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Serum concentrations of eicosanoids and lipids in dogs naturally infected with Babesia canis

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ABSTRACT

Canine babesiosis is a tick-borne disease with world-wide significance caused by intraerythrocytic protozoa of the genus *Babesia*. The eicosanoids, as inflammatory mediators, are involved in the regulation of the immune response and inflammatory reaction. Metabolism of lipids is of great importance in babesiosis. In this study it was aimed to investigate the dynamics of serum concentration of prostaglandin E_2 (PGE_2), thromboxane B_2 (TxB_2), leukotriene B_4 (LTB_4), triglycerides, total cholesterol (Chol), HDL- and LDL-cholesterol in dogs naturally infected with *Babesia canis* and healthy dogs. Both groups were measured for all parameters on the admission day and on the first, second and seventh day of the disease. Dogs that were included in this study had systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS). It was demonstrated that the level of LTB_4, PGE_2, TxB_2 in dogs naturally infected with *B. canis* significantly changed during the disease. The level of LTB_4 was significantly higher during the study, while the concentration of PGE_2 was significantly higher second, third and seventh day of disease in relation with healthy dogs. The level of TxB_2 was significantly lower at the beginning of the disease, but after seven days concentration was significantly higher. Both group of patients with SIRS and MODS had significantly higher level of LTB_4. Substained high concentrations of PGE_2 were observed in dogs with MODS after therapy but not in dogs with SIRS, and LTB_4 followed a similar tendency. On the other hand, increases in TxB_2 were only significant in dogs with SIRS. The lipid profile in naturally infected dogs with *B. canis* infection was significantly changed. Further studies are needed to assess the prognostic values of lipid mediators in dogs with *B. canis* infection, and the ability of these markers to predict the progress of SIRS and MODS.

1. Introduction

Canine babesiosis is a tick-borne disease of worldwide importance. The causative organism of canine babesiosis is either *Babesia canis* or *Babesia gibsoni*. Large *Babesia canis* was divided into three different species, namely *Babesia canis*, *Babesia rossi* and *Babesia vogeli* (Schetters, 2005). Canine babesiosis caused by *B. canis* is a very common cause of morbidity and mortality of dogs in Croatia (Beck et al., 2009; Brkljačić et al., 2010). The disease can be clinically classified into uncomplicated and complicated forms. Dogs with uncomplicated babesiosis are typically

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presented with signs relating to acute haemolysis, including pale mucous membranes, fever, anorexia, depression, splenomegaly and water hammer pulse (Taboada and Merchant, 1991). The clinical manifestations of the complicated form are variable and related to the complications developed. The main complications are the development of an excessive inflammatory response called "systemic inflammatory response syndrome" or SIRS (Bone et al., 1992) and also a multiple organ dysfunction syndrome or MODS (Jacobson and Clark, 1994; Taboada, 1998).

Besides cytokines, eicosanoids have also been suggested to play a key role in pathophysiology of SIRS (Müller-Werdan and Schuster, 2005). Eicosanoids are lipid mediators derived from phospholipase-released arachidonic acid that regulate different physiological processes and modulate inflammatory and immunological response in mammals (Funk, 2001; Brattig et al., 2006). They include prostaglandins, thromboxanes and leukotrienes. They are synthetized from an outset interaction between life agents and the host cells. Resulting from this interaction proinflammatory cytokines, including IL-1, TNF-α and IL-6 can trigger the release of eicosanoids from various cell types (Seeger et al., 2005). Eicosanoids have a wide variety of effects and sometimes these effects are antagonists. For example, prostaglandins causes vasodilation, and inhibits platelet aggregation; whereas thromboxanes cause vasoconstriction and promote platelet aggregation (Betz and Fox, 1991; Sala and Giarcarlo, 2001).There are some evidences of a relationship between babesiosis and changes in serum lipid metabolism in the host (Goodger et al., 1981; Wright and Goodger, 1988; Cunha et al., 2000). This could be influenced by the fact that parasites rely on a complex system of uptake and synthesis mechanisms to satisfy their lipid needs (Ramakrishnan et al., 2013) but also can be related with the inflammatory response that occurs in the host (Rosenson, 1993).

To the author's knowledge there is no published data about possible changes in serum eicosanoids and different lipids in dogs with *B. canis* as well their role in the development of possible complications of this disease such as SIRS and MODS and their evolution after treatment. The aim of this study is to investigate the serum concentration of eicosanoids and lipids in dogs naturally infected with *B. canis* analyzing their concentrations in infected dogs that developed SIRS or MODS and evaluating their behaviour after treatment.

2. Material and methods

2.1. Animals

Three groups of dogs were used in our study:

- Group 1 consisted of 27 dogs naturally infected by *B. canis*, admitted to the Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Croatia, with clinical signs of acute babesiosis. The clinical signs of these dogs including fever, lethargy, ticks found by the owner or veterinarian, pale mucous membranes, anaemia, jaundice, haemoglobinuria or haematuria, anorexia, splenomegaly, tachycardia and vomiting. Dogs in this group were of various breeds, between 6 months and 13 years of age, and 17 of them were males. The diagnosis was confirmed by demonstration of the parasites within the infected erythrocytes in Romanowsky-stained thin blood smears. One dose (6 mg/kg of body weight) of imidocarb dipropionate (Imizol®, Shering – Plough) was administered to all the dogs subcutaneously on the day of admission. All the dogs included in this study came from Zagreb area, in which no cases of leishmaniosis or ehrlichiosis have been reported. Therefore, serology for these diseases was not performed.

  On the basis of clinical manifestations and laboratory data the affected dogs were divided into two subgroups: one subgroup including those with SIRS and another subgroup with the dogs that had MODS. The SIRS criteria used in this study were established on the basis of the criteria proposed by Purvis and Kirby (1994) and Welzl (2000). The animal was classified as SIRS positive if two or more of the following 4 criteria were fulfilled: body temperature higher then 39.5 °C or lower then 38 °C, heart rate more then 160 beats/min, respiration rate more than 20 breaths/min and WBC count less than 6×10^9/L or more than 12×10^9/L or more than 10 percent band neutrophils.

  The animal was classified as MODS positive if two or more of the following 5 criteria were fulfilled: renal dysfunction (serum creatinine more than 180 μmol/L – reference values 44–140 μmol/L), liver dysfunction (alanine aminotransferase, ALT, more than 176 U/L – reference value less than 88 U/L, and alkaline phosphatase, AP, more than 360 U/L – reference value less than 156 U/L), central nervous system dysfunction (a modified Glasgow coma scale score less than 9) (Marshall et al., 1995), respiratory system dysfunction (radiographic pulmonary oedema, dyspnoea or blood/tinged frothily nasal discharge), muscular involvement (creatine phosphokinase, CPK, more than 600 U/L – reference value less than 160 U/L).
- Group 2 (healthy animals with treatment) consisted of 9 mongrel dogs of both sexes (4 females and 5 males), aged from 2 to 9 years. These dogs were considered healthy based on physical examination and haematological and biochemical data, and they come to the hospital to receive a subcutaenous dose of imidocarb dipropionate (6 mg/kg) as a preventive measure against babesiosis at the request of their owners.
- Group 3 (healthy animals) consisted of 8 mongrel dogs of both sexes (3 males and 5 females), aged from 1 to 9 years. These dogs come to the hospital for routine check up controls and were considered healthy based on clinical examination and haematological and biochemical data.

2.2. Samples

The blood samples for analysis from groups 1 and 2 were collected from the cephalic vein on the day of admission, and the first, second, and seventh days after the administration of imidocarb dipropionate. The samples were placed in tubes with EDTA for haematological analysis and tubes with no anticoagulant which were centrifuged at $1200 \times g$. A portion of the obtained serum was used for routine biochemical analysis while the remainder was stored at −80 °C

until it was used for analyzing lipid mediators. The blood samples for analysis from group 3 were taken once during routine visits to a Clinic. Permission to collect blood samples was obtained from each dog owner. Owners were informed about the use of the specimens and the aims of the research.

2.3. Blood analysis

The lipids analysed were triglycerides, total cholesterol (Chol), high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol and were measured according to standard methods using commercial kits in an automatic analyser (Olympus AU 600, Olympus Diagnostica GMBH, Germany).

2.4. Lipid mediators

Concetrations of the prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and thromboxane B_2 (TxB_2) in serum samples were analysed by commercial multispecies ELISA kits (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. All samples were measured in duplicate. Briefly, serum samples were tested at 1:50, 1:100 and 1:200 dilutions, respectively, for PGE_2. For TxB_2 and LTB_4 samples were tested at 1:400, 1:800 and 1:1600 dilutions, respectively. Optical densities (ODs) were measured at 420 nm in a spectrophotometer Multiscan MS version 3.0 (Labsystems, Helsinki, Finland). The calculation of concentrations was carried out following manufacturers' instructions. Using triplicate at three different dilutions we validated the precision of ELISA assay for canine sample types. Linearity was good over a three dilution measurements.

2.5. Statistical analysis

Statistical analysis was done with software package Statistica, version 6. Graphical presentation of data was made with box-and-whisker methods. All applied statistical tests were two-way. The correlation between the investigated parameters was determined using the Spearman rank order test. Comparison of two independent groups was performed using the Mann–Whitney *U* test. Comparison of three or more independent groups was performed using the Kruskal–Wallis ANOVA test with post hoc Steel-Dwass test for group comparisons. For all analyses, significance was set at $P < 0.05$.

No significant differences were found between the healthy animals with and without treatment in any of the analytes studied. Therefore the two groups were considered as a single healthy control group.

3. Results

3.1. SIRS and MODS frequency in the dogs with Babesia canis

Of the 27 dogs with acute canine babesiosis that were admitted during the study period, 21 (78%) presented SIRS, with 3 dogs having three criteria for SIRS, whereas the other 18 dogs had two of the criteria for SIRS. The number of dogs with MODS was 9 (33%), with two dogs having 2 criteria and seven dogs having 3 or more criteria of MODS. The most frequently identified organ dysfunction was liver compromise (10 cases). Six of 27 dogs diagnosed with babesiosis died (a mortality rate of 22%). Three dogs died second day, while 3 dogs died after seventh day after admission. All dogs that died had developed MODS.

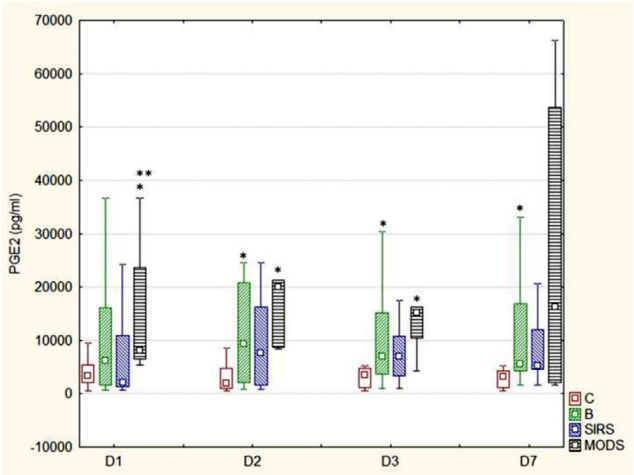

Fig. 1. PGE_2 concentration in control group (C) and in canine babesiosis group (B), SIRS group (SIRS) and MODS group (MODS) before (D1—first day) and after antibabesial treatment (D2—second, D3—third and D7—seventh day). (*) Indicates a significant difference ($P < 0.05$) between uninfected (control) and infected (canine babesiosis) group by the Mann–Whitney *U* test. (**) Indicates a significant difference ($P < 0.05$) between SIRS and MODS group by the Mann–Whitney *U* test. Box plots show the median (square within box), 25th–75th percentiles (box), non-outlier range (whiskers).

3.2. Prostaglandin E_2

PGE_2 values obtained from naturally infected dogs with *B. canis* are shown in Fig. 1. PGE_2 showed significantly higher values in infected than in control group at days 2, 3 and 7th ($P < 0.05$). Dogs with MODS had a significant increase in PGE_2 at day 1 compared with dogs with SIRS, and mantained high PGE_2 concentrations despite treatment. Whereas dogs with SIRS showed a tendency to decrease PGE_2 concentrations after treatment.

3.3. Leukotriene B_4

The concentrations of the LTB_4 found throughout the study in naturally infected dogs are shown in Fig. 2. LTB_4 significantly increased after the first day of the disease, reaching the maximum third day of the disease, and then decreased until the end of the study. There were no significant differences between dogs that had SIRS and MODS in LTB_4 during the study. Although dogs with MODS had higher values of LTB_4.

3.4. Thromboxane B_2

TxB_2 values obtained throughout the study in naturally infected dogs are shown in Fig. 3. The levels of TxB_2 significantly decreased at first and second day of disease ($P < 0.05$)

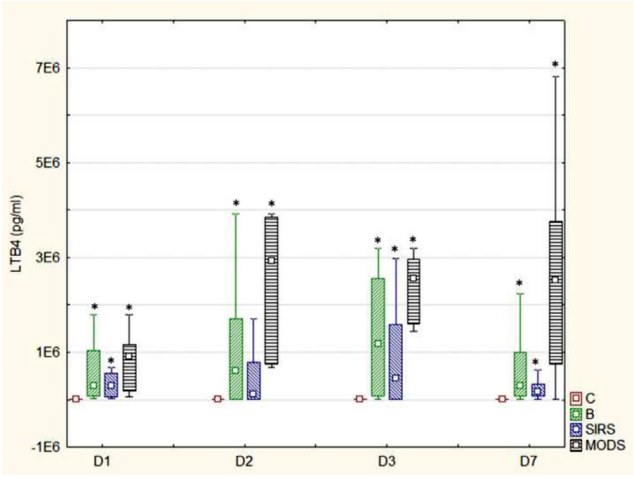

Fig. 2. LTB_4 concentration in control group (C) and in canine babesiosis group (B), SIRS group (SIRS) and MODS group (MODS) before (D1—first day) and after antibabesial treatment (D2—second, D3—third and D7—seventh day). (*) Indicates a significant difference ($P < 0.05$) between uninfected (control) and infected (canine babesiosis) group by the Mann–Whitney *U* test. Box plots show the median (square within box), 25th–75th percentiles (box), non-outlier range (whiskers).

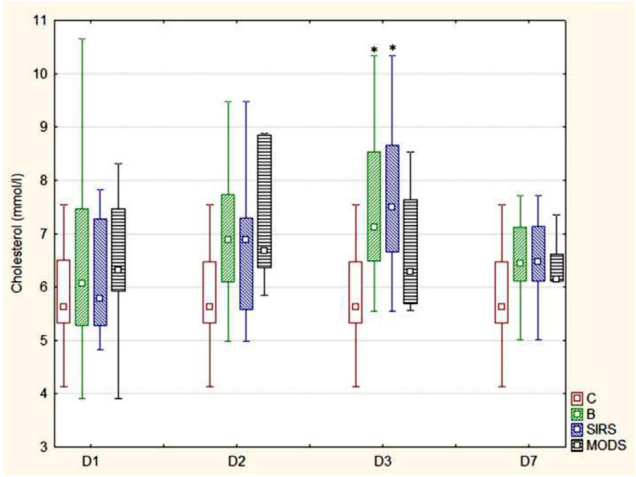

Fig. 4. Total Cholesterol concentration in control group (C) and in canine babesiosis group (B), SIRS group (SIRS) and MODS group (MODS) before (D1—first day) and after antibabesial treatment (D2—second, D3—third and D7—seventh day). (*) Indicates a significant difference ($P < 0.05$) between uninfected (control) and infected (canine babesiosis) group by the Mann–Whitney *U* test. Box plots show the median (square within box), 25th–75th percentiles (box), non-outlier range (whiskers).

when compared with healthy dogs. However at seventh day of the disease the level of TxB_2 significantly increased in relation with healthy dogs. This increase was mainly due to the dogs with SIRS that had significant higher values than healthy dogs ($P < 0.05$).

3.5. Serum lipid profiles in naturally infected dogs with B. canis infection

Total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol values obtained from naturally infected dogs are shown in Figs. 4–7. The levels of total cholesterol significantly increased at third day of disease ($P < 0.05$) when compared with healthy dogs. At third day of infection total cholesterol significantly increased in dogs with SIRS but not in dogs with MODS. Whereas triglycerides showed an increase at second day in all infected animals and did not show significant differences between dogs with SIRS and MODS.

All infected animals and specially dogs with MODS showed a significant decrease in HDL-cholesterol at day one, and during the rest of days showed lower values of HDL in comparison with SIRS and healthy dogs. Although the differences were not significant, dogs with MODS showed an increase in LDL-cholesterol and kept high values of this fraction during the first three days of the study.

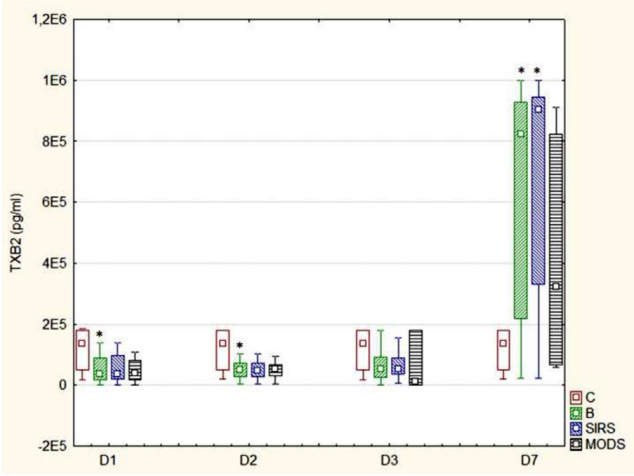

Fig. 3. TxB_2 concentration in control group (C) and in canine babesiosis group (B), SIRS group (SIRS) and MODS group (MODS) before (D1—first day) and after antibabesial treatment (D2—second, D3—third and D7—seventh day). (*) Indicates a significant difference ($P < 0.05$) between uninfected (control) and infected (canine babesiosis) group by the Mann–Whitney *U* test. Box plots show the median (square within box), 25th–75th percentiles (box), non-outlier range (whiskers).

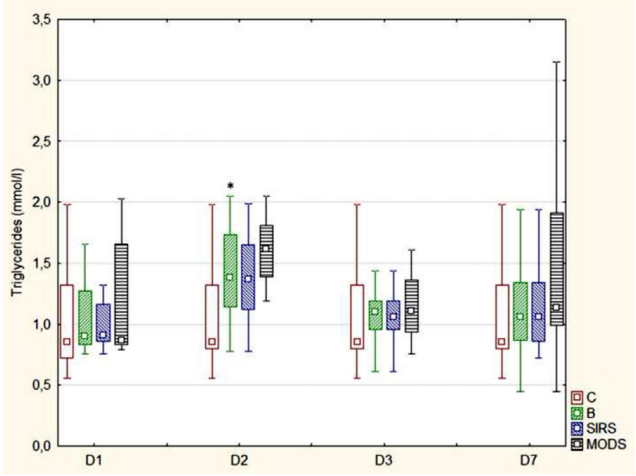

Fig. 5. Triglycerides concentration in control group (C) and in canine babesiosis group (B), SIRS group (SIRS) and MODS group (MODS) before (D1—first day) and after antibabesial treatment (D2—second, D3—third and D7—seventh day). (*) Indicates a significant difference ($P < 0.05$) between uninfected (control) and infected (canine babesiosis) group by the Mann–Whitney *U* test. Box plots show the median (square within box), 25th–75th percentiles (box), non-outlier range (whiskers).

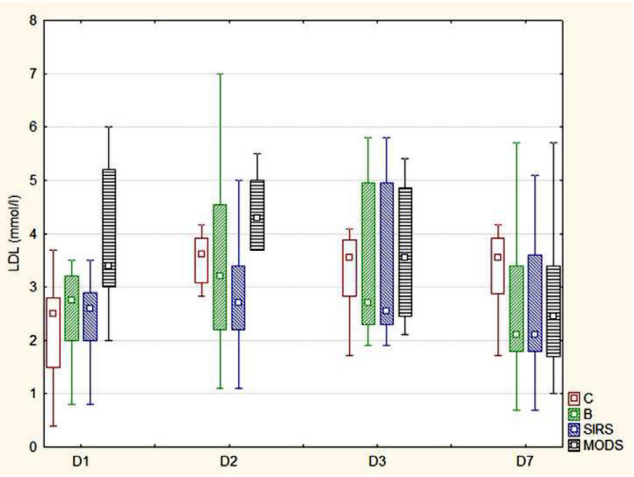

Fig. 6. LDL concentration in control group (C) and in canine babesiosis group (B), SIRS group (SIRS) and MODS group (MODS) before (D1—first day) and after antibabesial treatment (D2—second, D3—third and D7—seventh day). (*) Indicates a significant difference ($P < 0.05$) between uninfected (control) and infected (canine babesiosis) group by the Mann–Whitney *U* test. Box plots show the median (square within box), 25th–75th percentiles (box), non-outlier range (whiskers).

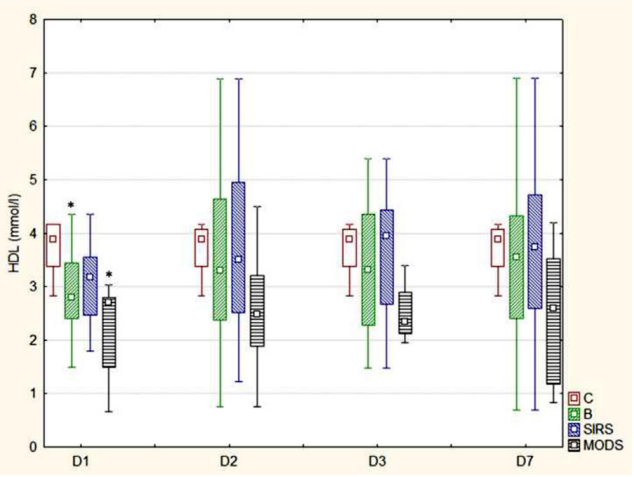

Fig. 7. HDL concentration in control group (C) and in canine babesiosis group (B), SIRS group (SIRS) and MODS group (MODS) before (D1—first day) and after antibabesial treatment (D2—second, D3—third and D7—seventh day). (*) Indicates a significant difference ($P < 0.05$) between uninfected (control) and infected (canine babesiosis) group by the Mann–Whitney *U* test. Box plots show the median (square within box), 25th–75th percentiles (box), non-outlier range (whiskers).

3.6. Correlation between lipid mediators and lipids in naturally infected dogs with B. canis infection

Correlation between lipid mediators and lipids on day of admission are shown in Table 1. There were significant positive correlations ($P < 0.05$) between LDL and LTB_4, PGE_2, and LTB_4, PGE_2, and LDL, cholesterol and LDL, HDL and triglycerides.

4. Discussion

Eicosanoids are produced by cells of the immune system that are implied in the elimination of parasites (Ali et al., 1999) and are considered as inflammatory mediators

Table 1
Correlation between the levels of lipid mediators, and lipids in dogs naturally infected with *Babesia canis* first day of disease (Spearman rank order) (*$P < 0.05$).

Diagnostic test	LTB_4	PGE_2	TxB_2	Chol.	HDL	LDL	Trig
LTB_4	1.00						
PGE_2	0.62*	1.00					
TxB_2	0.06	0.11	1.00				
Chol.	0.14	−0.04	0.08	1.00			
HDL	−0.30	−0.39	−0.03	0.38	1.00		
LDL	0.59*	0.46*	0.05	0.70*	0.03	1.00	
Trig.	−0.22	−0.34	0.14	0.26	0.55*	−0.11	1.00

being also involved in the development of tissue damage and hypoxia (Higgs et al., 1981; Higgins and Lees, 1984). To the authors' knowledge this is the first clinical study in which various eicosanoid concentrations in dogs with *B. canis* infection are measured, and our results showed that *B. canis* infection produces significant changes in the different eicosanoids studied.

Increases in PGE_2 and LTB_4 were found in dogs with babesiosis in our study being both analytes correlated. Babesia parasites produce toxin-like compounds that trigger inflammatory responses (Schetters, 2005). In this inflammatory process PGE_2 leukotrienes induces inflammation and modulates the host immune response (Kubata et al., 2007; Penrose and Austen, 1999; Gok et al., 2000). The high levels of PGE_2 and LTB_4 are consistent with the development of the inflammatory reaction which characterize *B. canis* infection (Matijatko et al., 2007). In fact, these eicosanoids enhanced vascular permeability and edema. Namely, prostaglandins synergize with other mediators (e.g., bradykinin, histamine) to elicit enhanced vascular permeability, while LTB_4 promotes adhesion to vascular endothelium through specific integrins, cause plasma leakage from postcapillary venules and enhance mucus secretion (Funk, 2001). But, leukotrienes are considered potentially more harmful than PGE_2 because they are potent neutrophil chemotactic and chemokinetic agents and can increase microvascular permeability. Overproduction of LTB_4 is associated with several pathological condition like lung edema, inflammatory bowel disease, eye and skin diseases (Stenson, 1990; Chari et al., 2001; Pace et al., 2004; Smith et al., 2004). Also, animal studies have shown an important role for LTB_4 release in promoting leukocyte infiltration and degranulation in the glomeruli, which are characteristic features of glomerular immune injury (Henderson, 1994). Recent investigations confirm that renal damage is deemed a common, yet poorly documented, complication in canine babesiosis (Defauw et al., 2012).

After an initial decrease, TxB_2 significantly increased compared to the control group at seventh day of disease when the parasite disappeared from blood. Thromboxane A_2 (TxA_2) is produced by platelets when activated and produces TxB_2 which is inactive. Our preliminary investigation indicate the activation of platelets in babesiosis through increased CD40L, and consequently through activated endothel (increased ICAM), increased chemokines (IL-8, MCP) and oxidative stress (Jennings, 2009; Barić Rafaj

et al., 2013; Crnogaj, 2012. PhD theses; Mayer, 2012. PhD theses). Probably low concentrations of TxB_2 first and second day of the study are associated with a low number of platelets that had the animals in our study, and the increase in TxB_2 could be due to recovery of the number of platelets that happened in our study (with increases of more than 10 fold at day 7 compared with day 1, data not shown), as indicated by Obata et al. (2002).

Main complications associated with canine babesiosis are a consequence of the development of SIRS and MODS; being described that SIRS precedes MODS and that it is likely that MODS is the result of a perpetual inflammatory response (Jacobson and Clark, 1994; Welzl et al., 2001; Matijatko et al., 2012). The presence of MODS had significant impact on outcome because six of 9 dogs that had MODS died. Dogs with MODS had higher concentrations of PGE_2 and LTB_4 than SIRS positive dogs, that in case of PGE_2 did not decrease after treatment. Since both eicosanoids are related with tissue damage, it could be postulated that the increase in these mediators could be related with the development of MODS in dogs with babesiosis.

According to our results serum lipid profile is significant changed in naturally infected dogs with *B. canis* infection. Namely, lipid metabolism is of great importance in babesiosis because *B. canis*, as the majority of haemoparasites can not synthesize their own lipids. They take them up from the host plasma (Valentin et al., 1991). Also, mechanisms involved in lipid changes may be partly related to an acute phase response, which is known to increase triglyceride values and to decrease HDL and LDL cholesterol values (Rosenson, 1993).

In the present study HDL cholesterol values were significantly lower first day of infection when compared with healthy dogs. These results were consistent with previous findings in canine babesiosis by Cunha et al. (2000). Similar changes in HDL have also been reported in dogs with visceral leishmaniasis (Durgut et al., 2012). In our study MODS positive dogs had also significantly lower levels of HDL cholesterol first day than healthy dogs, and concomitantly with the general improvement of the dogs the level of HDL cholesterol was slightly higher. The low concentrations of HDL cholesterol could be due to lipoprotein lipase hypoactivity (Nakamura, 1998) or due to a reduction in the liver function that was the most frequently identified organ dysfunction. Then increase in triglyceride concentrations that was found in the dogs with *B. canis* infection may be associated with increased hepatic production of triglycerides and/or a defect of triglycerides removal from circulation, since it has been demonstrated that increased levels of triglycerides in acute phase response are a part of the host response (Carpentier and Scruel, 2002). Unlike other drugs such as glucocorticoids (Martinez-Subiela et al., 2005), imidocarb dipropionate does not seem to produce an increase in lipid mediators and parameters of lipid profile.

In conclusion, the results of this study demonstrated that babesiosis in naturally infected dogs caused by *B. canis* produced significant changes in lipid mediators with: (1) significant increases in LTB_4 and PGE_2, that are of higher magnitude in dogs that develop MODS and that in case of PGE_2 does not decrease after treatment in dogs with MODS. (2) A decrease in TxB_2 which increases after the disease is resolved. In addition there are increases in triglycerides and total cholesterol, and decreases in HDL cholesterol. More studies would be needed to assess the prognostic values of lipid mediators in dogs with *B. canis* infection, and to assess the ability of these markers to predict the risk of presentation of SIRS and MODS.

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