Characterization and quantification of ceramides in the nonlesional skin of canine patients with atopic dermatitis compared with controls

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Conflict of Interest
No conflict of interest has been declared.

Abstract
As in humans, there is mounting evidence in support of an abnormal skin barrier contributing to the pathogenesis of canine atopic dermatitis (AD). Studies in people with AD have associated an abnormal skin barrier with deficiencies in ceramides, which represent important components of the stratum corneum (SC) intercellular lipid lamellae. Therefore, the goal of this study was to determine if the SC of dogs with AD is deficient in ceramides compared to normal dogs. Samples of SC were obtained from nonlesional skin of the caudal abdomen of 14 patients with AD and 14 age-, breed- and sex-matched healthy controls using a cyanoacrylate stripping procedure, and the subclass and relative amount of ceramides were assessed blindly by thin layer chromatography. Paired t-tests using R statistical computer software revealed the percentage amounts of ceramides 1 and 9 were significantly lower in nonlesional skin of AD dogs compared to controls (P = 0.034 and P = 0.047, respectively), and the cholesterol percentage amount was significantly higher in AD dogs than in controls (P = 0.016). Furthermore, the cholesterol/ceramide ratio was significantly higher in the AD group with respect to controls (P = 0.014). These findings suggest that decreased amounts of ceramides in the skin of dogs with AD may be involved in the impaired barrier function of their skin.

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Introduction
Atopic dermatitis (AD) is a chronic allergic dermatopathy frequently diagnosed in dogs1,2 and humans.3–8 The disease in both species has close similarity in many aspects. The symptoms typically start at an early age; chronic pruritus is the main clinical sign, lesion distribution is similar and dogs and humans with AD have a higher carriage rate of Staphylococcus organisms, and are very prone to develop secondary skin infections.9–17 Considerable research to elucidate the pathomechanism of canine AD has shown that affected dogs, like their human counterparts, have an abnormal immune reaction to environmental allergens that favours a T helper 2 response.18–28 Significant research in humans with AD has demonstrated an impaired stratum corneum (SC) lipid barrier function,29–47 Many of these studies have revealed a deficiency of ceramides in the SC of affected patients.32–38 The ceramides are the largest group of SC lipids.48,49 The other components include cholesterol, free fatty acids, cholesterol sulfate and cholesterol esters.48,49 The SC lipids form a barrier comprised of a broad, continuous, highly organized, repetitive multilamellar membranous structure known as the SC intercellular lipid lamellae.48–51 This epidermal barrier provides several important functions including control of percutaneous absorption of irritants and allergens (permeability barrier function) and regulation of transcutaneous water loss (water permeability barrier function).48–51 Ceramides are deemed to be the most important class of lipids that comprise the skin barrier, given that the barrier cannot be disrupted unless ceramides are removed.49–52 Ceramide deficiency has been specifically linked to increased transepidermal water loss (TEWL) and, consequently, reduced skin hydration values in humans with AD.32 Furthermore, it has been speculated that the constitutive barrier disruption related to decreased ceramides in the SC of people with AD may be associated with their predisposition to Staphylococcus aureus colonization.15 Indeed, this study by Arikawa et al. suggested that decreased levels of ceramides lead to decreased levels of sphingosine, a natural antimicrobial within the SC, and subsequent increased numbers of bacteria on the skin of people with AD. These findings explain, at least partially, the easily irritated and scaly appearance of the skin of individuals with this disease and their propensity to develop secondary skin infections. Moreover, the development of...
a ceramide-dominant barrier repair emollient was shown to recover diminished water barrier function after solvent treatment, 53 alleviate significantly the clinical signs of human AD, and restore the barrier as visualized by transmission electron microscopy (TEM), supporting the belief that an impaired lipid barrier function, mainly attributable to decreased SC ceramides, plays an important role in the pathogenesis of this disease. 45

There is mounting evidence in support of an abnormal skin barrier also contributing to the pathogenesis of canine AD. The direct application of house dust mites to the skin in occluded chambers (atopy patch test) has demonstrated that environmental allergens penetrate the skin and elicit inflammation in dogs with AD, but not in normal dogs. 54 Moreover, when house dust mite solution was applied to the cage floor of high IgE-producing beagle dogs and dogs with AD, it induced clinical signs and histological changes similar to the spontaneous disease, but no reaction was noted in normal dogs. 55 A recent study showed that the epicutaneous route of allergen exposure is the most important one in maintaining AD clinical signs in high IgE-producing beagle dogs compared to the oral and inhalatory routes. 56 Transmission electron microscopy performed on the nonlesional skin of dogs with AD revealed that the extracellular lipid lamellae of the SC were often abnormal in structure, and their continuity and thickness were less as compared to normal canine skin. 57 A recent study corroborates the above findings that nonlesional skin of dogs with AD have lamellar lipids that are disorganized and reduced in number, and furthermore demonstrates that application of a topical skin lipid complex results in production and secretion of endogenous SC lipids, thereby improving the epidermal barrier. 58 Moreover, TEWL in sensitized beagle dogs was increased in atopic predilection sites before and after allergen challenge but not in normal control dogs, suggesting an altered barrier function in atopic areas. 59 Finally, a recent study also documented increased TEWL in both lesional and nonlesional inguinal skin of AD dogs compared to controls, and showed that the relative amounts of ceramides were significantly lower at those same sites, providing further evidence of an alteration in the skin barrier of dogs with AD. 60

The purpose of the present study was to determine whether or not the nonlesional skin of dogs with AD is deficient in specific ceramides compared to the skin of sex-, age- and breed-matched control dogs as has been shown in people with AD.

Materials and methods

Study population

Privately owned dogs with year-round AD were recruited from patients of the Dermatology Service at the Veterinary Medical Center of the University of Minnesota. Inclusion criteria required a diagnosis of AD based on compatible history and clinical signs and exclusion of other pruritic diseases. 61,62 A food elimination trial of 8-week duration was performed on each dog prior to enrollment; however, other pruritic disorders such as sarcoptic mange and flea bite hypersensitivity were excluded on an individual basis as required. Before entering the study, dogs received appropriate therapy for any existing secondary bacterial and yeast skin infections. Antihistamines and essential fatty acids were withdrawn for 14 days, topical and oral glucocorticoids for 21 days, and injectable glucocorticoids and oral cyclosporine for 60 days prior to enrollment. Moreover, dogs were not allowed to be shampooed or have any topical products applied such as rinses, leave-on conditioners, powders, lotions, sprays, creams, ointments and gels for 1 week prior to obtaining samples. Dogs receiving allergen-specific immunotherapy were excluded from the study. Healthy dogs without a history or clinical signs of skin disorders were recruited from patients at the Veterinary Medical Center of the University of Minnesota to serve as age-, breed- and sex-matched controls. Client consent was required for participation in the study. The study was approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Collection of stratum corneum lipids

Stratum corneum samples were obtained by a stripping procedure using cyanoacrylate resin as previously described with minor modifications. 63,67 The samples were obtained from nonlesional skin localized to the caudal aspect of the abdomen. This site was chosen, as the caudal abdomen is an area typically affected in dogs with AD. 3,4,14 The site of SC removal was kept consistent by choosing an area directly ventral to the wing of the right or left ilium and 3 cm from the midline of the abdomen. Hair was shortened with scissors, taking care not to disturb the surface of the skin. The skin was then gently wiped with 70% ethanol to remove skin surface lipids. 67 Two drops of cyanoacrylate resin were placed on the skin and then a glass slide was positioned to adhere to a skin area of approximately 2 x 2 cm2 until dry. This sample, containing often numerous hair shafts, was discarded to avoid false alterations in the final lipid analysis of the SC. Hair shafts have been found to contain lipids including fatty acids, cholesterol and other sterols, and wax alcohols. 60 This stripping procedure was repeated a second time in the same location. An adequate amount of SC was still present after the first stripping as determined by biopsied haemotoxylin and eosin sections (data not shown). Immediately after collection the samples were coded with random numbers, put into a screw-cap test tube and placed on ice. Within 30 min of collection, the collection device containing the sample was rapidly flushed with nitrogen to remove oxygen and the cap replaced. The samples were stored at –20 °C until shipped overnight on ice to the laboratory of one of the investigators (PW) for lipid extraction and measurement.

Lipid extraction

Extraction of lipids from the SC was performed as described previously with minor modifications. 63,67 The cyanoacrylate resin was scraped from the slides using a razor blade and transferred to a glass screw-cap tube (Teflon-lined caps were used). The lipids were extracted in 4 mL of hexane/ethanol 95:5 (v/v) under sonication for 20 min. This extraction was repeated three times with centrifugation at 1000 x g after each extraction. The extracted SC lipids were filtered using a Millipore type HVLP 0.45 μm filter (Sigma Chemical Company; St. Louis, MO, USA), and dried under nitrogen.

Separation and measurement of lipids with thin-layer chromatograms

Samples were stored at –20 °C until prepared for thin layer chromatography (TLC) and the methodology was as previously detailed with some modification. 62 Extracted lipids were dissolved in 100 μL of chloroform:methanol (2 : 1 v/v). Silica gel G TLC plates activated at 110 °C were scored into lanes. 63 The prepared samples and standards were applied to the plates using glass capillaries. The chromatogram was developed first with chloroform:methanol:water phase, 40 : 10 : 1, to 10 cm, then chloroform:methanol:acetic acid, 190 : 9 : 1, to 20 cm, and finally hexane:ethyl ether:acetic acid, 70 : 30 : 1, to 20 cm. 64 After development, chromatograms were air-dried, sprayed with 50% sulphuric acid, and heated to 220 °C for 2 min. 65 Digitized images (TIFF) of the charred chromatograms were captured using a UMAGE MagicScan II flatbed scanner (Micro Direct International Corporation; Palo Alto, CA, USA), and the images were analysed using image analysis software (TNIMAGE). Human cholesterol and ceramide standards were included on each plate and were used as a guide to identification as well as to establish standard curves for quantification. 66 The lipid analyses were performed blinded by one of the investigators (PW).
Statistical analyses
A paired Student’s t-test using R statistical software (www.R-project.org)66 was used to compare the amount of individual ceramides, cholesterol and the cholesterol/ceramide ratio between matched control and AD dogs. All statistical comparisons were evaluated at a 5% level of significance (P ≤ 0.05). With a power of 80%, 10 dogs per group would be needed to detect a difference of approximately 0.8 standard deviations between the groups. We expected standard deviations of the differences in the ceramide levels between the AD group and the control group to be approximately equal to those reported in Table 2 of Bleck et al.38

Results
A total of 28 dogs were enrolled in the study, 14 dogs with AD and 14 age-, breed- and sex-matched controls. The signalments of the 14 matched pairs are presented in Table 1. The mean age of the AD dogs at the time of sampling was 4.6 years (range: 1.3 to 8.75) and in the control dogs, 4.5 years (range: 1.25 to 8.9). There were 10 spayed females and 18 neutered males. The breeds in each group included English setter (n = 1), golden retriever (1), German shepherd dog (1), soft-coated wheaten terrier (1), shih tzu (1), Staffordshire terrier (1), Gordon setter (1), Australian shepherd (1), Labrador retrievers (4), and Labrador retriever mixed breed dogs (2).

The mean percentage amounts and the standard deviations of the ceramide subclasses and cholesterol are shown in Figure 1. The percentage amount for ceramides 1 and 9 was significantly lower in AD dogs than in control subjects (paired t-test; P = 0.034 and P = 0.047, respectively), and the cholesterol percentage amount was significantly higher in AD dogs (paired t-test; P = 0.016). The cholesterol/ceramide ratio was significantly higher in the AD group compared to control subjects (paired t-test; P = 0.014).

Discussion
This study was designed to examine whether deficiencies of one or more ceramide subclasses exist in nonlesional skin of dogs with AD, as there is increasing awareness that epidermal barrier dysfunction is an important component of the pathophysiology of AD,29–47 especially ceramide deficiency.32–38 We identified that both ceramide 1 and ceramide 9 are decreased in nonlesional skin of atopic dogs compared with healthy matched controls. Decreased levels of ceramide 1 have been most consistently documented in people with AD, substantiating our findings.32,33,36–38 Ceramide 1, although the least abundant of the ceramides in humans, is the most vital component of an intact and functional SC lipid barrier.22,32–34,37,38,52,67 It serves as a molecular rivet in stabilizing the intercellular multilamellar lipid array.32,34,51,68 Ceramide 1 is a carrier of linoleate, and linoleate is important in barrier function.32,34,35 It provides fluidity and plasticity to the lipid lamellae.68 In dietary deficiency, linoleate becomes replaced by oleate which results in faulty lamellar bodies that can no longer form lamellar sheets, resulting in diminished water-barrier function.69,70 In support of this, Yamamoto et al. found a significant decrease in the proportion of ceramide 1 and increased levels of esterified oleate of ceramide 1 in the AD patients.32 It is therefore conceivable that the deficiency of ceramide 1 not only results in a structural instability of the lipid barrier, but that its alteration further leads to diminished barrier function.

Table 1. Breed, sex, and age distribution of matched pairs of dogs enrolled in the study

<table>
<thead>
<tr>
<th>Pair</th>
<th>Atopic dogs</th>
<th>Control dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breed</td>
<td>Sex</td>
<td>Age (years)</td>
</tr>
<tr>
<td>1</td>
<td>English setter</td>
<td>FS</td>
</tr>
<tr>
<td>2</td>
<td>Golden retriever</td>
<td>MN</td>
</tr>
<tr>
<td>3</td>
<td>German shepherd dog</td>
<td>FS</td>
</tr>
<tr>
<td>4</td>
<td>Soft-coated wheaten terrier</td>
<td>MN</td>
</tr>
<tr>
<td>5</td>
<td>Shih tzu</td>
<td>MN</td>
</tr>
<tr>
<td>6</td>
<td>Labrador retriever</td>
<td>MN</td>
</tr>
<tr>
<td>7</td>
<td>Labrador retriever mix</td>
<td>MN</td>
</tr>
<tr>
<td>8</td>
<td>Labrador retriever</td>
<td>FS</td>
</tr>
<tr>
<td>9</td>
<td>Labrador retriever</td>
<td>MN</td>
</tr>
<tr>
<td>10</td>
<td>Labrador retriever</td>
<td>MN</td>
</tr>
<tr>
<td>11</td>
<td>Staffordshire terrier</td>
<td>MN</td>
</tr>
<tr>
<td>12</td>
<td>Labrador retriever mix</td>
<td>FS</td>
</tr>
<tr>
<td>13</td>
<td>Gordon setter</td>
<td>FS</td>
</tr>
<tr>
<td>14</td>
<td>Australian shepherd</td>
<td>MN</td>
</tr>
</tbody>
</table>

FS, female spayed; MN, male neutered.

Figure 1. Mean percentages (standard deviation) of ceramides and cholesterol from the stratum corneum of patients with AD and control dogs. □ P < 0.05 in comparison with healthy canine controls.
Ceramide 9 was only recently discovered in humans with the advent of better techniques for isolating ceramides.\textsuperscript{49} Ceramide 9 is in the same subclass as ceramide 1 in that like ceramide 1, it is a carrier of linoleate.\textsuperscript{49} Its importance in the maintenance of the lipid lamellae has not yet been clarified, but perhaps its structural similarity to ceramide 1 indicates a similar important function. Future studies will likely shed light on its role in the lipid barrier.

In this study, cholesterol levels were markedly higher in atopic dogs in comparison to controls, and the cholesterol/ceramide ratio was also significantly higher. These findings have been previously demonstrated in humans with AD by DiNardo \textit{et al.}\textsuperscript{37} These investigators found decreased amounts of ceramide 1 and 3, increased cholesterol levels and an increase in the cholesterol/ceramide ratio in AD nonlesional and lesional skin. Mustakallio \textit{et al.}\textsuperscript{29} and Melnik \textit{et al.}\textsuperscript{35} also identified increased sterols in the SC of people with AD. Cholesterol is the primary sterol found in the SC, and it improves the plasticity and rigidity of the lipid lamellae.\textsuperscript{50} Cholesterol helps to maintain the fluid content of the lipid barrier, which enables the ceramides and free fatty acids to assume a tight and highly ordered configuration.\textsuperscript{71} Increased epidermal cholesterol synthesis has been shown to occur in response to elevated TEWL or disturbances of barrier function.\textsuperscript{38} It is possible, therefore, that accelerated sterologenesis simply occurs in an attempt to restore barrier function, as occurs in essential fatty acid deficiency and in skin that has been treated with detergents or lipid solvents.\textsuperscript{72}

Eleven different subclasses of ceramides have been identified in the SC of humans.\textsuperscript{49,50} Individual ceramides differ in their architecture, and ceramides with structural similarities are grouped under common codes according to the nomenclature system of Motta \textit{et al.}\textsuperscript{73} We only identified five ceramides in the SC of the dogs included in the study based on thin layer chromatogram mobility compared to that of humans. It is possible that dogs have fewer types of ceramide in their SC compared to humans or the samples and methods used in this study only allowed for the identification of five different subclasses. More sensitive quantification techniques may need to be utilized in the future.

The amount of fatty acids in our samples was too small to be quantified. Fatty acids are an important component of sebaceous (surface) and hair lipids and we took necessary precautions to remove the surface lipids by cleaning the skin with alcohol and discarding the outermost layers of the SC.\textsuperscript{51,52} The ceramides and cholesterol, however, are the major specific SC lipids and are clearly related to the barrier function of the skin, whereas the sebaceous lipids coat the hair and the skin surface, but do not contribute to barrier function.

A particular strength of our study design was the use of paired age-, breed- and sex-matched controls. We chose these stringent criteria in order to reduce, as much as possible, the many variables that play a role in the composition of the epidermal lipid barrier. For instance, ceramide content of the SC significantly declines with increasing age in people.\textsuperscript{33,74} Sex-dependent ceramide changes have also been identified in the SC of people.\textsuperscript{75}

We chose the cyanoacrylate stripping method to obtain the SC lipids, as the use of solvent-extraction methods is not optimal for ceramide extraction efficiency and consistency.\textsuperscript{33} We found this method to be a valuable, noninvasive and practical means of obtaining SC for lipid analysis. Taken together, the results of this study suggest that a deficiency in ceramide 1 and 9 along with an increase in cholesterol may be responsible for the lipid barrier defect that is present in dogs with AD. Moreover, the fact that these findings are observed in nonlesional skin further supports the view that barrier impairment represents a basic defect in AD. Whether it is the decrease in ceramides that leads to functional abnormalities in the SC, such as increased TEWL as demonstrated in a previous study, increased penetration of allergens, and the disarray of the lipid lamellae, needs to be further evaluated.

\section*{References}


Résumé  Comme chez l’homme, de plus en plus d’éléments sont en faveur d’un défaut de barrière cutanée dans la pathogénie de la DAC (dermatite atopique canine). Des études ont montré l’association d’un défaut de ceramides (constituants importants des lipides lamellaires intercellulaires de la couche cornée) avec un défaut de la barrière cutanée humaine. Ainsi, le but de cette étude était de déterminer si la couche cornée des chiens atopiques est déficiente en ceramides en comparaison avec les chiens normaux. Des échantillons de couche cornée non lésionnelle ont été prélevés sur l’abdomen caudal de 14 chiens atopiques et 14 chiens contrôles en utilisant une méthode de prélèvement à la colle. La sous-classe et le taux relatif de ceramides ont été évalués en aveugle par chromatographie en couche fine. Les tests statistiques ont montré un pourcentage de ceramides 1 et 9 significativement plus bas sur les peaux nonlésionnelles des chiens atopiques que sur les chiens contrôles (P = 0.034 et P = 0.047 respectivement), et le pourcentage de cholestérol était significativement plus élevé chez les chiens atopiques que chez les chiens contrôles (P = 0.016). En outre, le rapport cholestérol/ceramide était significativement plus élevé dans le groupe atopique (P = 0.014). Ces résultats suggèrent que des taux de ceramides plus bas dans la peau des chiens atopiques pourraient être impliqués dans leur défaut de barrière cutanée.

Resumen  De forma parecida a los humanos, existen evidencias acumuladas en favor de una barrera anormal de la piel contribuyendo a la patogénesis de la dermatitis atópica canina (AD). Estudios en personas con AD han asociado una barrera anormal de la piel a deficiencias en ceramidas, que representan componentes importantes en los lipidos lamelares intercelulares del estrato corneo (SC). Por tanto el propósito de este estudio fue determinar si el SC de perros con AD es deficiente en ceramidas comparado con perros normales. Las muestras de SC se obtuvieron de piel sin lesiones del abdomen caudal de 14 pacientes con AD y de 14 controles comparables en edad, raza y sexo, utilizando una impronta con cinta adhesiva. Se analizaron a ciegas las subclases y la cantidad relativa de ceramidas mediante cromatografía de capa fina. Pruebas t pareadas utilizando un programa R estadístico reveló que los porcentajes de ceramidas 1 y 9 eran comparativamente menores en piel sin lesiones de AD comparados con controles (P = 0.034 y P = 0.047, respectivamente), y el porcentaje de colesterol fue significativamente mayor en perros con AD que en controles (P = 0.016). Mas aún, la relación colesterol/ceramida fue significativamente mayor en el grupo de perros con AD respecto a controles (P = 0.014). Estos hallazgos sugieren que una cantidad disminuida de ceramidas en la piel de perros con AD puede estar implicada en la funcionalidad anormal de la barrera de la piel.

Die Unterkasse und die relativen Mengen der Ceramide wurden mittels Dünnchromatographie blind beurteilt. Gepaarte t-tests unter Verwendung von R statistischer Computer Software zeigten, dass die prozentualen Anteile der Ceramide 1 und 9 in nicht-läsionaler Haut der AD Hunde im Vergleich zu den Kontrolltieren signifikant niedriger waren ($P = 0.034$ bzw. $P = 0.047$), und dass der prozentuale Anteil an Cholesterol bei den AD Hunden signifikant höher war als bei den Kontrolltieren ($P = 0.016$). Darüber hinaus war der Cholesterol/Ceramid-Quotient bei der AD Gruppe im Vergleich mit den Kontrollen signifikant höher ($P = 0.014$). Diese Ergebnisse weisen darauf hin, dass erniedrigte Mengen an Ceramiden in der Haut von Hunden mit AD an der beeinträchtigten Barrierefunktion ihrer Haut beteiligt sein könnten.