

Evaluation of cutaneous and circulating (serum and exosomes) levels of chemokines (CCL17, CCL22, CCL27 and CCL28) in atopic dogs and their correlation with severity of the disease

Domenico Santoro , Linda Archer and Eric Chong

Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA

*Correspondence: Domenico Santoro, Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610, USA. E-mail: dsantoro@ufl.edu

Background – Canine atopic dermatitis (AD) is a complex multifactorial disease characterised by an exaggerated immunological response. Little is known about the role that cutaneous and circulating chemokines play in disease severity.

Objective – To evaluate the messenger (m)RNA and protein levels of selected chemokines in skin and serum of healthy and atopic dogs, and in the atopic group to determine whether there is a correlation with disease severity.

Materials and methods – Skin biopsies and blood samples were taken from 12 privately owned atopic [lesional (AD-L) and nonlesional (AD-NL) skin] and 12 privately owned healthy dogs. Circulating exosomes were extracted from the serum. Cutaneous and exosomal mRNA levels of CCL17, CCL22, CCL27 and CCL28 were quantified using quantitative real-time PCR. Protein levels were evaluated using canine-specific ELISA kits. The severity and extent of the clinical signs also were assessed in the atopic dogs using Canine Atopic Dermatitis Extent and Severity Index, 4th iteration (CADESI-04) and a validated pruritus Visual Analog Scale (pVAS).

Results – The expression of CCL28 exosomes in skin was greater in AD-L when compared to healthy ($P = 0.019$) and AD-NL ($P = 0.002$) samples. However, serum expression was lower in dogs with AD compared to healthy dogs ($P = 0.03$). A higher expression of CCL17 and CCL22 was seen in AD-L when compared to healthy skin ($P = 0.018$ and $P = 0.019$, respectively). There also was a positive correlation between clinical scores and CCL22 (AD-NL; $r = 0.6$, $P = 0.05$) and between the pruritus score and CCL22 (AD-L; $r = 0.6$, $P = 0.05$). Differences in CCL27 concentrations were not observed.

Conclusions and clinical importance – This study suggests that CCL17, CCL22 and CCL28 may play a role in the cutaneous inflammatory response in atopic dogs. They may be considered as markers of disease severity, although further studies are needed to validate these findings.

Introduction

Canine atopic dermatitis (AD) is a common chronic inflammatory skin disease that has been reported to affect as much as 25% of the canine population in the United States.¹ Canine AD has a multifactorial and incompletely understood pathogenesis and shares many clinical characteristics with its counterpart in humans.² It involves a complex interaction between environmental antigens, genetic predisposition, and a number of disparate cell and noncell types. Among others, lymphocytes, cytokines and non-cytokine factors (e.g. chemokines, lipids, antimicrobial peptides)³ play a significant role. This complex interaction results in the activation of both B and T lymphocytes,³ and the production of inflammatory mediators and alarmins.^{2,3} Once the

immunological cascade is activated, it leads to the production and recruitment of many inflammatory cells (e.g. eosinophils and lymphocytes) involved in the pathogenesis of AD.³ The clinical presentation of AD is the result of an imbalance between different T-helper (Th) cell subtypes (Th1, 2, 17 and 22), regulatory T cells (Tregs) and inflammatory mediators (e.g. cytokines, chemokines and lipids).⁴

Immune-modulatory molecules, such as chemokines, play a pivotal role in the recruitment of leucocytes to the site of inflammation.^{3,5} Several studies, in both human and veterinary medicine have shown contrasting results regarding the involvement and association of several chemokines and AD. Recent studies, in humans and dogs, have shown a strong positive correlation between the severity of AD and serum level of the chemokines CCL17^{5,6} and CCL22,⁵ highlighting the potential role of such molecules in the pathogenesis of AD. In addition, a higher mRNA expression of CCL17 and CCL28, and a lower mRNA expression of CCL27 has been shown to be present in lesional versus nonlesional canine atopic skin.^{7,8} Such chemokines have been selected over the

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years for their relevant role in the chemotaxis and skin-homing of inflammatory cells. In particular, both CCL17 and CCL22 bind to the CC chemokine receptor 4 (CCR4), selectively expressed on Th2 lymphocytes,^{9,10} and are highly represented in the blood¹¹ and lesional skin^{8,12} of atopic dogs. Likewise, CCL27 and CCL28, binding to CCR10, are essential for homing of memory T cells to the skin and play a fundamental role in cutaneous T-cell-mediated inflammatory diseases.^{13,14}

Over the years the assessment of the participation in cutaneous allergies of various inflammatory molecules has been determined by cutaneous or systemic expression of such molecules. Obviously, the *in situ* expression of such mediators has been considered the most relevant. However, because of ethical considerations and difficulties in extracting proteins from the skin of affected dogs, the serum has been most often used for such assessment. Unfortunately, serum concentrations of these mediators may not correlate with those in the skin, opening the way to misinterpretation.

Recently, the use of exosomes as a potential alternative to serum concentrations of biomarkers has been proposed in both human and veterinary medicine. Exosomes are small extracellular vesicles that have the semi-selective ability to package and transport diverse biological cargos such as proteins (e.g. cytokines and chemokines), messenger (m)RNAs, micro RNAs (miRNAs) and small molecules between cells.^{15–17} They have increasingly attracted attention as potentially improving the evaluation of circulating concentrations of inflammatory mediators in humans and dogs. This new platform has been shown to be extremely valuable in evaluating organ function based on blood concentrations (e.g. early liver damage), making exosomes a more realistic way to evaluate biomarkers. For example, a recent study in HIV-positive patients and drug abusers compared the levels of cytokines and chemokines free in the plasma and in plasma exosomes, and showed that the concentration with levels differed.¹⁸ Such results highlight the importance of evaluating exosomal protein/mRNA alongside plasma protein.¹⁸ Likewise, in dogs, a recent study showed how the exosomal concentrations of miRNA122 may be interpreted as a biomarker of early hepatic dysfunction in leishmaniotic dogs.¹⁷ Altogether this evidence suggests that the measurement of exosomal mRNA and protein potentially may be more reliable in the investigation of inflammatory biomarkers in canine AD.

To date, because of limited resources, very little information is available on the role that such chemokines play in canine AD. To the best of the authors' knowledge, no studies have been published simultaneously evaluating the expression of chemokines (CCL17, CCL22, CCL27, and CCL28) in serum, serum exosomes and skin (lesional and nonlesional) of atopic dogs. Thus, the main goal of this study was to evaluate the mRNA and protein expression of CCL17, CCL22, CCL27 and CCL28 in the skin and serum (free and in exosomes) of healthy and atopic dogs. A secondary goal was to determine if cutaneous and/or serum (free and in exosomes) concentrations of these chemokines correlate with the severity of AD.

Materials and methods

All dogs were recruited in the veterinary hospital at the authors' institution with previous approval from the Institutional Animal Care and Use Committee (IACUC) (no. 201810434) and a signed owner consent form.

Canine population

Twenty-four privately owned dogs (healthy: $n = 12$; atopic: $n = 12$) were enrolled for this study. Naturally affected, dogs with mild to moderate [Canine Atopic Dermatitis Extent and Severity Index, 4th iteration (CADESI-04): 10–59]¹⁹ nonseasonal ($n = 6$), seasonal ($n = 4$), or nonseasonal with seasonal worsening ($n = 2$) AD were enrolled in the study. Canine AD was diagnosed based on recently published guidelines.²⁰ All dogs were on monthly flea prevention and dogs with nonseasonal dermatitis had food allergy excluded with the performance of a diet trial and challenge. All dogs with non-seasonal clinical signs underwent a strict food trial for at least eight weeks.

Inclusion criteria

Dogs with an age between one and 10 years old and weight ≥ 10 kg were enrolled in this study. At the time of enrollment, healthy dogs had no history of skin disease and were clinically healthy.

Exclusion criteria

Pregnant and lactating dogs were excluded from the study. Dogs with clinical evidence or a history of malignant neoplasia, metabolic/endocrine, parasitic or other allergies (food/flea/contact) also were excluded. Dogs with active bacterial/yeast infections diagnosed based on published guidelines²¹ were excluded. All dogs followed drug withdrawal periods for oral and topical antibiotics and antifungal (four weeks), corticosteroids (eight weeks for deposit injectable, four weeks for oral, three weeks for topical), antihistamines (two weeks), ciclosporin (four weeks), lokivetmab (eight weeks) and oclacitinib (one week). Dogs receiving allergen specific immunotherapy for <1 year before enrollment also were excluded from the study.

Clinical evaluation

All dogs were scored by the same investigator on the day of enrollment using the validated CADESI-04 scoring system.¹⁹ Data on pruritus also were recorded using the validated pruritus Visual Analog Scale (pVAS).^{22,23}

Skin biopsy collection

One 8 mm punch biopsy was taken from skin between the shoulders (nonlesional atopic and healthy skin) and on the ventral thorax/abdomen (lesional atopic skin) using local anaesthesia (1 mL lidocaine hydrochloride 2%, subcutaneously (Hospira Inc.; Lake Forest, IL, USA)). Each biopsy was immediately divided in two halves, placed in 1.5 mL microfuge tubes, and flash frozen in liquid nitrogen. These samples were stored at -80°C until processing for molecular biology [quantitative real-time (qRT-)PCR] and protein evaluation were carried out.

Blood sample collection

Ten millilitres of blood were collected by jugular venipuncture on the same day as the biopsies were taken after ≥ 12 h fasting and placed in two tubes without anticoagulant agents. After clotting for 30 min at room temperature, the blood was centrifuged at 327 g for 15 min and the serum obtained was stored at -80°C until further analysis. One aliquot was used for the determination of free serum levels of CCL17 and CCL28. The other aliquot was used for exosomal isolation and determination of exosomal protein (CCL17 and CCL28) and RNA (CCL17, CCL22, CCL27 and CCL28) concentrations of the selected chemokines.

Total RNA isolation from skin biopsies

One half of the 8 mm biopsy punch was homogenised using a PowerGen 125 (Fisher Scientific; Pittsburgh, PA, USA) and then processed into RNA using the AllPrep RNA/protein kit (Qiagen; Valencia, CA, USA) reagents, according to the manufacturer's protocol. The total RNA concentrations were determined at 260 nm using UV NanoDrop1000 spectrophotometry (Thermo Scientific; Wilmington, DE, USA).

Exosome isolation

Exosomes from serum were isolated using qEV2 smart columns (Izon Science Ltd.; Medford, MA, USA) following the manufacturer's protocols. Once the sample was run through the column, a 50 µL aliquot was used for exosome quantification and quality using a Malvern NanoSight NS300 (Malvern Panalytical; Westborough, MA, USA) confirming the presence of exosomes identified as particles with a mean size of 140 ± 0.65 nm. The remainder of the flow-through was collected and concentrated using an Amicon Ultra-15 Ultracel-100 centrifugal unit (Sigma Aldrich; St Louis, MI, USA). Then the concentrated sample was divided in two aliquots for RNA (qEV RNA extraction kit; Izon Science Ltd) and protein (Total exosome RNA and Protein isolation kit, Invitrogen; Carlsbad, CA, USA) extractions using commercially available extraction kits. Once extracted, total RNA integrity, quality and quantity were assessed using a 2100 Bioanalyzer (Agilent Biotechnologies Inc.; Santa Clara, CA, USA). The protein extracts were quantified using a Micro BCA Protein Assay (Thermo Fisher Scientific;)

Quantitation of mRNAs by qRT-PCR

Total RNA (2 ng) was converted to complementary DNA (cDNA) by reverse transcription of mRNA using Superscript III (Invitrogen). All mRNA levels were quantified using ABI (Applied Biosystems Inc.; Foster City, CA, USA) qRT-PCR methodology (Applied Biosystems, User Bulletin #2). All samples were analysed in triplicate 25 µL RT reactions in a StepOne Plus Real Time PCR System (Thermo Fisher Scientific). The Taqman assay amplifications for CCL17, CCL27, CCL28 and RPL8 were carried out as follows: 95°C for 20 s followed by 50 cycles of 95°C for 3 s and 60°C for 30 s; reaction volume 25 µL. The exosome assays used 250 pg. cDNA per reaction, while the skin assays used 2 ng per reaction. The CCL22 and RPL0 application was carried out using a SybrGreen assay as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min followed by the melting curve cycle of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s; reaction volume 25 µL. The exosome assays used 250 pg. cDNA per reaction, whereas the skin assays used 2 ng per reaction. The results were analysed using the comparative C_T (cycle threshold) method (control versus treated) and the relative mRNA expression of each gene (Tables 1 and 2) was compared using the $\Delta\Delta C_T$ method ($2^{-[\Delta C_T \text{ experimental sample} - \Delta C_T \text{ control sample}]}$). All samples were normalised against the canine ribosomal protein L (RPL8 or RPL0) transcription levels.²⁴

Quantitation of protein levels by ELISA

Skin proteins were extracted using the AllPrep RNA/protein kit (Qiagen) reagents, according to the manufacturer's protocol. The protein concentration was determined at 280 nm using UV NanoDrop1000 spectrophotometry (Thermo Fisher Scientific), whereas exosomal protein concentration was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

The amount of CCL17 and CCL28 were measured using commercially available ELISA kits specific for canine CCL17 and CCL28 (LSBio; Seattle, WA, USA) following the manufacturer's instructions.

Table 1. Inventoried Taqman expression assays

Canine gene	Manufacturer	Product ID
CCL17	Invitrogen	[Cf02622129_m1]
CCL27	Invitrogen	[Cf02622473_g1]
CCL28	Invitrogen	[Cf02622497_m1]
RPL8	Invitrogen	[Cf02663822_m1]

Table 2. Primers used for SYBR Green expression assays

Canine gene	Primer sequences	Amplicon length
CCL22	Forward: GTATCTGCTGCCGGGACTAC Reverse: ACAGTTAGGAAGACCACGCC	109
RPL0	Forward: TTGTGGCTGCTGCTCCTGTG Reverse: ATCCTCGTCCGATTCTCCG	107

The skin samples were further diluted at 1:5 before running the ELISA. Each sample was run in duplicate and the mean was extrapolated and analysed for each group. Protein levels were analysed using the Cytation 1 Cell Imaging Multi-Mode Reader (BioTek; Winooski, VT, USA).

Statistical analysis

Data were tested for normality using the Kolmogorov–Smirnov test ($\alpha = 0.05$). All data were statistically analysed. Differences between the mRNA and protein levels of each chemokine (CCL17, CCL22, CCL27 and CCL28) in the serum, serum exosomes and in the skin (lesional and nonlesional) of atopic dogs were compared using one-way ANOVA or Kruskal–Wallis test, if data were not normally distributed. Either Tukey's or Dunn's multiple comparison test was used for *post hoc* analysis. Differences in chemokine levels in the serum (free or exosomal) of atopic and healthy dogs were compared using Wilcoxon–Mann–Whitney U-test (healthy versus atopic) tests.

The correlation between CADESI-04, pVAS and chemokine levels (mRNA and protein) in the serum, serum exosomes and skin was tested using a Spearman's correlation test and a correlation matrix was extrapolated. *P*-values of ≤ 0.05 were considered significant. PRISM 6 software (GraphPad Software Inc.; La Jolla, CA, USA) was used to compare all statistical data.

Results

Canine population and clinical evaluation

Twelve atopic dogs (six castrated males and six spayed females) and 12 healthy dogs (four castrated males, eight spayed females) were enrolled in this study (Wilcoxon–Mann–Whitney U-test: $P = 0.68$). The average age was 5.4 ± 2.2 years for the atopic dogs and 5.4 ± 2.9 years for the healthy dogs (unpaired Student's *t*-test: $P = 0.97$). The average weight was 24.5 ± 14.6 kg for the atopic and 20 ± 9 kg for the healthy dogs (unpaired Student's *t*-test: $P = 0.43$). Breeds represented in the atopic dog group included mixed breed dogs (seven), golden retriever (one), Brittany spaniel (one), great Dane (one), whippet (one) and papillon (one). Breeds represented in the healthy dog group included mixed breed dogs (seven), whippet (one), great Dane (one), Brittany spaniel (one), Bernese mountain dog (one) and papillon (one).

Lesions biopsied on the atopic dogs included erythema (seven), papules (one) or erythema with papules (four). An average pVAS score of 6 ± 2 and a median CADESI-04 score of 10 (range 4–22) were recorded for the atopic dogs.

Expression of mRNA

Skin. All four chemokines tested were detected in the skin extracts harvested from healthy and atopic dogs.

When compared to CCL17 expression of mRNA in healthy skin concentrations were 2.2- and 6.9-fold higher in nonlesional and lesional atopic skin, respectively.

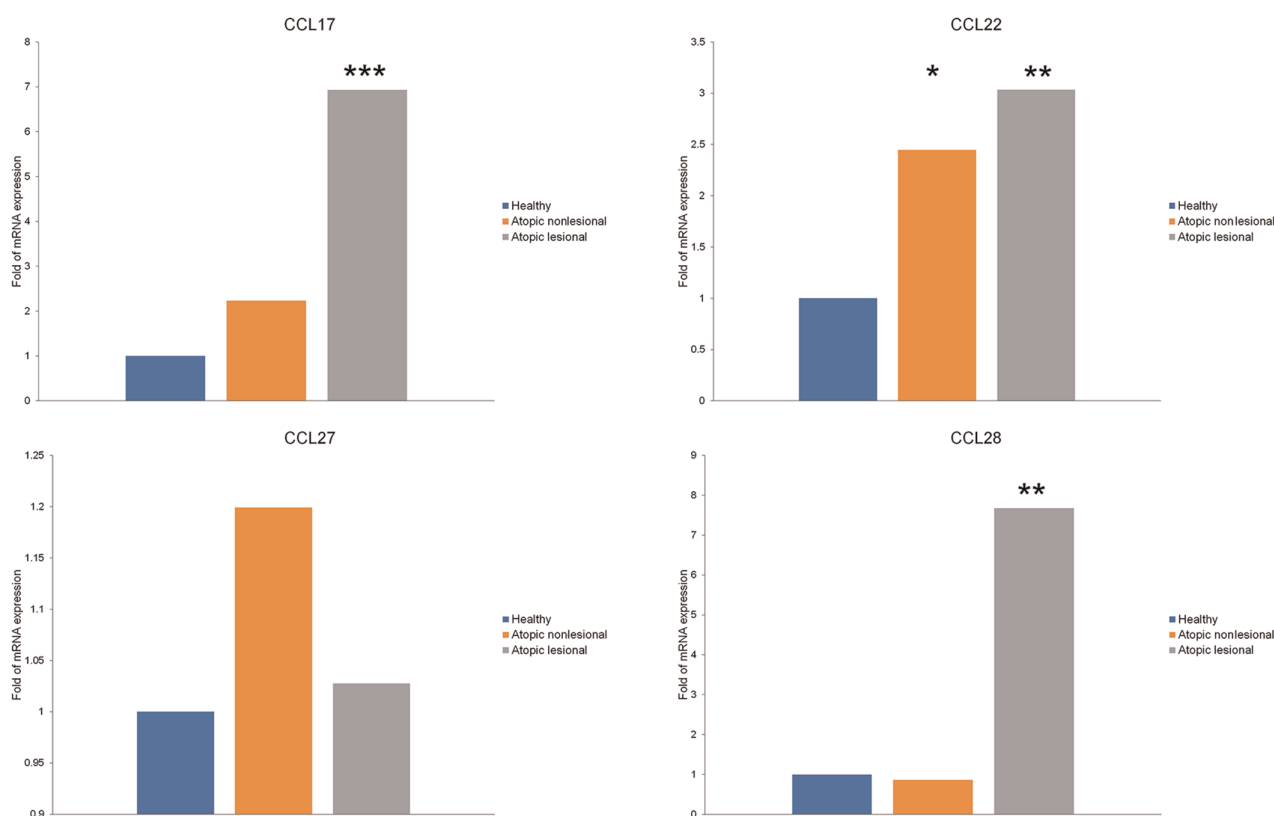


Figure 1. Relative mRNA expression for each chemokine in nonlesional (CCL22: $n = 12$; CCL17: $n = 11$; CCL27: $n = 11$; CCL28: $n = 11$) and lesional (CCL22: $n = 12$; CCL17: $n = 12$; CCL27: $n = 12$; CCL28: $n = 12$) atopic skin compared with normal (CCL22: $n = 10$; CCL17: $n = 11$; CCL27: $n = 12$; CCL28: $n = 11$) control skin. Groups were compared using Dunnett's multiple comparison test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

However, only the comparison between healthy and lesional atopic skin was statistically significant ($P = 0.0013$) (Figure 1).

Likewise, the mRNA expression of CCL22 in atopic skin was 2.4- (nonlesional, $P = 0.048$) and 3.0-fold (lesional, $P = 0.013$) higher than in healthy skin. Both comparisons (nonlesional versus healthy and lesional versus healthy) were statistically significant (Figure 1).

The expression of CCL28 mRNA had a similar trend when compared to the other cytokines. In particular, a 7.7-fold higher expression level of CCL28 was seen in lesional atopic skin when compared to healthy skin; this difference was statistically significant ($P = 0.013$) (Figure 1).

Finally, the expression of CCL27 mRNA was comparable in all groups. In fact, no difference was seen between atopic (nonlesional or lesional) skin and healthy skin ($P > 0.6$) (Figure 1).

Serum exosomes. Totals of 307 ± 146 pg./ μ L and 292 ± 156 pg./ μ L of total RNA were extracted from the serum exosomes harvested from healthy and atopic dogs, respectively ($P = 0.57$). Among the chemokines tested, only CCL27 was detected in the exosomal extract; CCL17, CCL22 and CCL28 were below the detection limit.

The CCL27 mRNA in atopic dogs was 1.5-fold higher than in healthy dogs, although the difference was not statistically significant ($P = 0.6$).

Expression of protein (ELISAs)

Skin. CCL17 was detectable only in the skin of three healthy, five nonlesional and three lesional skin samples. When the samples from atopic skin (lesional and nonlesional) was compared to the healthy skin samples, there was no difference in CCL17 expression ($P > 0.1$) (Figure 2).

CCL28 was detectable in the skin of all dogs, yet the values were above the curve for five healthy, seven nonlesional atopic and four lesional skin samples. When the remaining values were statistically analysed, a significantly lower amount of CCL28 was detected in the lesional (and not nonlesional) atopic skin when compared with the healthy skin ($P = 0.04$) (Figure 2).

Serum. CCL17 was not detectable in the serum of dogs of either group. CCL28 was detectable only in the serum of half the healthy ($n = 6$) and half the atopic ($n = 6$) dog serum. There was no significant difference in serum CCL28 (healthy: 87.1 ± 122.8 pg./mL versus AD: 32.2 ± 19.9 pg./mL; $P = 0.8$)

Serum exosomes. A total of 632 ± 383 μ g/mL and 642 ± 512 μ g/mL protein was extracted from the serum exosomes harvested from healthy and atopic dogs, respectively ($P = 0.85$). Both CCL17 and CCL28 were detected in the exosomal extract. Although there was no difference in CCL17 exosomal concentrations between the two groups, a significantly lower ($P = 0.03$) absolute

Chemokines in canine atopic dermatitis

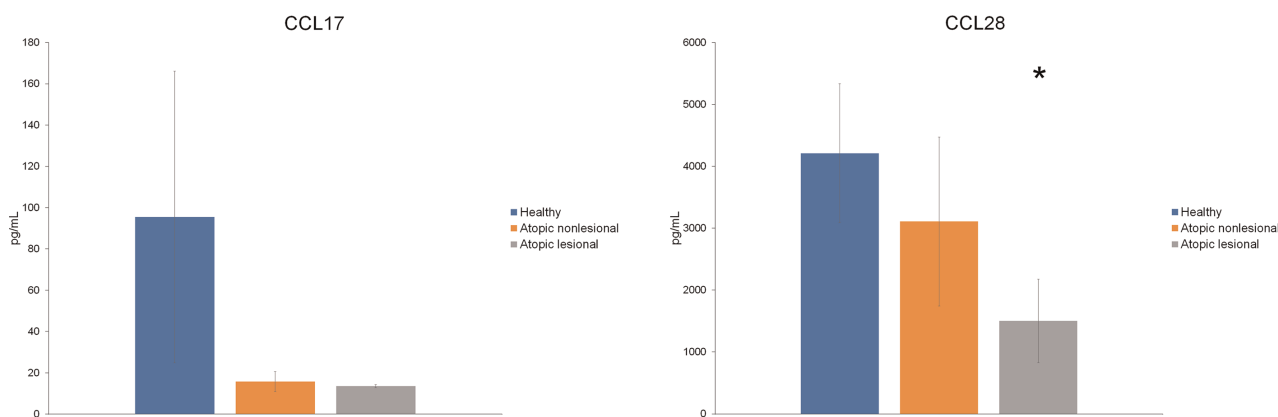


Figure 2. Protein concentrations for each chemokine in nonlesional (CCL17: $n = 5$; CCL28: $n = 5$) and lesional (CCL17: $n = 3$; CCL28: $n = 8$) atopic skin compared with normal (CCL17: $n = 3$; CCL28: $n = 7$) control skin. Groups were compared using Dunn's multiple comparison test (*, $P \leq 0.05$; bars indicate SEM).

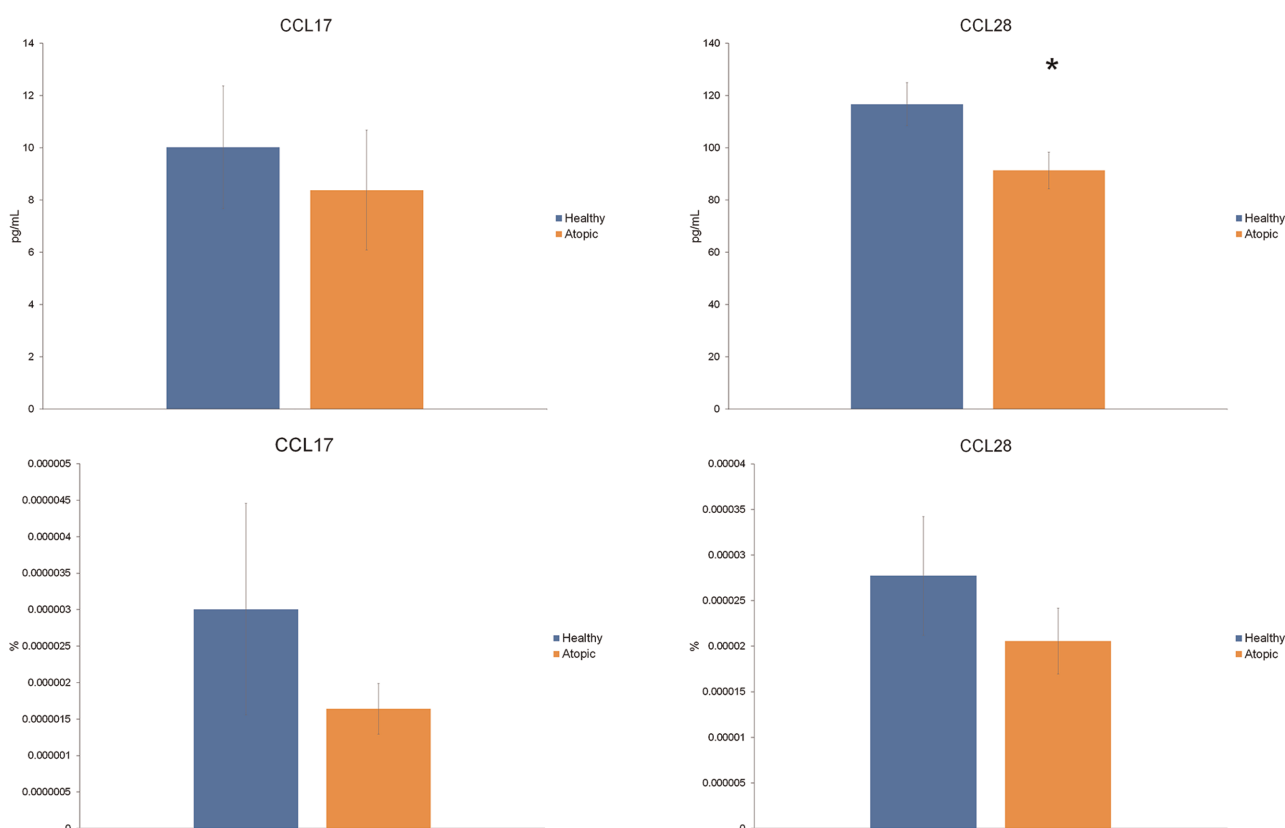


Figure 3. Protein concentrations/ percentages for each chemokine in serum exosomes of atopic (CCL17: $n = 9$; CCL28: $n = 10$) dogs compared with normal (CCL17: $n = 10$; CCL28: $n = 10$) control dogs. Groups were compared using the unpaired Student's t -test (*, $P \leq 0.05$; bars indicate SEM).

amount of CCL28 was present in serum exosomes harvested from atopic dogs (91.3 ± 22.2 pg./mL) as compared to healthy dogs (116.7 ± 26 pg./mL) (Figure 3). However, when the concentration of chemokines was normalised and expressed as a percentage of the total protein exosomal content there was no difference between the two groups for either CCL17 or CCL28 ($P > 0.2$).

Finally, when the amount of exosomal CCL17 was compared to CCL28, a significantly greater amount of CCL28 (absolute or as percentage of total protein) was

seen in both groups (healthy: $P = 0.002$ and AD: $P \leq 0.0004$) (Figure 4).

Correlation

A significant correlation was seen between cutaneous mRNA expression and protein concentrations, and serum exosomal protein expression and the clinical scores, CADESI-04 and pVAS.

In particular, CCL22 mRNA concentrations in nonlesional and lesional atopic skin were negatively correlated with the CADESI-04 ($r = -0.58$; $P = 0.05$) and the pVAS

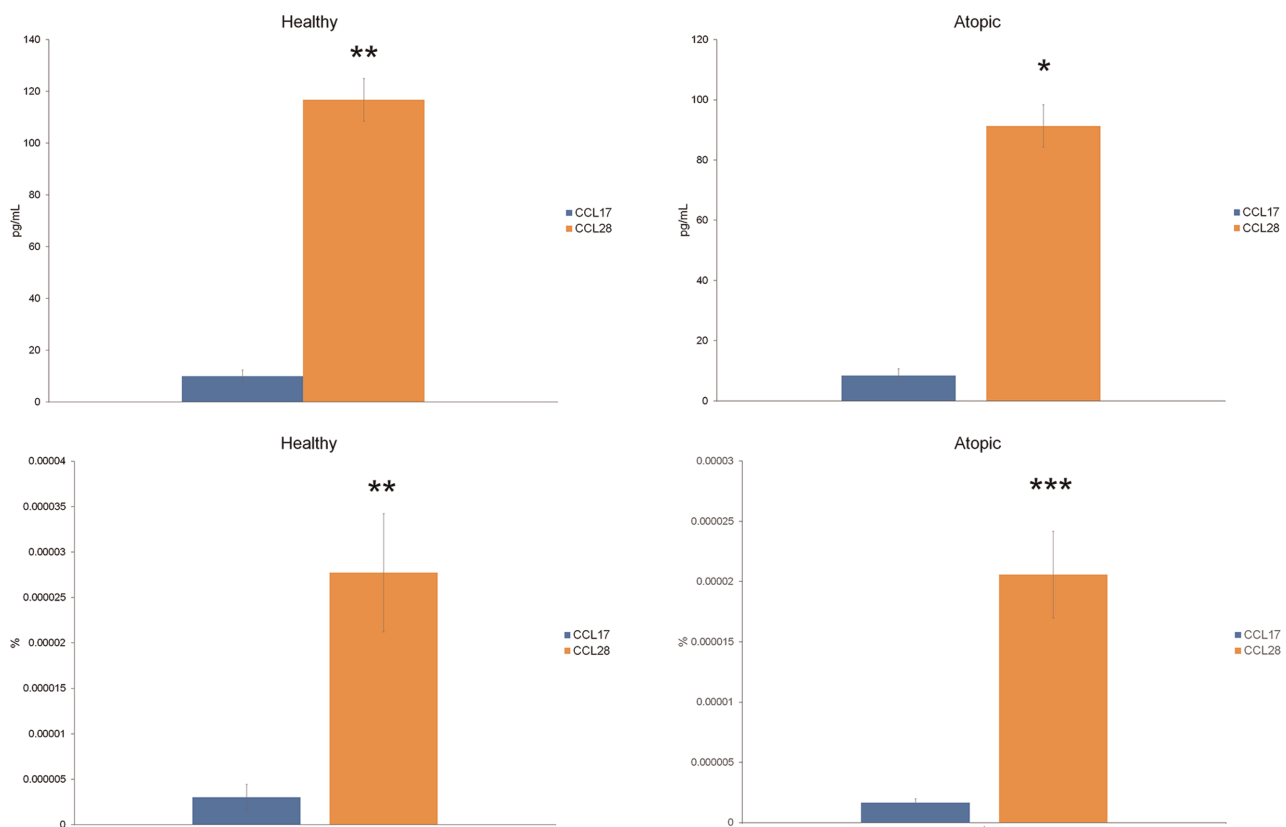


Figure 4. Protein concentrations/percentages for each chemokine in serum exosomes of either healthy (CCL17: $n = 10$; CCL28: $n = 10$) or atopic dogs (CCL17: $n = 9$; CCL28: $n = 10$).

Groups were compared using the paired Student's t -test or the Wilcoxon signed rank test (**, $P \leq 0.01$ and ***, $P \leq 0.001$; bars indicate SEM).

($r = -0.58$; $P = 0.05$) scores, respectively. Likewise, the protein concentrations of CCL28 in the nonlesional atopic skin negatively correlated with the CADESI-04 score ($r = -0.97$; $P = 0.03$). Conversely, the protein concentrations of CCL17 expressed in serum exosomes were positively correlated with the CADESI-04 score ($r = 0.7$; $P = 0.04$).

Discussion

This is the first study reporting the detection of chemokines in serum exosomes in dogs and comparing them to the chemokine mRNA and chemokine protein in the skin and serum of healthy and atopic dogs.

The results of this study showed that the mRNA expression of CCL17, CCL22 and CCL28 is significantly higher in lesional (nonlesional for CCL22 only) atopic skin when compared to healthy skin. However protein concentrations in the skin did not mirror these findings. Additionally, cutaneous concentrations of CCL22 and CCL28 negatively correlated with the clinical scores, while serum exosomal CCL17 protein concentrations positively correlated with the CADESI-04 score.

Chemokines such as CCL17, CCL22, CCL27 and CCL28 are considered essential mediators of inflammation in atopic skin because of their potent cutaneous chemotactic activity for immune cells.³ Such chemokines are produced mainly by keratinocytes and are the basis for skin homing for cells expressing CCR4 and/or CCR10 receptors. Our knowledge of the role that skin-homing

cytokines play in canine AD is based primarily on studies assessing the cutaneous mRNA concentrations of selected chemokines.^{7,8} From such studies, it is clear that, overall, atopic skin has an increased expression of such chemokines when compared to healthy skin. These results were confirmed by the present study in which mRNA was detected for three of the four chemokines tested: CCL17, CCL22 and CCL28. It should be noted that in this study lesional skin included erythema and/or papules which is more consistent with acute lesions rather than chronic changes reported previously.^{7,8} In addition, mRNA concentrations of CCL22 were significantly higher even in nonlesional atopic skin when compared to healthy skin showing a potential involvement of this chemokine in the early stages of the disease, as recently suggested in atopic human patients.²⁵

Contrary to the mRNA concentrations measured, a decreased expression of the protein CCL28 was seen in the lesional atopic skin when compared to healthy skin. This could be explained by protein degradation *in vivo* or an alteration in the transcription machinery.^{26,27} Protein levels of CCL17 and CCL28 for many dogs were higher than the limits of the ELISA detection used, leading to the loss of some data with potential influence on the results.

To the best of the authors' knowledge, previous studies have reported cutaneous mRNA expression of CCL17, CCL27 and CCL28, and no correlation with skin protein concentration has been made.^{10–12} Further studies would be required to validate our findings and explain this discrepancy.

A novel finding of this study was the identification of mRNA and serum and exosomal protein concentrations of some of the chemokines tested. Very recently, Asahina et al.⁶ reported that atopic dogs have a significantly higher expression of CCL17 when compared to healthy dogs using a custom-made ELISA. The magnitude of expression was in the order of ng/mL for atopic dogs and pg./mL for healthy dogs.⁶ Atopic dogs also had a variable CADESI-04 score ranging from 0 to 117 and a median of 12.⁶ These scores were very similar to the ones reported in the present study (median 10, range 4–22) although in our study the variance was much less. We did not detect CCL17 in the serum of any dog. The cause(s) of this discrepancy may include the use of a custom-made versus a commercially available ELISA, the difference in clinical scores, the breeds studied, and the difference between acute and chronic atopic lesions. It is possible that CCL17 increases with the progression of atopic flares and that the dogs enrolled in our study were in an acute phase.

On the contrary, CCL28 was detectable in the serum of healthy and atopic dogs, although at very low concentrations. While the serum from atopic dogs had less CCL28 than the serum of healthy dogs, when the two populations were compared to each other a statistically significant difference between them was not evident. These data are in discordance with recent publications both in atopic children and adults.^{28,29} In the former study, the authors were able to demonstrate a significant increase in the serum protein concentrations of CCL28 during a flare and the concentration was higher in a quiescence state of disease as compared to healthy children.²⁸ In that study the authors were able to show a positive correlation between the serum concentrations of CCL28 and the severity of AD, a correlation that was not found in the canine population analysed herein.

The results of our study show how detectable concentrations of mRNA and proteins can be harvested from exosomes to facilitate the investigation of circulating inflammatory mediators. Exosomes are small extracellular vesicles that contain genetic and protein material to be delivered to distant cells. Because of their mycelial structure, the exosomal content is preserved from enzymatic degradation and it may better reflect circulating levels of inflammatory mediators. In the present study, ≈ 600 μ g/mL total protein and ≈ 300 pg./ μ L total RNA were harvested from healthy and atopic dogs with no difference between the two groups. When chemokine-specific primers were used, the only chemokine detectable was CCL27; in fact, the other chemokines tested were below detection limits. The reasons behind this phenomenon are not clear, yet one potential explanation could be the presence of inactive or noncoding RNAs which can deliver genetic information in a distant location. When the concentration of CCL27 in atopic dogs was compared with that in healthy dogs, it did not reach significance although it was higher. Unfortunately, as a consequence of a lack of reagents, the evaluation of exosomal CCL27 was not possible at the protein level.

Contrary to the mRNA concentrations, the proteins CCL17 and CCL28 were detectable in serum exosomes. When the amounts (absolute and in percentage of total proteins) of both chemokines were compared with each

other, CCL28 was much higher than CCL17 in both healthy and atopic dogs. However, when the absolute amount of each chemokine was compared between healthy versus atopic dogs, a significant difference was seen only for CCL28 (lower in atopic than in healthy dogs). However, such significance was lost when the percentage of CCL28 over the total proteins was considered.

Finally, when the cutaneous and circulating chemokine mRNA and/or protein concentrations were correlated with the clinical signs, the cutaneous expression of CCL22 and CCL28 and the exosomal protein CCL17 were correlated with either the CADESI-04 or the pVAS. Among these, only the exosomal expression of CCL17 was positively correlated with CADESI-04, this being in line with a clinical correlation with serum concentrations of CCL17 showed previously.⁶ Contrarily, the cutaneous concentrations of CCL22 and CCL28 were negatively correlated with the severity of the clinical signs. These latter findings disagree with previous reports showing a positive correlation between serum CCL28 and severity of AD in children.²⁸ To the best of the authors' knowledge, no such studies have been published in atopic dogs.

This study has several limitations and further studies are warranted to determine the role that chemokines play in canine AD. In particular, the small sample size and the lack of reagents to assess the protein expression of all chemokines were factors. Commercially available ELISA kits were available only for CCL17 and CCL28, significantly limiting the array of chemokines that could be studied and compared with the PCR results. In addition, all of our dogs had mild AD and acute lesions, and this may not be comparable with chemokine expression in chronic atopic lesions and/or in moderate to severe AD. Finally, for several dogs, cutaneous protein concentrations of chemokines were much higher than the detection limit of the ELISA assay used, reducing the number of valuable samples tested.

In conclusion, our results suggest that cutaneous and circulating chemokine concentrations are extremely variable in dogs with mild and acute AD. Furthermore, this study highlights how molecular and protein assessment do not necessarily overlap and that proteomic data may not always be extrapolated from molecular data. In fact, in this study high mRNA concentrations of chemokines were associated with low protein expression of the same chemokines. An important finding in this study was the ability to isolate exosomes from the serum of healthy and atopic dogs, and to extract sufficient RNA and protein to assess chemokines expression. This study shows how exosomes can potentially represent a valuable alternative to other biomarkers regarding assessment of disease status. Overall, the results of this study suggest that CCL17, CCL22 and CCL28, and not CCL27, may play a role in the cutaneous inflammatory response seen in atopic dogs. Furthermore, exosomal CCL17 may be considered a good marker of disease severity, although further studies are needed to assess the specificity of such molecules and validate these results.

Author contributions

Domenico Santoro: Conceptualization; formal analysis; funding acquisition; investigation; methodology; project

administration; resources; supervision; visualization; writing – original draft; writing – review and editing. **Linda Archer:** Data curation; investigation; writing – review and editing. **Eric Chong:** Conceptualization; data curation; investigation; writing – review and editing.

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Résumé

Contexte – La dermatite atopique canine (DA) est une maladie multifactorielle complexe caractérisée par une réponse immunologique exagérée. On sait peu de choses sur le rôle des chimiokines cutanées et circulantes dans la gravité de la maladie.

Objectif – Évaluer les niveaux d'ARNm (messager) et de protéines de chimiokines sélectionnées dans la peau et le sérum de chiens sains et atopiques, et dans le groupe atopique afin de déterminer s'il existe une corrélation avec la gravité de la maladie.

Matériels et méthodes – Des biopsies cutanées et des échantillons de sang ont été prélevés sur 12 chiens atopiques [peau lésionnelle (AD-L) et non lésionnelle (AD-NL)] et 12 chiens sains de propriétaires privés. Les exosomes circulants ont été extraits du sérum. Les niveaux d'ARNm cutanés et exosomaux de CCL17, CCL22, CCL27 et CCL28 ont été quantifiés à l'aide d'une PCR quantitative en temps réel. Les niveaux de protéines ont été évalués à l'aide de kits ELISA spécifiques canins. La gravité et l'étendue des signes cliniques ont également été évaluées chez les chiens atopiques à l'aide de l'indice d'étendue et de gravité de la dermatite atopique canine, 4e itération (CADESI-04) et d'une échelle visuelle analogue de prurit validée (pVAS).

Résultats – L'expression des exosomes CCL28 dans la peau était supérieure dans AD-L par rapport aux échantillons sains ($P = 0,019$) et AD-NL ($P = 0,002$). Cependant, l'expression sérique était plus faible chez les chiens

atteints de MA par rapport aux chiens sains ($P = 0,03$). Une expression plus élevée de CCL17 et CCL22 a été observée dans AD-L par rapport à la peau saine (respectivement $P = 0,018$ et $P = 0,019$). Il y avait également une corrélation positive entre les scores cliniques et CCL22 (AD-NL; $r = 0,6$, $P = 0,05$) et entre le score de prurit et CCL22 (AD-L; $r = 0,6$, $P = 0,05$). Aucune différence dans les concentrations de CCL27 n'a été observée.

Conclusions et importance clinique – Cette étude suggère que CCL17, CCL22 et CCL28 pourraient jouer un rôle dans la réponse inflammatoire cutanée chez les chiens atopiques. Ils peuvent être considérés comme des marqueurs de la gravité de la maladie, bien que d'autres études soient nécessaires pour valider ces résultats.

Resumen

Introducción – La dermatitis atópica canina (AA) es una enfermedad multifactorial compleja caracterizada por una respuesta inmunológica exagerada. Poco se sabe sobre el papel que juegan las quimioquinas cutáneas y circulantes en la severidad de la enfermedad.

Objetivo – evaluar los niveles de proteína y RNA mensajero (mRNA) de quimioquinas seleccionadas en la piel y el suero de perros sanos y atópicos, y en el grupo atópico para determinar si existe una correlación con la gravedad de la enfermedad.

Materiales y métodos – se tomaron biopsias de piel y muestras de sangre de 12 perros atópicos de propietarios particulares [piel lesionada (AD-L) y no lesionada (AD-NL)] y 12 perros sanos también de particulares. Los exosomas circulantes se extrajeron del suero. Los niveles cutáneos y exosómicos de RNAm de CCL17, CCL22, CCL27 y CCL28 se cuantificaron mediante PCR cuantitativa en tiempo real. Los niveles de proteína se evaluaron utilizando kits ELISA específicos para caninos. La gravedad y el alcance de los signos clínicos también se evaluaron en los perros atópicos utilizando el índice de extensión y gravedad de la dermatitis atópica canina, cuarta revisión (CADESI-04) y una escala análoga visual de prurito validada (pVAS).

Resultados – la expresión de exosomas de CCL28 en la piel fue mayor en AD-L en comparación con muestras sanas ($P = 0,019$) y AD-NL ($P = 0,002$). Sin embargo, la expresión sérica fue menor en perros con DA en comparación con perros sanos ($P = 0,03$). Se observó una mayor expresión de CCL17 y CCL22 en AD-L en comparación con la piel sana ($P = 0,018$ y $P = 0,019$, respectivamente). También hubo una correlación positiva entre las puntuaciones clínicas y CCL22 (AD-NL; $r = 0,6$, $P = 0,05$) y entre la puntuación de prurito y CCL22 (AD-L; $r = 0,6$, $P = 0,05$). No se observaron diferencias en las concentraciones de CCL27.

Conclusiones e importancia clínica – este estudio sugiere que CCL17, CCL22 y CCL28 pueden desempeñar un papel en la respuesta inflamatoria cutánea en perros atópicos. Pueden considerarse como marcadores de la gravedad de la enfermedad, aunque se necesitan más estudios para validar estos hallazgos.

Zusammenfassung

Hintergrund – Die atopische Dermatitis des Hundes (AD) ist eine komplexe multifaktorielle Erkrankung, die durch eine übertriebene Immunantwort charakterisiert wird. Es ist wenig bekannt über die Rolle, die kutane und zirkulierende Chemokine beim Schweregrad der Erkrankung spielen.

Ziel – Eine Evaluierung der Messenger (m) RNA und der Proteinwerte von selektierten Chemokinen in der Haut und im Serum von gesunden und atopischen Hunden, sowie eine Untersuchung, ob in der atopischen Gruppe eine Korrelation mit dem Schweregrad der Erkrankung besteht.

Materialien und Methoden – Hautbiopsien und Blutproben wurden von 12 privaten atopischen Hunden [(läsionale (AD-L) und nichtläsionale (AD-NL) Haut] und 12 privaten gesunden Hunden genommen. Es wurden zirkulierende Exosome aus dem Serum extrahiert. Kutane und exosomale mRNA Werte von CCL17, CCL22, CCL27 und CCL28 wurden mittels quantitativer real-time PCR quantifiziert. Proteinwerte wurden mittels Hunde-spezifischer ELISA Kits evaluiert. Der Schweregrad und das Ausmaß der klinischen Zeichen wurden bei den atopischen Hunden ebenfalls mittels Canine Atopic Dermatitis Extent and Severity Index, 4te Auflage (CADESI-04) und einer validierten Juckreiz Visual Analog Scale (pVAS) beurteilt.

Ergebnisse – Die Expressierung von CCL28 Exosomen in der Haut war bei AD-L größer im Vergleich zu gesunden ($P = 0,019$) und AD-NL ($P = 0,002$) Proben. Nichtsdestotrotz war die Expressierung im Serum bei Hunden mit AD im Vergleich zu gesunden Hunden ($P = 0,03$) niedriger. Eine höhere Expressierung von CCL17 und CCL22 wurde bei AD-L im Vergleich zu gesunder Haut ($P = 0,018$ bzw. $P = 0,019$) gefunden. Es bestand auch eine positive Korrelation zwischen klinischen Werten und CCL22 (AD-NL; $r = 0,6$; $P = 0,05$) und zwischen den Juckreizwerten und CCL22 (AD-L; $r = 0,6$; $P = 0,05$). Es wurden keine Unterschiede bei den CCL27 Konzentrationen beobachtet.

Schlussfolgerungen und klinische Bedeutung – Diese Studie liefert Hinweise, dass CCL17, CCL22 und CCL28 eine Rolle bei der Entzündungsantwort der Haut atopischer Hunde spielen könnte. Sie könnten als Marker für den Schweregrad der Erkrankung betrachtet werden, obwohl noch weitere Studien nötig sind, um diese Ergebnisse zu validieren.

要約

背景 – 犬アトピー性皮膚炎(AD)は、免疫反応の亢進を特徴とする多因子複合疾患である。皮膚および血中ケモカインが疾患の重症化に果たす役割については、ほとんど知られていない。

目的 – 本研究の目的は、健康犬とアトピー犬の皮膚および血清中の選択したケモカインのメッセンジャー(m)RNAおよびタンパク質レベルを評価し、アトピーグループにおいては、疾患の重症度と相関があるかどうかを判断することであった。

材料と方法 – アトピー[病変皮膚(AD-L)および非病変皮膚(AD-NL)]のオーナー所有犬12頭と健康オーナー所有犬12頭から皮膚生検および血液サンプルを採取した。血清から循環エクソソームを抽出した。CCL17、CCL22、CCL27、CCL28の皮膚およびエクソソームmRNAレベルは、定量的リアルタイムPCRを用いて定量化した。タンパク質レベルは、犬特異的ELISAキットを用いて評価した。臨床症状の重症度および程度も、犬アトピー性皮膚炎重症度指数(CADESI-04)および有効なかゆみのVisual Analog Scale(pVAS)を用いて評価された。

結果 – 皮膚におけるCCL28エクソソームの発現は、健康($P = 0.019$)およびAD-NL($P = 0.002$)サンプルと比較して、AD-Lでより大きかった。しかし、血清中の発現は、健康犬と比較してAD犬で低かった($P = 0.03$)。健康皮膚と比較した場合、AD-LではCCL17とCCL22の高い発現が見られた(それぞれ $P = 0.018$ と $P = 0.019$)。また、臨床スコアとCCL22(AD-NL; $r=0.6$, $P=0.05$)およびそう痒スコアとCCL22(AD-L; $r=0.6$, $P=0.05$)の間に正の相関が見られた。CCL27濃度の差は観察されなかった。

結論と臨床的重要性 – 本研究は、CCL17、CCL22およびCCL28がアトピー犬の皮膚炎症反応に関与している可能性を示唆するものである。これらの所見を検証するためにさらなる研究が必要であるが、これらは疾患の重症度マーカーとして考えられるかもしれない。

摘要

背景 – 犬特異性皮膚炎(AD)は一種複雑の多因素疾病、表現為過度の免疫応答。我们对皮膚和循环趋化因子在疾病严重程度中的作用知之甚少。

目的 – 评价健康和特应性犬皮肤和血清中选定趋化因子的信使(m)RNA和蛋白水平,并在特应性组中确定是否与疾病严重程度存在相关性。

材料和方法 – 从12只私家特应性犬[病变(AD-L)和非病变(AD-NL)皮肤]和12只私家健康犬中采集皮肤活检和血液样本。从血清中提取循环外泌体。使用定量实时PCR定量CCL17、CCL22、CCL27和CCL28的皮肤和外泌体mRNA水平。使用犬特异性ELISA试剂盒评价蛋白水平。还使用犬特应性皮肤炎程度和严重指数第4版(CADESI-04)和经验证的瘙痒视觉模拟量表(pVAS)评估特应性皮肤炎犬中临床体征的严重程度和范围。

结果 – 与健康($P = 0.019$)和AD-NL($P = 0.002$)样本相比,AD-L皮肤中CCL28外泌体的表达更高。然而,与健康犬相比,AD犬的血清表达较低($P = 0.03$)。与健康皮肤相比,在AD-L中观察到CCL17和CCL22的较高表达(分别为 $P = 0.018$ 和 $P = 0.019$)。临床评分与CCL22(AD-NL; $r = 0.6$, $P = 0.05$)、瘙痒评分与CCL22(AD-L; $r = 0.6$, $P = 0.05$)也呈正相关。未观察到CCL27浓度的差异。

结论和临床重要性 – 本研究表明CCL17、CCL22和CCL28可能在特应性犬的皮肤炎症反应中发挥作用。它们可被认为是疾病严重程度的标志物,尽管还需要进一步的研究来验证这些发现。

Resumo

Contexto – A dermatite atópica canina (DA) é uma doença multifatorial complexa caracterizada por uma resposta imunológica exagerada. Pouco se sabe sobre o papel das quimiocinas cutâneas e circulantes na gravidade da doença.

Objetivo – Avaliar os níveis de RNA mensageiro (mRNA) e de proteínas de quimiocinas selecionadas na pele e soro de cães saudáveis e atópicos, e, no grupo atópico, determinar se há correlação com a gravidade da doença.

Materiais e métodos – Biópsias cutâneas e amostras de sangue foram coletadas de doze cães atópicos de proprietários [pele lesional (DA-L) e não lesional (DA-N)] e doze cães saudáveis de proprietários. Exossomos circulantes foram extraídos do soro. Os níveis cutâneos e exossômicos de mRNA de CCL17, CCL22, CCL27 e CCL28 foram quantificados utilizando o PCR real-time quantitativo. Os níveis de proteínas foram avaliados utilizando kits de ELISA específico canino. A gravidade e a extensão dos sinais clínicos foram também avaliadas nos cães atópicos utilizando o *Canine Atopic Dermatitis Extent and Severity Index*, 4th iteration (CADESI-04) e a escala visual analógica de prurido validada (pVAS).

Resultados – A expressão dos exossomos de CCL28 na pele foi maior em DA-L quando comparado a amostras de cães saudáveis ($P=0,019$) e de DA-NL ($P=0,002$). No entanto, a expressão sérica foi menor em cães com DA em comparação com cães saudáveis ($P=0,03$). Observou-se expressão mais alta de CCL17 e CCL22 em DA-L quando comparada à pele saudável ($P=0,018$ e $P=0,019$, respectivamente). Também houve uma correlação positiva entre os escores clínicos e CCL22 (DA-NL; $r=0,6$, $P=0,05$) e entre o escore de prurido e CCL22 (DA-L; $r=0,6$, $P=0,05$). Não foram observadas diferenças nas concentrações de CCL27.

Conclusão e importância clínica – Este estudo sugere que CCL17, CCL22 e CCL28 participam da resposta inflamatória cutânea em cães atópicos. Elas podem ser consideradas marcadores de gravidade da doença, apesar de ainda serem necessários mais estudos para validar estes achados.