Vitamin D shows in vivo efficacy in a placebo-controlled, double-blinded, randomised clinical trial on canine atopic dermatitis

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Abstract
Atopic dermatitis (AD) in dogs is among the most common skin diseases in small animal practice. It is an inflammatory disease based on a genetic predisposition to develop hypersensitivity against environmental and food allergens and typical clinical signs up exposure. Treatment sometimes can be difficult and associated with adverse effects. Previous studies evaluating cholecalciferol as treatment for human AD have shown promising results. With canine AD being a good animal model for its human counterpart, it was hypothesised that cholecalciferol might have beneficial clinical effects in dogs, too. In this randomised, placebo-controlled, double-blinded eight-week cross-over study, 23 client-owned dogs received either systemic cholecalciferol (n=16), a vitamin D receptor analogue (n=8) or placebo (n=13). Blood samples for ionised calcium were obtained regularly during the study, and Canine Atopic Dermatitis Extent and Severity Index and pruritus scores, blood levels of vitamin D metabolites, measurements of skin pH and transepidermal water loss were determined before and after. Pruritus and lesion scores decreased significantly in the cholecalciferol group versus placebo. No differences in water loss or skin pH were observed. An increase in serum 25-hydroxycholecalciferol strongly correlated with a reduction in pruritus. Systemic cholecalciferol may be a viable treatment option for canine AD.

Introduction
Atopic dermatitis (AD) in dogs is a frequent inflammatory and pruritic skin disease with characteristic clinical signs.1 2 A genetic predisposition to develop a hypersensitivity against environmental and food antigens is assumed.3 Environmental factors also play an important role in the development of AD in human beings and animals.4 These genetic and environmental factors lead to changes in the epidermal barrier function and the immune response.5 6 Canine AD is considered an excellent animal model for its human counterpart.6–9

The most prominent clinical sign of canine AD is pruritus; erythema or papules may be observed as the sole sign in early stages of the disease. Alopecia, crusts, hyperpigmentation and lichenification develop secondary to self-trauma and/or infection.10 Spontaneous remission is rare,11 and the disease prominently affects the owner’s quality of life,12 comparable to parents of atopic children.13 Treatment typically consists of allergen avoidance, allergen-specific immunotherapy and symptomatic therapy with anti-inflammatory and anti-pruritic agents either individually or in combination.14 The clinical course and treatment can be frustrating.15

Three major factors influence the pathogenesis of AD in human beings and dogs: a compromised skin barrier facilitates penetration of allergens into the skin,16 an exaggerated immune response to those allergens is responsible for cutaneous inflammation17 and the inability to control microorganisms on the surface leads to secondary microbial overgrowth.17 18 Vitamin D is involved in the barrier function, the immune response and the production of antimicrobial peptides (AMP),19 20 thus being instrumental in influencing bacterial growth on the surface of the skin.

Vitamin D is a highly potent hormone, and severe adverse effects such as hypercalcaemia have been associated with cholecalciferol therapy in human beings.21 To minimise these adverse effects, an entire spectrum
of vitamin D receptor (VDR) analogues has been developed which show a much better safety profile in human beings than cholecalciferol, while maintaining clinical efficacy. Paricalcitol is a VDR agonist with potent immunomodulatory efficacy used in human beings with a decreased potential to induce hypercalcaemia and hyperphosphataemia.

25-Hydroxycholecalciferol (25-OH-D3) is the first metabolite of cholecalciferol following hydroxylation in the liver and is one of the most reliable vitamin D metabolites in laboratory evaluation. Its serum concentration has been directly correlated to the severity of AD and atopic asthma in children. Limited data are available in the atopic dog regarding the metabolisation of cholecalciferol when administered at high doses, although one report concerning oncology patients identified only low adverse effects. The kidneys hydroxylate 25-OH-D3 to either the active 1,25-dihydroxycholecalciferol or to the 24,25-dihydroxycholecalciferol (24,25-OH-D3) metabolite. The latter is less likely to result in hypercalcaemia due to its weaker stimulation of osteoclasts. With high serum concentrations of 1,25-dihydroxycholecalciferol, the 25-OH-D3 is preferentially metabolised to the 24,25-OH-D3. The enzyme 1,25-24-hydroxylase is responsible for this metabolisation and a deficiency thereof induces nephro lithiasis in human patients.

The aim of this randomised, double-blinded, cross-over, placebo-controlled study was to evaluate whether vitamin D and the VDR analogue paricalcitol improve clinical signs and skin barrier function of canine AD. A further aim was to correlate serum concentrations of vitamin D and its metabolites to the clinical signs.

Methods

Study design

This study was originally planned as a three-arm randomised, double-blinded, controlled, cross-over trial comparing treatment of canine AD with cholecalciferol, paricalcitol or placebo with a washout period of eight weeks between treatments. Pre-results, the trial was approved by governmental authorities (Government of Upper Bavaria) under the number 55.2-1-54-2532-99-12. After the first 13 dogs had been included, the protocol was changed to a two-arm randomised, double-blinded, cross-over trial due to adverse reactions to paricalcitol.

Study objects

Twenty-three pruritic dogs with confirmed AD were included. The diagnosis was based on clinical history, examination and ruling out differential diagnoses. Owners had to sign a written consent form informing them about possible side effects and crucial criteria for the study. Additional inclusion criteria included flea control for at least eight weeks before the start of the study and a novel single-protein elimination diet for at least six weeks without any clinical improvement. All dogs were on the same commercially formulated diets before and for the duration of the trial. Ongoing therapy for AD was only permitted if it was unchanged and had been administered for at least four weeks before inclusion with antihistamines, fatty acid supplementation or topical therapy and for at least eight weeks before in the case of immunosuppressive drugs such as cyclosporine or glucocorticoids. Allergen immunotherapy had to be ongoing for at least 12 months, and during the last three months before inclusion the dose and frequency of injections had to be constant. A dose change was not permitted during the study for all medications. One dog was on long-term glucocorticoid therapy alone, another dog was on a combination of dexamethasone and allergen immunotherapy (more than two years), one dog was on a combination of cyclosporine and allergen immunotherapy (more than one year) and three dogs were on allergen immunotherapy alone for over two years. Three additional dogs were on long-term antihistamine therapy, apart from topical therapy such as shampooing or essential fatty acids (unchanged for more than six months). Supplementary sources of calcium such as bones or calcium powder were not permitted during the study to reduce the risk of hypercalcaemia.

Study intervention

Once daily, dogs received either placebo (palm oil), cholecalciferol (Vigantol-Oel, Merck KGaA, Darmstadt, Germany) at 300 IU/kg or the VDR agonist paricalcitol (Zemplar, Abbott Laboratories, Chicago, USA) at 0.02 µg/kg orally for eight weeks. The dosage was increased every two weeks based on the patient’s blood calcium concentrations and the severity of clinical signs, from approximately 300 IU/kg to 700 IU/kg to 1400 IU/kg cholecalciferol and from 0.02 µg/kg to 0.04 µg/kg to 0.1 µg/kg paricalcitol by week 4. In one dog, cholecalciferol was further increased to 2700 IU/kg in week 6 by the owner. If the ionised calcium concentration rose above 1.42 mmol/L the dosage was reduced to the previous well-tolerated dosage. If calcium levels were within the reference range (1.20–1.42 mmol/L) and clinical signs were not resolved, the study medication dose was increased. To ensure double-blinding in this study, the paricalcitol and cholecalciferol were both diluted in palm oil to achieve identical medication volumes to cholecalciferol by staff that was otherwise not involved in the study.

The primary study outcome was the decrease of Canine Atopic Dermatitis Extent and Severity Index-03 (CADESI-03) and Pruritus-VAS compared with placebo. Secondary study outcomes were changes in skin barrier function and patients’ general condition (see Fig 1).
Randomisation
The first 12 dogs were randomised to receive either placebo, cholecalciferol or paricalcitol. A computer-based randomisation matrix (Microsoft Excel 2011 for Mac, Microsoft, Redmont, USA) was used to determine the sequence of medications. Five dogs showed elevated ionised serum calcium, and after completion of that study arm treatment was unblinded for those dogs revealing paricalcitol as the cause of the adverse effects in four of the five dogs, the fifth dog developed hypercalcaemia on placebo. Consequently, the use of paricalcitol was discontinued and the remaining 11 dogs were only randomised to receive cholecalciferol or placebo.

Clinical evaluation
A validated lesion score (CADESI-03)\textsuperscript{31} was obtained on days 0 and 56 by a blinded veterinary dermatology specialist. Additionally, on those days a pruritus score (Pruritus Visual Analogue Scale) designed and validated to measure the severity of itch in dogs was determined by the owners.\textsuperscript{32} Owners were also asked for a global assessment (GA) of the treatment efficacy at the last visit. In this assessment, clients evaluated their dog’s general condition as slightly (+1) or clearly improved (+2), slightly (−1) or clearly deteriorated (−2) or unchanged (0) compared with the first visit.

Further evaluations
The transepidermal water loss (TEWL) was determined with a closed-chamber vapometer (Delfin Technologies, Kuopio, Finland) and the skin pH with a pH meter (Mettler Toledo, Schwerenbach, Switzerland), each by calculating the means of five individual measurements on each of four body sites (ventrum, tail base, inner pinna and between the shoulder blades) on days 0 and 56. The fur was clipped directly before measurement, and sampling was performed under adequate room temperature and humidity (according to the manufacturer’s recommendations).

Blood samples were obtained and ionised calcium concentrations determined with blood gas analysis (Siemens RapidPoint 405, Siemens Healthcare, Munich, Germany) on days 0, 7, 14, 28 and 56 to monitor for hypercalcaemia. If adverse clinical changes such as polyuria, polydipsia or anorexia were observed, serum analysis and additional calcium measurements were performed and patients’ medication was withdrawn for one day. If signs resolved, treatment was reintiated at the dose administered before the occurrence of the adverse effects. If signs did not resolve completely within the week, patients were to be withdrawn from the study.

At each visit, a complete dermatological examination was performed including cytology if needed.

Additionally, blood samples from day 0 and 56 were frozen at −80°C and sent frozen to a commercial laboratory (Labor Dr. Limbach & Kollegen, Heidelberg, Germany) for validated serum analysis of vitamin D metabolites, namely 25-OH-D3, 25-hydroxyergosterol (25-OH-D2), 24,25-OH-D3 and 3c-epimer-25-hydroxycholecalciferol (3c-E-25-OH-D3). Studies have shown liquid chromatography tandem mass spectrometry to be a reliable method of measuring vitamin D metabolites in human patients.\textsuperscript{33, 34}

Statistics
Based on a CADESI-03 SD of 18 (as reported in previous studies on canine AD), a power of 80 per cent and a significance level set at 0.05, it was calculated that 12 dogs per study arm were needed to detect a difference

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**FIG 1:** Study chart: tabular mapping of the study course with the three-arm protocol. CADESI, Canine Atopic Dermatitis Extent and Severity Index; GA, global assessment; PVAS, Pruritus Visual Analogue Scale; TEWL, transepidermal water loss.
of 15 points in the CADESI. After data collection, it was decided to only evaluate data of the arms using placebo and cholecalciferol due to the cessation of paricalcitol treatment during the study.

Data were evaluated for normality with a D’Agostino and Pearson omnibus normality test. Changes in CAD-ESI-03, pruritus and serum concentrations of vitamin D and its metabolites between groups were compared with a Mann-Whitney test. Correlations between medication serum concentrations and clinical scores were calculated with a Spearman correlation test. To evaluate potential cross-over effects, the lesion scores of dogs before treatment with placebo first were compared with those of dogs treated with placebo after a course of vitamin D and the eight-week washout period. A P value <0.05 was considered significant. Analysis was performed with a commercial program (GraphPad Prism 6 for Mac, GraphPad Software, La Jolla, USA).

Results

Study objects and adverse effects

Twenty-three atopic dogs were included in the study. Only 2 dogs completed all three treatments, owners of 10 dogs discontinued the study after two treatment arms and 11 dogs received only one arm. In total, 37 treatment courses were conducted: 16 dogs received cholecalciferol, 13 dogs palm oil as placebo and 8 dogs were administered the VDR agonist paricalcitol. Five of those early courses resulted in polydipsia, polyuria and hypercalcaemia. The clinical signs resolved within one day after discontinuation of the medication, and all five patients restarted at the earlier safe dosage and completed the study with normal calcium levels and without further adverse clinical effects. Unblinding in those individuals occurred after each of those dogs had completed the study arm. Four of the five dogs had received the VDR agonist, the remaining patient was in the placebo group. As a result of these 4 dogs’ reactions, the VDR agonist treatment arm was discontinued and the remaining 12 dogs were only treated with placebo and cholecalciferol. Most dogs responded well to 1000–1400 IU/kg. However, few dogs needed a higher dosage and in one dog 300 IU/kg was sufficient. There was no significant difference between the lesion scores of dogs before treatment with placebo first and those of dogs treated with placebo after a course of vitamin D, indicating no carry-over effects.

Clinical evaluation

After eight weeks of treatment, patients who received cholecalciferol showed significantly less pruritus than patients from the placebo group (P<0.0001, Fig 2). Also, 9 of 16 dogs (56.3 per cent) in the cholecalciferol group improved in their pruritus score by ≥50 per cent compared with 1 dog receiving placebo. The difference in decrease of CADESI scores from day 0 to 56 between placebo and cholecalciferol was highly significant (P=0.0069, Fig 3). GA scores did not change significantly. Also, 6 of 16 dogs (37.5 per cent) in the cholecalciferol group improved in their CADESI score by ≥50 per cent compared with 2 dogs in the placebo group. Mean, median and range of improvement in CADESI, pruritus and GA scores are listed in online supplementary table I.

TEWL and pH values

Neither TEWL nor pH changes were significant between any of the groups and time points. Mean, median and range of changes are listed in online supplementary table I.

Vitamin D metabolites

In total, 20 of the 23 participating dogs had 25-OH-D3 serum concentrations below 50 µg/L (17 of these even below 40 µg/L). Concentrations of serum vitamin D metabolites could have been measured in only 33 of the 37 clinical treatment courses. Two samples were lost during processing and in two cases there was not enough serum available for a validated analysis. Over the study period of eight weeks, 25-OH-D3 showed a mean increase of 80 µg/mL (250 per cent) in the cholecalciferol group (n=13; P=0.0007), while the
paricalcitol group 25-OH-D3 concentrations increased by an average of 36 µg/mL (n=7; 105 per cent). The placebo group showed a decreased vitamin D by an average of 14 µg/mL (n=13; −20 per cent). 24,25-OH-D3 mean concentrations increased in the cholecalciferol group by 30 µg/mL (1200 per cent; P=0.0009) and in the paricalcitol group by 11.9 µg/mL (460 per cent), whereas it was again decreased in the placebo group by an average of 4.5 µg/mL (−10 per cent). No significant changes were observed in 25-OH-D2 or 3c-E-25-OH-D3. There was a statistical correlation between the decrease in pruritus-VAS and the increase in 25-OH-D3 (P=0.005, r=0.484) as well as the increase in 24,25-OH-D3 (P=0.008, r=0.46, supplementary table II).

Discussion

In this study, oral vitamin D decreased both pruritus and also acute and chronic skin lesions in dogs with AD. The improvement in the dogs’ pruritus was significantly correlated with an increase of the most common skin diseases in small animal practice and is also of high prevalence in human beings. In human patients, vitamin D has been reported as an important factor in the development and clinical course of AD, often associated with changes in AMP expression. AMP are regarded as the first-line defence of the skin against secondary infections, especially in diseases with a reduced skin barrier function such as AD. Especially the upregulation of cathelicidins promoted via the VDR is considered to play a major role in atopic lesions. In human AD, the concentrations of cathelicidins are significantly decreased compared with human psoriatic skin. In dogs with AD, a first report identified similar changes in gene expression. A specific VDR haplotype gene polymorphism change has been described in human AD; this gene is considered responsible for the regulation of the epidermal barrier function. Furthermore, a study has shown an upregulation of transforming growth factor-β-dependent FoxP3- or interleukin-10-dependent regulatory T cells, which might also influence AD symptoms in dogs.

In this first, double-blinded study evaluating vitamin D in dogs with canine AD, pruritus was significantly reduced after eight weeks of therapy compared with treatment with placebo.

Many human atopic individuals have demonstrated vitamin D serum concentrations below the reference range. A recent study has shown an association between VDR gene polymorphism and severe AD in human adults, where a specific receptor haplotype is more common in atopic people. Based on an unpublished pilot study by one of the collaborators (HK), it was hypothesised that AD in dogs may be associated with decreased vitamin D concentrations as has been described for human beings. Before this study, the authors treated a case series of five atopic dogs with oral vitamin D (cholecalciferol) at 300–1400 IU/kg and assessed clinical adverse effects in addition to laboratory monitoring of serum Ca²⁺ and P levels. Four of the five dogs showed clinical improvement without any adverse reactions. None of the dogs showed laboratory abnormalities. The starting dose was determined due to earlier mice trials and calculations of comparable effects to cholecalciferol in human medicine.

In human beings, there is evidence for an association between atopy and asthma and decreased vitamin D concentrations in blood and skin. Half of the asthmatic children in one study had vitamin D concentrations below the normal range; those concentrations were inversely correlated to prick test responses and positively correlated with the forced expiratory volume. In another study, there was a significant association between the degree of vitamin D deficiency and the severity of AD in children. These studies point to a role of vitamin D in the pathogenesis of atopy. However, while several studies show improvement with vitamin D supplementation, sometimes in combination with heliotherapy or vitamin E, others have failed to identify clinical improvement.
VDR agonists with less toxicity and strong effects on 1,25-dihydroxycholecalciferol-induced AMP in keratinocytes and other skin cells in vitro have been developed to reduce the risk of vitamin D-related adverse effects such as hypercalcaemia in human beings.23 24 In human patients, the VDR agonist paricalcitol showed a low potential for hypercalcaemia in immune-mediated diseases with an additional lower risk for adverse effects such as atherosclerosis.25 26 Other receptor agonists exist, but paricalcitol was chosen for its availability, ease of administration and also for its comparatively low cost, which makes this agent a practical solution in dogs. Despite the good safety profiles in human beings, in this study hypercalcaemia was observed in half of the dogs in the VDR agonist group. Our results indicate that this VDR agonist in dogs probably has a more calcaemic effect than cholecalciferol. Although the total number of dogs treated with paricalcitol is small, the authors conclude that cholecalciferol is preferable to paricalcitol in dogs due to its apparently better safety profile. In addition, vitamin D showed a higher efficacy with respect to the lesions and pruritus of the dogs with AD.

Adverse effects with the placebo were observed in one dog, which developed hypercalcaemia with associated polydipsia and polyuria. In this case, the history and clinical examination were unremarkable and the client refused any further diagnostic work-up, besides the ionised calcium measurement which was mandatory due to the study protocol.

The number of dogs completing all three arms of this cross-over study was very small. The main reason for this lack of compliance was the time-consuming study protocol that required a multitude of visits, particularly in the first two weeks of each arm, leading to a large drop-out during the study after the first and particular the second arm.

A gradual increase in the dose was undertaken, and the final dose of cholecalciferol was frequently not reached until four weeks after inclusion. In future studies, a longer treatment duration could be considered. The increase in serum 25-OH-D3 during the study shows effective absorption of the supplemented cholecalciferol. No adverse clinical effects or serum electrolyte abnormalities were seen with cholecalciferol administration. Additionally, serum-24,25-OH-D3 levels also increased, indicating that the back-up mechanism was activated to metabolise cholecalciferol into the less active form and prevent hypercalcaemia.27 In three cases, 25-OH-D3- and 24,25-OH-D3-concentrations were measured 16 (n=1) and 20 weeks (n=2) after cessation of therapy, respectively. Serum concentrations were slightly elevated in only one case and in the upper reference range in the residual two cases, still indicating a possible prolonged effect of cholecalciferol, so monitoring for adverse effects may have to be continued beyond vitamin D administration. It is notable that despite this lack of clinical efficacy was observed during the eight-week washout period. Possibly, the study period was too short to cause more noticeable/measurable adverse effects. Thus, it is recommended that dogs on vitamin D should be monitored regularly until further studies evaluate long-term safety. The longest follow-up for two dogs treated with cholecalciferol beyond the eight weeks of this study was six months; no clinical adverse effects were noted during that time and hypercalcaemia was not present. In one of these dogs, a 12-week cessation of therapy resulted in disease recurrence with subsequent improvement again on therapy.

It may be possible that 24,25-OH-D3-measurement allows a prediction of long-term adverse effects such as calcium depositions without hypercalcaemia. Although 24,25-OH-D3 concentrations in this study remained elevated for several months in one dog, no adverse effects were noted with administration of cholecalciferol. Longer treatment periods are needed to answer this question. 25-OH-D2 and 3c-E-OH-D3 levels were not changed in this clinical trial.

A sole therapeutic cholecalciferol effect of compensating an increased consumption or even total lack of vitamin D in atopic individuals is possible, but additional effects are presumed. This is supported by an unpublished pilot study conducted by one of the collaborators (HK), suggesting lower vitamin D concentrations in atopic compared with healthy dogs and is suspected in atopic human patients.28

Some recent in vitro studies have shown the involvement of AMP in AD in both human beings and dogs.29 30 In human beings, the synthesis of AMPs is increased under the influence of vitamin D30 and decreased in vitamin D deficiency.31 An increase in AMP expression may contribute to the prevention of secondary microbial involvement. In a study recently performed by the authors’ group, candidate genes involved in the pathogenesis of canine AD were identified which had not been described previously.32 BCL3, a transcription factor that modulates AMPs, was upregulated with allergen-mediated inflammation.33 34 An increase in BCL3 leads to a decrease in AMPs. As vitamin D inhibits the expression of BCL3,35 this is one possible mechanism for the increase of AMPs and a plausible proposal to explain why vitamin D could be an effective treatment in AD.

TEWL as well as skin pH were measured to attempt to document the postulated improvement of skin barrier function in less lesional atopic dog skin.36 1,25-Dihydroxycholecalciferol upregulates genes encoding proteins relevant for the skin barrier function in human beings such as tight junctions, gap junctions and adhesive junctions helping to minimise the penetration of allergens.37 However, in this study, there was no significant change in TEWL or pH values. Either TEWL and pH were not reliable indicators for the skin barrier function, or the authors were not able to measure these changes in the cutaneous barrier with cholecalciferol at the dose and duration used.
Essential fatty acids are reported to be of benefit in dogs with AD by improving the skin barrier function and reducing inflammatory reactions.\textsuperscript{49} Palm oil contains linoleic acid, an essential fatty acid. Cholecalciferol and paricalcitol were both diluted in palm oil for the study blinding, but as there was clinical deterioration in the placebo group, it is unlikely that the results were influenced by the linoleic acid in the placebo.

This study only evaluated a small number of dogs and short-term treatment for eight weeks. However, results suggest that oral vitamin D may be potentially useful for the treatment of canine AD. As dogs are a good animal model for human medicine,\textsuperscript{57} this should be considered as a promising study for future treatment options in both veterinary and human medicine.

The results of this study suggest that systemic cholecalciferol may be a viable and effective treatment alternative for canine AD as it has been shown for human AD. Further studies will need to elucidate the possible beneficial effects of vitamin D on canine AD, evaluate long-term effects and elucidate the mechanisms of cholecalciferol in dogs with AD.

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Competing interests None declared.

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