The Effect of Ketoconazole on Blood and Skin Cyclosporine Concentrations in Canines

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By
Laura Leigh Gray

Graduate Program in Comparative and Veterinary Medicine

The Ohio State University
2012

Master's Examination Committee:
Dr. Andrew Hillier, Advisor
Dr. Lynette Cole
Dr. Paivi Rajala-Schultz
Copyrighted by
Laura Leigh Gray
2012
Abstract

Cyclosporine (CSA) (Atopica®; Novartis Animal Health, Greensboro, NC) is approved for treatment of canine atopic dermatitis (CAD). Cyclosporine is metabolized in the liver by cytochrome P450 enzymes, a process inhibited by ketoconazole (KTZ). Thus, concurrent administration of CSA and KTZ potentially reduces the dose of CSA required to maintain therapeutic blood and tissue concentrations in CAD. Specific aims were to determine skin and blood CSA concentrations when CSA was administered alone at recommended and subtherapeutic doses, and when administered at subtherapeutic doses concurrently with KTZ. We hypothesized that CSA skin and blood concentrations at the recommended dose of CSA (5 mg/kg) alone would not differ significantly from subtherapeutic CSA dosing (2.5 mg/kg) concurrently with KTZ (2.5 mg/kg or 5.0mg/kg).

In a randomized cross-over study design, six healthy research hounds received each of the following four treatments once daily for 7 days followed by a 14-day washout:  CSA 5.0 mg/kg (Treatment 1 [T1]); CSA 2.5 mg/kg (T2); CSA 2.5 mg/kg + KTZ 5.0 mg/kg (T3); and CSA 2.5 mg/kg + KTZ 2.5 mg/kg (T4). After the 1st, 4th and 7th dose of CSA or CSA/KTZ for each treatment, a skin sample (8mm-punch biopsy) was collected 4 hours (peak) and 24 hours (trough) after dosing to determine skin CSA concentrations, and a blood sample was collected 1.4 hours (peak) and 24 hours (trough) after dosing to determine whole blood CSA concentrations. CSA concentrations were
quantified by high-performance liquid chromatography tandem mass spectrometry. Data were analyzed using repeated measures approach with PROC MIXED in SAS (SAS Inst. Inc. Cary, NC). Pairwise comparisons between days and treatments were performed by obtaining least squares means and Tukey-Kramer adjustment for multiple comparisons. Correlation between skin CSA and blood CSA concentrations was assessed using Spearman Correlation Coefficients.

Mean blood CSA concentration in T1 (307.5 ng/ml) was not significantly different from T2 (169.41 ng/ml, P=0.287), or T4 (417.74 ng/ml, P=0.136), but was significantly less than T3 (644.83 ng/ml, P=.0002). Mean skin CSA concentration in T1 (0.6 ng/mg) was significantly greater than T2 (0.262 ng/mg, P=0.05), not significantly different from T4 (0.697 ng/mg, P=0.895), and significantly less than T3 (1.236 ng/mg, P=.0006). Correlation between blood and skin CSA concentrations was moderate across treatment groups (r=0.6679). There was no significant difference in the mean peak blood CSA concentration after the 1\textsuperscript{st} dose compared to the 7\textsuperscript{th} dose in any treatment group. There was a significant increase in mean peak and trough skin CSA concentrations from the 1\textsuperscript{st} dose to the 7\textsuperscript{th} dose in all treatment groups [e.g. T1 mean peak after 7\textsuperscript{th} dose (1.06 ng/mg) was significantly greater than mean peak after 1\textsuperscript{st} dose (0.272 ng/mg) P=.0001].

As there was no difference in mean blood or skin CSA concentrations when CSA was dosed at 5.0mg/kg once daily (T1) compared to when CSA was dosed at 2.5mg/kg once daily with 2.5mg/kg of KTZ once daily (T4), it is anticipated that similar clinical outcomes would occur with either dosing regimen.
Dedicated to John and Alice Gray for their love and support.
Acknowledgments

I would like to sincerely thank Dr. Andrew Hillier, Dr. Lynette Cole, and Dr. Wendy Lorch for creating and maintaining such a wonderful dermatology residency program. The tireless dedication to teaching and devotion to practicing the highest quality of dermatology exemplified by each one of you is what makes this residency program beyond compare. As a resident I have received endless support, instruction, and guidance in order to mold me into the most capable dermatologist possible. I will never forget to critically evaluate literature and the value of practicing evidence based medicine. I would especially like to thank my advisor Dr. Andrew Hillier for being a wonderful mentor and advisor – providing countless hours of advice and endless corrections that have improved my own attention to detail. I would like to extend an additional thanks to the co-authors and thesis committee members (Dr. Andrew Hillier, Dr. Lynette Cole, and Dr. Paivi Rajala Schultz) for their contributions towards our research/paper as well as their guidance and support during the process of thesis preparation and defense. I would also like to thank Natalie Tabacca, Michele Fox, Holly Roberts and Deb Crosier for their friendship and support.
Vita

2002..........................B.S. Nursing, Clemson University

2008..........................D.V.M., University of Georgia

2009 to present ..................Graduate Teaching and Research Associate,

The Ohio State University

Fields of Study

Major Field: Comparative and Veterinary Medicine

Studies in Dermatology
Table of Contents

Abstract..............................................................................................................ii

Dedication .........................................................................................................iv

Acknowledgement ..........................................................................................v

Vita .................................................................................................................vi

List of Tables ....................................................................................................ix

List of Figures .................................................................................................x

CHAPTER1 Introduction ..................................................................................1

CHAPTER 2 Literature review ........................................................................5

2.1 Atopic dermatitis .....................................................................................5
   2.1.1 Definition and pathogenesis of canine atopic dermatitis .............5
   2.1.2 Clinical signs and diagnosis .......................................................10
   2.1.3 Treatment ..................................................................................12
       2.1.3.1 Allergen specific immunotherapy ....................................12
       2.1.3.2 Glucocorticoids .................................................................14
       2.1.3.3 Essential fatty acids .........................................................17
       2.1.3.4 Antihistamines .................................................................18
   2.2 Cyclosporine in humans .......................................................................19
       2.2.1 Mechanism of action .............................................................19
       2.2.2 Uses .....................................................................................20
       2.2.3 History and formulation .....................................................21
       2.2.4 Pharmacokinetics .................................................................23
           2.2.4.1 Absorption ................................................................23
           2.2.4.2 Distribution .................................................................24
           2.2.4.3 Metabolism and elimination ....................................27
           2.2.4.4 Drug interactions .......................................................28
           2.2.4.5 Intra- and inter-patient variability of cyclosporine
absorption and pharmacokinetics………………………………..29
2.2.4.6 Adverse effects…………………………………………..31
2.2.4.7 Monitoring……………………………………………….34
2.3. Cyclosporine in canines………………………………………38
   2.3.1 Pharmacokinetics………………………………………38
      2.3.1.1 Absorption, distribution, metabolism, and elimination…38
      2.3.1.2 Drug interactions…………………………………….40
      2.3.1.3 Adverse Effects………………………………………40
      2.3.1.4 Monitoring…………………………………………41
2.4 Cyclosporine use in canine atopic dermatitis…………………42
2.5 Pharmacotherapeutic manipulation of cyclosporine concentrations…44
   2.5.1 Humans ……………………………………………………44
   2.5.2 Canines……………………………………………………45

CHAPTER 3  The effect of ketoconazole on blood and skin cyclosporine concentrations canines…………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………
List of Tables

Table 1. Cytochrome P450 substrates in humans and canines…………………………58

Table 2. Target and actual dosage range of cyclosporine administered solely or in combination with ketoconazole to six healthy hound mixes…………………………70

Table 3. Descriptive statistics for cyclosporine concentrations in whole blood and skin..............................................................................................................71

Table 4. Least squares means obtained from the mixed models for daily peak and trough whole blood cyclosporine concentrations......................................................72

Table 5. Least squares means obtained from the mixed models for daily peak and trough skin cyclosporine concentrations for six dogs................................................................73
List of Figures

Figure 1. Mean cyclosporine (CSA) concentrations in whole blood for six dogs for Treatments 1-4.................................................................84

Figure 2. Mean cyclosporine (CSA) concentrations in skin for six dogs for Treatments 1-4........................................................................85

Figure 3. Cyclosporine (CSA) concentrations in whole blood for six dogs during Treatment 3.................................................................86

Figure 4. Cyclosporine (CSA) concentrations in skin for six dogs during Treatment 3...87
Chapter 1: Introduction

Canine atopic dermatitis (CAD) is currently defined as “a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens”[1]. The pathogenesis of CAD is complex and likely multifactorial including but not limited to genetic/inherited predisposition, environmental allergen exposure, immunological abnormalities, abnormal barrier function, food, infection, self-trauma, and neuromediators. Though the true incidence and prevalence of CAD is unknown, reported estimates range from 3-15% [2]. Treatment for CAD is most often multi-faceted and focused on controlling the primary allergic response of CAD as well as symptomatic treatment for the relief of pruritus and control of secondary infections; with the most effective treatment options consisting of allergen-specific immunotherapy, Atopica® (cyclosporine) administration, and symptomatic treatment (low-dose corticosteroid, antihistamines, fatty acid supplementation, treatment/prevention of secondary infection, and a large spectrum of topical preparations) [3]. Treatments must be continued in some capacity for the life of the patient as CAD is a disease that is not cured but controlled.

Cyclosporine is a potent immunosuppressive and immunomodulatory agent, particularly for inflammatory and immune-mediated processes involving T cell activation and proliferation. Cyclosporine’s initial and current primary use in humans is for
prevention of rejection in solid-organ transplant recipients, whereas the primary use in canines is for the treatment of CAD[4, 5]. Cyclosporine is a lipophilic molecule that is generally poorly absorbed when administered orally, with an estimated bioavailability of approximately 30% with the modified micro-emulsified formulation [6]. Once absorbed, cyclosporine binds extensively to erythrocytes, leukocytes, and plasma proteins with extensive absorption into tissues, particularly in skin and fat [7-10]. Cyclosporine’s oral bioavailability and metabolism are primarily controlled by the cytochrome P450 enzyme system and the P-glycoprotein (PGP) efflux pump. Though both of these are present in smaller amounts in the small intestine and kidney where there is a small degree of metabolism and elimination, the primary location of metabolism is the liver[11, 12]. Nephrotoxicity, both acute and chronic, is generally regarded as the most serious adverse event associated with CSA administration in humans[13]. When used for the treatment of CAD; vomiting, diarrhea, and soft stools are the most commonly reported adverse events, occurring in 15-25% of patients[14]. Due to CSA’s potential for toxicity and narrow therapeutic index in humans for prevention of solid-organ transplantation rejection, appropriate monitoring of CSA is crucial. Adjustment of CSA dosing to achieve target peak concentrations based on sampling at 2 hours (C2) has been shown to decrease acute rejection and is therefore considered the best method [15-17]. Monitoring CSA levels in CAD is generally not recommended, though may be useful in the event of adverse reactions or perceived lack of efficacy[18, 19].

The use of CSA for the treatment of CAD and treatment for prevention of transplant rejection with CSA both require life-long therapy. As CSA can be cost
prohibitive, cost-saving measures are often explored. In humans ketoconazole has proven to be an effective and safe method for reducing the cost of CSA administration [20, 21]. The potency of KTZ inhibition of CSA is two-fold[22]. Ketoconazole in addition to being a potent inhibitor of the CYP 3A4 enzyme system, is also an inhibitor of the P-glycoprotein pump which allows up to a 70% reduction in CSA administration [23, 24]. Though itraconazole is not as potent of an inhibitor of the P450-enzyme system as ketoconazole, it has also been shown to increase CSA concentrations in transplant recipients and provide a cost reduction of 30% [22, 25]. Diltiazem, a calcium channel blocker and inhibitor of CYP 3A4, has also been used to pharmacotherapeutically increase the concentration of CSA in transplant recipients [26-29]. Grapefruit juice also inhibits the CYP 3A4 enzyme system, resulting in an increase in CSA concentrations[30]. In canines, ketoconazole has also been shown to be a potent inhibitor with capability of reducing CSA dosing by as much as 80%, with the majority of this data being generated from perianal fistula studies[31-34]. Metoclopramide and cimetidine have proven ineffective for increasing CSA concentrations, and grapefruit extract has shown variable response [35-37]. To the author’s knowledge, there are no published reports on the use of KTZ as a cost-saving measure in dogs with CAD treated with CSA, although it is frequently advocated for this purpose [38-42]. The specific aims of this research were to determine skin and whole blood CSA concentrations when CSA was administered alone at the recommended (5.0 mg/kg/day) and the sub-therapeutic (2.5 mg/kg/day) dose, and when administered at the sub therapeutic (2.5 mg/kg/day) dose concurrently with KTZ at two different doses (2.5 mg/kg/day or 5.0 mg/kg/day). We hypothesized that when CSA
was administered alone at the recommended label dose (5.0 mg/k/day), the skin and whole blood CSA concentrations would not differ significantly from those obtained with sub-therapeutic CSA dosing (2.5 mg/kg/day) concurrently with either dose (2.5 mg/kg/day or 5.0 mg/kg/day) of KTZ.
Chapter 2: Literature Review

2.1 Canine Atopic Dermatitis

2.1.1 Definition and Pathogenesis of Canine Atopic Dermatitis

Canine atopic dermatitis (CAD) is currently defined as “a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens”[1]. Patients with signs clinically indistinguishable from CAD that lack demonstrable IgE antibodies are assigned the diagnosis of atopic-like dermatitis (ALD)[1]. The pathogenesis of CAD is complex and likely multifactorial including but not limited to genetic/inherited predisposition, environmental allergen exposure, immunological abnormalities, abnormal barrier function, food, infection, self-trauma and neuromediators. Though the true incidence and prevalence of CAD is unknown, reported estimates range from 3-15%[2]. Due to a variety of postulated causes including increased urbanization, higher indoor allergen loads, exposure to infection at an early age, dietary changes, and others; the prevalence of atopic dermatitis (AD) in humans has risen in the past few decades estimated to exceed 30% of the population of developed countries[2]. As canine environments may evolve with those of their human counterparts, it is possible
that the prevalence of CAD may be on the rise as well. Though studies vary according to geographical region, CAD and flea allergy dermatitis are reported as the most common causes of canine pruritus[2].

The heritability of CAD appears to be multifactorial. CAD was initially believed to have a genetic component due to the fact that clinically certain breeds seemed to be more affected[43]. Earlier studies investigating the genetic component of CAD focused on heritability of genes responsible for production of IgE, though it is known that IgE elevation also occurs in clinically non-atopic canines and is not diagnostic for CAD [43]. A more recent microarray analysis of mRNA expression compared lesional and non-lesional skin of dogs with CAD to that of healthy controls and found 54 genes significantly differentially expressed in dogs with CAD versus controls[44]. These genes correlated with a multitude of functions including innate immune function, inflammatory cell responses, cell cycle apoptosis, barrier function and transcriptional regulation; some of these gene expression alterations mimicked those known to be abnormal in human AD[44]. A subsequent study evaluating gene expression in CAD evaluated correlation of some of the genes detected with clinical severity scores and positive reactions on intradermal allergy test (IDAT)[45]. Serum amyloid A1, S100 calcium binding protein A8, and plakophilin 2 were found to correlate with CADESI-03 scores; and mast cell protease I, serum amyloid A1, S100 calcium binding protein A8, and serine protease inhibitor kazal type 5 were found to correlate with IDT results[45]. However, further comparison of gene expression for CAD showed that breed as well as geographic location resulted in significant variation of gene expression[46].
Until recently, a direct cause and effect relationship between environmental allergen exposure and the pathogenesis of CAD had not been proven[47]. Presumed relevant environmental allergens included house dust and storage mites, pollens (trees, grasses, and weeds), mold spores, epidermal allergens, and insect allergens. The pathogenic role of environmental allergens in CAD was assumed to be relevant due to the fact that a large number of published studies and clinical experience documented CAD patients with positive reactions on intradermal tests (IDT) and elevated levels of serum IgE to environmental allergens[47]. Additional support for the pathogenesis of environmental allergens in CAD is suggested by resolution or improvement of clinical signs while on allergen-specific immunotherapy (ASIT). [47]. However, investigation of a particular allergen’s relevance in CAD by comparison of IDT results between patients is hampered by the lack of standardization of allergen extracts, use of allergens produced by multiple companies, variation of batches within companies, false positives and false negatives on IDT, and the fact that the concentration of allergens used in IDT is not standardized[47]. Similar challenges of the lack of standardization occurs with serologic testing for allergen-specific IgE, specifically standardization of the allergen extracts, accuracy/variation of detection reagents, lack of gold standards for quality control, and false positive/false negatives. Likewise investigation of the relevance of allergens based on response to ASIT is problematic due to lack of standardization in allergen extract used to formulate ASIT and the fact that ASIT most commonly includes administration of many allergens simultaneously rather than administration of single allergens[47].
Recent studies have established a more clear cause and effect relationship between allergen exposure and development or flares of signs indicative of CAD. A model for CAD using high-IgE Maltese/beagle dogs showed that epicutaneous application of *Dermatophagoides farinae* (Df) sonicated slurry induced clinical, serological, and microscopic changes mimicking spontaneous CAD[48]. A similar study showed that high-IgE producing beagles exposed to Df epicutaneously developed signs similar to spontaneous CAD, normal dogs did not react to epicutaneous application of Df, and dogs with known mite-sensitive CAD flared after epicutaneous mite application[49]. Additionally, a similar study with the same high-IgE producing beagles showed that oral, systemic, and epicutaneous exposure to Df all lead to clinical signs of CAD, although epicutaneous exposure may be the most important[50].

The integrity (or lack thereof) of the epidermal barrier contributing to epicutaneous allergen exposure has also been an area of investigation for CAD, likely due to recent evidence of its importance in human AD[51, 52]. Mutations in a gene encoding expression of a filament aggregating protein called filaggrin, a component of the epidermal barrier mainly responsible for keratohyalin granules in the outer nucleated layers of the epidermis, has been correlated with development of AD in humans [52, 53]. Evidence is beginning to mount that lipid defects of the epidermal barrier may be important for the development and/or progression of CAD [54-57].

Both the innate as well as the adaptive immune system (IgE) play an important role in the development and perpetuation of CAD, the details of which are beyond the scope of this review. Briefly, the innate immune system is very important as a first line
of defense against pathogens, and numerous defects in the innate immune system have been linked with AD in humans[58]. These defects in the innate immune response promote inflammation directly; in addition they predispose towards infection which can further exacerbate AD[58]. Research in CAD on the importance of these defects in the innate immune response has lagged that in human AD. Currently antimicrobial peptides are the most explored aspect of this subject in CAD, particularly β-defensins and cathelicidins. However, recent reports in humans provide contradicting evidence of whether these AMP’s are increased or decreased in AD[59]. Early studies showed a decrease in AMP’s associated with AD[58], but a more recent study has contraindicated those results and suggested that implication of AMP’s in AD as well as staphylococcal colonization warrants further research[59]. A recent study on these AMP’s in CAD showed an increased expression of some canine β-defensins and canine cathelicidin in lesional, non-lesional, and challenged CAD epidermis when compared to healthy controls[60], which was similar to findings in the most recent human study[59]. Conflicting results are likely due to the multifactorial etiology of the development and pathogenesis of AD.

Defects in the innate immune system, paired with epidermal barrier defects, and well as abnormalities in the acquired immune system are all intertwined[61]. T-cells (acquired immune system) are divided into two response patterns: cell-mediated Th1 response associated with IL-2, IL-12, Y-IFN, and IL-18; and humoral Th2 response associated with IL-4, IL-5, II-6, and IL-13, and IgE-antibody production. A Th2 response during the acute phase of AD, followed by a Th1 response in the chronic phase of AD has
been suggested. A simplified description of the possible pathogenesis of CAD is presented[61, 62]: Abnormal epidermal barrier allows for increased allergen (environmental and microbial) penetration allowing increased contact with IgE antigen specific epidermal antigen presenting cells, which travel to a local lymph node where they present processed antigen to T helper lymphocytes. Dogs with CAD may be genetically predisposed to have a Th2 dominated response. IgE synthesis is promoted by Th2 lymphocyte associated cytokines which also promote eosinophil survival, a source of further inflammatory mediators. Keratinocytes, in response to microbes and Langerhans cells, are activated to release inflammatory cytokines and chemokines. Mast cells armed with allergen-specific IgE degranulate on exposure to allergen, releasing inflammatory proteases, histamine, chemokines, and cytokines and a resultant influx of neutrophils, eosinophils, Th2 lymphocytes, and dendritic cells. Continued inflammation and pruritus secondary to inflammatory mediators/cytokines in addition to recurrent secondary infections may lead to a Th1 response during the chronic phase.

2.1.2 Clinical Signs and Diagnosis

There is no specific and accurate diagnostic test for CAD. Rather, diagnosis is based on a collection of historical fact and clinical signs and symptoms characteristic of AD as well as exclusion of other pruritic disease (cutaneous adverse food reaction, ectoparasitosis, bacterial and yeast infections) [62, 63]. Clinical signs primarily consist of pruritus starting at an early age (6 months to 3 years) involving the face, ears, paws, ventrum (particularly folded regions like axillae and inguinal regions) as well as recurrent bacterial and yeast skin and ear infections [64-67]. A set of diagnostic criteria amended
from that established for humans was published in 1986 by Willemse. A subsequent set of diagnostic criteria specifically for CAD came from Prelaud in 1998 after attempting to verify those established by Willemse, and consisted of five possible criteria with combination of any three leading to a sensitivity and specificity for the diagnosis of CAD of 79% and 81% respectively[68]. The criteria were as follows: Onset of signs between 6 months and 3 years, glucocorticoid-responsive pruritus, bilateral anterior interdigital erythematous pododermatitis, erythema of the concave ear pinna, and cheilitis. In 2010, Claude Favrot et al evaluated a large population of geographically diverse dogs in a prospective fashion to evaluate the sensitivity and specificity of the criteria established by both Prelaud and Willemse, and to determine if another set of criteria may be established[66]. This study established two new sets of criteria. Set 1 included: a) age of onset <3 years b) most time spent indoors c) corticosteroid-responsive pruritus d) chronic or recurrent yeast infections e) affected front paws f) non-affected ear pinnae g) non-affected ear margins h) non-affected dorso-lumbar area. A combination of 5 of the previous criteria had a sensitivity and specificity of 85% and 79%, respectively for differentiating non-AD from CAD[66]. The second set removed the corticosteroid criteria as that is somewhat subjective, and had a similar sensitivity (77%) and specificity (83%) with 5 of the criteria being filled. The sensitivity/specificity in Favrot’s study of Willemse’s criteria and Prelaud’s criteria were found to be 49%/80% and 74%/68% respectively. Additionally, Favrot’s study confirmed previous study results that the clinical presentation of canines with cutaneous adverse food reaction, referred to as food-induced atopic dermatitis in this study, was indistinguishable from that of CAD[67, 69].
Of note is the fact that if only Favrot’s set of criteria is used for the diagnosis of CAD, one in five patients will be misdiagnosed[70]. Therefore it is the recommendation of the International Task Force of Canine Atopic Dermatitis that Favrot’s criteria be used in the clinical setting as an aid in diagnosis while ruling out all other pruritic diseases and in a research setting to establish a uniform set of inclusion criteria[70].

2.1.3 Treatment

Treatment for CAD is most often multi-faceted and focused on controlling the primary allergic response of CAD as well as symptomatic treatment for the relief of pruritus and control of secondary infections[3]. Treatments must be continued in some capacity for the life of the patient as CAD is a disease that is not cured but controlled. General categories of treatment include: allergen avoidance (though this is rarely feasible or practical), skin barrier protection, controlling inflammation, ASIT, and controlling secondary infections[3]. The following sections will address known evidence for use and efficacy of the main categories of individual treatments for CAD. Cyclosporine for treatment of CAD will be discussed in depth in section 2.7.

2.1.3.1 Allergen Specific Immunotherapy (ASIT)

Allergen specific immunotherapy is defined as administration of increasing concentrations of relevant allergen extracts in order to decrease the allergic response of an individual to those allergens on subsequent exposures [71, 72]. Currently ASIT is administered via injection; oral (sublingual) allergen administration is still in experimental phases in canines[71, 73]. Indications for use of ASIT in CAD include
demonstration of elevated IgE via IDT or via serology for allergens that are pertinent to the patients’ disease seasonality, and where owners can administer and afford this therapeutic [72]. Though the exact mechanism of action of ASIT in human medicine is unknown, there is evidence as to its effect on antigen presenting cells, T cells, B cells, and number/function of effector cells which mediate the allergic response[71, 72]. Thus far, it has been shown that ASIT in canines may shift immune response to allergens to a Th1 response by increasing production of IFN-ϒ [74], and that ASIT increases production IL-10 with an increase in the percentage of Treg cells[75].

Evidence on the efficacy and mechanism of action of ASIT in veterinary medicine has largely been generated from uncontrolled and open studies generating data that is difficult to compare across studies[71, 76]. As mentioned previously, the lack of standardization for preparation and schedule of administration for ASIT makes comparison of studies especially challenging. Reported efficacy ranges from 50-100%[71]. The one prospective randomized placebo controlled study on the efficacy of canine ASIT did report a greater than 51% decrease in clinical signs in 59% of patients treated with ASIT and 21% with placebo, though is lacking in details of data and the results are questionable as 4/5 placebo treated dogs had complete remission of their disease[77]. A randomized double-blinded study comparing ASIT administered conventionally and rush ASIT found that a greater than 50% reduction in pruritus was observed in 45% of patients treated conventionally and 55% of dogs treated with rush ASIT [78]. One study has shown the importance of combination allergen specific therapy for the treatment of CAD as administration of one allergen alone
(Dermatophagoides farinae) in dogs with multiple IgE positives did not differ in efficacy from placebo[79]. Despite the lack of prospective blinded controlled studies, it is widely accepted that ASIT is an effective therapeutic for treatment of CAD [71, 72].

Reported side effects of ASIT in the canine literature consist primarily of an increase in pruritus both at the site of injection as well as generalized with reported incidence ranging from 5-25%[71, 72]. These frequently occur with an increase in the allergen concentration or frequency of administration, highlighting the importance of patient re-evaluation during immunotherapy and patient specific optimization of ASIT[72]. Systemic adverse reactions have been reported in approximately 1% of patients and include weakness, depression, lethargy, anxiety, sleepiness, diarrhea, vomiting, panting, urticaria/angioedema, collapse, and anaphylaxis[72]. Reported time to efficacy ranges from 2-12 months, and thus ASIT should be administered for at least 12 months before assessing the patients’ full response [72]. Though there are anecdotal reports of patients’ clinical disease remaining controlled after discontinuation of ASIT, these are sporadic and not part of a controlled study. As such, it is likely that ASIT if effective will be a life-long treatment[72].

2.1.3.2 Glucocorticoids

Glucocorticoids fall into the category of anti-inflammatory drugs for the treatment of atopic dermatitis. Glucocorticoid mechanism of action is primarily via its effects on gene transcription regulated by an intracellular receptor (GR) which is located in almost every tissue of the body[80]. Glucocorticoids activate GR which is then translocated into the nucleus where it recruits co-factor complexes and functions as a gene transcription
factor to influence (positively or negatively) transcription of targeted genes[80]. Of particular interest for the treatment of AD is the targeted suppression of a wide variety of pro-inflammatory proteins: IL-1B, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-18, Cyclooxygenase-2, E-selectin, inducible NO synthase, IFN-γ, TNF-α, intercellular adhesion molecule, monocyte chemo attractant protein 1, and vascular cell adhesion molecule[80]. In addition to suppression of genes that produce pro-inflammatory mediators, glucocorticoids also activate anti-inflammatory genes such as lipocortin-1 which inhibits phospholipase A2. Phospholipase A2 is an important enzyme responsible for the production of arachidonic acid, the precursor for many potent inflammatory mediators such as prostaglandins, prostacycline, thromboxanes, and lipoxygenase generated leukotrienes [80, 81].

Benefits of the use of glucocorticoids for the treatment of AD include their cost, effectiveness and efficacy. The majority of studies evaluating the efficacy of therapeutics for treatment of CAD employ glucocorticoids as the control or standard of care [81, 82]. The fact that glucocorticoids are used as a control implies their overall efficacy for controlling CAD. Specifically, one study compared two doses of oral prednisone with arofylline. Both the high dose of prednisolone (0.5mg/kg twice daily for a week, then once daily for a week, then every other day for two weeks) and the low dose of prednisolone (0.25mg/kg dosed similarly) significantly decreased pruritus within a week, with the total percentage reduction in pruritus at the end of the trial being 48% and 56% respectively [81, 83]. Methylprednisolone at an initial dose of 0.75mg/kg once daily was compared to CSA 5 mg/kg once daily in a blinded controlled study[84]. Both treatments
were found to significantly reduce pruritus by 36% (CSA) and 33% (methylprednisolone) and lesion score by 52% and 45%, with no statistically significant difference between the two groups. An additional blinded controlled study comparing prednisolone and CSA reported similar efficacy for treatment of CAD with glucocorticoids[85]. Topical glucocorticoids in the form of a 0.015% triamcinolone spray have been evaluated in one placebo controlled study for treatment of pruritus (not necessarily due to CAD). Sixty-nine percent of patients treated with the active ingredient had a greater than 50% reduction in lesion and pruritus scores[86]. An additional study on topical glucocorticoids in the form of 0.0584% hydrocortisone aceponate spray reported similar efficacy when compared to placebo, without changes in biochemical, complete blood count, or adrenal axis status after 70 days of spray administration and no apparent change in skin thickness after 56 days of administration [87]. When this same topical formulation (.0584% hydrocortisone aceponate spray – trade name Cortavance®) was compared with CSA in dogs with CAD in a single blinded randomized controlled clinical trial, there was no difference in the efficacy of pruritus control between the CSA group and the Cortavance® group[88].

Short, medium and long term side effects are the limiting factor for administration of glucocorticoids. The degree of side effects are typically dose, duration and patient dependent and include polyuria, polydipsia, polyphagia, obesity, alopecia, skin-thinning, hepatopathy, hyperglycemia, gastric ulceration, recurrent infections (skin and urinary tract), calcinosis cutis, and iatrogenic Cushing’s disease.
2.1.3.3 Essential Fatty Acid Supplementation

Essentially fatty acids (EFA) have multiple immunomodulatory and anti-inflammatory properties, which may contribute to their anti-inflammatory properties observed in the treatment of CAD. EFAs are classified as Omega-3 fatty acids (\(\alpha\)-linolenic acid, eicosapentanoic acid, and docosahexanoic acid) and Omega-6 (\(\gamma\)-linoleic acid and linoleic) fatty acids. Specific research on their mechanism of action in CAD still needs to be explored, although this has been documented in human literature. Identified mechanisms include modulation of production of cutaneous inflammatory prostaglandins and leukotrienes, decreased synthesis of inflammatory cytokines (such as IL-1, tumor necrosis factor, IL6, IL-2, and T-lymphocyte proliferation), as well as altering the epidermal lipid barrier resulting in decreased trans-epidermal water loss[89]. Though fatty acid supplementation has been used for the treatment of CAD since 1987, the vast majority of initial studies lacked controls and randomization and included a limited number of patients, making the determination of their efficacy challenging[89]. Since the CAD Task Force review in 2001, there have been further studies evaluating EFA supplementation for CAD. One in vitro study was unable to demonstrate changes in cytokine expression[90]. A pilot study showed that oral EFA containing Omega-6 and Omega-3 FA’s increased the free and protein bound lipid content found in the skin of dogs with CAD compared to controls[56]. A double-blinded placebo controlled study demonstrated that pruritus and lesional scores significantly decreased with administration of flax-seed oil as well as with a commercially available EPA/DHA supplementation in dogs with CAD[91]. There was no correlation of this improvement to total EFA dose nor
Omega 6 to 3 ratio[91]. Likewise, another double-blinded placebo controlled study compared administration of borage seed oil (Omega 6) and fish oil (Omega 3) to a placebo and found that this EFA combination had a corticosteroid sparing effect compared to placebo[92]. There has been limited investigation into topical application of EFAs (via a spot-on formulation) to date with no definitive indication of positive effect [93]. Thus there is still a lack of evidence for EFA dosage, ratio, or formulation that may provide the most (or any) benefit for treatment of CAD. EFA supplementation does seem to improve clinical signs associated with CAD, though it is unlikely to provide significant enough improvement when used as a monotherapy[62]. Side effects, if any, are generally mild and may consist of flatulence, diarrhea, or vomiting.

2.1.3.4 Antihistamines

Antihistamines are receptor antagonists of specific histamine receptors, primarily the H1 and H2 receptors. Pruritus, pain, and induced vascular permeability in humans related to histamine release are primarily mediated through H1 receptors, though it is unclear how important of a role H1 receptors play in the sensation of pruritus in canines[94]. Similar to EFA’s, evidence for the efficacy of antihistamines for the treatment of CAD is controversial due to historical poor study design[94]. A recent meta-analysis of 6 studies evaluating antihistamines showed that 0-30% of owners reported a good to excellent response (doxepin, loratidine, and clemastine), 10% reported an anti-pruritic effect (clemastine, cyproheptadine, and chlorpheniramine/hydroxyzine combo), and one study reported a greater than 50% reduction in pruritus in 27% or patients (diphenhydramine, hydroxyzine)[76].
2.2 Cyclosporine in Humans

2.2.1 Mechanism of Action

Cyclosporine, known as Cyclosporine A (CSA), is a neutral lipophilic cyclic 11-amino acid peptide, originally isolated from a soil fungus Hypocladium inflatum in search of new anti-fungal agents [4, 95, 96]. Due to its lipophilicity, CSA passes through lipid membranes (i.e. cells) passively and binds with high affinity to a cytosolic protein called cyclophilin, in particular to cyclophilin A, the most abundant cyclophilin in T cells [95-97]. The cyclophilin/CSA complex binds with another cytosolic protein called calcineurin (also known as PP2B) which is a protein serine/threonine phosphatase[97]. The process of T-cell activation involves intracellular calcium elevation which then activates calmodulin, which subsequently interacts with calcineurin to incite its phosphatase activity. This phosphatase activity is required for dephosphorylation of nuclear factor of activated T-helper cells (NFAT), which must be dephosphorylated in order to translocate into the nucleus and activate gene expression[4, 95, 97]. Binding of the cyclophilin/CSA drug complex to calcineurin inhibits this phosphatase activity, thus preventing NFAT transcriptional activation of genes for IL-2 (necessary for full activation of the T-helper cell pathway), IL-4, CD40L, IL-6, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF) [95, 97, 98]. The effective blockade of T-helper cell activation also indirectly affects growth and differentiation of B-cells, thus affecting the humoral immune system[98]. Additionally, CSA blocks mast cell degranulation, cytokine production (IL-3 and IL-5), and decreases mast cell survival time
CSA also inhibits the activity natural killer cells, antigen presenting cells (Langerhans cells in the epidermis), and eosinophils. The sum total of these mechanisms makes CSA a potent immunosuppressant and immunomodulatory therapeutic, particularly for inflammatory and immune-mediated processes involving T-cell activation and proliferation.

### 2.2.2 Uses

Cyclosporine’s initial and current primary use in humans is for prevention of rejection in solid organ transplant recipients, and was approved for this purpose by the US FDA in 1983 (Sandimmune) and 1995 (Neoral). Neoral® was subsequently approved for treatment of rheumatoid arthritis and psoriasis in 1997. Due to its potent immunosuppressive activity, CSA has been used off-label for countless diseases believed to have an immune-mediated or inflammatory component with variable success, though controlled randomized studies are generally lacking. Due to the numerous and serious adverse effects of CSA, its sustained use still remains focused primarily on transplant rejection for liver, kidney, heart, bone marrow, and lung transplantation. It is also used in humans for psoriasis and atopic dermatitis as a rescue treatment to induce rapid remission or resolution of signs in severe disease, as well as to serve as a bridge to other therapeutics.

### 2.2.3 History and Formulations

Sandimmune® (Sandoz) the original formulation of CSA available for humans approved for use in transplant rejection in 1983 is offered in a liquid suspension and a
soft gelatin capsule employing an oil and ethanol vehicle for the drug[104]. In an attempt to increase oral bioavailability, a modified formulation of the drug Neoral® (Novartis, formerly Sandoz) was introduced and approved for use in the US in 1995, also in a liquid and soft gel capsule with the drug in a micro-emulsion containing a surfactant, lipophilic solvent (corn oil), hydrophilic solvents (propylene glycol), and an anti-oxidant [104, 105]. There are currently numerous generic CSA formulations available; Gengraf (Abbott Laboratories) and Hexal (Hexal AG) were among the first, although availability varies among countries with most generic formulations being available in Europe[106, 107].

Due to the fact that CSA’s primary use in human medicine is the prevention of allograft rejection of solid organ transplants, the bioequivalence of aforementioned formulations is a topic of much importance and debate, particularly as CSA in these subjects has a very narrow therapeutic window [107]. The establishment of bioequivalence between drugs according to both European and US guidelines requires a single dose study in healthy individuals. Establishment of bioequivalence does not evaluate steady state conditions, patients with comorbidities, difference between sexes, or fasting/fed pharmacokinetic profiles[106, 107]. Due to intra- and inter-patient variability of CSA absorption, establishment of equivalent bioavailability by these measures is a poor measure of true drug performance, particularly in the most commonly used forum of transplant patients who have considerable comorbidities[106]. Extensive studies comparing Sandimmune® to Neoral® have been conducted with most reporting Neoral® to be superior in treatment outcome due to the known improved bioavailability and
decreased pharmacokinetic variability of Neoral® [104, 108, 109]. A meta-analysis of published studies showed the incidence of graft rejection for liver, heart, and kidney transplants to be significantly lower for patients treated with Neoral® versus Sandimmune[110]. A Collaborative Transplant Study has also shown treatment with Neoral® versus Sandimmune to provide a superior 4-year graft survival rate for patients receiving Neoral® [111].

The higher bioavailability of Neoral® and thus more predictable drug exposure has shown to be instrumental in decreasing graft rejection, increasing survival times, and decreasing costs[112]. This exemplifies the importance of predictable drug exposure on clinical outcome, thus highlighting the question of bioequivalence and particularly the fact that bioequivalence does not guarantee clinical outcome equivalence. Both Gengraf and Hexal generics were approved as bioequivalent to Neoral®, however, both drugs exhibited a statistically significantly lower AUC and Cmax when compared to Neoral® in pharmacokinetic profiles in humans[107]. To date there are no large scale analyses comparing Neoral® with any one generic, and the smaller scale studies currently published offer conflicting outcomes[108]. Comparison of these study outcomes is also confounded by the number of different generics employed for treatment. A retrospective report from the Collaborative Transplant Study base indicated a lower 1-year overall graft survival with generic CSA (78%) versus Neoral® (88%)[108]. Additionally, a retrospective analysis of cost showed that health care costs were higher in patients treated with generic CSA versus Neoral®[113]. The current recommendation for use of generic CSA in transplant patients is one of caution, particularly when switching a patient who is
well maintained on Neoral® to a generic, as small changes in drug availability of a drug with a narrow therapeutic window can significantly affect patient outcome [108].

2.2.4 Pharmacokinetics

2.2.4.1 Absorption

CSA is a lipophilic molecule that is poorly absorbed from the gastrointestinal tract, with an estimated bioavailability of approximately 30% for the microemulsified formulation and estimated absorption ½ life from the upper gastrointestinal tract of 1 hour[6]. Bioavailability varies greatly according to the formulation of CSA with wide inter- and intra-patient variability. Lipophilic molecules are generally absorbed in the proximal small intestine due to their dependence on emulsification by biliary secretion[105]. The secretions form micelles in which the lipophilic drug is solubilized, aiding its delivery to enterocytes, the process of which is often the rate-limiting step of lipophilic drug absorption[105]. Speed of solubilization is also therefore important as the GI transit time of the small intestines in humans is 3.5-4.5 hours. Thus the microemulsification of CSA improves absorption by 1) the presence of the lipid suspension stimulates biliary secretions necessary for solubilization (additional surfactants in the drug vehicle may also aid this process) and 2) lipids decrease gastric emptying time, therefore decreasing small intestinal transit time and allowing longer time for absorption[105]. Transporters located in the enterocyte membrane may aid or prevent the drug’s entry into the enterocyte. Of particular importance is the P-glycoprotein pump (PGP), an efflux pump associated with the multi-drug resistance gene, which decreases
drug delivery by pumping it back into the intestinal lumen[105]. PGP’s are also found in high concentrations of the apical surface of epithelial cells of the proximal tubules of the kidney and biliary canalicular membranes of hepatocytes; as well as the capillary epithelial cells of the blood brain barrier, testes, uterus, and placenta[22]. Once the drug enters the enterocyte, it is subject to metabolism by cytochrome p-450 enzymes (CYP 450), particularly the 3A4 (CYP 3A4) enzymes which constitutes more than 70% of the CYP 450 enzymes in the small intestine[105]. PGP is likely the rate-limiting step of CSA absorption, and contributes significantly to the first-pass elimination of CSA, equaling an estimated extraction ratio of approximately 60%[22, 114]

2.2.4.2 Distribution

Once absorbed, CSA binds extensively to erythrocyte, leukocytes, and plasma proteins with extensive absorption into tissues [7, 8]. The affinity of CSA for red blood cells is demonstrated by a blood to plasma CSA ratio of 2.0 in normal patients, and 1.32 and 1.62 in liver and renal transplant patients possibly due to decreased hematocrit[7]. An increase in hematocrit from 25% to 45% results in an increase in the blood CSA to plasma ratio[8]. Plasma/erythrocyte CSA binding is also influenced by temperature as a decrease in temperature from 37C to 21C causes a 50% shift of plasma bound CSA to erythrocytes. Approximately 80% of CSA in the plasma is bound to lipoproteins, the majority of which is bound to high density lipoproteins and low density lipoproteins with a very small portion in very low density lipoproteins and negligible amount in very low density lipoproteins[6, 115].
Considerable variation in time to peak concentration and peak concentrations achieved in blood and plasma were reported in initial publications, in part due to the CSA formulation (Sandimmune) used at that time[6]. The mean time to peak blood concentration after oral administration was reported to be 3.8hrs, but varied from 1-8 hours. Likewise the mean peak concentration was variable with a mean of 540ng/ml and a range of 240 – 1250 ng/ml [6]. A study in healthy volunteers showed the time of peak concentration to occur 1.4-2.1 hours after administration with Neoral®, while a study in kidney transplant recipients reported the range of 1.8-6 hours[104, 116]. Maximum concentration (Cmax) measured by radioimmunoassay (RIA) was dose dependent; Cmax for 200 mg CSA orally was 1026 ng/mL (+/-218), for 400 mg orally was 1558 ng/mL (+/-286), for 600mg orally was 1813 ng/mL (+/-400), and for 800mg orally was 2144 ng/mL (+/-576)[104]. Plasma half-life ranged from 4.4 to 13.9 hours[104].

There are few reports of CSA distribution into tissue, mainly consisting of animal and postmortem studies. One rat model evaluated a single oral dose and oral dosing for 21 days using radioactively-labeled CSA and measurement via high pressure liquid chromatography (HPLC)[10]. In the model with 21 days of repeated oral dosing as well as the single oral dose; tissue samples were taken at 4, 8, 24, 48, 96, and 240 hours after administration of the last dose. After a single IV dose the highest concentrations were in the skin, fat, and liver with the lowest concentrations in the brain, likely due to the blood brain barrier. Generally tissues to blood ratios were greater than one, supporting the extensive distribution of CSA into tissues. After repeated oral dosing for 21 days, when sampled 24 hours after the last dose, 12.64% of the total dose was found in the skin,
11.33% in fat, 7.51% in the liver, and 0.9% in the kidney. All tissue concentrations consisted of mainly parent compound (versus metabolites) and were higher after multiple dosing, increasing by a factor of 1.2 to 4.4 with levels accumulating most significantly in the skin. Levels of parent compound peaked at 8 hours in the skin and adipose, compared to 4 hours in the liver and kidney. CSA radioactivity was still detectable in all tissues after extended dosing 240 hours (10 days) after administration of the last dose, with skin representing the highest percentage at 2.4% of the dose. An additional rat study found the tissue-to-blood concentration ratio following single administration of 6mg/kg of CSA to be 11.0 for the liver, 10.4 for the kidney, 2.45 for skin, and 6.27 for fat[117]. A human study likewise confirmed that parent drug in the kidney exceeds that in blood by 5-10 times and was well distributed in both the kidney cortex and medulla[118]. Likewise skin concentrations in humans and canines are reported to be about six to ten times that found in blood[9, 119]. Results reported in combination for rats, mice, and rabbits detailed high tissue to blood ratios ranging from 2 to 10[8]. Tissue concentrations were reported to decline biphasically with a terminal half-life of 60-120 hours, with the parent drug identified as the major component of CSA in all tissues analyzed. Skin and fat were particularly prone to accumulate CSA and identified as a “storage depot”[8]. A post mortem study in seven human transplant patients receiving CSA likewise confirmed the high tissue concentrations of CSA with CSA and CSA metabolite concentrations in tissue exceeding their blood counterparts by 8-53 times. [120]. HPLC was employed to differentiate parent drug from metabolites and found that unlike other tissues where metabolite 17 predominated, fat and pancreas contained parent CSA. The pancreas
contained the highest CSA concentration (based on CSA per kg of tissue) followed by spleen, liver, fat, kidney, lung, bone marrow, heart, and whole blood[120].

2.2.4.3 Metabolism and Elimination

Cyclosporine’s oral bioavailability and metabolism are primarily controlled by the CYP 450 enzyme system and the PGP efflux pump. Though both of these are present in smaller amounts in the small intestine and kidney where there is a small degree of metabolism and elimination, the primary location of metabolism is the liver[11, 12]. Metabolism of CSA produces water-soluble metabolites from the lipophilic parent drug in order to aid in excretion, which occurs in two phases[121]. Phase I involves intra-molecular alterations of the parent drug via mixed function oxidases (CYP 450 enzymes). The CYP 450 enzyme sub-family 3A4 is the most noteworthy as it is the most abundant of the human cytochromes, and is most important for biotransformation of CSA, estimated to account for an additional initial 30% first pass extraction[114, 121]. Phase II reactions are those involving the glucuronyl transferase of sulfotransferase and involve attachment of a water-soluble polar group to the parent drug[121]. As many as 30 metabolites of CSA have been identified [11, 122]. Metabolites with only one modification, M-17, M-21, and M-1 have been shown to exhibit some immunosuppressive activity and are present in higher concentrations in tissue versus blood [123]. Generally metabolites with two modifications have less than 10% activity of that of CSA[123]. Metabolites are excreted primarily in the bile, with only 6% of the dose being excreted in the urine and 0.1% of the dose being excreted unchanged in the urine.
2.2.4.4 Drug Interactions

Drug interactions with CSA are extensive and are primarily due to PGP’s or CYP 450 enzyme systems. Drugs that are substrates of the PGP’s are subject to being effluxed via these pumps; the list of PGP substrates is extensive and includes CSA. The list of PGP inhibitors is also extensive and includes ketoconazole. Inhibition or induction of PGP primarily affects the rate of drug absorption[22]. As mentioned previously CYP 450 enzymes are responsible for Phase I metabolism and 90% of drug oxidation can be attributed to 6 main cytochromes: CYP 1A2, 2C9, 2C19, 2D6, 2E1, and 3A4[22].

Drugs can be classified as CYP 450 substrates, inducers, or inhibitor. CYP 450 substrates are drugs that are metabolized by CYP 450 an enzyme, the list of which is extensive and includes CSA (Table 1). Drugs that are inducers actually increase the amount and activity of CYP 450 enzymes. There is a period of “onset” and “offset” which is generally not rapid as onset requires transcriptional production to produce new enzymes or increase activity of existing enzymes and offset requires metabolism of the inducer as well as decay of the increased enzyme levels [22]. Though inducers are enzyme sub-family specific, some common inducers affecting multiple sub-families include barbiturates, rifampin, and corticosteroids (prednisone and dexamethasone)[22].

Generally inducers decrease the effectiveness of CYP substrate drugs; however substrate pro-drugs that need to be metabolized to an active metabolite can actually have increased activity.

Inhibition of CYP enzymes can occur through competitive inhibition, also known as reversible inhibition, quasi-reversible inhibition, and irreversible inhibition. Reversible
or competitive inhibition is the most common type of inhibition. This type of inhibition typically has a rapid onset and depends on the affinity of the inhibitor for CYP enzymes, the concentration of drug required for inhibition, as well as the half-life of the inhibiting drug[22, 124]. Substrates and inhibitors are also not mutually exclusive. Quasi-reversible inhibition occurs when conversion of the drug by CYP enzymes forms inhibitor metabolites that bind to the CYP enzymes and render them inactive [124, 125]. Quasi-reversible inhibition has thus far only been reversed in vitro, and in vivo is therefore classified as irreversible inhibition. Erythromycin and diltiazem are classic quasi-irreversible inhibitors with propranolol and chloramphenicol being classic irreversible inhibitors [124, 125]. The drugs with the highest degree of inhibition of CSA are erythromycin, clarithromycin, fluconazole, itraconazole, ketoconazole, ritonavir, saquinavir, nelfinavir, diltiazem, cimetidine, and grapefruit juice.

2.2.4.5 Intra- and Inter-patient Variability of Cyclosporine Absorption and Pharmacokinetics

Variability of CSA absorption between patients and in different patient populations and with different formulations of CSA has been well documented. As mentioned previously, the microemulsified formulation of CSA decreases intra-individual and inter-individual variability of absorption. Important factors impacting absorption have been detailed, primarily those focusing on administration with or without food, pediatric, patients, liver disease, and other comorbidities. Food consumption and time of CSA administration has been shown to have varying effects according to the formulation of CSA. Neoral® was shown to have a minor decrease in area under the
curve (AUC) of 15% and a maximum concentration (Cmax) decrease of 26% when administered with a fat-rich meal, compared to Sandimmune which had a 37% increase in the AUC when administered with a fat-rich meal[126]. Additionally pre-prandial Neoral® was shown to have a significantly higher AUC drug concentration compared to post-prandially administered Neoral®; pre-prandial administration was also correlated with improved clinical efficacy in a later study [127, 128]. Conversely, Hexal and Gengraf (commonly used generic formulations of CSA) show a 12% and 13% greater absorption when taken with food[107]. As liver function is important for fat uptake and fat metabolism, poor CSA bioavailability can occur in patients with liver disease and immediately post-operatively in liver transplantation[7]. Decreased bioavailability also occurs in patients with diabetes, gastrointestinal disease (malabsorption), cystic fibrosis, pediatric patients, and African Americans [7, 107]. Circadian variability has been implicated to contribute to intra-patient variability with their being a 20% variability of AUC during the day and 34% at night [129], as has the patients’ total and plasma lipoprotein concentration and composition[115].

Of recent interest on the subject of inter-individual variability is the subject of genetic variation. CSA is primarily metabolized by cytochrome P450 enzymes, specifically CYP 3A. The CYP 3A family has four isoenzymes: CYP3A4, CYP3A5, CYP3A7, and CYP3A43; with CYP3A4 and 5 being the most important for metabolism of CSA [130]. Polymorphisms of the CYP3A4 gene are rare, where conversely several polymorphisms of the CYP3A5 exist and are expressed in approximately 50% of the African American population and 30% of the Caucasian population [131]. A meta-
analysis of the effect of this genetic diversity indicated that renal transplant patients with the CYP3A45*3 polymorphism require lower doses of CSA to reach the desired target level [130]. Additionally, genetic variation in the form of single nucleotide polymorphisms of the multidrug resistance gene (MDR1, also known as ABCB1) affecting expression as well as function of PGP have been identified [132]. One study showed that renal transplant patients with MDR1 wild type for single polymorphism C1236T had a lower dose-adjusted peak CSA concentration and dose-adjusted area under the curve [133].

2.2.4.6 Adverse Effects

Cyclosporine has numerous adverse effects in humans including hypertension, malignancy potential, neurologic events, gastrointestinal events, gingival hyperplasia, cutaneous changes, hepatotoxicity, infections, and hyperlipidemia, acute nephrotoxicity, and chronic nephrotoxicity with the most serious health concern being chronic nephrotoxicity. Hypertension is one adverse effect with a reported incidence of 25% - 37% in transplant recipients, believed to be cause by a number of factors including its effects on the sympathetic nervous system, upregulation of angiotensin II receptors in vascular smooth muscle cells, increased plasma levels of endothelin-1, and effects on whole blood viscosity and plasma fibrinogen levels[122]. There are conflicting reports on whether the onset of hypertension is CSA dose related[103]. Current recommendations are to treat with a calcium channel blocker (amlodipine or isradipine) or to reduce the dose of CSA by 25-50% if possible [103]. The risk of neoplasia has been documented in transplant patients, though many of these patients are receiving multiple
immunosuppressive therapeutics simultaneously. A review of cardiothoracic transplantation showed an increase risk for development of lymphoproliferative disease associated with Epstein-Barr Virus[134]. People treated with immunosuppressive doses of CSA have been shown to be at increased risk for neoplasia of the skin (squamous cell and basal cell carcinoma), Kaposi’s sarcoma, and lymphoma when compared to the normal population, though the incidence of such neoplasia is similar to the rates reported for patient’s on conventional immunosuppressive therapy [135]. Some cases of reported lymphoma regressed rapidly with withdrawal of the CSA, supporting cause and effect relationship[103, 135]. Patient’s treated with lower doses of CSA for psoriasis have been shown to have increased incidence of squamous cell carcinoma, particularly those that had previously been treated with photochemotherapy (PUVA)[103].

Neurologic events consist of headaches, tremor, seizures, psychosis, paraesthesias, and sleep disturbances with total neurologic events and are reported in 12-51% of transplant recipients[122]. Gastrointestinal side effects (nausea, abdominal pain, diarrhea, flatulence, vomiting) are reportedly transient with mild to moderate severity and variable incidence 2%-81%[109]. Gingival hyperplasia has been reported in up to 30% of patients with the pathogenesis being uncertain, though may be associated with poor oral hygiene[103]. The most commonly reported cutaneous change is hirsuitism/hypertrichosis, reported in as high as 60% of patients; the mechanism is unknown[103]. Transplant patients have been shown to have increased incidence of cytomegalovirus infection, candidiasis, and herpes viral infections[122]. Hyperlipidemia, specifically hypertriglyceridemia and hypercholesterolemia, has been reported secondary
to CSA administration, and may contribute to atherosclerosis in renal transplant patients [103, 136, 137].

Hepatotoxicity has also been reported with varying incidence, as high as 58% in some studies[138]. The incidence of hepatotoxicity can be difficult to assess as most data on CSA is from transplant recipients, many of whom receive multiple drugs that can also affect liver function (prednisone, azathioprine, and tacrolimus). One retrospective study of patients receiving CSA for ocular disease reported elevation above normal reference values in one liver parameter for 58% of patients with previously normal liver values[138]. There were elevations of alkaline phosphatase, total bilirubin, and aminotransferase activities[138]. The elevations were generally mild, likely due to CSA induced cholestasis, and did not require discontinuation of CSA[138].

Nephrotoxicity is generally regarded as the most serious adverse event associated with CSA administration both in transplant recipients and patients being treated for autoimmune diseases, and can be divided into two categories; acute and chronic nephrotoxicity[13]. Acute toxicity presents as acute renal failure as evidenced by an elevated creatinine and resolves when CSA dose is reduced or discontinued[13]. This toxicity occurs secondary to an imbalance of renal constrictors and renal dilators. There is a marked increase in renal vasoconstriction, primarily occurring at the afferent arteriole, but also occurring within adjacent small arterioles in the glomerular tuft with subsequent variable decrease in glomerular filtration rate[139]. The mechanism of this intense renal vasoconstriction has not been fully elucidated, but potential causes include an increase in vasoconstrictor factors such as endothelin-1, thromboxane, and angiotensin
II, paired with a decrease in vasodilator factors such as prostacycline and nitric oxide[13]. Incidence of renal dysfunction with short-term therapy in psoriasis patients was reported to occur in 4-27% of patients and in 27-35% of transplant recipients [103, 122].

2.2.4.7 Monitoring

Due to CSA’s potential for toxicity, and narrow therapeutic index for prevention of solid-organ transplantation rejection, appropriate monitoring of CSA is crucial. CSA is classified as a critical dose drug which means a very small change in concentration may result in a change in efficacy or toxicity [15]. Since its implementation into medicine over thirty years ago, three main points of discussion/research regarding CSA monitoring have been elucidated: 1) What biologic fluid/tissue should be used for monitoring? 2) What time should monitoring be performed to optimize efficacy and reduce toxicity? and 3) What laboratory technique is preferred?

Initially, plasma or serum (versus whole blood) was the matrix recommended for CSA measurement as it was believed that plasma may be more reflective of the CSA concentration at the site due to CSA’s extensive tissue distribution[140]. The controversy over which matrix was optimal, plasma/serum versus whole blood, centered around the fact that the distribution of CSA in plasma/serum versus CSA in whole blood is temperature dependent with 73% CSA in plasma at 4 C, 93% at 20 C, and 97% at 37 C [6]. When temperature decreases, CSA rapidly diffuses from plasma into blood cells, a process that takes two hours to reach equilibrium[6]. Additionally, hemolysis may falsely increase plasma CSA levels [141]. The levels in whole blood may contain a portion of “erythrocyte-bound” CSA that may not be biologically active to tissues and contains
metabolites numbers 1 and 17 [6, 141]. In light of shipping samples and laboratory
techniques, whole blood eventually became, and remains, the medium of choice for CSA
analysis; the values are temperature dependent and hemolysis is not of concern[141-143].
As previously discussed, CSA tissue concentrations greatly exceed those found in plasma
and whole blood. Due to the invasive nature of obtaining tissue samples for
measurement (i.e. biopsy of the target organ) and the frequency of sampling required for
monitoring purposes, measurement of CSA concentrations in the target tissue has been
minimally investigated and is unlikely to ever become clinically useful [9, 118].

Monitoring is crucial for transplant patients, both for clinical outcome as well as
toxicity. Cyclosporine is typically administered every 12 hours with trough levels being
originally used as a measure of assessing drug safety, and not intended to be used for
clinical efficacy [15]. Because trough levels were being measured to assess for residual
CSA prior to the next dose, they were implemented into therapeutic monitoring and target
ranges were established [15]. Subsequent studies showed that AUC (sampling from
hours 0-12) was a better predictor of clinical outcome with the administration of Neoral®
[116, 144]. However, sampling for twelve hours is labor intensive, expensive, invasive to
the patient, and thus difficult to impossible to implement into routine practice. As such,
research trended toward investigating a time that would be most representative of AUC
or maximum concentration (Cmax). An abbreviated AUC sampling from hours 0-4 was
shown to correlate well to AUC (0-12) with Neoral® administrations, though this still
required four samples [145]. Sampling at 2 hours (C2) was then shown to correlate well
with AUC and decreased acute rejection in all organ types, leading to an improved
outcome in new transplant recipients as well as maintenance transplant recipients [15-17].

Monitoring at C2 with Neoral® therapy is considered to be the best option[15]. Outside
the realm of transplant recipients, CSA monitoring is less established and is primarily
intended to prevent toxicity. For the treatment of psoriasis, monitoring of trough levels
does not seem to be indicated if the dose of CSA is less than 3 mg/kg daily[146].

Monitoring is not routinely recommended during treatment for psoriasis or atopic
dermatitis, though could be used to assess patient compliance with treatment or to assess
for levels above the recommended dosage range (i.e. toxicity) [103].

Two main methodologies are available for CSA measurement; radioimmunoassay
(RIA) and high performance liquid chromatography (HPLC). Initially the debate over the
preferred methodology was intense[141]. HPLC has generally been the gold standard of
measure as it is highly specific for parent compound and does not cross-react with
metabolites. High performance liquid chromatography was generally employed for
calibration and validation of various RIA’s with its initial primary use being in the
research/laboratory forum [6, 11, 141]. High performance liquid chromatography was
not initially used for routing patient monitoring for the following reasons: 1) Extensive
sample preparation (lysis of whole blood and extraction of CSA); 2) Variable extraction
efficiency; 3) Complex and expensive instrumentation requiring employment of specialty
trained technicians; 4) Lengthy sample analysis time; and 5) Requirement of high
temperatures for chromatographic analysis, resulting in column failure in as short as 30
minutes[6, 141]. The initial benefits of RIA compared to HPLC were its ease of the test
kit performance (i.e. lacked technically trained staff), cost effectiveness and quicker turn
around time. The most notable drawback of RIA was that it cross-reacted with a significant portion of CSA metabolites, and was not specific for CSA parent compound, the portion of CSA with the most therapeutic and toxicologic importance[140, 141]. Radioimmunoassay initially used a radio-labeled white rabbit antisera conjugated against CSA, which cross-reacted by as much as 60% with the major human metabolite of CSA, metabolite 17, meaning that this RIA overestimated levels of CSA parent compound by as much as 60%[141]. A subsequently developed assay using sheep antisera had a 32% cross reactivity with metabolite 17[141]. Commercially available immunoassays continued to be developed and those commonly employed today include a monoclonal radioimmunoassay (mRIA), monoclonal fluorescence polarization immunoassay (mFPIA) and enzyme-multiplied immunoassay (EMIT). When compared with validated HPLC, CSA overestimation due to cross reactivity with metabolites ranged from 22-48%, 22-30%, and 8-30% respectively[142]. The overestimation of CSA was highest in liver transplant patients, likely due to altered CSA metabolism[147].

Though a consensus statement in 1995 decreed that methods used to evaluate CSA levels should be specific for parent CSA, RIA was generally regarded as sufficiently accurate and specific for day-to-day monitoring due to its advantages in accessibility, ease of test performance, and quick turn-around time [142, 143, 147]. The turn-around time should ideally be within one dosing period (12 hours in most cases), making in-house immunoassays appealing. However, this status quo was challenged by a study in which none of the immunoassays fulfilled the recommended accuracy and specificity recommended in the Lake Louise Conference [148]. A subsequent study comparing CSA
trough levels to C2 levels, showed that although immunoassay performance accuracy was improved with C2 levels versus trough, only one of the immunoassays recorded CSA levels that had a linear relationship with the HPLC control[149]. This study also concluded that the performance of immunoassays was not in accord with established Lake Louise guidelines, a topic of concern due to CSA’s narrow therapeutic index[149]. Though immunoassays are still widely in use, the availability and use of HPLC has increased, mainly due to validation of more rapid HPLC assays paired with mass spectrometry (HPLC-MS/MS, HPLC-LC/MS)[150, 151]. These assays are generally more sensitive and specific even than HPLC methods, require smaller sample sizes, and have the added benefit of being able to measure multiple immunosuppressant drugs from one sample (this is important as patients are often on combination therapy)[150, 152]. Though these assays require trained technical staff and are expensive to purchase, they are becoming more commonly utilized for routine patient monitoring[152].

2.3 Cyclosporine in Canines

2.3.1 Pharmacokinetics

2.3.1.1 Absorption, Distribution, Metabolism and Elimination

The pharmacokinetic profile (absorption, distribution, metabolism, and elimination) of CSA in canines is very similar to that in humans, detailed extensively in section 2.4.4 above [11, 12, 18]. Canines metabolize CSA more rapidly than humans, with 70-100% of metabolism occurring within 30 minutes[153]. As in humans, the CYP450 enzyme system is responsible for hepatic metabolism of CSA with CYP subsets
3A12, 3A26, and 2B11 being the primary enzymes in canines[154]. The same formulation of brand name CSA used in humans is used in dogs i.e. Novartis products Atopica®(canine) and Neoral® are identical[5]. The use of Atopica® in canines is currently only approved for treatment of CAD. The degree of inter- and intra-individual variation in CSA concentrations is less in canines than that in humans. Administration of the micro-emulsified formulation of CSA with food has been shown to increase variability of whole blood CSA concentrations and generally decrease bioavailability by 22%, thus the drug is recommended to be administered two hours before or after eating[18]. Griffiths noted a six-fold variation in trough levels when using Neoral® for treatment of perianal fistulas, a higher degree of variation than reported in most other studies[155]. Pharmacokinetic studies by Steffan noted a coefficient of intra-individual variation for AUC and Cmax of 11% and 24% coefficient of inter-individual variation for AUC[18]. Possibilities for inter-individual variation include the effects/variability of the PGP system as well as CYP 450 enzyme system. P-glycoprotein expression in relation to ABCB1 genetic diversity has been evaluated in dogs, though not in context with CSA metabolism [156, 157]. Mouatt reported an increased inter-individual variability in CSA concentrations when CSA was administered concurrently with ketoconazole (KTZ)[32]. The author is unaware of any in vivo studies evaluating CYP 450 enzyme genetic expression or degree of function in canines, though some in vitro studies indicate that polymorphisms of the canine CYP 450 enzyme system do exist and may be important in drug metabolism[158, 159].
2.3.1.2 Drug Interactions

There is little information in canine medicine about in vivo CSA drug interactions. Possible drug interactions as predicted by in vitro inhibition can be found in (Table 1)[154, 160]. Known in vivo drug interactions will be discussed in detail in sections 2.8.1 and 2.8.2.

2.3.1.3 Adverse Effects

The adverse effects of CSA administration in humans and canines have some similarities, though some noteworthy differences. A long-term toxicological study in canines evaluating the doses of 5 mg/kg/day, 15 mg/kg/day, and 45 mg/kg/day showed that when given at 45 mg/kg a generalized cutaneous papillomatosis and gingival hyperplasia developed in most dogs[161]. None of the dogs with cutaneous papillomatosis had evidence of malignant transformation. In marked contrast to humans; there was no evidence of nephrotoxicity, hepatotoxicity, or myelotoxicity. Two tumors (fibroma and basalioma) were reported in this patient population, which were deemed unlikely to be associated with CSA administration. Diarrhea, emesis, anorexia, and weight loss were also noted as adverse effects (frequency not noted). CSA was generally well tolerated with all adverse effects being fully reversible following withdrawal of the drug, and side effects were noted to be generally dose-dependent[161]. Due to development of cutaneous papillomatosis and gingival hyperplasia at 45 mg/kg/day, this study thus set the toxic dose at 15mg/kg/day[161]. A subsequent long term high-dose CSA study also confirmed lack of evidence of nephrotoxicity[162].
At 5 mg/kg once daily, as prescribed for the treatment of CAD, the most commonly reported adverse events are vomiting, diarrhea, and soft stools, occurring in 15-25% of patients[14]. Generally these events occurred during the first month of treatment, with repeated episodes of vomiting or extended episodes of diarrhea being rare[14]. Other reported adverse events with a frequency of less than 2.5% included bacterial skin infection, urinary tract infection, papillomatosis, gingival hyperplasia, and neurological disorders[14]. There are two case reports of neoplasia following chronic immunosuppression with CSA in canines, though clearly no cause and effect relationship could be established [163, 164]. A retrospective case-controlled analysis in felines receiving CSA post-renal transplant did report a higher incidence of malignant neoplasia in post-transplant patients receiving CSA, though likewise did not establish a cause and effect relationship [165].

2.3.1.4 Monitoring

Correlation of CSA levels in whole blood or tissue and treatment outcome or disease control in canines has not clearly been demonstrated. The body of evidence in canine research pertaining to the optimal time (peak, trough, day of administration) to monitor CSA levels, desired CSA levels, and optimal method of monitoring is generally lacking when compared to the breadth of research on monitoring in humans. This is likely, at least in part, due to the fact that CSA in canines does not have as narrow of a therapeutic window as in humans. As reported in humans, RIA in canines over-estimates the concentration of CSA in whole blood due to cross reaction with metabolites by 1.5 to 1.7 times when compared to HPLC [18, 34]. Analysis via HPLC is more often reported
in canines than RIA, likely due to the fact that most reports are in a research setting (not clinical patient monitoring), and HPLC is considered the gold standard in human medicine. Thus far, clinical monitoring of CSA levels has not been proven to correlate to patient outcome in CAD or in the treatment of perianal fistulas [18, 31, 32, 166]. It should however be noted that in the single study comparing the CSA levels and clinical outcome in CAD only evaluated one whole blood sample taken for CSA analysis within 24 hours of the patients’ last dose of CSA; the timing of sample collection and whether it reflected a peak or trough CSA level was not reported. One study investigated T-cell cytokines profiles in canines as a potential method for evaluating the immunosuppressive effects of CSA and found that IFN-gamma and IL-2 were significantly decreased when CSA was dosed at 10mg/kg every 12 hours, but there was no significant effect on IL-2 when CSA was dosed at 5mg/kg every 24 hours[167].The current recommendation for monitoring CSA levels in CAD is generally that monitoring is not necessary, although it may be useful in the event of adverse reactions or perceived lack of efficacy[18, 19].

2.4 Cyclosporine use in Canine Atopic Dermatitis

As in humans, CSA has been used for treatment of a wide variety of inflammatory and immune-mediated canine diseases, with a general lack of controlled trials to evaluate efficacy. As CSA in the form of Atopica® is labeled for the treatment of CAD, its efficacy in the treatment of this disease will be the focus of the following review. A randomized controlled trial proved 5mg/kg once daily to be effective for the control of signs of CAD, and 2.5mg/kg once daily to be ineffective for the treatment of CAD [168]. A meta-analysis from 2005 provides the best summary for the efficacy of CSA in the
form of Atopica for treatment of CAD[14]. Atopica was proven to be as effective in controlling signs of CAD as glucocorticoids, with an overall improvement in lesion scores of > 50% in 63-87% of dogs after 12-16 weeks of therapy[14]. When paired with 51-93% of dogs having a pruritus level designated as “mild” by week 16, this equated to a good to excellent global assessment score in 65-76% of patients. Of the ten studies included in this analysis, CSA administration could be decreased to every other day in 40-50% of patients after four weeks and to twice weekly in 20-26% of patients after 12-16 weeks. The likelihood of a better response during maintenance treatment was predicted by a >50% improvement of baseline lesion scores[14]. Further, adverse effects were generally mild and consisted most commonly of vomiting and diarrhea/soft stools in 25% and 15% of patients respectively. A retrospective analysis following long-term use of CSA for CAD (51 canines) found that 71% of owners were satisfied with CSA as a treatment for their atopic dog, but 10% discontinued CSA therapy as it was cost prohibitive[169]. Twelve canines (24%) experienced continued amelioration of the signs of CAD after discontinuing CSA for up to 12 months at the time of the study completion, a number which has not been corroborated with further studies and is difficult to interpret due to the retrospective nature of the study. The author is unaware of any studies comparing the efficacy of generic formulations of CSA to Atopica for the treatment of CAD. One study comparing human generic CSA to prednisone for the treatment of CAD in a small group of dogs reported no significant difference in the reduction of pruritus or lesion (CADESI-01) scores between groups treated with prednisolone and groups treated with human generic CSA[170].
2.5 Pharmacotherapeutic Manipulation of Cyclosporine Concentrations

2.5.1 Humans

As mentioned previously the list of possible drug interactions with CSA is extensive, and important to note for optimal patient care particularly in the arena of transplantation medicine in which patients with “poly-pharmacy” is common. Similarly to the treatment of CAD, treatment for prevention of transplant rejection is life-long and as such, cost-saving measures are often explored. CSA (even generic) is often prohibitively expensive. Manipulation of CSA concentrations by concurrent administration of known inhibitors of CSA metabolism via the P450 enzyme system thus became an appealing alternative in order to decrease long-term cost. Ketoconazole has proven to be an effective and safe method for reducing the dose and therefore cost of CSA administration and has the additional benefit of treating thrush (yeast infection) commonly associated with immunosuppression in humans [20, 21]. The potency of KTZ inhibition of CSA is two-fold[22]. Ketoconazole is a potent inhibitor of the CYP 3A4 enzyme system, and is also an inhibitor of the PGP, which allows up to a 70% reduction in CSA administration [23, 24]. Two studies showed no increase in hepatotoxicity (a potential side-effect of KTZ and CSA) following the long-term combined use of CSA and KTZ in humans [20, 21]. Though itraconazole is not as potent of an inhibitor of the P450 enzyme system as ketoconazole, it has also been shown to increase CSA concentrations in transplant recipients and provide a cost reduction of 30% [22, 25].

Diltiazem, a calcium channel blocker and inhibitor of CYP 3A4, has also been used to pharmacotherapeutically increase the concentration of CSA in transplant recipients[26].
Two studies even showed an improved outcome of patients treated with the combination of CSA and diltiazem versus patients treated with CSA alone. However, a further study showed a high degree of inter-patient variability in the dose of diltiazem required to elevate CSA to the optimal level [27-29]. Grapefruit juice has also been demonstrated to inhibit the CYP 3A4 enzyme system, resulting in an increase in CSA concentrations. The most potent inhibitor of the CYP 450 enzyme system in grapefruit juice is psoralen, although flavonoids may be potentially as potent as KTZ [22]. Due to the fact that grapefruit formulation is not controlled as are other drugs, its combination with CSA to boost CSA concentrations is recommended under caution and close medical supervision [22].

### 2.5.2 Canines

Administration of ketoconazole for manipulation of CSA levels in canines is the most researched interaction to date. To the author’s knowledge, there is no research into the interaction of CSA and KTZ for the treatment of CAD, although it is frequently advocated for this purpose [38-42]. Cyclosporine is metabolized in the liver by the cytochrome P450 enzymes CYP 2B11 and 3A12/26 in canines, and ketoconazole (KTZ) is a potent inhibitor of numerous cytochrome P450 enzymes, including CYP 2B11 and 3A12/26 [154]. A study in healthy research beagles showed that using KTZ at 13.6 mg/kg daily enabled a 75% reduction of CSA dose, maintaining a whole blood CSA trough level of 400-600 ng/mL, the target range of the study [33]. This reduced CSA administration equaled a monetary savings of 57.8%. Likewise, in this same study, KTZ at 4.7mg/kg daily reduced CSA dose by 38% and thus reduced cost by 23.8%. Another in
vivo study of the critical KTZ dosage range for CSA clearance inhibition in the dog determined that these effects were dose-dependent and maximized within the KTZ dosage range of 2.5-10 mg/kg daily [34]. These results have been clinically applied in studies on the efficacy of combination KTZ and CSA for the treatment of canine perianal fistulas, with excellent short-term results and a significant decrease in CSA dosing. One study involving 12 dogs with perianal fistulas showed that doses of KTZ between 5 and 11 mg/kg daily allowed a 50-75% dose reduction of CSA while maintaining target CSA trough levels [31]. All 12 dogs had at least short-term resolution of clinical signs, and this reduced dose equaled a monetary savings of 35-71% when compared to cost of treatment with CSA alone. Further studies in dogs with perianal fistulas had concurring results; a dose of 10mg/kg daily of KTZ allowed reduced CSA administration equaling >80% cost reduction, and doses of 5.3-8.9 mg/kg twice daily of KTZ enabled a reduction of CSA dose equaling a savings of 70% compared to CSA alone [32, 34]. A retrospective analysis of dogs administered KTZ showed vomiting, anorexia, lethargy, and diarrhea to be the most common adverse effects with drug induced hepatopathy being extremely rare but potentially fatal [171]. Adverse events, particularly those of gastrointestinal upset were reported more commonly with co-administration of CSA and KTZ [171].

The effect of cimetidine co-administration with CSA in canines has also been investigated, and was shown to increase the time to Cmax of CSA, although the Cmax itself was not elevated [36]. Likewise, metoclopramide administration was shown to have no effect on the pharmacokinetic parameters of CSA [35]. In concordance with human
studies grapefruit juice has been shown to variably affect CSA concentrations in canines [35, 37].
Table 1. Cytochrome P450 substrates in humans and canines

<table>
<thead>
<tr>
<th>Cytochrome P450 in People</th>
<th>Human Substrates</th>
<th>Cytochrome P450 in Canines</th>
<th>Canine Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Caffeine, Theophylline</td>
<td>Ortholog CYP1A2</td>
<td>Fluoroquiolones, Theophylline</td>
</tr>
<tr>
<td></td>
<td>Inhibited by Ciprofloxacin, Cimetidine</td>
<td></td>
<td>Induced by Omeprazole</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Propofol</td>
<td>CYP2B11</td>
<td>Propofol, Diazepam, Progesterone, Testosterone, Diclofenac, Cyclophospamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Induced by Phenobarbitol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibited by Chloramphenicol</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Phenytoin, Fluconazole, Diclofenac, Warfarin, Glipizide, Flubiprofen, Piroxicam, Ibuprofen, Celecoxib, Naproxen, Meloxicam</td>
<td>CYP2C21</td>
<td>Testosterone, Diclofenac</td>
</tr>
<tr>
<td></td>
<td>Induced by Barbbituates, Rifampin</td>
<td></td>
<td>Inhibited by Phenobarbitol</td>
</tr>
<tr>
<td></td>
<td>Inhibited by Cimetidine, Fluconazole, some NSAIDs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Codeine, Tramadol, Propanolol and other beta-blockers, Phenothiazones, Auinidine, Dettromethorphan, Chlorpheniramime, Imipramine, Fluoxetine, Amitryptylne, Chlorpromazine, Metoclopramide</td>
<td>CYP2D15</td>
<td>Celecoxib, Dextromethorpham, Imipramine, Metoprolol, Propanolol</td>
</tr>
<tr>
<td></td>
<td>Inhibited by Quinidine, Cimetidine, Diltiazem</td>
<td></td>
<td>Inhibited by quinidine</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Ketoconazole, Itraconazole, Cyclosporine, Tacrolimus, Erythromycin, Clindamycin, Clarithromycin, Cisapride, Diazepam, Midazolam, Diltiazem, Digoxin, Quinidine, Verapamil, Aflatoxin, Budesonide, Loratidine, Amitryptylne, Doxepin,</td>
<td>CYP3A12</td>
<td>Erythromycin, Tacrolimus, Cyclosporine, Midazolam, Diazepam, Progesterone, Testosterone</td>
</tr>
<tr>
<td></td>
<td>Induced by Rifampin, Barbituates, Dexamethasone, Grisocofulvin</td>
<td>CYP3A26</td>
<td>Induced by Phenobarbitol, Rifampin</td>
</tr>
<tr>
<td></td>
<td>Inhibited by Grapefruit juice, Ketoconazole, Itraconazole, Diltiazem, Cimetidine</td>
<td></td>
<td>Inhibited by Grapefruit juice, Ketoconazole</td>
</tr>
</tbody>
</table>
Chapter 3: The Effect of Ketoconazole on Blood and Skin Cyclosporine Concentrations in Canines

3.1 Abstract

Background: Cyclosporine (CSA) (Atopica®; Novartis Animal Health, Greensboro, NC) is approved for treatment of canine atopic dermatitis (CAD). CSA is metabolized by liver cytochrome P450 enzymes, a process inhibited by ketoconazole (KTZ). Hypothesis/Objectives: The aims of this study were to determine skin and blood CSA concentrations when CSA was administered alone at 5.0 mg/kg (Treatment 1: T1) and at 2.5 mg/kg (Treatment 2; T2); and when CSA was administered at 2.5 mg/kg concurrently with KTZ at 5 mg/kg (Treatment 3; T3) or at 2.5 mg/kg (Treatment 4; T4). We hypothesized that the skin and blood CSA concentrations in T1 would not differ from those obtained with T3 or T4. Animals: In a randomized cross-over study, six healthy research dogs received each of the treatments (T1, T2, T3, T4) once daily for 7 days. Methods: After the first, fourth, and seventh dose for each treatment a peak and trough skin punch biopsy sample and whole blood sample were collected and analyzed with high-performance liquid chromatography tandem mass spectrometry. Data were analyzed using a repeated measures approach with PROC MIXED in SAS. Pairwise comparisons were performed with least squares means and Tukey-Kramer adjustment for multiple comparisons. Results: Mean blood CSA concentrations in T1 were not different from T2
or T4, but were less than T3. Mean skin CSA concentrations in T1 were greater than T2, not different from T4, and less than T3. Conclusions and Clinical Relevance: Administration of CSA and KTZ concurrently at 2.5 mg/kg may be as effective as CSA alone at 5.0 mg/kg for treatment of CAD.

3.2 Introduction

Cyclosporine (CSA) is an immune-modulating drug currently labeled as Atopica® (Novartis Animal Health, Greensboro, NC) for use in the treatment of canine atopic dermatitis (CAD).[172] As a calcineurin inhibitor, CSA’s primary mechanism of action is prevention of transcriptional activation of genes responsible for interleukin-2 (IL-2) production, a necessary step for full activation of the T-helper cell pathway [95, 97]. The absence of IL-2 synthesis prevents the activation and proliferation of T cells in addition to the secondary synthesis of cytokines involved in CAD, such as IL-4, IL-5, IL-8, and IFNγ[95]. A systematic review and meta-analysis of 10 controlled clinical trials enrolling approximately 800 patients, provided strong evidence for the efficacy of CSA in the treatment of CAD, and concluded that the efficacy of CSA was comparable to that of oral corticosteroids[14]. The clinically effective dose of CSA for the treatment of CAD has been determined to be 5 mg/kg orally once daily [84, 168]. Treatment with CSA is typically maintained lifelong (as with all treatments for CAD), and 40-50% of dogs have continued control of their disease with every other day dosing at 5 mg/kg after four to eight weeks of induction daily dosing[14].

The cost of therapeutic agents as well as potential adverse effects are important factors for consideration, particularly when coupled with the fact that CAD commonly
manifests at a young age and requires lifelong treatment. Using the current manufacturer’s suggested retail price for Atopica®, the average annual cost for dogs receiving 5 mg/kg daily during a four to eight week induction phase followed by 5 mg/kg every other day maintenance phase would be approximately $650-750 per 10 kg body weight. Cost of Atopica® was listed as a reason for discontinuing treatment in 10% (5/51) of dogs with CAD being treated for at least 6 months in a study of long-term use of CSA [169]. The authors are not aware of any published studies assessing the effect of drug cost on owners’ initial choice of therapy for their dog with CAD.

Adverse effects of CSA administration for treatment of CAD are generally mild and include transient vomiting and diarrhea most commonly; with papillomatosis, gingival hyperplasia, hypertrichosis, and increased risk for bacterial infection reported in a smaller percentage of patients[5, 14]. Even when administered to dogs at 45 mg/kg daily for one year; no evidence of hypertension, hepatotoxicity, nephrotoxicity, or bone marrow toxicity was observed, all of which are concerns of CSA administration in humans[5, 161].

As the cost of Atopica® may be a limiting factor for its use, methods to reduce the dose of CSA without loss of clinical efficacy have been evaluated. Cyclosporine is primarily metabolized in the liver by cytochrome P450 enzymes 2B11 and 3A12/26 [154, 173]. Consequently, research has thus far focused on pharmacotherapeutic manipulation of this enzyme system by co-administration of drugs known to be P450-enzyme inhibitors. Recent studies indicate that neither metoclopramide nor cimetidine, both P-450 inhibitors, have any effect on the pharmacokinetic profile of CSA [35, 36].
Grapefruit juice and powdered whole grapefruit may variably affect the pharmacokinetic parameters of CSA in dogs [35, 37]. Ketoconazole (KTZ) is a potent inhibitor of numerous cytochrome P450 enzymes, including CYP 2B11 and 3A12/26[154, 173]. Numerous in vitro and in vivo studies have demonstrated that co-administration of KTZ with CSA results in increased whole blood CSA concentrations in canines[31-34, 166, 174]. The inhibitory effect of KTZ on CSA clearance from blood was determined to be dose dependent, with the critical KTZ dosage range identified as 2.5-10 mg/kg daily[34]. A study on normal research beagles investigating the dose of KTZ necessary to maintain whole blood CSA trough levels between 400-600 mg/ml showed that KTZ at 13.6 mg/kg daily enabled a 75% reduction of CSA dose with an estimated monetary savings of 57.8% at that time, and KTZ at 4.7mg/kg daily reduced CSA dose by 38% and thus reduced cost by 23.8%[33]. These results have been clinically applied in studies on the efficacy of combination KTZ and CSA for the treatment of canine perianal fistulas, with excellent short-term results and a significant decrease in dose of CSA required for clinical efficacy. One study involving 12 dogs with perianal fistulas showed that doses of KTZ between 5 and 11 mg/kg daily allowed a 50-75% dose reduction of CSA while maintaining target CSA trough levels, and all 12 dogs had at least short-term resolution of clinical signs[31]. This reduced dose equaled a monetary savings of 35-71% when compared to cost of treatment with CSA alone. Further studies in dogs with perianal fistulas had concurring results; a dose of 10mg/kg daily of KTZ allowed for an 80% reduction of CSA administration, and doses of 5.3-8.9 mg/kg twice daily of KTZ enabled a reduction of CSA dose equaling a savings of 70% compared to CSA alone[32, 166].
As CSA is a lipophilic drug, it distributes widely in tissue and has been reported in the skin at concentrations up to 10 times higher than blood concentrations in humans[9, 175]. An abstract of an unpublished study in dogs dosed with CSA at 3.8 mg/kg once daily for 14 days found that skin levels of CSA were 2.5 to 6.4 times higher than the whole blood CSA levels, and that depletion of CSA is slower from the skin than the blood[119]. Studies examining tissue levels of CSA are sparse and are primarily toxicological or post-mortem studies. One rat model evaluated a single intravenous dose, a single oral dose, and oral dosing for 21 days using radioactively-labeled CSA and measurement of CSA by high performance liquid chromatography (HPLC). After repeated oral dosing for 21 days, 12.64% of the total dose was found in the skin, 11.33% in fat, 7.51% in the liver, and 0.9% in the kidney[10]. All tissue concentrations consisted of mainly parent compound (versus metabolites) and were higher after multiple dosing, increasing by a factor of 1.2 to 4.4 with levels accumulating most significantly in the skin. A human study likewise confirmed that parent drug CSA in the kidney exceeds that in blood by 5-10 times and was well distributed in both the kidney cortex and medulla[118]. This accumulation of CSA in the kidney has been proposed to play a role in nephrotoxicity in humans and rats[118, 176].

Concurrent use of KTZ and CSA for treatment of CAD has been suggested by veterinary dermatologists at continuing education meetings [36, 38, 39, 177-179]. However, the authors are not aware of any published studies that have evaluated the effects of KTZ on CSA skin levels in dogs, or any published clinical trials of the efficacy of this combination of drugs for the treatment of CAD. Thus, the specific aims of this
study were to determine skin and whole blood CSA concentrations when CSA was administered alone at recommended (5.0 mg/kg/day) and sub therapeutic (2.5 mg/kg/day) doses, and when administered at sub therapeutic (2.5 mg/kg/day) doses concurrently with KTZ at two different doses (2.5 mg/kg/day or 5.0 mg/kg/day). We hypothesized that when CSA was administered alone at the recommended dose (5.0 mg/kg/day), the skin and whole blood CSA concentrations would not differ significantly from those obtained with sub therapeutic CSA dosing (2.5 mg/kg/day) concurrently with either dose (2.5 mg/kg/day or 5.0 mg/kg/day) of KTZ.

3.3 Materials and Methods

3.3.1 Animals

The experimental protocol was approved by the Institutional Animal Use and Care Committee (IACUC). Six clinically normal adult laboratory dogs (foxhounds) one year of age to four years of age were used in this study. All dogs had complete blood counts and serum biochemical profiles prior to study enrollment. Dogs were housed in the laboratory research facility at a veterinary college and were under the care of the University Laboratory Animal Resources (ULAR) staff. The animals were housed indoors, in individual concrete runs, in a temperature and humidity controlled environment. They were maintained on a diet of Iams Mini Chunks (The Iams Company; Cincinnati, OH) or Teklad 25% Lab Dog Diet (Harlan Laboratories; Indianapolis, IN), fed once a day, with occasional Iams dog treats, and water *ad libitum*. During the study periods, food was available from 2 p.m. until 7:30 a.m.
All treatments (CSA and CSA/KTZ) were administered at 9:30 a.m. to comply with the Atopica® label recommendation that the drug should be administered at least 2 h before or after feeding as bioavailability is better in fasted animals.\[18\] Vomiting for more than two consecutive dosing periods (48 h) was deemed criteria for withdrawal from the study, as were signs of systemic illness (lethargy, fever, changes in complete blood counts or chemistry values above or below the reference range). Anti-emetics were not permitted as most of these agents are also metabolized by (or affect) the cytochrome P450-enzyme system.\[154\] The dogs were visually monitored daily and any adverse events (including vomiting or diarrhea/soft stool as well as erythema, swelling, or discharge from biopsy sites) were recorded. Biopsy sites that became infected were to be treated topically with chlorhexidine gluconate solution 2% (Phoenix™ Pharmaceutical Inc.; St. Joseph, MO) and Triple antibiotic ointment® (E. Fougera and Co; Melville, NY) twice daily until completion of the study period, and then with 5.0-10.0 mg/kg generic cefpodoxime (Proxetil Putney Inc; Portland, ME) once daily until resolution of the infection. Antibiotic administration was not permitted during the study period to avoid increasing the likelihood of vomiting or diarrhea.

3.3.2 Sample collection

Whole blood samples for CSA analysis (1.0 ml) were collected via cephalic, lateral saphenous, or jugular venipuncture with a 22-gauge needle. The blood was placed into a lavender top tube (EDTA tube) and gently mixed, then transferred to Eppendorf tubes (VWR International, Radnor, PA) and stored frozen at -80º C until analysis.
Skin samples were collected from the dorsal and dorsolateral neck and trunk due to ease of access and maximal skin thickness for sample analysis. Local anesthesia was performed by injecting 0.5 mls of 2% lidocaine (Butler Schein™ Animal Health; Dublin, OH) subcutaneously with a 25-gauge needle. After 5 min, the skin sample was collected with an 8-mm biopsy punch (Medichoice® Tru Punch Disposable Biopsy Punch, Owens & Minor; Mechanicsville, VA), and the site was closed with a single cruciate suture using 3-0 absorbable monofilament (3-0 PDS, Ethicon ©, Novartis Animal Health; Greensboro, NC). All subcutaneous tissue was trimmed from the skin sample, and the skin was placed in an Eppendorf tube and stored frozen at -80 °C until analysis.

3.3.3 Study design

Prior to commencement of the study, whole blood and skin samples were taken for CSA analysis to ensure that each dog was beginning with CSA levels below the limit of detection. Completion of each of the four treatment periods detailed below was followed by a 14-day washout, at which time whole blood and skin samples were collected for CSA analysis to ensure that all CSA concentrations were below the level of detection prior to receiving the next treatment. Complete blood counts and serum biochemical profiles were performed on all dogs within 90 days prior to study enrollment as well as prior to (day 0) and immediately following completion (day 8) of Treatments 3 and 4.

The six dogs were randomly assigned via a computer-generated list (using the dogs’ research number) into two groups of three dogs each. One group was administered CSA at 5 mg/kg orally once daily for 7 days (Treatment 1), and the other group was
administered CSA at 2.5 mg/kg orally once daily for 7 days (Treatment 2). CSA was administered as Atopica® available as 10 mg, 25 mg, 50 mg and 100 mg capsules. Combinations of these capsule sizes were used to dose the subjects as close as possible to 2.5 and 5.0 mg/kg/day, and the calculated dose was rounded up if necessary to match the available capsule size(s). After the 1\textsuperscript{st}, 4\textsuperscript{th} and 7\textsuperscript{th} dose of CSA, a skin sample was collected at 4 h (estimated peak skin concentration) and at 24 h (estimated trough skin concentration) to determine skin CSA levels.[119] After the 1\textsuperscript{st}, 4\textsuperscript{th} and 7\textsuperscript{th} dose of CSA a whole blood sample was collected at 1.4 h (peak whole blood concentration) and at 24 h (trough whole blood concentration) to determine whole blood CSA levels[18]. Peak concentration samples were thus collected on days 1, 4, and 7; while trough concentration samples were collected on days 2, 5, and 8.

Following a 14-day washout period, the same two groups of three dogs were then randomly assigned to receive either CSA at 2.5 mg/kg and KTZ at 5.0 mg/kg orally once daily for 7 days (Treatment 3) or CSA at 2.5 mg/kg and KTZ at 2.5 mg/kg orally once daily for 7 days (Treatment 4). Ketoconazole (Teva; Sellersville, PA) was administered in the generic form of 200 mg tablets (or part thereof) or as a compounded solution of the tablets per Trissel’s formulary (prepared by the facility’s pharmacy) in order to ensure dosing as close to 2.5 mg/kg or 5.0 mg/kg as possible.[180] The compounded solution was not used for the 5.0 mg/kg KTZ dose due to the large volume required. The CSA and the KTZ were administered concurrently. After the 1\textsuperscript{st}, 4\textsuperscript{th} and 7\textsuperscript{th} dose of CSA and KTZ, a skin sample was collected at 4 h (estimated peak skin concentration) and at 24 h (estimated trough skin concentration) to determine skin tissue CSA levels. After the 1\textsuperscript{st},
4th and 7th dose of CSA and KTZ a whole blood sample was collected at 1.4 h (peak whole blood concentration) and at 24 h (trough whole blood concentration) to determine whole blood CSA levels. Peak concentration samples were thus collected on days 1, 4, and 7; while trough concentration samples were collected on days 2, 5, and 8.

3.3.4 Crossover

After a 14 day washout period, the two groups of dogs then followed a full crossover study design. Thus at completion of the study each of the six dogs had received each of the four treatments in random order.

3.3.5 Cyclosporine Analysis

Skin and whole blood samples were shipped overnight on dry ice to iC42 Bioanalytics (UC Denver, Denver, CO) for analysis. Samples were shipped, and analyzed in two batches; the first sent half way through the study and the second at study completion.

Cyclosporine in EDTA whole blood and tissue was quantified using high-performance liquid chromatography- tandem mass spectrometry (HPLC-MS/MS) following the procedures as previously described. [151] All tissues were weighed. Tissues were pulverized under liquid nitrogen and 100 mg of the frozen tissue powder was measured into 1 mL of the 0.5 mol/L potassium phosphate buffer (pH 7.4) and homogenized. Homogenates were prepared as 200 μL aliquots for extraction.

For protein precipitation, 800 μL of ZnSO₄·7H₂O (17.28 g/L) in 30:70 (v/v) HPLC grade water / methanol containing the deuterated internal standard cyclosporine-
D4 (50 ng/mL) was added to 200 μL aliquots of EDTA whole blood and tissue homogenates. Samples were vortexed for 2.5 min and then centrifuged at 13,000·g for 10 min at 4°C. The supernatant was transferred into glass HPLC vials for analysis.

The extracts were analyzed using an LC/LC-MS/MS system (HPLC Agilent 1100 Series, Applied Biosystems/Sciex API 4000 triple quadruple mass spectrometer, Carlsbad, CA). For on-line sample clean-up, 20 µL supernatant was loaded and cleaned on a 4.6 x 12.5 mm, 5µm, Eclipse XDB-C8 column (Agilent, Santa Clara, CA) using a mobile phase of 20% methanol / 80% 0.1% formic acid at a flow rate of 5mL/min for 1 min. Then the column switching valve (Rheodyne, Cotati, CA) was activated and the analytes were back-flushed onto the analytical column (4.6 x 150 mm, 5µm, Eclipse Zorbax XDB -C8, Agilent) that was kept at 65°C. A gradient was used from 87% methanol/ 13% 0.1% formic acid to 100% methanol in 2.0 min at a flow rate of 1mL/min and held at 100% for 1.5 min. The mass spectrometer was run in the positive MRM (multiple reaction monitoring) mode. The declustering potential (DP) was set to 131 V. Detection of the ions was performed by monitoring the transitions of m/z 1224.6 → 1112.4 for cyclosporine [M+Na⁺] and m/z 1228.6 → 1112.4 for the deuterated internal standard cyclosporine-D4 [M+Na⁺]. The collision energy (CE) was 85eV.

The lower limit of quantification for cyclosporine in EDTA whole blood was 5.0 ng/mL, and 25 ng/g (.025 ng/mg) for tissues. The assay was linear over three orders of magnitude. The inter-assay accuracy was between 85-115% and total imprecision was ≤ 17%. There were no matrix interferences, carry-over or ion suppression. Both batches of
samples were reported with assay specific quality control data and calculated inter-assay accuracy.

3.3.6 Sample size estimation

A pilot study with two dogs was performed prior to the study. Results from the pilot study were used for a power calculation using a power of 80% and significance level of 0.05, which indicated that six dogs would be needed in order to detect a significant difference in CSA concentrations in skin using the trough value from day 8 between treatment groups 1 and 2. The preliminary data suggested that differences when comparing the additional treatment groups were greater, and would require less than six dogs to achieve statistical significance (Stata 10, Stata Corp, College Station, TX).

3.3.7 Statistical analysis

Daily CSA blood and skin concentrations were considered the outcomes in the data analyses using repeated measures approach with PROC MIXED in SAS (v. 9.2, SAS Inst. Inc., Cary, NC). Compound symmetry covariance structure was used to account for the non-independence of the repeated observations from individual dogs. The effects of day, treatment, and order of treatments on skin and blood CSA concentrations were initially assessed and then also treatment-day interaction was tested. If the treatment-day interaction was significant, analyses were further stratified by treatment; and blood and skin CSA concentrations between the days were compared within each treatment, separately for days with presumed peak (days 1, 4 and 7) and trough (days 2, 5 and 8) values. Pairwise comparisons between days and between treatments were performed by obtaining least squares means and using Tukey-Kramer adjustment to account for
multiple comparisons. Correlation between skin CSA and blood CSA values was assessed using Spearman Correlation Coefficients. For all analyses, values with \( P \leq .05 \) were considered statistically significant.

3. 4 Results

All treatments were administered as intended (Table 2) except for one dog (# 38), that received medications in ~15 g of canned food as the dog was resistant to manual pilling. The inter-assay accuracy of the CSA HPLC-MS/MS according to comparison with internal quality controls was reported for the two batches of samples, with each batch containing samples of skin and whole blood from all four treatment groups. The first batch analysis showed that blood CSA detection accuracy ranged from 98.3 to 104\%, and skin accuracy ranged from 95.7 to 115\%. The second batch showed that blood CSA detection accuracy ranged from 86.7 to 102\%, and skin accuracy ranged from 93.6 to 117\%. None of the skin and whole blood samples collected prior to entry into the study or prior to each treatment (following the 14 day washout period) had detectable levels of CSA.

Descriptive statistics for the measured (unadjusted) whole blood and skin CSA concentrations for all treatment groups by day are presented in Table 3 and Figures 1 and 2. Using the repeated measures model and evaluating for fixed effects; treatment (Treatment 1, 2, 3 or 4), day (day 1, 2, 4, 5, 7, 8) and treatment-day interaction each had a significant effect on skin and whole blood CSA concentrations (F-test; \( P < 0.0001 \)). However, the order in which treatments were received did not have a significant effect on
whole blood or skin CSA concentrations (F-test; \( P = 0.854 \) and F-test; \( P = 0.756 \), respectively).

The adjusted mean whole blood CSA concentration across all days for Treatment 1 (307.5 ng/ml) was not significantly different from the adjusted mean whole blood CSA concentration for Treatment 2 (169.41 ng/ml, Tukey-Kramer; \( P = 0.14 \)) or Treatment 4 (417.74 ng/ml, Tukey-Kramer; \( P = 0.136 \)), when evaluated with a mixed model approach considering treatment, day, and treatment-day interaction. However, the adjusted mean whole blood CSA concentration for Treatment 1 was significantly lower than the adjusted mean whole blood CSA concentration for Treatment 3 (644.83 ng/ml, Tukey-Kramer; \( P = .0002 \)). The adjusted mean whole blood CSA concentration for Treatment 3 was significantly higher than adjusted mean whole blood CSA concentration for Treatment 4 (Tukey-Kramer; \( P = 0.0081 \)), and the adjusted mean whole blood CSA concentration for Treatment 2 was significantly lower than that for Treatment 3 and Treatment 4 (Tukey-Kramer; \( P < 0.0001 \) and \( P = 0.0040 \), respectively).

Utilizing least squares means obtained from the mixed models, the daily mean peak whole blood CSA concentrations did not differ significantly from each other within any treatment group (Table 4). Likewise the daily mean trough whole blood CSA concentrations were not significantly different within Treatment 3; however, they were lower on day 2 compared to day 5 and on day 2 when compared to day 8 in Treatments 1, 2 and 4 (Table 4).

Using the mixed model approach considering treatment, day, and treatment-day interaction, the adjusted mean skin CSA concentration for Treatment 1 (0.61 ng/mg) was
significantly higher than the adjusted mean skin CSA concentrations for Treatment 2 
(0.262 ng/mg, Tukey-Kramer; \( P = 0.05 \)), not significantly different from the adjusted 
mean skin CSA concentration for Treatment 4 (0.697 ng/mg, Tukey-Kramer; \( P = 0.895 \)), 
but was significantly lower than the adjusted mean skin CSA concentration for Treatment 
3 (1.236 ng/mg, Tukey-Kramer; \( P = 0.0006 \)). The adjusted mean skin CSA concentration 
for Treatment 3 was also significantly higher than the adjusted mean skin CSA 
concentration for Treatment 4 (Tukey-Kramer; \( P = 0.0024 \)), and the adjusted mean skin 
CSA concentration for Treatment 2 was significantly lower than that of Treatment 3, and 
Treatment 4 (Tukey-Kramer; \( P < 0.0001 \) and \( P = 0.0129 \), respectively).

Utilizing least squares means obtained from the mixed models, the mean daily 
peak skin CSA concentrations on day 4 were significantly higher than day 1 mean daily 
peak CSA concentrations in Treatments 1, 2 and 3, while day 7 mean daily peak CSA 
concentrations were significantly higher than those of day 1 within all four treatments 
(Table 5). The mean trough CSA concentrations on day 5 were significantly higher than 
those on day 2 for Treatments 1 and 4; day 8 mean trough CSA concentrations were 
significantly higher than day 2 mean trough CSA concentrations for Treatments 1, 2, and 
4; and day 8 mean trough CSA concentrations were significantly higher than those of day 
5 only in Treatment 4 (Table 5).

The correlation between whole blood and skin CSA concentrations combining 
values from all treatments was moderate (Spearman Correlation Coefficient; \( r^2 = 0.6689 \)). 
The correlation of whole blood and skin CSA values within each treatment groups were 
moderate for Treatments 1 and 2 (Spearman Correlation Coefficient of 0.786 and 0.6881,
respectively) and for Treatments 3 and 4 (Spearman Correlation Coefficient of 0.5623 and 0.5766, respectively).

One dog (#38) exhibited higher skin CSA concentrations than other patients, especially for the day 7 peak and day 8 trough of Treatment 3. Figures 3 and 4 show the whole blood CSA (Figure 3) and skin CSA concentrations (Figure 4) for patient number 38 (red) as well as the concentrations of the other five dogs obtained during Treatment 3. Statistical significance was not affected by these values.

Chemistry profile values and complete blood counts for all dogs were within normal reference range for the laboratory prior to study commencement and remained within the reference ranges following completion of Treatment 3 and Treatment 4. Gastrointestinal upset was the most commonly noted adverse event and was generally sporadic. Four of six dogs vomited during the study period; two dogs vomited once, one dog vomited three times, and one dog vomited four times. Only one patient (#97) vomited for two consecutive dosing periods (48 h) during Treatment 1, but did not continue vomiting and thus was not withdrawn from the study. Six of the nine vomiting episodes occurred during Treatment 1. Soft stool was reported from one dog on two occasions during Treatment 1, and two dogs experienced diarrhea on four and five total separate occasions during Treatments 1, 3, and 4.

3.4 Discussion

In this study we have established skin concentrations of CSA that are achieved in normal research dogs when administered CSA at 5.0 mg/kg and 2.5 mg/kg orally once daily, as well as skin CSA concentrations achieved when CSA is administered at 2.5
mg/kg orally once daily with concurrent KTZ at either 2.5 mg/kg or 5.0 mg/kg. The results from this study provide a baseline for comparison of CSA levels in skin and blood at two clinically relevant CSA dosages (5.0 mg/kg and 2.5 mg/kg). It also provides evidence that concurrent administration of a CYP 450 inhibitor (KTZ) significantly elevates CSA levels in skin as well as blood.

Whole blood peak and trough concentrations of CSA when administered at 5.0 mg/kg in this study were within the range of those documented in previous studies[18, 36]. Though 2 h blood CSA concentrations in humans are predictive of acute rejection and clinical outcome in solid organ transplantation, a study of 97 dogs with AD treated with CSA at a mean dose of 4.6 mg/kg orally once daily for 28 days found no significant correlation between clinical improvement and whole blood CSA concentrations[15, 17, 116]. As skin is likely the target organ for treatment of AD, the concentration of CSA achieved in the skin may be important in determining the clinical response to CSA therapy. This is further exemplified by the moderate correlation between skin and whole blood CSA concentrations in our study which indicates that whole blood concentrations do not accurately predict CSA concentrations in the skin. Additionally, skin concentrations in Treatment 2, a dosage of CSA known to be ineffective for the control of CAD, were significantly lower than those found in Treatment 1[168]. The lack of significant difference in whole blood concentrations between Treatment groups 1 and 2 provides further support that skin concentrations may be more representative of the concentration of CSA required for clinical efficacy. Skin concentrations achieved in Treatment 4 were not different than those obtained in Treatment 1. It would be a fair
expectation that clinically, treatment of CAD with either treatment regime would yield similar results. Treatment 4 would offer a considerable (50%) reduction in cost of CSA administration. However, as it is not yet known if skin CSA concentrations correlate to clinical efficacy, identical treatment outcomes cannot be assumed and should be verified in atopic dogs. While Treatment group 1 establishes anticipated skin CSA concentrations, CSA concentrations in dogs clinically affected with AD may differ from those found in research (normal) dogs, and may differ in clinically affected versus non-affected skin. The skin levels established in this study were those found in the dorsal neck, which may differ from those sites typically affected by CAD such as the inguinal and axillary regions, face, ears and ventral neck.

The highest CSA skin concentrations were obtained during Treatment 3, and were significantly higher than those found in both Treatment 1 and Treatment 4. If skin concentrations are in fact correlated to clinical efficacy, higher CSA skin concentrations found with Treatment 3 may provide additional clinical effect for the control of CAD. However, it is possible that the degree of elevation of CSA paired with 5.0 mg/kg of KTZ may provide higher concentrations of CSA than necessary for control of CAD. As previously discussed, the margin of safety for CSA administration in canines is much greater than in humans. However, side effects from CSA have been generally observed to be dose-dependent, supporting the importance of using the lowest effective dose. Though a recent retrospective analysis of the side effects of KTZ in dogs did not indicate that dose was a factor in the number or severity of side effects for KTZ, it has been shown that vomiting occurred more often with co-administration of CSA and KTZ[169, 171].
Vomiting in this study, however, occurred more frequently during Treatment 1. Additionally, inhibition of the CYP 450 enzymes by KTZ has been shown to be dose dependent, which was supported in this study with blood and skin CSA concentrations obtained in Treatment 3 being higher than those in Treatment 4[34]. As KTZ inhibition of CYP 450 can affect the metabolism of a countless other drugs and would be a lifelong therapeutic when paired with CSA for treatment of CAD, it may be important to use the lowest effective KTZ dose to potentially limit the degree of interaction with other drugs[154]. Further, it should be noted that in this study, CSA and KTZ were administered for only 7 days and the long-term effect of this drug combination on the whole blood and skin CSA concentrations is unknown.

The significant increases in mean skin CSA peaks on days 4 and 7 when compared to day 1 as well as significant increases in the mean skin CSA troughs on days 5 and 8 when compared to day 2 concur with previous studies indicating that CSA accumulates in skin after repeated CSA administration[8, 10]. It appears that skin CSA concentrations may have been approaching steady state in Treatments 1, 2, and 3 after 7 days of drug administration as there were no significant differences in the skin CSA concentration between days 4 and days 7, or between days 5 and days 8. However, this does not appear to be the case in Treatment 4 where day 4 peak skin CSA concentrations were less than those on day 7, and day 5 trough skin CSA concentrations were less than those on day 8, which may have been due to the higher KTZ dose. Further studies on CSA skin concentration after continuous daily treatment for greater than 7 days are necessary to accurately establish when true skin CSA steady state is achieved. The
accumulation or storage of CSA in skin may be a factor why every other day or even every three day administration of CSA can control signs of CAD, and may be associated with the time of drug administration required to see clinical effect for treatment of CAD. As CSA does accumulate in the skin with repeated administration and the skin is not a true compartment for drug distribution when considering pharmacokinetics, it is unlikely that skin CSA levels have a clearly defined peak or time of maximum concentration (Tmax). The Tmax is likely variable depending on the duration of treatment, dose, co-administration of drugs affecting CSA metabolism (such as KTZ) and individual patient factors. Four hours was chosen as an estimated time for peak skin CSA concentration in this study based on previous reports[10, 119].

The degree of inter-individual variability in whole blood CSA concentrations was comparable to those reported in previous studies[18, 36]. Inter-individual variation in whole blood CSA concentrations is a well-recognized phenomenon in humans and is associated with a variety of factors such as age, race, and comorbidities[107]. The genetic expression of a functional polymorphism of the CYP 450 enzyme responsible for CSA metabolism as well as ABCB1 genotypes have been the focus of recent research on the inter-individual variation of CSA concentration in humans[130, 133, 181]. ABCB1 genotypes are important because CSA is a substrate of the P-glycoprotein system. ABCB1 genetic polymorphisms have been identified in canines, though not in the context of CSA administration[156, 157]. One dog (#38) did have very high skin CSA concentrations particularly during Treatment 3, even though this dog’s blood CSA concentrations taken at the same time point fell within the normal range. Interestingly,
this is the dog that received medications in a small amount of food (due to difficulty in administration of the oral medications), which has been shown to increase variability in CSA concentrations[18]. However, there is no obvious explanation for why only the skin CSA concentrations were so significantly elevated with a normal blood CSA concentration. It is possible that certain dogs may accumulate CSA in their skin at higher than expected levels; which could correlate to degree of clinical efficacy, time taken to see clinical effect, ability to reduce CSA dosing, or adverse effects.

In conclusion, in this study we have established skin CSA concentrations in normal research dogs when administered CSA at 5.0 mg/kg/day, the known therapeutic dose for treatment of CAD; and at 2.5 mg/kg/day, a known sub-therapeutic dose for treatment of CAD. Additionally skin and whole blood CSA concentrations when 2.5 mg/kg/day of CSA was administered with 2.5 mg/kg or 5.0 mg/kg of KTZ were established. As there was no significant difference in mean blood or skin CSA concentrations when CSA was dosed at 5.0 mg/kg once daily (Treatment 1) when compared to CSA dosed at 2.5 mg/kg once daily with 2.5 mg/kg of KTZ once daily (Treatment 4), it is anticipated that administration of CSA and KTZ concurrently at 2.5 mg/kg/day may be as effective as CSA alone at 5.0 mg/kg/day for treatment of CAD.
Table 2. Target and actual dosage range of cyclosporine administered solely or in combination with ketoconazole to six healthy hound mixes.

<table>
<thead>
<tr>
<th></th>
<th>Treatment 1 (mg/kg)</th>
<th>Treatment 2 (mg/kg)</th>
<th>Treatment 3 (mg/kg)</th>
<th>Treatment 4 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine</td>
<td>Target Dosage</td>
<td>5.0</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Actual Dosage (Range)</td>
<td>5.0-5.3</td>
<td>2.5-2.9</td>
<td>2.5-2.9</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Target Dosage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Actual Dosage (Range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Target and actual dosage range administered for six dogs treated with 5.0 mg/kg of cyclosporine (CSA) once daily (Treatment 1), 2.5 mg/kg of CSA once daily (Treatment 2), 2.5 mg/kg of CSA and 5.0 mg/kg of ketoconazole (KTZ) once daily (Treatment 3), and 2.5 mg/kg of CSA and 2.5 mg/kg of KTZ once daily (Treatment 4).
Table 3. Descriptive statistics for cyclosporine concentrations in whole blood and skin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment 1</td>
<td>544 (0-895)</td>
<td>34.5 (0-73.2)</td>
<td>682.3 (447-798)</td>
<td>87.0 (44.4-199)</td>
<td>710 (191-160)</td>
<td>94.8 (47.9-182)</td>
</tr>
<tr>
<td>Skin</td>
<td>.27 (0-.53)</td>
<td>.27 (0.54)</td>
<td>1.21 (.78-1.96)</td>
<td>.69 (.33-1.03)</td>
<td>1.06 (.84-1.32)</td>
<td>.78 (.39-1.19)</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td>338.5 (144-422)</td>
<td>18.7 (10.3-33.5)</td>
<td>412 (309-553)</td>
<td>31.6 (14.6-57.5)</td>
<td>347 (53.1-586)</td>
<td>380 (112-814)</td>
</tr>
<tr>
<td>Skin</td>
<td>.16 (.12-.25)</td>
<td>.19 (.08-.61)</td>
<td>.44 (.26-.66)</td>
<td>.24 (.12-.41)</td>
<td>.43 (.25-.59)</td>
<td>.36 (.16-.75)</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td>738.7 (371-1420)</td>
<td>699.9 (423-1470)</td>
<td>981.4 (22.2-2130)</td>
<td>249.9 (95.1-522)</td>
<td>1451.3 (848-1950)</td>
<td>392.7 (112-814)</td>
</tr>
<tr>
<td>Skin</td>
<td>.48 (.19-.94)</td>
<td>.37 (.17-.60)</td>
<td>1.68 (.98-2.91)</td>
<td>1.16 (.96-1.57)</td>
<td>2.37 (1.25-4.57)</td>
<td>2.59 (1.26-8.11)</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td>821 (574-1010)</td>
<td>42.6 (21.7-65.9)</td>
<td>813.3 (151-1190)</td>
<td>102 (66.5-179)</td>
<td>1013.7 (710-1270)</td>
<td>131.6 (68.8-206)</td>
</tr>
<tr>
<td>Skin</td>
<td>.36 (.20-.49)</td>
<td>.30 (.20-.40)</td>
<td>.84 (.51-1.05)</td>
<td>.82 (.52-1.31)</td>
<td>1.37 (.97-2.44)</td>
<td>1.18 (.78-1.42)</td>
</tr>
</tbody>
</table>

Mean (and range) cyclosporine (CSA) concentrations in whole blood and skin for six dogs treated with 5.0 mg/kg of CSA once daily (Treatment 1), 2.5 mg/kg of CSA once daily (Treatment 2), 2.5 mg/kg of CSA and 5.0 mg/kg of ketoconazole (KTZ) once daily (Treatment 3), and 2.5 mg/kg of CSA and 2.5 mg/kg of KTZ once daily (Treatment 4). Blood concentrations are reported in ng/ml with skin reported in ng/mg. Days 1, 4, and 7 represent peaks with days 2, 5, and 8 representing troughs.
Table 4. Least squares means obtained from the mixed models for daily peak and trough whole blood cyclosporine concentrations.

<table>
<thead>
<tr>
<th></th>
<th>Peak blood CSA</th>
<th>Trough blood CSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 4</td>
</tr>
<tr>
<td>Treatment 1</td>
<td>544.00</td>
<td>710.00</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>338.50</td>
<td>412.00</td>
</tr>
<tr>
<td>Treatment 3</td>
<td>738.67</td>
<td>981.37</td>
</tr>
<tr>
<td>Treatment 4</td>
<td>821.00</td>
<td>813.33</td>
</tr>
</tbody>
</table>

Least squares means obtained from the mixed models for daily peak and trough whole blood cyclosporine (CSA) concentrations for six dogs treated with 5.0 mg/kg of (CSA) once daily (Treatment 1), 2.5 mg/kg of CSA once daily (Treatment 2), 2.5 mg/kg of CSA and 5.0 mg/kg of ketoconazole (KTZ) once daily (Treatment 3), and 2.5 mg/kg of CSA and 2.5 mg/kg of KTZ once daily (Treatment 4). Blood concentrations are reported in ng/ml. The same superscripts on the daily peak or trough values within a treatment indicate a significant difference between the days with the Tukey-Kramer adjusted P-values given. <sup>a</sup> P = 0.003, <sup>b</sup> P = 0.012, <sup>c</sup> P = .011, <sup>d</sup> P = .007, <sup>e</sup> P = .0008, <sup>f</sup> P < .0001
Table 5. Least squares means obtained from the mixed models for daily peak and trough skin cyclosporine concentrations for six dogs.

<table>
<thead>
<tr>
<th></th>
<th>Peak skin CSA</th>
<th>Trough skin CSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 4</td>
</tr>
<tr>
<td>Treatment 1</td>
<td>.27(^{a,b})</td>
<td>1.21(^{a})</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>.16(^{c,d})</td>
<td>.44(^{d})</td>
</tr>
<tr>
<td>Treatment 3</td>
<td>.48(^{e,f})</td>
<td>1.68(^{e})</td>
</tr>
<tr>
<td>Treatment 4</td>
<td>.36(^{g})</td>
<td>.84(^{h})</td>
</tr>
</tbody>
</table>

Least squares means obtained from the mixed models for daily peak and trough skin cyclosporine (CSA) concentrations for six dogs treated with 5.0 mg/kg of CSA once daily (Treatment 1), 2.5 mg/kg of CSA once daily (Treatment 2), 2.5 mg/kg of CSA and 5.0 mg/kg of ketoconazole (KTZ) once daily (Treatment 3), and 2.5 mg/kg of CSA and 2.5 mg/kg of KTZ once daily (Treatment 4). Skin concentrations are reported in ng/mg. The same superscripts on the daily peak or trough values within a treatment indicate a significant difference between the days with the Tukey-Kramer adjusted P-values given. \(^{a} P < .0001, ^{b} P = .0001, ^{c} P = .0008, ^{d} P = .0011, ^{e} P = .023, ^{f} P = .0013, ^{g} P = .0006, ^{h} P = .035, ^{i} P = .0010, ^{j} P = .0002, ^{k} P = .0083, ^{l} P = .0002, ^{m} P < .0001, ^{n} P = .0030\)
Figure 1. Mean cyclosporine (CSA) concentrations in whole blood for six dogs treated with 5.0mg/kg of CSA once daily (Treatment 1), 2.5mg/kg of CSA once daily (Treatment 2), 2.5mg/kg of CSA and 5.0mg/kg of ketoconazole (KTZ) once daily (Treatment 3), and 2.5mg/kg of CSA and 2.5mg/kg of KTZ once daily (Treatment 4). Blood concentrations are reported in ng/ml. Days 1, 4, and 7 represent peaks with days 2, 5, and 8 representing troughs.
Figure 2. Mean cyclosporine (CSA) concentrations in skin for six dogs treated with 5.0mg/kg of CSA once daily (Treatment 1), 2.5mg/kg of CSA once daily (Treatment 2), 2.5mg/kg of CSA and 5.0mg/kg of ketoconazole (KTZ) once daily (Treatment 3), and 2.5mg/kg of CSA and 2.5mg/kg of KTZ once daily (Treatment 4). Skin concentrations are reported in ng/mg. Days 1, 4, and 7 represent peaks with days 2, 5, and 8 representing troughs.
Figure 3. Cyclosporine (CSA) concentrations in whole blood for six dogs (patient numbers 32, 38, 62, 82, 94, 97) treated 2.5mg/kg of CSA and 5.0mg/kg of ketoconazole (KTZ) once daily (Treatment 3). Blood concentrations are reported in ng/ml. Days 1, 4, and 7 represent peaks with days 2, 5, and 8 representing troughs.
Figure 4. Cyclosporine (CSA) concentrations in skin for six dogs (patient numbers 32, 38, 62, 82, 94, 97) treated 2.5mg/kg of CSA and 5.0mg/kg of ketoconazole (KTZ) once daily (Treatment 3). Skin concentrations are reported in ng/mg. Days 1, 4, and 7 represent peaks with days 2, 5, and 8 representing troughs.
Chapter 4: Conclusions and Future Directions

In this study we have established skin concentrations of CSA that are achieved in normal research dogs when administered CSA at 5.0 mg/kg and 2.5 mg/kg orally once daily, as well as skin CSA concentrations achieved when CSA is administered at 2.5 mg/kg orally once daily with concurrent KTZ at either 2.5 mg/kg or 5.0 mg/kg. The known therapeutic dose of CSA for control of CAD is 5.0 mg/kg/day, with 2.5 mg/kg/day being a known subtherapeutic dose. As there was no significant difference in mean blood or skin CSA concentrations when CSA was dosed at 5.0 mg/kg once daily (Treatment 1) when compared to CSA dosed at 2.5 mg/kg once daily paired with 2.5 mg/kg of KTZ (Treatment 4), it is anticipated that administration of CSA and KTZ concurrently at 2.5 mg/kg/day may be as effective as CSA alone at 5.0 mg/kg/day for treatment of CAD. The combination therapy of 2.5mg/kg of KTZ paired with 2.5mg/kg of CSA could reduce cost of CSA administration by approximately 50%, a critically important factor for some patients. Reducing the cost of CSA administration is pertinent for not only maintaining dogs with AD on this therapy to control their lifelong disease, but also in overcoming the initial financial burden of placing a large patient (i.e. Labrador Retriever, Golden Retriever) on CSA to determine if it will be an effective therapeutic for controlling their disease. Treatment 3 blood and skin CSA concentrations were significantly higher than those in all other treatments. It is possible CSA at 2.5 mg/kg paired with 5.0 mg/kg of
KTZ may provide additional clinical benefit for the treatment of CAD, though may also increase the risk for adverse effects particularly with chronic administration.

Many avenues of additional research need to be explored for appropriate interpretation and application of this study. This study was performed in normal research dogs; it is possible that CSA concentrations in patients clinically affected by CAD may differ from those levels found here. Skin samples were taken at estimated peaks and troughs, though there is likely not a true peak or trough due to the pharmacokinetics of CSA. Additionally, the skin sites sampled in this study were not those that are clinically affected by atopic dermatitis. It is possible that CSA levels in the axillae, ear pinnae, interdigital, and inguinal regions may differ from the levels found in the dorsolateral neck/trunk of normal research dogs, and CSA skin concentrations may even differ in affected sites versus non-affected sites in dogs with CAD.

As skin is likely the target organ for CSA in the treatment of AD, the concentration of CSA achieved in the skin may be important in determining the clinical response to CSA therapy for CAD; however, it is currently unknown if skin CSA concentrations correlate to clinical efficacy. The concept of CSA skin concentrations correlating to clinical efficacy is supported by the fact that Treatment 1 CSA concentrations were significantly higher than those in Treatment 2 only in skin, not in blood, which correlates to reported clinical efficacy at the 5 mg/kg dose of CSA but not at the 2.5 mg/kg dose. Moderate correlation between skin and whole blood CSA concentrations in our study, indicating that whole blood CSA concentrations do not accurately predict CSA concentrations in the skin further supports the possibility that skin
CSA concentrations may be correlated to clinical response. It is possible that certain dogs (as evidenced by patient #38 – who had very high skin CSA concentrations particularly during Treatment 3, even though this dog’s blood CSA concentrations taken at the same time point fell within the normal range) may accumulate CSA in their skin at higher than expected levels. This accumulation could correlate to degree of clinical efficacy, time taken to see clinical effect, ability to reduce CSA dosing, or adverse effects.

Prior to investigating if skin CSA concentrations correlate to clinical efficacy, additional studies should be performed evaluating CSA concentrations in skin of different body regions, CSA skin concentrations throughout multiple time points in the day, as well as CSA concentrations after administration of CSA for longer than seven days. This study only evaluated the effect of KTZ on CSA concentrations for a period of 7 days. As CAD is a lifelong disease, the long term interaction and safety of this therapeutic combination is important to explore. Likewise CSA concentrations would need to be established in clinically atopic dogs versus normal research dogs, and ideally in lesional and non-lesional skin of atopics. The data provided by this study provides some normal ranges that would be pertinent for the above-mentioned research.

An additional avenue of research would be utilizing the results obtained here for instituting a clinical trial. Considering our findings, the most likely scenario would be comparison of clinical response of CAD to CSA and CSA/KTZ in two treatment groups with CSA once daily at 5.0mg/kg constituting one treatment and CSA at 2.5mg/kg paired with KTZ at 2.5mg/kg constituting the other.
Monitoring during the treatment of CAD with CSA is not currently recommended as the general perception is that there is no correlation between CSA blood levels and clinical efficacy. However, this belief is based on the results of a single study that measured whole blood CSA concentrations once at a poorly defined time point (samples were collected once within 24 hours of the last CSA dose), and it is as of yet unknown if skin concentrations correlate to clinical efficacy[18]. In the arena of transplantation rejection in human medicine, almost 30 years of monitoring CSA concentrations and research lead to the conclusion that C2 monitoring correlates well with clinical efficacy and reduced toxicity. Considering the much larger margin of safety for CSA in canines as compared to humans and the fact that CAD is non-lethal disease, it is unlikely that a similar body of research will ever be generated in canines. As discussed due to p-glycoprotein expression as well as CYP 450 expression, addition of KTZ to a CSA dosing regimen could potentially increase the inter-patient variability of CSA pharmacokinetics in an unpredictable manner, which may make monitoring CSA concentrations (blood and skin) pertinent particularly in the face of any adverse effects.
References


60. Santoro, D., et al., Expression and distribution of canine antimicrobial peptides in the skin of healthy and atopic beagles. Veterinary Immunology and Immunopathology, (0).


