only occasionally been reported to trigger AFR in dogs (Kang et al. 2012; Rostaher et al. 2017) (LoEIII). All food for which the allergen has been identified are listed in Table 1.2.
Studies reported that 35-48% of the dogs are allergic to more than one food allergen with an average of 2.4 ingredients (Harvey 1993; Jeffers et al. 1996; Paterson 1995) (LoEIII). Food allergies in dogs can also be induced by cross-reactive allergenic structures. Indeed, recently, the presence of cross-reactive IgE-binding epitopes has been demonstrated in dogs (Bexley et al. 2017) (LoEIII). In human literature, up to 93% of children with cow's milk allergy also have beef allergy (Ayuso et al. 2000; Martelli et al. 2002). It seems they have common allergens, like bovine serum albumin (BSA), myoglobin or bovine IgG (Ayuso et al. 2000; Fuentes et al 2004; Martelli et al. 2002). Bovine IgG seems to be the major allergen responsible for canine adverse food reactions (CAFR) to cow's milk and, since it can also be found in beef, it has been suggested that IgG can also be involved in CAFR to beef (Martin et al. 2004) (LoEIII). Patients allergic to chicken meat can experience reactions to eggs. In this case, serum

albumins, which are present in many tissues including chicken muscle tissue and egg yolk, are responsible for the double intolerance (Quirce et al. 2001).

Table 1.2. Food and their allergens identified in dogs with AFR.

Food	Food allergen	AFR	
		Spontaneous	Experimentally induced
Peanut	Ara h 1 (60kDa)		(Teuber et al. 2002)
	Not available	(Kang et al. 2012)	(Marsella 2015)
Beef	Bos d 6 (66kDa)[1]	(Ohmori et al. 2007)	
	Bos d 7 (56kDa)[2]	(Martín et al. 2004)	
Lamb	Bos d 7	(Martín et al. 2004)	
	Fosfoglucomutasi (51kDa)	(Martín et al. 2004)	
Milk	Bos d 7	(Martín et al. 2004)	
Walnut	Jug 2 (42kDa)		(Teuber et al. 2002)
	(40kDa)		(Teuber et al. 2002)
	Not available	(Rostaher et al. 2017)	
Brazil nut	Ber e 1 (7kDa)		(Teuber et al. 2002)
	(45kDa)		(Teuber et al. 2002)
Soy	(75 and 50kDa)		(Puigdemont et al. 2007)
	(31 and 20kDa)		(Puigdemont et al. 2007)
	Not available	(Adachi et al. 2009)	
Eggs	Gal d 1 (28kDa)[3]	(Shimakura et al. 2016)	(Shimakura et al. 2016)
	Gal d 2 (45kDa)[4]	(Shimakura et al. 2016)	(Shimakura et al. 2016)
	Ovotransferrin		(Shimakura et al. 2016)

[1]Bovine serum albumin; [2]Bovine heavy chain IgG; [3]Ovalbumin; [4]Ovomucoid.
For foods such as fish, wheat and chicken allergens have not been identified yet. Ovalbumin may also be responsible for chicken allergy.

Also in veterinary medicine studies aim to identify specific allergens responsible for canine cutaneous adverse food reactions and as in human some authors identified bovine IgG as a major allergen in cow's milk (Martin et al. 2004) (LoEIII).
Beside animal proteins, dogs can show IgE sensitizations against vegetable proteins, such as maize/potato granule-bound starch synthase-1 (GBSS-1), rice glutelin type B (GLUB), soybean glycin (Gly m6), soybean β-conglycinin α chain (Gly m5), soybean sucrose-binding protein (SBP), and potato aspartic protease inhibitor (Sol t 2) (Roitel et al. 2017) (LoEIII). It is not clear if fat sources, such as soy, corn, peanut and sesame oils, which can be very allergenic in humans, could be equally allergenic in dogs (Boyce et al. 2010). Interestingly, in human, the allergenicity of food oils increases if little of the food protein is removed during processing (Boyce et al. 2010).
Adverse food reactions to food additives, such as preservatives or colorants are possible immune and non-immune mediated in human (Hannuksela et al. 1987, Murdoch et al. 1987; Turner et al. 2012; Wuthrich et al. 1997). It is also believed to occur in dogs, even though there are no data confirming this (Roudebush et al. 1992; White 1986) (LoEV).
Allergenicity may vary among different variants of the same food, as for apples (Vieths et al. 1994), or be the same as for different cultivars of mango. During storage, ripening, some fruit, such as apple and peanut, increase their allergenicity (Chung et al. 2003). Removing a part of fruit or vegetables, as starch from potatoes or wheat, or peeling of peaches, reduces their allergenicity (Vieths et al. 1994).
Possible other food-related factor for the development of food allergy is the thermal process. For instance, heating can reduce the allergenicity of certain food proteins because it can denature proteins, modifying the tertiary protein structure and thus altering IgE-reactive conformational epitopes as reported for beef, ovine, hazelnut and eggs (Des Roches et al. 2006; Eigenmann 2000; Fiocchi et al. 1995; Joo et al. 2006; Lemon-Mule et al. 2008; Lopez et al. 2012; Mine et al. 2012, Peng et al. 1998; Urisu et al. 1997). However, in some cases the IgE binding of proteins can be increased by heating, as for peanut (Beyer et al. 2001; Chung et al. 2003; Maleki et al. 2000; Maleki et al. 2003). Recently, it has also been reported that cooking and processing of food reduces IgE sensitization in dogs suggesting that raw meat might be more allergenic than processed food (Richard et al. 2017) (LoEIII). Changing the pH could also reduce allergenicity of food, as for peanut (Kim et al. 2012). The association of proteins with

other food components can increase their allergenicity as in Maillard reactions, where the presence of sugars induces the production of compounds that are involved in IgE binding (Gruber et al. 2005).

Several food industries are interested in biochemical food processing methods, such as enzymatic hydrolysis, to decrease the allergenicity of food by disrupting potential epitopes. Enzymatic hydrolysis can diminish or enhance the allergenicity depending on which enzyme was used. Enzymes frequently used for this process are trypsin, elastase (with hazelnuts), actinase (with rice), proteases (with soybeans), bromelain (with wheat), transglutaminase (with casein and wheat) (Tanabe et al. 1996; Watanabe et al. 1990; Watanabe et al. 1994; Watanabe et al. 1995; Wigotzki et al 2000; Yamanishi et al. 1996; Yamauchi et al. 1992). These enzymes derive from many sources, including pig, bacteria or fungi. It has been reported that aminopeptidase from *Aspergillus oryzae* and alcalase from *Bacillus licheniformis* give a higher degree of hydrolysis than other tested enzymes, such as papain, trypsin and chymotrypsin (Humiski et al. 2007), resulting in too small peptides to be detected by the immune system. These techniques, much used in human for children with cow's milk allergy (Businco et al. 1999; Terracciano et al. 2002; von Berg et al. 2003), are now emerging in the feed industry (Cave et al. 2004; Loeffler et al. 2004; Loeffler et al. 2006; Marks et al. 2002).

Other studies have been tried to decrease food allergenicity by high intensity ultrasound (shrimps), pulsed electric field or pulsed ultraviolet light with good results with peanuts and shrimps or by γ-radiation and high pressure that seem to be ineffective in celery and hazelnuts (Chung et al. 2008; Kato et al. 2000; Li et al. 2006; Lopez et al. 2012; Shriver et al. 2011).

Biotechnology is being used to reduce or even remove allergens from common foods through at least three different approaches: removing the specific allergenic protein, altering the protein so the immune system cannot recognize it or reducing the allergen digestion stability (del Val et al. 1999).

Finally, dogs that are first sensitized by an inhaled allergen (*Cryptomeria Japonica),* may react to a cross-reactive allergen in raw food (fresh tomato) (LoEIV) (Fujimura et al. 2002). This type of food allergy is called oral allergy syndrome. The allergen in the food is fragile and does not resist digestion in the stomach, thus it loses its capacity to induce allergic reaction when cooked.

1.4. Types of food allergic reactions

The hypersensitivity mechanisms responsible for food reaction are poorly understood. The most extensively studied and best-defined food allergic reactions in people and laboratory animals is the IgE-mediated type I hypersensitivity. This involves an appropriate Th2 response to food antigens associated with interleukin production, such as IL-4, IL-13 and IL-5, which in turn activate B cells to produce allergen-specific IgE. After the initial phase of sensitization antigen-specific IgE bind to Fcε receptors (FcεRI) on the surface of mast cells and basophils. During a re-exposure, of the sensitized individual, allergen is bound by two or more IgE and this cross-link induces mast cell activation and secondary degranulation with rapid release of active mediators, including histamine, serotonin, tryptase, kallicreine, proteases and proteoglycans in seconds or minutes followed by other mediators derived from arachidonic acid, such as leukotriens, prostaglandins, thromboxanes and factors activating platelets. The clinical evidence of this immediate phase is oedema, wheal and pruritus due to vasodilatation, extravasation of inflammatory cells and sensitization of local nerve fibers. A late phase reaction develops 4-8 h after the immediate phase as a consequence of proinflammatory cytokines released by the mast cells, resulting in tissue infiltration of eosinophils, basophils and Th2 cells. In the skin, this late phase is characterized by swelling, pruritus and erythema. Production of food antigen-specific IgE antibodies can be measured during the phase of sensitization and after the oral food challenge (Jackson et al.2002; Puigdemont et al. 2006), suggesting these antibodies may have a role in the pathogenesis of adverse food reactions in dogs (Jackson et al.2002; Puigdemont et al. 2006; Serra et al. 2006) (LoEIII). Type I hypersensitivity seems to be the most common in dogs (Harvey 1993; Jeffers et al. 1991; Jeffers et al. 1996; Proverbio et al. 2010; Wilhelm et al. 2005), although this is questionable because it is impossible to demonstrate food antigen-specific IgE antibodies in dogs with proven food allergy (Jeffers et al. 1996; Paterson 1995) (LoEIII). Moreover, dermatologic and gastro-enteric signs could appear several hours after food allergen ingestion and pruritus does not disappear immediately after food avoidance. All these issues lead to suspect a possible role of delayed hypersensitivity in dogs with food allergy (Appleman et al. 2003; Chen et al. 1995; Cantor et al. 1967) (LoEIII). Type III and IV reactions have been described in humans (Appleman et al. 2003; Chen et al. 1995; Cantor et al. 1967). Type III hypersensitivity,

also called immune-complex hypersensitivity, is an IgG-mediated reaction where allergen exposure leads to IgG production and re-exposure to these allergens results in the formation of immune complexes with complement activation. Clinical signs manifest themselves within 24 hours of exposure. Type IV hypersensitivity is a T cell-mediated reaction characterized by sensitization to food allergens, which leads to the formation of antigen-specific T lymphocytes, above all Th1 cells. Second exposure to allergens triggers reactivation of those T cells, which release cytokines, such as IFNγ, and chemokines, which results in an increased expression of adherence molecules on the endothelia and hence promotes the recruitment of CD4+ cells, CD8+ cells, basophils and macrophages. Symptoms like erythema, papules and pruritus, occur 24-72 hours after allergen exposition.

In dogs, a major role for T cells has been confirmed by demonstrating that these cells proliferate when they are stimulated with culprit allergen extract, and that this proliferation correlates with the appearance of clinical signs and with their improvement after a restrictive diet (Fujimura et al. 2011) (LoEIII).

A proper understanding of the exact immune mechanism in canine food allergy is still lacking and highly required.

1.5. Signalement

There is no consistent breed predilection, however, a higher risk is reported for certain breeds, such as American Cocker spaniel, English Springer spaniel, Labrador and Golden Retriever, Collie, Miniature Schnauzer, Chinese Shar pei, Poodle, West Highland White terrier, Wheaten terrier, Boxer, Dachshund, Dalmatian, Lhasa apso, German Shepherd, Pug, Rhodesian ridgeback (Picco et al. 2008; Rosser 1993) (LoEIII). Adverse food reactions occur in dogs younger than 3 years (83%) (Scott et al. 2007) and in contrast to atopic dermatitis it may occur in dogs younger than 1 year of age (30-50%) and in dogs older than 7 years (Picco et al. 2008) (LoEIII). There is no sex predisposition (Carlotti et al. 1990; Chesney 2002; Denis and Paradis 1994; Verlinden et al. 2006) (LeEIII).

1.6. Clinical signs

Adverse food reactions may be difficult to diagnose due to the lack of pathognomonic signs. Non-seasonal pruritus represents the most common and first clinical signs. However, it can be episodic if offending allergens are fed periodically or seasonal if atopic dermatis is exacerbated by AFR (Hensel et al. 2015) (LoE III). Pruritus is mainly localized to the ventral area, in particular to the axillae, groin, paws on palmar or plantar and dorsal interdigital skin. Pruritus of the ears, muzzle, perianal and periocular area is also associated with AFR (Figure 1.9) (Rosser 1993, Maina et al. 2014) (LoE III). Especially with regard to some breeds, other areas, considered atypical for this condition, may be more frequently affected such as lips in Dalmatians and elbows, hind limbs and thorax in German shepherd dogs. The dorsolumbar area is also frequently affected in Shar-pei and West Highland white terrier dogs (Wilhem et al. 2010) (LoEIII).

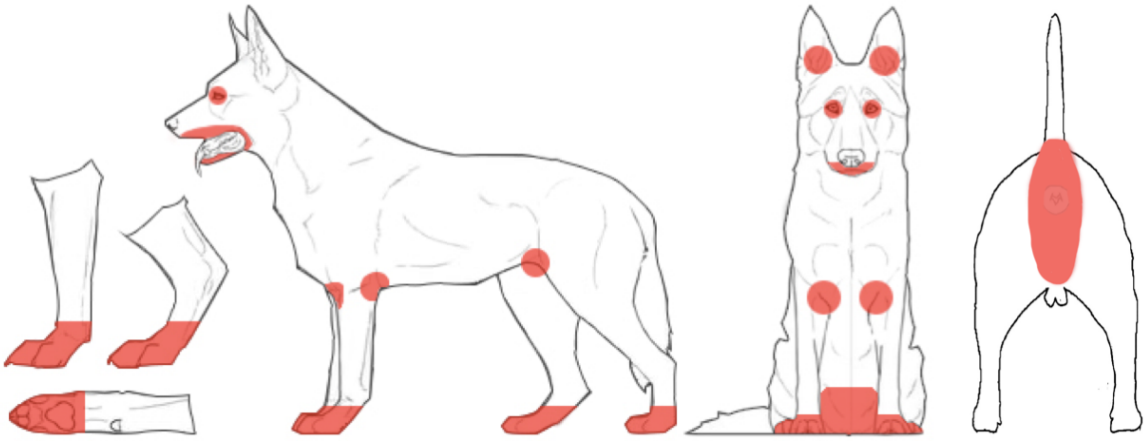

Figure 1.9: Distribution of clinical lesions and pruritus in dogs with AFR and/or AD

Other cutaneous clinical signs are erythema, papulae with a similar distribution as the pruritus, bilateral otitis externa with erythema of the pinnae and vertical canal (24%) (Rosser 1993) (LoEIII). Otitis externa may even occur unilaterally. Recurrent otitis externa is a common complaint and may be present in 56% to 80% of cases (Carlotti 1990; Chesney 2002; Harvey 1993; Rosser 1993) (LoEIII).

Secondary infections of the skin, such as superficial pyoderma or Malassezia dermatitis, are a common consequence of skin inflammation and lead to a more

generalized dermatitis and pruritus. A recurrent superficial pyoderma in the absence of pruritic skin disease has also been recognized in a few cases (Chesney 2002; Harvey 1993) (LoEIII). Secondary changes resulting from chronic pruritus include excoriations, lichenification, hyperpigmentation and extensive alopecia. Uncommon manifestation associated with AFR are: urticaria with or without signs of anaphylaxis, urticarial vasculitis and neutrophilic leukocytoclastic vasculitis (Declercq 2015; Min-Hee Kang et al. 2012; Nichols et al. 2001; Rostaher et al. 2017) (LoEIII). Finally, food can also trigger oral allergic syndrome (Fujimura et al. 2002) and erythema multiforme (Itoh et al. 2006) (LoEIV). Signs consistent with urticaria include wheals (hives), angioedema or both (Hill 2014; Miller et al. 2013; Prelaud 2008; Zuberbier et al. 2006) (LoEIII). Vasculitis manifests itself with poorly healing ulcers located in the center of the footpads, erosion, ulceration and crusting of the pinnal margin, elliptical lesions on the concave aspect of the pinna and urticarial vasculitis (Nichols et al. 2001) (LoEIII). The latter presents itself as urticaria-like lesions that do not blanche with diascopy and do not pit with pressure, distinguishing them from a true urticarial (Morris et al. 1999). Erythema multiforme is a clinically distinct lesion usually consisting of erythematous polycyclic or target-shaped macules that are nonpruritic or slightly elevated papules that spread peripherally and are clear centrally (Itoh et al. 2006) (LoEIV). Rarely reported clinical signs of adverse food reactions include seizures and respiratory signs, including bronchitis, rhinitis and chronic obstructive pulmonary disease (Carlotti 2014) (LoEIII). Beside dermatologic signs, AFR may cause also gut symptoms, such as chronic diarrhea and/or vomiting, or milder signs, such as a tendency to develop loose stools or frequent defecations. Abdominal discomfort, borborigmi and flatulence are also reported. Concurrent gastrointestinal and dermatological signs are seen in 6-44% of affected dogs (Johansen et al. 2017; Picco et al. 2008; Proverbio et al. 2010; Volkmann et al. 2017), but they are not pathognomonic for food-related diseases (LoEIII). Indeed, a large multicenter prospective study of dogs with atopic dermatitis reported a prevalence of gut symptoms of 26.3% among dogs with food induced atopic dermatitis (FIAD), but also in 10.5% in dogs with NFIAD (non-food induced atopic dermatitis) (Favrot et al. 2010) (LoEIII). Food responsive enteropathy (FRE) is a common chronic inflammatory enteropathy characterised by chronic intermittent or persistent diarrhea with a marked response to the elimination diet (Allenspach et al. 2003; Hall et al. 2010; Kennis 2006; Volkmann et al. 2016) (LoEIII). Generally, most FRE dogs respond within few days, but may take up to 14 days to clinically improve

(Marks et al. 2002, Allenspach et al. 2007) (LoEIII). About 30-75% can return eating the previous diet after a 12-week elimination diet (Allenspach et al. 2016; Luckschander et al. 2006; Mandigers et al. 2010) (LoEIII). An inherited gluten sensitive enteropathy has been reported in Irish setters with clinical signs of chronic diarrhea and weight loss and histological demonstration of mucosal inflammation (Hall et al. 1992; Daminet 1996; Garden et al. 2000) (LoIII). The disease is fully responsive to a gluten-free diet. Similarly, canine epileptoid cramping syndrome, described in Boston terriers and characterised by paroxysmal dyskinesia and mild to severe gastrointestinal signs, is also responsive to a gluten-free diet (Lowrie et al. 2015) (LoEIII). Conversely, a familial protein-losing enteropathy described in Soft Coated Wheaten Terriers with chronic diarrhea, vomiting and weight loss does not respond to gluten, but may improve with an elimination diet (Vaden et al. 2000) (LoEIII).

As stated above, cutaneous signs may mimic those of other pruritic dermatoses, such as atopy and flea allergy. It has been estimated that up to 40% of food allergic dogs have a CAD pattern and often CAD and AFR occur concurrently (Chesney 2002; Loeffler et al. 2006) (LoEIII). Studies report that 10% of food-allergic dogs also have CAD sensu stricto and up to one-third of dogs with CAD sensu stricto also have food hypersensitivity (Griffin and DeBoer 2001; Jackson et al. 2005) (LoEIII). Similarly, it has been suggested that up to 31% of dogs may have concurrent atopic dermatitis and flea-bite hypersensitivity (Carlotti and Costargent 1994) (LoEIII). Interestingly, it has been suggested that food and flea hypersensitivity are responsible for acute flares of CAD (Olivry et al. 2015b.) (LoEII). Moreover, it has been suggested that flea-allergic dogs may be predisposed to atopic dermatitis and also that atopic dogs are predisposed to FAD probably as a consequence of the inflammation caused by the flea bites and the introduction of antigens contained in flea saliva (Sousa and Halliwell 2001) (LoEIII).

Depending on the underlying immunologic mechanism in AFR, symptoms are detected within a few hours to 3 days, but if the allergen was excluded from the diet longer than one month, it can also last up to 7 days (Fadok 1994; Jefers 1991; Rosser 1993; Walton 1967) (LoEIII).

1.7. Diagnosis

As stated above, AFR may present itself with several different clinical signs and none are pathognomonic, therefore the list of differentials is long (Table 1.3).

Table 1.3: AFR differentials

Parasitic diseases	Cheyletiellosis
	Demodicosis
	Sarcoptic mange
	Pulicosis
	Otodectosis
	Trombiculiasis
Allergic diseases	Flea bite hypersensitivity
	Non-seasonal atopic dermatitis
	Contact allergy
Other diseases	Keratinisation disorders
	Adverse drug reactions
	Epitheliotropic lymphoma
	Autoimmune disease (foliaceus pemphigus)
	Any disease with secondary pyoderma or malassezia dermatitis

Among the differentials, the main are parasitic diseases associated with pruritus, such as sarcoptic mange, and allergic conditions, such as flea bite hypersensitivity and atopic dermatitis (Hensel et al. 2015) (LoEII). These conditions may all cause ventral pruritus and erythema (Figure 1.10 a,b,c). Although these conditions may also appear with suggestive type and distribution of lesions (Figure 1.10 g,h,i,l), none are pathognomonic and the diagnosis of AFR is reached per exclusionem of other differentials (Table 1.3). Ectoparasitic infestations and flea bite hypersensitivity have to be excluded by skin testing and by ectoparasite control. Secondary infections, such as bacteria and yeast infestations should also be treated because they may mask the initial signs. Once clinical signs are still present after ruling out parasitic and infectious diseases, then, diagnosis of allergic diseases is made. Differentiation between AFR and AD is more than challenging because AD, presents itself with clinical signs indistinguishable from those of AFR (Figure 1.10 a,b,d,e,g,h) and unfortunately, there

are no laboratory tests so sensitive to allow a reliable differentiation (Mueller and Olivry 2017) (LoEII).

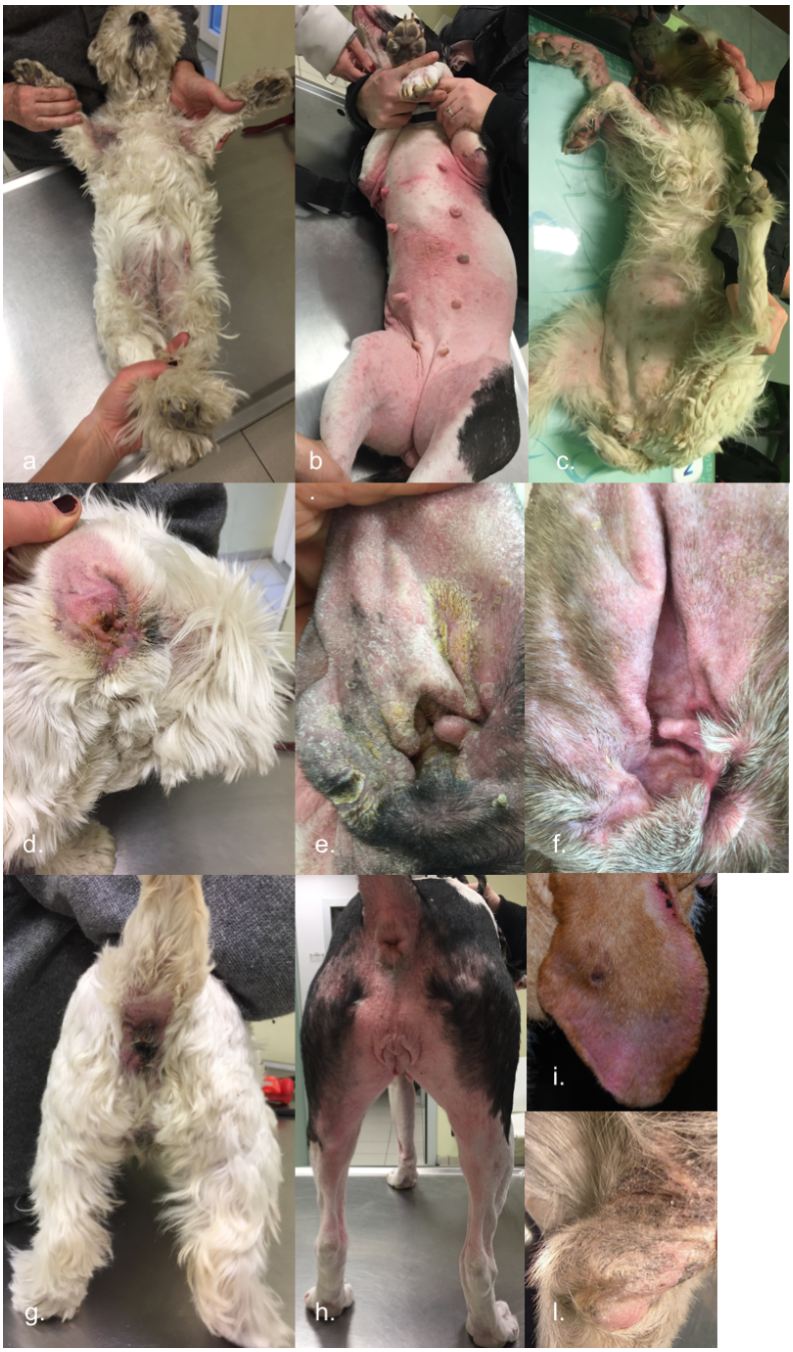

Figure 1.10: Distribution of dermatological lesions in dogs with atopic dermatitis (a,d,g), adverse food reaction (b,e,h) and sarcoptic mange (c,f,i). All can present pruritus, erythema and secondary lesions with similar ventral distribution (a,b,c) and ears involvement (d,e,f). However perianal pruritus is more frequently present in dogs suffering of allergic diseases rather than other dermatological conditions. Convex aspect of the pinna and its margins (i) and elbows (l) are more often affected by sarcoptes mange.

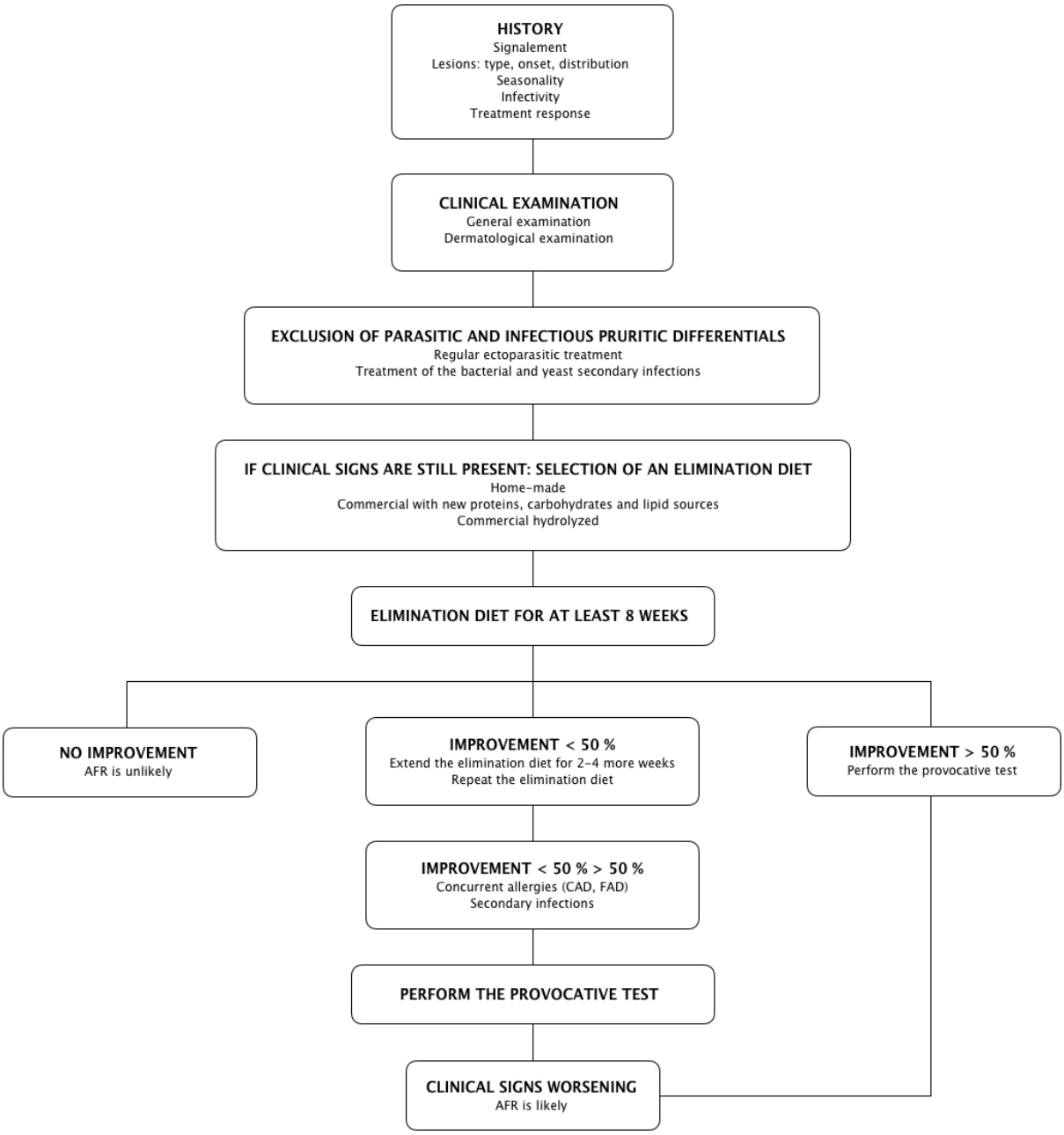

Figure 1.11: Schematic differential.

Definitive diagnosis is made by performing a food trial test, whose parts are elimination and provocative tests (Figure 1.11). Diagnosis of AFR is made with the alleviation of clinical signs when the animal is being fed an appropriate novel or hydrolyzed diet and recurrence of clinical signs when the patient is challenged with the previous diet (Fadok 1994; Guilford et al. 1996; Paterson 1995) (LoEIII). The goal of this test is to avoid contact with allergens in order to stop the allergic reactions and the relative

clinical signs bearing in mind that even small amounts can trigger reactions in sensitized patients (Roudebush et al. 1994) (LoEIII).

1.7.1. Food trial: elimination and provocative diets

1.7.1.1. Elimination diet

The choice of the elimination diet is an important step that can be hard if the dog has already been fed many diets, narrowing the ingredients to make the elimination diet (Gaschen et al. 2011). For this reason, it is very important that the nutritional history is as detailed as possible.

The majority of food allergens are proteins or glycoproteins, although there is evidence in humans that even carbohydrates or lipids can be allergenic (Cave 2006) (LoEIII). However, it is more likely that protein contaminants within the carbohydrates trigger the allergic reactions. For instance, corn contains proteins with a molecular weight between 20 and 23 kDa, which can induce allergic responses (Frisner et al. 2000). Similarly, vegetable oils and animal fats may contain lipophilic proteins (Crevel et al. 2000; Olszewski et al. 1998; Zitouni et al. 2000). In this context, an elimination diet should include, in addition to a new protein, new carbohydrate and lipid sources. Some food such as fish, lamb, chicken and rice should be avoided as these are present in the majority of maintenance commercial diets. Therefore, it is likely that the dogs may be already sensitized to those food (Brown et al. 1995) (LoEIII). Studies demonstrated that food such as beef, dairy products, chicken, wheat, eggs, soy and lambs are most allergenic for dogs (Mueller et al. 2016; Roudebush 2013) (LoEII). Finally, an elimination diet should avoid animal proteins sources that are taxonomically similar to those included in the dog's current diet to avoid cross reactions (Bexley et al. 2017) (LoEIII). When the owner reports an elimination diet was already performed, it is mandatory to ensure that ingredients, duration and exclusivity have been correctly followed. Moreover, it is important to guarantee if a correct protein was used, for a correct amount of time and without mistakes in the diet. The elimination diet can be either commercial or home-made. Recently, the use of a Cyno-DIAL test (Galileo Diagnostics by Genclis, Vandoeuvre- les-Nancy, France) was reported to select the best commercial diet to use as elimination diet (Favrot et al. 2017; Maina et al. 2018) (LoEIII). This test uses western blotting to detect in serum IgE specific to proteins in the current diet and the available choices for the elimination diets. It is interesting to

note that in three dogs allergic to diets commonly used in food trials, the clinical signs were alleviated when the diet was changed to one suggested by this test, allowing after the provocation diet the diagnosis of adverse food reaction (Favrot et al. 2017) (LoEIII).

1.7.1.2. Different types of elimination diet

Home-made diet

Many authors recommend the use of home-made diets as elimination diet, because this decreases the risk to erroneously introduce unwanted food components (Bloom 2005; Rosser 2014; Roudebush et al.1992) (LoEIII). Indeed, it may happen that dogs that tolerate ingredients present in a home-made diet may react to a commercial diet made with the same ingredients (Hensel et al. 2015) (LoEII). This can be due to the thermic process that changes the conformation of food allergens, the presence of additives, as well as a potential contamination of the commercial diets with protein sources not declared on the label (Leistra et al. 2001; Raditic et al 2011; Ricci et al. 2013; Rosser 1993; Rutgers et al. 1995) (LoEIII). Ricci and colleagues (2013) demonstrated by microscopic analysis and polymerase chain reaction (PCR) assay that 10/12 single protein commercial diets and in one hydrolyzed commercial diet, normally used for diagnosis of AFR, were contaminated with ingredients of animal origin not mentioned on the label (LoEIII).

Home-made diets have some disadvantages as well. These may be nutritionally imbalanced, causing gastrointestinal problems and weight loss. Indeed, in both North America and Europe most prescribed diets contain too much protein and fats and are poor in calcium, Ca:P ratio, essential fatty acids, vitamins A and E, copper and zinc (Hesta et al. 2002; Roudebush 1992, Streiff et al. 2002) (LoEIII). In the majority of home made elimination diets meat is often used as a single source of protein and it is frequently the dominant ingredient. As a consequence, these diets are too rich in protein, B vitamins, and phosphorus, but deficient in calcium. Even though clinical signs are unlikely to occur with a short-term calcium deficiency in an adult dog, clinical disease, such as fibrous osteodystrophy, can rapidly occur in a puppy within 6-8 weeks (Cave 2014; McMillan et al. 2006) (LoEIV). Furthermore, preparing a home-made diet is time consuming and expensive, especially for large breeds. In addition, some owners might not like cooking particular protein sources, like rabbit or horse. To be

noted, a home-made diet has also the advantage to be tailored to patients/owners needs (Hill 1999; Remillard et al. 2000) (LoEIV). Understanding the needs of the owner is mandatory to increase compliance.

Commercial diets

Commercial food diets can be dry food or canned and are made with ingredients never eaten before by the dog or with hydrolyzed food. Many owners find these diets convenient and handy. They are easy to find and practical to use, less time consuming and nutritionally balanced, resulting in a better compliance than home-made diets (De Jaham 2000; Roudebush et al. 1992; Tapp 2002; Vroom 1994) (LoEIII).

Commercial diets used for food trials are generally made from a single source of protein and therefore called limited antigen diet or “novel protein” source diet. They are also misleadingly called hypoallergenic diets as these preparations can decrease the allergenic reactions only if the dog is naïve for the protein contained in it. These diets contain protein sources such as venison, horse, duck, wild boar, pork, fish and others, often associated with new sources of carbohydrates such as potatoes, sweet potatoes, parsnip and barley. To address the need of new protein sources, exotic protein sources such as ostrich, buffalo and kangaroo are now available to the market. Even though these diets have been recommended in the past, their usage is now questionable because they might contain traces of undeclared protein sources (Horvath-Ungerboeck et al. 2017; Leistra et al. 2001; Raditic et al 2011; Ricci. et al 2012; Roudebush et al. 1992; Vroom 1994) (LoEIII).

Hydrolyzed diets are alternatives to prevent food allergic reactions. Proteins within these diets are degraded sufficiently to remove allergenic epitopes, preventing the immune system to recognize the culprit allergen. These diets, conversely to a limited antigen diet, may contain protein commonly eaten by the dog. Proteins consist of multiple amino acids linked together by peptide bonds, forming a long chain that is folded into a three-dimensional structure. The antigenicity of a protein can be decreased by (1) disrupting its three-dimensional structure, (2) altering the structure of amino acid side chains (e.g. conjugation of amino acids with sugars, oxidation of amino acids) or (3) cleaving the peptide bonds (hydrolysis). The latter forms the basis of hydrolyzed proteins used in canine diets. Hydrolysis disrupts the proteins in peptides of <5 kDa (partially-hydrolyzed formulas) or <3 kDa (extensively-hydrolyzed formulas). The resulting peptides are so small that they are not recognized by allergen-

specific IgE antibodies. Indeed, in humans with an IgE-mediated hypersensitivity to food, it seems that peptides have to be larger than 10 kDa to trigger a reaction (Cave 2006; Dupont et al. 2012; Lowe et al. 2013; Shah et al. 2012). Hydrolyzed diets are prescribed when the dog has already eaten any source of protein or when the nutritional history is unknown. Price and questionable efficacy are however the main disadvantages. A systematic review has shown that up to 50% of allergic patients included in three studies, which have been fed with partial hydrolyzed diets containing a protein source to whom dogs are allergic, experienced worsening of clinical signs (Olivry et al. 2010c) (LoEII). This could be explained by an incomplete hydrolysis resulting in the presence of larger peptides, small peptides (3-5 kDa) binding to two IgE molecules as haptens or even smaller peptides (0.5 kDa), which could trigger non-IgE mediated lymphocytic reactions. Indeed, it has been demonstrated that a high concentration of small peptides (<0.5 kDa) can activate T cells *in vitro* (Hemmer et al. 2000; Murphy 2011; O'Brien et al. 2008). Thus, it has been suggested to use only hydrolyzed diets that contain a protein source to whom the dog is not suspected to be allergic to (Olivry et al. 2010c) (LoEII). In a recent study, the allergenicity of an extensively-hydrolyzed formula containing chicken feathers was compared to a partially-hydrolyzed formula containing poultry liver. Forty percent of dogs fed with the partially-hydrolyzed diet experienced worsening of their clinical signs compared to none of the dogs fed with an extensively-hydrolyzed diet (Bizikova et al. 2016) (LoEIII). Therefore, it has been suggested to use extensively-hydrolyzed formulas during food trials. Unexpectedely, in the latest studies, 7-8% of dogs with confirmed AFR had specific IgE's against the above mentioned extensively-hydrolyzed diet (Favrot et al. 2017; Maina et al. 2018) (LoEIII). This diet, which contains extensively hydrolyzed poultry feather and purified cornstarch, has 95% of the peptides originating from poultry feathers below 1kDa (Bizikova et al. 2016). In addition, it does not contain cross contamination with undeclared animal proteins, but it does contain residual presence of granule-bound starch synthase-1 (GBSS-1), an amylogenesis enzyme protein from corn starch (*Zea mays*) that is not subjected to hydrolisation (Lesponne et al. 2017) (LoEIII). In line with previous findings, dogs with suspected AFR have been shown to produce specific IgE against GBSS-1 protein (Roitel et al.2017) (LoEIII). In confirmation of this, none of the dogs, negative to the oral food challenge with corn, included in the study of Bizikova et al. (2016) positively react to this extensively hydrolyzed diet (LoEIII). Unfortunately, if this IgE sensitization to

carbohydrate-derived proteins is associated with clinical signs of AFR in dogs is still to be demonstrated, until that time, this extensively hydrolyzed diet should be used only in non-corn allergic dogs.

Duration of elimination diet

The duration of a restrictive or elimination diet has been a matter of debate for a long time. In the beginning, it was recommended to perform an elimination diet for 3-4 weeks (Ackerman 1998; Harvey 1993; Jeffers et al. 1991; Muller et al. 1989; Walton 1967; Fadok 1994; Paterson 1995) (LoEIII). Later, Rosser (1993) pointed out that a food trial should last up to 10 weeks because only 25% of food allergic dogs could have been correctly diagnosed when the diet was continued for only 3 weeks, while the remaining part improved after 6-10 weeks (LoEIII). In the study of Denis and Paradis (1994), some cases needed 13 weeks to improve (LoEIII). Recently, Olivry and colleagues (2015) examined the veterinary literature to find relevant evidence about the duration of the elimination diet, agreeing that to diagnose 90% of case with AFR, the elimination diet should be last at least 8 weeks (LoEII).

1.7.1.3. Provocative diet

Dogs that improve during the elimination diet are then challenged with the original food with a provocative diet. Every food fed to the dog in the past has to be tested, one at a time for a period of 7-14 days each (Harvey 1993; Hill 1999; Jeffer 1991) (LoEIII). In those cases where the nutritional history is unknown or with a non-cooperative owner it is possible to first test those aliments that are commonly associated with AFR, such as beef, dairy products, chicken and wheat (Mueller et al. 2016) (LoEII). Depending on the underlying pathogenic mechanism, symptoms can appear upon a few hours to three days, but if the protein has been avoided for more than one month it may last up to 7 days to trigger clinical signs (Guaguère 1993; Furukwa 1991; Jeffer 1991; Rosser 1993; Walton 1967; White 1969; Wills 1992) (LoEIII). It is not clear if these delayed reactions would really require a longer challenge or if it would be enough to fed an appropriate amount of protein in one single administration and then monitor the patient for two weeks. If one-two weeks after re-introduction, an ingredient, present in the original diet, does not trigger any reaction, it is possible to test a new food component. Since some dogs develop allergies against multiple food components, it is advised to

test all foods fed to the dog. If symptoms occur on one specific food component, the challenge must be interrupted and the dog should be fed an elimination diet until clinical signs disappear. Then, a new food component can be tested. Every food component that triggers a reaction must be considered to be responsible for AFR-related symptoms and should therefore be avoided.

1.7.2. Other tests

Since food trials are very time-consuming, quite expensive and require both a compliant owner and dog, laboratory or other *in vivo* tests may be seen as attractive options.

1.7.2.1. Skin biopsy

A skin biopsy is not recommended as routine diagnostic tests for AFR because histopathological features of AFR are similar to many any other hypersensitivities. Lesions are non specific, usually characterized by chronic hyperplastic dermatitis with varying degrees of superficial perivascular dermatitis with mononuclear cells or neuthrophils infiltrating as seen in atopic dermatitis (Figure 1.12). Sometimes eosinophils can also be present and rarely eosinophilic vasculitis can be seen (Nichols et al. 2001) (LoEIV). Veenhof and colleagues found that CD8+ lymphocytes are the most representative cells in AFR (Veenhof et al. 2011), however these are also present in dogs with AD (Olivry et al. 1997) (LoEIII). Lastly, a case of AFR has been described that histologically was characterized by an epitheliotropic lymphocytic infiltrate mimicking an epitheliotropic lymphoma (Ghernati 1998) (LoEIII).

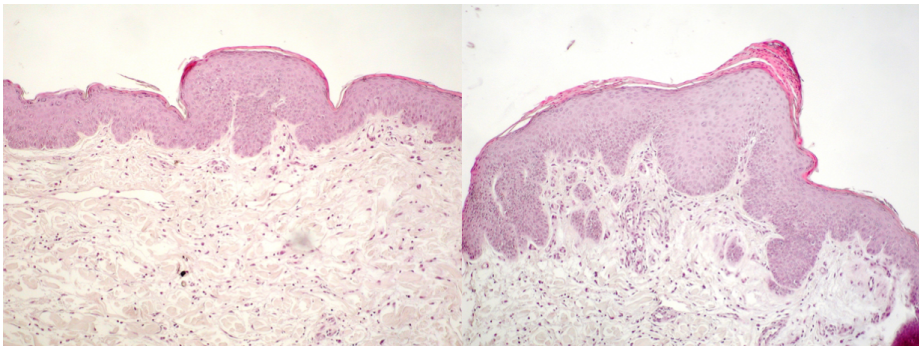

Figure 1.12: Chronic hyperplastic dermatitis with mild superficial perivascular and interstitial inflammation in a dog with AFR (left) and in a dog with AD (right) (courtesy of Noli C.).

1.7.2.2. Serological tests

Serological tests are commercially available for both human and dogs. In human, they are routinely used to assess if the patient is sensitized against a particular food and to confirm that the reaction is IgE mediated. However, specific IgE are unable to differentiate clinically relevant allergy from tolerance. Also in veterinary medicine, many labs promote these tests to diagnose AFR even though their use is not recommended because of poorly reproducible results and a low correlation with clinical signs and the results of a food trial, which remains so far, the "gold standard" for diagnosis of AFR (Hardy et al. 2014; Jeffer et al. 1991; Mueller and Olivry 2017; Mueller et al. 1998; Wilhelm et al. 2005) (LoEII). In dogs, many studies demonstrated that serological tests, such as Radio Allergosorbent Test (RAST) and Enzyme-Linked Immunosorbent Assay (ELISA), are characterised by a low sensitivity and a high specificity (Bethlehem et al. 2012; Jeffer et al.1991; Mueller and Olivry 2017; Mueller et al. 1998) (LoEII). In fact, even if immediate hypersensitivity reactions are responsible for a part of the allergic response, the principal pathogenic mechanisms seem to be cell mediated or not immune-mediated (e.g. food intolerance) (Day 2005; Ishida et al. 2004; Fujimura et al. 2011; Jeffer et al. 1991; Spergel 2006; Suto et al. 2015) (LoEIII). These tests might have a low sensitivity because of polyreactive IgE, a possible influence of food-specific IgG, or a poor allergen preparation (Halliwell et al. 2001; Peng et al. 1997; Zimmermann et al. 1980) (LoEIII). Because of this low sensitivity of ELISA, other techniques based on IgE detection, such as western blotting have been tested. Even though this test gave better results than ELISA, it is not recommended because it is still inaccurate (Bethlehem et al. 2012) (LoEIII). In addition, serological tests can yield false positive results in healthy dogs and in those with diseases different than AFR. This phenomenon called “asymptomatic hypersensitivity” has been demonstrated in many studies (Bethlehem et al. 2012; Halliwell et al. 2005; Foster et al. 2003; Mueller et al. 1998) (LoEIII). Production of higher concentrations of IgE specific against food components in clinically healthy dogs may be related to the breed, as demonstrated in West Highland white terriers (Day et al. 2005; Ermel et al. 1997; Roque et al. 2011a; Roque et al. 2011b; Teuber et al. 2002; de Weck et al. 1997) (LoEIII). Since IgG can appear against food antigens and as such serological tests based on IgG are not recommended in human nor in dogs (Boyce et al. 2011; Carr et al. 2012; Cianferoni et al. 2009; Gerez et al. 2010).

IgG seem to be more involved in mechanisms of tolerance than in the pathogenesis of AFR (Sampson 1999; Stapel et al. 2008). A study evaluated the concentrations of food-specific IgG and IgE in dogs with AFR, AD or in healthy dogs and showed that food-specific IgG were higher in dogs with AFR. However, they were also present in the other groups (Halliwell et al. 2005) (LoEII). Presence of food-specific IgG in dogs with AD may be explained by exposure to the antigens via the diet or a cross-reaction with environmental allergens. Bethlehem and colleagues (2012) found that the positive predictive value of an allergen-specific IgG ELISA was 34.8%, while the negative predictive value was 83.7%. Nevertheless, the results of this ELISA are not sufficiently reliable to be used routinely in clinical practice (LoEIII).

1.7.2.3. Lymphocyte proliferation test

This assay assesses the ability of lymphocytes to respond to mitogens or specific antigens. It is used to investigate cell mediated reactions starting from whole blood and is based on the assumption that lymphocytes of patients with AFR, which are sensitised to one or more allergens, would proliferate when stimulated *in vitro* with those allergens. The lymphocyte proliferation test (LPT) has been evaluated as a diagnostic test for AFR (Ishida et al. 2004) (LoEIII). In 82% of the cases with AFR the LPT results correlated with those of a food trial. Additional data from this study demonstrated that LPT is better than IgE testing and intradermal testing (Ishida et al. 2004) in detecting true positivities (LoEIII). Unfortunately, even though this test seems to discriminate healthy from allergic dogs, unfortunately, it does not differentiate between dogs with AFR or AD (Fujimura et al. 2011) (LoEII). The accuracy of this test is higher than other tests (81-90%), with a positive and negative predicatability of 63-75% and 88-99% respectively (Mueller and Olivry 2017) (LoEII). The main limitation of this assay is that it requires fresh blood speciments that have to be processed too quickly which makes this test not appropriate for clinical routine.

1.7.2.4. Food intradermal test

In this test, a small amount of an allergen is injected in the dermis and if immediate cutaneous reactions, such as a wheal or erythema, occur, the test is considered positive (Figure 1.13). This test, like the others mediated by allergen-specific IgE, is not useful for diagnosis nor to detect dogs that should follow a food trial. It has a

sensitivity of 33% and specificity of 50%, a low predictive value and a poor correlation with food trial results (Ishida et al. 2004; Jackson et al. 2003; Jeffer 1991; Kunkle et al. 1992; Mueller and Olivry 2017) (LoEII).

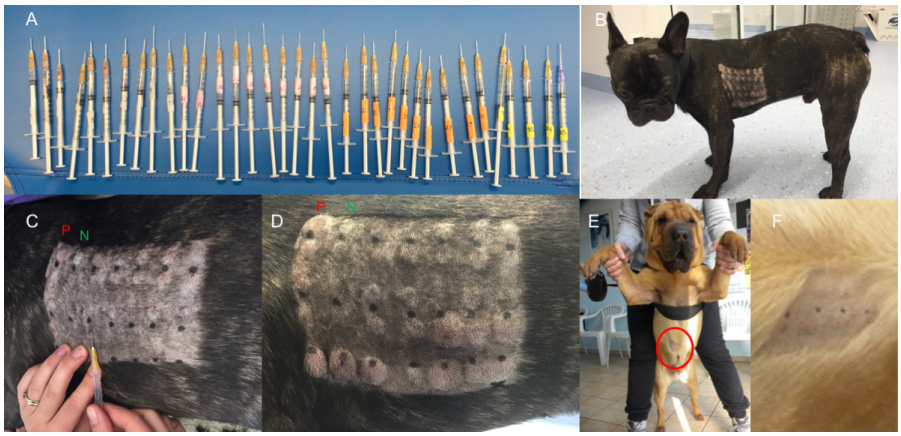

Figure 1.13: Small amount of allergen extracts (A) are injected into the skin with a syringe and a needle (C). The reading is performed after 10-15 minutes assessing the resulting wheals and erythema. One test with saline solution is used as a negative control (N) and one test with histamine as a positive control (P). The saline solution should cause no reaction. If it is positive it means that the skin of the patient is very reactive, and the rest of the tests can give false positive results. These are positive unspecific reactions, not really due to true allergic sensitisation. The histamine control must cause a positive reaction. If it is negative it means that the skin is not reacting, and the rest of the tests can give false negative results, although the patient is really sensitised. The wheal diameter of each allergen is compared to that of histamine and saline. For an objective evaluation, the test is considered positive when the wheal has a diameter bigger or equal to the diameter of histamine added to the diameter of control divided by two. This means that the dog is sensitised. If it is smaller, the test is considered negative, which means that the patient is not sensitised. Allergens used for AFR (E, F) are selected on the basis of dog's nutritional history and are often less than those used for AD (B).

1.7.2.5. Patch test

A patch test is used to determine if a specific food component can trigger a delayed reaction. To this end, tiny cups containing small amounts of a pure food component are taped to the dog's skin for up to 72 hours. The food components used in the test are selected based on the patient's nutritional history. After the incubation period, the patches are removed and the presence of erythema, papules, pustules or vesicles is evaluated (Figure 1.14). This test is very sensitive (96.7-100%) with a specificity of 89-100%, expecially for protein sources, rather than carbohydrates or dry dog food (Bethlehem et al. 2012; Johansen et al. 2017) (LoEIII). The negative and positive predictive values are 88-99% and 63-75%, respectively (Mueller and Olivry 2017) (LoEII). Positive results are not useful for diagnosis because there are too many false positives. On the upside, the negative results correlated well with the results of an oral food challenge, suggesting that the food components that do not trigger any reaction

are suitable to be used during the elimination diet (Bethlehem et al. 2012) (LoEIII). The downsides of the test are: it is impractical, time-consuming to prepare and to evaluate and it requires a high degree of compliance as the dogs should not remove the bandage and scratch themselves.

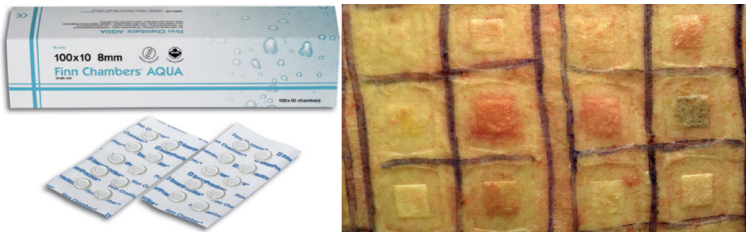

Figure 1.14: The patch test is performed by placing patches with allergens on the skin of the patient. These patches are removed after 48 hours and the final reading is performed after 72-96 hours. The skin is examined for reactions of redness, itching, papules or vesicles. If reactions appear, the test is positive, which means that the patient is sensitised to that specific substance.

1.7.2.6. Gastroscopic food-sensitivity testing

This test evaluates by endoscopical examination allergic reactions triggered by food allergen extracts applied on the gastric mucosa. An allergen solution is dropped onto the gastric mucosa and the area is observed for up to 5 minutes. Two-three minutes after the application, any changes such as local or diffuse erythema or swelling, as well as increased peristalsis are considered as positive reactions (Elwood et al. 1994; Guilford et al. 1994, Olsen et al. 1991; Vaden et al. 2000) (LoEIII). Reactive sites should be endoscopically biopsied to confirm the reactions with histology. These reactions correlate well with food trial results and no reactions have been seen with application of control substances (Guilford et al. 1994). Because of unsatisfactory accuracy, the test is not recommended for diagnose AFR in dogs (LoEIII).

1.7.2.7. Colonoscopy fecal IgE, hair and saliva testing.

Less frequently, other tests have been investigated for diagnose AFR in dogs such as colonoscopy, determination of canine fecal IgE, hair and saliva testing (Allenspach et al. 2006; Vaden et al. 2000) (LoEIII). Colonoscopic allergen provocation test showed a good accuracy being able to detect 73% of the culprit allergens (Allenspach et a. 2006) (LoEIII). Despite these promising results, the study was based on small uncontrolled case series. Moreover, future application of this technique in clinical practice is unlikely because it needs sedation and it may be too expensive. Fecal food-specific IgE and hair and saliva testing are not recommended as an alternative

to the food trial test because they could not differentiate allergic from non allergic dogs (Coyner and Schick 2016; Vaden et al. 2000) (LoEIII).

1.8. Treatment

There is no cure for AFR and strict avoidance of food allergens is the only way to prevent food-related reactions in previously sensitized patients. Once the food components to which a dog is sensitized have been identified through a food trial, then it is eliminated from the diet. Even though much attention is given to avoid culprit allergens, accidental exposures are still frequent. Some dogs are extremely skilled in scavenging or eating leftover food. This behaviour is so deeply ingrained in canines that it is difficult to stop. In addition, some diets may also be contaminated with undeclared proteins (Ricci et al. 2013) (LoEIII). Other explanations are that owners may hide giving some left-over food or treats to the dog. Alternatively, dogs may be allergic to multiple food components, making it very difficult to compose the maintenance diet (LoEIV).

When inadvertent exposure occurs, short-term relief may be gained with topical glucocorticoids, which are beneficial when the lesions are localized or with systemic treatment, such as oral glucocorticoids or oclacitinib when the lesions or pruritus are generalised (Table 1.4) (Olivry et al. 2010a) (LoEII). Oral prednisolone, prednisone or methylprednisolone given at 0.5 to 1.0 mg/kg per day, in one or divided into two doses, improve clinical signs of dogs with severe or extensive allergic dermatitis (Gadeyne et al. 2014; Olivry et al. 2003; Olivry et al. 2010a; Olivry et al. 2010b; Olivry et al. 2013; Taszkun et al. 2010) (LoEII). If remission occurs, the dose of oral glucocorticoids should be tapered until discontinuation. The anti-inflammatory effects of glucocorticoids are dependent on their ability to inhibit the activity of crucial transcriptional regulators of pro-inflammatory genes, including NF-κB and AP-1 (Clark 2007). Glucocorticoids therefore inhibit many of the initial events in an inflammatory response and they promote the resolution of inflammation by inhibiting vasodilation and increasing vascular permeability that occurs following inflammatory insult and decreasing leukocyte influx into inflamed sites and their differentiation.

Oclacitinib (Apoquel, Zoetis) is a JAK inhibitor that selectively inhibits JAK1-dependent cytokine secretion involved in allergy, inflammation and pruritus such as IL-31 (LoEIII). Oclacitinib can be prescribed at 0.4-0.6 mg/kg orally twice daily for up to 14 days to

rapidly reduce skin lesions and pruritus (LoEIII). Then if necessary it can be continued once daily without the need to taper (Olivry et al. 2015b) (LoEII). Short-term treatment with these medicaments appears safe (Cosgrove et al. 2013; Gadeyne et al. 2014; Little et al. 2015) (LoEIII). Furthermore, when the offending ingredients are not identified, the symptoms should be controlled for longer periods. Initially, flares should be treated with glucocorticoids or oclacitinib, which leads to a faster improvement than ciclosporin. Oral oclacitinib should be given at the same dosage as for acute flares twice daily for 14 days and then once daily thereafter. If glucocorticoids are prescribed, then they have to be replaced by oral ciclosporin or oclacitinib (Olivry et al. 2015b) (LoEII). These drugs are safer for long term use. Oral ciclosporin is a calcineurin inhibitor (Marsella et al. 2005). It is an immunosuppressive agent that inhibits T-cell activation, decreases the functions of mast cell and eosinophils, decreases cytokine secretion and hence the recruitment of eosinophils to the sites of allergic inflammation (Al-Dajari et al. 2002; Ciesek et al. 2005; Marsella et al 2001) (LoEIII). It should be administered at 5 mg/kg once daily until clinical signs are alleviated, which will usually take 4 to 6 weeks (Kovalik et al. 2011; Little et al. 2015; Olivry et al. 2010b; Olivry et al. 2013; Steffan et al. 2006) (LoEIII). Thereafter, in case a complete remission is obtained, ciclosporin can be tapered with a dose and frequency adjusted to maintain the remission of signs. Ciclosporin can be combined with oral glucocorticoids or oclacitinib for the first 3 weeks to accelerate the onset of clinical improvement. In an RCT study, ciclosporin, orally given at 5 mg/kg daily for 4 weeks, together with prednisolone at 1 mg/kg daily for 7 days followed by alternate day dosing for 14 days, led to a quicker improvement of skin lesions and pruritus scores than when ciclosporin was given alone (Dip et al. 2013) (LoEII). The prolonged concomitant administration of oral glucocorticoids, ciclosporin or oclacitinib in any combination is not recommended because of the theoretical higher risk of immunosuppression predisposing to potentially severe opportunistic infections of the skin or other organs.
The oral intake of essential fatty acids (EFAs), especially those rich in omega-6, either as supplement or in enriched diets, can influence superficial skin lipids and improving the quality of the coat. They are not useful to treat acute flares due to the length of time needed for any possible beneficial effect to occur. They are not suitable for monotherapy of canine AD, but since they have a glucocorticoid-sparing effect, EFAs intake is recommended for long-term therapies because it might allow for a further reduction in the dose and/or frequency of oral glucocorticoids, ciclosporin and perhaps

even oclacitinib, required to maintain remission of clinical signs of AD (Saevik et al. 2004) (LoEIII).

In an RCT study, pentoxifylline at a high dose of 20 mg/kg three times daily, either alone or in combination with oral EFA supplementation, was shown to improve skin lesions and pruritus. The effect seemed highest for dogs treated with the combination of pentoxifylline and EFAs (Singh et al. 2010) (LoEIII).

Recombinant canine interferon-gamma (IFN-γ; Interdog, Toray Industries) administered subcutaneously at 5,000 to 10,000 units/kg three times weekly for 4 weeks and then once weekly is effective to treat dogs with AD with minimal side effects (Iwasaki et al. 2006; Yasukawa et al. 2010) (LoEIII). Mild to no effects were shown for subcutaneous injections of recombinant feline IFN-ω (Virbagen Omega, Virbac) at 1 to 5 million units per dog, three times weekly for 4 weeks and then monthly thereafter (Carlotti et al. 2009; Litzlbauer et al. 2014) (LoEIII).

Type 1 antihistamines are not effective for treating acute flares nor as monotherapy for chronic allergic conditions. They should be given daily as preventatives to manage chronic AD. In combination with other antihistamines or other drugs, they may improve their beneficial effects although further studies are required to validate this (Olivry et al. 2010a) (LoEII).

Masitinib (Masivet/Kinavet, AB Science), a tyrosine-kinase inhibitor used in the treatment of mast cell tumors in animals, appears to offer some benefit in chronic cases, but the risk of severe adverse side effects makes this drug only a useful alternative for atopic dogs not responding to other approved drugs (Cadot et al 2011; Dajgle et al. 2010) (LoEIII).

Other drugs appear to provide little (misoprostol, tepoxalin, oral methotrexate) or no benefit (e.g. leukotriene inhibitors, capsaicin, dextromethorphan, low level laser therapy, selective serotonin re-uptake inhibitor (SSRI)) (Fujimura et al. 2014; Stich et al. 2014; Pin et al. 2012) (LoEIII). Others have not been studied in detail and require further proof of efficacy (high-dose oral pentoxifylline, oral low-dose once weekly methotrexate and the adjunctive effect of vitamin E to antihistamines) (Olivry et al. 2010a).

Every time acute flares are triggered by accidental ingestion of food allergens it is important to evaluate the presence of bacterial and yeast infections of skin and ear. Once they are diagnosed, they have to be treated with topical and/or systemic

antimicrobials following antimicrobial treatment guidelines established in the country of practice and/or in international consensus recommendations (Beco et al. 2010; Hillier et al. 2014; Olivry et al. 2010a) (LoEII).

Table 1.4: Treatment of AFR in dogs.

Acute/ chronic	Principle	Medicament*	Commercial name	Duration	Dosage
A/C	Improvement of skin and coat hygiene and care	non-irritating shampoo	Allermyl, Virbac	Short and long course	At least once weekly
A/C	Improvement of skin and coat hygiene and care	non-irritating shampoo	Douxo Calm, Ceva	Short and long course	At least once weekly
C	Improvement of skin and coat hygiene and care	Supplementation with oral EFAs	Megaderm/EFA-Z, Virbac	Long course	At least two months of supplementation.
A/C	Reduction of pruritus and skin lesions with pharmacological agents	topical glucocorticoids: hydrocortisone aceponate spray	Cortavance, Virbac	Short course Long course	1 spray, once to twice daily. 1 spray once to twice daily but intermittently
A/C	Reduction of pruritus and skin lesions with pharmacological agents	oral glucocorticoids: prednisolone, prednisone or methylprednisolone	Medrol vet, Pfizer; Prednicortone, Dechra.	Short course Long course	0.5 mg/kg once to twice daily 0.5 mg/kg once to twice daily until clinical remission, then tapered to the lowest dosage and frequency that maintains signs under control
A/C	Reduction of pruritus and skin lesions with pharmacological agents	oclacitinib	Apoquel, Zoetis	Short and long course	0.4– 0.6 mg/kg orally twice daily for up to 14 days than once daily.
C	Reduction of pruritus and skin lesions with pharmacological agents	Calcineurin inhibitors: Cyclosporine	Atopica, Novartis	Long course	5 mg/kg once daily until satisfactory control of clinical signs, then, tapered.

Medication that are reported in red colour should be used only for treating acute flares to avoid risk of side effects in the long term

*Only those medicaments for whom there is evidences of efficacy are listed

The therapeutic approaches listed above are based on evidence and have been recommended for atopic dermatitis. Since the clinical symptoms of AD overlap with AFR, the theapeutic approaches for AD may also be useful in AFR. However, dogs with AFR may be less responsive to corticosteroids or ciclosporin (and maybe to oclacitinib), probably due to a different mechanism of action. Lastly, concurrent allergies can influence the threshold for clinical symptoms in some animals (Roudebush et al. 2000) (LoEIII). Prevention of fleas and other causes of pruritus must be carried out (Kunkle 1995) (LoEIII).

1.9. Prognosis

The prognosis of AFR is very good when the offending food allergen is identified. The risk of relapses is associated with accidental ingestions or to new sensitization. Indeed, some dogs may become allergic to the new protein in their diet after 2–3 years (Fadok 1994) (LoEIII). When this happens, a new food trial has to be carried out to identify the new allergen in order to exclude it and to make a new ad hoc diet (De Jaham 2000) (LoEIII). In human, there has been a notion that strict avoidance would hasten recovery. This notion is based upon the theory that lack of exposure will result in deletion of immunologic memory. In a study of 23 adults with allergies to a variety of foods who underwent baseline double-blind, placebo-controlled food challenges,10 patients had positive challenges for 13 foods. These patients were placed on a strict dietary avoidance of the offending food for 1-2 years and then re-challenged. Five (38%) of the 13 previously offending foods were well tolerated. This particular study supports the conclusion that strict avoidance may have helped tolerance development in a subset of these adult patients with food allergy. It has been estimated that one-third of the persons that strictly avoided the offending food component during 1–2 years, tolerated new exposure to the food allergen (Pastorello et al. 1989). According to Muller et al. (1989) natural hyposensitisation rarely occurs (LoEIV). Since there is a concern that accidental ingestions can delay or prevent tolerance development, this might explain why natural hyposensitisation rarely occurs in dogs (Muller et al. 1989). Recently, the therapeutical role of strict avoidance has been questioned because of conflicting data and because avoidance may trigger increased reactivity (Allen et al. 2009). Indeed, if a food component that is tolerated is avoided for a long period, it may trigger severe acute reactions upon re-introduction (Barbi et al. 2004; Larramendi et

al. 1992). On the contrary, when immunotherapy is used to treat AFR in humans, it increases the threshold dose for allergic reactions and decreases the risk of severe reactions after inadvertent ingestion of the allergens. Thus, challenging the conventional wisdom of strict avoidance.

2. Allergen-specific immunotherapy

2.1. Introduction

Allergen-specific immunotherapy (ASIT), which is also called allergen immunotherapy, hyposensitization therapy or desensitization, is a form of long-term treatment that decreases the symptoms of patients with allergic conditions by reducing their sensitivity to allergens. It is highly effective in the treatment of IgE-mediated diseases, such as allergic rhinitis, conjunctivitis and venom hypersensitivity. It is the only treatment which leads to a life-long tolerance against previously disease-causing allergens due to restoration of the normal immunity (Bonifazi et al. 2005; Bousquet et al. 1998; Durham et al. 1999; Kussebi et al. 2003). It is also used in asthma and in bronchial hyper-responsiveness. Its use in atopic dermatitis is questionable as the evidence for effectiveness is weak. Allergen-specific immunotherapy is also used to prevent the onset of new sensitizations (Pajno et al. 2001) and to reduce the development of asthma in patients with rhinitis caused by airborn allergens (Eng et al. 2002; Moller et al. 2002). Based on the results of recent studies, ASIT appears to be effective in inducing short-term desensitization in patients with AFR. However, in the latter ASIT is not yet ready for implementation in a clinical setting and randomized, placebo-controlled studies are needed to determine whether these strategies induce long-term tolerance.

2.2. Historical perspective of ASIT in humans

Allergen-specific immunotherapy is not a new method to treat allergic disorders. The first attempts of allergen-specific immunotherapy date back to the antiquity when king Mithridates VI from Pontos (120-63 BC) ingested increasing doses of snake venom to make himself immune against this toxin. It is unknow if this immunotherapy worked, because he killed himself with a sword when the Romans conquered his kindom. Although Leonard Noon is credited as the pioneer of the immunotherapy, before him, in the late 1800s, Blackley, Curtis and Dumbar performed the first self-based investigations into the nature of pollen allergy and treatment. However, Noon has been the first to perform a clinical trial using grass pollen extracts to desensitize patients suffering of allergic rhinitis. Even though he did not know the pathogenesis of the disease and the mechanism of immunotherapy, he was the first to publish a successful

human trial of immunotherapy in 1911. Erroneously, he believed that “hay fever was caused by a soluble toxin” and the patients present the idiosyncrasy because of their sensitivity to this toxin”. Noon hypothesized that inoculations of pollen extract led to the production of antibodies against toxins in the pollen. Despite the wrong rationale he succeeded to demonstrate that prophylactic subcutaneous inoculation with a grass pollen extract was effective in suppressing immediate sensitivity to grass pollen (Noon 1911). Unfortunately, Noon died a few years later (1914) and his incomplete work was carried on by Freeman who reported in 1914 results of the first immunotherapeutic trial with 84 patients treated with grass pollen extracts during a three-year period. This protocol showed successful outcomes and acquired immunity lasting at least one year after the treatment was discontinued (Freeman 1914). In 1930, he published the first rush immunotherapy protocol (Freeman 1930) in which injections of pollen vaccines were given at 1.5 to 2 hours intervals over a daily 14-hour period under close observation in a hospital setting. In view of these findings the concept of pollens as toxins was replaced by pollens as allergens.

In 1915 Cooke introduced the intracutaneous skin test technique, which permitted practical identification of a wide variety of allergenic substances (Cooke 1914). The development of methods to extract allergens from food, airborne and environmental materials was extensively pursued by Wodehouse (1917), Walker (1917) and Coca (1922). From now on a variety of injectable materials become available for the treatment of allergic patients. In 1921 Kern recognized house dust as an important environmental allergen, stressing the role of non-seasonal allergens in the pathogenesis of allergic diseases. The high prevalence of positive reactions in the intradermal skin test to house dust initiated a widespread use of a house dust vaccine for the treatment of perennial rhinitis and asthma. Noon, Freeman and Coca’s methods were rapidly taken up by physicians trying to treat allergic diseases and hospital and clinics devoted to diagnosis and treatment of allergic disorders were established (Cohen 1983). However, allergen-specific immunotherapy (ASIT) was administered empirically without control group for many years with non-standardized extracts. The first controlled randomized trial with pollen extract was published by Frankland et al. in 1954. In this study, 200 patients with seasonal hay fever were enrolled and divided in four groups to receive whole grass pollen extract, purified pollen protein, the corresponding ultrafiltrate that contained no protein and a phenol-containing diluent. Only the whole grass pollen extract and the purified grass pollen proteins were

effective to decrease symptoms. At the end of the study, 79% of the patients had a good to excellent response to the treatment. Frankland (1955) also demonstrated that hyposensitization with injections of grass pollen extract was significantly more effective for treating hay fever when given in a higher dose than a lower dose. Parallel studies were also conducted by Cooke (1914), one of the creators of the term « atopy », introduced immunotherapy in the Unites States calling it « active immunisation » in 1914. Later, in 1922, he proposed the term « hyposensitization » as a better name for this type of treatment (Cooke 1922). Cooke was aware of the problems of variations in allergenic activity of the vaccines due to non-standardized extraction methods, which he tried to solve together with the chemist Stull. They developed an assay based on the measurement of protein nitrogen content as representation of residual stable activity of allergenic fractions (Stull et al. 1933). In 1965 Lowell and Franklin published the first double-blinded study about the effectiveness and specificity of immunotherapy in treating ragweed hay fever (Lowell and Franklin 1965). They demonstrated that a single allergen (ragweed) in a multi-allergen mixture was effective in reducing seasonal allergic symptoms. Philip Norman and Larry Lichtenstein in 1978 demonstrated the allergen specificity of ragweed immunotherapy in patients with dual sensitivity to ragweed and grass pollen (Norman and Lichtenstein 1978). Hunt and colleagues demonstrated the efficacy of purified venom over whole insect body extracts in patients with anaphylaxis to the stings of hymenoptera (Hunt 1978). The long-term benefits of allergen immunotherapy, with a persistent efficacy of several years after discontinuation, have been illustrated for both venom (Golden et al 2000) and grass pollen immunotherapy (Durham et al 1999; Durham et al 2010). In 1986, the UK Committee on Safety of Medicines established that subcutaneous immunotherapy with respiratory allergens was not safe, as it could induce life threatening adverse reactions (CSM update 1986). From then on, researches aimed to find a safer route of administration and in 1998 the sublingual route, which was investigated for the first time in 1986 in a randomized, double-blind, placebo-controlled trial (Scadding et al. 1986), was mentioned as a possible alternative to subcutaneous immunotherapy in a World Health Organization position paper (Bousquet et al. 1998). Its application in clinical practice was then confirmed in subsequent articles (Bousquet et al. 2001; Bousquet et al. 2008). Even though the initial study reported conflicting results, recent meta-analyses led the Word Allergy Association, the Cochrane Collaboration, and the Johns Hopkins University Evidence-

Based Practice Center to conclude that SLIT is a safe and efficacious treatment (Bousquet et al 2009; DeBoer et al 2016; Lin et al. 2013; Radulovic et al 2011). In 2014 the first two SLIT products received United States regulatory approval for treatment of human pollen-induced allergic rhinitis (Oralair, Stallergenes, Norwell, MA, USA and Grastek, Merck, Whitehouse Station, NJ, USA).

2.3. Genesis of ASIT in dogs

The first report of successful allergen-specific immunotherapy in dog goes back to 1941 when Wittich treated a dog with symptoms of hay fever (Wittich 1941). Since then, immunotherapy has been applied for the treatment of canine atopic dermatitis for nearly 70 years. SCIT has been used for several years as the only approach to desensitize dogs suffering from atopic dermatitis. Two SCIT methods are mainly used. In North America, dogs are subcutaneous injected with increasing doses of saline-phenol preserved allergen extracts. In Europe, allergen extracts are injected with adjuvants such as alum to provide a slow release formulation in order to decrease the frequency of the injections. Conversely to human, where administration of sublingual immunotherapy (SLIT) goes back to 1986 (Scadding et al 1986), the first trial in dogs was reported in 2016 (DeBoer et al. 2016).

2.4. Mechanisms proposed of ASIT

Over the century, several mechanisms have been proposed to explain the effect of ASIT, but the immunological changes that occur during allergen-specific immunotherapy are still not completely understood. The main goals of immunotherapy are to induce peripheral T cell tolerance, to regulate allergen-specific antibodies and to modulate the threshold for the activation of mast cells, eosinophils and basophils. For many years it was wrongly believed that an allergic patient was characterized by a Th2 hyperpolarisation and that a successful immunotherapy should promoted a shift from a Th2 to a Th1 polarisation (Arzl et al. 2011). We now know that allergic conditions are associated with a Th2 cytokine profile in the acute phase and with Th1 cytokines in the chronic phase. A successful immunotherapy induces a shift toward peripheral T cell tolerance, which is achieved by the generation of allergen-specific regulatory T cells (Tregs). Tregs can be naturally occurring or induced. The latter,

called iTregs, were shown to play an important role in allergen tolerance preventing unwanted immune responses to non-pathogenic environmental allergens in healthy individuals. Regulatory T cells work by means of the two cytokines IL-10 and TGF-β. The former downregulates MHC-II molecules on APCs, skews the allergen-specific IgE response to an IgG4 dominant response and decreases the release of pro-inflammatory cytokines and their receptors. IL-10 also downregulates eosinophilic activity, suppresses IL-5 and GM-CSF production and CD40 expression by activated eosinophils and enhances eosinophil cell death (Treter et al. 2000). TGF-β on the other hand, inhibits B-cell proliferation and differentiation, decreases immunoglobulin production and further promotes the conversion of naïve CD4+CD25- to CD4+CD25+ Tregs. The latter are also able to control allergic inflammation by suppressing Th2 immune responses, inhibiting mast cell degranulation directly by OX40-OX40L interaction, and downregulating costimulatory molecules on DCs, which prevents their ability to activate naïve CD4+ T cells.

ASIT induces a transient increase in allergen-specific serum IgE, followed by a gradual decrease during the months or years of treatment. It is interesting to note that the improvement of allergic clinical signs does not match with the decrease in allergen-specific serum IgE levels of patients under ASIT treatment. Improvements occur earlier than the reduction in serum IgE levels. Curiously, the decrease in the IgE/IgG4 ratio during ASIT seems to correlate with a skewing from allergen-specific Th2 to Treg cell responses. IgG4 is considered a non-inflammatory protective isotype. It acts as a 'blocking antibody' by competing for the same epitopes as IgE thus preventing IgE-dependent allergic responses by increasing the threshold required for mast cell and basophil degranulation, IgE-mediated antigen uptake and development of memory IgE production (Flicker et al. 2003). The ability of IgG4 antibodies to inhibit these allergic reactions relies not only on the quantity of the blocking antibody, but also on their affinity and specificity. Allergen-specific immunotherapy also decreases the recruitment of mast cells, basophils and eosinophils to the skin, nose, eyes and bronchial mucosa after exposure to allergens and reduces the release of mediators, such as histamine, from basophils and mast cells (Canadian Society of Allergy and Clinical immunology 2010; Joint Task Force on Practice Parameters et al. 2007; Frew 2010).

The mechanism by which immunotherapy exercise its effect in dogs is still unclear. As one might expect, it probably depends on the route of administration. In dogs, as in

humans, subcutaneous specific immunotherapy increases the number of Tregs and IL-10 levels in atopic dogs. It also increases the IgG levels and promotes a shift to a Th1 cell response (Fraseret al. 2004; Hou et al. 2008; Keppel et al. 2008; Hites et al. 1989; Shida et al. 2004) (LoEIII).

2.5. ASIT as a treatment for AFR in humans

Apart from pollinosis, airway allergy and venom hypersensitivity, allergen-specific immunotherapy is also used to treat food allergy. Prevalence studies in humans indicate that around 10% of the Western population suffers from food allergy, with the highest occurrence in children and with an increasing incidence over time (Branum et al. 2009; Sicherer et al. 2010). For egg, milk, wheat and soy allergens, spontaneous clinical tolerance can occur. Children allergic to peanut, tree nuts and seafood allergens rarely show spontaneous tolerance (Carr 2005; Vickery et al. 2011). Only a strict dietary avoidance can prevent the occurrence of allergic reactions in these patients (Moran et al. 2013; Sicherer et al. 2010; Vickery et al. 2011). Even though avoidance of food stuffs is an effective “treatment” for food allergies, it still leaves patients at risk of life-threatening reactions if accidental exposure occurs. This risk causes anxiety and decreases the quality of life (Moran et al. 2013; Rabjohn et al. 1999; Sicherer et al. 2010). Driven by these concerns, allergen-specific immunotherapy has also been tested as an alternative therapy for food allergies. This involves treating patients with escalating doses of the offending food over a period of several months. Incremental exposure to a given antigen can render an individual temporarily less reactive to that antigen by increasing the reactivity threshold (e.g. desensitization) or, less frequently, it may result in sustained tolerance (Kulis et al. 2011).

The first administration route to be explored for immunotherapy in food allergies was the subcutaneous one in 1992. Peanut allergic patients were injected subcutaneously following an initial rush schedule and then by weekly maintenance dosing for 4 weeks (Oppenheimer et al. 1992). Despite the promising results, one patient in the placebo group received a maintenance peanut dose due to a formulation error and died. Systemic adverse reactions also occurred in another study during the rush build-up and maintenance phase. Because of the high risk of life-threatening adverse

reactions, SCIT was considered unsafe and not a recommended route to use (Nelson et al. 1997). Studies on SCIT were therefore discontinued until 2008 when Carp parvalbumin (Cyp c 1), the molecule which contains the most cross-reactive IgE epitopes in the family of homologous fish parvalbumin, was modified rendering it hypoallergenic. Parvalbumin is the allergen to which the majority of fish-allergic patients are sensitized (Griesmeier et al. 2010; Lopata et al. 2009; Van Do et al. 2005). After pre-clinical testing (toxicology testing and efficacy in mouse models), which show good safety profile, SCIT with alum-absorbed hypoallergens Carp parvalbumin entered in phase I/II^{a} and II^{b} randomized double-blind placebo-controlled (DBPC) clinical trials (Zuidmeer-Jongejan et al. 2012; Zuidmeer-Jongejan et al. 2015).
Despite these promises, many groups of research are investigating alternative routes safer than SCIT, such as oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) (Jones et al. 2014). Most studies assessed oral immunotherapy (OIT), which involves a daily administration of the offending food allergen mixed with a food vehicle in gradually, but constantly increasing doses (varying from milligrams to grams) over the course of several months to years until a pre-specified maintenance dose is reached (mostly 4 gr) (Le et al. 2014). If this maintenance dose is successfully and safely achieved, the patient is desensitized. After that, the maintenance dose may be discontinued for a pre-specified amount of time and the patient rechallenged with the offending food. If the subject does not react, he or she has achieved sustained tolerance. The mechanism of action seems to overlap with aero-allergen immunotherapy: increased regulatory T cells, decreased allergen-specific IgE levels, increased protective allergen-specific IgG4 levels and basophil anergy (Fuentes-Aparicio et al. 2014; Kulis et al. 2011; Thyagarajan et al. 2012).
OIT is currently experimental and not FDA approved. Interestinghly, a large, Phase 3 trial for AR101, an oral immunotherapy treatment containing a characterized peanut protein profile, met all the endpoints set out by the Food and Drug Administration. With AR101, patients eat a measured amount of peanut powder (held in a capsule, then opened and sprinkled in food) on a daily basis. The amount of peanut protein powder in the capsule gradually up-dosed from 0.5 to 300 mg/day.

OIT protocols are mainly adapted from those conducted for aero-hypersensitivities. OIT protocols for food allergy typically comprise three phases: an initial dose escalation that takes place over the course of 1-2 days with an minute dose (e.g., 0.1

mg of food protein), rapidly increased by doubling doses to a maximum but still subthreshold level of food protein (10–25 mg), in an attempt to identify the maximum tolerated dose of a food allergen that can be safely given at home. Patients continue to eat this dose daily at home for 2 weeks. Then, a build-up phase is performed with weekly to biweekly dose escalations over 6–12 months until a target dose is reached. The last phase is the maintenance phase with daily home dosing that occurs over the course of several months or years (Jones et al. 2014). The starting doses for patients participating in OIT are selected to be low enough not to cause reactions and are either chosen for each subject according to a threshold identified in an initial OFC or are fixed for the entire study population (Beyer 2012). There is no standard set for length of escalation (a few months to years) and maintenance dose (300 mg to over 4000 mg of food protein). This will depend on the food item used or protocol being followed. Alternatives to conventional OIT protocols include rush protocols, in which the food allergen is administered in increasing doses several times per day for several days (Longo et al. 2008; Staden et al. 2008). The majority of protocol require daily administration of the food allergen. Interestingly, an Italian study demonstrated that a maintenance regimen with milk given twice weekly was as effective as a regimen requiring daily administration of the same amount of milk (Pajno et al. 2013). Common side effects are oral pruritus, abdominal pain, vomiting, and nausea (75%), while more severe side effects are eosinophilic esophagitis (2.7%) and anaphylactic reactions, which require intramuscular injection of epinephrine/adrenaline (25%) (Anagnostou et al. 2014; Calatayud et al. 2014; Keet et al. 2012; Lucendo et al. 2014). A modified protocol demonstrated that a lower maintenance dose could decrease side effects without impacting on the efficacy (Bird et al. 2015).

Studies have been shown that OIT can induce desensitization in patients allergic to peanut, eggs or milk. The rate of desensitization to peanut ranges between 62 and 100%, while sustained tolerance is achieved in 50% of cases (Anagnostou et al. 2014; Jones et al. 2009; Varshney et al. 2011; Vickery et al. 2014). To enhance the tolerogenic effect of the immunotherapy to peanut allergy, such as induction of Treg and Th1 responses, allergens have been combined with probiotic strains, such as *Lactobacillus GG*. This probiotic strain can modulate the peanut-specific immune response inducing possible sustained unresponsiveness (Tang et al. 2015). In patients allergic to eggs, OIT leads to desensitization in 55-94% of the patients, while sustained tolerance is achieved in 28-31% of the cases. Desensitization was also

achieved in 71-80% of patients with cow milk allergy (Sanchez-García et al. 2012; Vazquez-Ortiz et al. 2013). However, a higher variability for tolerance induction (35 to 90% of treated patients) was reported (Martorell et al. 2011; Pajno et al. 2010). Unfortunately, long-term effectiveness of OIT in cow milk allergy remains challenging and hardly lasts for more than 5 years. Indeed, only half of the succesfully desensitized patients continued to tolerate one serving of milk per day (Keet et al. 2013). OIT has also been attempted for multiple food allergies and its safety seems to be comparable to the safety of single food immunotherapy (Begin et al. 2014).

To facilitate rapid desensitization in protocols with single and multiple allergens, anti-IgE therapy, such as Omalizumab, has been used (Begin et al. 2014; Nadeau et al. 2011; Schneider et al. 2013; Wasserman et al. 2014). It functions by depleting free IgE and downregulating FcεRI on effector cells, such as mast cells and basophils, and on antigen-presenting cells. Hence, it minimizes IgE-mediated side effects and accelerates build up dosing (Begin et al. 2014; Nadeau et al. 2011; Shankar et al. 2013; Schneider et al. 2013). Even thought OIT appears to be effective, adverse events are frequent, thus limiting its clinical utility. Thus, other administration routes, such as the sublingual route, have been investigated in immunotherapy.

2.6. Sublingual immunotherapy

2.6.1. Introduction

Sublingual immunotherapy is a treatment where allergen extracts are applied to the oral mucosa underneath the tongue for up to several minutes and then spit out or swallowed. In SLIT, the oral mucosa is exposed to undigested antigens. This is in contrast to OIT, where allergens are exposed to gastric digestion. The first double-blind, placebo-controlled SLIT trial was performed for hazelnut allergy in 2005. Twenty-three adults were included in the study to receive either hazelnut extract (n=12) or placebo (n=11). After 8-12 weeks of treatment up to 45% of patient were able to ingest 20g of hazelnut in a post treatment oral food challenge without any symptoms (Enrique et al. 2005). Since then, other clinical trials investigating the safety and efficacy of SLIT have been performed for hazelnut, peach, milk and peanut allergy (Burks et al. 2015; Chin et al. 2013; Enrique et al. 2005; Garrido-Fernandez et al. 2014; Keet et al. 2012; Kim et al. 2011; Narisety et al. 2015).

In 2011, SLIT was used to treat children with peanut allergy. Eighteen children received peanut extract (n=11) or placebo (n=7) over a period of 12 months (Kim et al. 2011). The protocol was considered effective and safe with only 0.26% of peanut doses required. In 2013, a bigger randomized trial was performed, including 40 peanut-allergic patients. In this study, 44% crossover subjects were responders and side effects, mainly oral/pharyngeal, were infrequent (Fleischer et al. 2013).

2.6.2. Sublingual immunotherapy versus oral immunotherapy

SLIT, as OIT, takes advantage of allergen exposure through the oral mucosa, which is thought to be tolerogenic. Unlike OIT doses that range from milligrams to grams, SLIT doses typically range from micrograms to milligrams at maintenance. Studies have been conducted in order to compare the efficacy and safety of SLIT versus OIT. A retrospective study analysed two protocols of immunotherapy for peanut allergy. Twenty-three subjects were treated with OIT and 27 received SLIT over a 2-year period. The amount of peanut protein tolerated was higher in the OIT group. Unfortunately, safety was not compared (Stacy et al. 2013). Later, in a double-blind, placebo-controlled pilot study, children with peanut allergy were randomized to receive active SLIT/placebo OIT or active OIT/placebo SLIT. All children who finished the study had a greater than 10-fold increase in challenge threshold after 12 months. The increased threshold was significantly greater in the active OIT group. Changes in skin test results and peanut-specific IgE and IgG4 levels were also higher with OIT. However, the higher efficacy of OIT was associated with more adverse reactions (Narisety et al. 2015).

Despite a poorer efficacy, SLIT is safer than OIT. Therefore, SLIT may ultimately represent a better method of immunotherapy for patients with a history of severe allergy who cannot tolerate OIT (Chin et al. 2013; Keet et al. 2012).

The difference in efficacy and safety between the groups is likely related to the dose of allergen; the cumulative dose that the subjects in the SLIT group received in the aforementioned study was at least 140-fold lower than the minimum cumulative OIT dose. The lower dose used in SLIT depends on the small volume that can be given sublingually. On the one hand, this decreases side reactions, but on the other hand it results in a limited maximum dose that can be administered. It has been speculated

that higher SLIT doses might result in an improved efficacy (de Boissieu et al. 2006; Enrique et al 2005).

2.6.3. Properties of the sublingual mucosa

The sublingual mucosa has unique characteristics that make it suitable for immunotherapy. It is a stratified non-keratinized squamous epithelium, which is particularly thin, highly vascularized and permeable and thus it allows frequent contact of antigens with local immune cells. Mucosa-associated lymphoid tissue (MALT) is absent from the oral mucosa, therefore DCs in the epithelium play a key role in the oral immunity by taking up antigens. There are three subsets of dendritic cells (DC): 1) the Langerhans cells present in the mucosa itself, 2) myeloid dendritic cells located in the lamina propria and 3) plasmacytoid dendritic cells residing in the submucosa. In humans, the myeloid and plasmacytoid dendritic cells are rarer than the langerhans cells. These three types DCs can all promote a tolerogenic environment through many mechanisms. They can release immunosuppressive cytokines such as IL-10 and TGF-β; stimulate differentiation of Tregs or yet, express inhibitory molecules or immunosuppressive enzymes such as indoleamine 2,3-dioxygenase (IDO). Why oral mucosa has tolerogenic properties is still under investigation. Hitherto, studies have been shown that human oral LCs display higher expression of Toll-like receptors 2 and 4 (TLR2 and TLR4) in comparison to epidermal LCs in steady state (Allam et al. 2010). Stimulation of these receptors induces increased expression of co-inhibitory molecules B7-H1 and B7-H3 and decreased expression of the co-stimulatory molecule CD86 (B7-2). As a consequence, there is a polarization towards development of Tregs and secretion of IL-10 and TGF-β by stimulated T cells (Allam et al. 2008). Interestingly, it has been demonstrated that oral LCs express higher levels of maturation marker CD83 compared to cutaneous LCs indicating a state of partial maturation (Lutz et al. 2002). Partial maturation is associated to tolerance and characterized by the absence of production of inflammatory cytokines. This suggests that oral bacteria, which constantly stimulate TLR on oral DCs, act in a tolerogenic fashion by inducing a semi maturation state. It is likely that commensal bacteria act in a tolerogenic fashion to maintain immune homeostasis and only when dangerous signals properly stimulate TLRs on DCs, it induces fully maturation/activation of DCs with a consequent production of IL12 enabling T cell-mediated immunity.

Another important feature that makes the oral mucosa special is that mast cells and eosinophils are present only in a low number (Brandtzaeg et al. 2005; Calderon et al. 2012; Kim et al. 2011; Moingeon et al. 2012; Mascarell et al. 2009; McGhee et al. 2012; Nurmatov et al. 2012; Rachid et al. 2012; Scadding et al. 2009; Song et al. 2009). Last but not least, plasma cells within the salivary glands produce secretory IgA, antibodies that line the oral mucosal surfaces catching antigens and preventing them to attach to the mucosal surface.

2.6.4. Specific immunologic changes after sublingual immunotherapy

The immunotherapy consists of a controlled contact with an increasing concentration of allergens and modulates the immune response (Favrot 2009; Moran et al. 2013). It exerts its action not only on T cells, mast cells, basophilic and eosinophilic granulocytes, but also on immunoglobulins and cytokines.

2.6.4.1. The effect on T cells

In humans, SLIT promotes the development of Tregs. These cells are a heterogeneous family which include thymus-derived or inducible regulatory T cells. The latter play a key role in suppressing aberrant allergen-specific effector T cell responses following allergen-specific immunotherapy via SCIT and oral immunotherapy (OIT).

Healthy individuals have a high Treg activity for the most important allergens (Akdis et al. 2004). Individuals who develop allergies however show a deficient Treg response, resulting in a predominant Th2 cell activity with a high production of IL-4, IL-5 and IL-13 (Akdis et al. 2004; Durham et al. 1998; Secrist et al. 1993; Umetsu et al. 2003). Induction of Tregs appears to play a central role in the mechanism of SLIT. Through their production of IL-10 and TGF-β, Tregs induce a shift from IL-4, IL-5 and IL-13 producing Th2 cells to IFN-γ producing Th1 cells (Bohle et al. 2007; Jutel et al. 2006; Secrist et al. 1993; Yacoub et al. 2012). Since Th1 cells are pro-inflammatory cells, one could expect that the increase in Th1 activity would lead to increased tissue inflammation and as a consequence an increased severity of the allergic symptoms. It is assumed that this inflammatory response is prevented because IL-10 can cause anergy in effector T cells (Bellinghausen et al. 2001). In addition, Tregs also affect

immunoglobulin concentrations, cytokine production, cell-cell interactions and activation of pro-inflammatory cells.

In a study with SLIT in humans, Tregs played an important role in suppressing the allergic reactions during the first weeks of therapy. However, after one year of therapy no more Treg activity was detected (Bohle et al. 2007). This corresponds with the fact that induced Tregs have a short lifespan (Vukmanovic et al. 2006). Still the allergic response remained suppressed. Hypotheses for the immunologic mechanism in this stage are the occurrence of clonal deletion of allergen-specific T lymphocytes (Bohle et al. 2007) or the induction of anergy in the allergen-specific T lymphocytes by high levels of IL-10 (Akdis et al. 1998; Akdis et al. 1999). In accordance with these findings, it has been suggested to divide the immunologic effects of SLIT in an early and a late phase. In the early phase, there is a predominant role for Tregs, while in the late phase there is a specific non-reactivity of allergen-specific T cells (Bohle et al. 2007). Since SLIT is generally started at low doses during the build up phase and reaches high doses during the maintenance phase, there is speculation whether it is time or dose or both that decide which mechanism of allergen-specific immunosuppression predominates. There are indications that low doses of antigens induce active suppression by Tregs, while high doses lead to clonal deletion or anergy (Burks et al. 2008; Chen et al. 1994; Howard et al. 1997).

2.6.4.2. The effect on cytokines

As described above, SLIT induces production of two suppressive cytokines, IL-10 and TGF-β. IL-10 is a potent suppressor of IgE (total and specific) and promotes the production of IgG4, a blocking antibody that antagonize the allergic inflammation cascade resulting from antigen recognition by IgE (Akdis et al. 1998). The resulting shift in balance between IgE and IgG4 is a key phenomenon for successful allergen-specific immunotherapy. TGF-β induces another important immunoglobulin: IgA, which contributes to rebuild mucosal tolerance by dampening immune responses (Sonoda et al. 1989). Concurrently, SLIT induce Th1 cytokine production. Thus, IFN-γ, which is enhanced with SLIT, inhibits Th2 cells (Jutel et al. 2006; Secrist et al. 1993; Yacoub et al. 2012).

2.6.4.3. The effect on immunoglobulins

In allergic individuals IgE is an important mediator of the allergic immediate and late phase reactions (Olivry et al. 2001). As stated above, SLIT induce an isotype shift, downregulating allergen-specific IgE production and stimulating allergen-specific IgG and IgA production in humans and dogs (Akdis et al. 1998; DeBoer et al. 2010; Keppel et al. 2008; Marsella et al. 2013; Moingeon et al. 2006; Sonoda et al. 1989). Allergen-specific IgG and IgA can function as blocking antibodies by binding the allergen before it can reach IgE. Through this competition there is a reduction in the amount of degranulating mast cells and basophils. Due to the blocking effect of IgG and IgA, less allergen can reach IgE carrying antigen-presenting cells. This way less lymphocytes will be activated by allergen presentation (Akdis et al. 2006; Mothes et al. 2003; van Neerven et al. 1999; Wachholz et al. 2003). However, allergens can still bind IgG (IgG1-3) forming immune complexes, that can be taken up by APC, which will become activated and present the allergen to T cells. Furthermore, IgG and IgA can also bind the low affinity FcγRIIb receptor of mast cells and basophils in mice and humans. With regard to the production of these immunoglobulins, in the first months of immunotherapy, there is an initial increase in allergen-specific serum IgE concentration in treated patients (Kim et al. 2011; Kostadinova et al. 2013; Nowak-Wegezyn et al. 2011), which is then followed by a significant decrease. IgG also increases at the beginning of immunotherapy without showing a subsequent drop like for IgE (Enrique et al. 2005; Kim et al. 2011). In humans, mainly the IgG4 subclass is increased during ASIT (Akdis et al. 1998; Akdis et al. 2006; Chin et al. 2013; Keet et al. 2012; Kim et al. 2011; Pradalier et al. 1999; van Neerven et al. 1999; Wilson et al. 2005). In contrast to IgG1 to 3, IgG4 and IgA do not activate complement nor induce antibody-dependent cell-mediated cytotoxicity. Therefore, the upregulation of IgG4 in humans contributes to the anti-inflammatory mechanism of immunotherapy (Akdis et al. 2006; Olivry et al. 2010). The effectiveness of immunotherapy-induced allergen-specific IgG4 depends on the IgE/IgG4 ratio. The decline in this ratio is due to the increase in IL-10, which ensures an increase in IL-4 induced IgG4 production and a decrease in the conversion of IgE from IgM (Rachid et al. 2012). An additional advantage of using SLIT is the rise of allergen-specific IgA. This could result in increased levels of secretory IgA (SIgA), which provides protection at mucosal

surfaces, where it interferes with the antigen penetration through the mucosae (Allam et al. 2011; Goddeeris 2009; Moigeon et al. 2012; Rolland et al. 2009;).

2.6.4.4. The effect on pro-inflammatory cells

In humans, a significant decrease in the recruitment and activation of inflammatory cells, such as mast cells in the oral mucosa and basophils in the blood, occurs several months after using SLIT (Allam et al. 2011; Kim et al. 2011; Kostadinova et al. 2013; Moingeon et al. 2012; Nowak-Wegrzyn et al. 2011; Rachid et al. 2012). This decrease correlates directly with the reduction in IgE antibodies, which are important to mediate mast cell activation and degranulation, and indirectly by the effect of increased IgG4 levels, which can inhibit mast cell and basophil activation through binding of the inhibitory FcγRIIb receptor (Flicker et al. 2003; Wachholz et al. 2003;). The threshold to activate mast cells and basophils is also increased by the action of SLIT on T cells which produce less cytokines (Treter et al. 2000). SLIT can also prevent the recruitment of eosinophils (Marcucci et al. 2001; Passalacqua et al. 1998) and neutrophils to the inflamed tissue by suppressing the expression of intercellular adhesion molecule-1 (ICAM-1) (Passalacqua et al. 1999). Moreover, the suppression of Th2 cell activity by IL-10 reduces the production of IL-5 and therefore reduces eosinophil differentiation and activation (Bousquet et al. 1998; Durham et al. 1998; Jutel et al. 2006; Yacoub et al. 2012).

2.6.5. SLIT preparation and administration

Identification of the allergens responsible for hypersensitivity is required for the initiation of immunotherapy. This identification may occur by a prick test, intradermal test or detection of serum-specific IgE for atopic dermatitis and by a food trial for AFR. Once the culprit allergens are identified, they can be formulated as a solution or a tablet for administration in SLIT. Tablets consist of a rapidly dissolving matrix when held under the tongue. Solutions are usually administered as drops, which are held under the tongue for a specified period of time and then swallowed. Sublingual immunotherapy comprises two phases: the induction and the maintenance phase. In the former SLIT is administered on a daily basis with dose escalation until a certain target dose is reached. As a result of increasing doses, side effects are more likely to

occur and the patient has to be carefully monitored. The symptoms are mainly of local nature: swelling and itching of the lips, the sublingual area and oropharynx. Systemic reactions, such as urticaria, angioedema and asthma, rarely occur. When severe anaphylactic reactions are expected or when a rush protocol is applied, the process takes place in a controlled environment, such as a hospital. When the target dose is reached, the maintenance phase starts. During this phase, the target dose is administered on a daily basis for several months or even years.

2.6.6. SLIT in dogs

Conversely to the extensive literature in human medicine, in dogs, there are only few studies investigating the effect of SLIT in dogs with atopic dermatitis. This paucity may reflect the difference in safety of SCIT between human and dogs. Indeed, SCIT in dogs is effective and has a good safety profile. However, since some owners are reluctant to give injections to their dogs, the painless sublingual route has recently been investigated in dogs. Boer and Morris performed an uncontrolled multicentric (DeBoer and Morris 2012) study where 217 owned dogs were treated over at least 6 months with SLIT twice daily concurrently with other medications, such as glucocorticoids and cyclosporine. Over half of dogs (55%) that finished the study (124/217) had a good-to-excellent response to SLIT (LoEIII). Interestingly, around half of the dogs that previously did not respond to SCIT, were successfully treated with SLIT. The reason for this phenomenon is unknow but likely due to the tolerogenic environment in the oral mucosa or the increased patient/owner compliance. The first placebo controlled blinded study was reported in the same year with the authors evaluating the effects of SLIT administered daily for one year on clinical signs and immunological parameters in laboratory dogs sensitized to aeroallergens (Marsella et al. 2012) (LoEII). Unexpectedly, besides the 66% of dogs in the active group that improved, also 50% of dogs in the placebo group showed more than 40% of improvement of clinical signs. This can be explained with the lack of allergen exposure for a year. Proving the efficacy of SLIT, IL-10 and TGF-β were significantly increased in the active group when compared with the placebo and interestingly, some of the immunological changes decreased once the SLIT treatment was discontinued. No significant changes were noted in allergen-specific IgE. In 2016 an uncontrolled SLIT pilot study was conducted on dust mite-sensitive dogs with spontaneous atopic

dermatitis (LoEIII). The allergen concentration was increased every two months and the dose of oral methylprednisolone received at the beginning of the study was concurrently decreased. SLIT significantly decreased both clinical lesions and pruritus (with a decrease in the median Canine Atopic Dermatitis Extent and Severity Index (CADESI) score from 76.5 to 59, while the median Pruritus Visual Analogue Scale (PVAS) score declined from 65 to 37) and decreased the amount of medications needed to control the clinical signs of AD (mean methylprednisolone used in the first two months of the study to the final two months declined from 10.2 to 4.3 mg/kg/2 months). Clinical improvements were further confirmed by a decrease in allergen-specific IgE and an increase in allergen-specific IgG (DeBoer et al. 2016).

Recently, an uncontrolled rush protocol was investigated to treat atopic dermatitis (LoEIII). Dogs with atopic dermatitis were treated with SLIT comprising a 48 hours rush build-up phase followed by a 6-month maintenance phase. This SLIT protocol decreased the CADESI score with more than 30% in 76% of the dogs, but topical steroids were allowed. No side effects were reported (Fujimura and Hishimaru 2016).

Even though studies on allergen-specific SLIT in dogs are few and mostly uncontrolled, SLIT appears to be safe because adverse reactions are absent to very mild. Moreover, SLIT has an easier and painless administration route and is generally accepted as a worthy alternative to the conventional SCIT.

Chapter II:

Aims of the study

Aims of the study

Adverse Food Reactions (AFR) are reactions to apparently harmless food antigens with an unknown etiology. These AFRs have become increasingly prevalent in the past decades (Osborne et al. 2011). AFR is an important health concern in humans because it is often associated with life-threatening reactions (Nwaru et al. 2014). On the contrary, in dogs, adverse events are mostly milder, mainly limited to different degrees of pruritus and erythema and/or gastrointestinal disorders (Carlotti et al. 1990; Denis et Paradis 194; Hillier and Griffin 2001; Picco et al. 2008, White 1986). Even though AFR is less dangerous in dogs, small amounts of the offending allergen can trigger flares in an allergic dog, causing discomfort and suffering. In addition, these flares lead to frustration, an increased workload and expenses for the owner, thus decreasing the quality of life of both. Therefore, effective treatments to alleviate or cure the symptoms of AFR are really necessary.
To provide a better treatment rather than the limited current standard of care based on food avoidance, new therapeutic strategies, such as immunotherapy, are being investigated in humans. Food-specific sublingual immunotherapy (FA-SLIT) represents a potential etiological treatment, which was demonstrated in many randomized controlled trials (RCTs) to be safe and effective in the treatment of food allergy.

Against the background of the paucity in the knowledge of sublingual immunotherapy in dogs, the present thesis aimed at:

1. Investigating for the first time the use of FA-SLIT in healthy dogs, assessing its tolerability, safety and sterility (**Chapter 3**).

2. Evaluating whether FA-SLIT could be equally well tolerated and safe in dogs with AFR and to assess the efficacy by desensitizing dogs with AFR (**Chapter 4**).

3. To investigate if the clinical efficacy of FA-SLIT paralleled an immunological shift toward tolerance induction (**Chapter 5**) and to assess if T cell

subpopulations overexpressed in dogs with AFR may be used as biomarkers for the diagnosis of AFR or to detect the culprit allergens (**Chapter 6**)

4. To investigate whether nutritional lifestyle factor such as the consumption of vegetable oil, is associated with a higher prevalence of AFR in dogs (**Chapter 7**).

Chapter III:

Food-specific sublingual immunotherapy is well tolerated and safe in healthy dogs: a blind, randomized, placebo-controlled study

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3.1. Abstract

Background: Food allergies are common chronic conditions in dogs but no treatment strategies are currently available to cure them. Over the past decade, experimental food allergen-specific sublingual immunotherapy (FA-SLIT) has emerged as a potential treatment for food allergies in human medicine. However, FA-SLIT has not been investigated in dogs. Therefore, the objective of this study was to prospectively evaluate the safety, tolerability and dispenser sterility of FA-SLIT in healthy dogs before testing it in food allergic dogs.

Methods: Eight experimental healthy beagle dogs, never orally exposed to peanut, were randomized in two groups to receive SLIT with peanut or placebo for 4 months. Subjects were monitored daily for local and systemic adverse effects. Blood samples for complete blood count and serum biochemistry, and urine for urinalysis were collected and the dogs' body weight was recorded at day 0, 35 and 119 of the SLIT treatment. Sera for the determination of peanut-specific IgG and IgE were collected at day 0, 35, 49, 70, 91, 105 and 119. Intradermal tests were performed before (day 0) and after (day 119) the experiment. The content of each dispenser used to administer treatment or placebo was tested for sterility after usage. In order to assess the presence or absence of sensitization, dogs were challenged 6 months after the end of the study with 2000 µg of peanut extract daily for 7 to 14 days.

Results: All dogs completed the study. The treatment did not provoke either local or systemic side effects. Peanut-specific IgG significantly increased in the treatment group. Even though a significant increase in peanut-specific IgE was also seen, intradermal tests were negative in all dogs before and after the experiment. The challenge test did not trigger any adverse reactions in the treated dogs, which shows that the protocol did not cause sensitization to peanut, but nevertheless primed the immune system as indicated by the humoral immune response. All dispenser solutions were sterile.

Conclusions: Our results demonstrate that the used peanut-SLIT protocol is well tolerated and safe in healthy dogs. Further studies should evaluate tolerability, safety and efficacy in dogs with food allergy.

3.2. Introduction

Food allergy is a relatively rare but progressive problem in both humans and dogs (Chesney et al. 2002; Maina et al. 2014; Nowak-Wegrzyn et al. 2011; Proverbio et al. 2010). The treatment involves strict avoidance of allergen intake and, if necessary, it is combined with symptomatic therapy. However, it is not possible to cure food allergy in dogs. Researchers have shown that allergen-specific immunotherapy may be a potential curative treatment for food allergy in humans (Kim et al. 2011; Kostadinova et al. 2013; Moran et al. 201). Immunotherapy entails frequent contact with the specific allergen, starting from a low dose that gradually increases. This leads to a modification of the immune response, with an increased threshold value at which clinical symptoms occur. In humans, different approaches exist according to the route of administration: subcutaneous immunotherapy (SCIT), oral immunotherapy (OIT), sublingual immunotherapy (SLIT) and only recently epicutaneous immunotherapy (EPIT). Although SCIT has been successfully used in the treatment of patients with atopic dermatitis, its use in food allergy is dissuaded because of a high risk for severe side effects such as itching, urticaria, angioedema and symptomatic bronchoconstriction (Nelson et al. 1997). To overcome these problems, OIT and SLIT have been used (Compalati et al. 2013). The former comprises the daily consumption of milligrams to grams of allergen in a food vehicle. The latter involves dispensing small amounts (micrograms to milligrams) of allergen extract under the tongue. Both these therapeutic approaches provide progressively increasing amounts of allergen over weeks to months, until an established maintenance dose is reached. Adverse events consisting of multisystem, upper and lower respiratory tract and gastrointestinal symptoms, are reported for both administration routes, however, in humans, the safety profile of SLIT seems superior to that of OIT (Keet et al. 2012). Oropharyngeal itching is the most common SLIT-related side effect, which typically occurs during the build-up phase and mostly resolves without any treatment. Systemic side effects are very rare. Studies examining SLIT for specific food in humans are limited to hazelnut,

peanut, cow's milk, peach and kiwi (De Boissieu et al. 2006, Enrique et al. 2005; Fernandez-Rivas et al. 2009; Fleischer et al. 2014; Kim et al. 2011; Mempel et al. 2003). The aims of SLIT are to achieve desensitization by increasing the threshold for clinical reactivity to the culprit food and later to induce/restore tolerance induction, which refers to the ability to ingest the food without allergic reaction after discontinuation of the therapy. Thus far, available evidence suggests that SLIT is able to induce desensitization in the majority of patients (between 52 and 100% of treated patients) with only one study reporting a lower percentage (10%) (De Boissieu et al. 2006; Enrique et al. 2005; Fernandez-Rivas et al. 2009; Fleischer et al. 2014; Keet et al. 2012; Kim et al. 2011). Only one study evaluated the capacity of SLIT to induce tolerance between 10 and 50% of patients (Keet et al. 2012). In contrast to the extensive literature describing the use of immunotherapy in humans with food allergy, no studies have been performed in dogs. Therefore, the purpose of this study was to evaluate the safety, tolerability and dispenser sterility of SLIT with peanut allergen in a prospective, randomized, blinded, controlled study in healthy dogs.

3.3. Methods

Study design

The study was approved by the Ethical Committee of the University of Ghent, Belgium (EC 2014/144 (experiment); EC 2014/121 (Intradermal test)). This study was a randomized, blinded, placebo-controlled study using escalating doses of peanut extract in healthy dogs.

Randomization and blinding procedures

Subjects were allocated to a treatment group or placebo group (four dogs per group), following simple randomization by flipping a coin: the side of the coin (heads or tails) determined the assignment of each subject. Ten equally looking dispensers were prepared by the principal investigator during the experiment. Five dispensers, named Group 1 and numbered from 1 to 5, contained different concentrations peanut extract solution, and five others (named Group 2 and also numbered from 1 to 5) contained only placebo, as described further. A second investigator, responsible for administering the solution to the dogs, and the animal care takers were blinded to the treatments.

Animals

Eight clinically healthy laboratory raised beagle dogs were included: four intact females, one neutered female, one intact male and two spayed males. Median age was 6.25 years (±3.15) (range 2–10 years) and median weight 10.5 (± 1.44) (range 8.2–12.3 kg) (Table 3.1). To our knowledge all subjects never received peanut in their diet. Clinical histories were evaluated and the dogs underwent an accurate clinical examination to rule out presence of allergy or other diseases, before the inclusion.

Table 3.1. Signalment and assigned group of eight beagles dogs included in the study.

Group	No.	Sex	Age (years)	Weight
P	1	MC	9	9.8
P	2	M	10	8.2
P	3	MC	3	10.2
P	5	F	7	10.1
T	4	F	7	12.3
T	6	FS	3	12.3
T	7	F	9	11.7
T	8	F	2	9.7

Abbreviations: F, intact female; FS, female spayed; M, male; MC, male castrated; P, placebo group; T, treatment group.

Housing

Dogs were housed in kennels in a research facility at the Faculty of Veterinary Medicine, Ghent University. Each kennel consisted of an inner part (90 cm × 473 cm) and an outer part (90 cm × 300 cm). All dogs had their own equipment (e.g. toys and bowls) and the animal care takers were properly trained not to mix these materials among dogs.

Peanut and placebo sublingual drops

The treatment group received lyophilized peanut extract (Greer®, Lenoir, NC, USA) fully dissolved in 50% glycerinated saline to a maximum peanut protein concentration of 20,000 µg/ml. Normal sterile glycerinated saline solution served as placebo. Both solutions (peanut extract and the placebo solutions) were poured into dark dispensers.

As described previously, 5 dispensers per group (numbered from 1 to 5) were prepared. All dispensers addressed to the placebo group contained only sterile glycerinated saline solution. Dispensers for the treatment group contained increasing concentrations of peanut extract solution with number 1 being the least concentrated (Table 3.2). Dilutions were made with glycerinated saline under sterile conditions. Peanut extracts and dispensers containing placebo or treatment solutions were kept at 4°C during the study period.

SLIT protocol

Before and during the study, dogs were fed a strict peanut-free diet. The solution was administered sublingually, by hooking a dispenser tip over the lower teeth and dispensing from 50 to 250 µL (1 push dispensed 50 µl) of solution into the oral cavity under the tongue. This was performed once a day at the same time for all dogs, at least 1 hour after the meal (Fig. 3.1). Dogs were not allowed to eat and drink for 30 min after peanut or placebo administration. After each administration, the oral cavity of the dogs was carefully examined to rule out accidental injuries by the dispenser. After the starting dose of 0.25 µg peanut protein, doses were increased on day 2 and again on day 3. Then weekly increases by 25 to 100% occurred until the daily maintenance dose of 2000 µg peanut protein was reached (Table 3.2). This maintenance dose was continued daily for 2 months. Subjects were monitored by the primary investigator several times during the hour following each administration. After each dose increase and the subsequent day dogs were monitored for an additional 2 h in the morning and 2 h in the evening to monitor onset of pruritus, which could have been masked by the dogs' excitement during the short daily visits. Furthermore, animal caretakers were also instructed to monitor on a daily basis for adverse effects (e.g. vomiting, diarrhoea, urticaria, angioedema and oral pruritus) and to record it.

Tolerability assessments

Definition

Tolerability is referred to as absence of SLIT-related local adverse events.

Clinical evaluation

The muzzle, mouth and the oral cavity of the dogs were examined in detail by the principal investigator.

Table 3.2: Peanut SLIT dosing schedule for the treatment group (Adapted from Kim et al. 2011).

Week	Days	Dilutions	Dispenser n°	Pumps*	Protein (µg)
1					
	1	1:4000	1	1	0.25
	2	1:4000	1	5	1.25
	3	1:400	2	1	2.5
	4	1:400	2	1	2.5
	5	1:400	2	1	2.5
	6	1:400	2	1	2.5
	7	1:400	2	1	2.5
2		1:400	2	2	5
3		1:400	2	4	10
4		1:40	3	1	25
5		1:40	3	2	50
6		1:40	3	4	100
7		1:4	4	1	250
8		1:4	4	2	500
9		1:1	5	1	1000
10		1:1	5	2	2000
11		1:1	5	2	2000
12		1:1	5	2	2000
13		1:1	5	2	2000
14		1:1	5	2	2000
15		1:1	5	2	2000
16		1:1	5	2	2000
17		1:1	5	2	2000

Five dispensers (1–5) with increasing concentrations were used as well as variable number of pumps to come to increasing amounts of protein administered sublingually. The amount of protein dispensed ranged from 0.25 µg to 2000 µg. Placebo (only glycerinated solution was administered) was administered according to the same protocol

* Each pump dispensed 50 µl of solution.

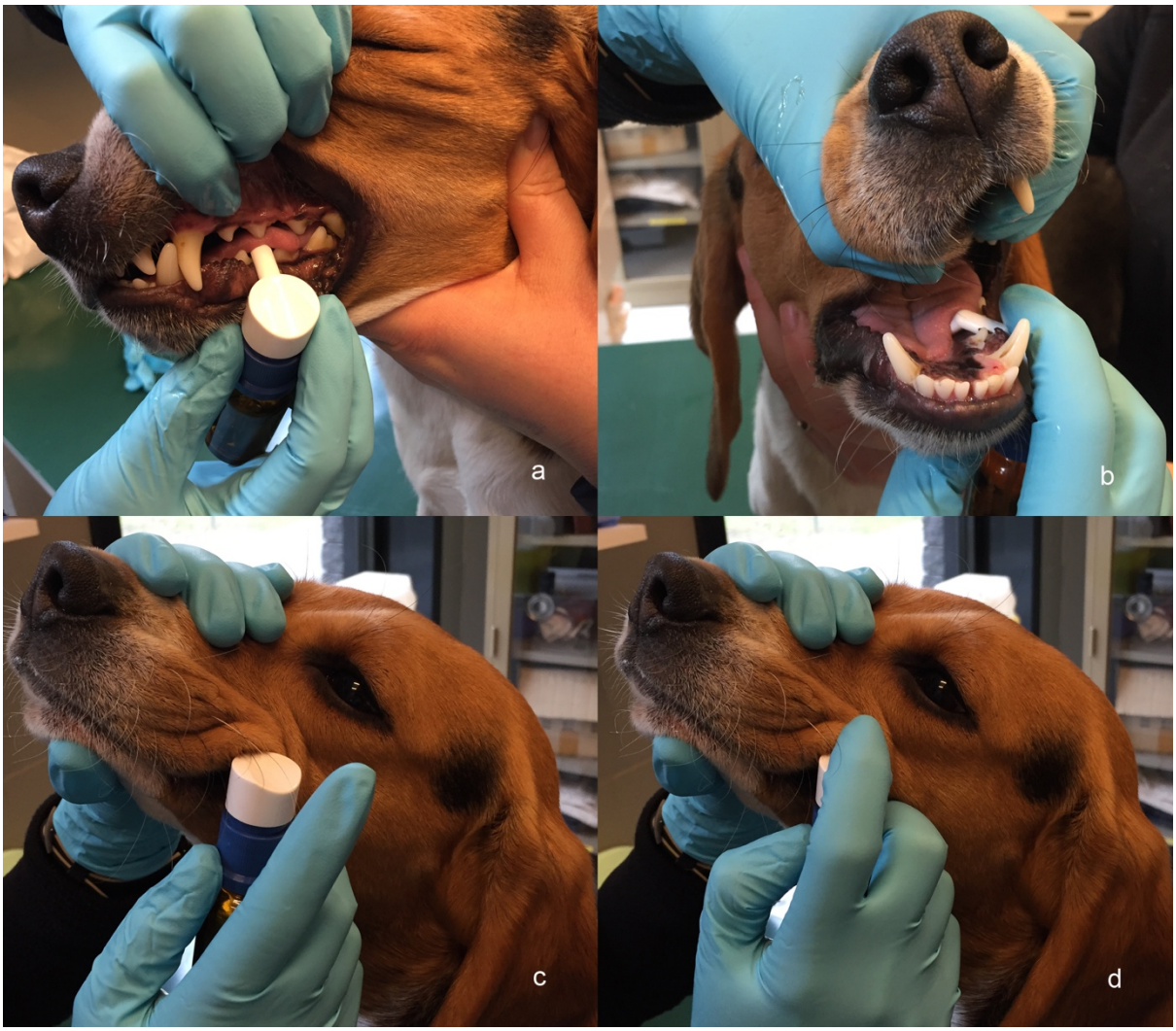


Figure 3.1: SLIT administration in a dog. Dispenser tip is hooked over the lower teeth, into the oral cavity, under the tongue a, b. A drop of solution is then dispensed by pushing the cap of the dispenser c, d.

All changes observed after the first visit (day 0) (e.g. erythema, swelling, vesicles and ulcerations, immediate or delayed oral or muzzle itching, sialorrhea, continuous chewing and vomiting) were recorded and the possible relation with the treatment was assessed by the Naranjo adverse drug reaction probability scale (Naranjo et al. 1981).

Safety assessments

Definition

Safety refers to SLIT-related reactions that occur far from the site of administration and include both life-threatening and non life-threatening systemic adverse events (Cox et al. 2010).

Clinical adverse events and concomitant medications administered

All observed adverse events that occurred during the study period or within 14 days after the end of the experiment were recorded (e.g. diarrhoea, abdominal pain, urinary tract infection/cystitis, facial urticaria, erythema and pruritus on the axillae, groins paws and perianal area, pyoderma, otitis, epilepsy, somnolence, anorexia and anaphylaxis). Onset, duration, severity and treatments were noted. Naranjo Adverse Drug Reaction Probability Scale was used to assess the likelihood of a real adverse drug reaction (Naranjo et al. 1981).

Clinical laboratory tests

Complete blood count analysis was performed before and at the end of the experiment. Blood samples for serum chemistry, and urine for urinalysis (free catch) were collected just before the first administration at day 0, at day 35 and again at day 119, the end of SLIT. Hepatic functions were evaluated by measurement of alanine transferase (ALT), aspartate transferase (AST) and alkaline phosphatase (ALP), and urinary functions by determining creatinine, total protein and urea concentrations. Urinalysis was performed by urine dipstick testing for pH, protein, glucose, bilirubin, specific gravity, blood, ketones, nitrite, urobilinogen and ascorbic acid.

Body weight change

Dogs were meticulously weighted during each visit. Any change in the body weight (BW) was recorded.

Intradermal test

Intradermal testing was performed at the end of SLIT (day 119) by intradermal injection of 20 µg peanut protein (0.05 ml of a 1:1000 w/v dilution of peanut protein) in the ventral lateral area of the abdomen. The wheals induced were measured after 15 min, 24 h, 48 h and 72 h. Saline solution was used as a negative control and a dilution 1:10 of histamine phosphate (0275 mg/mL) (Greer Laboratoires, Lenoir, NC, USA) was used as positive control. Peanut extract and histamine were diluted with saline solution.

Challenge testing

In order to assess late occurring sensitization, the four treated dogs were challenged 6 months after the end of the study with sublingual administration of 2000 µg of peanut extract daily for 7 days and in one dog the challenge was even prolonged for a week. All dogs were monitored for 14 days. Onset of pruritus or any other clinical signs were recorded.

Tolerance induction assessment

Peanut-specific IgG and IgE enzyme-linked immunosorbent assay (ELISA)

To probe the induction of suppressive IgG antibodies rather than potentially sensitizing IgE antibodies in subjects undergoing peanut-specific SLIT, the peanut-specific IgG and IgE responses were analysed. Sera were obtained from all dogs (active and placebo) at day 0, 35, 49, 70, 91, 105 and 119 of the SLIT treatment and then frozen at -20°C until processed. Circulating concentrations of peanut-specific IgG and IgE were determined by ELISA. Briefly, Nunc MaxiSorp® flat-bottom 96- well plates were coated overnight at 4°C with solutions of the peanut protein at 0.05 mg/ml in bicarbonate buffer, whereafter they were blocked at room temperature with 2% gelatine from cold fish water skin (Sigma-Aldrich®, Steinheim, Germany) in bicarbonate buffer. In subsequent steps performed at room temperature, wells were first incubated with serum samples (diluted 1/2.5 and 1/50 for IgE and IgG, respectively, in bicarbonate buffer with 2% gelatine from cold water fish skin) for 2 hours, then with polyclonal goat anti-canine heavy and light chain IgG (125 ng/ml) (Bethyl, Montgomery, USA, A40-123P) or polyclonal goat anti-canine IgE (125 ng/ml) (Novus Biologicals, Cambridge, UK, NB7346) HRP-conjugated antibodies for 1 hour and finally with a solution of ABTS (Roche Diagnostics, Mannheim, Germany). The OD was measured at 405 nm (Tecan Spectra Fluor Fluorescence and Absorbance Reader) and analyzed with XFluor™ software. In between steps, plates were washed from three to five times, using 0.05% Tween®20 in PBS.

Sterility testing at the final container

The sterility testing was performed following the method described in the fifth Edition of the International Pharmacopoeia. Briefly, before and after first using all dispensers (placebo and treatment groups), 1 ml content was added to 10 ml of Soybean-Casein Digest sterilized Medium (Tryptone Soya Broth (TSB), Oxoid, Thermo scientific, UK) and 1 ml to 10 ml of Nutrient broth (Nutrient Broth, Difco, BD, USA). Dispenser 1 for

both placebo and active treatment was also tested at day 119 to evaluate the sterility of its content overtime. TSB was incubated at 22.5 °C and Nutrient Broth at 37 °C for 14 days. Cultures were assessed daily. In case of increased turbidity due to growth of potential contaminants, such as fungal, yeast, aerobic and anaerobic bacteria, further identification occurred.

Statistical analysis

Data were analysed with statistical software SPSS Statistics 21 (IBM, New York, United States). Haematological parameters, serum biochemistry and urinary parameters (specific gravity and pH) were compared between groups, before the experiment (day 0), at day 35 and at the end of the SLIT (day 119). The data were subjected to analysis of variance (ANOVA) in the context of general linear models at a significance level of 0.05 (Mendenhall et al. 1996). Summary statistics (mean and SD) for BW and percentage change from baseline were calculated at each time point. Significant differences in serum peanut-specific IgG and IgE between the two groups were calculated using a Mann-Withney U test. A p-value lower than 0.05 was considered significant.

3.4. Results

SLIT administration

All dogs completed the study. The administration of SLIT was easy and well accepted by the dogs. The dispensers, with their hooked nozzle, did not hurt their mucosa. During and after administration of the solutions, the dogs did not show any changes in their behaviour.

Tolerability assessments

Clinical evaluation

At the time of the inclusion, all dogs were healthy and no lesions were noted on the skin and/or mucosae. All dogs but one did not show any adverse effects during or after the SLIT. Only one dog (placebo group) vomited once during the induction phase. However, according to the Naranjo scale, which estimates the probability of adverse

drug reactions, this case could be classified as a 'doubtful' reaction to one of the components of the placebo (Naranjo score -1).

Safety assessment

Clinical adverse events and concomitant medication administered

No adverse effects were recorded and therefore no additional treatment was given to the dogs during the study period.

Laboratory tests

Administration of peanut-specific immunotherapy had no significant effect on haematology, on indices of hepatic and renal functions nor on urinalysis between groups and over time. The values for all these parameters were within the normal laboratory reference ranges for each analyte at all time points, showing that they were not affected by the administration of either treatment or placebo (Table 3.3). Comparison between groups did not show any significant difference.

Body weight changes

When compared with the baseline values (placebo group: mean = 9.58 kg (± 0.93); treatment group = 11.5 kg (± 1.23)), body weights remained relatively constant during the study. The mean BW at the end of the study was 9.58 kg (± 0.93) in the placebo group and 11.6 kg (± 1.11) in the treatment group.

Intradermal challenge test

Intradermal testing of dogs with peanut extract at the end of SLIT did not provoke any positive reactions.

Sublingual challenge test

Sublingually challenging the treated dogs with peanut extract 6 months after the end of the immunotherapy did not provoke any clinical signs.

Table 3.3. Complete blood count, serum chemistry, and urinary values (mean±SD) and range for eight beagles dogs included in the study at different time points.

Parameters	Mean±SD						Range
	Day 0		Day 35		Day 119		
	P	T	P	T	P	T	
Complete blood count values							
RBC	6.97±0.72	6.39±0.23	-	-	7.39±0.88	6.94±0.04	6.20-8.70 milj/µl
Haematocrit	47.8±5.56	44.33±1.99	-	-	47.45±	46.03±1.66	43.0-59.0 %
Haemoglobin	16±2,21	14,88±0,25	-	-	8.04	15,98±0,49	14,0-20,0 g/dl
MCV	16±2.21	14.88±0.25	-	-	16.68±	15.98±0.49	14.0-20.0 g/dl
MCHC	33,37±0,78	33,58±1,55	-	-	2.09	34,7±0,56	30,0-36,0 g/dl
%Reticulocyte	68.57±1.96	69.38±2.23	-	-	63.95±	66.33±2.12	63.0-77.0 fl
WBC	7556,67± 2091,28	7157,5± 1088,8	-	-	4.09	8180±1047	6000-16000 /µl
Neutrophil	33.37±0.78	33.58±1.55	-	-	35.4±2.05	34.7±0.56	30.0-36.0 g/dl
Lymphocyte	0.43±0.15	0.35±0.19	-	-	0.78±0.55	0.55±0.33	2-0 %
Monocyte	7556.67±	7157.5±	-	-	6815±	8180±1047	6000-16000 /µl
Eosinophil	2091.28	1088.8	-	-	2775.81	7,28±3,36	0,0-8,0 %
Basophil	66.23±7.83	68.68±1.41	-	-	62.88±	64.88±4.84	55.0-77.0 %
Platelet	395666,67± 137587,55	321500± 80172,73	-	-	10.23	248500± 29949,96	164000-510000

Biochemical values							
Urea	2.35±0.68	2.45±1.05	3.2±1.10	3.55±1.95	2.7±0.42	3.1±1.70	2.5-9.6 mmol/L
Crea	28.5±14.18	23.5±8.54	32±11.78	26±9.80	24.5±7.19	32±28	44-159 mmol/L
ALT	24±4.90	26.5±5.26	33.5±8.22	35±8.25	10±0	10±0	10-100U/L
AST	11.5±5	11.5±5.97	13±2.58	26±10.95	13.5±5	20±7.48	0-50 U/L
ALKP	108±64.60	45±6.63	132± 114.32	97± 43.50	122±106.62	91.5±69.19	23-212 U/L

Urinary values							
SG	1018.75±4.79	1018.75± 2.5	1017.5± 2.89	1018.75± 2.5	1017.5±5	1018.75± 2.5	1015-1060
Proteins (mg/dL)	neg	neg	neg	neg	neg	neg	neg
Glucose (mg/dL)	neg	neg	neg	neg	neg	neg	neg
pH	6.13±0.48	6.125±0.25	6.125± 0.25	6.125±0.25	6.25±2.89	6±0	5.5-7.0
Blood	neg	neg	neg	neg	neg	neg	neg
Ketones	neg	neg	neg	neg	neg	neg	neg
Nitrite	neg	neg	neg	neg	neg	neg	neg
Urobilinogen	neg	neg	neg	neg	neg	neg	neg
Ascorbic acid	neg	neg	neg	neg	neg	neg	neg

Abbreviations: ALT, alanine aminotransferase; ALKP, alkaline phosphatase; AST, Aspartate transaminase; Crea, creatinine; MCHC, mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; P, placebo group; RBC, red blood cells; SG, specific gravity; T, treatment; WBC, white blood cells.

Tolerance induction assessment

Peanut-specific IgG ELISA

At day 105 and 119, the treatment group showed a significant increase in peanut-specific serum IgG in comparison to the placebo group ($p = 0.0267$ and 0.0369, respectively). Although not significant at all time points, starting from day 70, the treatment group showed consistent higher peanut-specific IgG concentrations in comparison with the placebo group (Fig. 3.2).

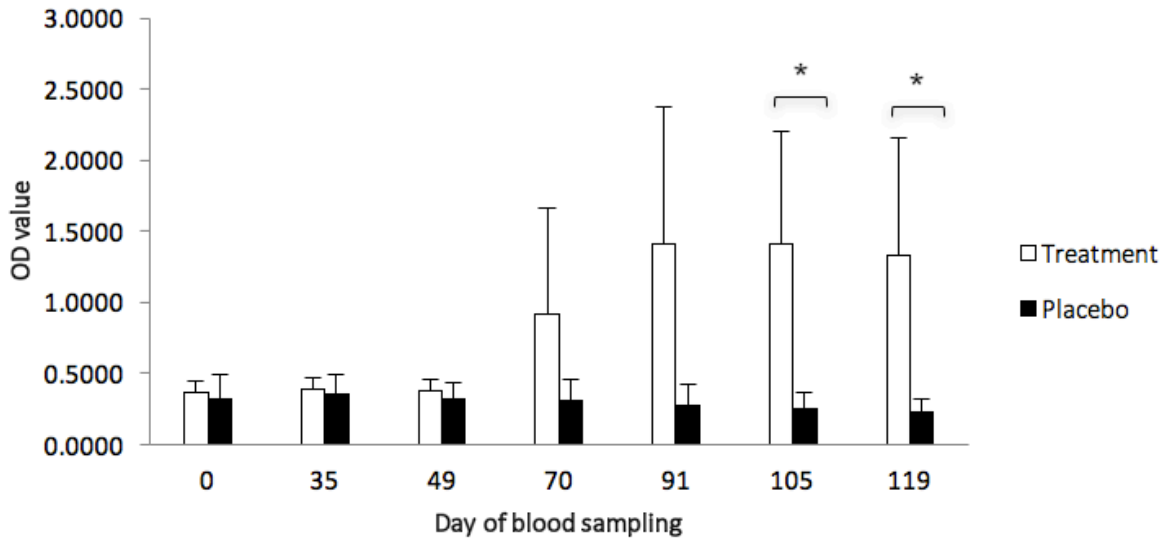


Figure 3.2: Mean increase in O.D. for peanut-specific IgG in the treatment (white bars) and placebo groups (black bars) ± SD. SLIT ended at day 119. Asterisk shows that at day 105 and 119, the increase in the treatment group was significant in comparison with the placebo group (p = 0.0267 and 0.0369, respectively)

Peanut-specific IgE ELISA

At day 91, 105 and 119, statistically significant differences in peanut-specific IgE were seen when the treatment group was compared to the placebo group (p = 0.0298, 0.00735 and 0.0245, respectively) (Fig. 3.3).

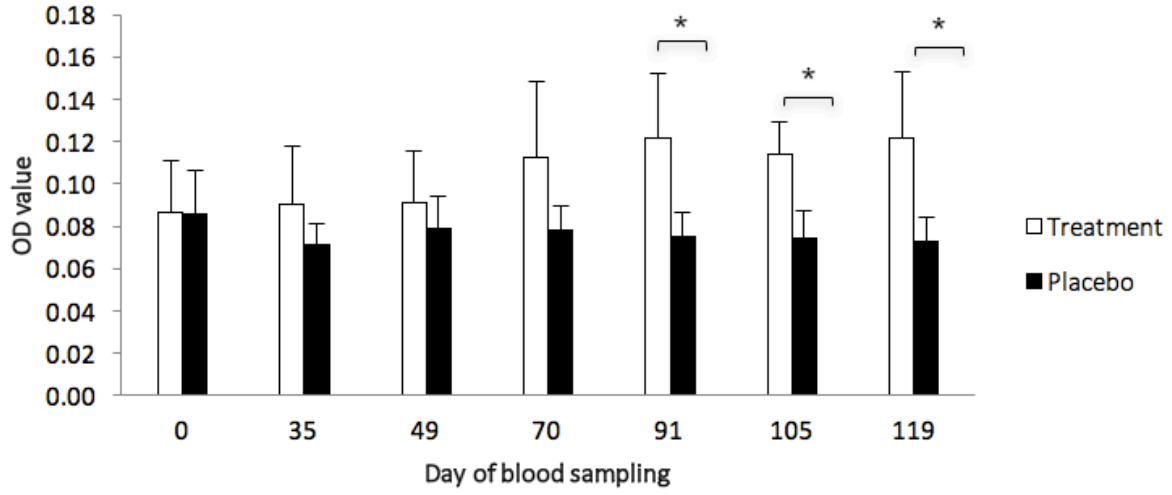
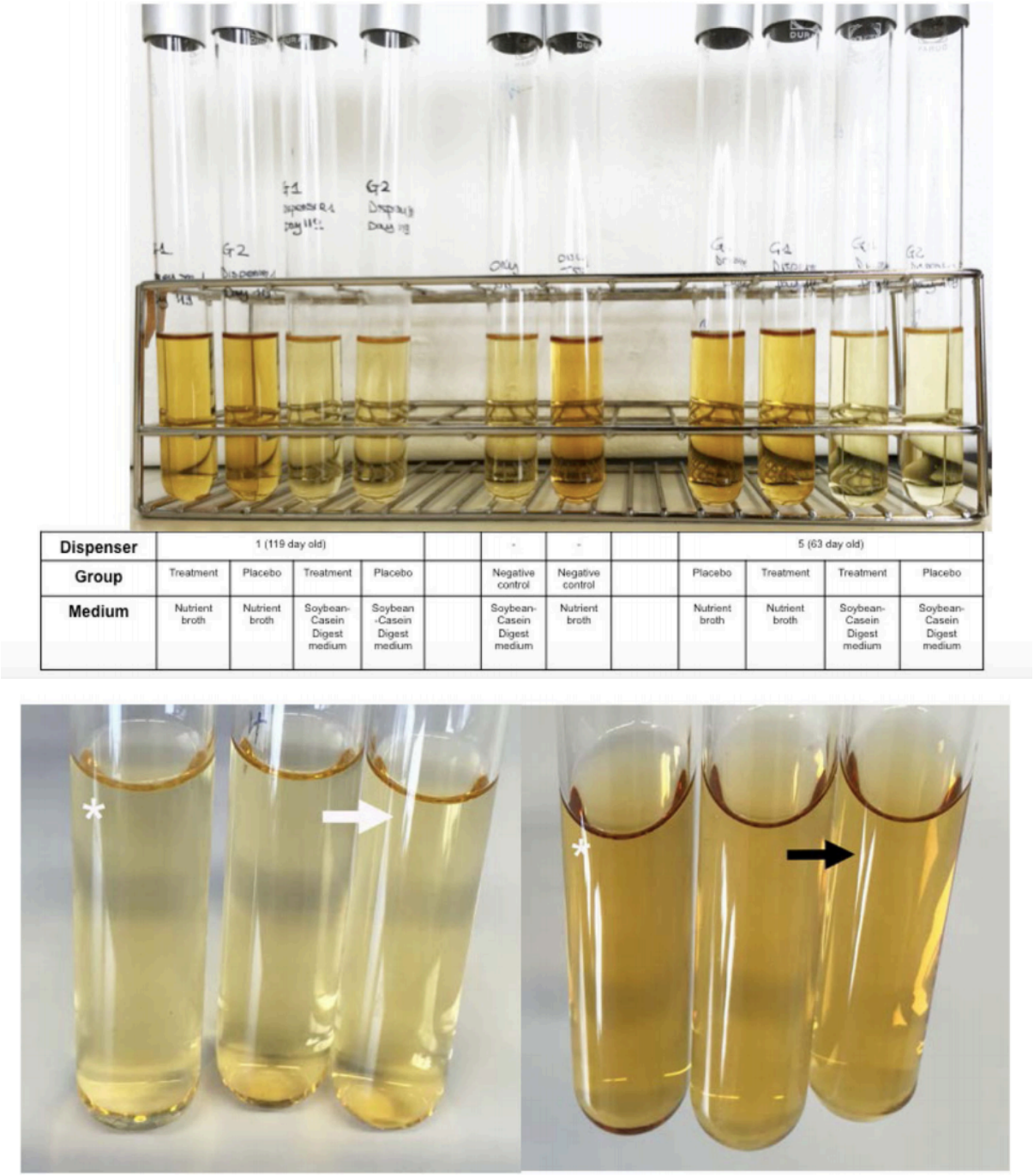


Figure 3.3: Mean increase in OD for peanut-specific IgE in the treatment (white bars) and placebo groups (black bars) ± SD. Asterisk shows that at day 91, 105 and 119, the increase in the treatment group was significant in comparison with the placebo group (p = 0.0298, 0.00735 and 0.0245, respectively)

Sterility testing at the final container

None of the culture media became turbid after incubation with 1 ml dispenser solution, confirming the sterility of the tested solutions (Fig. 3.4).



Dispenser	1 (119 day old)					-	-		5 (63 day old)			
Group	Treatment	Placebo	Treatment	Placebo		Negative control	Negative control		Placebo	Treatment	Treatment	Placebo
Medium	Nutrient broth	Nutrient broth	Soybean-Casein Digest medium	Soybean -Casein Digest medium		Soybean-Casein Digest medium	Nutrient broth		Nutrient broth	Nutrient broth	Soybean-Casein Digest medium	Soybean-Casein Digest medium

Figure 3.4 Sterility testing. Top: The content of all tubes was clear and no turbidity was seen after 14 days of incubation. Bottom: Magnifications of the tubes inoculated with content of dispenser 1 showing no turbidity after incubation for 14 days as described in material and methods (from left to right: Tryptone Soya Broth and peanut (*), Tryptone Soya Broth and placebo and only Tryptone Soya Broth (white arrow); Nutrient broth and peanut (*), Nutrient broth and placebo, only Nutrient broth (black arrow))

3.5. Discussion

This is the first prospective, randomized, blinded, placebo-controlled trial of peanut-SLIT in dogs. This study successfully demonstrated the safety and tolerability of the peanut-specific sublingual immunization in healthy dogs. Indeed, none of the dogs used in this study experienced either systemic or local side effects. Other studies that have been performed in humans with different food extracts, including kiwi, hazelnut, peach, cow's milk and peanut, have shown that SLIT has a very good safety profile (Nowak-Wegrzyn et al. 2011; Moran et al. 2013). Most likely, testing different food extracts in dogs will lead to a similar conclusion. Side effects are rarely reported in human patients and when present, they are mainly of local nature, such as swelling and itching of the lips, inflammation of the area under the tongue and the oropharynx, and often do not require treatment (Kim et al. 2011; Fleischer et al. 2014). Skin itch is also reported, but it was most commonly present in the placebo group (Kim et al. 2011). Systemic reactions such as urticaria, angioedema and asthma rarely occur. The occurrence of side effects is mainly allergen and dose dependent and is mostly limited to the induction phase. There are only few communications reporting the safety of SLIT in dogs. A first pilot study investigated the effect of SLIT in 10 mite-sensitive dogs with atopic dermatitis (DeBoer et al. 2010). This study was followed by a multicenter open trial evaluating 124 dogs (DeBoer et al. 2012). Although no side effects have been reported, it is worthwhile noting that many dogs included in these studies received concurrent medications in order to control symptoms or secondary infections. Unfortunately, there are no studies about food-specific sublingual immunotherapy in dogs. As the avoidance of the offending factors is more difficult in atopic dermatitis, it is clear that more efforts are done in searching new treatments for atopic dermatitis than for food allergy. In fact, the prognosis for food allergy is generally good when the offending food allergen is identified and the dog is fed with a diet in which this allergen is absent. Strict avoidance of the offending allergen is necessary to avoid relapses. However, accidental reactions are common, as allergens can be hidden in various foods or contaminate commercial food (Ricci et al. 2013). Lack of family member's compliance or inappropriate food access can lead to undesired relapses. The induction of tolerance against offending allergens might prevent such relapses and as such be an important therapy in food allergy. To be noted, while in humans one-third of the people that strictly avoided the offending food component for

1–2 years could tolerate it after such a time span, in dogs, natural desensitization rarely occurs (Muller et al. 1989; Pastorello et al. 1989). Furthermore, even when the diet is strict, dogs, as human, can become allergic to another food component present in their diet after 2–3 years (Fadok 1994). In humans, the sensitization to other food stuffs, also called “allergic cosensitization” or “collateral priming” has been shown to be prevented through a regulatory T-cell dependent mechanism induced by an early allergen-specific immunotherapy (Baron-Bodo et al. 2012 ; Des Roches et al. 1997; Holt et al. 2004; Holt 2008; Holt et al. 2007; Jacobsen et al. 2007; Marogna et al. 2004; Marogna et al. 2008; Mondoulet et al. 2015; Novembre et al. 2004; Purello-D’Ambrosio et al. 2001). Unfortunately, this has not been assessed in dogs yet. The goal of this study was to evaluate the safety and the tolerability of food-specific sublingual immunization in healthy dogs. Almost none of the dogs, in both the placebo and treatment group, manifested any systemic or local adverse effect. Only one dog in the placebo group vomited once; however, there was no correlation between placebo administration and clinical signs. Especially noteworthy is the extreme easiness of using the pump-type hooked-dispenser bottles which allowed a fast and safe administration. In fact, we carefully examined the oral cavity and no lesions were found. In addition, absence of changes in dog’s behaviour during the study and in particular at the time of each administration suggests that the daily administration did not affect their well-being. This is an important point which must not be overlooked because, indirectly, it may increase the compliance to treatment and consequently, also the efficacy of the SLIT. This study was limited to healthy dogs for two reasons: first, since the main aim of this study was to evaluate the safety of the protocol, it was necessary to use healthy dogs in order to rule out irritation by the allergen used rather than a real allergic reaction. Secondly, we wanted to understand if this protocol could induce sensitization against peanut allergen, which would not have been possible in dogs which were already allergic. Peanut (Arachis hypogaea) was chosen because none of the included dogs had previously eaten this protein and because commercial diets for dogs normally do not contain peanut as ingredient. We must emphasize that even in case of induced allergy, the avoidance of this protein would have been easier compared with other proteins. The most striking aspect of this protein-choice is that peanut causes the most severe and typically permanent hypersensitivity reactions to foods in humans, and therefore, it has been extensively studied in human literature (Sicherer et al. 2010). Currently, 17 proteins, namely, Ara h 1 to Ara h 17, have been

identified as peanut allergens (WHO/IUIS Allergen Nomenclature Sub-Committee, 2015-07-07) (WHO/IUIS Allergen Nomenclature Sub-Committee 2015). These have been further classified as major or minor allergens based on their ability to elicit an IgE response in >90% of allergic patients (Marsh et al. 1986)]. Ara h1, 2, and 6 are known as major allergens and they retain their IgE reactivity after heating and enzymatic digestion, probably due to their stable and homotrimeric structure, which protects the catalytic sites within the protein (Koppelman et al. 1999; Lehmann et al. 2006; Maleki et al. 2000; Suhr et al. 2004; Vijay et al. 1987). Since the stability and potency of allergen extracts and consequently the efficacy of the immunotherapy may be affected by contamination, solutions and dispenser preparations were made under sterile conditions (Baron-Bodo et al. 2012; Des Roches et al. 1997; Marogna et al. 2004; Marogna et al. 2008; Purello-D'Ambrosio et al. 2001). Moreover, glycerin, which is a stabilizer and also a preservative, was added to allergen extract solutions to prevent loss of allergens by sticking to the glass vials and to inhibit microbial growth. No microorganisms could be cultured from the dispensers' content even 119 days after preparation and when used for oral administration. Interestingly, the use of only glycerin and no other preservatives such as phenol, which is commonly used in vaccine preparations, is sufficient to maintain the solution sterile. It should be borne in mind that phenol, which is a good preservative, could denaturate allergens even when stabilised in 50% glycerine (Grier et al. 2007; Niemeijer et al. 1996; Soldatova et al. 2000; Weber et al. 2008). It could be questioned that a four months sublingual contact might not be enough to sensitize dogs. It is impossible to estimate how long this study should need to have lasted to really exclude induction of allergy, as dogs may develop food allergy spontaneously between the age of 4 months to 14 years (Roudebush et al. 2000). However, high concentrations of food-specific IgE were already detected in 77.8% and in 100% of the experimentally induced food allergic beagles included in the study of Puigdemont, respectively, at day 57 and at day 85 of the sensitization protocol, showing that sensitization can occur earlier. In addition, food-specific intradermal testing was also positive in all sensitized dogs at day 85 (Puigdemont et al. 2006). Even though the concentration of peanut-specific IgE was increased significantly in our experiment, the intradermal test at the end of the experiment and provocative diet challenge were negative. To note, a rise in IgE has also been demonstrated in human studies during the initial months of immunotherapy and it does not lead to an increase in adverse reactions if a simultaneous rise in allergen-specific

IgG occurs (Kim et al. 2011; Varshney et al. 2011). Interestingly, in humans, as in our study, allergen-specific IgG concentrations showed a simultaneous and more extensive increase than IgE, suggesting a good tolerance induction (Kim et al. 2011; Jones et al. 2009; Varshney et al. 2011). Indeed, there are many articles reporting significant increase in serum concentration of food-specific IgG and IgE after allergy-specific immunotherapy (ASIT). These increases have been associated with successful oral and sublingual immunotherapy, desensitization and induction of tolerance for specific food allergens. This has been extensively reported in human literature for both atopy and food allergy and in veterinary literature only for canine atopic dermatitis (Hites et al. 1989; Hou et al. 2008; Hourihane et al. 1997Kim et al. 2011; James et al. 1992; Savilahti et al. 2010; Skripak et al. 2008; Vickery et al. 2013). Therefore, this is the first article reporting an increase in serum concentration of food-specific IgG after administration of a new protein in naïve dogs. It is acknowledged that the skin test is not a diagnostic assay for food allergy and the oral food challenge (OFC) is still considered the gold standard test. Therefore, we performed an OFC with 2000 µg of peanut extract 6 months after the end of the experiment. None of the dogs showed any signs of allergic sensitization, further confirming that our protocol did not sensitize dogs against peanut. In a study designed to determine the minimum dose of peanut protein capable of eliciting an allergic reaction in sensitized individuals, clinical signs were evident after ingestion of 2000 µg of peanut (Taylor et al 2002; Vickery et al. 2013). Administration of peanut during the oral food challenge lasted 7 days for 3 dogs because they were already included in a new experiment which did not allow peanut administration. It has been reported that the OFC should be continued for 7-14 days (August 1985; Harvey 1993; Jeffers et al. 1991; Miller et al. 2013) because a small percentage of allergic dogs may require more days to show clinical signs after being fed the culprit protein. In dogs, it is unclear if these delayed reactions require the OFC to be carried out over several days or if it is enough to administer a weight-appropriate dose of protein in a single day, as it is routinely done in human medicine, followed by monitoring the patient the following 14 days, as we meticulously did in this experiment.

3.6. Conclusions

To conclude, we demonstrated that sublingual administration of escalating doses of peanut extract in healthy dogs is a safe and well tolerated protocol. Given the premises, this food-specific SLIT protocol might be a suitable treatment to desensitize dogs with food allergy. Future research should focus on testing the same protocol in dogs with proven food allergy.

Chapter IV:

A double-blinded, randomized, placebo-controlled trial of the efficacy, quality of life and safety of food allergen-specific-sublingual immune-therapy in client-owned dogs with adverse food reactions: results of a pilot study.

Adapted from: Maina E, Cox E, 2016. A double-blinded, randomized, placebo-controlled trial of the efficacy, quality of life and safety of food allergen-specific sublingual immunotherapy in client-owned dogs with adverse food reactions: results of a small pilot study. Vet Dermatol; 27: 361-e91.

4.1. Abstract

Background: Food allergen-specific sublingual immunotherapy (FA-SLIT) has emerged as a novel and successful approach to desensitize human patients to specific food allergens. However, it has not been tested in dogs. Therefore, we sought to investigate the efficacy, quality of life (QoL), tolerability and safety of FA-SLIT in dogs with adverse food reactions (AFR).

Methods: Dogs with proven AFR were randomized to treatment (T group; n=7) or placebo (P group; n=6) to receive either FA-SLIT (based on the results of a food elimination trial) or glycerinated saline, respectively. The treatment was continued daily for six months with fortnightly dosage escalations. To evaluate the treatment pVAS, CADESI-04, QoL, fecal consistency scores, owner assessment, overall tolerability score urine and blood analyses were assessed.

Results: Eleven dogs completed the study, while two dogs in the T group were withdrawn by the owner after FA-SLIT exacerbated clinical signs of AFR. Statistical tests showed significant protection against food challenge-induced clinical signs following FA-SLIT therapy as indicated by reduced pVAS and CADESI scores ($P<0.05$): the QoL did not differ between groups. The treatment was rated as effective or quite effective by 80% of the owners, whereas placebo was rated as ineffective by all owners.

Conclusions: FA-SLIT was effective, well tolerated and safe. No severe adverse events were recorded; erythema and pruritus were reported in association with only 0.7% of the dispensed doses. Larger clinical trials with more extended maintenance immunotherapy periods will be needed to provide more precise estimates of efficacy and frequency of adverse events.

4.2. Introduction

Food allergy, an immune-mediated reaction to food, can be IgE-mediated or non-IgE-mediated. In some dogs it is impossible to demonstrate food antigen-specific IgE antibodies and, consequently, the term “adverse food reactions” (AFR), which includes immunological and nonimmunological causes, is preferred. It has been estimated that approximately 7.6–12% of dogs suffer from AFR (Chesney 2002; Proverbio et al. 2010). Treatment for AFR currently relies on strict avoidance of the responsible allergens, as there is no known cure for the disease. However, accidental ingestion is possible and avoidance is not always easy in dogs. In addition, spontaneous resolution has never been described. However, additional sensitizations can occur over time. When contact with the allergen cannot be prevented, a dog may be treated symptomatically with anti-inflammatory and antipruritic drugs. Symptomatic treatment is, by definition, orientated towards control of clinical signs, but does not resolve the disease. Recently, several studies have described the treatment of human food allergy with allergen-specific immunotherapy (Skripak et al. 2008; Keet et al. 2012; Narisety et al. 2015). Desensitization of the patients was obtained in randomized and controlled trials. Different routes of administration have been investigated, including subcutaneous, sublingual, oral, epicutaneous and intra-lymphatic routes (Hylander et al. 2013; Senti et al. 2012; Senti et al. 2011; Von Moos et al. 2011). The sublingual route has gained increasing acceptance because of its ease of administration and its safety profile as compared to the others (DeBoer et al. 2010; DeBoer et al. 2012; Wilson et al. 2005; Grosclaude et al. 2002). Food allergen-specific sublingual immunotherapy (FA-SLIT) involves administration of small amounts of allergen extract under the tongue with increasing doses over time. Once the solution is applied sublingually, the allergens make contact with the oral mucosa where they are captured and processed by dendritic cells. These cells migrate to the sub-mandibular lymph nodes where allergen-specific regulatory T cells (Tregs) are induced. These Tregs are responsible for the desensitization by deletion or induction of anergy of allergen-specific effector T cells (Burks et al. 2008; Weiner et al. 2011; Sun et al. 2007; Vickery et al. 2009). Although studies have been reported which assess the effect of SLIT for atopic dermatitis (AD) signs in dogs, to the best of the authors’ knowledge, there are no reports on its use to control canine AFR (DeBoer et al. 2010; DeBoer et al. 2012;

Fujimura and Ishimaru 2016; Marsella and Ahrens 2012.). In a randomized, placebo-controlled study we have demonstrated that FA-SLIT is a well tolerated and safe treatment modality in healthy experimental dogs (Chapter 3). Consequently, we hypothesized that FA-SLIT may safely desensitize dogs with AFR, as has been shown for people with food allergy. The purpose of the following randomized, double blinded, placebo-controlled pilot study was to determine the efficacy and safety of the FA-SLIT treatment in dogs with AFR. Specifically, as a primary outcome, we wanted to evaluate whether FA-SLIT could decrease pruritus and lesion scores in these dogs during challenge with the provocative diet. As secondary outcome measures, we assessed the impact of FA-SLIT on the quality of life (QoL) of both owners and dogs, evaluated the effect of the treatment on the faecal consistency and determined if allergen-specific IgE titres decreased.

4.3. Methods

Study subjects

Client-owned dogs with a definitive diagnosis of AFR were recruited from five referral clinics in northern Italy. The AFR diagnosis was obtained through dermatological examination and recheck visits using standard procedures. Dogs with pruritus and/or gastrointestinal signs were assessed for ecto- and endoparasitic infestations and flea bite hypersensitivity by faecal flotation examination, intradermal testing, and by ectoparasite control with the application of a topical spoton containing imidacloprid and moxidectin (Advocate, Bayer Animal Health; Milan, Italy) every 14 days for three doses. Dogs were excluded if ecto- and endoparasitic infestations or flea bite hypersensitivity were diagnosed. Secondary skin infections with bacteria and/or yeast were resolved prior to inclusion. Dogs with a nonseasonal allergic phenotype and a negative response to antiparasitic treatment were fed an elimination diet. Home cooked or commercial diets were given, consisting of a mixture of a carbohydrate and a protein source not previously fed. Hydrolyzed diets were used when no new proteins were available for the dog. The restriction diet was fed for at least 8 weeks without any other food or flavoured supplements. Some details of the diets are provided in Table 4.1. If the dogs improved on the restriction diet, they were challenged by re-feeding separate food components from the former diet. This provocation testing lasted 7–14 days (or less, if signs relapsed) for each food component to assess any relapse of

clinical signs. Dogs were diagnosed with AFR if they improved when given the restriction diet, subsequently showed cutaneous and/or gastrointestinal clinical signs during the challenge period and then improved again with reintroduction of the restriction diet. The food responsible for the development of the clinical signs in each dog is reported in Table 4.1. In order to exclude dogs with possible concurrent AD and to avoid the need for anti-pruritus medications, only dogs which showed complete resolution of clinical signs while being fed the restriction diet were enrolled into the study.

Table 4.1. Signalment, body weight, allergy and restriction diet of the 13 dogs included in the study.

Case no.	Group	Age (years)	Sex	Breed	Weight (Kg)	Allergic to:	Restriction diet
1	P	0.5	M	French bulldog	6	W	Ra, Po
2	P	0,.5	M	German shepherd	23	C, B, R	H, Po
3	P	0.5	M	German shepherd	19	C	H, Po
4	T	2	F	French bulldog	12	Pk,R,W,B,C,Mi	H, Po
5	T	2.5	M	Dachshund	7	W,B	z/d
6	T	2	F	WHWT	10	C,Fi	H, Po
7	P	1.5	FS	Dobermann	27	B	F, Po
8	T	13	M	Italian shepherd	42	C	Ra, Po
9	T	0.9	F	Shih tzu	7	C	F, Po
10	P	2	M	Sharpei	25	R,C	Ra, Po
11	T	3	F	French bulldog	10	C,W,B	z/d
12	P	1	F	Golden retriever	20	C	H, Po
13	T	3	F	French bulldog	9,5	Fi	z/d

B, beef; C, chicken; F, female; Fi, fish; FS, female spayed; H, horse; M, male; Mi, milk; P, placebo group; Pk, pork; Po, potatoes; R, rice; Ra, rabbit; T, treatment group; W, wheat; WHWT, West Highland White Terrier; z/d, z/d ultra (Hill's z/d Ultra allergen free®).

Randomization and masking

Dogs were randomized to one of two experimental groups in a 1:1 ratio using IBM SPSS v21.0 software (IBM Corporation; Armonk, NY, USA): FA-SLIT (T group) or placebo (P group). Blocking was based on the order of enrolment in the study. A person not involved in the clinical examination of the dogs maintained the randomization list. The study was blinded to both the investigator and the dog owner. For each subject, either a placebo or treatment set in identical brown glass dispensers was prepared and properly labelled with case and concentration numbers (from 1 to 4 with increasing concentration). The pet owner administered the appropriate treatment after demonstration by the investigators.

Food allergen-specific immunotherapy and placebo

Fifty percent glycerinated food allergenic extracts (crude defatted pork meat, chicken meat, beef, fish, cow's milk and crude defatted rice, wheat and corn) were provided by Greer Laboratoires (Lenoir, NC, USA). The treatment group received a solution containing one or more food extracts, based on results of the restriction diet trial and food specific challenges. The starting dose was 12.5 µg/mL and the maximum protein concentration was 20,000 µg/mL per allergen type. Sterile glycerinated saline solution served as placebo. Treatment dilutions were made in glycerinated saline under sterile conditions. Food extracts and dispensers containing placebo or allergen extract were stored at 4°C until use.

SLIT administration and study schedule

During the study period, which lasted at least 6 months, dogs were maintained on a restriction diet. The solution was administered once a day under the tongue by hooking the dispenser tip over the lower second and third premolar teeth and spraying 100 µL of the solution into the oral cavity (Figure 4.1). The administration occurred each day at the same time, at least one hour after a meal. Thereafter, dogs were restricted from eating or drinking for 30 min. An investigator carefully monitored each dog for at least one hour following the first two doses to assess for evidence of adverse reactions. Starting from 1.25 µg of protein in 100 µL, doses were increased every 2 weeks by 50–100% until a maintenance dose of 2000 µg in 100 µL was reached (Table 4.2). In case of onset of pruritus or skin lesions during the administration of the treatment, a more dilute solution (25–50% less concentrated) was administered to the dog for

fourteen days, whereafter the dose was gradually increased again following the original protocol.

Table 4.2. Food antigen sublingual immunotherapy dosing schedule.

Weeks	Dilution	Dispenser	Pumps	Protein (µg)
1	1:4000	1	1 pump	1.25
2	1:4000	1	1 pump	1.25
3	1:4000	1	2 pumps	2.5
4	1:4000	1	2 pumps	2.5
5	1:4000	1	4 pumps	5
6	1:4000	1	4 pumps	5
7	1:500	2	1 pump	10
8	1:500	2	1 pump	10
9	1:250	2	2 pumps	20
10	1:250	2	2 pumps	20
11	1:166	2	3 pump	30
12	1:166	2	3 pump	30
13	1:100	3	1 pump	50
14	1:100	3	1 pump	50
15	1:50	3	2 pumps	100
16	1:50	3	2 pumps	100
17	1:25	3	4 pumps	200
18	1:25	3	4 pumps	200
19	1:20	4	1 pump	250
20	1:20	4	1 pump	250
21	1:10	4	2 pumps	500
22	1:10	4	2 pumps	500
23	1:5	4	4 pumps	1000
24	1:5	4	4 pumps	1000
25	1:2.5	4	8 pumps	2000
26	1:2.5	4	8 pumps	2000

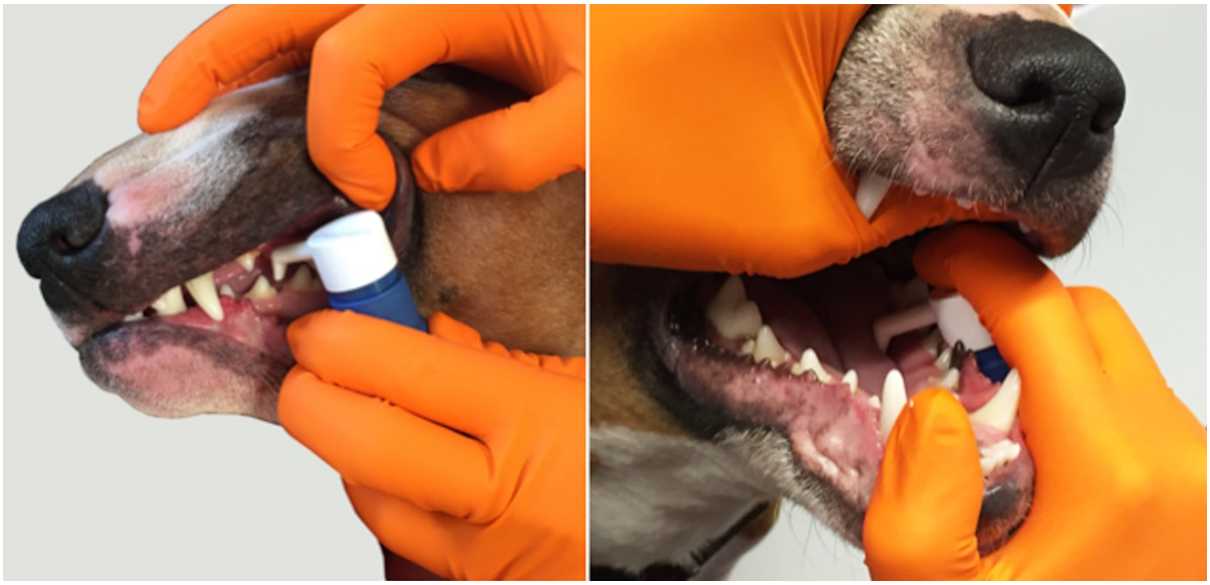


Figure 4.1. Correct placement of the dispenser tip for administration of sublingual immunotherapy. The picture shows the right hooking of the dispenser tip over the lower teeth, between second and third premolars, under the tongue.

Concurrent treatment

Flea control measures with topical imidacloprid and permethrin (Advantix, Bayer; Milan, Italy) were provided every 3–4 weeks during the study to all dogs and this was the only treatment allowed during the study.

Efficacy assessment

During the study, dogs were re-examined by the investigator on a monthly basis. For each dog, QoL, pruritus and skin lesions scores were assessed before and at the end of the study in case of a dietary challenge (Figure 4.2). The dietary challenges were both performed with the administration of 50-100 grams/ml daily (dogs $\leq$ 25 kg and dogs $\geq$ 25 kg, respectively) of the culprit food for 14 days. Data were recorded on day 14. Quality of life was assessed by the owners by means of a validated questionnaire consisting of 15 questions (Noli et al. 2011a; Noli et al 2011b). The questions were divided into three subgroups as follows: one question on the general severity of the disease (S, range 0–3); seven questions on the QoL of the dog (QoL1, total score range 0–21) and seven questions on the QoL of the owner (QoL2, total score range 0–21). Each question had four possible answers classified as: not at all (score 0), a little bit (score 1), quite a bit (score 2) and very much (score 3). Scores of each subgroup of questions where then summed to give a total QoL questionnaire score

(range 0–45). Pruritus was assessed by means of a validated 10 cm long visual analog scale (pVAS) (Hill et al. 2007). Lesions were assessed by means of the Canine Atopic Dermatitis Extent and Severity Index (CADESI-04) (Olivry et al. 2014). In short, erythema, lichenification, excoriation and/or alopecia were evaluated on a scale from 0 to 3 (0 = none, 1 = mild, 2 = moderate and 3 = severe) at 20 different body areas and summed to give a clinical total score, which ranged from 0 to 180. Owners were asked to score their dog's faecal consistency on a scale of one to seven using a Purina® (St. Louis, MO, USA) faecal scoring chart at four different time points as depicted in Figure 4.2 Faecal consistency scores (FCS) recorded while the dogs were on the restriction diet (i.e. prior to the intervention) were compared to scores recorded during the trial and during provocative food challenges which were performed both before and after the trial period, in order to assess the effect of FA-SLIT on faecal consistency. In addition, owners were asked to rate the effectiveness and the ease of treatment administration by completing a four-question survey (Table 4.3).

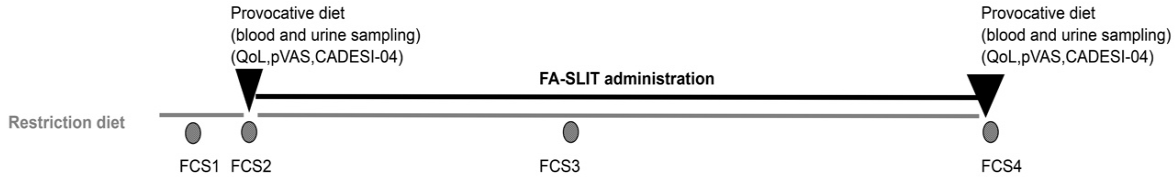


Figure 4.2. Study protocol for food antigen sublingual immunotherapy (FA-SLIT) and its evaluation (QoL, quality of life; pVAS, pruritus Visual Analog Scale; CADESI-04, Canine Atopic Dermatitis Extent and Severity Index; FCS, faecal consistency score).

Table 4.3. Owner assessment of effectiveness and ease of treatment.

Questions	Scoring systems
How do you rate the effectiveness of the treatment?	0 = poor, 1 = fair, 2 = good, 3 = excellent
How difficult was it for you to administer the treatment?	0 = easy, 1= feasible, 2 = difficult, 3= very difficult
How annoying was it for you to administer the treatment on a daily basis?	0 = easy, 1= feasible, 2 = difficult, 3= very difficult
Would you rather have preferred a subcutaneous treatment with injections but less frequent administration (1 injection every 2-3 weeks)?	sublingual, subcutaneous.

Serological testing

Serum food allergen-specific IgE concentrations were evaluated by enzyme linked immunosorbent assay (ELISA). Blood was collected by cephalic or jugular venipuncture during the two provocative phases of dietary challenge (before and after treatment). Sera were frozen at - 20°C until tested. The ELISA was performed using Nunc MaxiSorp®flat-bottomed 96 well plates coated overnight at 4°C with allergens at 0.05 mg/mL in bicarbonate buffer. The following allergen extracts were coated in triplicate: pork, chicken, beef, cow's milk, fish, rice, wheat and corn. After blocking with 0.5% gelatin from cold water fish skin (Sigma-Aldrich®; Steinheim, Germany) in phosphate buffered saline (PBS), wells were subsequently incubated with two-fold dilutions of the serum samples in 0.05% Tween®20 in PBS (PBST), followed by a canine IgE-specific polyclonal antibody (horseradish peroxidase-labelled, Novus Biologicals; Cambridge, UK, NB7346) and a solution of ABTS (Roche Diagnostics; Mannheim, Germany). Between each step, the plates were washed with PBST. Finally, the optical density (OD) was measured at 405 nm (Tecan Spectra Fluor Fluorescence and Absorbance Reader, Tecan Group Ltd; M€annedorf, Switzerland). All steps, except for the coating, were performed at room temperature. Pre- and post-treatment results were compared and differences between time points per allergen and per dog were recorded. Differences between allergens included in the treatment and those not included were compared in order to demonstrate an association between treatment and changes in IgE OD values.

Statistical analysis

Evaluation of efficacy was based upon response in two co-primary end-points: the percentage reduction at 6 months from baseline for both owner-assessed pVAS and CADESI. In particular, the treatment was considered successful if the percentage reduction of the CADESI and the pVAS scores were equal to or greater than 50% at the end of the study as compared to the initial value at baseline. Secondary end-points included (i) changes in mean subgroup scores and total QoL scores at the end of the study as compared to the baseline, (ii) FCS changes between study time points and (iii) changes in allergen-specific IgE values at 6 months as compared to the baseline. Data were analysed with SPSS Statistics 21 (IBM; New York, USA). The distribution of the data was assessed by a normality test. pVAS and QoL data were normally distributed and the difference between groups for these parameters was analysed by

an independent t-test. The data related to FCS scores were not normally distributed and the differences between groups from FCS 1 to FCS 3 and from FCS 2 to FCS 4 were analysed with a Mann–Whitney U test. CADESI data were analysed by Wilcoxon signed rank test. Differences between pre- and post-treatment total scores of the QoL of all dogs were analysed by the paired samples t-test. Correlations between initial pVAS and CADESI scores with the presence of adverse events, the minimum dosage to induce adverse effects and the number of different allergens included in the dispenser were analysed with a Spearman correlation test. Differences in serum IgE OD values for each allergen extract included in the treatment were determined by subtracting the OD measured at baseline from the OD measured at the end of the experiment. These differences where then compared by a Mann–Whitney U test with those for allergen extracts not included in the treatment of that dog. These differences were further compared between groups. For all comparisons, a value of $P < 0.05$ was considered to be significant. Data were analysed by per protocol analysis. Only data concerning presence/absence of adverse events were analysed by both: per protocol and by intention-to-treat (ITT) analysis using data collected from all enrolled subjects who received the treatment, whether or not they completed the study.

Safety assessments

In order to rule out the onset of local adverse effects, the muzzle, mouth and the oral cavity of the dogs were examined by the investigator several times within the hour following the first two administrations of sublingual solutions. All changes were recorded. The owners were asked to monitor their dog for all reasonably anticipated local adverse effects for at least sixty minutes after each dose, including perilabial erythema, urticaria and angio-oedema, intra-oral swelling, wheals, vesicles or ulcerations, immediate or delayed oral or muzzle pruritus, hyper-salivation, continuous chewing or vomiting. These data were analysed and compared between groups. Nonlocal treatment-related effects that occurred during the study period or within 7 days after the end of the experiment were also recorded. These included: diarrhoea, abdominal pain, urinary tract infection/cystitis, urticaria, erythema, pruritus, pyoderma, otitis, epilepsy, somnolence, anorexia and anaphylaxis. The onset, duration and severity (categorized as 0 = none, 1 = mild, 2 = moderate and 3 = severe) and any necessary treatments were also noted. The Naranjo adverse drug reaction probability scale was used to assess the likelihood of a real adverse drug reaction (Naranjo et al.

1981). Hepatic and urinary function were evaluated before and after the experiment by measuring alanine transferase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP), creatinine, total protein and urea. Urinalysis was performed by urine dipstick testing, which measured the following variables: pH, protein, glucose, bilirubin, specific gravity, blood, ketones, nitrite, urobilinogen and ascorbic acid. Dogs were weighed before starting the study and at the end by the investigator and every week by the owner. All body weight (BW) data were recorded and any change from the baseline was reported. At the end of the study, the owners were asked to complete a survey and rate the tolerability of the treatment. The questionnaire comprised eleven questions covering the behaviour of the dog after the administration of the immunotherapy/placebo, behavioural changes over time in relation to consecutive administrations, the onset of local cutaneous signs or vomiting, weight loss, changes in frequency or consistency of faeces, and presence or changes in bowel sounds or flatulence over the treatment period. A four-point scale (0 = none, 1 = mild, 2 = moderate and 3 = severe) was used (Table 4.4). The tolerance scores per dog were summed to give an overall tolerance score, which ranged from 0 to 33.

Table 4.4. Owner assessment of the tolerability of the treatment.

Questions:
1. Did the dog experience excessive salivation right after the administration of the treatment?
2. Did the dog show continuative licking at the lips, chewing or foaming at the mouth right after the administration of the treatment?
3. Did the dog experience perioral or oral pruritus after the administration of the treatment?
4. Did the dog experience perioral erythema after the administration of the treatment?
5. Did the dog turn his head away during the solution administration? Did you encounter increased reluctance to the treatment day after day (did the dog try to hide? Did he show aggressive behaviour?)
6. Did the dog lose weight during the study period?
7. Did the faecal consistency change during the study period?
8. Did the defecation frequency change during the study period?
9. Did your dog have abnormal bowel sound during the study period?
10. Did your dog have flatulence during the study period?
11. Did the dog vomit during the study period?

Scoring system: 0 = no, 1 = mild, 2 = moderate, and 3 = severe

Owner compliance assessment

Owner compliance was assessed during weekly telephone calls and by measuring the volume of fluid left in the dispensers at the end of the study. In short, in order to verify that the correct dose of treatment/placebo was dispensed, all dispensers were retrieved after their use and the solutions left were then collected, measured and compared with the amount that should have remained. The latter was calculated by multiplying the microlitre dispensed per day by the number of days during which the solution was administered.

4.4. Results

Study subjects

Thirteen dogs were enrolled in the study. Signalment, weight, identified allergens and restriction diets are listed in Table 4.1. Seven dogs were randomized to the treatment group (dogs 4, 5, 6, 8, 9, 11 and 13) and six to the placebo group (dogs 1, 2, 3, 7, 10 and 12). Two subjects from the active treatment group (dogs 5 and 13) were withdrawn from the study. In each case, pruritus and erythema occurred after dosage escalation, and the owners elected to withdraw their pets (at days 146 and 136) rather than dilute the solutions. Because data from these dogs could not be collected at the end of the study (i.e. no food rechallenge performed), they were excluded from the primary analysis; however, adverse events for these two dogs were included in an ITT analysis. The study lasted an average of 182 (±0.41) and 240 days (±63.30) for the placebo and treatment groups, respectively. The longer time needed to complete the protocol in the treatment group was due to the occurrence of pruritus and/or erythema in four dogs after sublingual administration.

Except for the two dogs withdrawn at the beginning of the study, all dogs completed the study. The administration of SLIT was easy and well accepted by the dogs. The dispensers, with their hooked nozzle, did not hurt their mucosa. During and after administration of the solutions, the dogs did not show any changes in their behaviour.

Efficacy assessment

Table 4.5 lists the CADESI, pVAS, FCS and QoL scores of all dogs at the beginning and end of the trial. There was no significant difference in baseline pVAS and CADESI

scores between the treatment groups, indicating appropriate randomization. At the end of the study, during the dietary challenge, the percentage reduction of the pVAS scores were 4.51% (±7.31) and 21.84% (±12.93) in the placebo and treatment groups, respectively (Table 4.6). Improvement was significantly greater (P = 0.04) for the dogs in the treatment group (Figure 4.3), where they showed up to 35% reduction of the initial pruritus score. However, none of the dogs in the treatment group reached the primary end-point of at least 50% reduction from the baseline pVAS score after the treatment. The CADESI scores decreased by 1.29% (±16.02) and 40.44% (±13.91) in the placebo and treatment groups, respectively (Table 4.6). Improvement was significantly greater (P = 0.04) for dogs in the treatment group. In the treatment group, two of five dogs (40%) reached the primary end-point of a ≥ 50% reduction of baseline CADESI at 6 months (Figure 4.4). There were no significant differences between groups in regard to secondary end-points (P = 0.46; Figure 4.5). QoL scores decreased by 12.42% (±14.83) and 18.53% (±18.26) in the placebo and treatment groups, respectively (Table 4.6). Differences between FCS2 and FCS4 in the placebo group showed that two out of six dogs had a one point improvement in FCS at the end of the study, whereas four dogs did not experience a change. In the treatment group, two out of five dogs improved by at least two points and the fifth dog have a one point improvement. Although 40% of the dogs with an improved FCS in the treatment group were considered to be a treatment success as compared to none of the dogs in the placebo group, there were no significant differences between FCS2 and FCS4 (P = 0.17) between the groups. In addition, there were no significant differences between FCS1 and FCS3 (P = 0.19). Indeed, FCS worsened by one point in one dog from the placebo group and improved by one point in one dog from the treatment group. One owner from the treatment group rated effectiveness to be good, whereas three rated it as fair and one as poor. In the placebo group, all owners rated the effectiveness of the treatment as poor. There was a statistically significant difference between groups (P = 0.007). Ten of the eleven owners (91%) rated the sublingual administration as feasible, whereas only one considered it difficult. One out of eleven (9%) considered the daily administration to be non-annoying, eight (73%) as fairly annoying and two (18%) rated it as moderately annoying. Only one owner would have preferred a less frequently administered subcutaneous option. There were no significant differences between groups. Serum allergen-specific IgE titres were high at baseline for the

proteins to which dogs were reactive on provocative challenges. However, allergen-specific IgE was also high for proteins to which the dogs did not react (Table 4.7). At the end of the study, there were no differences between allergen-specific IgE titres for proteins that had or had not been included in the treatment sets.

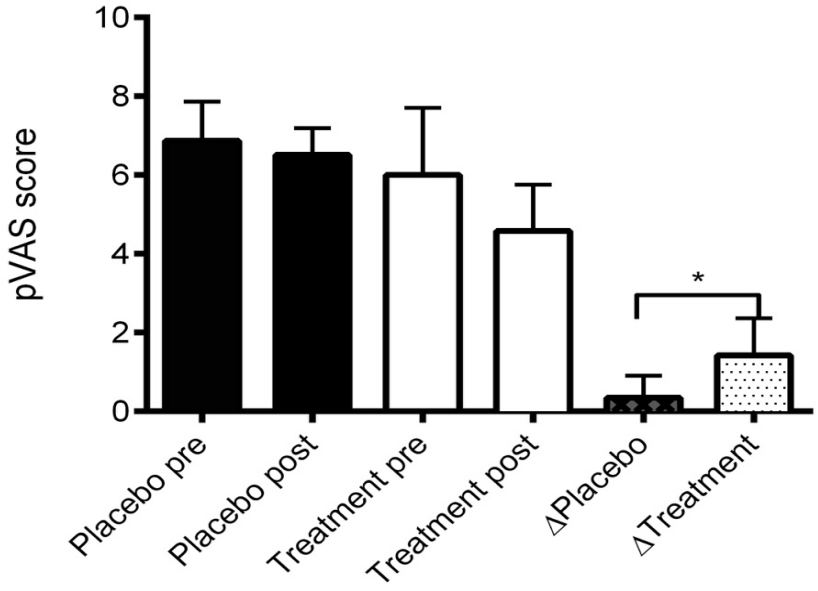


Figure 4.3: Mean pruritus Visual Analog Scale (pVAS) scores before and after the experiment in placebo and sublingual immunotherapy treatment groups. Differences between mean pVAS score before (pre) and after (post) for both groups (Δ = post–pre) are also reported. Error bars denote SD. Asterisk denotes a significant difference between conditions ($P < 0.05$).

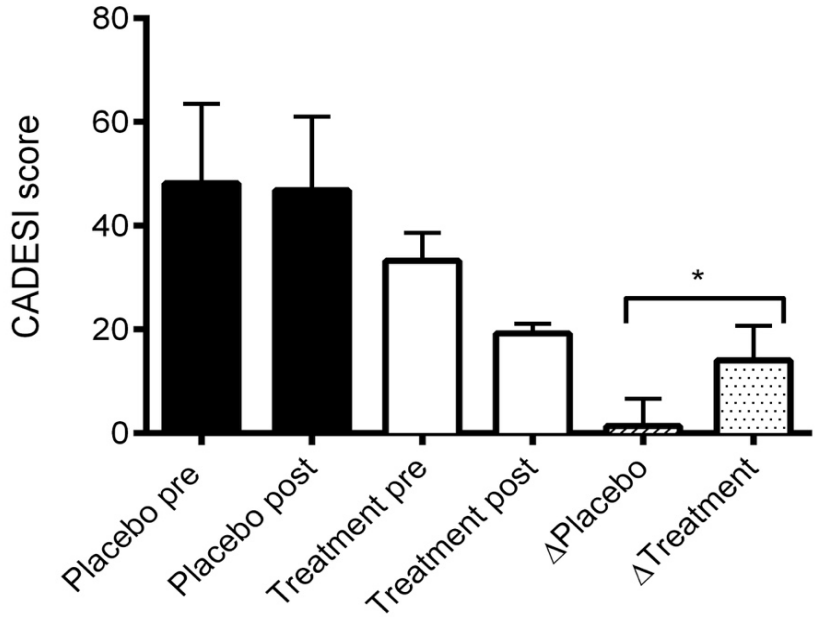


Figure 4.4: Mean Canine Atopic Dermatitis Extent and Severity Index (CADESI-04) scores before (pre) and after (post) the study for placebo and sublingual immunotherapy treatment group. Differences between mean CADESI-04 score pre and post for both groups (Δ = post–pre) are also reported. Error bars denote SD. Asterisk denotes a significant difference between conditions (P < 0.05).

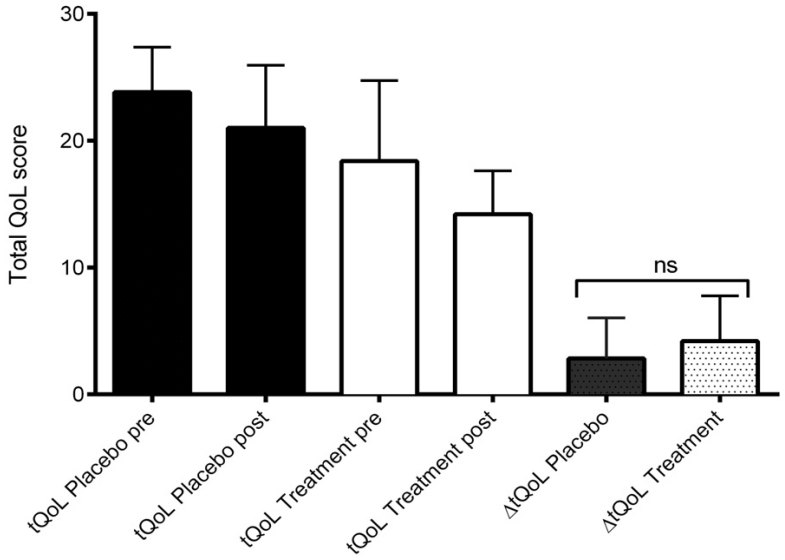


Figure 4.5: Mean total quality of life (QoL) recorded before and after the study for placebo, sublingual immunotherapy treatment group (95% confidence interval) and for all dogs together. Differences between before (pre) and after (post) mean scores were calculated for Placebo and Treatment groups (Δ = post–pre). Error bars denote SD. Differences between placebo and treatment groups are not significant (ns).

Table 4.5. Pruritus VAS, CADESI-04, owner-assessed severity (S), quality of life of dogs (QoL1) and life of owners (QoL2), quality of life total scores (QoLt), scores of all dogs during provocation at the beginning (pre) and the end (post) of the trial and their differences (D). Fecal consistency score (FCS) at 4 different time points and difference between FCS1 and FCS3 (D FCS1-FCS3) and between FCS2 and FCS4 (D FCS2-FCS4). Five dogs were treated and six served as control.

Group	Case no	PVAS			CADESI-04			S			QoL1			QoL2			QoLt			FCS					
		Pre	Post	D	Pre	Post	D	Pre	Post	D	Pre	Post	D	Pre	Post	D	Pre	Post	D	FCS 1	FCS 2	FCS 3	FCS 4	D FCS1-FCS3	D FCS2-FCS4
P	1	7.2	7.1	0.1	51	46	5	2	2	0	8	8	0	11	10	1	21	21	0	2	3	2	3	0	0
P	2	8.7	7.5	1.2	57	56	1	2	2	0	12	10	2	10	8	2	24	22	2	3	6	4	5	-1	1
P	3	6.1	6.2	-0.1	30	39	-9	3	3	0	14	14	0	9	7	2	26	27	-1	2	3	2	3	0	0
P	7	6.2	6.5	-0.3	35	30	5	2	2	0	9	7	2	8	3	5	19	12	7	3	5	3	4	0	1
P	10	6.2	5.7	0.5	72	70	2	3	3	0	14	13	1	12	7	5	29	23	6	2	2	2	2	0	0
P	12	6.8	6.1	0.7	44	40	4	1	1	0	13	12	1	10	7	3	24	21	3	2	3	2	3	0	0
T	4	7.5	6.4	1.1	32	21	11	2	2	0	9	3	6	5	4	1	16	13	3	2	3	2	3	0	0
T	6	5.6	4	1.6	37	16	21	3	1	2	11	2	9	7	7	0	21	13	8	2	3	2	2	0	1
T	8	5.6	4	1.6	31	20	11	2	2	0	7	5	2	11	7	4	20	16	4	2	2	2	2	0	0
T	9	7.8	5.1	2.7	40	19	21	3	2	1	13	9	4	10	5	5	26	19	7	3	4	2	2	1	2
T	11	3.6	3.5	0.1	26	20	6	0	0	0	3	3	0	6	4	2	9	10	-1	3	6	3	3	0	3

Table 4.6. Reduction (%) of the score in placebo and treatment group at the end of the treatment period.

Group	Case number	pVAS	CADESI-04	QoL
P	1	1.39	9.80	0.00
P	2	13.79	1.75	8.33
P	3	-1.64	-30.00	-3.85
P	7	-4.84	14.29	36.84
P	10	8.06	2.78	20.69
P	12	10.29	9.09	12.50
Average		4.51	1.29	12.42
Standard deviation		7.31	16.02	14.83
T	4	14.67	34.38	18.75
T	6	28.57	56.76	38.10
T	8	28.57	35.48	20.00
T	9	34.62	52.50	26.92
T	11	2.78	23.08	-11.11
Average		21.84	40.44	18.53
Standard deviation		12.93	13.91	18.26
Difference between groups significance		p= 0.04	p=0.01	p=0.46

Table 4.7. Food-specific IgE on individual dog level: individual mean OD values recorded before (pre) and after (post) the study.

Group	Dogs	Food extracts														Proven allergy
		pork		rice		wheat		chicken		beef		fish		milk		
		pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	
P	1	0.203	0.230	0.447	0.394	0.891	0.470	0.206	0.337	0.203	0.212	0.467	0.323			W
P	2	0.189	0.219	0.208	0.235	0.227	0.227	0.174	0.172	0.184	0.185	0.185	0.177			C, B, R
P	3	0.178	0.111	0.287	0.147	0.237	0.126	0.220	0.113	0.200	0.104	0.234	0.113			C
P	7	0.331	0.142	0.225	0.193	0.349	0.188	0.429	0.178	0.312	0.241	0.270	0.164			B
P	10	0.261	0.333	0.308	0.269	0.340	0.313	0.272	0.268	0.258	0.284	0.223	0.325			R,C
P	12	0.258	0.145	0.264	0.186	0.307	0.213	0.247	0.134	0.844	0.480	0.364	0.157			C
T	4	0.181	0.184	0.244	0.272	0.284	0.327	0.195	0.242	0.177	0.179	0.186	0.119	0.257	0.217	Pk,R,W,B,C Mi
T	6	0.312	0.331	0.310	0.225	0.291	0.349	0.445	0.429	0.258	0.312	0.222	0.270			C,Fi
T	8	0.236	0.165	0.248	0.330	0.235	0.304	0.170	0.243	0.216	0.130	0.211	0.238			C
T	9	0.221	0.174	0.260	0.169	0.252	0.174	0.227	0.163	0.223	0.162	0.242	0.182			C
T	11	0.276	0.281	0.731	0.692	0.241	0.443	0.382	0.363	0.227	0.233	0.233	0.252			C,W,B

B, beef; C, chicken; Fi, fish; Mi, milk; Pk, pork; R, rice; W, wheat.

Safety assessments

In total, 99% of the 2,294 doses administered were free of adverse effects (0% of the 1,093 doses in the placebo group and 1.4% of the 1,201 doses in the treatment group). No adverse effects were observed by the investigators following the first two administrations. There was no significant difference between the placebo (1.5 ± 1.05) and treatment (2.6 ± 1.14) groups in terms of the mean scores assigned by owners for overall tolerance ($P = 0.13$). The most common reaction, although sporadic and self-limiting (less than three times during the study) was vomiting, which was reported in four of 11 dogs (36%), however, there was no difference between groups. Two dogs in the treatment group (dogs 6 and 9) and two dogs in the placebo group (dogs 7 and 10) had episodes of vomiting. One subject (Dog 6) of the treatment group had three episodes of hypersalivation after administration. One other subject (Dog 9) licked her lips, chewed and foamed at the mouth immediately following treatment administration. Three dogs in the placebo group and one in the treatment group showed increased reluctance to receive the solution over time, which was rated as fair in three and moderate in one. However, no significant difference was observed between the groups. No life-threatening events occurred during the sublingual treatment and there were no clinical laboratory abnormalities or changes in body weight for any dog. The most common adverse events positively associated with FA-SLIT, as determined by the Naranjo test, were pruritus and erythema. Although treatment did not induce local clinical signs in the oral cavity or perilabial area, it did induce these signs at distant sites on at least one occasion after administration in four of five dogs receiving FA-SLIT. The clinical signs observed were similar to their responses to the provocative food challenge. Three of the dogs experienced moderate pruritus and the other mild pruritus, whereas two showed moderate erythema and two mild erythema. None of the dogs in the treatment group experienced severe pruritus or erythema. Dog in the placebo group did not exhibit these signs. However, the difference between groups was not statistically significant. It is important to note that the two dogs withdrawn from the study had moderate pruritus and erythema; data used for ITT analysis became significantly different between groups for both pruritus ($P = 0.01$) and erythema ($P = 0.02$; Table 4.8).

Table 4.8. Presence and description of side effects of food allergen-specific sublingual immunotherapy.

Group	Dog case number	Side effects		Min dose (µg)	Max dose (µg)	N° of relapses with side effects	N° of additional dilutions required	Study duration (days)
		pruritus	erythema					
P	1	0	0	0	0	0	0	182
P	2	0	0	0	0	0	0	182
P	3	0	0	0	0	0	0	182
P	7	0	0	0	0	0	0	183
P	10	0	0	0	0	0	0	182
P	12	0	0	0	0	0	0	182
T	4	2	2	50	500	5	5	325
T	6	2	1	200	1000	8	8	290
T	8	1	1	250	500	1	1	197
T	9	2	2	200	1000	3	3	207
T	11	0	0	0	0	0	0	182
Tw	5	2	2	180	200	-	-	withdrawal
Tw	13	2	2	80	100	-	-	withdrawal

(0=none, 1=fair, 2=moderate, 3=severe), minimal (Min) and maximal (Max) µg of single allergen extract generating side effects, duration of the study, number of relapses and resulting additional dilutions required to avoid side effects in order to finish the study (w=withdrawn)

Assessment of owner compliance

The owner of dog 6 made a mistake during the first week of the protocol increasing the dosage every day instead of every 2 weeks. This was confirmed by measuring the solution in the first dispenser. Dose validation suggested that all other owners correctly followed the protocol. The average dispensation of the solutions was 105.6% (±2.85%) and 104.1% (±2.94%) for the placebo and treatment groups, respectively.

4.5. Discussion

This study has demonstrated that FA-SLIT is able to significantly decrease pruritus and clinical signs of AFR as compared to a placebo treatment. Foods provoked milder reactions following 6 months of FA-SLIT therapy. This suggests that the threshold of sensitivity to food allergens was increased and might be associated with partial

desensitization. Although this level of desensitization was not enough to induce complete tolerance, it might be clinically relevant if it is able to protect against accidental ingestion of small amounts of allergens. The same amount of each food was used for provocative challenges performed both before and after the FA-SLIT trial. In order to compare our results with those reported in human patients we would need to have compared changes in the amount of protein tolerated before and after therapy, and between the placebo and treatment groups (Chin et al. 2013; De Boissieu et al. 2006; Enrique et al. 2005; Fernandez-Rivas et al. 2009; Fleisher et al. 2013; Keet et al. 2012; Kim et al. 2011). Unfortunately, in the initial design of the study this was not taken into account and once the study started the amount of protein tolerated at the baseline was not available anymore. According to the primary end-points, the success rate of this study was 0% and 40% for pruritus and clinical lesions, respectively. Although all subjects receiving FA-SLIT displayed improvements in both pruritus (up to 22%) and clinical lesions (ranging from 40 to 80%), these results are not consistent with a "cure" for AFR. However, it is possible that with a longer maintenance phase the efficacy might increase, as has been demonstrated in humans (McGowan et al. 2014). Additional studies are needed to identify the best protocol to provide significant relief of pruritus and improvement of clinical signs. As secondary end-points we defined the ability of FA-SLIT to improve the QoL and FCS. Unfortunately, there was no significant difference between groups in regard to either parameter. Nevertheless, the majority (60%) of dog owners in the treatment group reported a decrease in the QoL2 score as compared to only one owner from the placebo group. This suggests that FA-SLIT may improve the QoL of the pet owner (Linek et al. 2010; Noli et al. 2011a). Faecal consistency also improved in more dogs (40%) in the treatment group than the placebo group (0%). The lack of statistical significance for secondary outcome measures could be due to an underpowered study, because group numbers were small. Allergen-specific IgE was evaluated in this study because titres have been reported to decrease significantly (compared to placebo) in human patients after 4 months of FA-SLIT (Kim et al. 2011). Although titres generally decreased over the course of the study, there was no difference between the treatment and placebo groups nor did titres vary between foods that were or were not included in the treatment sets. This observation is consistent with the assumption that IgE-mediated reactions are not the main mechanism causing AFR in dogs (Day et al. 2005; De Weck et al. 1997; Roque et al. 2011a; Roque et al. 2011b; Teuber et

al. 2002). Some dogs expressed high levels of IgE against proteins to which they were not reactive, which is consistent with the assertion that serological testing for allergen-specific IgE is not a good diagnostic tool for AFR due to low sensitivity and specificity (Bethlehem et al. 2012; Foster et al. 2003; Halliwell et al. 2005; Mueller et al. 1998; Jeffer et al. 1991). Data from this study suggest that FA-SLIT may be well tolerated by dogs. The rate of local adverse events was very low, with only 0.14% of dispensed doses being associated with an adverse event. This contrasts with reports from human patients where up to 18% of dispensed doses cause oropharyngeal symptoms (Burks et al. 2015). However, four of five dogs in the treatment group did manifest (adverse) clinical signs when the concentration was increased, suggesting that reactions occur primarily during the escalation phase. This study protocol was adapted from a trial in children which showed a high rate of efficacy (100%) and a low rate of withdrawals (0%) (Kim et al. 2011), and the approach was to decrease the concentration of the sublingual solution until (adverse) clinical signs abated without other treatment. Based upon this, we believe that to improve compliance and efficacy the dose escalation should, at a maximum, occur at a rate of a 25–50% increase in dose every 2 weeks. The number of allergens included in treatment sets was not associated with an increased risk of adverse effects, but low case numbers limit statistical interpretation. The rare occurrence of vomiting and hypersalivation, which was similar between groups, suggests that these effects were not allergen dependent. This study has several limitations, the most significant being the small study group sizes. It is possible that uncommon, but biologically significant effects (either positive or negative) could have been missed. Post-treatment pruritus and erythema became statistically significant in the treatment group only when final observations from two withdrawn subjects were included in an ITT analysis. It is questionable whether this is the best technique for ITT analysis, as data imputation is recommended for more robust analyses (Moher et al. 2010). Therefore, the authors chose to interpret the primary outcome measures of the trial following a per-protocol analysis. The majority of the treated dogs benefited from FA-SLIT as indicated by improved pVAS and CADESI scores, and it is possible that the withdrawn subjects might have benefitted had they been permitted to continue with more dilute solutions. Another potential weakness is that the provocative food challenges did not utilize the same antigen source as the FA-SLIT treatment. However, the FA-SLIT solutions triggered allergic-type reactions

in six of seven dogs, so it seems likely that the food challenges properly identified sensitivities and that the allergenic solutions contained the relevant allergens.

4.6. Conclusions

In conclusion, this double-blind, placebo-controlled, randomized clinical trial of sublingual immunization in a small group of dogs with challenge-proven AFR has determined FA-SLIT to be generally safe and effective in decreasing pruritus and clinical signs in some subjects. These data support the feasibility of initiating a larger clinical trial which could conceivably provide more precise estimates of efficacy and adverse events. It is recommended that future studies utilize a more conservative escalation protocol and a more extended maintenance immunotherapy period.

Chapter V:

Changes in cytokines and phenotypic profile of T cells in peripheral blood of dogs with adverse food reactions subjected to food allergen-specific sublingual immunotherapy.

Adapted from: Maina E, Devriendt B, Cox E, 2017. Changes in cytokine profiles following treatment with food allergen-specific sublingual immunotherapy in dogs with adverse food reactions. Vet Dermatol; 28: 612-e149.

5.1. Abstract

Background: Food allergen-specific sublingual immunotherapy (FA-SLIT) is considered to be a novel, safe and effective approach in dogs with adverse food reactions (AFR). Here, we sought to investigate changes in key cytokines and T cell subpopulations associated with FA-SLIT.

Methods: Eleven dogs received either dose escalation of FA-SLIT or placebo over a six-month period. Oral food challenge was performed at the beginning and end of the study, along with clinical examinations and collection of skin surface, bacterial cytology and blood. Peripheral blood mononuclear cells were stimulated with the culprit food antigen. Cells and supernatants were used for flow cytometric and cytokine ELISA analyses (IL-10, IFN-γ, IL-4 and IL-17A), respectively.

Results: IL-10 and IFN-γ levels were significantly increased at the end of the study in the treatment group (T), compared with the placebo group (P), whereas no changes were found in IL-4 levels. IL-17A levels were decreased in both groups (but more profoundly in T). Bacterial scores on the skin were positively correlated with IL-17A and inversely correlated with IL-10 concentrations. The treatment significantly increased the percentage of proliferating CD4-CD8- T cells, while the percentage of CD4-CD8+ and CD4+CD8+ T cells was decreased. Both interleukins and T cells were not correlated with clinical scores.

Conclusions: FA-SLIT may modulate the allergic response toward Th1 and Treg cell phenotypes, and induction of tolerance in dogs with AFR. Therefore, FA-SLIT may be a tool to desensitize dogs with AFR. However, more data on a larger number of cases and a broader panel of cytokines are needed to corroborate these findings, and to elucidate the mechanism of action for responses to FA-SLIT by dogs with AFR.

5.2. Introduction

Currently, the only known treatment for adverse food reactions (AFR) is avoidance of offending allergens and administration of anti-inflammatory medications on accidental exposure. Given the prevalence of AFR and the significant impairment in the quality of life for both dogs and owners, the development of a safe and efficacious therapy targeting AFR is highly desirable. Sublingual immunotherapy (SLIT) is a novel approach used in the treatment of people with food allergy by inducing desensitization and eventually tolerance (Kim et al. 2011). With SLIT, small amounts of allergen extract are delivered sublingually as drops or tablets. Although the exact mechanisms of desensitization with allergen-specific immunotherapy are still not fully understood, it is known that skewing of T-cell responses from allergen-specific effector T cells toward regulatory T cells (Tregs) is an essential event in the development of healthy immune responses to allergens and is correlated with successful allergen-specific immunotherapy (ASIT) in people (Akdis et al. 1998). Interleukin (IL)-10 is thought to be a key cytokine driving the differentiation of Tregs, suppressing Th2 cells, mast cells, basophils and eosinophils, and inducing a class switch from IgE to IgG and IgA production by B cells (Akdis et al. 1998). In humans, other cytokines also have been evaluated as candidate biomarkers to monitor immunotherapy, but the results are inconsistent. IL-5 and IL-13 decrease, TGF-β transiently increases, IFN-γ increases but not consistently, IL-2 does not change, and IL-4 and IL-17 are undetectable (Fuentes-Aparicio et al. 2014; Kim et al. 2011). There are numerous studies available on immunotherapy for food allergy in humans, but studies in veterinary medicine are lacking. We have reported on the results of a study of food allergen (FA)-SLIT as a treatment for AFR in dogs (Chapter 4). It was observed that FA-SLIT had a favourable safety profile associated with decreased pruritus and clinical signs in some subjects. In the present study, we aimed to investigate if the clinical improvements were accompanied by changes in the allergic immune response. Recent studies in human medicine have shown that children with food allergy have increased levels of IL-17A expression compared to non-food allergic children (Qamar et al. 2013). IL-17A is produced by several immune cells in response to allergic inflammation and it correlates with severity of clinical lesions (Koga et al. 2008). IL-17A has also an

important role in the defense against bacterial infections, such as *Staphylococcus aureus*, supporting neutrophil recruitment and survival (Ishigame et al. 2009). The majority of allergic patients are permanently colonized with *Staphylococcus aureus*, which is positively correlated with the severity of the disease (Hofmann et al. 2016). Lack of equal knowledge in allergic dogs has driven us to assess the correlation between IL-17A with clinical lesions, pruritus and bacterial overgrowth.

5.3. Methods

Animals and sampling

Dogs were enrolled as reported previously (Maina and Cox 2016) (Chapter 4). Briefly, dogs with proven AFR and no other concurrent allergic conditions were randomized to receive either treatment (T group) with FA-SLIT (n = 7) or placebo (P group) consisting of glycerinated saline (n = 6). During the study dogs were fed a restrictive diet. Clinical signs were provoked by feeding the culprit diet before and at the end of the study. Severity of clinical signs was assessed with a pruritus Visual Analog Scale (PVAS) and the fourth iteration of the Canine Atopic Dermatitis Extent and Severity Index (CADESI-04) (Hill et al. 2007; Olivry et al. 2014). The treatment was continued daily for at least six months with fortnightly dose escalations. Peripheral blood samples were collected by cephalic or jugular venipuncture during the two provocative food exposure phases (before and after treatment). Concurrently, in order to evaluate bacterial presence and and skin infection, an adhesive tape-strip smear was obtained by direct impression of skin from the axillary and groin areas of the dogs. Samples were stained with a Romanowsky-type stain (Hemacolor, Merck; Darmstadt, Germany) and evaluated using high power microscopy fields (HPF: x1,000 magnification) for the presence of bacteria. Presence of bacteria and number of bacteria engulfed by neutrophils was evaluated empirically using a 0–4 severity scale (0, none seen; 1, ≤ 1/ HPF; 2, 1–5/HPF; 3, 5–10/HPF; 4, ≥ 10/HPF).

Lymphocyte isolation, stimultation, cytokine ELISA and flow cytometry

Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood was performed as described previously (de Bruin et al. 2007). Briefly, to increase the density of the polymorphonuclear cells and monocytes and hence to improve the

separation of these cells from the lymphocytes, blood (10 mL) was incubated with 2 mL of arabic gum/carbonyl iron solution at 37°C for one hour and gently turned every 10 min. Upon Ficoll density gradient centrifugation (600g, 30 min, RT), the interphase was collected, diluted in an equal amount of Alsever's solution (2.05% D-dextrose (Serva, Heidelberg, Germany), 1% heat inactivated fetal calf serum (hi-FCS) (Integro, Zaandam, The Netherlands), 100 µg/ml streptomycin/100 U/ml penicillin (P/S), 0.8% trisodium citrate dihydrate (Merck, Darmstadt, Germany), 0.055% water-free citric acid (UCB Pharma, Leuven, Belgium) and 0.42% NaCl (VWR prolabo chemicals, Leuven, Belgium) in ultra pure water, pH 6.1) and centrifuged at 550g for 15 min. RBC were lysed with lysis buffer (0.747% NH4Cl (VWR) and 0.206% Tris (VWR) in distilled water, pH 7.2) and the remaining cells were washed with phosphate buffered saline (PBS) and finally resuspended in 1 mL PBS. The cell concentration was determined with a hemocytometer and the cell suspension was adjusted with PBS to a concentration of $5x10^6$ cells/mL.

Lymphocyte re-stimulation, cytokine ELISA and flow cytometry staining

To detect proliferating lymphocytes, cells were incubated with CellTrace Violet (CellTrace™ Violet Cell Proliferation Kit, Life technologies, Carlsbad, CA, USA) following the manufacturer's instructions. The labelled cells were resuspended in complete medium (RPMI 1640 (Life technologies, Gibco®, Ghent, Belgium) with 10% hi-FCS, 1% P/S, 1% L-glutamine (Life technologies), 1% Non-Essential Amino Acids (Life technologies,), 1% sodium pyruvate (Life technologies), 0.5% gentamycine (Life technologies) and 0.1% β-mercaptoethanol (UCB Pharma)) at a concentration of $5x10^6$ cells/mL and 100 µL of this cell suspension was added to a flat-bottomed 96-well plate (Greiner bio-one, Frickenhausen, Germany) (de Bruin et al. 2007). Cells were then cultured in triplicate in flat-bottomed 96-well microtitre plates with allergen extracts (pork, chicken, beef, cow's milk, fish, rice, wheat and corn at concentrations of 25 µg/mL; Greer laboratories; Lenoir, NC, USA), concanavalin A (10 µg/mL; Amersham Pharmacia Biotech; Freiburg, Germany) or plain media. Cells stimulated with Con A and medium were used as positive and negative controls, respectively.

The PBMCs were then incubated at 37°C in a 5% CO2 humidified atmosphere for five days. The supernatant of the food allergen-stimulated cells was collected and the concentration of IL-10 (Treg), IFN-γ (Th1), IL-4 (Th2) and IL-17A (Th17) were

determined with canine-specific ELISA kits (DuoSet ELISA, R&D Systems; Minneapolis, WI, USA) following the manufacturer's instructions. The cells were instead stained with a mixture of three fluorochrome-conjugated primary anti-dog antibodies (AbD Serotec, Oxford, UK). This mixture contains FITC-conjugated anti-dog CD3 (mouse IgG1), R-Phycoerythrin-conjugated anti-dog CD4 (rat IgG2a) and Alexa Fluor 647-conjugated anti-dog CD8 (rat IgG1). As a control, PBMCs were also stained with fluorochrome-conjugated isotype-matched antibodies (AbD Serotec). After dead cell exclusion with the live/dead marker 7-aminoactinomycin D (7-AAD Life technologies), the CD3+ T cell subpopulations were analyzed using a BD FACSAria™ III Cell Sorter (BD Biosciences, San Jose, CA, USA) and BD FACSDivaTM software (BD Biosciences) (Figure 5.1).

Statistical analyses

For between-group comparisons, the Kruskal–Wallis test was employed, whereas for paired analysis of parameters before and after intervention within groups the Wilcoxon signed-rank test was used. Correlation between cytokine concentrations, T cells subpopulations and both clinical scores and bacterial overgrowth were analysed with a Spearman correlation test (q). Statistical analyses were conducted with SPSS Statistics 24 software (IBM; Armonk, NY, USA). A P-value < 0.05 was considered significant.

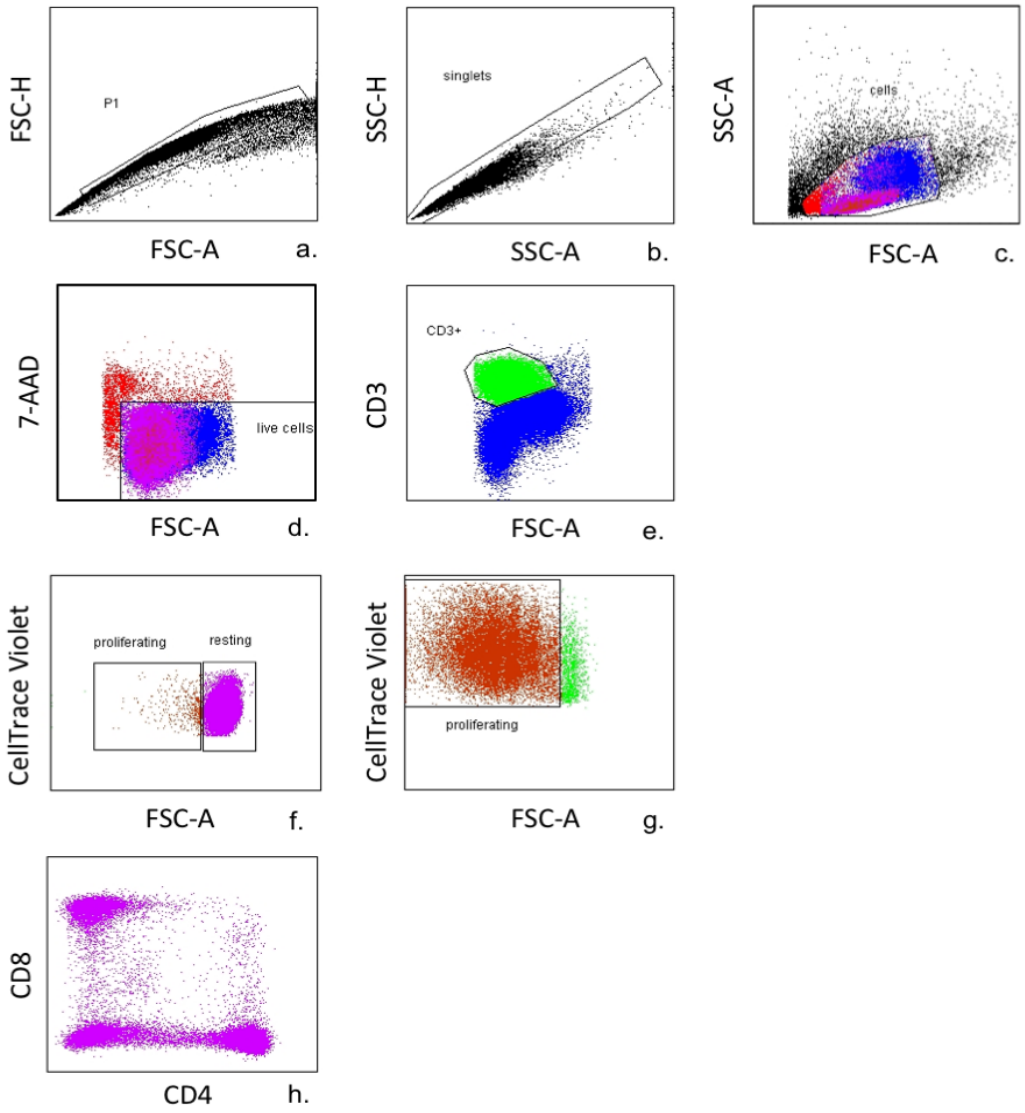


Figure 5.1. Flow cytometry gating strategy to identify proliferating T cell subsets.
a, b. Doublet cells were discriminated in forward scatter area (FSC)-A/FSC-H and side scatter area (SSC)-A/SSC-H dot plots. c. Based on their size and granularity, the lymphocytes within the singlet cell population were gated in a FSC/SSC dot plot. d. Living lymphocytes were gated based on a 7-AAD staining. e. Within the living lymphocyte gate, CD3+ T cells were selected. f. g. Based on CellTrace Violet, proliferating cells were identified. h. within proliferating and resting T cells, four subsets were identified based on CD4 and CD8 expression.

5.4. Results

FA-SLIT modulates the cytokine secretion profile of lymphocytes in AFR dogs

Changes in the cytokine secretion profile of allergen restimulated PBMCs in the placebo (P group) and treatment group (T group) are summarized in Figure 5.2, whereas the data of the individual dogs are presented in Figure 5.3. IL-10 was undetectable in samples from all dogs at the beginning of the study and increased significantly after FA-SLIT in samples of four dogs. The within-group IFN-γ concentration significantly increased by FA-SLIT (as compared to baseline), but not by placebo treatment. However, there was no statistical difference between the two groups at the end of the study. IL-4 could be detected in several samples in each group prior to treatment, but no significant changes were detected within or between groups. At the start of the study, IL-17A was detectable in all animals of the T group and four out of six animals of the P group. IL-17A concentrations decreased significantly after the experiment in both groups (placebo: $P < 0.05$; treatment: $P < 0.01$), but the effect was more pronounced in the treatment group as compared to the placebo group ($P < 0.05$). IL-17A levels were inversely correlated with IFN-γ at the beginning (q -0.61; $P < 0.05$), but not at the end of the study.

FA-SLIT affects T cell subset distribution and proliferation in AFR dogs

To assess if FA-SLIT affects the distribution and proliferation of T cell subpopulations, peripheral blood lymphocytes of dogs in the treatment group, were stimulated with food allergens in a recall assay and the resulting phenotype of the CD3+ T cell subpopulations was investigated by flow cytometry. The percentage of the (proliferating and non proliferating) CD4+CD8- and CD4+CD8+ T cells significantly decreased after the treatment ($p=0.007$ and $p=0.004$), while the percentage of CD4-CD8- T cells increased ($p=0.009$). As shown in Figure 5.4, proliferating CD4-CD8- T cells were significantly increased after the treatment (pre: 37.99%, post: 77.95%; $p=0.001$), while the percentage of proliferating CD4-CD8+ and CD4+CD8+ T cells was significantly decreased after the treatment ($p=0.002$ and $p=0.019$, respectively). There was no difference in the percentage of proliferating CD4+CD8- T cells. Percentages of the total and proliferating T cell subpopulations and their changes after therapy in the treatment group were however not correlated with changes in clinical scores (pVAS and CADESI) nor in IgE concentrations.

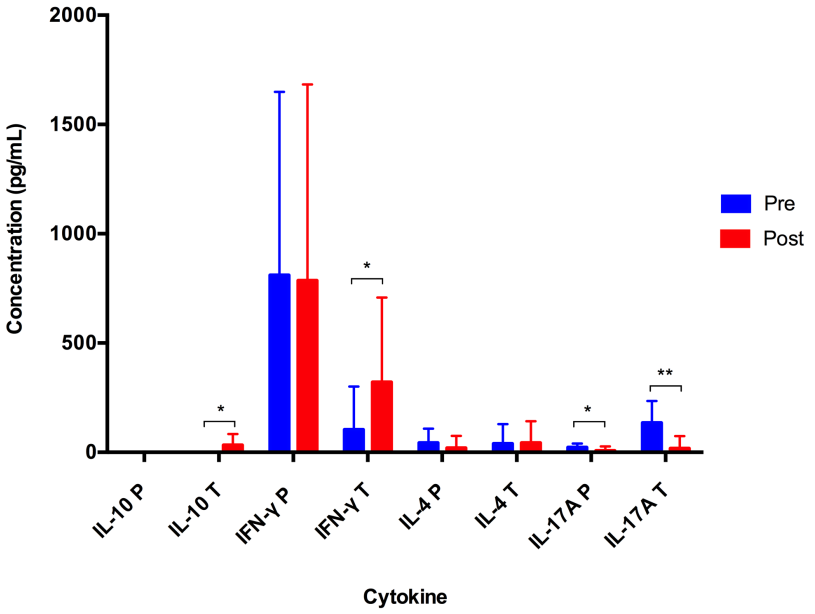


Figure 5.2: Cytokine [Interleukin IL-10, IFN-γ, IL-17A, and IL-4] concentration in the supernatant of allergen-restimulated peripheral blood mononuclear cells of the placebo (P) and treatment group (T) before (Pre) and after (Post) the study. Statistical analysis: * $P < 0.05$, and ** $P < 0.01$.

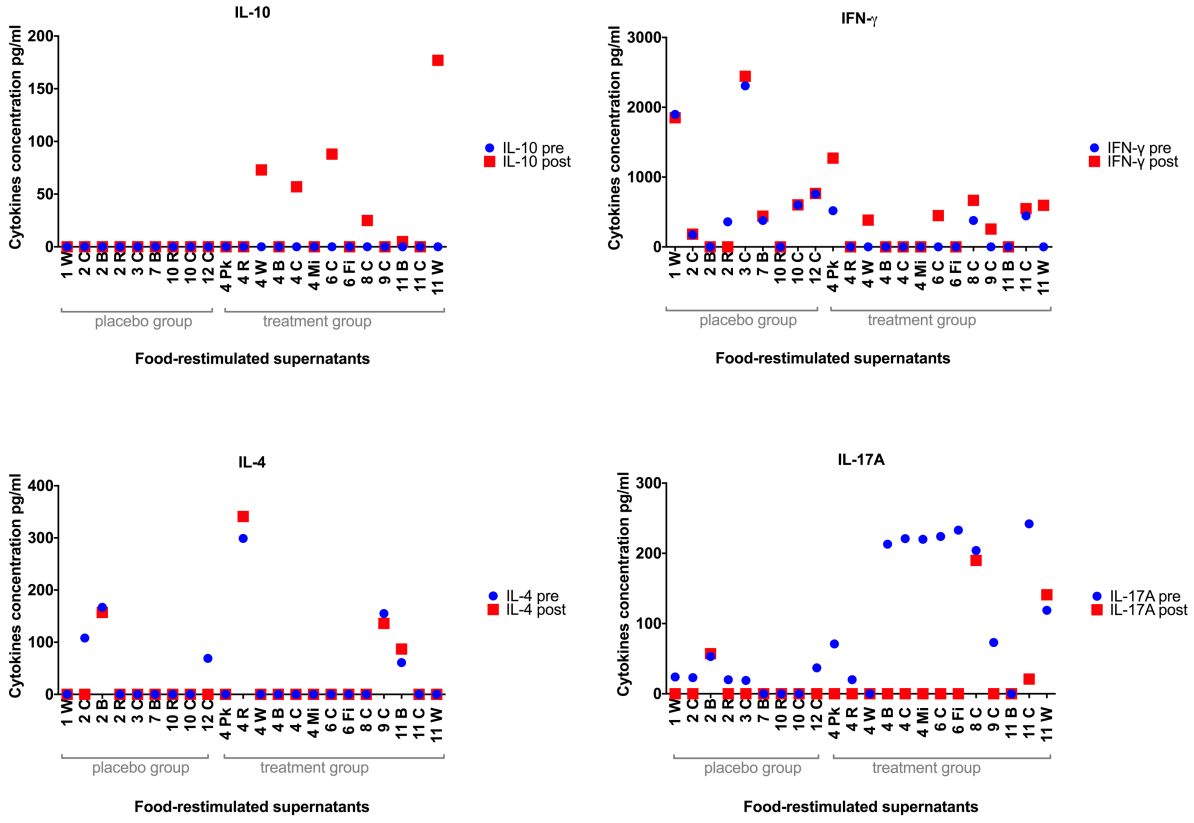


Figure 5.3. Cytokine concentrations in the supernatant of food allergen-restimulated peripheral blood mononuclear cells of individual dogs at the beginning (pre) and end (post) of treatment. Values for Case 5 are missing because the dog was withdrawn during the study (Chapter 4: Table 4.1).
B, beef; C, chicken; Fi, fish; Mi, cow's milk; Pk, pork; R, rice; W, wheat.

Cocci, often engulfed by neutrophils, were the most frequent bacteria found on cytology. A clear decrease in bacterial score was seen in four out of five treated dogs (Table 5.1). The difference between pre and post-treatment bacterial scores correlated with the difference between pre and post-treatment IL-17A concentrations (q 0.84; P = 0.001) and the bacterial scores were inversely correlated with IL-10 concentrations post-treatment (q -0.66; P < 0.05). The latter was in parallel with the improvement of PVAS and CADESI scores. This was not seen in the placebo group (Table 5.2).

Table 5.1. Bacterial scores of food allergic dogs pre- and post-treatment, receiving sublingual immunotherapy, by case.

Group	Case number	Bacterial score		
		pre	post	Difference
Placebo	1	4	3	1
Placebo	2	4	4	0
Placebo	3	2	3	-1
Placebo	7	2	2	0
Placebo	10	3	3	0
Placebo	12	3	2	1
Treatment	4	2	1	1
Treatment	6	3	0	3
Treatment	8	2	2	0
Treatment	9	3	1	2
Treatment	11	2	0	2

Results before and after therapy and their differences. The presence of bacteria and number of bacteria engulfed by neutrophils was evaluated using a 0–4 severity scale (0, none seen; 1, < 1/HPF; 2, 1–5/ HPF; 3, 5–10/HPF; 4, > 10/HPF). P, placebo; T, treatment.

Table 5.2. Individual pruritus Visual Analog Scale (PVAS) and Canine Atopic Dermatitis Extent and Severity Index, fourth iteration (CADESI-04) scores, and IL-10 and IL-17A concentrations, before (pre) and after (post) therapy and their differences (D) in dogs with adverse food reactions.

Group	Case	VAS			CADESI-04			IL-10 (pg/mL)			IL-17A (pg/mL)		
		Pre	Post	D	Pre	Post	D	Pre	Post	D	Pre	Post	D
P	1	7.2	7.1	0.1	51	46	5	0	0	-	24.4	0	24.4
P	2	8.7	7.5	1.2	57	56	1	0	0	-	31.94	19	12.94
P	3	6.1	6.2	-0.1	30	39	-9	0	0	-	19	0	19
P	7	6.2	6.5	-0.3	35	30	5	0	0	-	0	0	0
P	10	6.2	5.7	0.5	72	70	2	0	0	-	0	0	0
P	12	6.8	6.1	0.7	44	40	4	0	0	-	37	0	37
T	4	7.5	6.4	1.1	32	21	11	0	21.66	21.66	124.2	0	124.2
T	6	5.6	4	1.6	37	16	21	0	44	44	228.6	0	228.6
T	8	5.6	4	1.6	31	20	11	0	3	3	203.88	189.8	14.08
T	9	7.8	5.1	2.7	40	19	21	0	0	-	72.59	0	72.59
T	11	3.6	3.5	0.1	26	20	6	0	60.34	60.34	0	0	0

P, placebo group; T, treatment group.

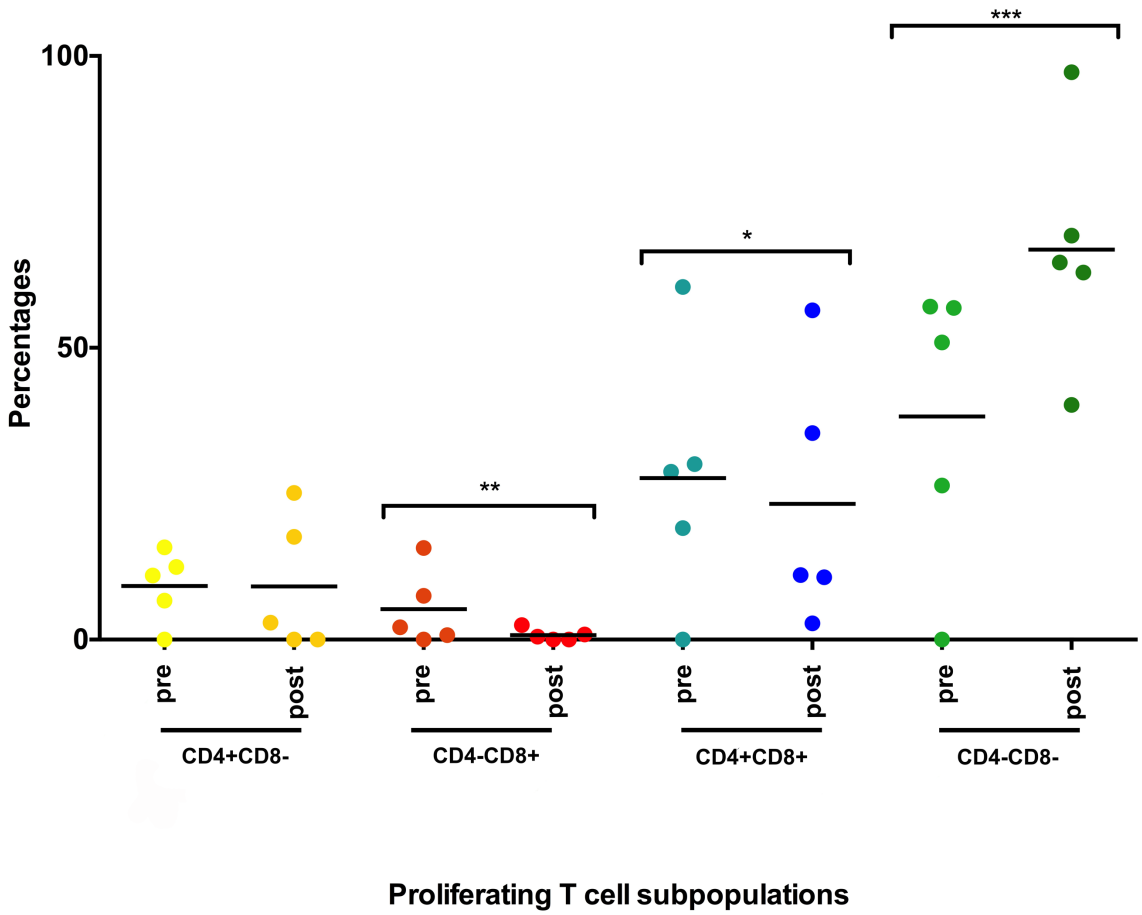


Figure 5.4: Enumeration of the percentages of proliferating CD3+ T cell subpopulations before and after the treatment in culprit allergen-restimulated PBMCs of dogs in the treatment group (cases 4, 6, 8 ,9 and 11). Since the responses were the same irrespective of the allergen, for dog allergic to more than one food component only the average of all culprit allergen is displayed (for dog 4 is displayed the average of the values for Pk,R,W,B,C, and Mi; for dog 6 the average of the values for C and Fi; for dogs 8 and 9, allergic to only one food component [C], no average was made; and for dog 11 the average of the values for C,B, and W is displayed). Abbreviations: B, beef; C, chicken; Fi, fish; Mi, cow milk; Pk, pork; R, rice; W, wheat. The horizontal lines correspond to the median value. Statistical analysis: Wilcoxon signed-rank test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

5.5. Discussion

To the best of the authors' knowledge, this is the first study to investigate the effect of immunotherapy for canine AFR and the associated cytokine expression and the phenotypic profile of T cells of allergen-stimulated PBMCs. A significant increase in IL-10 was observed for dogs undergoing FA-SLIT. Human studies have led to variable results, where IL-10 increased after FA-SLIT, but not in all patients (Kim et al. 2011). The results reported here reflect those observed in atopic dogs where ASIT induced increased IL-10 levels (Keppel et al. 2008). Our results also suggest that FA-SLIT

enhances IFN-γ concentrations without affecting IL-4 levels, which would be consistent with the promotion of Th1 differentiation and a shift to a Th1 dominant state. These findings are supported by previous human studies, although increased IFN-γ is not always a consistent feature (Akdis et al. 1998). A previous canine study reported higher IL-4 mRNA expression as compared to controls in both lesional and non-lesional skin of dogs with AFR; however, there were no differences in PBMCs (Veenhof et al. 2011). Interestingly, our findings mirror those of a previous study that showed an increase in IFN-γ levels and no changes in IL-4 in atopic dogs receiving ASIT (Shida et al. 2004).

This is also the first report to describe IL-17A production in PBMCs of dogs with AFR. IL-17A producing cells have been demonstrated in healthy dogs and in dogs with leishmaniosis or naturally occurring chronic inflammatory diseases. In humans, it has been demonstrated that IL-17A is involved in immune defence against bacterial infections and in the development of various immune-mediated diseases including psoriasis and allergic diseases (e.g. asthma, allergic rhinitis and atopy). Although we have shown that IL-17A levels were more profoundly decreased in treated dogs (versus placebo) at the end of the study, this was not correlated with the severity of clinical lesions nor with the intensity of pruritus (Table 5.3). This finding contradicts a previous report which argued that the percentage of Th17 cells was correlated with the severity of atopic eczema in people (Koga et al. 2008). However, in agreement with that study, the IFN-γ and IL-17A levels were correlated in our canine subjects.

In addition, the decrease in IL-17A concentrations after FA-SLIT treatment was significantly correlated with a decrease in bacterial counts, suggesting that microbial stimuli may activate IL-17A secretion (Eyerich et al 2009). However, IL-10 levels were inversely correlated with bacterial counts. We therefore hypothesize that the increase in IL-10 levels suppressed inflammatory responses against culprit allergens which, in turn, decreased susceptibility to bacterial colonization. To prove this order of events it would be necessary to control bacterial colonization by treatment with antimicrobials and observe if the same change in IL-10 occurs at the end of SLIT.

In our study, we also analysed the phenotypic profile of T cells and found that the percentage of CD3+CD4-CD8- T cells significantly increased after the treatment. This T cell subset has been reported to have a dual function: an inflammatory function being involved in the development of autoimmune diseases and infections or a suppressive

function preventing allograft rejection, graft-versus-host disease, and autoimmune diabetes. Their expansion after the treatment was combined with a decreased percentage of CD8+ T cells and the expression of IL-17A and with the increase of IFN-γ and IL-10 expression. Even though we are aware that additional markers would be important to better distinguish this subpopulation from other CD4-CD8- T cells, on account of these findings we speculate that this CD4-CD8- T cell subset, which increased after immunotherapy in our study, are regulatory T cells (Tregs). Double negative (DN) Tregs have first been described in 1989 by Strober et al. who showed that CD4-CD8- T cells from murine spleens were able to suppress allogeneic mixed lymphocyte reactions (Strober et al. 1989). Since then several other reports showed that DN Tregs are unique antigen-specific regulatory cells able to suppress CD8+ T cell responses and to control the immune response of other cells, such as CD4+ T cells, B cells, NK cells, and dendritic cells (DCs), employing multiple mechanisms, including the secretion of IFN-γ and IL-10 (Chen et al 2003a; Chen et al. 2005, Chen et al. 2007; Ford et al. 2002; Gao et al. 2011; He et al. 2007; Hillhouseet al. 2010; Ma et al. 2007). We previously underlined that successful immunotherapy is associated with an upregulation of regulatory T cell (Treg) activity. In our study, the CD4+ T cells were not increased by the treatment. On the contrary, we found a significant increase of DN T cells which might be responsible for the production of IFN-γ and IL-10 and for the suppressive action on CD8+ T cells. The role of this T cell subset in tolerance induction has been investigated only in one study. Raker and colleagues demonstrated that DN T cells are involved in mediating the suppressive effects of immunotherapy by inhibiting IgE production and attenuating clinical signs in a mouse model of type I allergy (Raker et al. 2015). In our study, the increased percentage of DN Treg was not followed by significant changes in IgE concentration, but it was concurrent with a significant decrease of the CD8+ T cells percentage implying that in dogs tolerance induction by DN Tregs may follow a different pathway, such as the suppression of CD8+ T cells. To the best of our knowledge this is the first study describing the change in DN T cells before and after immunotherapy and even though our results are preliminary and the DN T cell subpopultion should have been better characterized, we believe that they will inspire ongoing investigations in the coming years.

Interestingly, also the double positive (DP) CD4+CD8+ T cells were affected by the

treatment. Extrathymic DP T cells have been described in several pathological conditions as well as in healthy individuals in humans and recently also in healthy dogs (Heiner et al. 2015; Parel et al. 2004). In human, these cells may have suppressive and/or cytotoxic activities depending on the disease. For instance, in humans, they have a regulatory function in systemic sclerosis and inflammatory bowel disease, a cytotoxic activity in viral disease or even both in cancer (Bagot et al. 1998; Das et al. 2003; Desfrançois et al. 2010; Kitchen et al. 2004; Kitchen et al. 2005; Parel, et al. 2007; Sarrabayrouse et al. 2011). Although it has been established that DP T cells in dogs have an activated phenotype, their function remains unclear. In our study, we found that dogs with AFR had a higher baseline value than that reported in healthy dogs (about 2.4%) and this baseline value decreased after the treatment suggesting that DP T cells may have a role in the pathogenesis of AFR (Bismarck et al. 2012; Bismarck et al. 2014; Hoshino et al. 2008; Kato et al. 2007). This hypothesis is supported by a recent report demonstrating that the absolute number of DP T cells in lungs and mediastinal lymph nodes increased after immunization with OVA in mice with ovalbumin (OVA)-induced allergic asthma compared to healthy subjects. This suggests that the recruitment of DP T cells to sites of allergen-induced inflammation may be involved in the pathogenesis of allergic asthma (Zuśka-Prot et al. 2016).

5.6. Conclusions

FA-SLIT appears to modulate the immune system where it is associated with a functional switch from an activated profile to a regulatory one, suggesting tolerance induction. We were the first to report that IL-17A and DP T cells are increased in dogs with adverse food reactions, and that the food-specific immunotherapy can decrease these values and increase the percentage of DN T cells. However, future studies with more cases should be performed to better elucidate the role of these cells and their cytokines in the pathogenesis of AFR and to understand if IL-17A has a role in the pathogenesis or development of AFR or acts against infections which are common in allergic dogs.

Chapter VI:
CD4+CD8+ double positive T cells in healthy and allergic dogs. A pilot study.

Adapted from: Maina E, Devriendt B, Cox E, 2017. CD4+CD8+ double positive T cells in dogs with allergic diseases and in healthy dogs. A pilot study. (Manuscript in preparation)

6.1. Abstract

Background: The expression of CD4 and CD8 co-receptors on T cells is considered a thymic developmental stage of T cells. However, recent studies have shown that CD4+CD8+ double positive (DP) T cells are also present in low numbers in the peripheral blood of healthy and sick humans and dogs. In humans, DP T cells play a cytotoxic or suppressive role depending on the disease, while in dogs their function is still unknown.

Objectives: To further investigate the presence of this T cell population in a higher number of dogs with adverse food reactions (AFR), to describe differences in percentages of DP T cells among dogs with AFR, dogs with non-food-induced atopic dermatitis (NFICAD), and healthy (HTY) dogs and lastly, to understand if DP T cells may be used to develop a screening test to distinguish CAFR from NFICAD.

Methods: Peripheral blood samples were collected from dogs with AFR, NFICAD or healthy dogs and peripheral blood mononuclear cells (PBMC) were then isolated for flow cytometric analysis of T cell subpopulations. Total and proliferating percentages of non restimulated (medium) and restimulated (culprit allergens based on dietary trial) lymphocytes subsets were compared among groups.

Results: DP T cells were present in a higher percentage in dogs with AFR than in healthy dogs. Both the CAFR and NFICAD group showed statistically significant higher percentages of total and proliferative DP T cells than healthy dogs. Interestingly, dogs with AFR had more proliferating DP T cells than dogs with NFICAD in a recall assay. DP T cells proliferated when stimulated with the right culprit food allergens in 43% of the dogs. Unfortunately, 18% of non-culprit allergens also induced proliferation. DP T cells proliferated when stimulated with food allergens only in 25% of the dogs with NFICAD.

Conclusions: DP T cells are present in higher percentages in peripheral blood of dogs suffering from allergic conditions than in healthy individuals. DP T cells, when

stimulated with culprit food allergens, proliferate more in dogs with AFR than in those with NFICAD. However, overlapping proliferation between groups and the small sample size lead us to discourage the use of this test as a screening test to differentiate between healthy from allergic dogs.

6.2. Introduction

During the development in the thymus, thymocytes transiently express the co-receptor CD4 and CD8 simultaneously on their surface. However, upon maturation peripheral T cells stop the expression of one of the two co-receptors and become CD4+ or CD8+ single positive. More than 20 years ago, CD4+CD8+ double positive (DP) T cells were identified in peripheral blood. Like their thymic progenitors they express CD4 and CD8 simultaneously, but in contrast they have a longer lifespan and can express memory markers (Bagot et al. 1998; Nascimbeni et al. 2004). Even though the majority of studies suggest that peripheral DP T cell originates from mature CD4+ T cells, some evidence suggest these cells might derive from mature CD8+ T cells. Furthermore, studies aimed at elucidating their function presented contradicting results (Blue et al. 1986; Flamand et al. 1998; Kitchen et al. 1998; Luhtalaet al. 1997; Macchia et al. 2006; Molteni et al. 2002; Parel et al. 2007; Sullivan et al. 2001;). DP T cells are present in low numbers in healthy subjects (Blue et al. 1985; Kay et al. 1990; Colombatti et al. 1998; Patel et al. 1989). Interestingly, their frequency increases in various diseases, such as viral infections, where they seem to have a cytotoxic potential, autoimmune diseases, where they display a suppressive potential or in neoplastic diseases in which they show both cytotoxic and suppressive cytokine profiles (Bagot et al. 1998; Das et al. 2003; Desfrançois et al. 2010; Kitchen et al. 2004; Kitchen et al. 2005; Sarrabayrouse et al. 2011; Szczepanik et al. 2005).

In addition to humans, DP T cells have been recognized in many other species such as rat, mouse, monkey, swine, chicken and dogs (Alexandre-Pires et al. 2010; Otani et al. 2008; Zuckermann 1999). In contrast to the considerable amount of literature published on human DP T cells, very little is found in dogs. Canine DP T cells are present in healthy dogs in a small percentage (2.4%), but can increase to 20% when stimulated. The proportion of DP T cell increases in visceral leishmaniosis and after viral and bacterial stimulation (Schütze et al. 2009; Bismarck et al. 2012). We recently demonstrated that DP T cells are present in dogs with adverse food reactions and also that dogs with AFR had a higher baseline percentage of DP T cells (non restimulated) (4.11%; range: 0-19%) than that reported in healthy dogs (2.4%; range 1.4-4.2%) (Chapter 5). These findings are in line with previous results in human medicine where

atopic patients showed a higher frequency of DP T cells than healthy individuals (Bang et al. 2001). However, if they are beneficial or detrimental in hypersensitivities is still unknown. In the literature, there are only two articles describing DP T cells in allergic disease: one in human describing an increased percentage of activated CD4+CD8+ T cells in both skin and peripheral blood of atopic patients compared to healthy individuals and one in mice describing that the absolute number of DP T cells in lungs and mediastinal lymph nodes increased after immunization of mice with ovalbumin (OVA) upon OVA-induced allergic asthma compared to healthy subjects. This suggests that the recruitment of DP T cells to sites of allergen-induced inflammation may be involved in the pathogenesis of allergic conditions (Bang et al. 2001; Zuśka-Prot et al. 2016). Based on our findings in the previous chapter, where we demonstrated that dogs with AFR have a higher baseline percentage of DP T cells than healthy dogs which decreased after food allergen specific immunotherapy, we speculate that DP T cells may have a role in the pathogenesis of AFR in dogs. Therefore, this study aimed to further investigate the presence of this T cell population in a higher number of CAFR cases, to describe differences in percentages of DP T cells among dogs with AFR, dogs with non-food-induced atopic dermatitis (NFICAD), and healthy (HTY) dogs and lastly, to assess if DP T cells may be used to develop a screening test to distinguish CAFR from NFICAD.

6.3. Methods

Groups selection

The study was approved by the Ethical Committee of the University of Ghent, Belgium (EC 2013/189 (healthy) and EC 2013/198 (allergic dogs) and by the Deontological Committee of the Belgian government (232663/13_11_2/14). Three groups of dogs were included, namely healthy dogs (HTY), dogs with adverse food reactions (AFR), and dogs with non-food-induced atopic dermatitis (NFICAD). Eight clinically healthy laboratory raised beagle dogs were included in this study. They were sheltered in a research facility at the Faculty of Veterinary Medicine, Ghent University. Clinical histories were evaluated and prior to the inclusion the dogs underwent an accurate clinical examination to rule out the presence of allergy or other diseases. Dogs showing a history and clinical signs compatible with non-seasonal atopic dermatitis, such as pruritus and/or clinical signs of inflammatory skin diseases, were recruited

from several clinics in Belgium and Italy and underwent a diagnostic investigation that included assessment of ectoparasitic infestation (skin scrapings, trichography, coat brushing and trial insecticidal or acaricidal therapy where indicated) and microbial skin infection (microscopy of cytological specimens, bacterial and fungal cultures, and trial antimicrobial therapy where indicated). Dogs with an allergic phenotype and a negative response to anti-parasitic/bacterial/fungal treatments were fed an elimination diet. A home-cooked diet or commercial diets with a single meat and single carbohydrate source never fed before was attempted based on the dietary history. If new proteins were not available for the dog, a commercial hypoallergenic diet was selected. The restriction diet was fed for at least 8 weeks without any other food or flavoured supplements. If the dogs improved on the restriction diet, they were challenged by feeding separate food components such as pork, chicken, beef, cow-milk, fish, lamb, rice, corn and wheat, particularly those protein or carbohydrate sources from the former diet. This provocation test lasted 7-14 days (or less, if signs relapsed) for each food component to assess any relapse of clinical signs. Allergic dogs that failed to respond to the dietary trial, but whose clinical signs were consistent with atopic dermatitis and that fulfilled the diagnostic criteria for NFICAD were allocated to the group NFICAD (DeBoer et al. 2001; Favrot et al. 2010; Olivry 2010). Those dogs, that improved when given the restriction diet, subsequently showed cutaneous and/or gastrointestinal clinical signs during the challenge period, and then improved again with reintroduction of the restriction diet were allocated to group CAFR. At the time of inclusion, dogs had received no glucocorticoids, ciclosporin, oclacitinib or antihistamine within the previous month.

Peripheral blood samples (10 mL each) were collected from dogs from the cephalic vein into heparinized tube (dogs with clinical signs and/or pruritus were sampled during the acute phase). Blood was diluted (1:1) in phosphate-buffered saline (PBS). Peripheral blood mononuclear cell (PBMC) were isolated from whole blood, labelled with CellTrace Violet (CellTrace™ Violet Cell Proliferation Kit, Life technologies, Carlsbad, CA, USA) follwowing the manufacturer's instructions, and then transferred to a 96-well flat-bottomed cell culture microplate (Greiner bio-one, Frickenhausen, Germany) and stimulated with food allergen extracts or concanavalin A as described in **Chapter 5**. Subsequently, PBMC were incubated at 37°C in a 5% CO_2 humidified atmosphere for 5 days. After the incubation, the supernatant was removed, and the cells collected.

Flow cytometry

Mononuclear cells (10^6 cells) were stained with a mixture of three fluorochrome-conjugated primary anti-dog antibodies (AbD Serotec®, Oxford, UK). This mixture contains FITC-conjugated anti-dog CD3 (mouse IgG1), R-Phycoerythrin-conjugated anti-dog CD4 (rat IgG2a) and Alexa Fluor® 647-conjugated anti-dog CD8 (rat IgG1). Fluorochrome-conjugated isotype-matched antibodies (AbD Serotec®, Oxford, United Kingdom) were used to control non-specific binding. Stainings were performed at 4°C in the dark for 30 min. After extensive washing, 7-aminoactinomycin D (Sigma-Aldrich) was added and the CD3+ T cell subpopulations were analysed using a BD FACSAria™ III Cell Sorter (BD Biosciences, San Jose, CA, USA) and BD FACSDiva™ software (BD Biosciences). Doublet cells were excluded and viable 7-aminoactinomycin D-stained cells were analysed for CD3, CD4 and CD8 expression (for the gating strategy the reader is referred to **Chapter 5**).

Statistics

The non-parametric unpaired Kruskal–Wallis and Mann–Whitney U tests were used to compare groups for the flow cytometric enumeration of different T cell subpopulations (to compare three and two groups respectively). The final percentages of CD4+CD8+ lymphocytes were calculated after the percentage of background proliferation (non-restimulated cells) was subtracted. Proportions were considered statistically significant with a P-value of <0.05.

6.4. Results

Forty-four dogs (28 CAFR, 8 NFICAD, 8 HTY) of various breeds were included in the study and are summarized in Table 6.1 (no statistical difference in age between groups). Baseline percentages of non-restimulated DP T cells (medium) were 3.87% (0-22.03%), 1.28% (0.01-3.9%), and 7.18% (1.74-18.15%), and in the CAFR, NFICAD, and healthy group, respectively. The median percentage of total (proliferating and non proliferating) CD4+CD8+ cells (stimulated with food) in the CAFR, NFICAD, and healthy groups were 0.28 (0-24.93), 0.15 (0-1.70) and 0.95% (0-1.83%), respectively.

Table 6.1. Descriptive data

Group	Breed	Sex	Age (years)	Weigh
CAFR	Boston terrier Cavalier King Charles spaniel Cross-bred dog (x8) Dachshund Dobermann English setter French bulldog (x4) German Shepherd (x2) Golden retriever Italian Shepherd Jack russel terrier Poodle (x2) Sharpei Shih tzu West Highland white terrier York Shire terrier	9 Females 6 Females spayed 12 Males 1 Male neutered	mean 3.40 (0.5 - 13)	mean 15.31 (4 - 42)
NFICAD	Bull terrier Dalmatian English bulldog French bulldog German Shepherd Labrador retriever Labrador retriever3 Spitz	2 Females 3 Females spayed 2 Males 1 Male neutered	mean 3.50 (1 - 8)	mean 21.13 (6.5 - 35)
HTY	Beagles	4 Females 1 Females spayed 1 Males 2 Male neutered	mean 6.25 (2 - 10)	mean 10.54 (8.2 – 12.3)

Abbreviations: CAFR, canine adverse food reactions; HTY, healthy group; and NFICAD, nonfood-induced canine atopic dermatitis.

As previously stated, these data were corrected by subtracting the medium background values. Flow cytometric enumeration of total (proliferating and non) CD4+CD8+ cells was statistically different between the three groups ($p< 0.001$). There were statistically significant differences between the CAFR and the healthy groups ($p < 0.001$) and between the NFICAD and the healthy group ($p= 0.001$), but not between the CAFR and the NFICAD group ($p= 0.097$). Flow cytometric enumeration of proliferating CD4+CD8+ cells was statistically different between all three groups ($p< 0.001$) (Figure 6.1): CD4+CD8+ T cells proliferated more in the CAFR group than in the healthy groups ($p< 0.001$), more in the NFICAD group than in the healthy group ($p= 0.003$), and more in CAFR group than in the NFICAD group ($p= 0.042$). The mean

percentage of proliferating DP T cells restimulated with food allergens in the healthy group was used as a cutoff value to evaluate if proliferating DP T cells of allergic dogs (CAFR and NFICAD group) correlated well with those from oral food challenge (OFC). Twenty-eight dogs in the CAFR group were allergic to a total of 42 different food constituents. The percentage of proliferating DP T cells exceeded the cut-off in 12 of 42 (28.57%) allergens in 10 on 28 (37.71%) dogs. The percentage of proliferating DP T cells exceeded the cut-off value 10 times for the wrong allergens in 5/28 (17.86%) dogs. Only in 2/8 (25%) dogs in the NFICAD group the cut-off was exceeded and these dogs were not allergic to food.

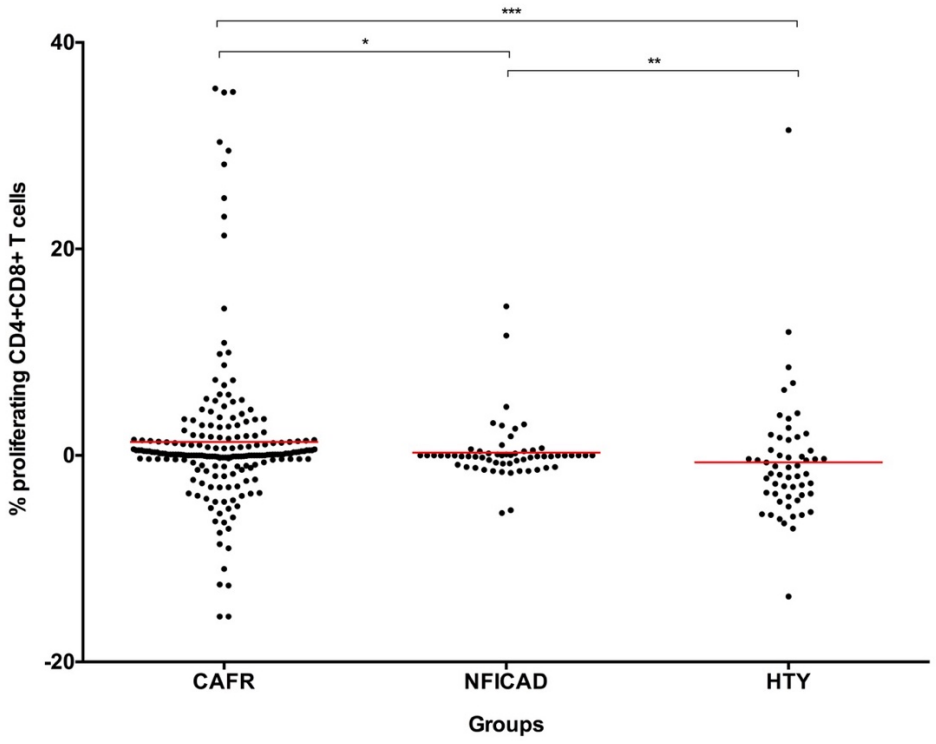


Figure 6.1. The percentage of proliferating CD4+CD8+ T cells in peripheral blood in the three groups. Abbreviations: CAFR, canine adverse food reactions; HTY, healthy; and NFICAD, nonfood-induced canine atopic dermatitis; the horizontal lines correspond to the median value. Statistical analysis (Kruskal-Wallis and Mann-Whitney, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Table 6.2. List of dogs with AFR (CAFR) and non-food induced atopic dermatitis (NFIAD), culprit allergens (bold) and all food that have been tested. Percentages of proliferating double positive T cells for each dog and each food allergens tested. Positive results are reported in red colour.

CAFR											
Dog	**Ag**	**%**	**Dog**	**Ag**	**%**	**Dog**	**Ag**	**%**	**Dog**	**Ag**	**%**
1	rice	0	9	beef	0	15	fish	0	22	**chicken**	1.46
1	chicken	0	9	milk	0.4	15	corn	0	22	**beef**	0.19
1	pork	0	9	fish	1	16	pork	1.91	22	milk	4.44
1	**wheat**	0	9	corn	0.7	16	rice	1.05	23	pork	0.1
2	**pork**	0	10	pork	0.1	16	wheat	1.39	23	rice	0
2	**rice**	0	10	rice	0	16	chicken	1.62	23	wheat	0
2	**wheat**	0	10	wheat	0	16	beef	1.42	23	chicken	0
2	**chicken**	0	10	chicken	0	16	milk	1.33	23	beef	0
2	**beef**	0	10	beef	0	16	**fish**	3.48	23	milk	0
2	**milk**	0	10	milk	0	16	**corn**	2.92	23	**fish**	0.3
2	fish	1.01	10	**fish**	0.3	17	pork	0.4	23	corn	0.5
3	pork	0	10	corn	0.5	17	**rice**	1.1	23	lamb	0.1
3	rice	0.08	10	lamb	0.1	17	wheat	0	24	pork	1.2
3	wheat	0	11	pork	0	17	chicken	0	24	rice	0.1
3	**chicken**	0	11	rice	0	17	beef	0	24	wheat	0.2
3	beef	0	11	wheat	0	17	milk	0	24	**chicken**	0
3	milk	0.07	11	chicken	0	17	fish	0	24	beef	0.1
3	fish	0	11	beef	0	17	corn	0	24	milk	0.1
4	pork	21.3	11	milk	0.1	18	**pork**	5.3	24	fish	0.2
4	rice	35.16	11	fish	0	18	rice	0	24	corn	1
4	wheat	29.52	11	corn	0	18	wheat	0.5	25	pork	3.41
4	chicken	28.2	11	**lamb**	0.1	18	chicken	0.6	25	rice	1.97
4	**beef**	35.21	12	**pork**	9.83	18	beef	0	25	wheat	3.52
4	milk	35.54	12	rice	0.41	18	milk	0.3	25	chicken	0
4	fish	30.36	12	wheat	0.4	18	fish	0	25	**beef**	5.48
5	porc	0	12	chicken	3.53	18	corn	0	25	milk	0.08
5	rice	0	12	beef	6.8	19	pork	0	25	fish	0
5	wheat	0	12	milk	4.76	19	rice	0	25	corn	1.53
5	**chicken**	1.73	12	fish	0	19	wheat	0	26	porc	0.86
5	beef	0	12	corn	4.25	19	chicken	0	26	rice	0
5	milk	0	13	pork	3.65	19	**beef**	0	26	wheat	0
5	**fish**	5.37	13	rice	0	19	milk	0	26	**chicken**	5.88
6	porc	3.27	13	wheat	1.91	19	fish	0	26	beef	0
6	rice	0	13	**chicken**	5.2	19	corn	0	26	milk	1.43

6	wheat	1.78	13	beef	2.91	20	pork	1.2	26	fish	24.92
6	**chicken**	4.02	13	milk	2.25	20	rice	0	27	porc	2.42
6	beef	2.8	13	fish	1.22	20	wheat	0	27	**rice**	0.68
6	milk	1.8	13	corn	1.31	20	chicken	0	27	wheat	0
7	pork	0.8	14	**pork**	5.92	20	beef	0	27	**chicken**	8.74
7	rice	0.2	14	rice	2.93	20	**milk**	10.9	27	beef	0.59
7	wheat	0.3	14	wheat	1.27	20	fish	0	27	milk	3.69
7	**chicken**	0.4	14	chicken	0.32	20	corn	0	27	fish	23.13
7	beef	0.7	14	beef	2.73	21	pork	0	28	pork	0
7	milk	0.5	14	Milk	1.88	21	rice	0.17	28	rice	0
7	fish	0.5	14	**Fish**	7.28	21	**chicken**	0	28	**wheat**	0
7	corn	1.5	14	corn	7.32	21	beef	0	28	**chicken**	0
8	**rabbit**	9.97	15	**pork**	4.45	21	fish	0	28	**beef**	0
8	**chicken**	14.22	15	rice	0	22	porc	0.33	28	milk	0
9	pork	0.2	15	wheat	0	22	**rice**	0.05	28	fish	0
9	rice	0.5	15	chicken	0	22	wheat	0			
9	wheat	0.8	15	beef	0						
9	**chicken**	0.1	15	milk	0						

NFICAD											
Dog	**Ag**	**%**	**Dog**	**Ag**	**%**	**Dog**	**Ag**	**%**	**Dog**	**Ag**	**%**
1	pork	0	3	pork	0	5	pork	0	7	pork	0
1	rice	0.7	3	rice	14.43	5	rice	0	7	rice	0.2
1	wheat	1	3	wheat	0	5	wheat	0	7	wheat	0
1	beef	0	3	chicken	0	5	chicken	0	7	chicken	0.1
1	milk	0	3	beef	0	5	beef	0	7	beef	0
1	fish	0	3	Milk	0	5	Milk	0	7	Milk	0
2	pork	0	3	Fish	0	5	Fish	0	7	Fish	0
2	rice	0	4	pork	0.04	5	corn	0	7	corn	0
2	wheat	0	4	rice	0	6	pork	0	8	pork	0
2	chicken	0	4	wheat	0	6	rice	0.4	8	rice	4.7
2	beef	0	4	chicken	0	6	wheat	0.2	8	wheat	0.5
2	milk	0	4	beef	3.13	6	chicken	0	8	chicken	2.6
2	fish	0	4	Milk	1.84	6	beef	0	8	beef	2.9
			4	Fish	11.6	6	Milk	0	8	Milk	3
						6	Fish	0.1	8	Fish	0.6
						6	corn	0	8	corn	0.4
						6	lamb	0			

6.5. Discussion

In this study, we reconfirmed that non re-stimulated DP T cells are present in a higher percentage in dogs with AFR than in healthy dogs. Contrary to our expectations, the median percentage of DP T cells in healthy dogs included in our study was higher than that detected in CAFR group. This rather contradictory result may be due to the sample collection. Indeed, healthy dogs were recruited directly from the University, therefore, the isolation time was shorter and as a consequence the cells were better preserved and probably more proliferating. For this reason, statistical analyses were performed after the percentage of background proliferation was subtracted.

The present study was also designed to describe differences in percentages of DP T cells among dogs with AFR, NFICAD, and HTY dogs. The percentage of total and proliferating re-stimulated CD4+ CD8+ T cells was higher in CAFR and in NFICAD groups than in the healthy group. The present findings seem to be consistent with other studies which found a significant increase of DP T cells in asthmatic mice and in atopic human patients (Bang et al. 2001; Zuśka-Prot et al. 2016). Taking the above into consideration and the fact that DP T cells decreased after food-allergen specific immunotherapy (**Chapter 5**), it could be hypothesised that these cells may be involved in the development of allergic diseases. However, the elucidation of their role in the pathogenesis of these diseases requires further investigations.

Interestingly, when considering only proliferating DP T cells we found that DP T cells when stimulated with food allergens proliferated more in the CAFR group than in NFICAD group. Unfortunately, due to the overlap between proliferating DP T cells in these groups, there is no clearly definable cut-off, thus this test cannot be used as a screening tool to distinguish CAFR from NFICAD. Another downside is that due to the high amount of false positive results in the CAFR group we cannot even use this as a test to detect culprit food allergens. In addition, with the small sample size, additional caution must be applied, as the findings might not be transferable to all patients. Surprisingly, looking at the data (Figure 6.1), DP T cells seem to proliferate more in some dogs, regardless of which food is used to stimulate them. Sample freshness may influence their proliferation. For this purpose, it would be interesting to repeat the study with the same time interval between sampling and in vitro analysis. Another explanation may be due to the individual tendency to produce DP T cells which may

reflect a more activated or chronic status and/or another immune mechanism involved in the pathogenesis of CAFR. Finally, it would be interesting to perform a Lymphocyte Stimulation Test in both CAFR and NFICAD with both food allergens and aeroallergens to see if the NFICAD group, when stimulated with aeroallergens, proliferate more than the CAFR group.

6.6. Conclusions

To conclude, in this study we observed that DP T cells are present in higher percentage in peripheral blood of dogs suffering from allergic conditions than in healthy subjects and demonstrated that when stimulated with culprit food allergens, DP T cells proliferate more in dogs with AFR than in those with NFICAD.
However, more research on this topic needs to be undertaken to better characterize DP T cells phenotype and function and their role in canine allergic conditions. In case of different DP T cells cytokines profiles, data could be used for designing a novel diagnostic assay to discern food allergy from non-seasonal atopic dermatitis.

Chapter VII:
Exploring the association between vegetable oil supplementation and adverse food reactions.

Adapted from: Maina E, Cox E, 2017. N-6 polyunsaturated fatty acid (PUFA)-rich oil, home-made diet and treats intake may enhance the susceptibility to develop adverse food reactions in dogs. (Article submitted to Veterinary Dermatology).

7.1. Abstract

Background: The prevalence of adverse food reactions (AFR) in dogs can be as high as 24% of all skin diseases (Olivry and Mueller 2016). It is therefore important to explore novel causal pathways. Results of studies in human and mice have shown that a high intake of n-6 PUFA-rich oils in the diet enhances the risk to develop allergic diseases and the severity of allergic reactions (Bolte et al. 2001; Sausenthsaler et al. 2006; Van den Elsen et al. 2015). The aim of this study was to investigate the association of AFR with the supplement of vegetable oils rich in n-6 PUFA in dogs.

Methods: Data on dietary intake of 459 privately owned dogs with skin disease were obtained from a food survey. Data on 420 dogs were eligible for statistical analysis.

Results: The use of oil was recorded in 54 of 420 (12.85%) dogs included in the study. In 9 of 33 (27.27%) dogs with AFR, in 7 of 22 (31.81%) dogs with concurrent AFR and atopic dermatitis (CAD), in 14 of 94 (14.89%) with CAD, and in 45 of 385 (11.60%) dogs with other conditions. The frequency of vegetable oil supplementation in dogs with ARF or concurrent AFR and CAD was significantly higher than that in dogs with other skin diagnoses ($P<0.05$; $P<0.001$). No other disease was significantly associated with vegetable oil supplementation.

Conclusions: Vegetable oil was supplemented more frequently in dogs with AFR than with other dermatological diseases. This is the first study to evaluate the supplementation of vegetable oil in dogs with skin disease.

7.2. Introduction

Adverse food reaction (AFR) is defined as any clinically abnormal response attributed to the ingestion of a food or food additive. Adverse reactions to foods can be broadly divided into those with an immune basis – food allergies – or those without an immune basis – food intolerances. AFR is a common condition in dogs that affects up to 24% of dogs with skin diseases (Olivry and Mueller 2016). Numerous studies have reported that AFRs are also increasingly common in humans and are considered as a major concern for food safety (Nwaru et al. 2014). The prevalence of true AFR is difficult to measure because many studies are relying on self-reporting questionnaires, based on perceptions of food allergy and they do not include an oral food challenge (OFC), the diagnostic gold standard. Clear is that food allergy is more common in children (3.9-8%) than adults (3.7%) (Branum et al. 2009; Gupta et al. 2011; McGowan et al. 2013; Skypala et al. 2011; Venter et al 2016). This is lower than the prevalence of intolerance to food and food additives in human, which is estimated to be between 5-20% (Hodge et al. 2009; Lessof et al. 1983; Zopf et al. 2009).

Giving the life-threatening problem in humans, considerable efforts are being made to understand why the incidence of AFR is increasing (Kool et al. 2016; Mullins et al. 2015; Rudders et al. 2014; Turner et al. 2015). Epidemiological studies suggest that the increasing rate of AFR is related to changes in lifestyle habits, such as increased hygiene and prosperity (the hygiene hypothesis states that exposure to microbes and parasites may be preventive), the timing and route of exposure to foods (increased risk for prolonged allergen avoidance, because oral tolerance would be bypassed with possible environmental sensitization) (Kotz et al. 2011; Lack et al 2012; Untersmayr et al. 2008). Other potential rectifiable risk factors include co-morbid atopic dermatitis which is also increasing, vitamin D insufficiency, reduced consumption of antioxidants, increased use of anti-acids (reducing digestion of allergens), obesity (being an inflammatory state), and changes in the intake of dietary fats (Visness et al. 2009). In particular, speculations on the contribution of changes in dietary habits during the past decades in Western countries have been made (Black et al. 1997; Devereux 2006). Indeed, in these countries an increased consumption of vegetable oils and margarines, rich in omega-6 polyunsaturated fatty acids (PUFA) has coincided with the decreased consumption of oily fish and fish products rich in omega 3 PUFA.

Several studies have evaluated the association between changes in the consumption of n-3 and n-6 PUFA and the risk to develop an allergic disease. Long chain n-3 PUFAs are largely found in oily fish which also contains vitamin D. Both n-3 PUFAs and vitamin D have been shown to decrease the risk of allergic diseases through their immunomodulatory properties and favorably influence the immune system to be refractory to allergy and to improve symptoms associated with asthma (Devereux et al. 2005; Farjadian et al. 2016; Furuhjelm et al. 2011; Horrobin et al. 1987; Jenmalm et al. 2013; Makino et al. 2005; Miles et al. 2015; Muehleisen et al. 2013; Nagakura et al. 2000 ; Okamoto et al. 2000; Palmer et al. 2013; Van den Elsen et al. 2013 ; Wendell et al. 2014). On the contrary, n-6 PUFA, such as linoleic acid (LA) and arachidonic acid (AA), are considered to be pro-inflammatory, because their metabolism leads to the formation of several inflammatory eicosanoids, such as PGE2, which promotes the production of IgE (Calder et al. 2000). Evidence suggests that the use of excessive amount of proinflammatory n-6 PUFAs and the increased n-6/n-3 ratio increases the risk to develop allergic diseases (Black et al. 1997; Jenmalm et al. 2013; Kankaanpaa et al. 1999; Simopoulos 2011; Wendel et al. 2014).

Since the prevalence of AFR in dogs varies widely from one study to another, it is possible that besides large variations in study methodology, environmental factors may also be important. So far, it is unknown whether n-6 PUFA-rich dietary vegetable oils may influence the risk of developing AFR in dogs. We observed that the prevalence of AFR is higher in Italy than in other countries and therefore we hypothesized that in Italy, which is a country with a well-known passion for the culinary arts and a predilection for oil as a seasoning, it is possible that owners easily and frequently enrich their dog's diet with oil (Maina et al. 2014; Proverbio et al. 2010). The aim of this study was to investigate the association of AFR with the supplementation of vegetable oils rich in n-6 PUFA in dogs.

7.1. Methods

Study population

The study population consisted of referral dog owners presenting their pets for a dermatological examination to ten different clinics in Northern Italy between 2014 and 2016.

The first author, working as a specialist in veterinary dermatology in these clinics, collected the information from dietary intake interviews and performed a dermatological visit at the initial consultation and re-examinations, when needed, during the following months. Additional information was collected by telephone when some data were missing. For the purposes of the specific dermatological analysis, all client-owned dogs with a definitive diagnosis of a dermatological disease were included sequentially and no active selection was undertaken based on historical features or clinical signs. Definitive diagnoses were obtained by means of a complete dermatological examination and recheck visits using standard procedures. Dogs with unknown or unclear diagnosis were excluded.

Survey

Dietary intake was assessed by using a survey comprising 11 questions. The format of the questions ranged from multiple choice, fill in the blank or choose from a list (Table 7.1).

The questions covered a wide range of information pertaining to the dog's diet intake. In particular the dog's owners were asked:

1. To describe what type of diet the dog was receiving daily at the time of the interview. This current diet was categorized into commercial (veterinary prescription diet or maintenance diet), home-made (cooked or raw animal protein and/or other ingredients, prepared at home), and combinations of these categories.
2. To meticulously list ingredients and recipes of the rations.
3. To indicate whether any diet change occurred before the dermatological visit. If owners responded yes, they were similarly questioned regarding the dog's previous diet category and whether the diet changed from commercial diet to home-made diet types and *vice versa*.
4. About the inclusion of vegetable oils into the diet and if so, the type, quantity and frequency of their administration.
5. Correlation between beginning of this inclusion and the onset of clinical signs.
6. About any dietary supplements containing essential fatty acids (EFA) supplemented daily to the dog's diet.

A specific numerical score (between 0 and 5) was given to the different frequencies for oil integration: 0 = never, 1 = a few times/year, 2 = a few times/month, 3 = a few times/week, 4 = daily, and 5 = multiple times/day. Information about the dog's

signalment, history and previous treatments and additional supplementation was obtained during the interview.

Table 7.1. Questionnaire about dietary intake posted to owners to complete before their referral consultation.

	Questions	**Scoring systems**
Dietary type and changes	What type of diet is fed daily to the dog?	Commercial pet food, Home-made diet, Commercial and home-made diet
	Is the commercial diet a veterinary prescription diet or a maintenance diet?	Veterinary prescription diet, maintenance diet
	Please, list all the ingredients (proteins and carbohydrates) in dog's diet.	(list)
	Has the diet of your dog been changed before or after clinical signs onset?	Before, After
Oil supplementation	Do you add any oil to your dog's diet?	No, yes
	Which type of oil do you add to your dog's diet?	Olive, olive- extravirgin, corn, mix seeds, sunflower
	How many tea-spoon of oil do you add per ration?	1,2,>2
	How often do you integrate oil to your dog's diet?	1 = a few times/year, 2 = a few times/month, 3 = a few times/week, 4 = daily, 5 = multiple times/day
	Did you start the oil integration before or after the onset of clinical signs?	Before, After
EFA supplementation	Do you feed any dietary supplement based on EFA to your dog?	No, yes
	Did you start the dietary supplement based on EFA before or after the onset of clinical signs?	Before, After

Statistical analysis

Data analyses were performed using SPSS 25.0 (IBM Corporation, Armonk, NY,USA). Chi-square test (Bonferroni correction) and Fisher's exact test were used to assess the association between the frequency of vegetable oil supplementation and the

different clinical diagnoses. Descriptive statistics assessed the patient demographics. Mean and standard deviation were reported for patients' ages and weight, whereas prevalence and frequencies described gender, age, breed and for the following categorical data: diagnoses, current primary diet categories, diet changes, oil and EFA supplementation. A type a error was set at 0.05, while a type b error was set at 0.15 (power of 85%). Dogs whose diet was changed before the onset of clinical signs or supplemented with oil after the onset of clinical signs were not included in the statistical analysis. The dose of vegetable oil that the dog received daily was calculated by dividing the amount of ml received daily (1 teaspoon = 4.93 ml) by the weight of the dog. Based on this supplementation of vegetable oils per day, dogs were then divided in four groups: group 1: 0.08-0.31 ml/kg/daily, group 2: 0.33-0.61 ml/kg/daily; group 3: 0.66-0.99 ml/kg/daily; group 4: 1.09-9.2 ml/kg/daily). Groups were finally compared with the diagnosis to see if a higher amount of supplemented oil was more associated with AFR. Knowing that veterinary prescription diets are richer in EFA and that some dogs may have received dietary supplements containing EFA, we assessed the association between these diets and EFA supplementation with AFR.

7.2. Results

Study population

Initially 550 dogs were enrolled in the study, of which 91 dogs were excluded because no final diagnosis was achieved. Signalment of the remaining 459 dogs is summarized in Table 7.2. An equal number of males and females, representing 69 different breeds, were enrolled in the study.

Table 7.2. Gender, age, weight and breed of 459 dogs included into the study. Only dog breeds represented by three or more animals are mentioned in the table.

		% of total dogs (459)	**n**
Gender	Females	47.49	218
	Females intact	17.74	86
	Males	52.51	241
	Males intact	44.23	203
Age	Mean ± SD= 6.07± 3.87 years (5m-17y)		

Weight	Mean ± SD= 19.14± 12.97 kg (1-38.5kg)		
Breed	Mongrel	26.58	122
	Labrador retriever	6.75	31
	German shepherd	5.45	25
	Dachshund	4.79	22
	Jack Russell terrier	4.14	19
	French bulldog	3.49	16
	Staffordshire bull terrier	3.27	15
	Miniature pinscher	2.83	13
	English bulldog	2.83	13
	Shih tzu	2.40	11
	Lagotto	1.96	9
	Boxer	1.96	9
	English cocker spaniel	1.74	8
	Bull terrier	1.74	8
	Golden retriever	1.53	7
	Chihuahua	1.53	7
	Breton	1.53	7
	Pug	1.53	7
	Poodle	1.53	7
	West Highland white terrier	1.31	6
	English setter	1.31	6
	Cavalier King Charles spaniel	1.31	6
	Yorkshire terrier	1.09	5
	Beagle	0.87	4
	Corso dog	0.87	4
	Argentine dogo	0.87	4
	Rottweiler	0.87	4
	Maltese	0.65	3
	Shar pei	0.65	3

Pomeranian dog	0.65	3
Pointer	0.65	3

SD, Standard deviation

Diagnoses

Fifty different diagnoses were determined for the 459 dogs enrolled in the study; number and the percentage of dogs for each dermatological diagnosis are listed in Table 7.3. The diagnoses with a higher prevalence were: atopic dermatitis (CAD) (22.66%), AFR (8.06%), primary superficial idiopathic pyoderma (7.84%), concurrent CAD and AFR (5.88%), demodicosis (5.88%), skin tumours (4.29%) and localized deep pyoderma (4.29%). For each diagnosis, the number and percentage of dogs supplemented with oil are also reported in Table 7.3.

Table 7.3. Dermatological diagnoses of 459 dogs included into the study. Number and percentages of dogs supplemented with oil among animals with the same diagnosis.

Diagnosis	**Oil**		
	tot n.	**n.**	**%**
CAD	104	15	14.4
AFR	37	11	29.7
Primary superficial idiopathic pyoderma	36	5	13.9
CAD + AFR	27	9	33.3
Demodicosis	27	4	14.8
Skin tumour	24	2	8.3
Localized deep pyoderma	24	3	12.5
Sarcoptes mange	13	1	7.7
Keratinization defects	13	1	7.7
Hormonal disease	12	1	8.3
FAD	11	1	9.1
Hair follicle dysplasia	11	1	9.1
Otitis (no underlying cause determined)	9	1	11.1

Recurrent flank alopecia	9	1	11.1
Leishmaniasis	8	1	12.5
Pemphigus foliaceous	7	0	0
Dermatophytosis	6	1	16.7
Intertrigo	6	1	16.7
Metatarsal fistulae	6	0	0
Vasculitits	6	0	0
Pulicosis	6	1	16.7
Lupus complex	5	0	0
Acral lick dermatitis	4	1	0
Actinic keratosis	4	1	25
Cheyletiella mange	4	0	0
Non-neoplastic neoformation	4	0	0
Eosinophylic furuncolosis	3	0	0
Kerion	3	0	0
Reactive Histiocytosis	2	0	0
Perianal Fistulae	2	0	0
Hereditary cutaneous hyaluronosis	2	0	0
CAFR	2	0	0
Alopecia X	2	0	0
Migratory necrolytic dermatitis	2	0	0
Behavioural disorders	2	0	0
Lupoid onychodystrophy	2	1	50
AFR + FAD	2	1	50
CAD + FAD	1	1	100
Phalangeal fractur	1	0	0
Trombiculosis	1	0	0

Vitiligo	1	0	0
Vogt-Koyanagi-Harada Disease	1	0	0
Zinc responsive dermatosis	1	0	0
Pannicolitis	1	0	0
Foreign body	1	0	0
Arthropods bite	1	0	0
Canine Dermatomyositis	1	0	0
Viral papilloma	1	0	0
Dermanyssus gallinae	1	0	0
Total case numbers	459	65	-

AFR, Adverse food reactions; C. diet, Commercial diet; C+H diet, Commercial and home-made diet; CAD, Canine atopic dermatitis; CADR, Canine adverse drug reaction; FAD, Flea allergic dermatitis; H. diet, Home-made diet; n., number of dogs.

Survey

The number and percentage of dogs for each dietary variable are listed in Table 7.4. The majority of dogs were fed commercial pet food (59.69%), 31.37% of dogs received both a commercial and home-made diet and only 8.93% of dogs were fed exclusively home-made food. Commercial diets and a combination of commercial and home-made diets consisted of 91.24 and 95.14% of maintenance diets and 8.76 and 6.94% of veterinary prescription diets, respectively. The main fed protein was chicken (73.86%) followed by beef (31.59%), fish (31.37%) and lamb (5.66%). Rice was the most prevalent carbohydrate source in their diet (62.75%) followed by potatoes (5.66) and wheat (4.79%). 14.16% of dogs received oil as an ingredient of their diet. Only a minority of dogs received dietary supplements with EFA (2.40%) and of these 81.82% were supplemented after the onset of clinical signs. The supplementation of oil began before the onset of clinical signs in the majority of the dogs (90.77%). The most common type of oil was extravirgin olive oil (70.77%) followed by corn (10.77%), mix seeds (10.77%), olive (4.62%), and sunflower (3.08%). Oil was supplemented at least once daily in 73.85% cases. The supplementation of oil was started after the onset of clinical signs in 9.23% of dogs and the diet was changed before the onset of clinical

signs in 7.41% of dogs. Therefore, these dogs were not included in the analysis. Statistical analysis was consequently performed on 420 dogs.

The use of oil was recorded in 54 of 420 (12.85%) dogs included in the study, in 9 of 33 (27.27%) dogs with AFR, in 7 of 22 (31.81%) dogs with concurrent AFR and CAD, in 14 of 94 (14.89%) with CAD; in 45 of 385 (11.60%) dogs with other conditions.

The frequency of oil administration was significantly higher in dogs with AFR and in those affected by concurrent AFR and CAD than in dogs with other diagnoses ($P<0.05$; $P<0.001$ respectively) (Figure 7.1). There was no significant association between oil administration and other dermatological conditions. An increased oil supplementation was not associated with an increased frequency of AFR (group 1: 35%; group 2: 92%; group 3: 56%; group 4: 40%). The frequency of AFR was higher in dogs fed with veterinary prescription diets (13.04%) than in those fed with maintenance diets (5.06%) ($P<0.001$).

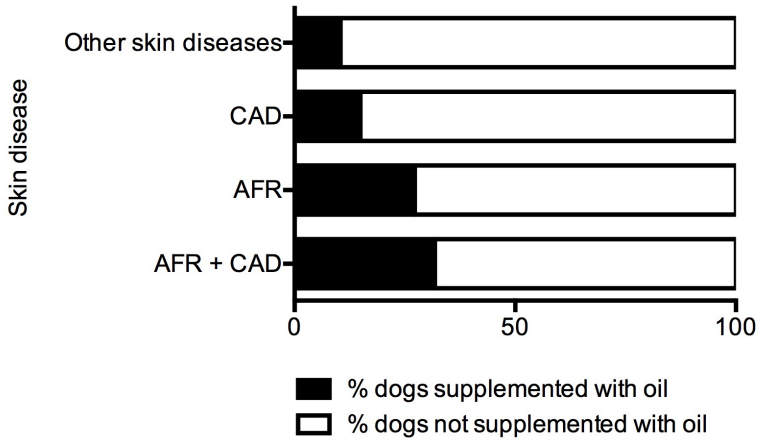


Figure 7.1. Percentages of dogs assuming vegetable oil among animals with AFR, concurrent AFR and CAD, CAD and other skin diseases. Number of dogs under each condition is reported in Table 7.3. Only dogs supplemented with vegetable oil before the onset of clinical signs are reported in the graphic.

Table 7.4. Dietary intake data of 459 dogs included into the study.

		%	n
All dogs		**100**	**459**
Diet			
Dietary type	Commercial pet food	59.69	274
	Maintenance diet	91.24	250
	Veterinary prescription diet (all conditions)	8.76	24

	Veterinary prescription diet for skin problems	6.93	19
	Hydrolyzed Veterinary prescription diet	0.73	2
	Home-made diet	8.93	41
	Commercial and home-made diet	31.37	144
	Maintenance diets	95.14	137
	Veterinary prescription diet (all conditions)	6.94	10
	Veterinary prescription diet for skin problems	6.94	10
	Hydrolyzed Veterinary prescription diet	2.78	4
Diet changes before clinical signs onset	From commercial food to home-made diet	5.01	23
	From home-made to commercial food diet	2.44	11
Main protein/carbohydrate in the diet (Number of dogs is reported for each protein/carbohydrate represented by 3 or more animals)	Chicken	73.86	339
	Rice	62.75	288
	Beef	31.59	145
	Fish	31.37	144
	Lamb	5.66	26
	Potatoes	5.66	26
	Pork	4.79	22
	Wheat	4.79	22
	Turkey	3.49	16
	Duck	1.74	8
	Eggs	1.09	5
	Hydrolyzed food	1.09	5
	Dairy products	0.87	4
	Only vegetal	0.87	4
	Rabbit	0.65	3
Oil supplementation		**14.16**	**65**
Type of oil	Olive- extravirgin	70.77	46
	Corn	10.77	7
	Mix seeds	10.77	7

	Olive	4.62	3
	Sunflower	3.08	2
Amount of tea-spoon of oil per ration	1	64.62	42
	2	30.77	20
	>2	4.62	3
Frequency of oil supplementation	1 = a few times/year,	0	0
	2 = a few times/month	13.84	9
	3 = a few times/week	13.31	8
	4 = daily	50.77	33
	5 = multiple times/day	23.08	15
Onset of oil supplementation/onset clinical signs	Supplementation started before clinical signs	90.77	59
	Supplementation started after clinical signs	9.23	6
Dietary supplements containing EFA		2.40	11
	Supplementation started before clinical signs	18.18	2
	Supplementation started after clinical signs	81.82	9

7.3. Discussion

Our aim was to analyse the association between vegetable oil inclusion in the diet and adverse food reactions (AFR). We demonstrated that the regular supplementation of vegetable oil is associated with AFR. To the best of the authors' knowledge, this is the first study in veterinary literature specifically evaluating the association between oil supplementation and AFR in dogs. In contrast to the extensive literature describing the effects of vegetable oil in humans, no attention has been paid to it in dogs. A high dietary intake of margarine and vegetable oil rich in n6-PUFA has been associated with an increased incidence of asthma, allergic rhinitis, eczema and allergic sensitization (Bolte et al. 2001; Haby et al. 2001; Nagel et al. 2005; Sausenthaler et al. 2006; Trak-Fellermeier et al. 2004; von Mutius et al. 1998; Winkler et al.1992). N-6 PUFA enhances the risk of atopic disease in children compared with non-atopic

children (Dunder et al. 2001).

It is unknown whether n-6 PUFA-rich dietary vegetable oils may also influence the risk of developing food allergy in humans. However, a recent study has shown that increased consumption of n-6 PUFA-rich oil suppresses tolerance induction and enhances the severity of the allergic effector response in a murine model of cows' milk allergy (Van den Elsen et al. 2013). N-6 PUFA might influence allergic disease by promoting formation of inflammatory mediators (Wendell et al. 2014). Indeed, n-6 PUFA contains linoleic acid, a precursor of arachidonic acid, which in turn generates pro-inflammatory eicosanoids, such as prostaglandin E2 and D2 (PGE2, PGD2). Diets supplemented with omega-6 rich vegetable oil lead to changes in the omega-6/omega-3 ratio disrupting the proper balance of pro- and anti-inflammatory agents, which in turn results in increased systemic inflammation. Indeed, omega-3 has been demonstrated to have an anti-inflammatory properties competing as a substrate with the pro-inflammatory arachidonic acid for cycloxigenase and 5-lipoxygenase. Changes in the ratio, in favour of omega-6, not only increase the production of pro-inflammatory eicosanoids (LTB4, PGE2), but also decrease the competing action of omega-3, thus resulting in a stronger inflammatory response. As a consequence, dendritic cells are activated, shifting T-helper balance from type 1 to type 2, which consequently may increase the incidence of IgE-mediated allergic diseases (Black et al. 1999; Gosset et al. 2005; Kankaanpaa et al. 1999; Kompauer et al. 2004; Prescott et al 2004; Theiner et al. 2006). It has also been demonstrated that n-6 PUFA decreases the IFNγ/IL4 ratio in mice and humans (Mizota et al. 2009). N-6 PUFA acts mainly by increasing the humoral response against allergens. Interestingly, a well-known IgE-based allergic disease, such as canine atopic dermatitis, does not seem to be affected by the inclusion of n-6 PUFA in the diet. Indeed, in our study no association was found between CAD and oil administration. It may depend on lack of enzymatic activity of Δ6-desaturase and Δ5-desaturase in the epidermis of atopic dogs, which makes them less susceptible to diet supplementation with n6-PUFA (Campbell et al. 1993; Fuhrmannnet al. 2006; Schlotter et al. 2008). Moreover, this difference between AFR and CAD may be due to a different sensitization route. In CAD, the epicutaneous route is more important (Marsella et al. 2006), while sensitization against food allergens occurs mainly through the gastrointestinal tract (Iweala et al. 2016). It has been demonstrated that high fat foods, such as those containing long chain

triglycerides (LCT), like n-6 PUFA, slow the gastric emptying rate and thus prolong the exposure time and increase the dose of allergen presented to the immune system (Mackie et al. 2012). Dietary LCT promote chylomicron-dependent intestinal absorption of food proteins to mesenteric lymph nodes (MLN) and a consecutive systemic dissemination into the blood. Even though mesenteric lymph nodes play an important role in the induction of oral tolerance (Worbs et al. 2006), dietary LCT also promotes chylomicron-dependent absorption and transport of bacterial lipopolysaccharides (LPS) through the MLN. LPS is a potent immune activator via activation of Toll-like receptor 4 that could contribute to T- and B-cell activation and thus increase the risk of sensitization (Ghoshal et al. 2009). Furthermore, chylomicrons are phagocytosed by macrophages and this could facilitate antigen uptake (Mamo et al. 1996; Elsegood et al. 2006). Besides, n-6 PUFA-rich oils in the diet might enhance the allergic effector response without increasing the level of IgE by increasing the sensitivity of mast cells to degranulate or by modulating the release of mediators by mast cells (Van den Elsen et al. 2013).

Our data also pinpointed that the frequency of AFR was higher in dogs fed, before the onset of clinical signs, with veterinary prescription diets than in dogs fed with maintenance diets. The former are diets that likely contain a higher amount of essential fatty acids, both n-3 and n-6 PUFA. Why this type of diet was fed to clinically healthy dogs is unclear, but we assume that it may be related to breed predisposition to develop allergies or to breeder advice, or yet, because presence of initial doubt clinical signs in these dogs. As a major source of uncertaintly and a main weak point of this study, the composition of diets was not investigated in detail. Indeed, dogs can also get EFA from their diet in a different amounts and proportion depending on the type of diet/recipes and treats/leftover intake. Therefore, even though we did demonstrate an association between oil supplementation and AFR, we cannot assess their causal effect. As a second limitation of this study, the amount and ratio of PUFA contained in the different types of oil was not determined. Knowing in detail the type, amount, ratio and frequency of the essential fatty acids intake of the dogs could have shed more light on its association with AFR and further corroborate or reject our hypothesis. Lastly, the prevalence of CAD and AFR reflect the dermatological referral case population of the author, and not the general population, further limiting the ability to extrapolate these results. Ideally, the design of this study should have also included

healthy dogs and dogs with non-dermatological diseases to assess the frequency and the effect of oil supplementation in other populations.

Notwithstanding these limitations, our study, unpretentiously aimed to find an association between vegetable oil supplementation and adverse food reactions, leaving the role to assess the causal relationship between these variables to further research.

7.4. Conclusions

Despite its exploratory nature, this study offers some insights into the relationship between AFR and vegetable oil supplementation. Once again, we would like to stress that although these data are interesting, they only show an association and do not provide evidence of a causal relationship. Therefore, these data must be interpreted with caution and further research is needed to verify these associations.

Chapter VIII:
General discussion & future perspectives

8.1. General discussion

Adverse food reactions (AFR) are a prevalent dermatological problem that may affect up 24% of dogs with skin diseases (Olivry and Mueller 2016). Itchiness is a common sign. Dogs suffering AFR are scratching, rubbing, scooting and above all licking to relieve themselves. The skin is primarly erythematosus and it quickly becomes hyperpigmented and lichenified because of self-induced trauma (**Chapter 1**). The diagnosis is based on the medical history, clinical signs, exclusion of other pruritic conditions and response to a food trial. Given the limitations of current testing modalities, a food trial is considered the diagnostic gold standard. Once the culprit food allergen is identified it can be excluded from the diet. Since there is currently no cure for adverse food reactions, strict avoidance of food allergens is the only way to prevent a reaction and clinical symptoms (**Chapter 1**).

In human, there are many strategies for helping patients and their families to manage and prevent adverse food reactions. Management involves counselling of patients and their family to strictly avoid culprit food allergens and, in case of IgE mediated anaphylactic reactions, to carry emergency treatment, such as an epinephrine injecting pen, at all times in case of accidental exposure. The challenge of strict avoidance, together with the fear of potential severe reactions any time food is consumed, causes considerable stress for patients and their families. Although it may seem very easy to avoid culprit allergens, incredibly, it has been reported that up to 75% of children with peanut allergy accidentally consume peanuts (Vander et al. 2000) due to incorrect assumptions about ingredients, misreading of labels or being given food by another adult.

Percentages of accidental exposure have not been reported in dogs, but are probably higher than in children because of the dog's nature to gather food, eat out of the garbage or beg for food, and because, contrary to children, they are not supervised all the time. Since food-related reactions in dogs are rarely life-threatening, owners are less vigilant. Finally, labelling of pet food products is less transparant than food intended for human consumption resulting in the presence of trace amounts of undeclared ingredients. To complicate things, very small amounts of the food suffice to cause a reaction, but this is often doubted by the owner who continues to feed culprit

allergens to their dog. Even though relapses result in non life-threatening reactions, these are unpleasant and a source of stress for both dogs and owners, diminishing their quality of life. As such there is strong interest to develop long-term therapeutic solutions for AFR (Primeau et al. 2000). So far there are no curative treatments, but only symptomatic ones that merely eliminate the symptoms but fail to provide an effective cure.

One promising approach aims to induce tolerance to specific allergens using immunotherapy (**Chapter 1**). Immunotherapy uses limited exposure to an allergen to promote tolerance. Traditional subcutaneous immunotherapy (SCIT) has proven unsafe for food allergy (Nelson et al. 1997; Oppenheimer et al. 1992), however, mucosal targeted immunotherapeutic approaches, such as oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) have shown promise in Phase I and early Phase II trials to desensitise or even induce tolerance in patients (Anagnostou et al. 2014; Chin et al. 2013; Jones et al. 2009; Kim et al. 2011; Varshney et al. 2011). At the time of writing only one peanut-OIT (AR101) finished phase III and currently is pending FDA approval.

Since food-specific immunotherapy has not been investigated in dogs and as SLIT has a reported efficacy and a better safety profile, we aimed in this PhD thesis to evaluate if FA-SLIT could be safely **(Chapter 3)** and effectively **(Chapter 4 and Chapter 5)** used in dogs. We tested a sublingual immunotherapy protocol that was shown to safely induce clinical desensitization in food allergic children, in healthy laboratory dogs in order to asses if it was safe and well tolerated also in this species (**Chapter 3**). This first clinical trial phase was then followed by a second clinical trial in owned dogs with AFR to assess clinical and immunologic changes induced by FA-SLIT administration (**Chapter 4 and Chapter 5**). While we demonstrated that FA-SLIT was able to skew the cytokine and T cell profile toward a tolerogenic response, we identified a new interesting T cell subset: double positive T cells, suspected to have a role in the pathogenesis of AFR. Therefore, we further investigated the frequency of these cells in a larger group of dogs with AFR and in dogs with CAD and in healthy dogs (**Chapter 6**). Geography revealed to be very important for finding AFR dogs suitable for our studies and this led to the suspicion that vegetable oil supplementation in dog's diets might be associated with AFR in Italy (Figure 8.1).

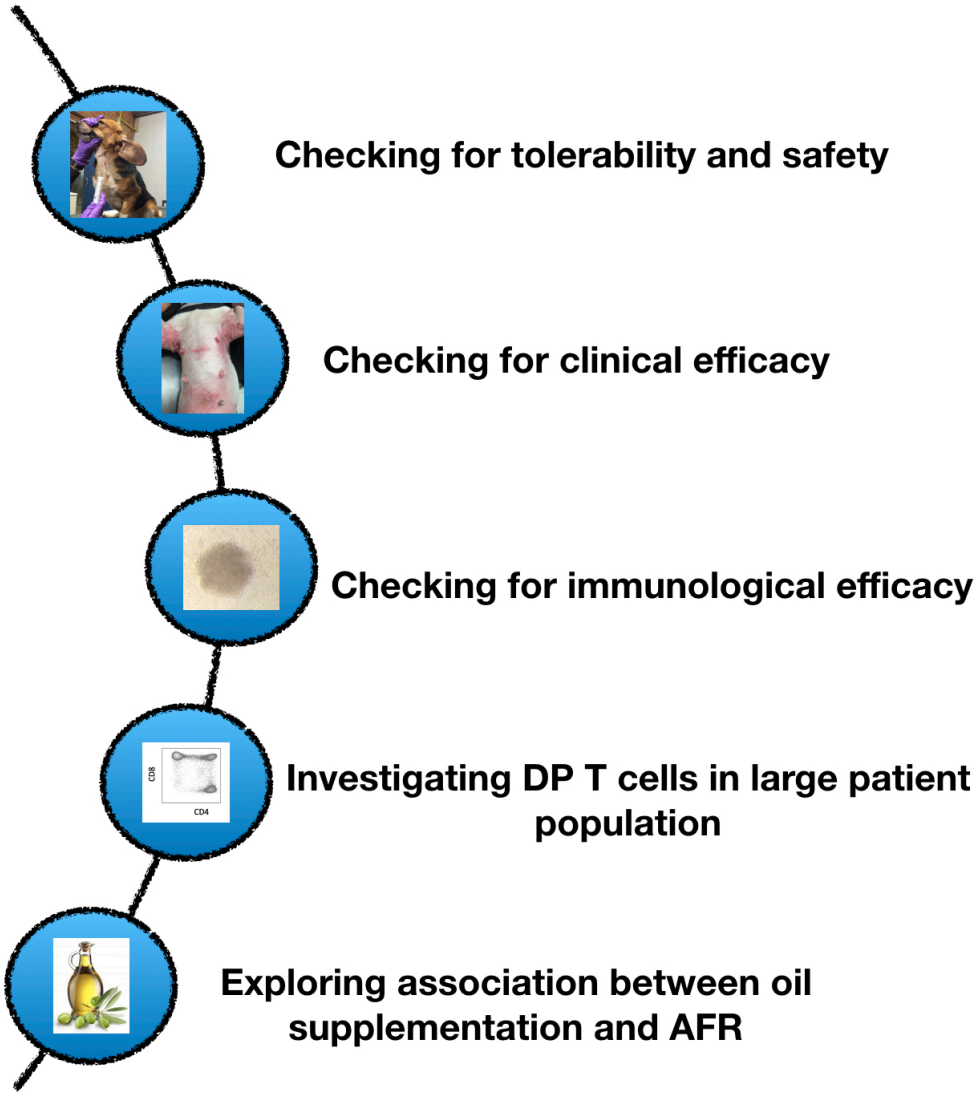


Figure 8.1: Multi-stage study design.

8.1.1. Sublingual immunotherapy: a new approach to treat adverse food reactions in dogs.

8.1.1.1. A safe and effective method for AFR in dogs

In the **third chapter**, food-specific immunotherapy was used for the first time in dogs. In a randomized, blinded, placebo-controlled trial, eight healthy dogs, never orally exposed to peanut, received SLIT with peanut or placebo for four months. We showed that a pump-type dispenser bottle with a hook to dispense the solution was easy to be used, harmless to the oral mucosa during the treatment administration and well

accepted by the dogs over time (Figure 8.2). The administration was easier in all dogs regardless of their skull/mouth conformation or behaviour (Figure 8.3).

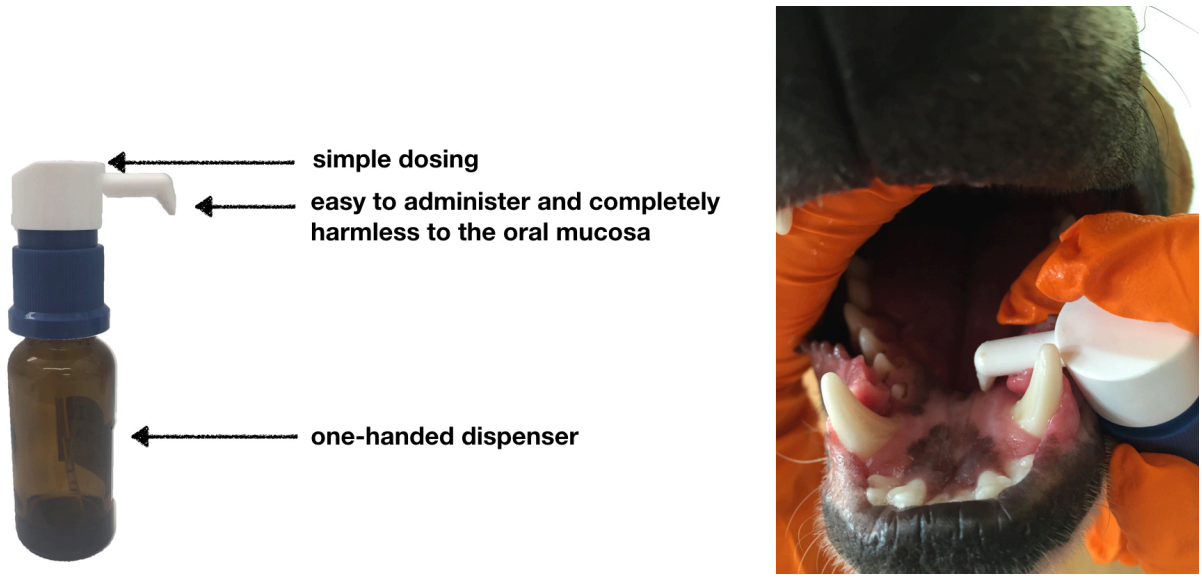


Figure 8.2: FA-SLIT dispeser: The pros.

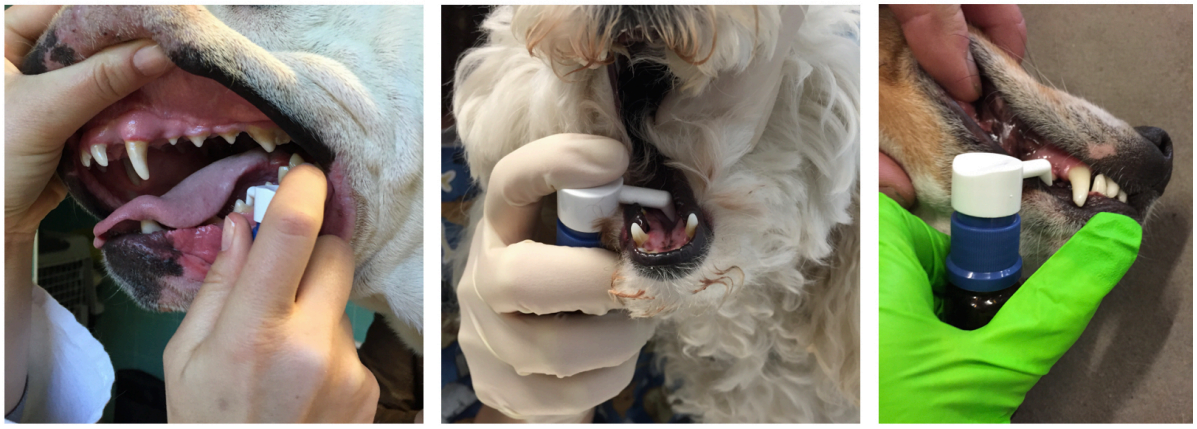


Figure 8.3: FA-SLIT administration. The hook of the dispenser can be easily inserted over the teeth, under the tongue, ensuring efficient delivery of the solution in all dogs.

To improve efficacy of a long-term treatment in dogs, it is mandatory to have a good compliance of both dogs and owners and as such the administration has to be as easy as possible. In a previous study a pump-type dispenser bottle with a hook to easily dispense the solution over the lower teeth, under the tongue, was used to administer an immunotherapeutic treatment to dogs. Authors reported that the treatment was well tolerated and interestingly, some dogs that did not respond to SCIT improved with

SLIT (Deboer et al. 2012). This suggests that a better compliance, due to the ease of administration, could have been the reason for this improved response with SLIT.

SLIT is reported to have a very good safety profile in humans. Even though local reactions, such as oral itching and/or swelling or itching of lips, swelling of the tongue, mouth or tongue ulceration occur frequently in allergic human patients that underwent FA-SLIT, these reactions are very mild and often do not require any treatment (Gidaro et al. 2005; Passalacqua et al. 2013). In our first study (**Chapter 3**), performed on healthy dogs we did not record any side effects except for one single episode of transient vomiting in the placebo group. We are aware that local and systemic allergic reactions in healthy dogs are unlikely, but with this experiment we ruled out irritation caused by the dispenser, allergen, vehicle or placebo. This is important to understand if reactions in patients are due to allergic reactions rather than an irritation to the SLIT administration. Importantly, the protocol used in our preliminary study did not sensitize dogs against the protein used. Indeed, dogs challenged with 2000 μg of peanut extract 6 months after the end of the experiment did not show allergic symptoms and they did not react positively to the intradermal test. In addition, administering increasing amounts of allergen with SLIT in naïve dogs induced higher levels of allergen-specific IgG as compared to IgE. This was suggested to be indicative of good tolerance induction (Jones et al. 2009; Kim et al. 2011; Varshney et al. 2011).

Since tolerance induction is associated with a successful sublingual immunotherapy, we then tested the same protocol in dogs with spontaneous AFR (**Chapter 4**). In our randomised blinded study, dogs spontaneously sensitized to food were randomized to receive SLIT treatment or placebo daily for at least 6 months. The active treatment for the sublingual immunotherapy was prepared based on the results of the food elimination and provocation trial. Similar to another study (Deboer et al. 2016), the dogs only experienced mild, transient local reactions to the administration without differences between treatment and placebo group. Conversely to humans, dogs did not experience any evidence of urticarial reactions nor lesions or itching at the oral cavity or perilabial area. In our study (**Chapter 4**), the most common clinical signs positively associated with FA-SLIT were pruritus and erythema at distal body sites. Why dogs show allergic reactions so far from the allergen-contact sites is unknown, but it may be linked to the higher number of mast cells in predilection sites for canine atopic dermatitis, such as interdigital skin and pinnae (Auxilia and Hill 2000). Another

hypothesis proposes that clinical reactions may depend on target organ hypereactivity. Indeed, individuals with history of skin sensitive to irritants are more likely to develop AD, when sensitized to food (Hanifin et al. 1980). Similarly, children with airway hyperreactivity have often asthma. In addition, another hypothesis to account for a different manifestation of AFR is the T cell homing of food-specific T cells to a specific site. There is evidence that the site of antigen recognition influences the migration pattern of T cells (Figure 8.4). Dendritic cells from the gut induce $\alpha 4\beta 7$ integrin and CCR9 chemokine receptor on activated T cells that in the presence of retinoic acid increase their capacity to home to the intestinal mucosa (Briskin et al. 1997; Kunkel et al.2000). On the contrary, dendritic cells at the skin surface induce high levels of lectin cutaneous leukocyte antigen (CLA) in combination with the chemokine receptors CCR4 and CCR10 on T cells homing to the skin (Campbell et al. 1999; Clark et al. 2006; Homey et al. 2002; Picker et al. 1994) (Figure 8.4). This imprinting generates long-lived antigen-specific memory T cells programmed to home to a specific surface for immune surveillance. In humans, percutaneous sensitization through a disrupted skin barrier is increasingly being recognized as an onset risk for food allergies. Once beyond the skin barrier, allergens promote local IgE-mediated reactions. In a study of human patients with milk-induced AD, the expression of CLA in peripheral blood lymphocytes that were *in vitro* stimulated with casein was significantly higher that the expression of patients that did not had cutaneous manifestation of milk allergy (Abernathy et al. 1995). In contrast, in dogs, the gastrointestinal route seems to be more important and it is more often associated to systemic reactions. Moreover dogs, conversely to humans, very rapidly swallow their food and therefore the dispensed dose of allergen may be absorbed into the gut rather than sublingually.

In **chapter 4**, we demonstrated the efficacy of the SLIT protocol to decrease clinical signs and pruritus in dogs with AFR during the challenge with the provocative diet. Indeed, both CADESI-4 and PVAS validation scores declined in the treatment group as compared to placebo group. FA-SLIT was however more effective in decreasing clinical signs than pruritus. Curiously, when SLIT was used to treat dogs with AD, it was more effective in controlling pruritus than clinical lesions (Deboer et al. 2016).

A comparison of the two studies reveals that baseline median CADESI score of the above mentioned atopic dogs was higher (CADESI-3: 76.5 (49.0– 95.4); than that of

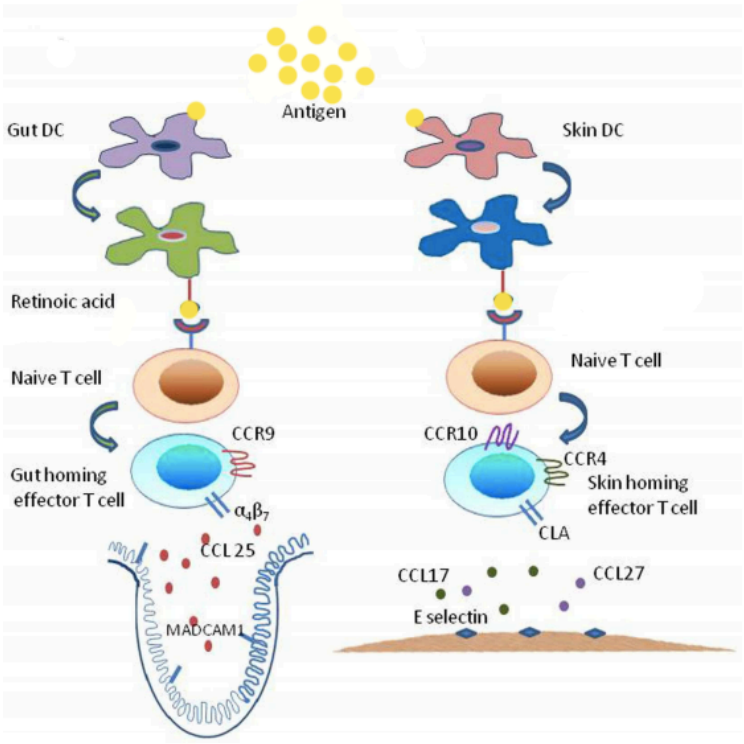


Figure 8.4. Imprinting mechanisms for gut- and skin-specific T cells. Naive T cells become activated when they encounter a cognate antigen presented by mature DCs. Peyer's patch DCs and MLN DCs express crucial enzymes for metabolizing vitamin A (retinol) into RA. The encounter between T cell and antigen in the presence of DC-derived RA induces gut homing receptors and suppresses skin homing molecules. When T cells are activated in skin-draining peripheral lymph nodes, they are exposed to DCs that cannot synthesize RA. The T cells then upregulate skin homing molecules CCR4, CCR10 and CLA. Adapted from Tufail et al. 2013.

dogs included in our study (CADESI-4: 32.2 (26-40)). Unfortunately, this observation does not lead to any significant conclusions as the evaluation was carried out with two different score systems (CADESI-3: highest score achievable: 1240, evaluating 62 body sites; CADESI-4: highest score achievable: 180, evaluating 20 body sites).

In the latter article, it was shown that SLIT allowed to decrease or even to discontinue glucocorticoids used to control clinical signs or pruritus. In our study, medication was not necessary because food allergens can be excluded in contrast to environmental allergens responsible for allergic manifestation in AD. The majority of clinical studies on FA-SLIT in humans evaluate the efficacy by comparing the amount of protein that an individual can ingest without triggering clinical signs before and after the treatment (Enrique et al. 2005; Fleisher et al. 2013; Kim et al. 2011). Unfortunately, we did not measure this difference, but only the different effect given by feeding the same amount of culprit food before and after the treatment. As successful treatment, we had put forward a percent reduction of the CADESI and pVAS scores equal to or greater than

50% at the end of the study as compared to the initial baseline value. Although all dogs treated with the active component had milder reactions once challenged at the end of the experiment, the improvements in both clinical lesions and pruritus reached the predefined cut-off in only a minority of the patients. Although we could not provide statistical evidence that the FA-SLIT protocol improved faecal consistency in dogs, 40% of dogs treated with the active component nevertheless showed improved FCS compared to none of the dogs in the placebo group. These milder reactions during the challenge test and improved FCS suggest that FA-SLIT was able to increase the threshold of sensitivity to food allergens as a consequence of partial desensitization. Unfortunately, this protocol was unable to induce tolerance. This is in line with human literature where it has been reported that the majority of patients can be desensitized to food allergens with SLIT, but only a minority develop tolerance or sustained unresponsiveness (Berin et al. 2013). The exact definition of sustained unresponsiveness is somewhat arbitrary, but generally refers to a lack of clinical reactivity to the ingested food for 1 to 6 months following therapy (Moran et al. 2015). In contrast to SLIT, many articles report the efficacy of oral immunotherapy (OIT) to induce tolerance (Buchanan et al. 2007; Burks et al. 2012; Syed et al. 2014; Staden et al. 2007; Vickery et al. 2010; Vickery et al. 2014), which is estimated to range between 25-50% of the subjects when assessed within 1-3 months of discontinued OIT therapy. However, when the allergen is avoided for longer periods this percentage drops significantly, suggesting that the clinical efficacy of OIT is generally transient and that regular consumption of the allergen is necessary to maintain tolerance. In 2012, Keet et al. directly compared SLIT and OIT demonstrating that desensitization and tolerance induction are more likely to occur with OIT than SLIT. The difference in efficacy and safety between the groups is likely related to the dose of allergen given; the cumulative dose that the subjects in the SLIT group received was at least 140-fold lower than the minimum cumulative OIT dose. Taking into account that a higher efficacy has also been associated with a higher rate of adverse reactions, a decreased percentage of desensitization and tolerance induction by SLIT is a compromise to accept. A possible future strategy to improve SLIT efficacy would be to use higher doses (Abramson et al. 2010; Radulovic et al. 2010) or to dispense multiple doses per day (Sicherer et al. 2014). Indeed, the protocol with the highest tolerance rate also used the highest concentration of culprit allergen (Vickery et al. 2014). Interestingly, this study lasted up to 5 years. These results seem to suggest that SLIT outcomes

may be improved by prolonging the protocol. On the one hand, it may increase the percentage of desensitized patients and it may promote tolerance induction. On the other hand, a longer protocol would allow a slower dosage escalation, which in turn would decrease the rate of side effects, as observed in our study (**Chapter 4**). Finally, as desensitization can be lost within one week of allergen avoidance (Keet et al. 2012), it may be reasonable to think that once desensitization is reached, the patient should continue to ingest the "culprit" antigen on a regular basis. However, when asked to judge the administration modality of FA-SLIT up to 91% of the owners indicated they did not like the daily administration/task. To improve the efficacy of FA-SLIT dispensing a more concentrated solution or longer treatments would be a better choice in dogs as compared to dispensing multiple doses per day. Even better would be to deploy a buccal delivery system containing the culprit allergens allowing a slow release of small amounts of food allergens during the day.

8.1.1.2. What have we learned from canine FA-SLIT? is this really worth it?

While a decrease in clinical symptoms alone may be an unacceptable outcome of SLIT for a person with a high risk of anaphylactic reactions due to AFR, in dogs, where allergic reactions are all in all milder, a partial desensitization might be an acceptable result. Indeed, one of the reasons that led us to test SLIT in dogs with AFR was the impossibility to prevent accidental exposures to the culprit antigen. An increased threshold might protect dogs with AFR against accidental ingestion of small amounts of allergens or decrease the severity of lesions when larger amounts of allergens are ingested as we demonstrated in our study. While our optimistic attitude may seem somewhat paradoxical, the reader should look very closely to dog n. 11 included in our study (**Chapter 4**) (Figure 8.5). This is the dog, in the treatment group, which improved less. If we look at the percentage reduction of clinical scores (23.08 CADESI-4; 2.78 pVAS) this treatment should not be recommended, but if we look at figure 8.5 we can easily understand that this dog had a benefit from this treatment and therefore FA-SLIT should be advisable.

AFR is a chronic disorder that has impact on the quality of life (QoL) of both the dogs and their owners. Noli et al. (2011b) demonstrated with a validated method that

diseases associated with more pruritus had the worst QoL scores. When dogs suffer from

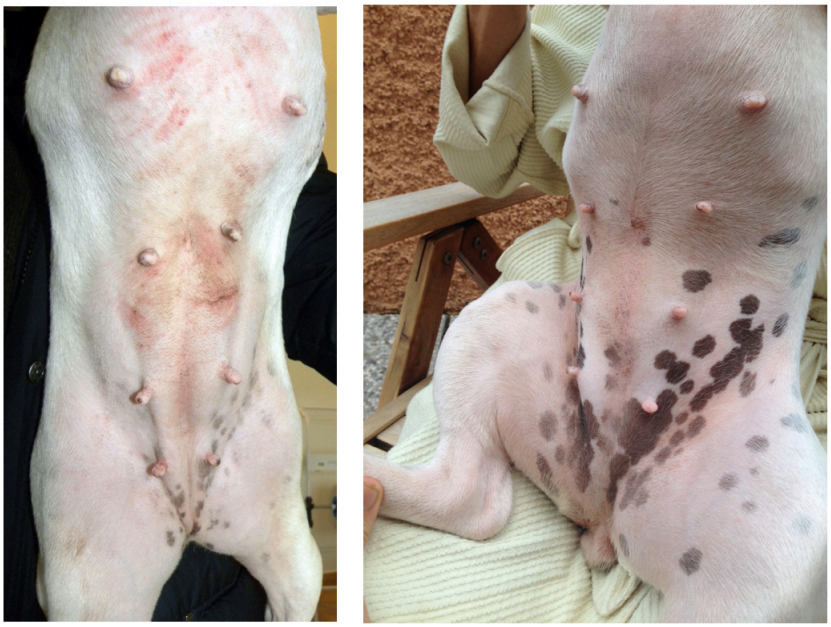


Figure 8.5. Dog 11 with erythema and excoriations on the ventral area during the first provocative test (at the beginning of the study, left) and with milder clinical signs during the second provocative test (at the end of the study, right). This dog had unchanged pVAS score at the end of the study. He was still scratching with the same frequency but not with the same intensity, causing less self-induced excoriations.

allergy, their behaviour and mood changes, resulting in more lethargic, anxious or aggressive behaviour, they play less and their welfare is also impacted by the burden caused by continuous therapies. On the other hand, time loss, emotional and physical distress together with increased household's expenses due to allergy-related issues (diets, drugs, veterinary consultations…), negatively affects the owner's quality of life (Noli et al. 2011a).

Studies have shown that SLIT improves the quality of life of patients with allergy (Blaiss et al. 2011; Ciprandi et al. 2010; Dider et al. 2011; Durham et al. 2012; Nelson et al. 2011; Woody et al. 2012). With a questionnaire, we showed that SLIT may also improve the QoL of the owners of dogs with AFR.

To answer the question that we have been asking above: is FA-SLIT really worth it? Yes, it is.

8.1.1.3. Shift towards a regulatory/suppressor T cell response as a key event during FA-SLIT.

We demonstrated that FA-SLIT in dogs is safe and a quite effective approach to treat dogs with AFR (**Chapter 3 and 4**), probably due to a desensitization in these dogs. However, we were uncertain whether this protocol might have modulated the immune system toward a non-allergic phenotype. Therefore, we analysed the cytokine secretion and the phenotypic profile of T cells of allergen-stimulated PBMCs isolated from blood samples of dogs collected during the two provocative tests before and after FA-SLIT. We showed that FA-SLIT increased the production of IL-10 and IFN-γ, while it decreased the number of allergen-specific CD8+ T cells. In addition, the percentage of double negative T cells (DN T cells) was increased upon FA-SLIT and might have a pivotal role in tolerance induction. Indeed, these cells have been characterized as unique antigen-specific regulatory cells able to suppress CD8+ T cells responses employing different mechanisms, including the secretion of IFN-γ and IL-10 (Barwig et al 2010; Raker et al. 2015). In mice, DN T cells are induced following allergen exposure and they play a role in mediating the suppressive effects of immunotherapy by inhibiting IgE production in a mouse model of type I allergy. In our study, the higher concentration of DN T cells did not correlate with changes in IgE concentration, but it was concurrent with a decreased CD8+ T cell percentage. This might imply that in dogs suppression of CD8+ T cells may be the mechanism of tolerance induction occurring in the course of food allergen specific immunotherapy.

Interestingly, in our study both CD8+ and DP T cell percentages were increased in dogs with AFR (**Chapter 5** and **Chapter 6**). The expression of CD4 and CD8$\alpha\beta$ co-receptors on T cells has for a long time been considered as a temporary developmental stage in the thymus. We know now that small numbers of DP T cells are also present in peripheral blood of healthy humans (Blue et al. 1985; Colombatti et al. 1998; Kay et al. 1990; Patel et al. 1989) and dogs (Alexandre-Pires et al. 2010; Otani et al. 2008). Mature CD8+ T cells have been described as one of the lineages from which these CD4+CD8+ DP T cells in dogs originate (Bismarck et al. 2014). The frequency of these cells increases with viral infections, autoimmune diseases and with atopic dermatitis in humans (Das et al. 2003; Desfrançois et al. 2010; Kitchen et al. 2004; Kitchen et al. 2005; Sarrabayrouse et al. 2011; Szczepanik et al. 2005), and in dogs with bacterial stimulation and leishmaniosis (Bismarck et al. 2012; Schütze et al.

2009). Their function is however still a subject of debate. It is unclear if they are beneficial or detrimental, but it has been demonstrated that DP T cells can acquire distinct functions depending on the pathological event, location (Overgaard et al. 2015) or even origin (Rothe et al. 2017). Rothe et al. (2017) recently showed that DP T cells are phenotypically and functionally heterogeneous and speculated that this difference may be related to their origin. For instance, DP T cells developed from CD8+ T cells are more likely to have an activated phenotype than a regulatory one. Since these cells have been found in the target organs of autoimmune diseases, it may suggest their involvement in the pathogenesis of these conditions (Parel et al. 2004; Parel et al. 2007). Especially of greater interest for this thesis, a high percentage of these cells have been recorded in cultured T cell lines derived from skin of patients with AD and in their peripheral blood (Bang et al. 2001). Therefore, it is a fair assumption that the recruitment of these cells at inflammatory sites is linked with the pathogenesis of these allergic disorders. To support this hypothesis, the percentage of DP T cells significantly decreased after FA-SLIT (**Chapter 5**), suggesting that these cells are involved in the pathogenesis of AFR and that immunotherapy induces peripheral desensitization or tolerance by decreasing these cells. We would like to speculate, knowing the limits of our study, that immunotherapy might promote the differentiation of regulatory DN T cells, which, in turn, produce IL-10 and IFN-γ to decrease the number of circulating CD8+ T cells. Less CD8+ T cells would be responsible for a decreased percentage of DP T cells (Figure 8.6).

Figure 8.6: Assumed cascade of cellular events induced by food-specific sublingual immunotherapy in dogs

8.1.1.4. Interleukin-17A in dogs with adverse food reaction: piece of the puzzle.

Besides DP T cells, IL-17 producing Th cells (Th17 cells) have also been proposed as an additional T cell subset which contributes to the pathogenesis of allergic diseases.

A hallmark of Th17 cells is their production of IL-17A, which is essential in the protection against extracellular pathogens, since it promotes proliferation, maturation and chemotaxis of neutrophils, and production of pro-inflammatory cytokines. The contribution of IL-17A to allergic diseases, such as asthma, allergic rhinitis and atopic dermatitis, has recently been confirmed (Hashimoto et al. 2005; Jutel et al. 2012; Nembrini et al. 2009; Oboki et al. 2008). This cytokine seems to potentiate allergic inflammation by triggering the pro-inflammatory properties of neutrophils, which in turn produce and release several mediators of the phlogosis. IL17 cytokine levels were also higher in patients with food allergy (during the symptomatic phase) (Qamar et al. 2013; Zbikowska-Gotz et al. 2016). IL-17 producing cells have been demonstrated in healthy dogs and in those with Leishmaniosis or naturally occurring chronic inflammatory diseases. We were the first to show that IL-17A is also produced in dogs with AFR and its concentration decreased after FA-SLIT. Increased levels of IL-17A were not correlated with the severity of clinical lesions nor with the intensity of pruritus, but with bacterial overgrowth, suggesting that, in agreement with human literature, microbial stimuli may activate IL-17 secretion (Eyerich et al. 2009). This in turn can trigger inflammation by stimulating innate immunity to mediate neutrophil recruitments, implicating the potential role of IL-17A as an aggravating factor in AFR (Figure 8.7). Importantly, the current study found that IL-10 levels were inversely correlated with bacterial overgrowth suggesting that immunotherapy promotes increased production of IL-10 which suppresses inflammatory responses against culprit allergens. As a consequence a less inflamed skin may decrease the susceptibility to colonization by opportunistic bacteria, leading to decreased IL-17A levels (Figure 8.7).

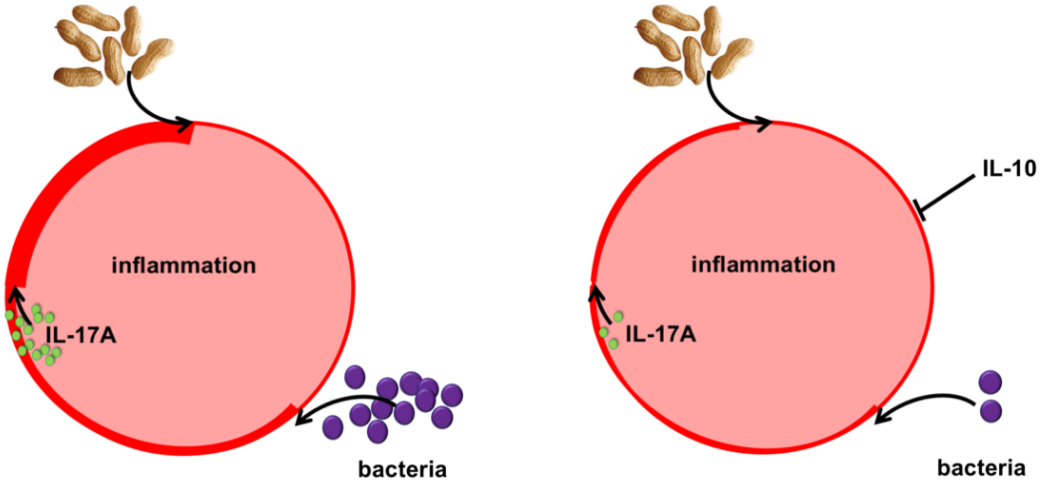


Figure 8.7. Assumed potentiating mechanism of IL-17A in CAFR and the effect of FA-SLIT on them. Skin inflammation secondary to AFR may predispose to bacterial infection. In response to bacteria, they induce T cells to release IL-17A which in turn potentiate the allergic inflammation by stimulating innate immunity to mediate neutrophil recruitments and survival. The former leads to a vicious circle of self-perpetuating factors. FA-SLIT increases the production of IL-10 which suppresses inflammation and reduces the burden of bacteria thus decreasing IL-17A levels.

8.1.2. Double positive T cells: new players in CAFR.

CD4+CD8+ DP T cells are increased in dogs with AFR (**Chapter 5** and **chapter 6**) and their frequency decreases after FA-SLIT suggesting that they are more likely to be involved in the pathogenesis of AFR rather than in tolerance induction. DP T cells are not pathognomonic of AFR since they can also be present also in blood of dogs with atopic dermatitis. Similarly to humans, also dogs with AD show a higher increase of DP T cells, which is not surprising considering that these are lymphocytes with features of activated cells and both AD and AFR are hypersensitivity disorders. Interestingly, when DP T cells are not stimulated no significant differences were present between these allergic groups. On the contrary, when stimulated with food allergens DP T cells of AFR dogs proliferated more than those of atopic dogs. Although this higher reactivity cannot be used as a screening tool to distinguish CAFR from CAD, it may helpful in selecting patients suitable for a food trial, because they more likely have AFR than AD. Unfortunately, proliferation of DP T cells against food allergens cannot be used to detect culprit allergens, because these cells erroneously proliferate upon stimulation with food components to which the dogs are not sensitized.

8.1.3. Scratching the surface: vegetable oil supplementation is associated with adverse food reactions in dogs.

CD8+ T cells and DP T cells from dogs with AFR respond vigorously to harmless antigens. Why the development of tolerance is impaired or an existing tolerance is broken down in these dogs, is still unknown. A possible explanation comes from the observation that allergies have increased strikingly in Western countries during the last decades, in parallel with a decreased consumption of saturated fat and an increased consumption of poly-unsaturated fat. Indeed, in the last century the consumption of butter, food rich in n-3 PUFA, has declined, while that of margarine (n-6 PUFA) has increased. Therefore, the change in dietary fatty acid composition was suggested to influence the risk of developing allergies. Indeed, omega-3 poly unsaturated fatty acids (PUFAs) were proven to decrease the risk of developing allergies. On the other hand, omega-6 PUFAs, such as LA and AA, have a pro-inflammatory activity, because their metabolism leads to the formation of inflammatory eicosanoids, such as prostaglandins. Therefore, an increased intake of n-6 PUFAs with a decreased intake of n-3 PUFAs may contribute to the development of allergies. A parallel might exist in dogs as most pet owners provide their food to the dog as home-made diet or occasionally as leftovers. It is therefore reasonable to think that dog's diets are affected by their owner's nutritional habits. Indeed, in **Chapter 7** we demonstrated that the inclusion of vegetable oils, rich in n-6 PUFAs, in the dog's diet was associated with AFR. It is important to bear in mind, that we established an association without proving a causal relationship between oil consumption and the development of an adverse food reaction. Therefore, these findings should be interpreted with caution.

Linoleic fatty acid and other n-6 fatty acids present in vegetable oil are a substrate for arachidonic acid, which in turn is the major substrate for eicosanoid synthesis. Eicosanoids include prostaglandins, in particular PGD2 and PGE2, thromboxane A2 and leukotrienes. PGD2, produced mainly by mast cells and activated macrophages, is a potent bronchoconstrictor, promotes vascular permeability and activates eosinophils and a Th2 response. PGE2 is a vasodilator, inhibits the production of Th1-type cytokines and primes naïve T cells to produce IL-4 and IL-5. It also promotes Ig class switching in uncommitted B cells toward the production of IgE. Thromboxane A2 is a bronchoconstrictor and stimulates the release of acetylcholine and leukotrienes, in particular LTB4, which is chemotactic for leukocytes. In addition, this molecule

increases vascular permeability, induces the release of lysosomal enzymes and the production of reactive oxygen species by neutrophils and inflammatory cytokines by macrophages and promotes IgE production by B cells. These mediators have been shown to predispose to or increase the severity of IgE-mediated allergies. Curiously, in our study (**Chapter 7**), atopic dermatitis, which is IgE-mediated, was not associated with vegetable oils in the diet. It is tempting to speculate that a lack of activity of fatty acid delta-6 and delta 5-desaturases in atopic dogs, which may decrease the amount of proinflammatory eicosanoid generated by omega-6 metabolism (Campbell et al. 1993; Fuhrmannnet al. 2006; Schlotter et al. 2008). It has been demonstrated that the type of dietary fat might determine antigen absorption. N6-PUFA, which are long chain triglycerides (LCT), slow the gastric emptying rate and thus prolong the time of exposure of the immune system to food allergens (Mackie et al. 2012). In addition, they stimulate lymphatic chylomicron transport, which in turn significantly enhance intestinal absorption of dietary antigens (Ghoshal et al. 2009). Food allergens in chylomicrons are carried to MLN and then to the bloodstream. In contrast, dietary medium chain tryglicerids are absorbed via the portal blood and do not promote antigen absorption. This transport from the intestine to the MLN is normally associated with tolerance induction but, since LCT also promote chylomicron-dependent transport of bacterial lipopolysaccharides, a potent immune activator, to the MLN, it may result in T and B cell activation.

In conclusion, we demonstrated for the first time that food-specific sublingual immunotherapy is well tolerated in dogs. It is effective in decreasing clinical lesions and pruritus when administered daily for at least 6 months. The tested protocol, inducing partial desensitization, protects dogs upon accidental exposure by decreasing the severity of allergic reactions. This partial desensitisation is a result of the tolerogenic modulation of the immune system induced by FA-SLIT, which increases the percentage of double negative T cells and IL-10 production, and decreases the percentage of CD8+ T cells and double positive T cells. The latter, likely involved in the pathogenesis of AFR, are present in higher percentages in dogs with allergy than in healthy dogs. While we demonstrated there is a significant difference between dogs with an AFR and those that are healthy, unfortunately, these findings cannot be used for diagnostic purposes. Finally, dogs suffering of AFR are characterized by a hyperactive immune system toward harmless antigens and the

nutritional lifestyle, in particular the use of n6-PUFA rich vegetable oils, of their owner may contribute to sensitization (Figure 8.6).

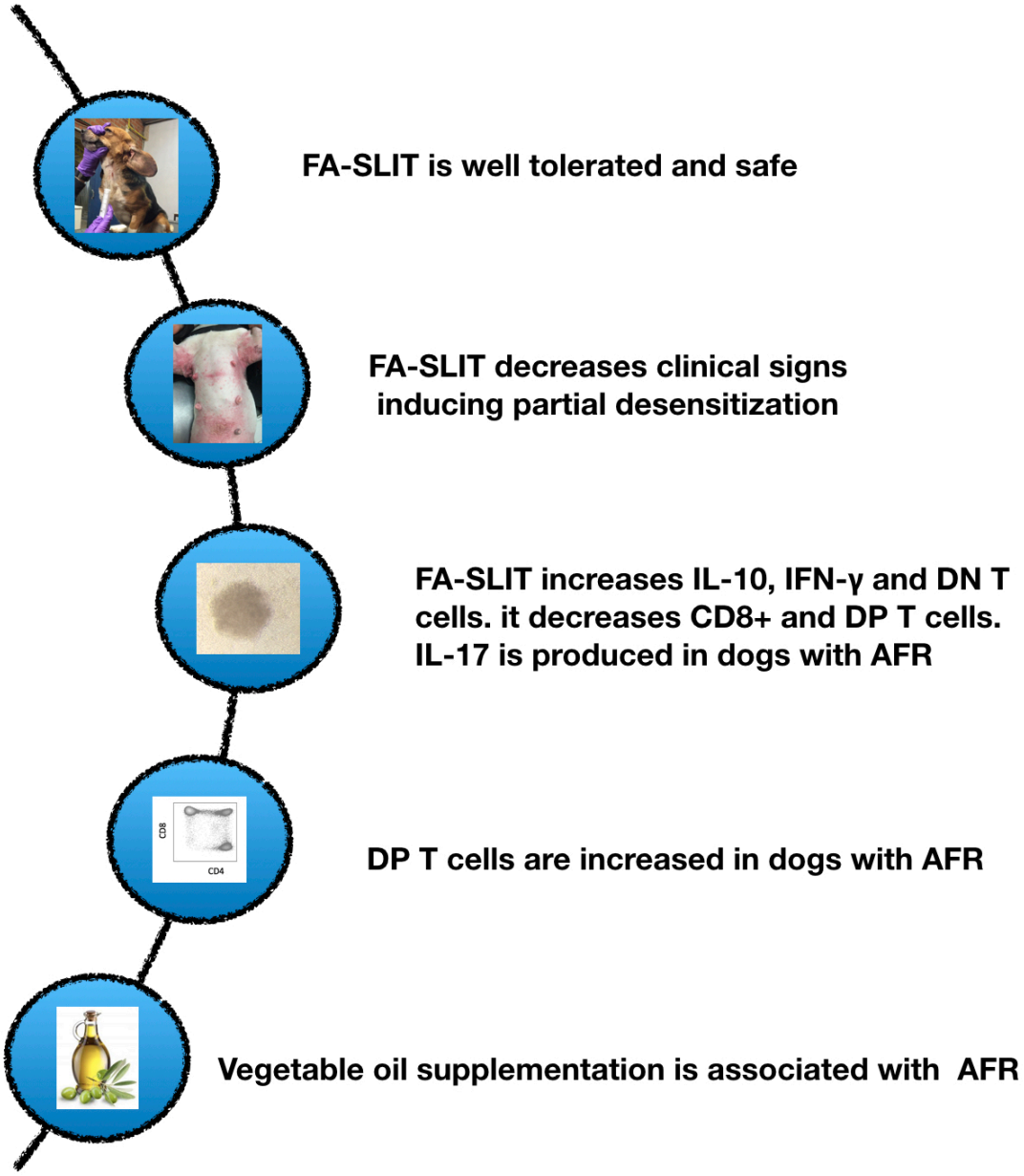


Figure 8.6: Research findings.

8.2. Future perspectives

In the present thesis, we describe the preparation, administration, safety and clinical and immunological efficacy of food-specific sublingual immunotherapy against adverse food reactions in dogs. However, future studies are warranted to establish efficacy in a larger group of dogs. These studies should improve the design of the study, taking into account the findings, weak points and suggestions this thesis has generated. For example, to avoid relapses occurring at the dosage escalation, the improved protocol should last longer allowing to increase the dosage every two weeks by 25% instead of 50-100%. Indeed, by administering a less concentrated solution we did not have any clinical allergic reactions in those dogs that did show allergic reactions with dosage escalations of 50-100%. In addition, in human, a longer protocol correlated with a higher tolerance rate. Moreover, since tolerance or desensitization can be lost when an allergen is avoided too long and this might result in more severe reactions when reintroduced, we suggested to continue the treatment for the rest of life of the patient or, to start feeding the protein responsible of their original problems, directly with the diet once they reach desensitization.

We think it may be interesting for the readers to know that one year after the end of the study one out of five dogs treated with the active treatment (Dog 6 in **Chapter 4**) is able to eat a commercial diet containing the proteins it was sensitized to. These data have not been included in the study because follow up one year after the end of the study was not prospectively designed and not all owners tried to feed small amounts of home-prepared culprit food. One of the observations from our study was that owners did not like the daily administration, therefore, newly designed studies should find a solution to administer allergens less often. For instance, employing buccoadhesive polymers which contain mucoadhesive agents might prolong and improve the contact between allergen and the mucosa (Figure 8.7).

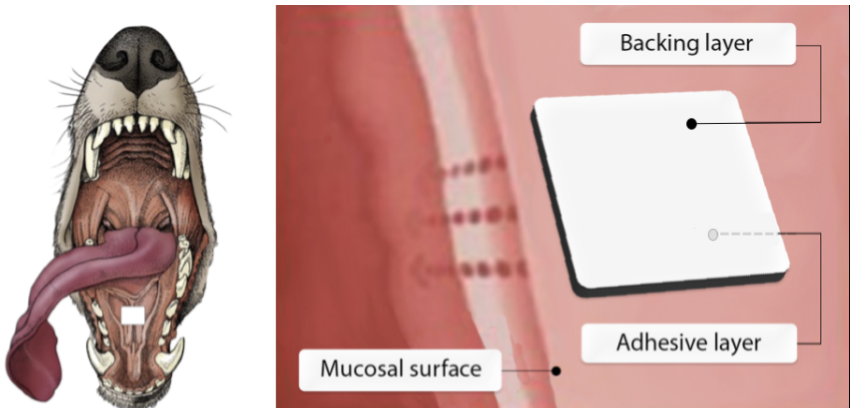


Figure 8.7: Mucosal mucoadhesive delivery system for sublingual immunotherapy.

These systems are interesting for immunotherapy because they can improve the efficacy of FA-SLIT protocols. Indeed, beyond the mucoadhesive polymers which increase the residence time of the allergens in the oral mucosa, they contain a penetration enhancer to improve allergen permeation across the mucosa and enzyme inhibitors to protect the allergens from degradation by mucosal enzymes. Some of these devices have an impermeable backing layer on top of the external surface which protects the allergen destined to be released from being lost due to swallowing. There are different drug delivery systems available. Forms can be either solid, semi-solid or liquid and all of them are already used for drug delivery in the oral cavity. In dogs, patches may be preferred over tablets, gels, ointments, or films because they have the advantage to have an impermeable backing layer, important to control the direction of drug release, prevent drug loss, and minimize deformation and disintegration of the device during the application period. In addition, they have a drug-containing reservoir layer from which the drug is released in a controlled manner and a mucoadhesive surface for mucosal attachment. In future investigations, it might be possible to use this new delivery system to ensure unidirectional diffusion of food allergens towards mucosal surfaces in dogs with adverse food reactions. Furthermore, to potentiate the effect of SLIT and to protect the allergens from oral degradation (when orally administered, antigen is highly exposed to enzymatic or pH-dependent destruction), allergenic extracts or single allergens can be encapsulated within nanoparticles. Poly(anhydride)-nanoparticles loaded with food proteins are an interesting tool to protect the antigen from exposure to extreme pH conditions and offers a controlled release platform that provides an adequate supply of the loaded compound to its site of absorption or action. In addition, these particles can promote tolerance induction being naturally captured by mucosal antigen-presenting cells as part of their duty as

sentinels and by acting as adjuvants to induce Th1 immune responses (Gómez et al. 2006; Gómez et al. 2007; Tamayo et al. 2010). In detail, these poly(anhydride)-nanoparticles triggering TLR2 or TLR4 signalling in oral LCs enhance their capacity to induce IFN-γ secreting Th1 cells as well as IL-10 and/or TGF-β producing Tregs (Moingeon 2012; Moingeon 2013). Furthermore, TLR agonists carried by nanoparticles influence the induction of protective long-term memory cells. (Kasturi et al. 2011; Lycke et al. 2010). Encapsulation of food proteins into poly(anhydride)-nanoparticles has been demonstrated to enhance their immunogenic properties by intradermal and oral immunization in mice (De Souza Rebouças et al. 2012; De Souza Rebouças et al. 2014)

With this thesis, we add further evidence that food-specific IgE is not the main immunopathogenic mechanism in CAFR. On the contrary, our data suggest an important role for CD8+ and double positive T cells. The obtained information could be used to design a novel diagnostic assay to discern food allergy from non-seasonal atopic dermatitis. To improve the predictive value we could decrease the number of false positive reactions. These may be due to the recognition of cross-reactive allergens, such as cross-reactive carbohydrate determinants (CCD), recognised as foreign by the immune system, but responsible for non-clinically relevant reactions. Repeating the experiment with recombinant purified allergens instead of allergen extracts may be helpful to decrease the number of false, not pathogenic reactions. Analysis of our result indicates that only a part of our AFR cases responded to the allergen stimulation. Indeed, the percentage of false negative reactions was high. This led us to question why some dogs reacted too much and why some did not react at all. A delay between sampling and T cell isolation may have decreased the lymphocyte proliferation in those dogs that reacted less. Unfortunately, blood specimens need to be processed very quickly after sampling, ideally within 24 hours which is very difficult when sampling and cell isolation are not performed in the same place. Concerning this, many critical points have to be improved to preserve cell viability such as: sampling, storaging, refrigeration and delivery phase.

Double positive T cells are an interesting population of which we still have to evaluate their surface markers and their cytokine profile in order to understand their origin, activation status and their role in canine allergic conditions. In detail, to better characterize the phenotype and function of CD4+CD8+ DP T cells, canine peripheral

blood cells could be first stained for CD4 and CD8α to distinguish different subgroups such as $CD4^{dim}CD8^{bright}$, $CD4^{bright}CD8^{bright}$, and $CD4^{bright}CD8^{dim}$ (Bismark et al. 2012). After that, the activation status can be assessed by evaluating the CD25 expression. Bismark et al. 2012 demonstrated that the $CD4^{dim}CD8^{bright}$ subpopulation has the lowest and the $CD4^{bright}CD8^{bright}$ subset the highest frequency of CD25+ cells pointing to the $CD4^{bright}CD8^{bright}$ subset as the one with the highest activation status. DP T cells might be also stained for CD44 and CD62L expression to identify naive ($CD44^{low}$ $CD62L^{high}$), central-memory ($CD44^{high}$ $CD62L^{high}$) and effector memory T cells ($CD44^{high}$ $CD62L^{low}$) (Rothe et al. 2017). Finally, expression of IFN-γ and granzyme B, which were found previously only in the $CD4^{dim}CD8^{bright}$ DP T cell subset, will give an indication of the cytotoxic potential of DP T cells (Rothe et al. 2017). We added new insights in the mechanism of immunotherapy, suggesting that double negative T cells and IFN-γ and IL-10 are required for desensitization, however, further work is required to establish if IFN-γ and IL-10 are produced by DN T cells. Moreover, these interesting populations should be better characterized.

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Summary

Summary

The umbrella term “adverse food reactions” is used to describe any undesirable physical responses to an ingested food product. These reactions can be divided into subcategories, such as food intolerances and food hypersensitivity/allergic reactions. AFR is a common and increasing problem in dogs. When an adverse reaction to food occurs, symptoms usually arise a few days later. Symtoms may include cutaneous signs, such as erythema and pruritus, or less frequently, gastrointestinal problems. Since most of the time there is not a temporal relationship between ingestion of a particular food and the onset of symptoms it is often impossible to use these data to diagnose food allergy. In addition, because of the lack of reliable diagnostic tests, results of food challenges in combination with clinical history and exclusion of other differentials are still considered the gold standard for the diagnosis of AFR in dogs.
The current treatment of AFR involves strict elimination of the offending allergen and continuous owner education on how to prevent and respond to accidental ingestion and reactions. Accidental ingestions are likely to occur in dogs due to their nature to eat everything. As a consequence, recurrent symptomatic treatments, such as antipruritic medications and, if secondary infections occur, antibiotics and antimicotic drugs, are needed to control AFR symptoms. In turn, dog owners experience great stress and difficulty in managing the dog’s clinical signs relapses. Altogether, these factors change the quality of life of both the dog and the owner in a profoundly negative way. Therefore, treatment options to cure AFR are needed. A significant amount of research has focused on the use of immunotherapy for the treatment of food allergy in humans. Several different routes for administering the immunotherapy have been studied and among these, SLIT offers a good compromise between safety and efficacy. Since there are no studies about SLIT in dogs with AFR, with this thesis we sought to assess if SLIT is as safe and effective in canine AFR.

Chapter 1 reviews the literature by giving a comprehensive overview of adverse food reactions and allergen immunotherapy. In particular we provided a solid background about the prevalence, pathogenesis, clinical signs, diagnosis, treatment and prognosis of AFR in dogs. We also covered the main milestones in the development of immunotherapy and further described important issues, such as immunology of the

allergic patient and how tolerance develops. In addition, we provided a knowledge on the key mechanisms of immunotherapy and in particular of sublingual immunotherapy.

Chapter 2 describes the aims of the doctoral thesis which were to evaluate the safety and efficacy of food-specific sublingual immunotherapy as a treatment option for AFR in dogs, to understand the immunological mechanisms underlying the efficacy of FA-SLIT and to identify nutritional factors predisposing to AFR.

In **Chapter 3** we investigated for the first time the use of FA-SLIT in a randomized, blinded, placebo-controlled study using healthy dogs. We used peanut extract as active treatment in laboratory beagles never exposed to peanut. Dogs were randomized to receive peanut solution or placebo for 4 months with weekly dosage escalations. FA-SLIT was a well tolerated and safe treatment, as none of the dogs had side effects nor developed sensitization against peanut. On the contrary, FA-SLIT primes the immune system, increasing food-specific IgG toward tolerance induction. We also demonstrated that the administration with a pump-type hooked-dispenser bottle was easy and safe and that the allergen solution extract remained sterile throughout the study. Altogether, these findings suggest that the FA-SLIT protocol might be a suitable treatment to desensitize dogs with AFR.

Chapter 4 is a pilot study assessing the possible use of FA-SLIT in dogs with AFR. Dogs with proven AFR and detected offending allergens were randomized to receive a solution containing the offendig allergen/s or a placebo for 6 months with fortnightly dosage escalations. After the treatment, clinical signs were provoked and dogs that were in the treatment group had milder lesions, less intense pruritus and improved consistency of the stool than those that only received placebo. No severe adverse events were recorded. FA-SLIT exacerbated clinical signs in a minority of the dispensed doses, when the dosage was increased. However, dosage dilution sufficed to control clinical signs allowing to continue the administration of the treatment till the end of the study. Finally, even though the protocol could not induce tolerance but only partial desensitization, we believe this is a relevant result because it might protect dogs from accidental ingestion of small amounts of culprit food.

Chapter 5 probed the immunological mechanisms underlying the efficacy of FA-SLIT in dogs with AFR. PBMCs of dogs included in Chapter 4 were collected during the two

provocative tests and stimulated with the culprit food antigen. Cytokine secretion and the phenotypic profile of T cells of allergen-stimulated PBMCs were assessed by ELISA and flow-cytometry, respectively. FA-SLIT increased the production of IL-10 and IFN-γ, and decreased the percentage of $CD8^+$ and $CD4^+CD8^+$ double positive T cells (DP T cells). Since FA-SLIT is able to induce desensitization, these cells or their progenitors are likely to be involved in the pathogenesis of AFR. The $CD4^-CD8^-$ double negative T cells (DN T cells), reported to act as regulatory cells, increased after the immunotherapy, implying they might have a key role in tolerance induction. We also demonstrated that IL-17A levels were reduced after FA-SLIT, but since this decrease is associated with bacterial overgrowth rather than with clinical lesions or pruritus, it is plausible to speculate that increased levels of IL-17 are a consequence of AFR rather than a cause. We believe IL-17A aggravates and/or perpetuates AFR symptoms in dogs rather than participates in its pathogenesis.

In **Chapter 6**, we investigated the presence of DP T cells in a larger number of dogs with AFR and in healthy and atopic dermatitis dogs. Once stimulated with the offending food allergens, DP T cells did not proliferate in healthy dogs but only in dogs with an allergic phenotype. Interestingly, they proliferated more in dogs with AFR than in those with atopic dermatitis. Even though this proliferative capacity overlaps with results of the gold standard challenge test, it cannot be used to detect culprit allergens because these cells erroneously proliferate in response to food components to which dogs are not sensitized.

Chapter 7 describes a study designed to understand if the prevalence of AFR might be influenced by geographical nutritional lifestyle, such as the use of vegetable oils. We collected data on dietary intake of 459 dogs with skin diseases with a food survey. This showed that n6-PUFA-rich oils in the diet are associated with AFR.

In **Chapter 8**, the general discussion and the future perspectives of our findings are presented. In general, the experiments show that FA-SLIT, applied for the first time in the treatment of AFR in dogs, led to good responses and it is well tollerated and safe. However, data suggest that considerably more work is needed to confirm efficacy, decrease allergic reactions and to reduce the workload of the owner. Moreover, we speculate DN T cells and DP T cells are involved in the tolerance induction and pathogenesis of AFR, respectively. Future research should however confirm our

findings and better characterize their phenotype and function. Finally, we demonstrated that AFR occur more frequently in dogs that have been supplemented with vegetable oils.

Samenvatting

Samenvatting

De parapluterm “adverse food reactions” (AFR) word gebruikt om elke ongewenste fysische reactie tegen voedsel te beschrijven. Deze reacties kunnen onderverdeeld worden in subcategorieën, zoals voedsel intoleranties en hypergevoelige/allergische reacties tegen voedsel.

AFR is een algemeen en stijgend probleem bij honden. Wanneer een ongewenste reactie tegen voedsel optreedt, dan kunnen de symptomen enkele dage later al opkomen. Deze symptomen kunnen huid problemen, zoals erythema en jeuk, of minder frequent gastro-intestinale problemen omvatten. Aangezien in de meeste gevallen er geen relatie is tussen het tijdstip van opname van een bepaald voedsel product en het begin van de symptomen, kunnen deze data meestal niet gebruikt worden om voedsel allergie te diagnosticeren. Omdat betrouwbare diagnostische testen ontbreken worden bovendien de resulaten van een voedsel provocatie test in combinatie met klinische geschiedenis en de uitsluiting van andere differentialen nog altijd als de gouden standaard beschouwd voor de diagnose van AFR in honden.

De huidige behandeling van AFR omvat de strikte onthouding van het voedsel allergeen en de continue opleiding van de eigenaar om toevallige opname van voedsel allergenen te vermijden en te reageren op eventuele reacties. Toevallige opnames van voedsel allergenen komen veelvuldig voor bij honden, aangezien ze van nature op alles kauwen en opeten. Bijgevolg zijn terugkerende symptomatische behandelingen, zoals anti-jeuk medicijnen of, in het geval van secundaire infecties, antibiotica en antimycotica nodig om de symptomen van AFR te behandelen. Hierdoor ervaren de eigenaars veel stress en vinden ze het moeilijk om te gaan met de terugval in klinische symptomen bij de honden. Samen kunnen deze factoren de levenskwaliteit van zowel de honden als de eigenaars negatief beïnvloeden. Daarom is er nood aan behandelingen om AFR te genezen. Veel onderzoek heeft zich gericht op het gebruik van immuuntherapie om voedsel allerieën bij mensen te behandelen. Verschillende toedieningswegen voor deze immuuntherapie werden onderzocht en van deze biedt sublinguale immuuntherapie (SLIT) een goed evenwicht tussen veiligheid en effectiviteit. Aangezien er geen studies bestaan over SLIT bij honden met

AFR, werd in deze doctoraatsthesis onderzocht als SLIT veilig en effectief is in honden met AFR.

Hoofdstuk 1 geeft een uitgebreid overzicht van de literatuur omtrent ongewenste voedsel reacties en allergeen immuuntherapie.in het bijzonder geven een achtergrond informatie over het voorkomen, de pathogenese, de klinische symptomen, de diagnose, de behandeling en de prognose van AFR bij honden. We behandelen ook de belangrijkste mijlpalen in de ontwikkeling van immuuntherapieën en bespreken belangrijke thema's, zoals de immunologie in de allergische patiënt en de ontwikkeling van tolerantie. Daarnaast geven we ook informatie over de sleutel mechanismen van immuunetherapy en sublinguale immuuntherapie in het bijzonder.

In **hoofdstuk 2** beschrijven we de doelstellingen van deze doctoraatsthesis. Deze omvatten de evaluatie van de veiligheid en effectiviteit van voedsel-specifieke sublinguale immuuntherapie als optie om AFR in honden te behandelen, het ontrafelen van de immunologische mechanismen die aan de basis liggen van FA-SLIT en de identificatie van nutritionele factoren die aanleiding kunnen geven tot AFR.

In **hoofdstuk 3** onderzochten we voor de eerste keer het gebruik van FA-SLIT in een gerandomiseerd, geblindeerd en placebo gecontrolleerd experiment in gezonde honden. Pindanoot extract werd gebruikt als actieve behandeling voor laboratorium beagles, die nog nooit aan pindanoot waren blootgesteld. De honden werden gerandomiseerd om pindanoot of placebo te krijgen gedurende vier maanden met wekelijkse dosis stijgingen. FA-SLIT werd goed verdragen en was een veilige behandeling, aangezien geen enkele hond neveneffecten vertoonde en niet gesensitiseerd werd tegen pindanoot. In tegenstelling, FA-SLIT activeerde het immuunsysteem, waarbij de voedselspecifieke IgG moleculen stegen om tolerantie op te wekken. We toonden ook aan dat de toediening van de allergeen extract oplossing met een pomp-type haak-dispenser fles eenvoudig en veilig was en dat deze oplossing steriel bleef gedurende de studie. Samengevat suggereren deze bevindingen dat het FA-SLIT protocol een geschikte behandeling zou kunnen zijn om honden met AFR te desensitiseren.

Hoofdstuk 4 omvat een kleine pilootstudie om het gebruik van FA-SLIT in honden met AFR te onderzoeken. Honden met AFR en gekende allergenen werden gerandomiseerd om een allergeen oplossing of placebo te krijgen gedurende zes maanden met twee-wekelijkse dosis stijgingen. Na de behandeling werden klinische symptomen uitgelokt. Dieren in de behandelingsgroep vertoonden mildere laesies, minder intense jeuk en een verbeterde consistentie van de stoelgang in vergelijking met dieren in de placebo groep. Er werden geen ernstige neveneffecten waargenomen. FA-SLIT verergde de klinische symptomen bij een minderheid van de toegediende dosissen, wanneer deze verhoogde werden. Een verdunning volstond echter om de klinische symptomen te controleren, waardoor de behandeling kon verder gezet worden tot het einde van de studie. Niettegenstaande het FA-SLIT procotol geen tolerantie, maar enkel een gedeeltelijke desensitisatie kon induceren, geloven we dat deze resultaten relevant zijn, aangezien het honden zou kunnen beschermen tegen een toevallige opname van kleine hoeveelheden van het voedsel allergeen.

De immunologische mechanismen die aan de basis liggen van deze gedeeltelijke desensitistie in honden met AFR werden onderzocht in **Hoofdstuk 5**. PBMCs, geïsoleerd na de twee uitlokkende diëten uit bloed van de honden ingesloten in de pilootstudie (hoofdstuk 4), werden gestimuleerd met de voedsel allergenen. Cytokine secretie en het fenotype van de T cellen werden bepaald met respectievelijk ELISA en flow cytometrie. FA-SIT verhoogde de secretie van IL-10 en IFN-g en verlaagde het percentage $CD8^+$ en $CD4^+CD8^+$ dubbel positieve T cellen (DP T cellen). Aangezien FA-SLIT in staat is om een partiële desensitisatie op te wekken, zouden deze cellen of hun voorgangers betrokken kunnen zijn in de pathogenen van AFR. De $CD4^-CD8^-$ dubbel negatieve T cellen (DN T cellen), die als regulatorische cellen kunnen optreden, verhoogden na de immuuntherapie. Dit wijst erop dat deze cellen een sleutelrol zouden kunnen spelen in de inductie van tolerantie. We toonden ook aan dat de IL-17A concentratie verlaagd was na FA-SLIT. Aangezien deze verlaging geassocieerd was met bacteriële overgroei in plaats van klinische laesies of jeuk, speculeren we dat verhoogde IL-17A concentraties een gevolg zijn van AFR eerder dan een oorzaak. We vermoeden dat IL-17A AFR symptomen in honden verergert of bestendigt in plaats van een rol te spelen in de pathogenese.

In **Hoofstuk 6** werd de aanwezigheid van de DP T cellen in een groter aantal gezonde honden, honden met AFR of atopische dermatitis onderzocht. Na stimulatie van de voedsel allergenen prolifereerden enkel de DP T cellen bij honden met een allergie. Bovendien was deze proliferatie sterker bij honden met AFR dan bij honden met atopische dermatitis. Niettegenstaande deze proliferatie capaciteit overeenstemt met de resultaten van de gouden standaard provokatie test, kunnen we deze proliferatie niet gebruiken om voedsel allergenen te identificeren, aangezien deze cellen ook prolifereren na stimulatie met voedsel componenten waartegen de honden niet gesensitiseerd zijn.

Hoofdstuk 7 beschrijft een studie om na te gaan als de prevalentie van AFR beïnvloed wordt door geografische nutritionele levensstijl, zoals het gebruik van plantaardige oliën. Met een voedsel enquete verzamelden we data over het diet bij 459 honden met een huidziekte. Dit toonde aan dat oliën met n6-onverzadigde vetzuren in het dieet geassocieerd waren met AFR.

De algemene discussie en de toekomstperspectieven van onze bevindingen worden beschreven in **Hoofdstuk 8**. De experimenten in de vorige hoofdstukken toonden voor de eerste keer aan dat FA-SLIT veilig is, goed verdragen werd en gebruikt zou kunnen worden om honden met AFR te behandelen. Verder onderzoek is echter nodig om de effectiviteit om allergsiche reacties te verminderen en om de werklast voor te eigenaar te verminderen. Gebaseerd op onze resultaten speculeren we dat DN en DP T cellen betrokken zijn in respectievelijk de inductie van tolerantie en de pathogenese van AFR. Verder onderzoek moet echter onze bevindingen bevestigen en het fenotype en de functie van deze cellen in AFR verder uitdiepen. Als laatste toonden we ook aan AFR meer voorkomt in honden, waarvan het dieet gesupplementeerd was met n6-onverzadigde vetzuren.

Curriculum Vitae & Bibliography

Curriculum Vitae

Elisa Maina was born on June 6th, 1981 in Lovere, Italy. In 2008, Elisa was graduated from the Faculty of Veterinary Medicine, Milan university, Italy. She worked as a generalist until 2010 discovering that her main area of interest was dermatology. Therefore, in the same year, she followed the course of dermatology of the European School of Advanced Veterinary Studies ESAVS (Austria). In the same year, she completed a dermatology externship at the College of Veterinary Medicine, University of Florida in 2010 and then followed a three-years Residency program in Dermatology (Italy) and became European Diplomate in Veterinary Dermatology (Dip ECVD) (European Diploma of Specialist in Veterinary Dermatology) in 2015. In 2012, he was awarded a BOF Grant of four years from UGent. In November 2012, Elisa was enrolled for a four-years PhD in the Laboratory of Immunology, at the Faculty of Veterinary Medicine of Ghent University, where she studied food allergy in dogs. She completed three-years of small Animal Pathology and Clinical Medicine School of Specialization, graduating with honours in 2018 at the Faculty of Veterinary Medicine, Milan. She is currently working as a referral dermatologist in Switzerland and in Italy. She has published papers in reputed journals and presented in national and international meetings.

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