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Comparison of circulating CD4$^+$, CD8$^+$ lymphocytes and cytokine profiles between dogs with atopic dermatitis and healthy dogs

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ABSTRACT

Introduction: Canine atopic dermatitis (cAD) is an inflammatory skin disease characterized by impaired immune function. Changes in the proportions of CD4$^+$ and CD8$^+$ lymphocytes and the serum concentrations of cytokines in the pathogenesis of cAD have been described.
Objectives: To assess whether the changes in the ratio of CD4$^+$ and CD8$^+$ lymphocytes in the peripheral blood of atopic dogs at the time of diagnosis are related to the severity of the disease. Furthermore, we determined whether the changes in the serum concentrations of the cytokines IL-31, IL-34, IL-10, IFN-γ, and TNF-α were different between atopic and control dogs.
Procedures: Fifty-six client-owned dogs with atopic dermatitis and 53 healthy control dogs were used. The percentages of CD4$^+$ and CD8$^+$ lymphocytes were determined by imaging flow cytometry. The index of CADESI-03 was calculated. Serum cytokine levels were analyzed using ELISA.
Results: Atopic dogs showed a higher percentage of CD8$^+$ lymphocytes, a lower CD4$^+$/CD8$^+$ ratio than healthy dogs, and a positive correlation with CADESI-03. Atopic dogs also showed higher serum IL-31 and IL-34 levels and lower IL-10 levels. A moderate positive correlation was found between serum IL-31 and CADESI-03.
Conclusions: The CD4$^+$/CD8$^+$ ratio may be a sensitive parameter that positively correlates with the severity of cAD, and elevated serum levels of IL-31 and IL-34 may facilitate diagnosis of the disease.

1. Introduction

Canine atopic dermatitis (cAD) is a genetically predisposed inflammatory and pruritic skin disease that is widely distributed in the canine population (Marsella et al., 2012; Mazrier et al., 2016). Many environmental allergens and risk factors, such as exposure to high levels of smoke or being reared in an urban environment, are etiological factors for cAD (Bizikova et al., 2015; Harvey et al., 2019). Many studies have attempted to explain the complex pathogenesis of AD, including the roles of immunological cells, cytokines, and chemokines. AD development may manifest with changes in the circulating lymphocyte subsets. Human AD shows increased levels of cluster of differentiation CD4$^+$CD25$^{+bright}$FoxP3$^+$ regulatory T cells (Tregs) (Gáspár et al., 2015), and similar findings were recently reported in client-owned atopic dogs (Hauck et al., 2016; Lee et al., 2020) and in an experimental model of cAD (Rostaher et al., 2018). Tregs have been found in both CD4$^+$ and CD8$^+$ subsets of skin explants in cAD (Jassies-van der Lee et al., 2014) but the percentage of Tregs was similar in cAD and control dogs. However, contradictory results have been reported in the veterinary literature. While Majewska et al. (2016) found elevated levels of CD8$^+$ and a low CD4$^+$/CD8$^+$ ratio in atopic dogs compared to control dogs, other researchers (Martins et al., 2018) found greater circulating CD4$^+$ levels and lower CD8$^+$ levels originating from an elevated CD4$^+$/CD8$^+$ ratio, which is the hallmark of atopic animals.

Cytokines are produced by a broad range of cells and control major physiological functions at the cellular level. They are also involved in a wide variety of pathological conditions and may help predict disease progression and severity (Baghdadi et al., 2017; Ge et al., 2015). Thus,

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these molecules play important roles in the pathogenesis of cAD. The early acute phase of cAD involves predominant secretion of pro-inflammatory cytokines such as interleukin (IL)-31, while the chronic phase shows T helper type 1 (Th1) inflammatory reactions with interferon (IFN)-γ, a characteristic cytokine of this phase (Marsella et al., 2012; Berker et al., 2017). Allergen sensitization induces T helper type 2 (Th2) cell changes and the cytokines IL-4, IL-5, IL-13, and IL-31, which are implicated in the development of cAD, particularly in the early phases and in acute lesions (Pucheu-Haston et al., 2015). IL-31 is a crucial cytokine involved in the development of atopic disease and pruritus, (Furue et al., 2018; Trier and Kim, 2018) and it has been shown in experimental dogs injected with IL-31 and in the serum samples from client-owned dogs with cAD (Gonzales et al., 2013, 2016; McCandless et al., 2014). A strong correlation between IL-31 serum levels and severity scores for atopic dermatitis (AD) have been demonstrated in children (Ezzat et al., 2011; Raap et al., 2012; Ozceker et al., 2018). This correlation has also been shown in a study evaluating clinical signs by CADESI-03 scores in an experimental model of cAD (Marsella et al., 2017). IL-34 is a cytokine involved in the development of various human diseases including autoimmune disorders, infectious, inflammation, and cancer (Baghdadi et al., 2017; Ge et al., 2015; Lelios et al., 2020). In humans, it has been demonstrated that IL-34 show specific expression in the skin by keratinocytes and the brain by neurons at the resting state, being fundamental in the maintenance of Langerhams cells and microglia (Baghdadi et al., 2017). Also, the expression of IL-34 is especially evident in the spleen, skin and brain (Baghdadi et al., 2017; Ge et al., 2019). In human patients with AD, IL-34 expression was lower in the lesional epidermis compared to controls (Esaki et al., 2015). In veterinary medicine, a strong correlation was found between clinical scores and serum IL-34 concentration in cAD (Gow et al., 2020).

Other important cytokines involved in the pathogenesis of cAD include IL-10, IFN-γ, and tumor necrosis factor (TNF)-α. IL-10 is an anti-inflammatory cytokine produced by Treg cells with an important role in the maintenance of a healthy immune response in allergic conditions (Palomares et al., 2017). The role of IL-10 in AD patients is controversial because both increased and decreased serum levels of this cytokine have been reported (Auriemma et al., 2013). An increase in the serum concentration of IL-10 as a consequence of immunotherapy in allergic dogs has been reported (Keppel et al., 2008; Maina et al., 2017). IFN-γ is a Th1 characteristic cytokine that can contribute to the exacerbation of allergic diseases, especially in the chronic phase (Berker et al., 2017). IFN-γ performs multifactorial functions in allergic diseases and its expression values are often variable or directly contradictory (Pucheu-Haston et al., 2015). Higher levels of gene transcripts of IFN-γ and TNF-α in atopic lesional skin have been detected in atopic dogs (Nuttall et al., 2002).

Since the pathogenesis of cAD is not well known due to its complexity and the contradictory results in the literature, the aim of this study was to compare some essential immunological parameters involved in cAD development. To this end, we analyzed the peripheral blood subset of CD4 and CD8 lymphocytes using image flow cytometry and their related cytokines in serum samples from healthy and client-owned atopic dogs. Another objective was to assess whether any of these parameters could serve as biomarkers for diagnosis and follow-up of allergic conditions.

2. Materials and methods

2.1. Ethics

This study was included under Project License PI54/17 approved by the Ethic Committee for Animal Experiments for the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. Written consent forms were obtained from the owners of the animals evaluated in this study.

2.2. Animals

A total of 56 client-owned dogs with AD were included in this study. Dogs were diagnosed with cAD at the Veterinary Hospital. The diagnosis of cAD was made on the basis of the clinical history and the type and distribution of skin lesions, and after a protocol of rouling out other causes of pruritus with similar cutaneous pattern. In all cases, tests were performed to confirm/exclude bacterial and *Malassezia* overgrowth, bacterial folliculitis sarcoptic mange, demodicosis, flea hypersensitivity, and food hypersensitivity (skin scrapping, trichography, cytology, strict antiparasitic control with pyrethrins spot on, and elimination diet trial during 8 weeks). Finally, the diagnostic protocol was completed, checking if the case met five clinical signs of Favrot et al. (2010). Dogs included in this study did not have bacterial overgrowth, bacterial folliculitis or yeast overgrowth, or in any case, the microorganisms were under control by topical therapy. Dogs were excluded from the study if they had major concurrent systemic diseases, such as metabolic, endocrine, or chronic diseases. Control dogs ($n = 53$) were greater than one year of age, had a body condition score of 3–3.5 on a 5-point scale, did not show any cutaneous process or other diseases that would affect other organic systems, and showed normal findings on hematological and biochemical analyses. Only dogs that had not been treated for pruritus or those that had not received any medications related to immune-related diseases were included. Every control dog was fed a standard canine diet, and every atopic dog was on the hypoallergenic diet used in the exclusion protocol for the diagnosis of pruritus. Among the dogs included in this study, 87.5%/12.5% were purebred/crossbred, while 57%/43% were male/female. The mean age of the animals was 6 years (range, 1 to 14 years).

2.3. Blood samples

Five milliliters of blood from the jugular veins was obtained for two tubes: 1 ml with EDTA as anticoagulant in one tube and the other 4 ml in a tube without anticoagulant to obtain serum. The tubes were kept at 4 °C for hematological analysis and evaluation of $CD4^+$ and $CD8^+$ T cells by imaging flow cytometry. Serum was obtained by centrifugation (3000 rpm, 4 °C for 15 min) and then maintained at −20 °C until analysis of cytokines by ELISA. Red blood cell counts were routinely analyzed using a veterinary counter (Idexx ProCyte Dx Hematology Analyzer; Las Rozas, Madrid, Spain).

2.4. Measurement of $CD4^+$ and $CD8^+$ T cells by imaging flow cytometry

The levels of $CD4^+$ and $CD8^+$ T cells were evaluated in peripheral blood mononuclear cells (PBMCs) by using imaging flow cytometry. Briefly, 1 ml of blood with EDTA was lysed with 10 ml of erythrocyte lysis buffer (150 mM NH_4Cl, 8 mM $KHCO_3$, 2 mM EDTA, pH 7). The lysed blood was centrifuged at 300 ×*g* for 5 min at 4 °C to separate the debris. The sample was then washed three times with PBS. A vial with 1 × 10^6 cells in PBS was labeled with 10 μL of rat anti-dog CD4:FITC/CD8:RPE (clone YKIX302.9/YCATE55.9; Biorad, Hercules, CA, USA) at 4 °C for 45 min. The stained cells were fixed with 20 μL of paraformaldehyde before cytometric analysis. Image flow cytometry was conducted using an ImageStream®X cytometer (Amnis®, Seattle, WA, USA). Cell sample acquisition (10,000 events) was performed with the INSPIRE® software and analyzed using the manufacturer's software (IDEAS v6.2). The results are expressed as the percentage of positive cells within the selected gates for cell surface markers. Fig. 1 shows picture obtained by ImageStream®X cytometer.

2.5. Serum concentrations of cytokines

The serum levels of the cytokines IFN-γ, TNF-α, IL-10, IL-31, and IL-34 were determined using ELISA kits. Canine IFN-γ, TNF-α, and IL-10 kits were obtained from DuoSet® ELISA Development Systems (R&D

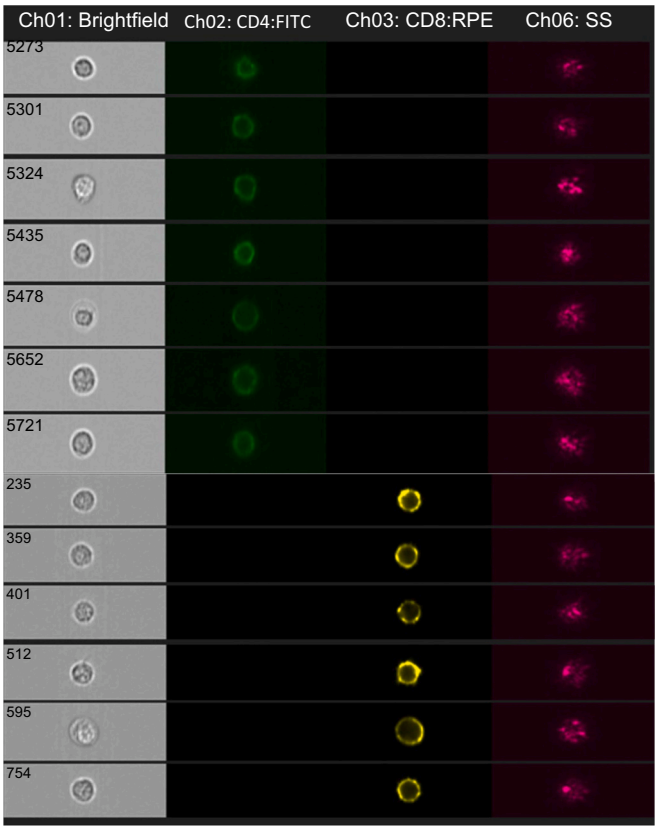

Fig. 1. Picture was obtained with image flow cytometry of the circulating T lymphocytes from a dog with cAD. Ch01 is the image of each lymphocyte isolated from peripheral blood; Ch02 are $CD4^+$ lymphocytes stained with rat anti dog CD4:FITC; Ch03 are $CD8^+$ lymphocytes stained with rat anti dog CD8:RPE. Pictures taken with a 40× objective of ImageStream®X cytometer (Amnis®, Seattle, WA, USA).

Systems, Inc., MN, USA; detection limits, 31.3 pg/ml, 5.6 pg/ml, and 31.3 pg/ml, respectively). The concentrations of serum IL-31 and IL-34 were determined using the canine IL-31 ELISA kit and canine IL-34 ELISA kit (MyBioSource, San Diego, CA, USA; detection limit, 50 pg/ml and 15.6 pg/ml, respectively). All samples and controls were analyzed in duplicate, in accordance with the manufacturer's instructions. Absorbance values were read at 450 nm (reference wavelength) using an automatic microELISA reader (Microplate Photometer, HiPo MPP-96; Riga, Latvia). Cytokine levels were calculated by interpolation from a standard curve obtained with the controls provided in the kits. Each test was performed by a different researcher who was blinded to the results for the other cytokines.

2.6. Statistical analysis

SPSS software (version 22.0; IBM Corp., Armonk, NY, USA) was used for statistical analysis. A Kolmogorov-Smirnov test was performed to determine if the data were normally distributed. Comparisons between groups (control and allergic dogs) were tested using the non-parametric unpaired Mann-Whitney *U* test and unpaired Student's *t*-test when the data were normally distributed. A Fisher's exact probability test for IFN-γ, TNF-α and IL-10 frequency analysis was performed. Correlation analysis between immunological and clinical parameters was performed using Spearman's rank correlation coefficient. Data for leukocytes and a subset of lymphocytes were expressed as percentages and absolute numbers. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Hematological and clinical parameters

Differences in the hematological parameters, CADESI-03 index, and pruritus score in atopic and healthy dogs are shown in Table 1. The hemogram values of allergic dogs were lower than those of the control dogs ($p < 0.001$). Although allergic dogs showed leukocytosis with a higher neutrophil count, they showed lower lymphocyte percentage and absolute count. The two groups showed no significant differences in eosinophil percentage or total count ($p > 0.05$).

3.2. Subset of $CD4^+$ and $CD8^+$ lymphocytes

Fig. 1 shows pictures of the FITC and RPE stained lymphocytes identified as $CD4^+$ and $CD8^+$ T cells. The gating strategy for analysis of $CD4^+$ and $CD8^+$ T cells are shown on Fig. 2. Table 2 shows the percentages and absolute counts of $CD4^+$ and $CD8^+$ T cells in the peripheral blood. Allergic dogs had a higher percentage of $CD8^+$ cells than control dogs ($p < 0.01$), but the total count of circulating $CD8^+$ T cells did not show statistically significant differences ($p > 0.05$). The $CD4^+/CD8^+$ ratio was lower in allergic dogs than in control dogs ($p < 0.01$). This $CD4^+/CD8^+$ ratio was calculated from the percentage of $CD4^+$ and $CD8^+$ T cells. When it was calculated using total count, statistically significant differences were also obtained (control dogs 3.78 ± 1.9, allergic dogs 2.71 ± 1.1; $p < 0.01$).

3.3. Serum cytokine levels in control and atopic dogs

Many animals presented concentrations below the detection limit when the serum levels of TNF-α, IL-10 and IFN-γ were analyzed. A Fisher's exact test to check significant differences in the detection of these cytokines between control and atopic dogs was performed (Table 3). TNF-α levels were almost undetectable in control dogs (50/53,

Table 1
Hematological and clinical parameters in healthy control and atopic dogs. The number of each analysis is included in brackets.

Parameter	Control dogs	Atopic dogs	Reference values	*p* value
Erythrocytes (10^6/μl)	(53) 7.54 ± 0.86	(56) 6.59 ± 0.83	5.65–8.87	0.000^{T}
Hemoglobin (g/dl)	(53) 17.1 ± 1.7	(56) 14.5 ± 2.2	13.1–20.5	0.000^{MW}
Hematocrit (%)	(53) 48.9 ± 6.8	(56) 43.2 ± 5.9	37.3–61.7	0.000^{MW}
Leukocytes (/μl)	(53) 8254 ± 2428	(52) 10935 ± 601	5050–16,760	0.003^{T}
Neutrophils (%)	(50) 67.6 ± 7.8	(52) 71.8 ± 10.1		0.022^{T}
Lymphocytes (%)	(50) 23.6 ± 7.1	(52) 15.9 ± 8.26		0.000^{T}
Eosinophils (%)	(49) 3.79 ± 1.99	(51) 3.96 ± 2.46		0.917^{MW}
Monocytes (%)	(50) 3.51 ± 3.32	(52) 2.96 ± 3.6		0.091^{MW}
Neutrophils (/μl)	(50) 5401 ± 1555	(47) 6531 ± 2246	2950–11,840	0.013^{MW}
Lymphocytes (/μl)	(49) 1820 ± 698	(46) 1419 ± 702	1050–5100	0.006^{T}
Eosinophils (/μl)	(49) 308 ± 209	(45) 367 ± 254	60–1230	0.334^{MW}
Monocytes (/μl)	(49) 244 ± 196	(45) 223 ± 257	160–1120	0.197^{MW}
CADESI-03 index	–	(48) 35.1 ± 40.1		–
Pruritus	–	(51) 5.37 ± 2.76		–

Data are means ± SD. MW: Mann-Whitney test; T: Student's t-test. Reference values according to Idexx ProCyte Dx Hematology Analyzer.

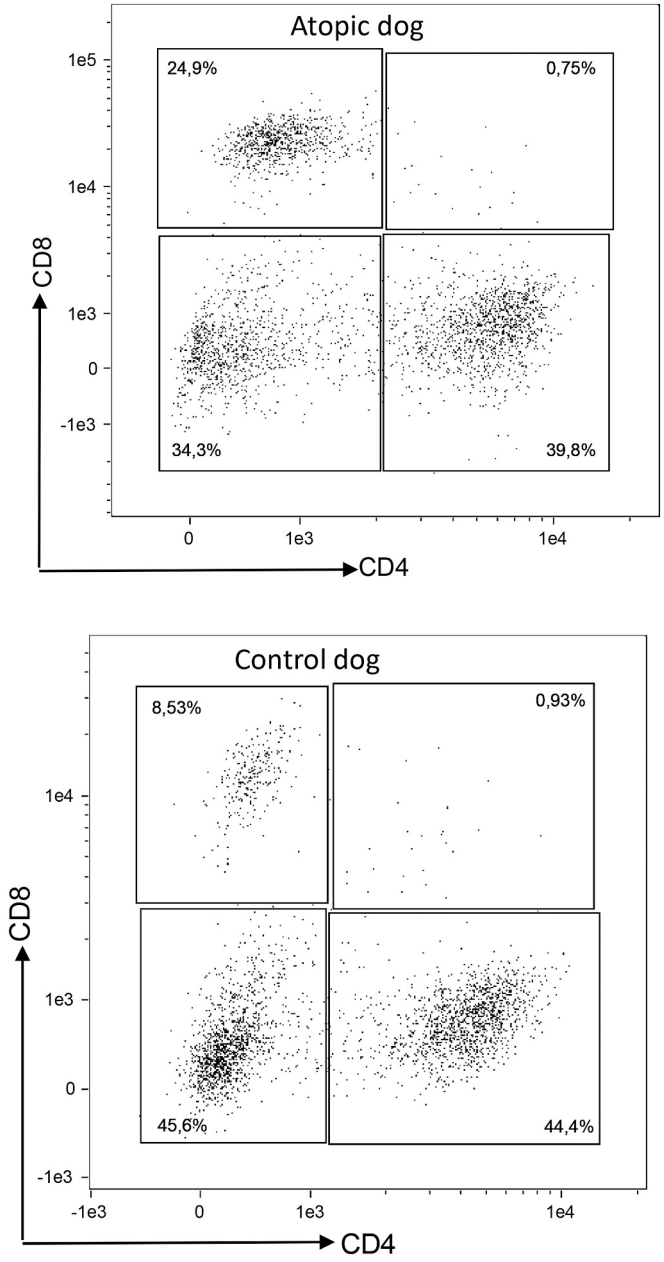

Fig. 2. Gating strategy for analysis of CD4 and CD8 T cells in atopic and control canine lymphocytes by imaging flow cytometry. Canine lymphocytes were gated for living cells. Percentages of T cell subpopulation are depicted in each quadrant corner. The upper right quadrant are lymphocytes stained with FITC and RPE and the lower left quadrant are lymphocytes that have not been labeled with any of the fluorochromes.

Table 2
$CD4^+$, $CD8^+$ T cells levels in circulating blood from healthy control and atopic dogs. Data are expressed as a percentage (%) and as absolute values (cells / µl). The number of each analysis is included in brackets.

Parameter	Control dogs	Atopic dogs	*p* value
$CD4^+$ (%)	(50) 45.28 ± 10.5	(51) 47.45 ± 15.9	0.298^T
$CD8^+$ (%)	(50) 14.41 ± 6.9	(47) 20.43 ± 11.6	0.004^{MW}
$CD4^+$ (/ µl)	(48) 763 ± 239	(46) 702 ± 333	0.218^T
$CD8^+$ (/ µl)	(50) 279 ± 190	(41) 284 ± 190	0.707^{MW}
Ratio $CD4^+/CD8^+$	(49) 3.73 ± 1.8	(47) 2.98 ± 1.74	0.014^{MW}

Data are means ± SD. MW: Mann-Whitney test; T: Student's t-test.
Ratio $CD4^+/CD8^+$ was calculated from the percentage of $CD4^+$ and $CD8^+$.

Table 3
Fisher's exact test for the detection of TNF-α, IL-10 and IFN-γ in control and atopic dogs. The detection limits of the cytokines are shown in the materials and methods section.

Cytokine	Dog	Above detection limit	Below detection limit	*p* value
TNF-α	Control	3/53	50/53	0.0001
	Atopic	24/56	32/56	
IL-10	Control	7/53	46/53	0.0616
	Atopic	16/56	40/56	
IFN-γ	Control	5/53	48/53	0.7621
	Atopic	7/56	49/56	

94.33%) *versus* atopic dogs (32/56; 57.14%, $p < 0.001$). For IL-10 and IFN-γ there were no significant differences between the two groups ($p > 0.05$), although IL-10 was detected in a higher proportion (16/56; 28.57%) in atopic dogs than in controls (7/53; 13.2%). The serum concentrations of cytokines in dogs in this study are summarized in Table 4. Atopic dogs had higher serum levels of IL-31 and IL-34 ($p < 0.01$) than the control dogs. Atopic dogs also showed lower serum levels of IL-10 ($p < 0.05$). There were no statistically significant intergroup differences in the TNF-α and IFN-γ levels ($p > 0.05$).

3.4. Correlations between clinical and immunological parameters

Correlations of the clinical severity and pruritus scores with the circulating levels of $CD4^+$, $CD8^+$, IL-31, IL-34, IL-10, IFN-γ, and TNF-α in the atopic dogs were studied. Fig. 3 shows all the statistically significant correlations detected by Spearman's rank correlation coefficient ($p < 0.05$). All the correlations found were considered moderate (r values 0.30–0.50) following the Cohen (1988) criteria for the interpretation of the correlations. The correlation found between the CADESI-03 index and PVAS ($r = 0.543$) was considered strong. The primary correlations were found between the CADESI-03 index, PVAS, T lymphocyte subsets, and leukocyte differential counts. Only the serum IL-31 concentration showed a positive and significant correlation ($p < 0.05$) with lymphocyte and eosinophil counts. In addition, a positive correlation was observed between the IL-31 concentration and the CADESI-03 index. No correlations ($p > 0.05$) were found between IL-34, IL-10, TNF-α, and IFN-γ and clinical or lymphocyte subsets.

4. Discussion

This paper presents new results regarding the immunological status of dogs with allergic dermatitis that could be useful for diagnosing and evaluating this condition. Analytical methods for assessing immunological and clinical status are more practical than other invasive methods, such as skin biopsy. The red blood cell counts showed that atopic dogs had lower erythrocyte, hemoglobin, and hematocrit values than control dogs; however, these results were within the reference ranges for dogs. Martins et al. (2018) and Chaudhary et al. (2019) did not find differences in these parameters between healthy dogs and dogs with cAD. Our results, which were consistent with those of other studies, confirm that cAD has little impact on the hematological parameters in atopic dogs.

Table 4
Serum cytokines levels from healthy control and atopic dogs. The number of each analysis is included in brackets.

Parameter	Control dogs	Atopic dogs	*p* value
IL-31 (pg/ml)	(45) 175 ± 96	(50) 246 ± 126	0.003
IL-34 (pg/ml)	(51) 335 ± 268	(51) 439 ± 214	0.001
TNF-α (pg/ml)	(3) 45.2 ± 52.1	(24) 39.8 ± 43.9	0.537
IL-10 (pg/ml)	(7) 130 ± 65	(16) 68.3 ± 17.7	0.050
IFN-γ (pg/ml)	(5) 190 ± 172	(7) 139 ± 157	0.291

Data are means ± SD. MW: Mann-Whitney test.

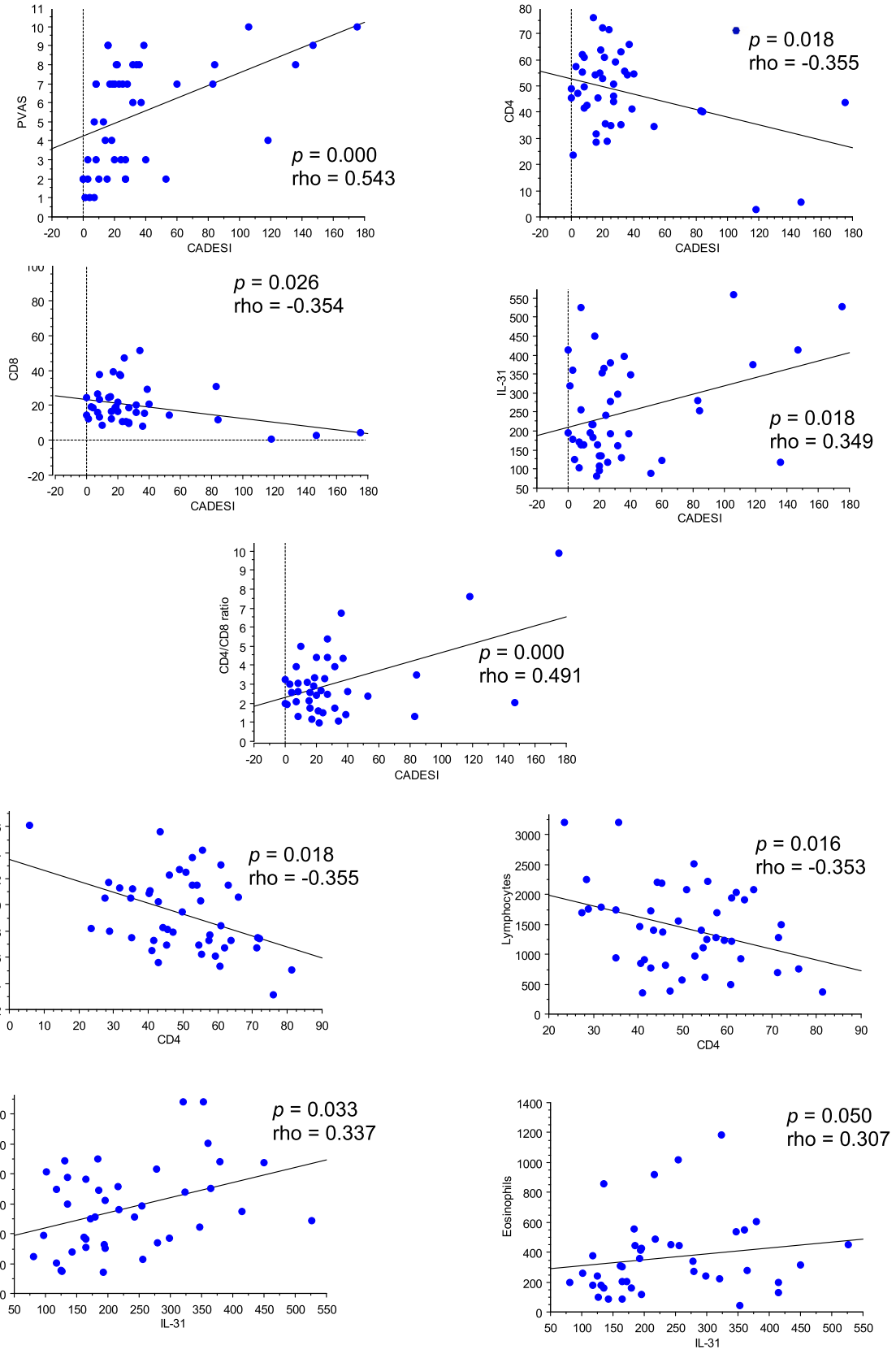

Fig. 3. Statistically significant correlations ($p < 0.05$) between the clinical findings and the immunological data in atopic dogs. The Spearman's correlation (rho) and p values are shown in the graphs.

Atopic dogs had higher leukocyte and neutrophil counts and lower lymphocyte concentrations, while circulating eosinophil counts were similar in both healthy and atopic dogs. Thus, allergic diseases in dogs caused changes in the level of circulating leukocytes, but the results reported in the literature review are contradictory. Majewska et al. (2016) found no changes in peripheral blood lymphocyte percentages in atopic dogs, unlike our findings. Other studies have confirmed our findings, but Chaudhary et al. (2019) reported a significantly higher eosinophil count in atopic dogs, and Martins et al. (2018) also found neutrophilia and lymphopenia, similar to our study. These discrepancies in the results may be due to differences in experimental variables across studies, including the number of dogs or disease severity. The clinical pathological changes observed in atopic dogs were limited to an increase in leukocyte count, mainly the neutrophil count and a decrease in lymphocyte concentration, which is a characteristic of the infection. Many dogs with cAD show secondary bacterial and *Malassezia* infections, which could explain the results obtained.

Alterations in the normal balance of T lymphocytes are common in the peripheral blood of allergic dogs, and imbalances in T cell populations characterize different stages of the disease: Th2 predominance in the acute phase and Th1 in the chronic phase (Marsella et al., 2012; Berker et al., 2017). T cells are important in the adaptive immune response of the animals. T helper cells are phenotypically characterized by their co-receptor CD4, which regulates other cells of the immune system by cytokine production. Cytotoxic T lymphocytes are characterized by the co-receptor CD8 and can kill cells presenting with MCH-I. The changes in the subsets of lymphocytes in the peripheral blood of allergic dogs have been reported in a few reports, and these studies reported contradictory results. Our study yielded similar findings to some of these studies (Taszkun et al., 2013; Majewska et al., 2016) with no changes in the $CD4^+$ subset and a significantly higher percentage of $CD8^+$ T cells in atopic dogs in comparison with a healthy group. In other recent studies, Martins et al. (2018) reported opposite results to ours, with significantly higher $CD4^+$ T cells and lower $CD8^+$ T cells and an increase in the $CD4^+/CD8^+$ ratio. Beccati et al. (2016) did not observe significant differences in the $CD4^+/CD8^+$ ratio in healthy or atopic dogs treated with citrine, and reported only small non-significant elevations without significant differences in canine atopic patients (Koury et al., 2019). However, due to the relative lymphopenia in allergic dogs, the total number of $CD8^+$ T lymphocytes, obtained by multiplying the percentage of $CD8^+$ by the total number of total lymphocytes, was not significantly different from that in the control group (Table 2). The higher percentage of $CD8^+$ T cells suggests that the cellular suppressive mechanisms of the immune response are involved in the pathogenesis of cAD. An increased percentage of $CD8^+$ T cells, which originate from a low $CD4^+/CD8^+$ ratio, has been recorded in complicated cAD (Taszkun et al., 2013) and in dogs with immune diseases (Watabe et al., 2012).

The percentages of $CD4^+$ and $CD8^+$ T cells were correlated with clinical signs in our atopic dogs (Fig. 3). The severity of clinical signs (CADESI-03 index) was negatively and moderate correlated with the percentage of $CD4^+$ and $CD8^+$ T cells and positively and moderate correlated with the $CD4^+/CD8^+$ ratio in our study. Martins et al. (2018) found similar results in their correlation analysis. This result can be explained by differences in the severity of the disease, which is reflected in the CADESI index: in the study by Martins et al. (2018) the CADESI index was 42.4, similar to ours (35.1), while Lee et al. (2020) reported a CADESI index higher than 120, indicating very severe disease. Hauck et al. (2016) also observed a positive correlation between the CADESI-04 index and Tregs. Determination of the $CD4^+/CD8^+$ ratio in our study was easier and faster because it used only a single laboratory reaction, while two cell surfaces and one intranuclear marker are needed for the analysis of Tregs (Gáspár et al., 2015; Beccati et al., 2016). High numbers of both $CD4^+$ and $CD8^+$ T cells in the lesional and nonlesional atopic epidermis have been reported in the skin biopsies of atopic dogs (Pucheu-Haston et al., 2015). Also, a higher relative number of $CD8^+$ T cells has been shown by immunofluorescence histochemistry of skin sections of atopic dogs compared with healthy controls (Jassies-van der Lee et al., 2014). These finding could explain the negative correlation observed between T cells and disease severity, because dogs with a high CADESI-03 index would have more recruited T cells in their skin and lower circulating T cells in their peripheral blood.

Important changes have been reported in the cytokine concentrations in cAD, and the serum levels of cytokines have been used to determine the prognosis, evolution, or the effectiveness of treatment against cAD (Gonzales et al., 2013, 2016; McCandless et al., 2014; Marsella et al., 2017). T cells are an important source of IL-31 production (McCandless et al., 2014), being a pruritogen cytokine stimulating sensory neurons, which has been identified to be an important marker in this regard (Trier and Kim, 2018). Augmented IL-31 expression has been found in the lesional skin and PBMCs of cAD patients in comparison with healthy controls (Furue et al., 2018), while significant positive correlations among CADESI-03 index, lymphocytes, eosinophils, and IL-31 serum levels were detected in atopic dogs in this study. Similar results have been reported in both human AD, mainly in children (Ezzat et a., 2011; Raap et al., 2012; Byeon et al., 2020), and in atopic dogs (Gonzales et al., 2013; Marsella et al., 2017; Chaudhary et al., 2019). These results are probably due to the level of pruritus and self-induced skin lesions expressed through the CASEDI-03 index in this study as a result of the action of the known pruritogen cytokine IL-31 (Trier and Kim, 2018). In this study, no correlation was found between the other cytokines analyzed (IL-34, IFN-γ, IL-10 and TNF-α) with the clinical signs of the disease, which could indicate that the analysis of IL-31 is the only cytokine indicative of cAD. The positive correlation found between IL-31 serum concentration and eosinophil levels has also been reported by Byeon et al. (2020) in children, indicating that IL-31 may play a role in regulating eosinophil concentration in peripheral blood. This correlation observed in atopic patients is related to the greater release of IL-31 by eosinophils compared to IL-31 released by CD4 T cells (Bağci and Ruzicka, 2018). In our study, IL-31 was detected in all most all control dogs and in all dogs with AD. However, Gonzales et al. (2013) did not find serum IL-31 in healthy, client-owned Beagle dogs sensitized to house dust mite, which can be attributed to the complex and multifactorial causes of cAD that present with different molecular or cellular changes. Other surprising results were reported by Marsella et al. (2017) who did not detect differences between IL-31 serum concentrations at the first challenge of Beagle dogs epicutaneously sensitized to *Dermatophagoides farinae* and 28 days later. Our results could reflect the upregulation of IL-31 in AD and may be a potentially useful marker for the detection of atopic dogs and the severity of cAD. Canine AD shows a clear Th2 mode response to allergens, and IL-31 is produced after exposure of Th2 polarized cells to allergen (McCandless et al., 2014), which agrees with the results of our study. The higher serum levels of IL-31 found in cAD confirm the important role of this cytokine in the development and pathogenesis of canine inflammatory diseases of the skin, such as has been described in human patients (Bağci and Ruzicka, 2018).

IL-34 has received less attention in veterinary medicine; however, a more thorough understanding of its role in the pathogenesis of cAD or its use as a biomarker for diagnosis or monitoring of disease severity is essential, as has been done in human medicine. IL-34 is released in response to inflammatory stimuli and IL-34 mediated tolerance helping to control of inflammation in skin diseases and prolongs survival of the implanted grafts (Baghdadi et al., 2017; Ge et al., 2019; Lelios et al., 2020). In veterinary dermatology, a recent study by Gow et al. (2020) showed an increase in the serum levels of IL-34 in atopic dogs in comparison with healthy controls, such as in our study. High levels of serum IL-34 have been reported in many human inflammatory or autoimmune diseases with inflammatory lesions (Baghdadi et al., 2017) and inflammatory lesions are frequently observed in cAD. Positive correlation between serum IL-34 concentration and clinical severity and pruritus scores was found in dogs by Gow et al. (2020), contrary to our study in which we did not find any correlation between serum IL-34 levels and

clinical parameters. IL-34 expression has been shown to correlate negatively with disease severity in the lesional skin in human atopic dermatitis in comparison with the normal epidermis (Esaki et al., 2015). These discordant IL-34 data, and the persistence of elevated IL-34 levels after treatment of atopic dogs with corticosteroids or oclacitinib (Gow et al., 2020), suggest that further studies are needed to test its potential usefulness as a biomarker and an indicator of disease progression. In addition, the potential therapeutic applications of IL-34 should be investigated, such as those suggested for human atopic dermatitis (Baghdadi et al., 2017; Ge et al., 2019).

We evaluated the serum concentrations of the main cytokines involved in cAD to determine their levels in allergic patients in comparison with those in healthy controls. In the present study, many dogs, healthy or atopic, did not show detectable levels of cytokines by ELISA tests. However, there were more atopic dogs that had TNF-α and IL-10 levels above the detection limit than controls, which may imply a higher activity of these cytokines in allergic dogs. A reason for the low percentage of samples with detectable levels of IL-10 is that the detection limit of the ELISA kit used was higher (31.3 pg/ml) than in the study of Majewska et al. (2017) in which it was detected in almost all samples with a detection limit of 5.9 pg/ml. Other reason is the existence of dogs with low or levels below the detection limit of IL-10 associated to clinical conditions such as leishmaniosis (Solano-Gallego et al., 2016). Nevertheless, cytokine profiling showed a significant decrease in IL-10 serum levels and a slight decrease in the Th1 cytokines IFN-γ and TNF-α. IL-10 is an anti-inflammatory cytokine produced mainly by Treg cells (McCandless et al., 2014; Palomares et al., 2017) that can down-regulate the immune response and plays a very important role in the induction and maintenance of healthy immune response in allergy, although interpretations of its role can be complicated (Pucheu-Haston et al., 2015). Previous studies in veterinary medicine yielded controversial findings regarding this topic. Keppel et al. (2008) did not find differences in IL-10 concentration at baseline between control dogs and allergic dogs, and no differences in IL-10 gene transcription in the lesional, non-lesional, or healthy canine skin (Nuttall et al., 2002). In contrast, higher levels of IL-10 were reported by Taszkun (2013) in the serum of atopic dogs. However, other studies showed a significant decrease in serum IL-10 levels in atopic dogs (Majewska et al., 2016), as in our study, or lower levels but without statistical significance in allergic dogs, as reported by Koury et al. (2019). Significantly elevated levels of IL-10 have been shown in healthy humans in comparison with individuals suffering from allergic rhinitis, since low IL-10 levels are associated with under-regulation of the immune response and allergic symptoms (Palomares et al., 2017). Similarly, we considered that the lower values of IL-10 in our atopic dogs in comparison with the control group could be due to the fact that in atopic individuals, Treg1 cannot inhibit allergic responses to different allergenic sources (Palomares et al., 2017). These different results reflect the complex mechanisms involved in the regulation of IL-10 in cAD and the ability of IL-10 to promote Th2 or regulatory immune responses (Pucheu-Haston et al., 2015).

This study also found that the concentration of TNF-α and IFN-γ were lower in atopic patients compared with healthy controls, but the result was not statistically significant. IFN-γ and TNF-α are secreted by Th1 cells and are considered to be key cytokines in the pathogenesis of allergic diseases. Increased transcription of IFN-γ has been reported in the lesional atopic skin of dogs (Nuttall et al., 2002; Jassies-van der Lee et al., 2014). TNF-α is a potent pro-inflammatory cytokine and was only detected in three healthy dogs, with levels slightly higher than atopic dogs. Perhaps these three dogs had an inflammatory disease other than cAD that was not diagnosed at the time of admission. To know exactly the role of TNF-α in cAD, it would be necessary to conduct studies comparing serum levels of TNF-α in dogs with cAD with dogs with other inflammatory processes. In our study, almost no IFN-γ levels were found above the detection limit in both healthy control and atopic dogs, but probably many dogs had serum levels of the cytokine, so the results should be interpreted with caution. An explanation could be that the detection limit of the ELISA kit is high, as in the study carried out by Majewska et al. (2016) in which they were only able to detect 2/20 samples using a kit with a detection limit of 60 pg/ml, higher than ours (31.3 pg/ml). Probably, another technique or ELISA kit with a lower detection limit can detect more dogs with serum IFN-γ and thus be able to perform a more secure comparison of results. Another possibility is of the presence of IFN-γ producer and non-producer dogs, situation that it has been described in other diseases such canine leishmaniosis (Solano-Gallego et al., 2016), a disease which the immune response plays an important role in controlling the infection.

However, these low levels of detection support the hypothesis that IFN-γ is associated with a chronic disease state in dogs (McCandless et al., 2014). The serum samples for IFN-γ in our study were collected at the first cAD diagnostic visit, that is, in the acute phase of the condition and probably in which it is more difficult to detect serum levels of IFN-γ. In this sense, it has been found that in human patients with atopic dermatitis display decreased IFN-γ production compared the healthy controls (Berker et al., 2017). Additionally, other studies have not shown significant differences in the serum concentrations of IFN-γ and TNF-α in atopic dogs (Koury et al., 2019), or described the effect of *ex vivo* boosted immune cell (EDIC) therapy in atopic dogs in comparison with baseline data (Bae et al., 2018). No variation in IFN-γ serum concentration, and a significant increase in serum TNF-α of atopic dogs has been reported (Majewska et al., 2016). Also, higher levels of mRNA TNF-α have been seen in lesional compared to non-lesional and healthy canine skin (Nuttal et al., 2002). These results seem to indicate the state of atopic inflammation in cAD and highlight the complexity of the immunological regulation in the pathogenesis of the condition. The high percentage of detection of TNF-α in atopic dogs compared to control dogs clearly indicates that of the two pro-inflammatory cytokines analyzed, TNF-α is easier to detect in atopic dogs than IFN-γ, which was practically undetectable, as in the study by Majewska et al. (2016). In general, there are few and poorly standardized assays to evaluate immunity responses in dogs in different diseases including cAD and other clinical conditions, being the cytokine profile in dogs frequently fragmentary.

In conclusion, data from our study indicate that an increase in the $CD8^+$ percentage and a lower $CD4^+/CD8^+$ ratio in cAD can be a parameter that correlates with clinical severity. Higher serum concentrations of IL-31 and IL-34 were associated with a greater disease frequency and severity and may be used in the diagnosis of the disease. The use of client-owned atopic dogs involves a high variability of conditions that cannot be well controlled (breed, age, origin, environment where they live). Nevertheless, further research is needed, with control dogs from a colony, to understand the roles of cytokines in the pathogenesis of cAD and to determine whether cytokines, mainly IL-31 and IL-34, can serve as predictive biomarkers or possible therapeutic targets, which has been suggested for human medicine.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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