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MARKERS OF INFLAMMATION, IMMUNITY AND RENAL DAMAGE IN DOGS WITH CANINE LEISHMANIASIS

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SUMMARY

1. INTRODUCTION

1.1 Canine leishmaniasis

Canine Leishmaniasis is a protozoal disease of dogs and humans sustained by *Leishmania Infantum*.[1]

Canine Leishmaniasis is an endemic disease that is spreading over wide areas bordering on the Mediterranean sea[2] but, because of climate changing, touristic movement of dogs, dogs adoption from southern areas, canine leishmaniasis is gradually moving toward northern country.[3]

In Italy, canine leishmaniasis is endemic all along Tyrrhenian, Ionian and Adriatic coast, but new endemic foci developed even in North Italy such as in Veneto (Verona), Emilia-Romagna (Bologna), Piemonte (Torino, Asti, Ivrea and Casale), Trentino Alto Adige (Trento) and Lombardia.[4-6]

Affected dogs may show a broad spectrum of clinical signs, both cutaneous and systemic, due to both the direct action of parasites and the host immunologic response, that makes the management of this disease particularly challenging.[7]

Sandflies bear the leishmania promastigotes with bites, usually in a small quantity (with a mean of 100-1000 amastigotes per bite).[8]

The first protective lines of the body, in particular neutrophils and complement factors, immediately kill the great part of promastigotes. The next step of antigen presentation in lymph nodes mediated by dendritic cells leads to phagocytosis by macrophages, but in predisposed dogs the lysosome-phagosome fusion is prevented and the infection will continue. A crucial point in this process is the cytokine pattern expressed during the early stage of infection. Th1 lymphocytes, in fact, induce an autocrine IL-2 mediated mechanism that leads to lymphokine production (IL-2, INF-γ, TNF-α e IL-12) and subsequent effective protection against leishmaniotic infection.[9,10]

Instead, sick dogs show a Th2 cytokine pattern that lead to enhanced humoral response that is not effective against the infection and moreover it may induce immune-mediated damage and the development of clinical manifestations of the disease.

Canine leishmaniasis usually has a subacute or chronic progression, and show many different clinical signs, affecting mainly the skin (typical dermatitis with scales and ulcers), joint (polyarthritis), eye (uveitis, blepharitis, keratoconjunctivitis etc) and kidneys (glomerulonephritis, chronic renal failure) depending on immune pattern and localization of immune complexes deposition.[11]

Diagnosis is based on proving the presence of parasite in lesions with cytological, histopathological or molecular (PCR) techniques, typical clinical pathological findings (hypergammaglobulinemia, hypoalbuminemia, proteinuria, high antibody titers). There is a general consensus concerning diagnostic criteria[11,12] and therapeutic standard,[13] right now (see an example in figure 1.1).

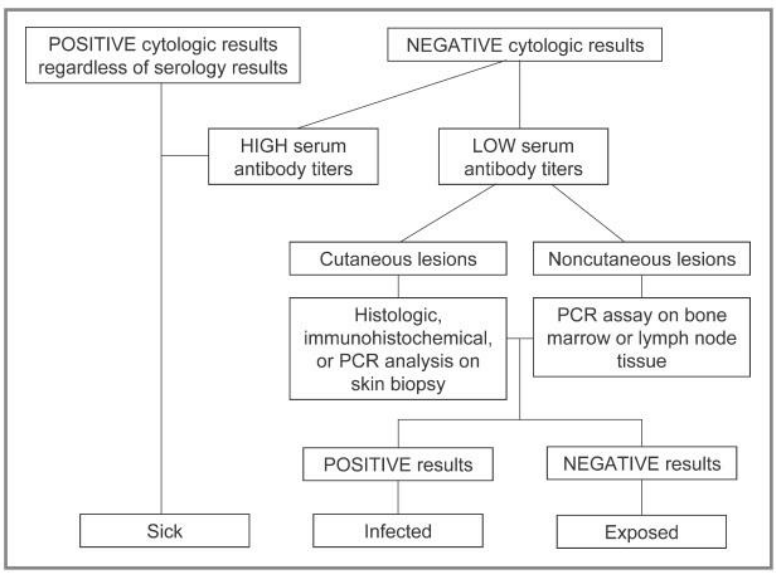

Figure 1.1; diagnostic algorithm for canine leishmaniasis[11]

Instead, there are just few studies regarding how to monitor the follow-up. Even the guidelines for monitoring dogs treated for canine Leishmaniasis are based on a consensus of experts rather than on structured scientific publications.[14,15] For this purpose, in routine practice, the approaches followed by the clinician are mainly based on a clinical assessment associated with electrophoretic evaluation, and the recovery of clinical pathological abnormalities (including serology, CBC, biochemical profile, PU/CU ratio) that were present before therapy, with particular emphasis on monitoring renal functions.[14-16]

Since, as stated above, this approach has not been standardized, the great variability in checking the effectiveness of treatments is still a major problem both in clinical practice and in the study of drugs efficacy and therapeutic protocols.

Furthemore, there are no prognostic markers that permit to predict the clinical outcome of the therapy before starting it or early in the course of treatments. In this regard, since most of the changes detectable in sick dogs actually depends on an inflammatory/immune reaction, the serum concentration of acute phase proteins (APPs) and of electrophoretic fractions was proposed in the past as a possible tool for monitoring.[17-20] Although the utility of these biomarkers may be promising, none of them seems to normalize particularly early during the treatment, ultimately decreasing their clinical utility.[17-20]

1.2 Paraoxonase, oxidants and lipid metabolism

In people, paraoxonasis enzymes (PON1) are synthesized from liver and are released in the blood flow bound at HDL lipoproteins[21.] Paraoxonase is a family of calcium containing proteins with enzymatic activity that work as rapid negative acute phase proteins. To date, three isoforms (PON1, PON2 and PON3) are recognized. Whilst PON2 is expressed ubiquitously in the body but confined inside the cells, where probably works restraining the cell apoptosis, PON1 and PON3 are mostly expressed in liver and excreted in blood

circulation bound to HDL to prevent LDL oxidative damage.[22]

In people, PON1 is synthesized from liver and is released in the blood flow bound at HDL lipoproteins[21] which work as antioxidant.[22]

Antioxidant properties of PON depend on their lactonasic action that prevents oxidation of LDL lipoproteins (LDL) and permits the metabolism of cholesterol-derived peroxide.[21] In atherosclerosis, for example, oxidation of even low dose of LDL stimulates MCP-1 (monocyte chemotactic protein-1), adhesion molecules (selectins, VCAM-1 and ICAM-1) and M-CSF (macrophage colony-stimulating factor) from endothelial cells.[23] The PON1-HDL complex, inhibits indirectly the MCP-1 production to prevent LDL oxidation.[24] A strong correlation between HDL levels and inflammatory responses, severity of clinical manifestation, plasmatic concentration of TNF, IL-1, IL-6 and IL-8 was demonstrated.[21,25,26]

Decreased PON1 activity was shown in many human disease characterized by oxidative stress, such as obesity, diabetes mellitus, neurologic diseases (autism, Alzheimer, Parkinson, SLA, depression),[27-29] autoimmune disease (Behçet disease, systemic lupus erythrmatosus, rheumatoid arthritis),[30-31] liver diseases[32-35] and renal insufficiency.[36] In addition, changes, in concentration and/or activity of PONs is ruled by lipoproteins and their metabolites, organic macromolecules, drugs, diet and lifestyle.

Antioxidative activity of PON1 is also useful in preventing carcinogenesis, and low levels of this enzymes are related to enhanced risk for prostatic carcinoma,[37] lung carcinoma,[38] gastric and pancreatic carcinoma,[39-40] neural tumors,[41] ovaric carcinoma.[42-43]

The antioxidative activity of PON1 is strongly related with its antinflammatory activity and in the acute phase response both the inhibition of hepatic synthesis and the inactivation of preexisting PON1 molecules occurs.[44] Therefore, PON1

works as a negative acute phase protein.

HDLs have antioxidant and antinflammatory activity and suppress the adhesion molecules of endothelial cells that are induced by cytokines (CAM).[44] As stated above, a strong correlation between serum HDL levels and the inflammatory response against endotoxic agents, the severity of clinical signs and plasmatic concentration of TNF, IL-1, IL-6 and IL-8 was reported. Furthermore, the expression of MCP-1 increases in animals with lower levels of HDL if compared with animals with normal level of HDL.[45] The interaction between PON1, lipid oxidation and inflammation is controlled by MCP-1 that modulates the monocyte migration in tissues and their differentiation in macrophages.[46] Even minute amounts of oxidized LDLs stimulate MCP-1 synthesis. PON1, fencing in vitro the LDLs from oxidative damage, indirectly inhibits MCP-1 production, that is stimulated by oxidized LDL.[24] Moreover, PON1 increases the cholesterol outflow from macrophages and the corresponding uptake by the HDL molecules, respectively, lowering the risk of atherosclerosis.[26]

During the acute phase response, a change of properties and composition occurs in HDL molecules. Among acute phase proteins, C-reactive protein (CRP), serum amyloid (SAA) and ceruloplasmin interact with lipoproteins: CRP binds B apolipoproteins, but SAA and ceruloplasmin adhere to HDL molecules causing a substitution of apolipoprotein-A, esterified cholesterol and their associated enzymes, such PON1, with free cholesterol, trigyicerides ad free fatty acids that lead to losing the antioxidative activity of HDLs.[36,47,48]

Despite the numerous reports available in human medicine literature, there are few paper about the role of PON1 in veterinary medicine, mainly in bovine clinic and laboratory animals.[49-51] In dogs, two techniques are validated for PON1 measurement[52,53] and PON1 seems useful as negative acute phase protein also in this species.[53]

1.3 Oxidative stress

Oxidative stress occurs when there is an imbalance between production of reactive oxigen species (ROS) or reactive oxigen metabolites (ROMs) and their removal by antioxidative molecules, thus leading to an increased levels of ROS.[54]

Free radicals are unstable molecules produced in oxygen combustion that hold one unpaired electron in the outermost shell. This configuration is very unstable and this electron can easily move to other molecules oxydating them in a chain reaction, with final production of energy stored as ATP molecules. Thus, oxygen is necessary for normal metabolism but, otherwise, is a dangerous element in auto-oxidative reaction.[55] All these chemical species are named ROS, and among their function, those that are important for this study are represented by signaling during inflammation (fibroblasts, smooth muscle in vessels, endothelium), protection against pathogens (*respiratory burst* in leucocytes), cellular damage.[56]

1.4 Oxidative stress and leishmaniasis

Canine leishmaniasis is characterized by great changes of oxidative metabolism of inflammatory cells. Such changes are bidirectional: on one hand, the parasites are able, in the early stage, to inhibit the oxidative metabolism of macrophages, defending themselves from intracellular oxygen-depending killing[57] and keeping asymptomatic the infection for a long span of time. The production of oxidants, targeted to kill the parasites via respiratory burst, is inhibited by parasite penetration into the cell, and, furthermore, the LPG (lipophosphoglycan) makes up a barrier all around the parasite that is able to catch the free radicals preventing the phagosoma-lysosoma fusion. On the

other hand, in the simptomatyc stage, the cell-mediated defenses disrupt and then inflammatory lesions, characterized by an intense tissue activation of phagocytes with highly increased oxidative metabolism.

Recent papers showed that d-ROMs levels in sick and healthy animals are quite similar, so, perhaps the two phenomena balance each other. This different oxidative response was assessed measuring the reactive oxygen metabolites (ROMs), that decreased in some animals in asymptomatic stage and increased in some other animals with symptomatic illness.[59]

The information about paraoxonase activity in leishmaniotic dogs are, to date, limited and partial[69] and we lack information about the role of HDL cholesterol and lipoproteins and, most of all, how the lipoproteins, the oxidative conditions, and the paraoxonase activity are related among them.

A preliminary study showed that paraoxonase may be a good early predictive marker of clinical recovery, but again we lack of thorough information on possible prognostic role of the whole oxidative pattern both regarding the clinical recovery and regarding the assessment of parasitic load.[53,59]

1.5 Urinary markers of renal damage

Proteinuria is the most sensitive marker of renal damage in clinical practice, especially when combined with GFR, but both these tests have some limitations especially for the identification of tubular damage. Hence, early, more sensitive, biomarkers are required. Recently, promising biomarkers have been identified for detecting renal injuries and to discriminate between tubular and glomerular damages.[60]

Among them, since the late ’80s, GGT has been proposed as urinary marker of tubular damage. But, even though it seemed very promising marker, this

enzyme was almost discarded because of supposed within-day variation and technical limitations. Thus, after an initial interest, this marker was almost abandoned.[61]

GGT is an enzyme widely spread thorough the body and its main activity, besides its involving in leukotrien and glutathione metabolism (GGT work sas a cellular antioxidant), is the transferring of aminoacids across the cellular membranes.[62]

This specific function is particularly important for GGT present in kidney on the membrane of tubular cells. Increases in urine NAG and GGT/UC ratio allow for earlier detection of renal tubular damage in dogs. Such early detection would be very useful for the clinicians.[63,64]

GGT seems to have some advantages. First, GGT does not undergo glomerular filtration, so serum levels of GGT do not affect urinary levels of this enzyme. Second, GGT is a membrane associated enzyme, and this is a very important point. In fact, when a renal injury causes tubular cells damage, the resulting membrane disruption lead to an increased release of this enzyme in the ultrafiltrate. This gives evidence of rapid increase of GGT levels in urine when a nephrotoxicity occurs. Third advantage, urinary GGT is easy to measure, given the same techniques used for serum samples work well on urine. Of course, given the specific gravity of urine affects the urinary GGT levels it's needed to normalize this value with UC ratio.[64, 65]

Unfortunately, urinary GGT has a big disadvantage that is its instability especially at low pH values. Moreover, freezing quickly decreases the activity of urinary GGT, and these two reasons make sending of urinary sample to a reference laboratory very difficult for this analytical purpose.[66]

Since 1997 urinary GGT is known to be useful as a index of renal damage in canine leishmaniasis, but in the published paper no comparison between GGT

and a “gold standard” method were done.[67] Even though renal biopsy represents the actual gold standard in assessing renal injuries, we used sodium dodecyl sulphate electrophoresis as reference method because it’s easier to perform, less invasive, and it has been reported to well correlate with results of renal biopsy.[68]

2. AIM OF THE THESIS

Despite the pathogenesis of canine leishmaniasis has been investigated through several studies, some aspect of the host-parasite interaction that lead to overt diseases and typical inflammatory lesions have not yet been understood. Among these, the oxidant-anti-oxidant mechanisms that occur in dogs with the clinical disease are particularly interesting since on one hand they can provide to us useful information on the pathogenesis of the disease, which can also be a model for investigating immune-mediated chronic inflammatory conditions occurring also in other species on the other hand the identification of changes in molecules involved in oxidative stress may serve as a potential target for ancillary treatments or as markers of disease severity and progression.

Similarly, most of the researches done until now have been focused on renal damage in dogs with canine leishmaniasis, since the progression of renal disease may be life threatening in affected dogs. Therefore, many studies investigated the serum or urine level of biomarkers that roughly identify a renal damage, such as creatinine, urea, urine specific gravity and quantification of proteinuria. Nevertheless, only a few studies were focused on markers of tubular damage, despite the development of tubulo-interstitial nephritis is associated with more advanced stages of the disease.

Therefore, the aim of this PhD thesis is to investigate on one hand the possible diagnostic or prognostic role of metabolites involved in oxidative stress associated with inflammation and of urinary biomarkers of renal tubular damage. To this aim, four separate studies have been done:

- Study 1: Role of paraoxonase-1 (PON-1) as a tool for assessing disease severity and response to conventional treatments in dogs with canine leishmaniasis

- Study 2: Role of high density lipoprotein (HDLs) and PON-1 as a marker of oxidative stress associated with canine leishmaniasis at clinical diagnosis and over treatment
- Study 3: Changes of PON-1 and HDLs in dogs receiving conventional treatments and supplementation with a stimulator of cell mediated immunity
- Study 4: Urinary gamma-glutamyl transferases (GGT) as a marker of tubular damage in dogs with canine leishmaniasis, using sodium dodecylsulphate electrophoresis as a reference method.

3. STUDY 1: Role of paraoxonase-1 (PON-1) as a tool for assessing disease severity and response to conventional treatments in dogs with canine leishmaniasis

3.1 Aim of the study

The aim of this study is to determine the possible prognostic role of paraoxonase (PON1), a fast negative APP recently studied in dogs[52,53,70] in comparison with traditional marker of inflammation and immunity (C reactive protein and electrophoretic fractions). Specifically we investigated whether the serum activity of PON1 correlates with the severity of the disease and whether PON1 better reflects the therapeutic response to conventional treatments for Canine leishmaniasis than the other biomarkers included in this study

3.2 Materials and methods

3,2,1 Animals and study design

This study was performed on serum and urine sampled from 39 dogs at first diagnosis of leishmaniasis that fulfilled the following inclusion criteria:

- Absence of previous treatments against leishmania
- No current anti-inflammatory treatments administered
- Absence of symptoms or of laboratory changes consistent with metabolic or endocrine diseases potentially able to interfere with the results (e.g. diabetes mellitus, hyperadrenocorticism).
- Absence of information about the possible presence of other vector-borne diseases (with particular emphasis on canine ehrlichiosis)
- Presence of a written informed consent of the owners about the inclusion in the study. According to the regulation of our Institution, in the presence

of an informed consent of the owners it is not necessary to require a formal approval from the Institutional Ethical Committee if samples are performed for diagnostic or monitoring purposes, as for this study.

Additionally, 20 control dogs (i.e. clinically healthy dogs without laboratory abnormalities as revealed by a basic hematological and biochemical workup similar to that described below for leishmaniotic dogs) were included in this study. All these dogs were sampled during routine wellness visit under informed consent of the owners.

At the first visit each dogs with leishmaniasis received a complete physical examination. Particular emphasis was paid to the symptoms typically consistent with leishmaniasis (furfuraceous dermatitis, alopecia and other skin lesions, lymphoadenomegaly, weight loss, epistaxis etc) in order to classify the dogs according to the severity of the disease according to the guidelines for diagnosis and clinical classification of canine leishmaniasis[11,12] as described below. As recommended by these guidelines, the diagnosis of leishmaniasis was based on the detection of amastigotes in cytological samples of skin lesions, enlarged lymph nodes or bone marrow (n=37). In the case of negative cytology in a dog with clinical suspicion of leishmaniasis, the diagnosis was based on positive serology (antibody titer higher than 4 fold compared with the threshold of positivity of the laboratory) and on positive PCR on bone marrow or enlarged lymph nodes (n=2).

After the first visit, all the dogs enrolled in the study received the same treatment: N-methylglucamine antimoniate (Glucantime©) 100mg/Kg once a day for 30 days and allopurinol (Zyloric©) 10mg/Kg twice a day for 30 days as recommended in the guidelines for treatment of canine leishmaniasis[12,13].

Samplings were scheduled at the first visit, before the first administration of treatments (T0), and after 3 (T1), 7 (T2), 14 (T3), 21 (T4), 28 (T5), 35 (T6) and 42 (T7) days. Unfortunately, it was not possible to follow the complete sampling

scheme for all the dogs included in the study. Specifically a lower number of samples was collected when the dogs died during the 42 days of observation or when the owners declined to perform the sequential samplings during the follow up. Therefore 26 dogs were sampled from T0 to T3, 22 dogs were sampled until T5 and, 18 dogs were sampled until T7. When possible, the time of complete clinical remission (i.e. the recovery of each lesion observed at the presentation) of dogs that responded to treatments was recorded.

At each visit, blood was collected from the cephalic vein and immediately transferred in part in tubes containing EDTA and in part into tubes without anticoagulant (Venoject, Terumo Italia Srl, Rome, Italy) Just after sampling, routine hematology was performed with an automated impedance instrument (Mindray BC-2800 veterinary hematology analyzer) on blood collected in EDTA, to assess the health status of each patient, and serum was obtained by centrifugation (1,100g x 8 min) of blood collected in plain tubes. After collection and separation of serum, a basic panel of biochemical tests focused on assessing the health status of the patients was performed on an automated biochemistry analyzer (Mindray BC-120). This panel included 14 test (TBil, Creatinine, Glucose, Total Protein, BUN, ALP, ALT, AST, tCa, Cholesterol, Triglycerides, Amylase, GGT, Phosphorus). Then the remaining serum was transferred to Eppendorf tubes and frozen at -20°C for a maximum of 6 months.

To complete the evaluation of the clinical condition, urine samples were also collected at first visit and randomly during the follow up, to assess and quantify the possible presence of proteinuria, as described in a previous study.[69]

Based on the results of physical examination and of the basic laboratory workup dogs affected by leishmaniasis were classified in the two groups included in the guidelines for clinical classification of canine leishmaniasis.[11]

- Group A included sick dogs (stage C of the guidelines mentioned above): infected dogs showing one or more clinical signs common to leishmaniasis

(see above), including hematologic, biochemical, and urinary alterations consistent with leishmaniasis.

- Group B included severely sick dogs (stage D of the guidelines mentioned above): sick dogs with a severe clinical condition such as severe proteinuric nephropathy with chronic renal failure, ocular disease (keratitis/uveitis) or severe joint disease impairing mobility and suggestive of systemic immune-complex disease, presence of concurrent vector-borne infections that requires additional therapies.

Finally, the health status was assessed for at least 6 months after therapy through periodical veterinary visits. When this was not possible, information about the health status of each dog was retrieved through a telephone interview with the owners. This “long term follow up” allowed us to determine which dogs had relapses or complications potentially associated with the disease (e.g clinical signs consistent with canine leishmaniasis or clinicopathological changes, mainly affecting the electrophoretic pattern) during the follow up.

3,2,2 Biochemical tests and serum protein electrophoresis

Total serum proteins were measured using an automated analyzer (Cobas Mira, Roche diagnostic, Basel, Switzerland) and a commercial kit (Real Time Diagnostic System, Viterbo, Italy) based on the biuret method.

Serum protein electrophoresis was performed on agarose gel using the automated analyzer Hydrasis (Sebia Italia Srl, Bagno a Ripoli, Florence, Italy) and the specific manufacturer’s reagents (Hydragel 15 β1-β2, Sebia Italia Srl). Specifically, the procedure was performed using 0.8% agarose gel in Trisbarbital buffer, pH 8.6. Migration time was 7 minutes at 800V, and gels were then stained with amidoschwarz, destained, and dried for scanning by the appropriate gel scanner. Densitometric analysis of scanned gels was

performed using the software provided by the manufacturer (Phoresis, Sebia Italia Srl). Total serum protein and the percentage of electrophoretic fractions were used to calculate absolute protein concentrations (g/L) for each electrophoretic fraction. The serum concentration of the two most important electrophoretic fractions associated with inflammation/immunity (namely α_2- and γ-globulins), the serum concentration of serum albumin, that during inflammation work as a negative acute phase protein, and the A/G ratio were then included in this study.

The serum concentration of C-reactive protein (CRP) was determined using the Cobas Mira instrument mentioned above and an immunoturbidimetric kit (Real Time Diagnostic System, Viterbo, Italy) already validated in dogs.[58]

The activity of paraoxonase (PON1) in serum was measured spectrophotometrically with the automated Cobas Mira spectrophotometer using the paraoxon based method recently validated in dogs.[53] Briefly, 6 μL of samples are incubated at 37°C, with 89 μL of distilled water, and 100 μl of a glycine buffer (0.05 mM, pH 10.5) containing 1 mM of paraoxon-ethyl (purity > 90%, Sigma-Aldrich, Saint Louis, MO, USA) and 1 mM of $CaCl_2$. The rate of hydrolysis of paraoxon to p-nitrophenol was measured by monitoring the increase in absorbance at 504 nm using a molar extinction coefficient of 18,050 L• mol-1 • cm-1 The unit of PON activity expressed as U/mL was defined as 1 nmol of p-nitrophenol formed per minute under the assay conditions.

3.2.3 Statistical analysis

Statistical analysis was performed in an Excel (Microsoft Corp, Redmond, WA, USA) spreadsheet using the Analyse-it software (Analyse-it Software Ltd, Leeds, UK).

Results from controls, sick dogs and severely sick dogs were compared to each other using a non parametric ANOVA test (Kruskal Wallis test), followed by the Bonferroni test that was used as a post-hoc test to evaluate the possible presence of significant differences between controls and the two groups of dogs with leishmaniasis.

For each analyte included in this study, results obtained at admission in dogs that died during therapy were compared with those of dogs that survived the treatment protocol using a non-parametric t-test for unpaired sets of data (U Mann Withney). The same test was used to compare the results at admission of dogs that recovered in less than 2 weeks with those of dogs that recovered in more than 2 weeks as well as the results of dogs that had a satisfactory long-term follow up with those of dogs that had relapses or complications during the follow up.

A non-parametric ANOVA test for paired samples (Friedmann test) was used to compare to each other the results obtained during the sequential time samplings. Nevertheless not all the dogs received the full set of time samplings, as specified above. Therefore, ANOVA for paired samples was first performed on the 26 cases for which samples fromT0 to T3 were available, then for the 22 cases for which samples from T0 to T5 were available and finally, for the 18 cases for which samples from T0 to T7 were available. A non-parametric t-test for paired samples was used in all these comparison to assess the possible differences between T0 and each of the sequential samplings. Such a comparison between sequential sampling was then repeated only on samples that for each analyte had abnormal values at first admission, to assess the actual response to treatments in terms of normalization of abnormal values, since it is unlikely that samples that were normal at first sampling had significant changes during the follow up.

3.3 Results

3.3.1 Case selection and group composition

At first admission, 23 dogs with leishmaniasis were classified as “Sick” (stage C according to GLSC guidelines[11]) and 16 dogs as “Severely sick” (stage D) because they had severe emaciation (n=8, in 4 cases associated with poliarthritis, concurrent infections, uveitis or severe renal and hepatic insufficiency), polyarthritis (n=3) severe proteinuric renal disease (n=2), co-infection with other thick borne pathogens (n=2), uveitis (n=1).

Five dogs died during the observation period. All these dogs were classified as severely sick at admission since presented severe emaciation (n=2) severe proteinuric renal disease (n=2) or uveitis (n=1).

The remaining 34 dogs (23 classified as stage C and 11 as stage D) were still alive at the end of the sampling period. In 28 cases information about the time of remission were available: 9 dogs (6 in stage C and 3 in stage D) had a complete clinical remission in one week and 12 dogs (10 in stage C and 2 in stage D) in two weeks; four dogs had a complete clinical remission in 3 weeks (3 were in stage C and 1 in stage D); the remaining 3 dogs (1 in stage C and 2 in stage D) had a complete clinical remission in 4 weeks.

The long term follow up was available for 26 dogs, 20 of which did not have relapses or sequelae during the follow up (14 were classified as stage C and 6 as stage D at admission), while 6 dogs (5 classified as stage C and 1 as stage D) had a poor prognosis characterized by relapses or by sequelae such as persistent and severe proteinuric renal disease.

3.3.2 Laboratory results

Results recorded at admission are summarized in figure 3.1.

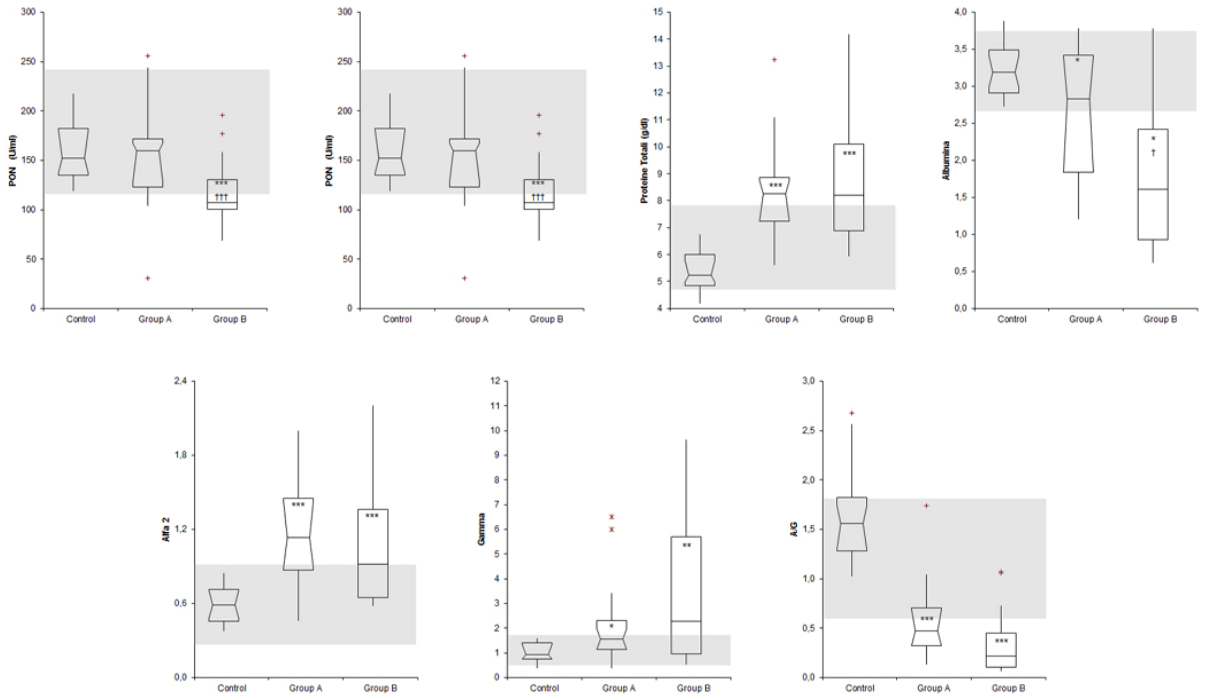

*Figure 3.1: Results obtained at admission in controls and in dogs affected by leishmaniasis classified as sick (group A) or severely sick (group B). The boxes indicates the I-II interquartile range (IQR), the horizontal line indicates the median values, whiskers extend to further observation within the I quartile minus 1.5*IQR or to further observation within the III quartile plus 1.5*IQR.Red symbols "+" and "asterisks" indicate respectively near outliers (values exceding the IIII quartile plu 1.5*IQR) and far outliers (values exceding the III quartile plus 3.0*IQR). The shaded area indicate the reference interval of the laboratory. Black asterisks indicate significant differences (* = p<0.05; ** = P<0.01; *** = P<0.001) compared with controls; The symbols † indicates significant difference († = P<0.05; ††† = P<0.001) compared with group A*

In summary, CRP and electrophoretic parameters were significantly altered in dogs with leishmaniasis compared with controls, irrespective on the severity of the disease. Conversely, PON-1 activity significantly decreased, compared with controls, only in dogs with signs of systemic inflammation associated with immune complex deposition.

No significant differences regarding PON1 activity, serum concentration of CRP and albumin and A/G ratio were found at admission between dogs that died during treatment and dogs that were alive at the end of treatment (figure

3.2), between dogs that recovered in less than 2 weeks and dogs that recovered in 3 or 4 weeks (figure 3.3) and between dogs that had a poor long term prognosis and dogs that had a good long term prognosis (figure 3.4)

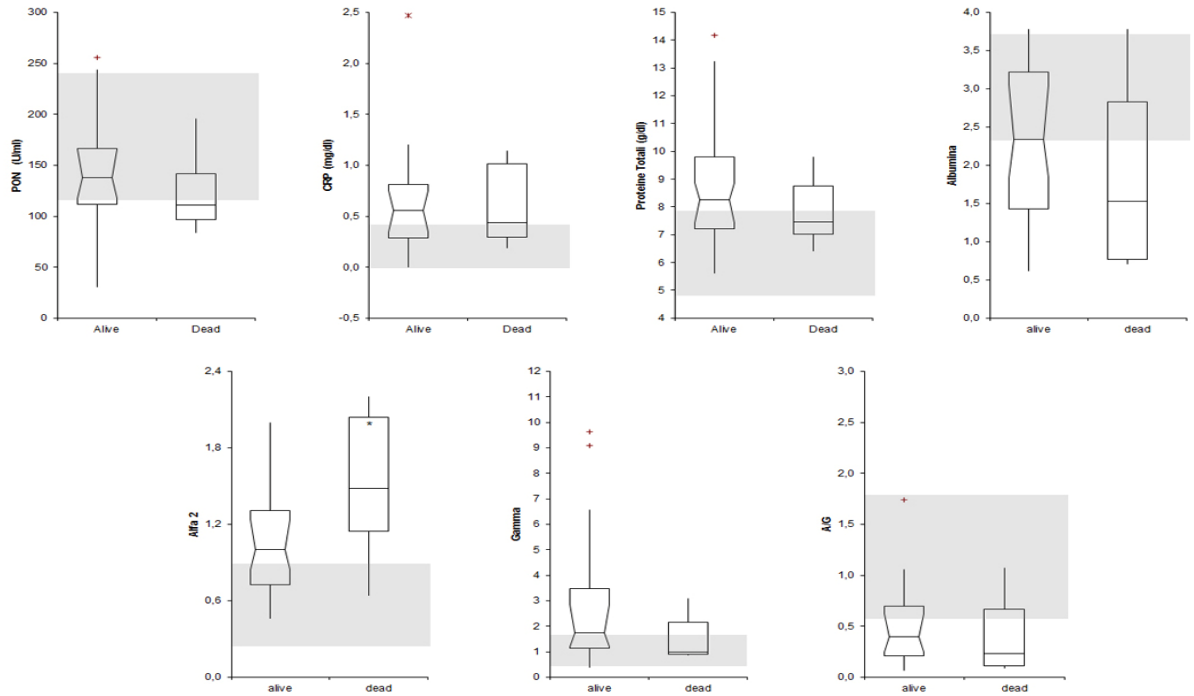

Figure 3.2:results obtained in dogs that survived or dead during the treatment. The interpretation of box and whiskers graphs is reported on figure 3.1 legend

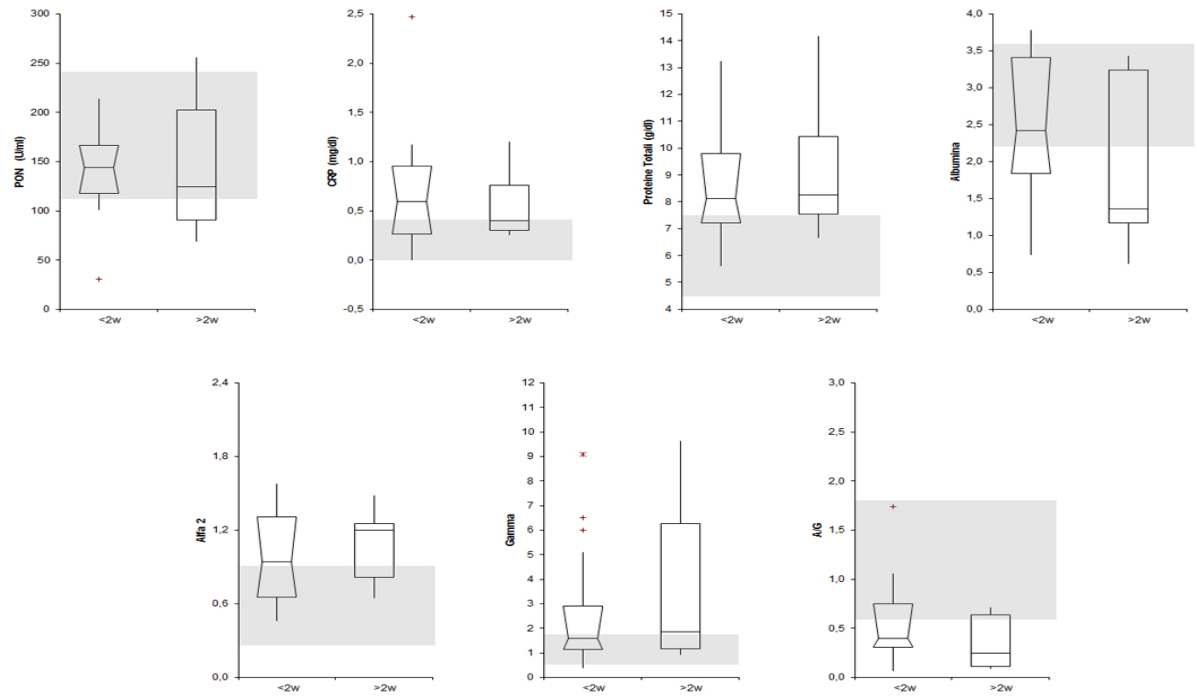

Figure 3.3: results obtained in dogs grouped according of the time of recovery (less than two weeks compared with more than two weeks). The interpretation of box and whiskers graphs is reported on figure 3.1 legend

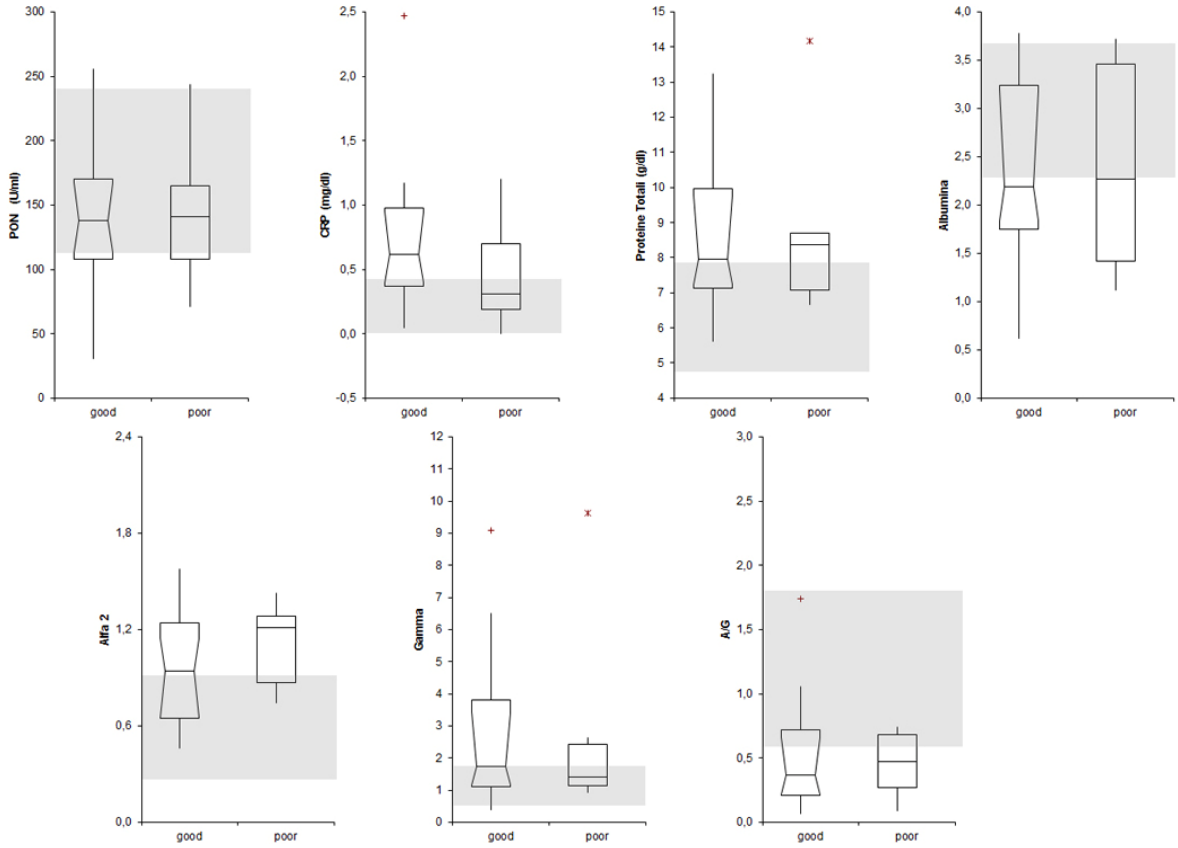

Figure 3.4: results obtained in dogs that had a poor long-term prognosis (relapses or sequelae) or a good long-term prognosis (no relapses of sequelae in the 6 months following the treatment. The interpretation of box and whisker graphs is reported on figure 3.1 legend

Conversely, the serum concentration of α2-and γ-globulin were respectively significantly higher and lower in dogs that died during treatment compared with dogs that were alive at the end of treatment.

Results recorded on the whole group of dogs during the follow up did not show any significant difference between sequential samplings (data not shown). Conversely, when only dogs with abnormal values at admission were considered, significant differences were found as follows:

- The comparison of results regarding samplings from T0 to T3 (figure 3.5) revealed a significant increaser of PON1 activity at T2 and T3 compared with T0. In these two time samplings in most of the dogs that had abnormal values

at T0 PON1 activity returned within the reference intervals. No significant differences were recorded for the other inflammatory markers that in most of the dogs remained outside the reference intervals until T3

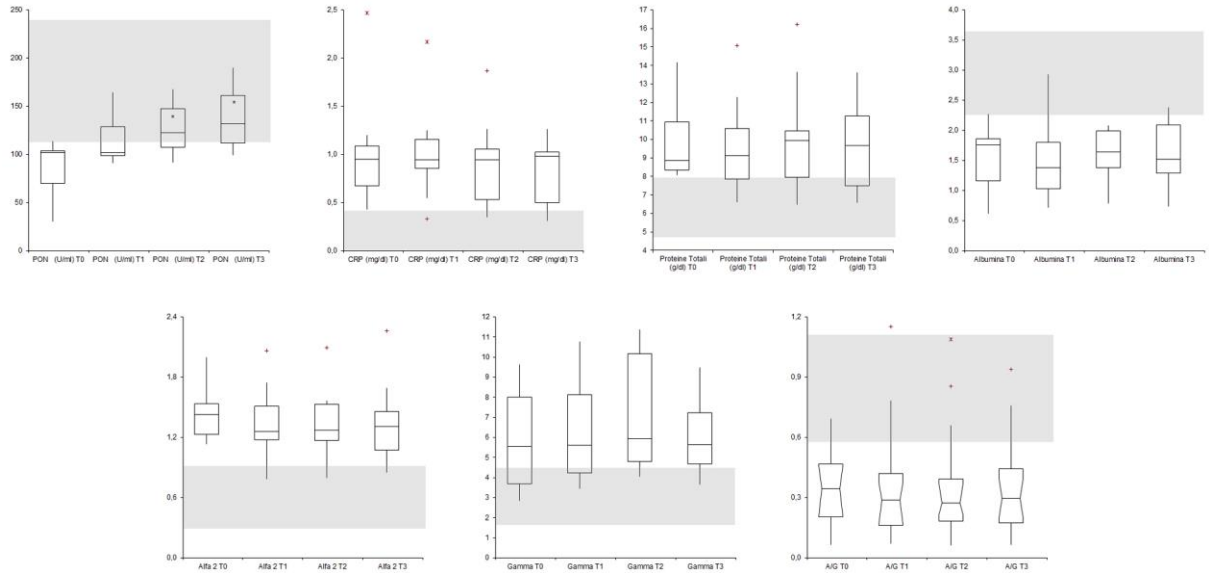

Figure 3.5: results recorded from T0 to T3 in dogs that had abnormal results at admission. the asterisks indicate a significant difference versus T0. (PON-1, n=9; CRP, n=15; total protein, n=15; albumin, n=12; α_2-globulins, n=13, γ-globulins, n=8; A/G ratio, n=20)

- The comparison of results regarding T0 to T5 (figure 3.6) confirmed a rapid normalization of PON1 values, with significant differences compared with T0 recorded at T3, T4 and T5. Significant differences compared with T0 were found at T4 and T5 for CRP and at T5 for total protein and g-globulins. However, for all these parameters even at the time samplings that were significantly different from T0 most of the dogs still had abnormal values compared with the reference intervals.

- The comparison of results regarding T0 to T7 (figure 3.7) did not show significant differences regarding PON1, basically due to the low number of samples (n=4) that did not allow to observe significant differences in spite of an evident normalization of values from T2 to T7. The serum concentration of CRP was significantly different from T0 at T6 and T7, when almost all the values returned with the reference interval. Significant differences compared

with T0 were found also for total proteins at T5, T6, T7 and for albumin, g-globulin and A/G ratio at T6 and T7. At these time samplings, however, most of the dogs still had abnormal values for all the inflammatory markers. As regards, g-globulin, also a transient and significant increase compared with T0 was found at T2. No significant differences regarding a2-gloublins were found and a2-globulin values remained outside the reference interval until T7.

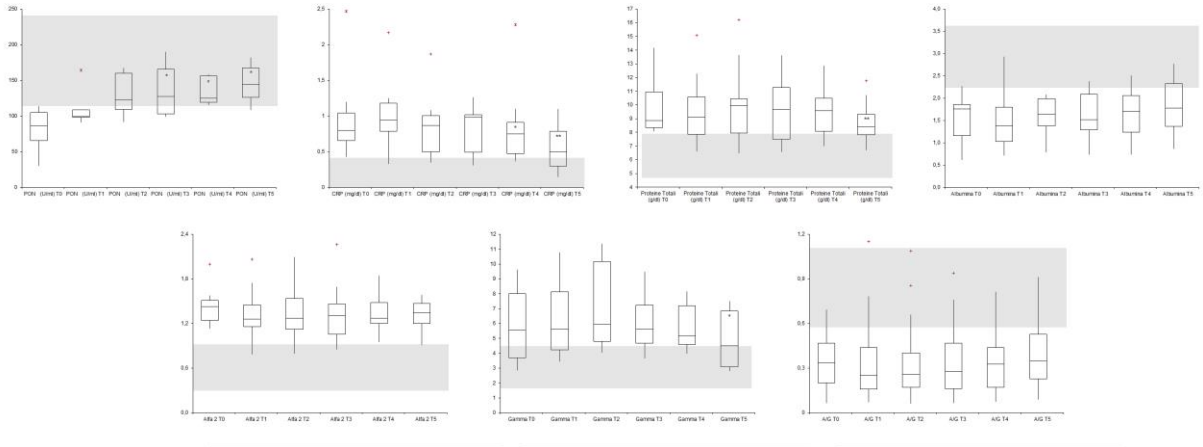

Figure 3.6: results recorded from T0 to T5 in dogs that had abnormal results at admission. the asterisks indicate a significant difference versus T0. (PON-1, n=6; CRP, n=13; total protein, n=15; albumin, n=12; α_2-globulins, n=11, γ-globulins, n=8; A/G ratio, n=19)

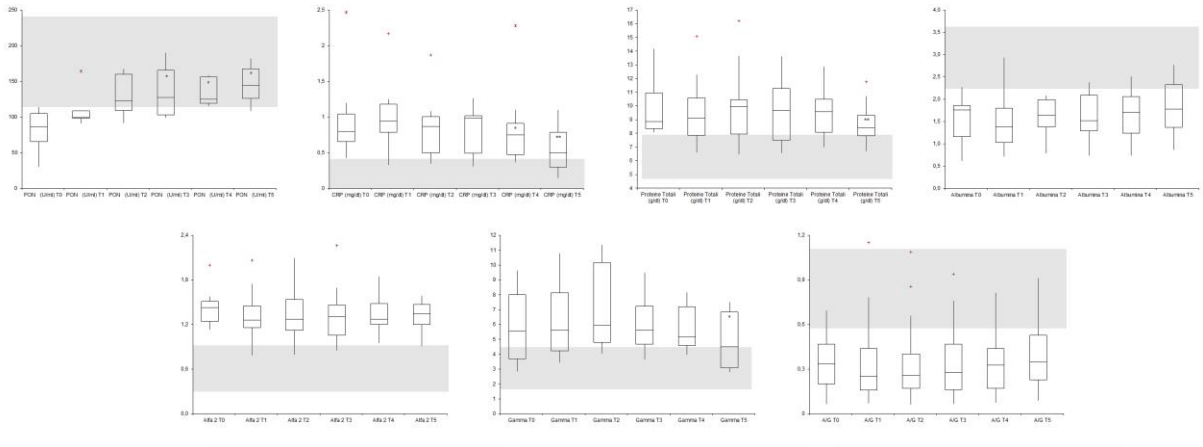

Figure 3.7: results recorded from T0 to T5 in dogs that had abnormal results at admission. the asterisks indicate a significant difference versus T0. (PON-1, n=6; CRP, n=13; total protein, n=15; albumin, n=12; α_2-globulins, n=11, γ-globulins, n=8; A/G ratio, n=19)

3.4 Discussion

Results recorded at admission revealed that clinical leishmaniasis is associated with an inflammatory reaction detectable using traditional markers of inflammation, as already reported in previous studies[11,12,14-15,17-20] but not using PON1 activity as a possible biomarker. Conversely, PON1 activity seems to be an indicator of systemic involvement, being decreased only in dogs with clinical signs associated with changes consistent with diffuse type III hypersensitivity reaction. Based on the current knowledge on the pathogenesis of canine leishmaniasis[11] and on PON1 metabolism, this finding is not surprising since it is very likely that the magnitude of oxidative damage, which may actually be responsible of a decreased PON1 activity[21] associated with leishmaniasis increases with the severity of the disease, whilst oxidative metabolism might even be reduced in early stages of infection or when the disease is limited to selected organs or systems[9] and when anti-oxidative mechanisms of the parasites[58] may be more intense than the local inflammatory reaction. However, PON1 activity seems to not be a good predictor of the clinical evolution of the disease since values at admission did not differ between dogs that had a favorable short-term or long- term follow-up and dogs that did not survived to the treatment or that had a slow recovery time or a poor long term prognosis. As short-term mortality (e.g. mortality during treatment), however, it should be noticed that median values recorded in the group of dead dogs were lower than those of dogs that survived the treatment, and the lack of significant differences between groups seems to depend mostly on the low number of dogs that died during treatment and to the wide individual variability in both groups.

Conversely, the intensity of the inflammatory reaction as determined by the serum concentration of CRP or globulin fraction, was sufficiently high to be detectable in serum also in the absence of severe diseases. In other words, the simple presence of reactivity against Leishmania increases the serum

concentration of CRP and of α2- or γ-globulins and decreases the serum concentration of albumin (and consequently also the A/G ratio decreased). This latter change, however, may depend either on a decreased hepatic production due to the role of albumin as a negative acute phase protein[70] or to the albumin loss subsequent to glomerular lesions typical of leishmaniasis.[71] Surprisingly, also values regarding CRP, albumin and A/G ratio recorded at admission appeared to not be associated with mortality during treatment, with the duration of clinical signs or with the long-term prognosis. For all these parameters, in fact, values were largely outside the reference intervals in all the groups of dogs. This seems to contrast with previous reports that reported higher level of acute phase proteins and lower albumin concentrations in dogs with a more severe clinical disease.[72] Among the traditional inflammatory markers, only a high serum concentration of α2-globulins seem to be a potential predictor of short-term mortality, likely reflecting a more intense inflammatory response in dogs that die during treatment. Previous studies showed that CRP should reflect the severity of the inflammatory response earlier and better than serum protein electrophoresis.[70] Therefore, the possible prognostic role of increased a2-globulins may depend on the simultaneous increase of other acute phase proteins (especially haptoglobin) that have been shown to increase in canine leishmaniasis.[17-20]

The lack of differences during the follow up of the whole group of dogs is not surprising since the whole groups of dogs included dogs with normal values of all the analytes. Therefore it is unlikely that fluctuations of these markers within normal limits are characterized by significant differences compared with T0, and, additionally, the presence of these “normal” dogs may actually mask the possible differences detectable in dogs with abnormal results at admission. This hypothesis was confirmed by the analysis of results from dogs that had abnormal values at admission.

This analysis revealed, although with some differences in the level of statistical significance, likely due to the different size of the groups sampled at sequential time sampling, a peculiar kinetic of inflammatory markers that may be summarized as follows: PON1 shows a rapid return to normal values with significant differences after about 1 week, CRP significantly decreases in 3 weeks-1 month but the complete normalization of values requires additional 1 or 2 weeks, total proteins normalize in 1-1.5 months, mostly due to the decrease of γ-globulins, which, however, have a transient increase during the treatment. No changes in the concentration of α2-globulin are found over time. Taken together these results suggests that during treatment, inflammatory stimuli still persists and maintain a high serum concentration of CRP and a-globulins, for at least a couple of weeks. Then, CRP decreases and normalizes whilst α2-globulins remain elevated, likely because this electrophoretic fraction includes other moderate APPs (Hp, and Cp) that need more time to return to normal values, with a more gradual decline than CRP.[70] Similarly, the normalization of γ-globulins requires a long time likely due to the half life of antibodies released in blood during infection and within the first week after the beginning of treatment. The rapid normalization of PON1 activity may depend on the pathophysiology of this enzyme: its decrease during inflammation is due either to a decreased hepatic production and to an increased consumption due to oxidative phenomena.[21,73] From this perspective, it is very likely that although inflammation may continue during treatment (as suggested by the kinetic of CRP described above), oxidative phenomena are rapidly reduced in the first days of treatment. Therefore, the rapid increase of PON1 activity may be due to a decreased peripheral consumption associated with a decreased oxidation rate rather than to an increased hepatic production of PON1. This hypothesis needs to be supported by additional studies on oxidants, lipoproteins and other molecules interacting with PON1 in dogs with leishmaniasis. Independently on its pathophysiological significance, the rapid normalization of PON1 activity in

treated dogs may have a practical utility, since it may work as an early biomarker of normalization in treated dogs.

Finally, in spite of a significant increase compared with T0, albumin and A/G ratio do not return within normal values in most dogs, likely due to the persistent proteinuria that occurs in leishmaniotic dogs.[11,71]

3.5 Conclusion

In conclusion, the results of the present study indicate that changes in PON1 activity may be an indicator of severity of canine leishmaniasis, possibly reflecting the unbalanced equilibrium between oxidative and antioxidant phenomena. The measurement of inflammatory markers included in this study, however, does not allow to predict the outcome of the disease either on a short-term or a long-term basis. Conversely, monitoring over time inflammatory markers allow to identify an early normalization of PON1 activity in the first week, likely due to a rapid decrease of oxidative phenomena associated with the symptomatic phase, followed by a normalization of CRP in about one month and then by an incomplete normalization of electrophoretic fractions, that, however, in most dogs were still altered at the end of the study period. On a practical point of view, monitoring of PON1 activity may be useful to early assess the response to treatments in the first weeks while CRP and electrophoretic fractions should be used later. Independently on this practical information about the prognostic role of serial measurement of inflammatory markers, the results of this study open interesting perspectives on the study of pathogenic mechanisms of canine leishmaniasis and of the changes that occur during treatment. Among these, the oxidative/antioxidant responses associated with infection and treatments, suspected on the basis of changes of PON1 activity, merit to be further investigated in the future.

4. STUDY 2: Role of high density lipoprotein (HDLs) and PON-1 as a marker of oxidative stress associated with canine leishmaniasis at clinical diagnosis and over treatment

4.1 Aim of the study

The information about paraoxonase activity in canine leishmaniotic dogs are, to date, limited and partial.[53] Study 1 investigated fluctuations of PON1 activity in dogs with leishmaniasis at first presentation and after treatment with antimonials and allopurinol. This revealed that PON1 activity decreases only in severely sick dogs, possibly related to the presence of severe oxidative stress, and that PON1 activity restores more rapidly than other markers of inflammation in dogs that successfully respond to treatments, likely because the level of oxidants rapidly decreases aftyer treatment. However, we lack information about the role of HDL cholesterol and lipoproteins and, most of all, how the lipoproteins, the oxidative conditions, and the paraoxonase activity are related among them. Therefore, better knowledge of this mechanism during the course of the disease would yield new information on the pathogenesis of leishmaniasis and even new tools for staging and monitoring the disease.

The aim of study 2 is to investigate the relationship between PON1 activity, oxidative markers (dROMs) and lipids associated with inflammation (total and HDL cholesterol, the latter measured by colorimetry and by electrophoresis of lipoprotein) in dogs with leishmaniasis at diagnosis and during the follow up.

4.2 Material and methods

4.2.1 Caseload and study design

This study has been performed on dogs selected as in Study 1: Specifically, dogs at first presentation were selected and grouped according to the severity

of the disease (sick or severely sick) following the staging system proposed by the Canine Leishmaniasis Working Group (CLWG).[11]

Then, dogs received a standard treatment with anitmonials and allopurinol, following the guidelines for treatment of leishmaniasis released by the CLWG[14] and were sampled after 3, 7, 14, 21, 28, 35 and 42 days. At each time sampling, a basic panel of biochemical test, routine hematology and serum protein electrophoresis were performed. PON1 activity was measured in all samples as described in Study 1. Then, dROMs, total and HDL cholesterol and lipoprotein electrophoresis were performed in selected samples, depending on the volume of serum available. Therefore, not all the samples received the whole panel of tests. Details of the samples available and of the tests performed in this study are reported in table 4.1 and the number of dogs receiving each single test is reported below when the single methods are described.

Dog N°	d-ROMs	HDL	Lipoproteins	Dog N°	d-ROMs	HDL	Lipoproteins
1	X	x	x	15	x		x
2	X	x	x	16	x		x
3	X	x	x	17			x
4	x	x	x	19	x		x
5	x	x	x	20	x		
6	x	x	x	21	x		
7	x	x	x	22	x		
8	x	x	x	26	x		
9	x	x	x	27	x		
10	x	x	x	28	x		
12	x		x	31	x		
14	x		x				

Table 4.1: list of samples and of tests performed on each sample

4.2.2 Assessment of oxidative status (dROMs)

The serum concentration of dROMs was measured in 22 dogs, at first presentation and throughout the course of therapy, with a global amount of 136 samples analyzed. The test in based on a spettrophotometric techique validated for the use in dogs.[58]

The results are expressed in “CARRATELLI” units (“CARR U”, from the name of the inventor of this test) and each unit is equivalent to 0,08 mg/100ml H_2O_2. The d-ROMs kit products by Diacron S.r.l. and Cobas Mira spectrophotometer were used for this study.

4.2.3 Measurement of HDL cholesterol

Total cholesterol and HDL cholesterol were measured at University Veterinary Hospital of Lodi with the ILab 300 Plus analyzer (Instrumentations Laboratory, Milano, Italia), on 22 samples collected on first presentation and then on 48 samples collected during the follow up. However, technical (storage) artifacts did not allow to use the results of 12 dogs at first presentation. Therefore, only 10 cases were included in the study. An immunoenzymatic technique was used to measure HDL cholesterol and the total cholesterol/HDL cholesterol ratios (HDL percentage) was calculated.

4.2.4 Serum lipoprotein electrophoresis

Electrophoresis of lipoproteins was performed on 113 samples (16 collected at first presentation, 97 during the therapy) with the semiautomatic instrument Hydrasis (Sebia Italia Srl, Bagno a Ripoli, Firenze, Italia) and its specific kits (Hydragel 15 Lipoprotein, Sebia Italia Srl). When migrating on agarose gel, the lipoproteins split into these 3 fraction, listed in ascending order of mobility:

chylomicrons, β-lipoproteins or LDL, pro-β-lipoproteins or VLDL and α-lipoproteins or HDL (that migrate as β_2-, β_1- and α_2-globulins, respectively).

4.3 Results

4.3.1 Total cholesterol and colorimetric HDL

4.3.1.1 Values at first presentation

Among the 10 dogs included in this part of the study, 7 dogs belong to group A (sick) and 3 to group B (severely sick) according to the CLWG staging system.[11] Two dogs belonging to group B died during the follow up, 6 dogs belonging to group A recovered in less than 2 weeks (but one of these developed a persistent proteinuric nephropathy), the last dog of group A had a much longer time of recovery and a worse prognosis.

At first presentation, no significant differences between dogs belonging to group A and group B were found for total cholesterol (P=0.909) HDL cholesterol concentration (P=0.833) or HDL percentage (P=1.000) (*Figure 4.1*).

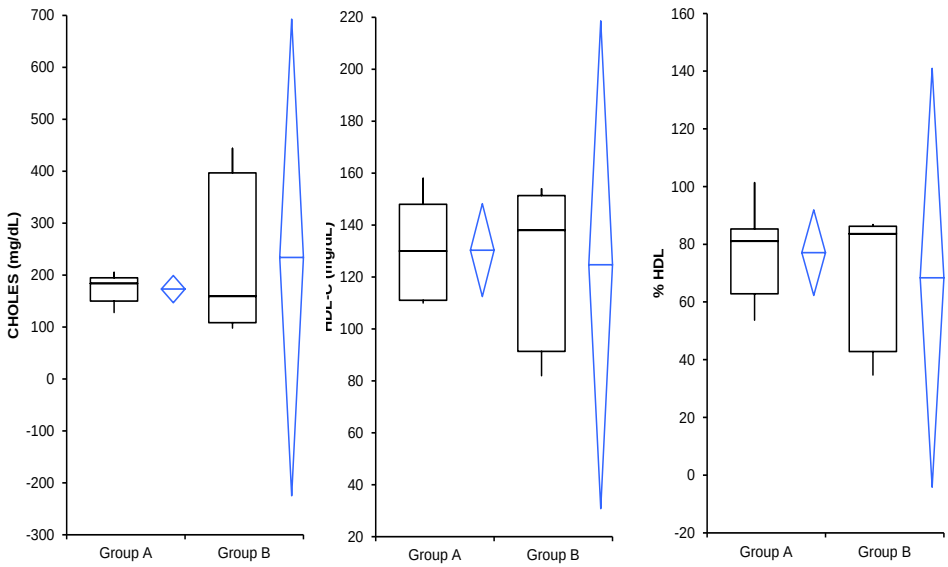

Figure 4.1: Box plots related to total cholesterol, HDL concentration, HDL cholesterol percentage recorded in dogs from group A (sick) and B (severely sick). For interpretation of box and whiskers refers to figure 3.1

Furthermore, no significant differences were found between dogs alive/dead after treatment, neither for total cholesterol levels (survivors: mean ± DS 163.5 ± 37.3 mg/dL, median 171,0 mg/dL; dead dogs: 301,5 ± 201,5 mg/dL; 301,5 mg/dL; P=0.400), nor for HDL cholesterol concentration (survivors: 124.2 ± 24.7 mg/dL; 123,0 mg/dL; dead dogs: 146.0 ± 11.3 mg/dL, 146.0 mg/dL; P=0.400), nor for HDL cholesterol/Total cholesterol ratio (survivors: mean ± DS 77.9 ± 15.1%, 81.6%; dead dogs: 60.7 ± 36.8%, 60.7%. P=0.833). Nevertheless, nearly all dogs had total cholesterol levels within reference interval (135-270 mg/dL)[74] whilst among dead dogs many animals showed both lower value, perhaps because a bad general condition, and higher value, perhaps due to nephrotic syndrome. No reference interval for HDL concentration are available, to date, in dogs.

Finally, even the comparison between animals with good prognosis and animals with poor prognosis showed no significant difference neither for total cholesterol levels (good: 166.2 ± 31.7 mg/dL, 158,0 mg/dL; poor: 146.5 ± 68.6 mg/dL, 146.5 mg/dL; P=0.857), nor for HDL cholesterol (good: 123.2 ± 17.1 mg/dL, 116,0 mg/dL; poor: 120.0 ± 53.7 mg/dL, 120.0 mg/dL; P=1.000), nor for HDL cholesterol percentage (good: 76.7 ± 19.5%, 82.3%; poor: 82.3 ± 1.9%, 82.3%, P=1.000).

These data suggest that lipid metabolites are not prognostic marker for leishmaniasis as well as other inflammatory markers are. Results are similar to that of PON1, that reaches lower values only in severely sick dogs.

4.3.1.2 Correlation between lipid parameters and inflammatory and oxidative markers

No correlations between HDL concentration, total cholesterol and HDL percentage and traditional markers of inflammation and immunity (α_2-globulins,

γ-globulins, albumin – negative acute phase protein – A/G ratio, C reactive protein) or PON1 activity were found in samples collected at first presentation (*Table 4.2*).

Total cholesterol			HDL			%HDL		
	P	R		P	R		P	R
vs. dROMs	0.798	-0.10	vs. dROMs	0.527	0.24	vs. dROMs	0.575	0.22
vs. alb	0.365	0.32	vs. alb	0.529	0.20	vs. alb	0.881	-0.05
vs α_2-glob	0.488	0.25	vs α_2-glob	0.724	0.13	vs α_2-glob	0.777	-0.10
vs γ-glob	0.907	-0.04	vs γ-glob	0.083	-0.57	vs γ-glob	0.328	-0.35
vs A/G	0.932	0.03	vs A/G	0.428	0.30	vs A/G	0.308	0.38
vs. CRP	0.698	-0.15	vs. CRP	0.290	-0.40	vs. CRP	0.341	-0.36
vs. PON1	0.467	-0.26	vs. PON1	0.519	-0.23	vs. PON1	0.173	0.47

Table 4.2: results of Spearmann correlation between traditional inflammatory paramters, dROMs, PON1 and CRP on one hand, total cholesterol, HDL concentration and percentage on the other

The absence of correlation may be due to the little number of samples examined. When all data collected in serial sampling are considered (i.e including data collected during the follow up), some significant correlation can be found. In particular: total cholesterol concentration does not correlate with other routine inflammatory marker, but it shows a significant negative correlation (P=0,027; r=-0,42) with γ-globulins (*Figure 4.2*).

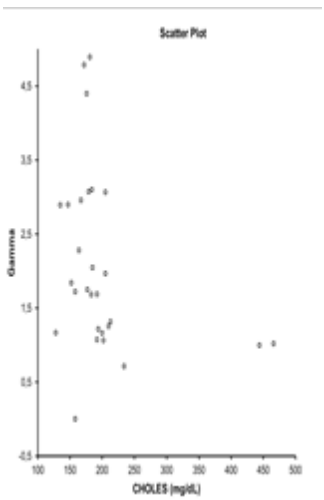

Figure 4.2: Spearman correlation between concentration of total cholesterol and γ-globulins.

The only explanation for the absence of biological relationship between these parameters is that total cholesterol and *γ*-globulins levels reflect the presence of the disease: the more the animals are sick, the more the γ-globulins rise and cholesterol decreases because the poor body condition. In human visceral leishmaniasis this finding is well documented in pediatric patients.[75-79]

The presence of immune-mediated dyslipidemia maybe an additional possible explanation.[80] High concentration of immunoglobulins can interfere with lipoprotein metabolism via different ways:

- Autoantibodies can bind to HDL forming immune complexes that accelerate HDL catabolism.[77]
- Immunoglobulins decreases the receptor mediates clearance of chylomicrons of IDL (Intermediate Density Lipoprotein) and LDL
- Alterations in lipoprotein-lipase activity

Another explanation is a low activity of lecithin-cholesterol acyltransferase enzyme (LCAT) that decreases the serum HDL concentration. This mechanism is well documented in human visceral leishmaniasis.[76,79] but not in canine leishmaniasis. Further studies are thus needed to investigate the relationship between immune system and cholesterol levels in canine leishmaniasis.

Finally, high cytokines levels (especially IL-6) can increase the LDL receptor activity[81] and the occurrence of hypertriglyceridemia may decrease both lipoprotein-lipasi production and liver lipasic activity, leading to a delayed clearance of VLDL. Tumor necrosis factor (TNFα) may play an important role in this mechanism because its level is usually elevated in chronic infection.[82]

On the whole set of samples (i.e. including both samples at first presentation and those collected over the follow up), the concentration of HDL shows a significant correlation with γ-globulins (P=0.010, r=-0.47), and with PON1 activity (P=0,002; r=0,55) and a mild correlation with A/G ratio (P=0,019,

r=0,44). The p-value is close to the significant level when CRP levels are considered (P=0,058), with a negative correlation (r = -0,37) (*Figure 4.3*).

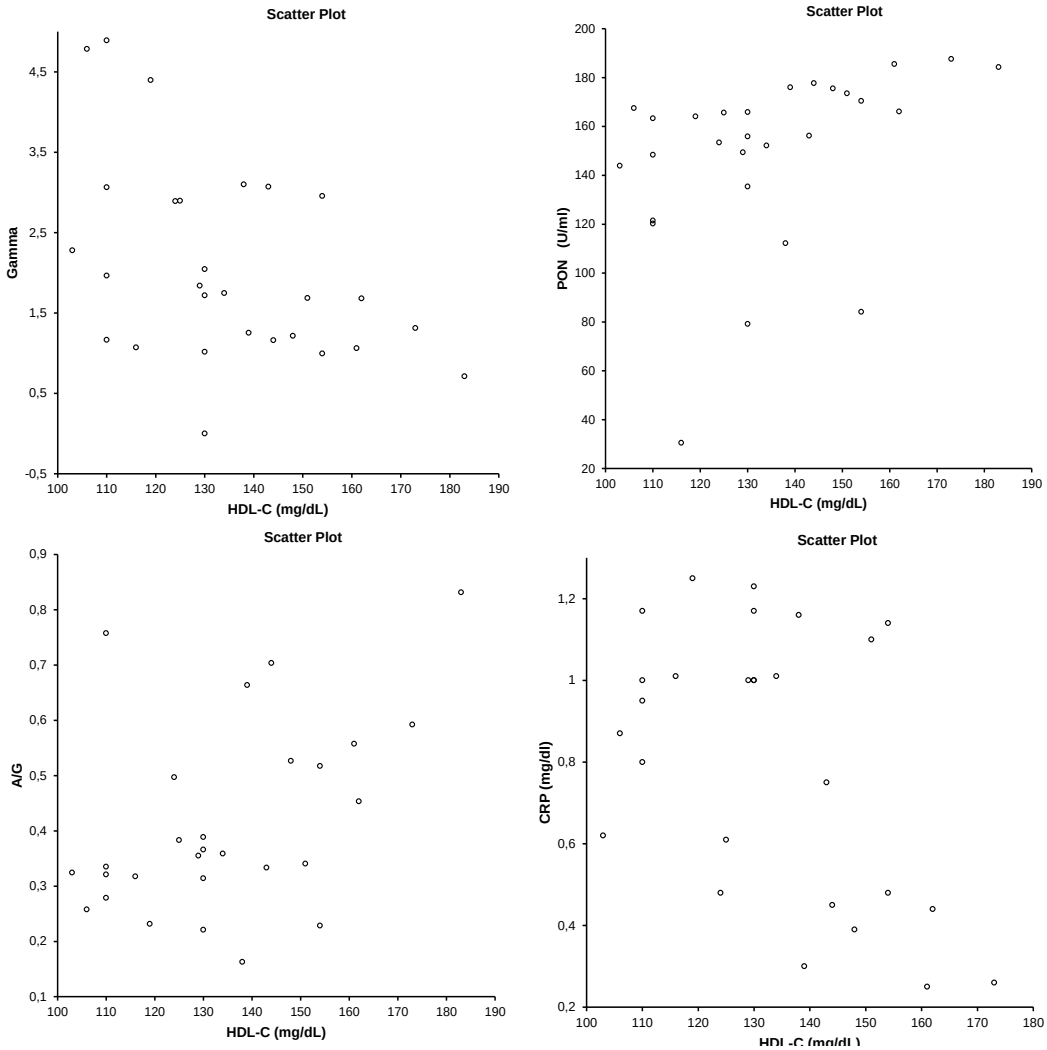

Figure 4.3: Spearmann correlation between HDL and γ-globulins, HDL and PON1, HDL and A/G ratio and HDL and CRP

These data support the previous hypothesis regarding the possible relationship between total cholesterol and health status (γ-globulins, A/G ratio). Moreover, these data point out that HDL reduction may reflect an inflammatory response (week significance of negative correlation with CRP) and enhanced oxidative activity (as suggested by decreased levels of PON1). Surprisingly, there is no correlation between the concentration of HDL and dROMs levels, suggesting that oxidants other than dROMs may be involved in oxidative stress during

leishmaniasis. However, this aspect will be discussed during the presentation of data regarding concentrations of dROMs in leishmaniotic dogs (see below).

Instead, when the HDL percentage is considered on the whole set of samples (i.e. including samples collected at first presentation and samples collected during the follow up), the negative correlation with CRP and α_2-globulins become significant (respectively P=0.014, r-0.47; P=0.008, r=-0.49). Furthermore, the percentage of HDL is positively correlated with PON1 (P=0.019; r=0.44), A/G ratio (P=0.000; r=0.61), and dROMs (P=0.034; r=0,42) (*Figure 4.4*). These results of this correlation analysis confirm the relationship between the reduction of HDL percentage and the presence of an inflammatory response (based on the negative correlation with CRP and α_2-globulins), as well as the potential role of oxidative metabolites (based on the positive correlation with PON1), although also in this case the lack of correlation with dROMs suggests that other oxidants may be involved

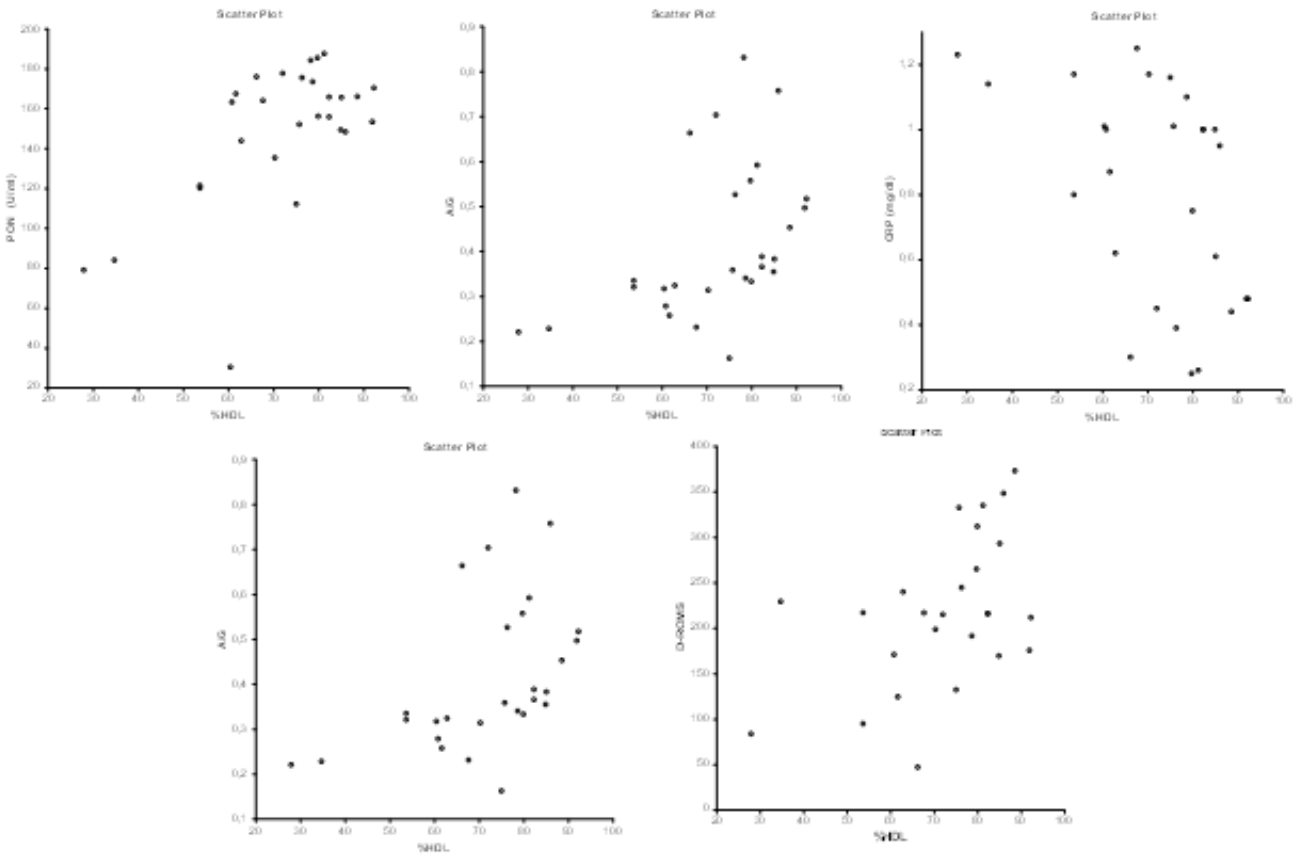

Figure 4.4: Spearman correlation between HDL cholesterol and PON1, A/G ratio, CRP, albumin and dROMs

4.3.1.3 Correlation between lipid parameters and inflammatory and oxidative markers

Analysis of total cholesterol in serial samples yielded no significant results regardless of the duration of the follow up, maybe due to the low number of dogs sampled (*Figure 4.5*).

Instead, HDL concentrations (*Figure 4.6*) showed a significant difference up to day 3(P=0,578), 14 (P=0,033), and 28 (P=0,024). Results at day 42 (P=0,366) were not significant because data were few and highly dispersed. This suggests that the colorimetric measurement of HDL may be useful to monitor the recovery of inflammatory and/or oxidative reaction when combined with clinical and clinico-pathological evaluation of leishmaniotic dogs.

Finally, the HDL percentage had an ascending trend during therapy alongside the clinical improvement (*Figure 4.7*) although there was no statistical significance at days 3 (P=0,937), 14 (P=0.875), 28 (P=0.305), and 42 (P = 0.265).

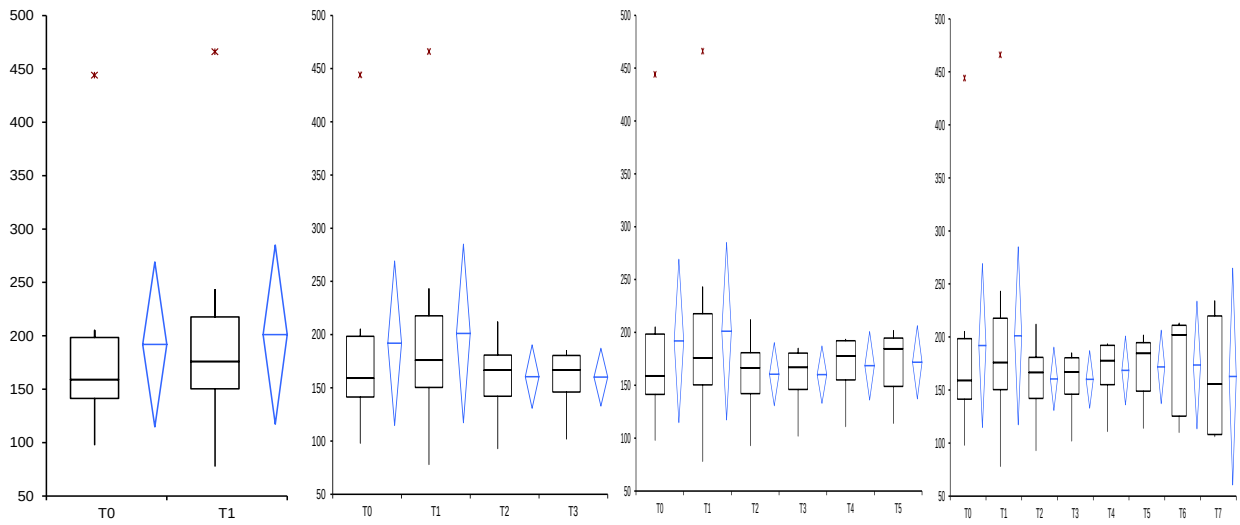

Figure 4.5: Comparison of values of total cholesterol obtained from day 0 to days 3 T_1 (n=9), 14 (n=7); , 28 (n=6), 42 (n=4). For interpretation of box and whiskers see figure 3.1

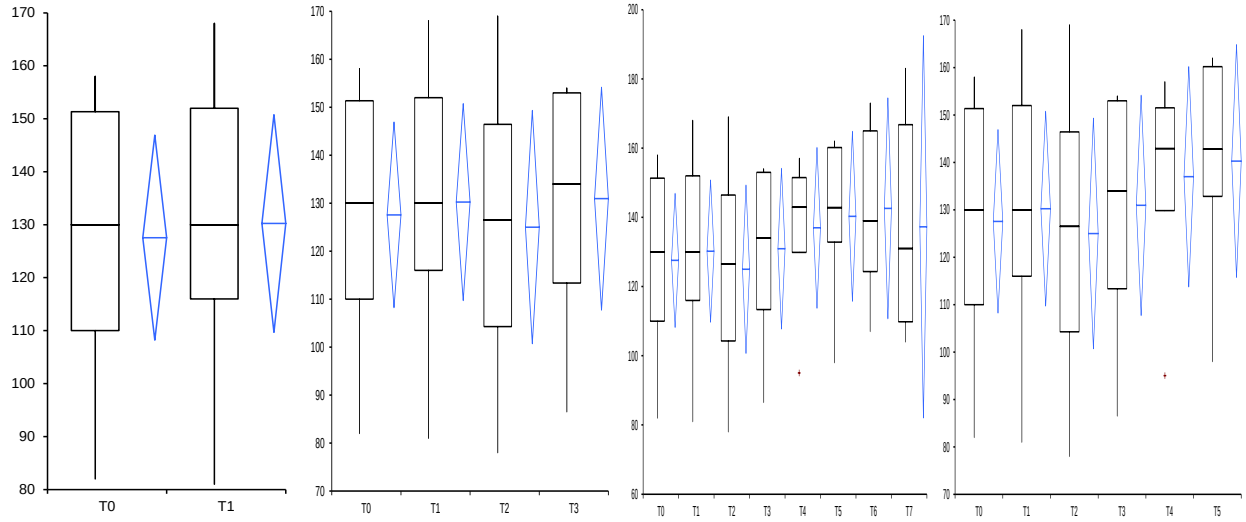

Figure 4.6: Comparison of values of HDL cholesterol obtained from day 0 to day 3 (n=9), 14 (n=7); 28 (n=6), 42 (n=4). For interpretation of box and whiskers see figure 3.1

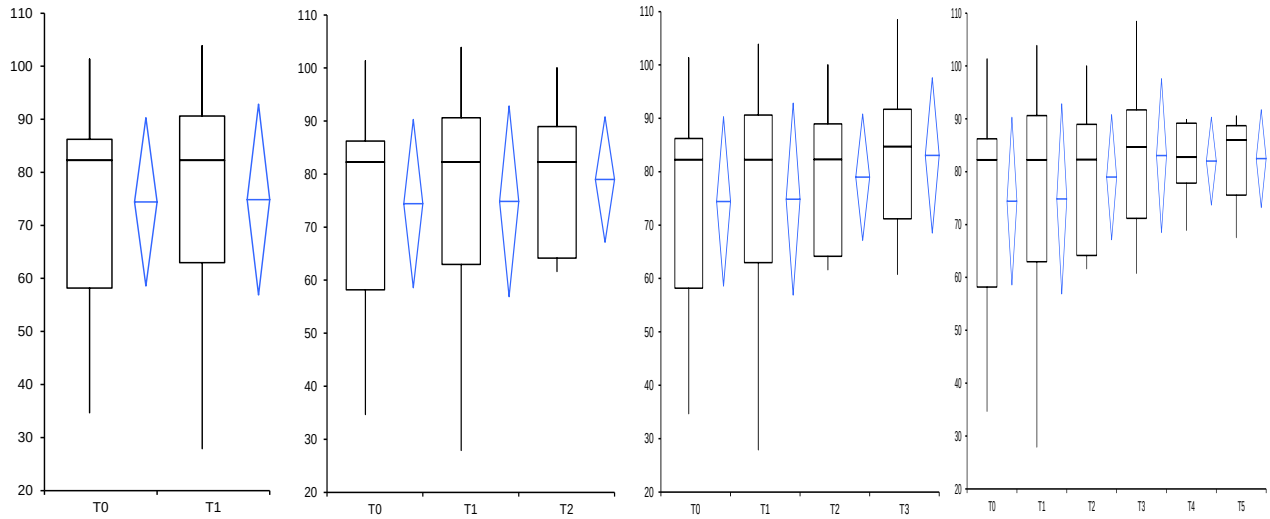

Figure 4.7: Comparison of values of HDL cholesterol obtained from day 0 to day 3 (n=9), 14 (n=7); 28 (n=6), 42 (n=4). For interpretation of box and whiskers see figure 3.1

4.3.1.4 Summary of results regarding total and HDL cholesterol

HDL cholesterol measurement works as inflammatory marker as well as PON1 in other species; on the other hand, HDL and PON1 activity can be used to detect and to monitor the oxidative phenomena and the inflammatory response

in course of leishmaniotic disease in dogs, although they are not useful to explain the pathogenesis of this processes.

The measurement of HDL lipoprotein does not work as marker of severity for canine leishmaniosis. Dogs affected with different degrees of severity show similar results, and no correlation with prognosis or with recovery time was found. HDL cholesterol shows variation during therapy very similar to that of PON1 (see Study 1) with normalization at T_3. Many differences are visually recognized in the diagrams but statistical analysis did not yield significant results, likely because too few cases were available. Instead, the difference between HDL and other parameters were statistically significant. The analysis of individual data permits to argue that HDL cholesterol percentage is particularly useful in assessing the relationship between HDL, oxidation and inflammation, because other pathologic changes such hepatic failure do not affect this parameter but absolute HDL concentration, that is kept on low values despite recovery of inflammatory/oxidative phenomena.[83] This hypothesis has to be confirmed with much larger samplings.

4.3.2 Serum lipoprotein electrophoresis

Serum lipoprotein electrophoresis provided several analytical and interpretative problems. In all samples a clear band at the deposition points, likely due to chilomicrons, was detectable and hampered the correct separation of the other lipoprotein fractions (figure 4.8)

All the electrophoretograms were thus corrected manually but this introduced a possible operator-dependent variable that may interfere with the evaluation of results. Samples collected at admission had very variable results, with an evident prevalence of HDLs in some patients and, conversely, with a prevalence of other electrophoretic fractions in other patients (figure 4.9).

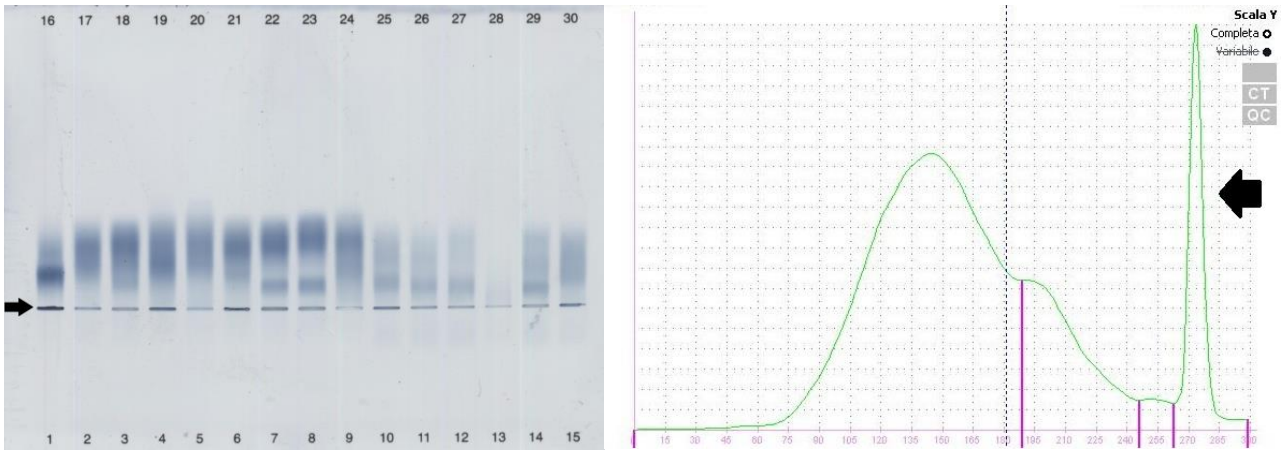

Figure 4.8: example of a gel, and a densitometric scan of lipoprotein electrophoresis, with the band or peak corresponding to chilomicrons evidenced by an arrow

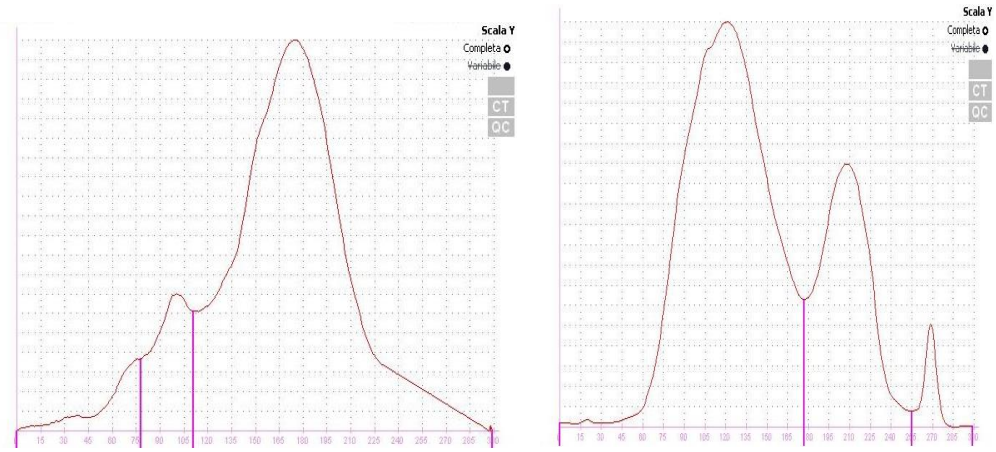

Figure 4.9: examples of electrophoretic profiles with very different peak distribution at first presentation

Although this may reflect a different inflammatory/oxidative status, the visual analysis of electrophoretograms in patients with severe or mild leishmaniasis revealed that some of the dogs with severe inflammation did not have a low HDL peak as expected and viceversa in dogs without severe inflammation. Similarly, only in rare cases HDL increased during successful treatments, as demonstrated by the colorimetric measurement of HDL described above. This discrepancy between electrophoretic and colorimetric evaluation of HDLs suggests that electrophoretic lipoprotein analysis may have been biased by the

technical limits described above. Therefore, results of serum lipoprotein electrophoresis have not been further interpreted in this study.

4.3.3 Measurement of oxidative radicals (dROMs)

4.3.3.1 Values at first presentation

The concentration of dROMs was measured at first presentation in 23 dogs, and eventually during the follow up. This animals were included on clinical basis with the purpose of obtaining homogeneous group, but the duration of follow up was also considered to compare the higher number of samples.

Of these dogs, 13 were from group A (sick) and 10 from group B (severely sick). 3 dogs of B group died during the follow up. Of the 20 survived dogs, 14 achieved clinical remission within 2 weeks, and 5 dogs recovered later. Long time prognosis of 17 dogs was known; 14 dogs had no relapse or complications after 12months but 3 dogs did.

Statistical analysis of first presentation did not show significant differences between group A and group B (P=0.496, *Figure 4.10*).

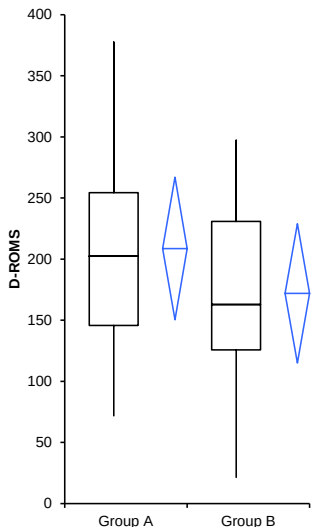

Fig 4.10: dROMs, values at first presentation. Comparison between group A and group B. For interpretation of box and whiskers see figure 3.1

The dROMs concentrations measured in samples of group A (mean ± DS = 208.6 ± 91.8 U CARR; median 202.25 U CARR; reference interval 71.7-377.6), and of group B (171.9 ± 79.6; 162.8; 21.4-297.2), in most dog was higher than reference interval (20.1-126.7 U CARR), determined in a previous study.[59] Thus, this finding suggest that at first presentation oxidation occurs regardless of disease severity.[58,59] Because in this study a control group was not included, we are unable to determine the importance of dROMs measurement at first presentation. Nevertheless, the very high levels of dROMs detected in some animals permit to argue that very strong oxidative phenomena occurs.

Anyway, no significant differences were found between dead and surviving dogs (P=0.811), between dogs recovered within two weeks and dogs recovering in more than 2 weeks (P=0.702), and finally between dogs with good and poor prognosis (P=0.239) (*Figure 4.11*).

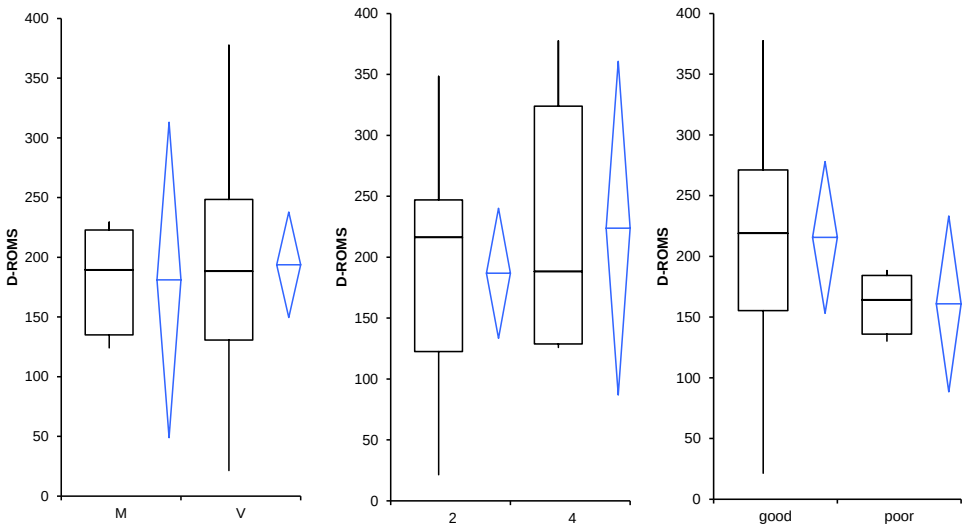

Fig 4.11: Comparison of dROMs values in, respectively, dead/survived dogs, early/late recovery, good/poor prognosis.For interpretation of box and whiskers see figure 3.1.

Therefore, markers of oxidation at first presentation doesn't work neither as prognostic factor nor as indicator of severity in canine leishmaniasis.

4.3.3.2 Correlation between dROMs and inflammatory markers

The dROMs concentration did not correlate with any of inflammatory parameter taken into account in this study(*Table 4.3*)

Nevertheless, when the whole dataset is analyzed, including samples collected during the follow up, some significant statystical correlation became detectable, between dROMs values and PON1 activity (P=0.003; r=0,26), albumin level (P=0,000; r=0.33), A/G ratio (P=0,000; r=0,30) (*Figure 4.12*), whereas correlation between dROMs and CRP (P=0,990; r=0,00) α_2-globulins (P=0.400; r=0,07), γ-globulins (P=0,096; r=-0,15) remained not significant.

	P	R
dROMs vs PON	0.279	0.24
dROMs vs CRP	0.599	-0.12
dROMs vs albumin	0.124	0.35
dROMs vs α_2-globulins	0.818	0.05
dROMs vs γ-globulins	0.245	-.026
dROMs vs A/G	0.088	0.38

Table 4.3: results of Spearmann correlation between dROMs and inflammatory parameters

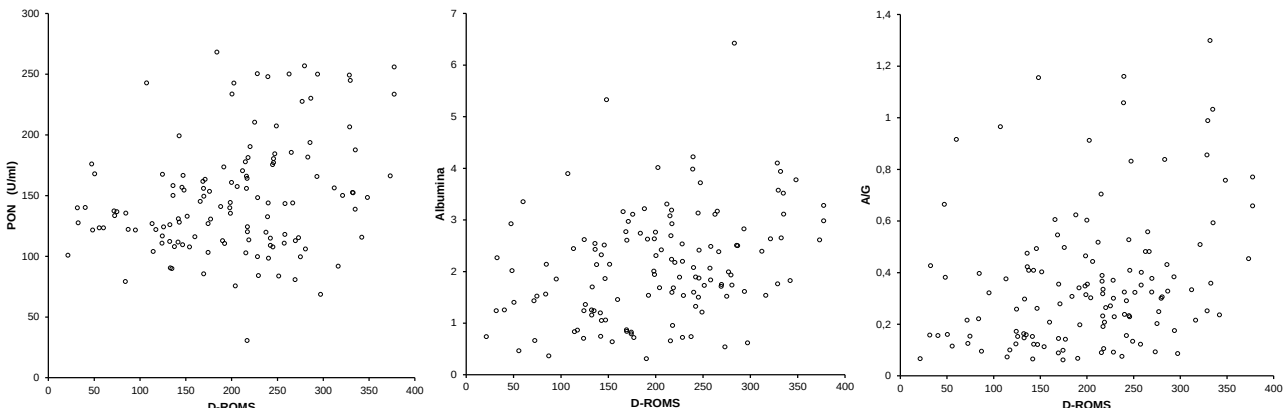

Fig. 4.12: Spearmann correlation between HDL cholesterol percentage and, respectevely, PON1 activity, A/G ratio, and albumine concentrazion.

Surprisingly, there are no correlations between dROMs and other parameters obviously associated with inflammation characterized by oxidative phenomena (e.g. CRP and α_2-globulins). A negative correlation between albumin and dROMs would be expected as well, because albumin works as a negative acute phase protein. The positive correlation detected may be caused by hypoalbuminemia from other origin than inflammation, such glomerulonephiritis, but a different explanation is that oxidative molecules other than dROMs are present. The direct correlation between dROMs and PON1 activity hold up this latter hypothesis.

4.3.3.3 Fluctuations of results in serial samples

The analysis of serial samples regarding the dROMs concentration yielded no significant results from 20 dogs on which samples collected at day 0 and 3 were available (P=0,079), although a slight increase of median value occurred at day 3. The same increase occurred at day 14 for 19 dogs (P=0.830), whereas at day 28 (P=0.889) and 42 (P=0.671) a visual decrease of dROMs levels occurred after the previously mentioned initial increase (*Figure 4.13*).

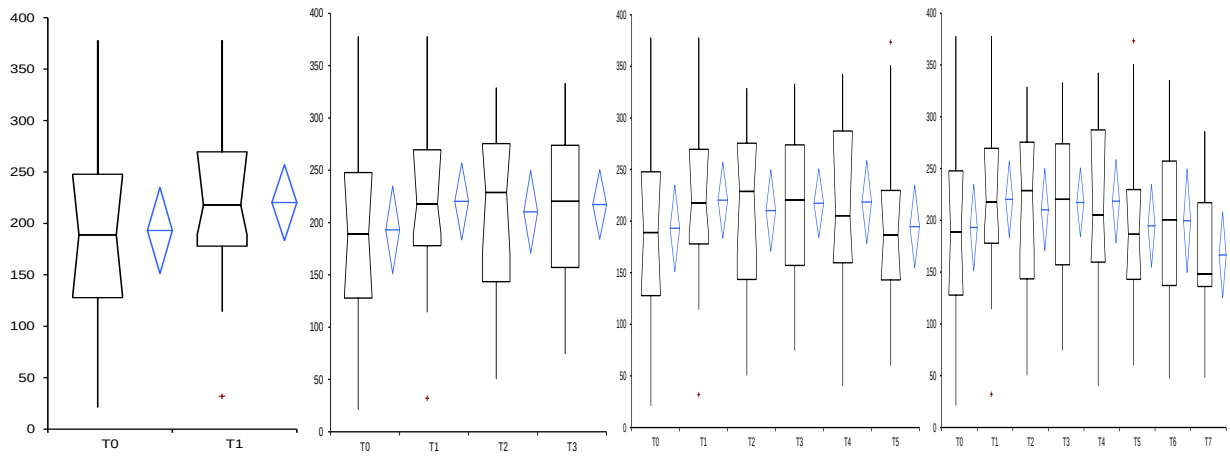

Fig 4.13: Comparison of dROMs levels in serial samples. For interpretation of box and whiskers see figure 3.1

The persisting high level of dROMs despite the course of therapy can be explained with a continuous production of oxidative radicals. Probably only in dogs with abnormal values at first presentation a progressive ameliorating can occur, whereas the animals with dROMs levels close to normal reference interval show only irrelevant biological fluctuations. The HDL trend previously described suggests that oxidative phenomena disappear within 7-10 days, as well as the PON1 trend (see Study 1). Instead, CRP values suggest the persistence of inflammatory stimuli for a further 2 weeks. Only α_2-globulins and, first of all, γ-globulins still remain abnormal after 7 weeks of therapy.

In the light of these data we can argue, regardless of the actual significance of all values found at first presentation, that probably the stronger inflammatory and oxidative reactions occurs only at the beginning of therapy, when PON and HDL reach the lowest values.

Constant dROMs high levels can be explained with persistence of inflammatory reaction over the first 2 weeks and beyond, when oxidative molecules not measured in this study disappears and only dROMs sustain a mild inflammatory-related oxidative process, (and in fact only α_2-globulins remain at high level whereas CRP levels decrease, as previously reported).

Another possible explanation, suggested by high dROMS and γ-globulins levels at day 42, is that inflammation detected at the end of therapy is related to a type III hypersensitivity reaction with synthesis of immune complexes.

Regardless of pathogenic mechanism, the dROMs does not work as a marker to monitor the evolution of inflammatory/oxidative reactions in course of canine leishmaniasis since they remains at high level for long time after starting therapy. . Moreover, dROMs levels are very dispersed at any observation time but, in contrast with HDL (*Figure 4.14*).

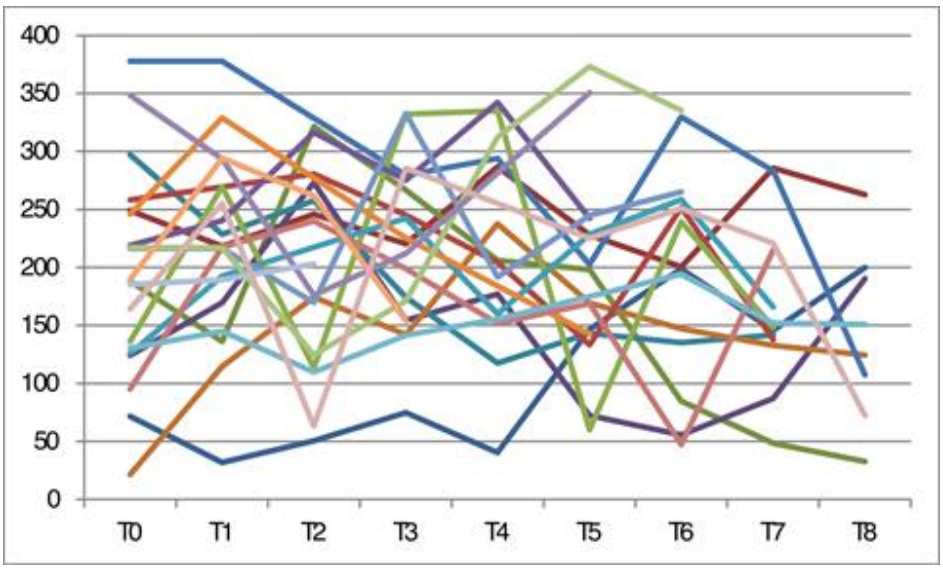

Fig 4.14: Values of dROMs in serial samples of each dog from day 0 to day 42

4.3.3.4 Summary of results regarding dROMs

Measurements of dROMs yielded unexpected results because they show pathological levels (suggesting the existence of oxidation) regardless the severity if the disease, the long time or short time prognosis, and the recovery time. Moreover, they remained persistently high all along the therapy, with a tendency to reduction only in the very final sampling times. This finding has to be confirmed using a control group to compare with. The dROMs levels does not correlate with PON1 activity and other inflammatory or oxidative marker, thus this parameter is not useful in monitoring the disease or as a prognostic factor. These findings suggest that a wider study is needed to detect which oxidative molecules are involved in oxidative processes during leishmaniasis.

4.4 Conclusions

In conclusion, this study provide interesting information about the role of lipid parameters and ROS in canine leishmaniasis and about their relationship between inflammatory markers and the serum activity of the antioxidant enzyme paraoxonase (PON1). Conversely the evaluation of lipidograms

obtained with serum lipoprotein electrophoresis require some modification of the laboratory protocol recommended by the manufacturer. As regards lipid parameters, the most striking feature is the possible role of HDL, either in terms of absolute values and of percentage on total cholesterol, as a biomarker of inflammation and of successful response to treatments. Specifically, the behavior of HDL parallel that of PON1, confirming the strict interactions between these two parameters and suggesting that both they are involved in oxidative responses associated with inflammatory reactions typical of leishmaniasis. Conversely, oxygen radicals seems to contribute only in part to the oxidative phenomena that characterize this inflammatory condition and are not useful in the diagnostic or prognostic approach to canine leishmaniasis.

5. STUDY 3: Changes of PON-1 and HDLs in dogs receiving conventional treatments and supplementation with a stimulator of cell mediated immunity

5.1 Aim of the study

Aim of this study was to evaluate whether the supplementation with a stimulator of cell mediated immunity modifies the inflammatory/oxidative responses in dogs with leiahsmaniasis. In particular, the effects of the addition of domperidone on conventional treatments for canine leishmaniasis (described in Study 1 and 2) were investigated. domperidone is an anti-dopaminergic drug that potentiates innate and cell-mediated immunity through the release of prolactin, which, in turn, interacts with cells of innate/cell mediated responses.[84] In dogs it has been demonstrated that domperidone induces an increased phagocytic and oxidative responses of neutrophils[85] and macrophages.[86] In a clinical trial, domperidone administration decreased the seroprevalence of canine leishmaniasis and the rate of clinical cases, and activated cell-mediated immunity as evidenced by leishmanin skin test and lymphocyte blastogenesis.[87] Therefore, domperidone is recommended for prevention of canine leishmaniasis or for treatment of mild cases by potentiating cell-mediated immunity.[88] However, no studies investigated how the addition of domperidone to conventional treatment may modulate the inflammatory/oxidative responses in dogs with leishmaniasis

5.2 Material and methods

This study has been performed on 20 dogs with leishmaniasis selected as in the former studies 1 and 2.

Then, dogs received a standard treatment with anitmonials and allopurinol, following the guidelines for treatment of leishmaniasis released by the CLWG.[14]

Domperidone was also administered at a dosage of 0,5 mg/kg every 24 h for 1 month.[88]

As in Study 1 and 2, dogs were sampled after 3, 7, 14, 21, 28, 35 and 42 days. At each time sampling, a basic panel of biochemical test, routine hematology and serum protein electrophoresis were performed. PON1 activity, CRP concentration, total and HDL cholesterol were measured, and serum protein electrophoresis was performed. All these tests were done using the methods described in studies 1 and 2. Although all the samples have been collected in each dog, the present thesis includes only preliminary results related to T0 to T6 of 8 dogs (T0 to T7 for HDLs), except for serum protein electrophoresis and CRP, that were assessed in all the dogs only in samples T0, T1 and T2 and only in 5 dogs (CRP) or 4 dogs (serum protein electrophoresis) from T0 to T6.

Results collected in sequential samplings were compared to each other using the same software used in previous studies (Analyse-it Software Ltd, Leeds, UK). Specifically, a non-parametric ANOVA test for paired samples (Friedmann test) was used to compare to each other the results obtained during the sequential time samplings. This test was first performed on all the samples by comparing results from T0 to T2, then the test was repeated on the samples that had the whole panel of tests from T0 to T6. A non-parametric t-test for paired samples was used in all these comparison to assess the possible differences between T0 and each of the sequential samplings.

For CRP and PON1 all these tests were then repeated only on the samples that had abnormal values of at first admission, to assess the actual response to treatments in terms of normalization of abnormal values, since it is unlikely that samples that were normal at first sampling had significant changes during the follow up.

5.3 Results

5.3.1 PON-1

The analysis of results collected in the 8 dogs that were analyzed from day 0 to T6 did not evidence significant differences between samplings, nor an evident trend over time (figure 5.1). However, when the analysis was repeated in the only 3 samples that had abnormal values at admission, a significant trend to increased values ($P<0.05$) was found, although the low number of observation did not allow us to assess at which time sampling significant differences from T0 were present. This analysis seems to suggest that also in dogs treated with domperidone oxidative phenomena associated with inflammation decrease over time. However, the visual analysis of the data recorded in the 3 dogs with abnormal values at admission, evidences that contrarily to what occurred in Study 1 in dogs treated with conventional therapy (see figures 3.5, 3.6, 3.7), on which PON1 had a constant increase over time, in dogs treated also with domperidone PON1 activity increased just after treatment, transiently decreased at T3 (2 weeks after the first administration of treatments) and finally had an increase in the last samplings. This suggests that transient oxidative phenomena may occur during treatment, maybe due to the potentiation of oxidative burst exerted by domperidone on phagocytes.[85,86]

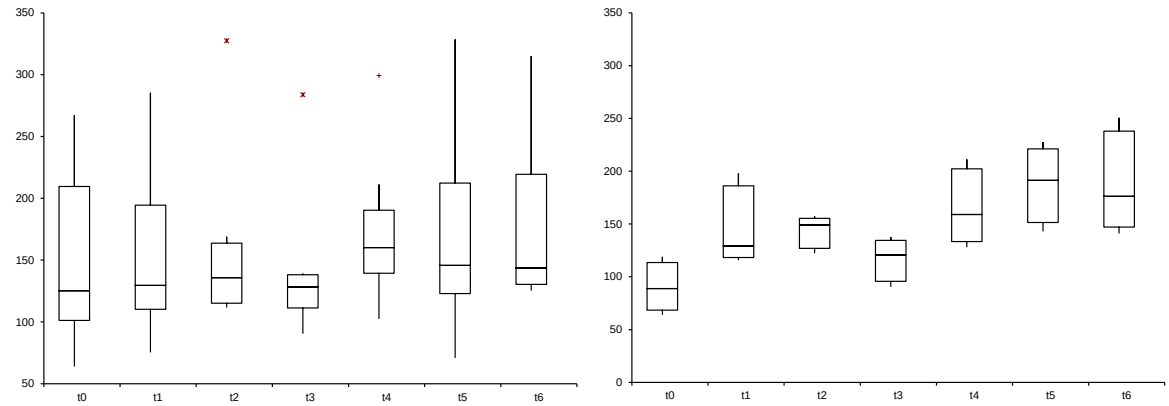

Fig 5.1: Comparison of PON1 activity recorded over time in the 8 dogs included in this part of the study (left panel) or in the 3 dogs that had abnormal values at admission (right panel) For interpretation of box and whiskers see figure 3.1

5.3.2 HDL

Also HDL concentrations increased over time (P<0.001) (figure 5.2), and significant differences compared with T0 were found from T3 (2 weeks after treatment). This confirms the hypothesis that oxidative phenomena decrease over time. In this case the trend recorded over time parallels, in term of magnitude and of time of significance, the one recorded in study 2 in dogs receiving conventional treatments but not domperidone. This seems to contrast with the transient decrease of PON1 activity recorded at T3. In the case this discrepancy will be confirmed by the analysis of additional samples, this results would confirm the hypothesis raised during study 2 about the possible presence of oxidant molecules with different oxidative pathways in dogs with leishmaniasis.

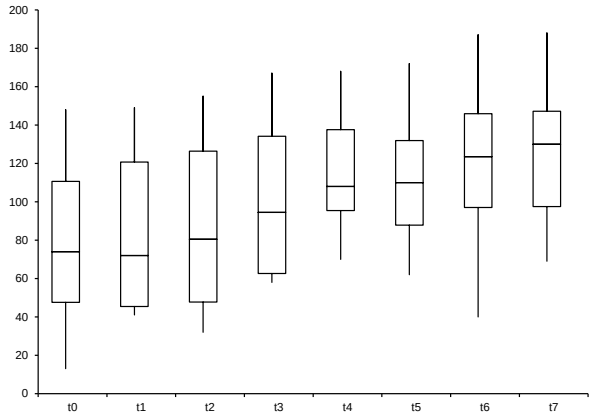

Fig 5.2: Comparison of HDL values recorded over time in the 8 dogs included in this part of the study. For interpretation of box and whiskers see figure 3.1

5.3.3 CRP

Unfortunately, a complete set of CRP values were available only for 5 animals, 3 of which had abnormal values at admission. In both cases no significant differences were recorded over time, probably because the number of cases was so low and the individual variability was so high to not allow to evidence the possible significance between the different time samplings. In terms of visual analysis of data (figure 5.3), however, CRP concentrations decreased

over time, although a high individual variability was present, especially at first samplings. This decrease became more evident when only samples with abnormal values at T0 were analyzed. However, also in this case a transient peak at 3 was found, supporting the hypothesis raised above on the possible activation of inflammation associated with domperidone administration.

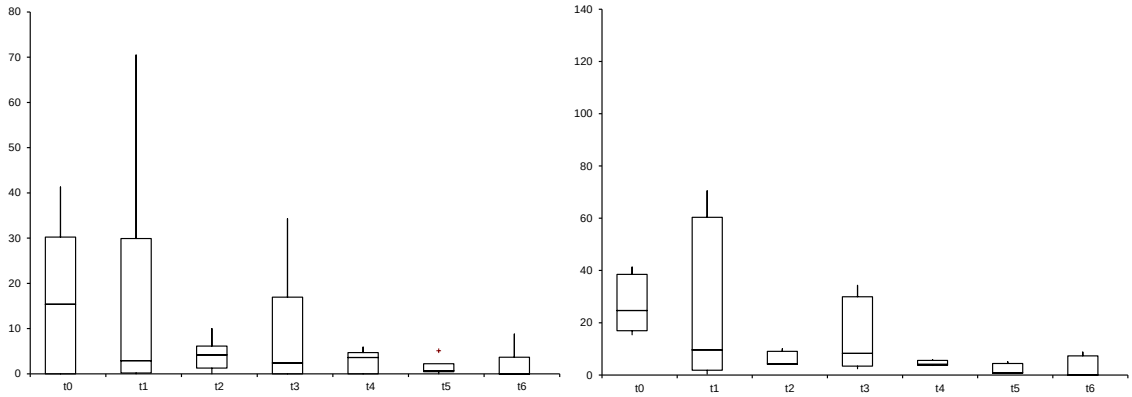

Fig 5.3: Comparison of CRP concentration recorded over time in the 8 dogs included in this part of the study (left panel) or in the 3 dogs that had abnormal values at admission (right panel) For interpretation of box and whiskers see figure 3.1

5.3.4 serum protein electrophoresis

Results regarding the electrophoretic fractions more directly associated with inflammation or immunity (albumin, that works as a negative acute phase protein, α_2- β_2 and γ-globulins) and of the albumin:globulin ratio recorded in the 5 dogs that were analyzed from T0 to T6 are reported in figure 5.4. Statistical analysis did not reveal any significant difference. On one hand, also in this case results statistical analysis may have been biased by the low number of observation and by the high individual variability. On the other hand, it should be reminded that also in the absence of domperidone no significant differences were recorded over time were observed for albumin and α_2-globulins and for the A/G ratio and that γ-globulins had only a slight significant decrease at T5 but never returned into the normal range (see figures 3.5, 3.6, 3.7). Also in the case of electrophoretic fractions, however, the visual analysis of data distribution evidences a difference with what recorded in dogs that did not

receive domperidone (study 1). In particular, in the former study, when no significant differences were recorded over time, the mean and median values were quite constant over time, while in this study a moderate increase of globulin fractions, followed by a decrease from T3 to T6 was visually evident.

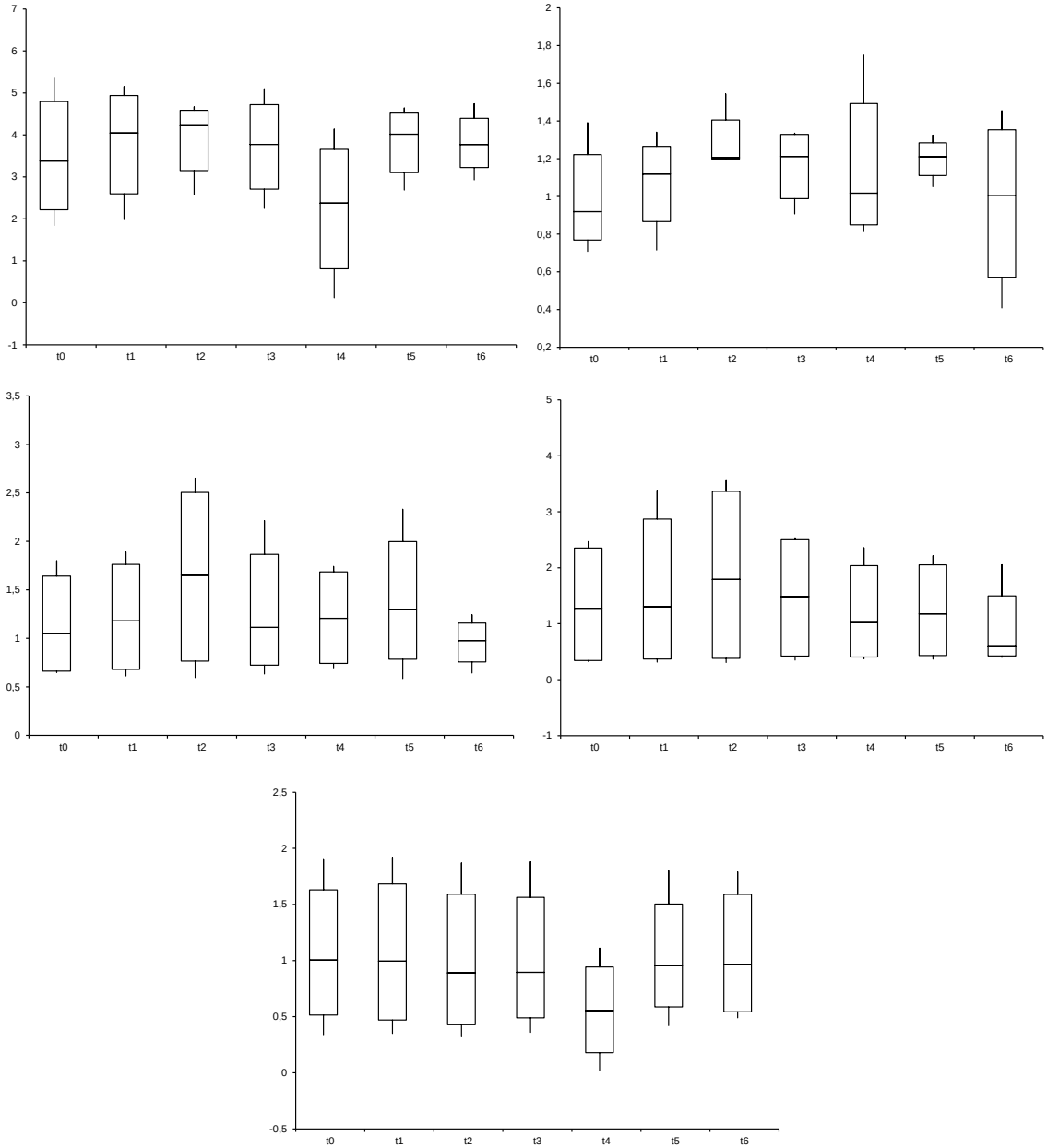

Fig 5.4: Comparison of electrophoretic results recorded over time in the 5 dogs included in this part of the study. The graphs display results regarding albumin (first row, left panel), α_2-gglobulin (first row, right panel), β_2-globulin (second row, left panel), γ-globulin (second row, right panel) and A/G ratio (third row). For interpretation of box and whiskers see figure 3.1

This transient increase becomes much more evident when the results recorded from T0 to T2 in the 8 dogs that were analyzed at these time sampling are compared to each other (figure 5.5)

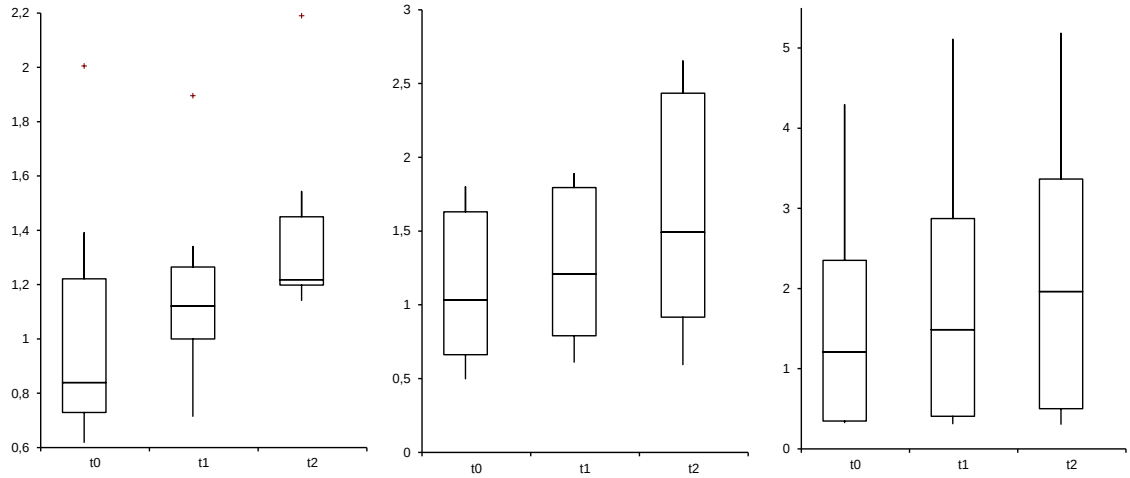

Fig 5.5: Comparison of electrophoretic results recorded over time in the 8 dogs sampled from T0 to T2. The graphs display results regarding α_2-gglobulin (left), β_2-globulin (center) and γ-globulin (right). For interpretation of box and whiskers see figure 3.1

The results regarding α_2, β_2 and γ-globulin, in fact, were significantly different over time ($P<0.05$ for all), with a significant increase compared with T0 recorded either at T1 ($P<0.05$ for all the analytes) and at T2 (for all the analytes).

Also this result support the hypothesis of an increased inflammatory response in the first samplings after treatments, that was not recorded in study 1, when conventional treatment was administered without domperidone

5.4 Conclusion

Although incomplete and absolutely preliminary, the results of study 3 suggest that the addition of domperidone to the standard treatments induces a transient potentiation of the inflammatory response in the first two weeks after treatment. This is evidenced by a simultaneous transient increase of globulin fractions involved with inflammation or immunity and of CRP and by a corresponding decrease of PON-1 activity, likely depending on the potentiation of oxidative responses of phagocytes induced by domperidone. This transient stimulations

of the host response seems to be effecting in increasing the responsiveness against the parasite, since, based on the results recorded from T3 to T6, inflammation and oxidation decrease over time. However, the rapid restoration of HDL levels that occurs also when the potentiation of inflammatory responses mentioned above is active, suggest that oxidants other than those involved in inflammatory responses are cleared by circulation independently on the pharmacological action of domperidone.

Based on these results, it seems that the modulation of innate/cell mediated responses induced by domperidone seem to potentiate the anti-leishmania defenses, as already demonstrated in vitro,[85,86] and as supposed based on results of studies on prevention of leishmaniasis in dogs treated with domperidone.[87,88] However, these results should be integrated with the additional tests to be performed on the other animals sampled during the PhD period and verified on a clinical basis to assess whether this supposed potentiation of inflammatory and immune responses is coupled with a more rapid recovery or a better prognosis in treated dogs.

6. STUDY 4: Urinary gamma-glutamyl transferases (GGT) as a marker of tubular damage in dogs with canine leishmaniasis, using sodium dodecylsulphate (SDS) electrophoresis as a reference method

6.1 Aim of the study

The aim of this study is to determine the usefulness of GGT, a simple and quick biochemical test, as renal biomarker of tubular damage in leishmaniotic and nephropatic dogs comparing this analyte with a more time consuming and expensive gold standard method such as SDS electrophoresis.

SDS is a technique widely used in veterinary medicine to separate protein fractions in urine sample based on molecular mass instead of electric charge as in conventional serum electrophoresis. SDS linearizes proteins because of denaturation of secondary e tertiary structures, and causes a negative charge of the molecules, thus the migration rate is affected only by molecular masses.

Using a central albumin band the gel lane is split in two zones. Tubular proteins have high molecular mass and migrate in the upper zone, instead proteins of glomerular origin are smaller and migrate in the lower half. In mixed glomerular and tubular proteinuria bands in both upper and lower zones are seen.

6.2. Material and methods

6.2.1 Sampling and pre-processing

Urine samples from 21 leishmaniotic dogs were analyzed. Samples were collected by cystocentesis and transferred to the laboratory. Five mLs of sample were centrifuged to perform sediment analysis according to standard procedures.[69] Supernatants were removed, immediately used to measure GGT activity as described below, and frozen at -20°C.

6.2.2 Measurement of urinary proteins and calculation of the urinary protein to creatinine (UPC) ratio

The analyses were performed using the automated spectrophotometer Cobas Mira (Roche Diagnostic, Basel, Switzerland). UPs were measured using pyrogallol red (total proteins high sensitivity, BEN s.r.l., Milan, Italy). This method is linear up to 250 mg/dL, thus samples with UPs higher than 250 mg/dL were manually diluted 1 to 5 with distilled water and re-analyzed. A quality control was performed before any work session with the specific control material, included in the kit (purified bovine serum albumin 100 mg/dL).

The UC was measured with a modified Jaffe method (Real Time Diagnostic System, Viterbo, Italy). Based on a previous study,[69] samples were diluted 1 to 20 with distilled water to fit linearity of the method. Particularly concentrated urine samples were further diluted to 1:100 to fit the linearity of the method. Quality control was performed before any work session with two levels (normal and high) of control serums (Normal Control Serum and Pathological Control Serum, Ben s.r.l., Italy) for creatinine and with bovine albumin (100 mg/dL) for urinary proteins. The UPC ratio was calculated using the formula UP/UC.

6.2.3 Measurement of urinary GGT and calculation of the GGT/UC ratio

GGT was measured just after sampling, with standard colorimetric methods on the Mindray BS-120 analyzer. The GGT/UC ratio was then calculated.

6.2.4 Sodium dodecylsulphate electrophoresis (SDS)

After thawing, samples were processed using SDS-AGE techniques using commercially available kit (Proteinurie 5 – Sebia Italia S.r.l., Bagno a Ripoli, FI,

Italy) on automated electrophoretic equipment (Hydrasis, Sebia Italia). Specifically, SDS-AGE was conducted as follows. Each urine sample was mixed (4:1 v/v) with the diluents included in the kit containing SDS and bromophenol blue. The instrument was prepared by mounting the sponges embedded with the buffer (pH 8.5) on the electrodes and by placing the gel on the migration chamber. Five ml of urine diluted as above were placed into dug wells of the gel and the procedure was started using the specific program (Proteinuria 1*5). Migration takes place under conditions of constant power (10 W) at 20°C to accumulate 60 Volt per hour (V/hr; approximately 15 min). The gel was then transferred to the staining module and automatically stained with acid violet, washed, treated with glycerin, and dried. Gels were then visually analyzed. Proteinuria was defined as glomerular (G, n=6), mixed (M, n=9) or tubular (T, n=6) based on the results of sodium dodecylsulphate (SDS) electrophoresis of urinary proteins, as summarized in figure 6.1.

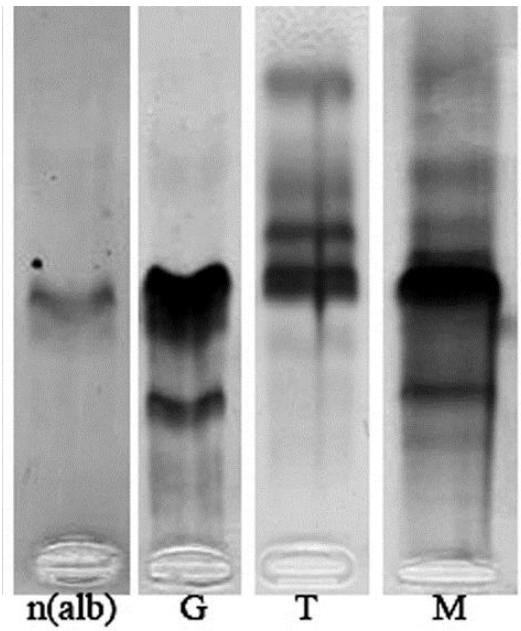

Fig 6.1 Example of SDS gel electrophoresis. Glomerular proteinuria (G) was defined when bands corresponding to proteins with molecular weight (MW) higher than that of albumin were present. Tubular proteinuria (T) was defined when bands corresponding to proteins with MW lower than that of albumin were present; Mized proteinuria (M) was defined when bands corresponding to proteins with MW higher and lower than that of albumin were present. Samples without ands or with bands corresponding to the MW of albumin were considered as negative (n(alb)

6.2.5 Statistical analysis

Results regarding the GGT/UC ratio from glomerular/negative (G/N), mixed (M) and tubular (T) groups were compared to each other using a Friedmann test followed by a Wilcoxon signed rank test for comparison of pairs of groups.

Then, data from mixed and tubular groups were merged in a single group characterized by tubular involvement (M/T) and compared with those of glomerular dogs or of negative dogs (i.e. “non tubular involvement” or NT) using the Wilcoxon test mentioned above. The results of dogs with pure tubular proteinuria (T) were then compared with those of a single group of dogs without pure tubular proteinuria (NT). To determine the discriminating power of GGT/CU and the best cut-off to identify dogs with tubular proteinuria alone (T) or associated with glomerular proteinuria (M/T), receiver operating characteristics (ROC) curves were designed.

6.3. Results

The GGT/UC ratio of M and T proteinuric dogs (median values of GGT/UC ratio: 2.3 and 10.9) significantly higher than that of glomerular/negative proteinuric dogs (GGT/UC ratio: 0.8) (figure 6.2). Similarly, the GGT/UC ratio of the group M/T, with tubular involvement (GGT/UC ratio: 3.6) was significantly higher than that of glomerular/negative dogs (figure 6.3). Finally, the GGT/UC of pure tubular proteinuric dogs (T) was significantly higher also than that of glomerular or mixed proteinuric dogs (GGT/UC 1.5) (figure 6.4).

The ROC curves show that GGT/UC have a good discriminating power to differentiate both dogs with tubular or mixed proteinuria (M/T) (figure 6.5) and dogs with pure tubular proteinuria (T) (figure 6.6). In both case the ROC curve was significantly different from the no discrimination line ($P<0.001$) and the area under the curves (AUCs) were respectively 0.92 and 0.89.

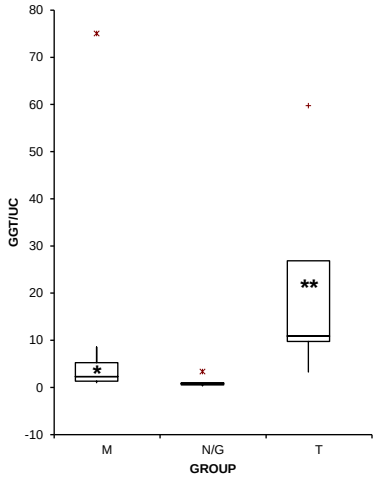

*Fig 6.2 GGT/UC of dogs with Mixed (M), glomerular/negative (N/G) or tubular (T) proteinuria. For interpretation of box and whiskers refer to figure 3.1; * = P<0.05 vs. N/G; ** = P<0.001 vs N/G*

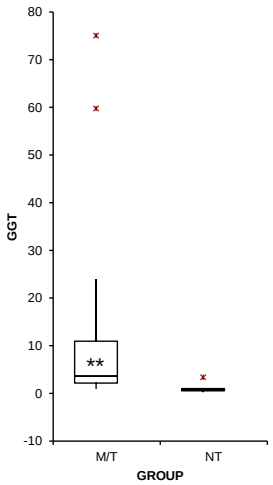

*Fig 6.3 GGT/UC of dogs with tubular involvement (M/T), comared with dogs without tubular involvement (NT) For interpretation of box and whiskers refer to figure 3.1; ** = P<0.01 vs N/G*

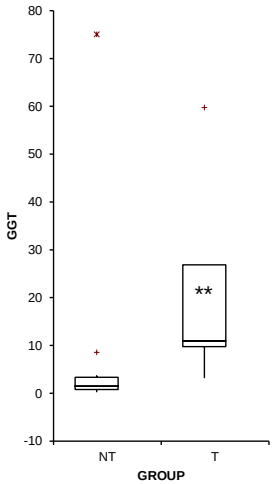

*Fig 6.4 GGT/UC of dogs with tubular proteinuria (T), compared with dogs with all the other types of proteinuria (NT) For interpretation of box and whiskers refer to figure 3.1; ** = P<0.01 vs N/G*

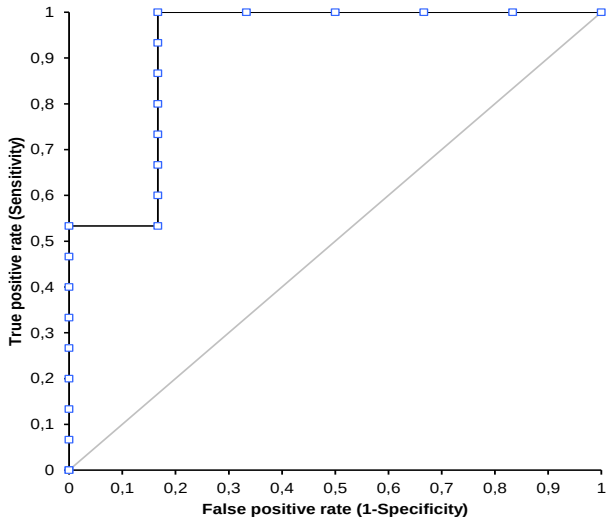

Fig 6.5 ROC regarding the ability of GGT/UC to differentiate dogs with tubular involvement (M/T), from dogs without tubular involvement (NT)

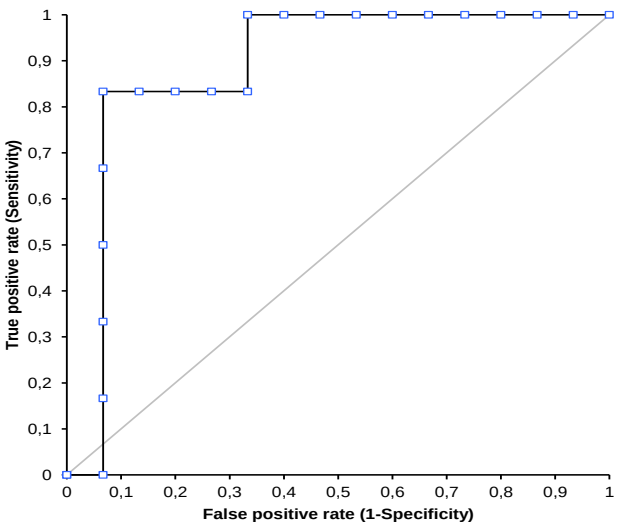

Fig 6.6 ROC regarding the ability of GGT/UC to differentiate dogs with tubular involvement (M/T), from dogs without tubular involvement (NT)

Based on ROC curves, the best cut-off to detect a tubular component of proteinuria (tubular or tubular and glomerular – mixed – proteinuria) is 1.49 (80% sensitivity, 83% specificity, 4.8 likelihood ratio). Therefore, the probability that a sample with GGT/UC values greater than 1,49 have a tubular component (alone or associated with glomerular proteinuria), is 4,8 times higher than the probability that the sample does not have a tubular component (i.e. that is a negative sample or a sample with glomerular proteinuria alone).

The best cut-off to discriminate a pure tubular proteinuria from mixed and glomerular proteinuria is 3.59 (83% sensitivity, 87% specificity, 6.25 likelihhod ratio). Therefore, the probability that a sample with GGT/UC values greater than 3.59 have a pure tubular proteinuria is 6.25 times higher than the probability that the sample does not have pure tubular proteinuria (i.e. that is a negative sample or a sample with glomerular proteinuria, or mixed proteinuria).

6.4. Discussion

The results of this study demonstrate that GGT/UC may differentiate different types of proteinuria in dogs with leishmaniasis. Although renal biopsy is the most accurate and sensitive method to identify the type and localization of kidney damage, in some comparative studies, renal diseases can be suspected by measuring the concentration of single urinary markers such as albumin, C-reactive protein, retinol-binding protein, and N-acetyl-b-D-glucosaminidase[60]. GGT may be one of these markers and, compared with the others listed above, has the advantage to be rapid and cheap, although it suffers from preanalytical artifact such as storage either after refrigeration or after freezing[66]. Information on multiple proteins in a single urine sample can be achieved using electrophoretic techniques. Specifically, it was found that sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), a noninvasive method to localize the origin of urinary proteins based on their molecular weight, provides a diagnostic sensitivity comparable to results obtained by kidney biopsy[68]. Therefore, SDS-PAGE is considered the gold standard method for qualitative analysis of urinary proteins. After the use of SDS, all the proteins are denatured and negatively charged, and as a consequence, they migrate in polyacrylamide gel electrophoresis based on their molecular weight, thus allowing the differentiation of glomerular versus tubular proteinuria[89]. Although the specificity of SDS-PAGE is low[68] and its correlation with the severity of renal damage is moderate[90] SDS-PAGE is

considered a very sensitive tool to discriminate between glomerular, tubular, or mixed proteinuria, and it is commonly used to monitor renal patients and to assess therapeutic responses in human medicine. In routine practice, however, SDS–agarose gel electrophoresis (SDS-AGE) is preferred to SDS-PAGE for its superior ability to separate large proteins and for its lower toxicity as shown in a previous study[91]. Based on these considerations, in this study SDS electrophoresis was used as a reference method to classify dogs as affected by glomerular, tubular or mixed proteinuria.

The measurement of GGT/UC showed to provide result that highly comparable to those provided by SDS-AGE and in particular to well differentiate dogs with tubular components of proteinuria or even dogs with pure tubular proteinuria This information may be relevant, in the management of leishmaniotic dogs, since in the pathogenesis of the disease renal tubular proteinuria, alone or associated with interstitial changes, occurs later during the course of the disease and it may be interpreted as a risk factor for the progression and worsening of the disease. Based on our results, the simple measurement of GGT and the calculation of GGT/UC ratio, that may be performed just after sampling and centrifugation, with low costs and without needs of expensive equipments, may provide in a short time relevant information to identify dogs with more advanced diseases and therefore with guarded prognosis.

6.5. Conclusion

In conclusions, urinary GGT in non proteinuric or glomerular samples is virtually absent and, conversely, increased urinary GGT levels are strictly associated with tubular damage. Thus, the GGT/UC ratio identifies leishmaniotic dogs with tubular proteinuria. This information is important in managing leishmaniotic patients since mixed/tubular proteinuria characterizes advanced stages of renal disease associated with canine leishmaniasis.

7. CONCLUSION

The results provided by the 4 studies included in this thesis allowed us to improve our knowledge about the role of inflammatory and oxidative phoenomena in canine leishmaniasis at first diagnosis and after treatment, and provided new insights about potential markers to be used for monitoring inflammation, oxidation, and renal damage in dogs affected by this disease and/or treated with conventional therapies eventually associated with stimulators of innate/immune responses.

As regards inflammatory/oxidative responses, this thesis confirmed the presence of oxidative phenomena associated with the inflammatory profiles of infected dogs and suggested that reactive oxygen species released by inflammatory cells are not the only oxidative mediators involved in this disease. This hypothesis is supported by the lack of consistency between changes in antioxidant molecules such as PON1 and HDL, on one hand, and the serum concentration of reactive oxygen metabolites (ROMs), on the other. Additionally, modulation of oxidative responses using domperidone had an evident effect on inflammation and PON1 activity but not on HDL concentration, that normalized also when oxidative responses associated with inflammation were potentiated by domperidone administration.

Independently on the speculation on the pathogenesis of inflammatory and oxidative patterns evidenced by the comparison of results from sick and severely sick animals or from dogs treated with the different treatment protocols, the serial analysis of markers of inflammation or oxidation defined the possible role of these markers as prognostic factors, especially after treatment. More specifically, antioxidant molecules such as PON1 and HDL may play a role as early markers of remission after treatment since they quickly normalize in dogs that respond to treatments, while conventional markers of inflammation take a longer time to return within reference intervals. Although

both markers may provide similar information in dogs undergoing conventional treatments, HDL seems to be more efficient in detecting responsive dogs when domperidone is used as an ancillary treatment: results of Study 3, in fact, evidenced that PON1 may not be a good marker in dogs receiving this treatment since its normalization occurs later than in dogs receiving only conventional treatment or, additionally, may increase in the first 2 weeks after domperidone administration.

As regards renal markers, we evidenced the possible role of GGT as a simple and inexpensive marker of tubular damage in dogs with leishmaniasis and we defined the ideal cut-offs to differentiate dogs affected by tubulointerstitial complications, which likely have a worst prognosis, from dogs with pure glomerular damage.

In conclusion, based on the results of this thesis, oxidation plays an important role in the pathogenesis of inflammation induced clinico-pathological changes in canine leishmaniasis and therefore the measurement of PON1 and HDL in blood and of GGT on fresh urine may be recommended as practical, cheap and rapid tools for monitoring the course of leishmaniasis and of response to treatments.

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9. LIST OF PUBLICATIONS DURING THE PhD

Results of study 1 have been published on a peer reviewed international journal.[1] Part of the results of study 2 have been presented at an international meeting[5] and then published on an international peer reviewed journal.[4] Results of study 3 are still in progress and will be completed soon and sent for publication. Results of study 4 have been presented at an international meeting[7] and will be integrated with those of GGT-SDS in diseas other with leishmaniasis to define the possible role of GGT in predicting SDS patterns in different canine diseases characterized by tubule-interstitial damage. In addition, Dr. Ibba during the PhD period, completed the publication on a case report on a rare feline parasitic disease, and participated to a study on the determination of feline reference intervals. Both these studies have been published on a peer reviewed international journal.[1,3] Finally, Dr. Ibba presented at a National meeting a case report on leishmaniasis in a dog,[8] and contributed to a study on osmotic fragility of RBCs in dogs with chronic kidney disease that was presented at an international meeting.[6]

The complete list of papers and abstract produced during the PhD period is reported below

International peer reviewed journals

[1]Paltrinieri S., Ibba F., Rossi G. (2014) Hematological and biochemical reference intervals of four feline breeds. Journal of Feline Medicine and Surgery 16:125-136, DOI: 10.1177/1098612X13499337

[2]Rossi G., Ibba F. Meazzi S., Giordano A., Paltrinieri S. (2014). Paraoxonase activity as a tool for clinical monitoring of dogs treated for canine leishmanias. The Veterinary Journal 199:143-149, DOI: 10.1016/j.tvjl.2013.10.007

[3]Ibba F., Lepri E., Veronesi F., Di Cesare A., Paltrinieri S. (2014) Gastric cylicospirurosis in a domestic cat from Italy. Journal of Feline Medicine and Surgery 16:522-526 DOI: 10.1177/1098612X13505577

[4]Ibba F., Rossi G., Meazzi S., Giordano A., Paltrinieri S. (2014) Serum concentration of high density lipoproteins (HDLs) in leishmaniotic dogs Accepted by Research in Veterinary Sciences

International meetings

[5]Rossi G., Meazzi S., Giordano A., Paltrinieri S. (2013) Serum concentration of high density lipoproteins (HDLs) in leishmaniotic dogs. 15th ESVCP/ECVCP Congress, 6th-9th November 2014, Berlin, Germany

[6]Scarpa P., Russo A., Vitiello T., Ibba F., Paltrinieri S. (2013) Red blood cell's osmotic fragility in dogs and cats with chronic kidney disease. 23rd ECVIM-CA Congress, Liverpool, 12th-14th September 2013

[7]Ibba F., Stranieri A., Xenoulis V., Paltrinieri S. (2014) Urinary gamma-glutamyltransferase (GGT) as a marker of tubular damage in dogs with leishmaniasis, using sodium ddodecylsulphate (SDS) electrophoresis as a reference method – 16° ESVCP/ECVCP congress, Milan, Oct 1st-4th 2014, page 30

National meetings

[8]Ibba F. (2013) Mysdiagnosis of lymphoma in a leishmaniotic dog. Congresso Internazionale SCIVAC- Leishmaniosi canina e malattie trasmesse da vettori. A che punto siamo? Palazzo dei Congressi - Pisa, 8-10 Mar 2013