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Short communication

Detection and dynamics of tumor necrosis factor alpha in the diagnosis and treatment of canine heartworm disease

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Abstract

The aim of this study was to determine the concentration of TNF-alpha (TNF-α) in dogs naturally infected with *Dirofilaria immitis* (*D. immitis*) and to assess whether there are any changes in TNF-α concentration and their dependence during therapy for heartworm disease (HWD). For this study, 14 client-owned dogs with HWD were selected. Clinical and parasitological examinations (modified Knott test for circulating microfilariae and SNAP Test IDEXX for circulating *D. immitis* antigen) had been used for diagnosing *D. immitis* and HWD. All dogs were treated with an alternative therapy for HWD (oral doxycycline 10 mg/kg b.w., once daily for 6 weeks, then alternately 4 weeks without and 2 weeks with the medication, and oral ivermectin 6-14 µg/kg b.w., every 2 weeks). The dogs blood sera at the moment of HWD diagnosis, during and at the end of therapy were frozen for further quantifying of TNF-α (Canine TNF-alpha ELISA kit, Thermo scientific). At the moment of HWD diagnosis TNF-α was detected in 9 dogs (7.21±12.44 pg/ml). Concentration of TNF-α was not significantly change during the therapy, neither related to the level of *D. immitis* antigen nor to antigen level changes. The alternative therapy for HWD has no influence on TNF-α concentration dynamics.

Keywords

Dogs, Heartworm disease, Tumor necrosis factor alpha

Heartworm disease represents the clinical and pathological findings associated with heartworm (*Dirofilaria immitis, D. immitis*) infestation. The parasite is a filarial nematode that resides in the pulmonary arteries, and occasionally in the right heart (Kittleson, 1998). As the disease is transmitted by mosquitoes, it is distributed worldwide, and has tremendous clinical importance in dogs, due to its serious and fatal complications (McCall et al., 2008; Simón et al., 2012).

Pathological changes in HWD are various and complex (Simón et al., 2007; Morchón et al., 2009; Venco, 2009; Genchi et al., 2012), being localized to the lung and cardiovascular system (cardiopulmonary dirofilariosis) with the possibility of changes in distant organs (glomerulonephritis, reactive polyarthritis) (Dunn, 2000). The initial pathological changes, described as villous myointimal proliferations, occur in the pulmonary arteries where the parasite resides. The characteristics of these changes are endothelial cell swelling, widening of intracellular junctions, increased endothelial permeability, and periarterial oedema. Damage of endothelial cells in the pulmonary arteries enables a genesis of arterial thrombi (pulmonary thromboembolism, PTE), while increased endothelial permeability leads to periarterial pulmonary oedema, and cellular infiltrations with neutrophils and eosinophils. Smooth muscle cells proliferate within the media and migrate into the intima. These lesions can be identified by electron microscopy even 4 days after the presence of adult worm or by angiography in 2 to 3 weeks (Schaub et al., 1981; Keith et al., 1983; Knight, 1980). Thickening of pulmonary arteries intima and media and lung blood flow obstruction increase pulmonary vascular resistance and lead to pulmonary hypertension (PH). As a result, right heart afterload is increased, causing right ventricular hypertrophy and right-sided heart failure (RHF, *cor pulmonale*) **(**Dunn, 2000, Ware 2011**).**

Numerous diagnostic procedures can be used in monitoring HWD and its most serious complications, such as PH, PTE, RHF and caval syndrome (CS), with blood and urine analyses, thoracic radiography and echocardiography being most useful for assessment of its severity (AHS 2020, ESDA 2017). Recently, cardiopulmonary biomarkers

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have been investigated and proposed as objective parameters for measuring and quantifying pathological processes in HWD. As indicators of PH, C-reactive protein (CRP) (Venco et al., 2014) and endothelin-1 (ET-1) (Uchide and Saida, 2005) can be used, while D-dimer can be used as a indicator of PTE in dogs with HWD (Carretón et al., 2011). In addition, troponin I, myoglobin and natriuretic peptides (ANP and NT-pro BNP) (Carretón et al., 2017; 2011; Kitagawa et al., 2000; Takemura et al., 1991) can assess myocardial injury and heart failure.

Generally speaking, in veterinary clinical practice, N-terminal pro-brain natriuretic peptide (NT-pro BNP) and cardiac troponins I and T (cTnI and cTnT) are more frequently used as the cardiac biomarkers, as they show the highest predictability for cardiac diseases. On the other hand, ANP, ET-1, tumor necrosis factor alpha (TNF-α) and C-reactive protein (CRP) are less frequently used due to their lesser specificity (Baisan et al., 2016).

The most important effects induced by TNF-α in cardiovascular system, such as inflammatory gene induction, leukocyte recruitment, barrier loss, cytoskeleton reorganization, proliferation, migration, apoptosis, constriction, decreased contractility and hypertrophy, are realized in endothelial cells, smooth muscle cells and cardiac myocytes (Urschel and Cicha, 2015).

Although the pathological changes caused by *D. immitis* and the cardiovascular effects of TNF-α overlap, the significance of TNF-α as a cardiac biomarker in HWD has not been determined yet. With the aim to broaden the knowledge about TNF-α, this study was performed to determine the concentration of TNF-α in dogs naturally infected with *D. immitis* and to assess whether there are any changes in TNF-α concentration during a therapy for HWD and in relation to the parasite burden changes caused by applied treatment for HWD.

Before enrolling a dog into the study, a written consent was signed by an owner of each dog. According to the national regulation, an ethical approval for clinical case study is not required. Fourteen privately owned dogs of different breeds with HWD were included in this study. The breeds of dogs were: Belgian Malinois, Labrador Retriever, Golden Retriever, Dogo Argentino, Hungarian Vizsla, Epagneul Breton, Rottweiler and mixed breed dogs. There were 8 male and 6 female dogs. The average age of dogs was 4.89±3.65 years (1-15 years). At the moment of testing for*D. immitis* infection all dogs were older than 7 months, exposed to at least one mosquito season and never used prevention for HWD.

For parasitological examination and the diagnosis of *D. immitis* infection, blood samples (whole blood and blood sera) were taken from each dog. Whole blood samples were tested by modified Knott test for identification and quantification of circulating microfilariae (mf) (Genchi et al., 2007; Bazzocchi et al., 2008), while Canine Heartworm Antigen Test Kit-Snapp HTWM (IDEXX, USA) was used for detection of circulating *D. immitis* antigen (Ag). The test is semiquanitative and able to differentiate high (Ag++) and low (Ag+) level of *D. immitis* Ag. The *D. immitis* infection was diagnosed in case of detection of either Ag or mf of *D. immitis* or both. Besides parasitological examinations, relevant clinical examinations were performed in order to establish the class of HWD (AHS 2020, Ware 2011).

All dogs were treated with an alternative therapy for HWD. The alternative protocol for HWD consisted of oral doxycycline (10 mg/kg b.w., once daily for 6 weeks, then alternately 4 weeks without and 2 weeks with the medication), and ivermectin administrations (6-14 µg/kg b.w., every 2 weeks) until antigen negativization, but not longer than 9 months (Bazzocchi et al., 2008). At the moment of diagnosis (the beginning of the alternative therapy) and after each month of the therapy control clinical and parasitological examinations were performed. On these occasions blood samples were taken and sera were frozen for further TNF-α analysis. The number of samples per each dog varied since the duration of the alternative therapy was not the same among them.

Concentrations of TNF-α were determined in sera by ELISA using dog specific test (Canine TNF-alpha ELISA kit, Thermo scientific) and read by an ELISA reader (ELx800 Absorbance Microplate Reader, BioTek, USA) at 450 nm wavelength. The detection limit of TNF-α was 2.87 pg/ml. ELISA was performed according to the instruction of the kit producer. For the purpose of TNF-α concentration comparison during the alternative therapy, the values at the beginning, at the middle and at the end of the therapy were used from each dog.

A commercial software package Statistica TIBCO was used for statistical analyses. All quantitative variables were reported as the mean±standard deviation, with minimum and maximum range. The t-test for dependent samples was used to assess TNF-α changes during therapy, while the t-test for independent samples was used to evaluate difference between TNF-α concentration in dogs with different HWD class. The TNF-α concentration changes in relation to different Ag level (Ag++, Ag+, Ag 0) were analyzed by the Wilcoxon matched pairs test. Concentrations of TNF-α according to the different Ag level in dogs during the therapy are correlated mutually. Dependence of TNF-α concentration changes and Ag level changes was tested by the Fisher probability test.A probability value of $P<0.05$ was considered as significant.

Out of 14 dogs with the diagnosis of HWD, Ag was detected in 12 dogs (7 dogs with Ag ++ and 5 dogs with Ag+), while mf were detected in 9 dogs. The dogs belonged to class 1 (6 dogs), class 2 (5 dogs) and class 3 (3 dogs), without any dog classified in class 4 (CS). At the moment of HWD diagnosis, TNF-α was detected in 9 dogs with the

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average value of 7.21±12.44 pg/ml (0-44.80). However, during therapy, TNF-α was detected in two more dogs in which initially TNF-α was not detected. Besides, in one dog in which TNF-α was detected at the beginning of the therapy, later on during the course of therapy it was not detected. There were variations in TNF-α concentration during the therapy, with the average values of 7.21±12.44 pg/ml (x_1) at the beginning, 4.69±11.64 pg/ml (x_2) in the middle and 5.65±9.05 pg/ml (x_3) at the end of the therapy.However, these changes were not statistically significant. There were no statistically significant differences in TNF-α concentration changes in relation to different Ag levels. Changes of TNF-α concentration were not related to the changes of Ag level during the therapy. However, correlation between TNF-α concentration for Ag++ (8.14±13.26 pg/ml) and TNF-α concentration for Ag+ (8.87±14.20 pg/ml) in dogs during the therapy was significant (r=0.84) (figure 1). Concentrations of TNF-α in dogs with different HWD class were 5.94±9.01 pg/ml (class 1), 3.51±3.29 pg/ml (class 2) and 15.89±25.08 pg/ml (class 3), with no statistically significant difference.

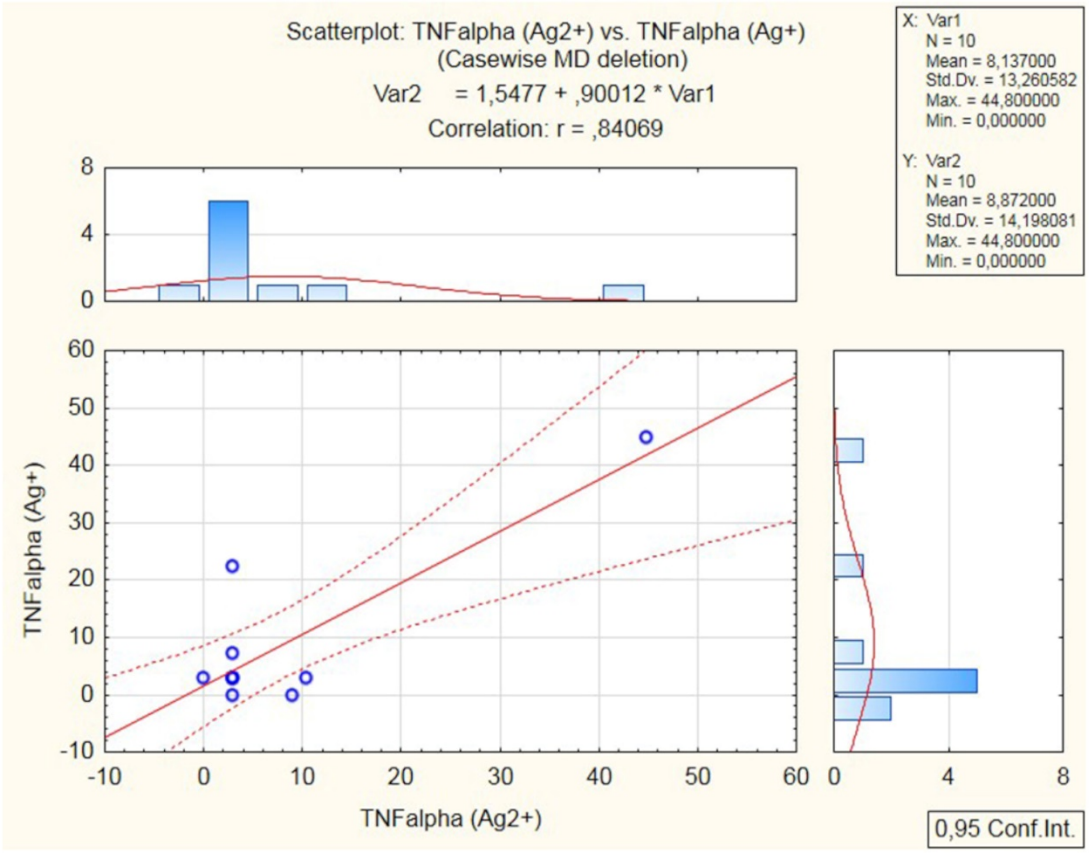

Figure 1. Correlation of TNF-α concentrations for high (Ag++) and low (Ag+) level of D.immitis Ag in dogs during the therapy.

Knowledge about the importance and clinical implications of cytokine measurements in the diagnosis and management of various diseases is constantly expanding. Evaluation of cardiopulmonary and inflammatory biomarkers in dogs with HWD highlighted the importance of D-dimer, interleukin-6, CRP, cardiac troponin I, myoglobin and NT-proBNP (Yoon et al., 2017, Carreton et al., 2017), without any given insight about TNF-α. According to our knowledge, this is the first report on TNF-α concentrations in canine HWD.

First described as a circulating antitumorogenic cytokine, TNF-α was thought to be produced by immune cells such as activated macrophages and lymphocytes (Carswell et al., 1975). However, further studies have proved its expression in smooth muscle cells (Warner et al., 1989), epithelial cells (von Asmuth et al., 1994), cardiac myocytes (Doyama et al., 1996) and endothelial cells (Neuhaus et al., 2000). In the cardiovascular system, TNF-α activates signal transduction pathways which contribute to vascular dysfunction, atherogenesis, hypertension, and adverse cardiac remodeling after myocardial infarction. Tumor necrosis factor alpha, which exists as soluble or membrane-bound, activates two different TNF receptors (TNFRs), TNFR1 and TNFR2 (Urschel and Cicha, 2015). Both receptors are found in equal proportions in the normal myocardium and TNF-α binds with equal affinity to both receptors (Smolens et al., 2000). Proinflamatory and proatherogenic effects of TNF-α, are mediated by TNFR1, while cardioprotective immunomodulatory responses are mediated by TNFR2 (Urschel and Cicha, 2015). Similar to the expression of β-adrenergic receptors in heart failure, the expression of myocardial TNFRs is also down-regulated in the presence of heart failure (Anguita et al., 1993; Fann et al., 1997). The fact that circulating or soluble forms of TNFRs are elevated in patients with heart failure postulates possible shedding of these receptors from the myocardial cells (Akasaka et al.,

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1998). In addition, the circulating TNFRs can neutralize the biological effects of circulating TNF-α (Pagani et al., 1992).

The reference value of TNF-α in dogs is not established, but its values in healthy dogs of particular and various breed have been published (Nielsen et al., 2013; O'Neill et al., 2013; Kilpatrick et al., 2014; Bastien et al., 2015; Frank et al., 2015; von Pfeil et al., 2015). Additionally, the studies on the subject of TNF-α concentration in different diseases have been reported (Richter et al., 2018). In dogs with babesiosis, TNF-α was detected in 6 out of 11 studied dogs (Zigner et al, 2014) with the average value of 14.7 pg/ml. Similarly, in our research, TNF-α was detected in 9 out of 14 dogs with the average value of 7.21±12.44 pg/ml. On the other hand, TNF-α was not quantified in myxomatous mitral valve disease (Kim et al., 2016). Concentrations of TNF-α in the dogs with babesiosis were in positive correlation with azotemia, fractional excretion of sodium and duration of the disease, and in negative correlation with systolic, mean and diastolic blood pressure, urinary creatinine to serum creatinine ratio, and urine specific gravity (Zigner et al., 2014). In our research no relation was found between TNF-α and the level of *D. immitis* Ag.

According to the literature from canine medicine, studies on the subject of TNF-α were aimed at understanding its role in pathogenesis of different diseases. In human medicine, however, studies are expanded from the diagnostic field to its possible therapeutic role (Urschel and Cicha, 2015). Circulating levels of TNF-α and soluble TNFRs are independent predictors of mortality in humans with heart failure (Mann et al., 2002). However, global TNF-α inhibition dysregulates both adverse and protective TNF-α signaling, leading to dramatic worsening of cardiac function. Therefore, novel therapeutic strategies are focused on blocking the responses specific to TNFR1 (Urschel and Cicha, 2015). In our research significant changes in TNF-α concentration during the alternative therapy for HWD were not found, and changes of both TNF-α concentration and Ag level were not in a mutual relation during the therapy. Thus, quantification of TNF-α solely, without insight on its receptors, does not contribute to the diagnosis and treatment of HWD in dogs. Further investigations of the influence of TNF-α on other complications observed in canine HWD, as well as analyses of TNFRs together with TNF-α in HWD, are needed in order to understand its role in the pathogenesis of this disease, and implementation of these findings into diagnostic, prognostic and therapeutic approach of canine HWD.

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