Haemostatic abnormalities in canine spirocercosis

By

Paolo Pazzi

Submitted to the Faculty of Veterinary Science, University of Pretoria, in partial fulfilment of the requirements for MMedVet (Med) degree.

Pretoria, October 2012

TABLE OF CONTENTS

SUMMARY

Spirocerca lupi (*S. lupi*) is a nematode that infects the dog's oesophagus resulting in an inflammatory fibroblastic nodule that progresses to a sarcoma in approximately 25% of cases. Inflammation, coagulation and cancer are exquisitely intertwined and inflammatory changes are known to lead to coagulation abnormalities. The nature and degree of haemostatic alterations in canine spirocercosis are unknown. Evidence of inflammation in dogs with clinical spirocercosis is provided by pyrexia, leucocytosis, increased serum interleukin 8 and C-reactive protein as well as severe inflammatory infiltrates on histopathology of nodules. This study aimed to determine if haemostatic abnormalities exist in canine spirocercosis, and hypothesised that the severity of abnormalities could be used to differentiate non-neoplastic from neoplastic spirocercosis.

Thirty-nine client-owned *S. lupi*-infected dogs and 15 healthy age- and sex-matched control dogs were included in this study. Blood samples were collected at the time of diagnosis. A complete blood count, prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen concentration, antithrombin (AT) activity, D-dimer concentration and thromboelastography (TEG) analysis were performed. Hypercoagulability was based on the maximum amplitude (MA) value derived from TEG. Inflammatory parameters were also determined and included C-reactive protein (CRP) and fibrinogen concentrations. The *S lupi*-infected dogs were divided into a non-neoplastic group (n=24) and a neoplastic group (n=15). Data were compared using the Kruskal-Wallis Test and Dunn's multiple comparisons applied post-hoc. Correlation was determined using Spearman's correlation.

Hypercoagulabilty was found in the neoplastic and non-neoplastic spirocercosis cases. In addition, the neoplastic group was significantly more hypercoagulable than the non-neoplastic group, and the non-neoplastic group was significantly more hypercoagulable than the control group. The median fibrinogen concentration was significantly higher in the neoplastic group compared to the non-neoplastic group, but there was no significant difference between the non-neoplastic and control group. The median CRP concentration was significantly higher in the neoplastic group compared to the non-neoplastic group, with no significant difference between the non-neoplastic and control group. Compared to the control group the median AT activity was significantly

decreased in both the non-neoplastic and neoplastic groups. No significant difference was found between the infected groups. Across the non-neoplastic and neoplastic groups, MA showed positive linear correlation with CRP and fibrinogen.

The study showed that spirocercosis is associated with a hypercoagulable state that becomes progressively more severe with neoplastic transformation. Overlap did exist between the median MA values of the non-neoplastic and neoplastic groups, but an MA of >76 mm provided a specificity of 96% and sensitivity of 73% for the differentiation of disease state. Thromboelastography might therefore be used as an adjunctive assay to support the suspicion of neoplastic transformation of the oesophageal nodule as well as to determine the overall haemostatic status of the patient. The MA correlated positively with the indicators of inflammation (CRP & fibrinogen) supporting the hypothesis that an inflammatory state induced by the *S. lupi* nodule is at least partly responsible for the hypercoagulability.

The link between inflammation, coagulation and neoplastic transformation in spirocercosis warrants further investigation to elucidate the exact factors resulting in the hypercoagulable state, whether clinically relevant complications develop and whether or not specific therapy should be instituted to prevent thrombotic sequelae.

ACKNOWLEDGEMENTS

I would like to thank the following:

Prof. Eran Dvir, my promotor, for providing invaluable guidance, support and patience during the project.

Prof. Amelia Goddard, my co-promotor, for providing the idea for this project and the invaluable guidance on all aspects of the project as well as financial support for the research.

Prof. Annemarie T. Kristensen, my co-promotor, for providing objective and important insights into the research project from beginning to end of the project.

The Faculty of Veterinary Science, the Department of Companion Animal Clinical Studies, Duncan Campbell Memorial Fund of the South African Veterinary Foundation (SAVF) and the *Spirocerca lupi* research fund/project for financial support that made this research possible.

My amazing wife, Michelle, who endured my frustrations but never wavered in her love, support and belief in me.

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

1.1 General Introduction

Spirocerca lupi (S. lupi) is a nematode that is primarily a parasite of dogs, although other animals, particularly carnivores may be affected.[1,2] Canine spirocercosis has a worldwide distribution, but is most prevalent in warm subtropical climates.[1] Infection occurs after the ingestion of the intermediate host, coprophagous beetles, or the paratenic/transport hosts (lizards, poultry, rodents, hedgehogs, rabbits).[3] The larvae penetrate the gastric mucosa of the final host, migrate within the walls of the gastroepiploic arteries to the caudal thoracic aorta where they remain for approximately three months before finally migrating through the mediastinum to the submucosa or muscular layer of the oesophageal wall. Final maturation occurs within the oesophageal wall and a fibrous nodule forms around the worm.[4] Mature oesophageal nodules may undergo neoplastic transformation, in 14 to 26% of cases, into sarcomas of which osteosarcoma is the predominant form.[4-6]

The most common presenting complaints are weight loss, regurgitation and anorexia, although a myriad of initial signs may be seen including pyrexia, hypersalivation, sialodenosis, dysphagia, dyspnoea/coughing, anaemia and sudden death (due to aortic or other large arterial vessel rupture secondary to aneurysm).[6-8] The nodule may also be an incidental finding on thoracic radiography or during post mortem. Aberrant migration of *S. lupi* occurs and may be difficult to diagnose as the presenting complaints can be very atypical.[6]

1.2 Diagnosis of spirocercosis in the dog

Diagnostic modalities are usually complementary in the final diagnosis of typical spirocercosis and can be made through one, or a combination of the following techniques:

1. Faecal floatation: The modified sodium nitrate ($NaNO_3$) centrifugal method showed that more eggs were detected when compared to the routine method, but the sensitivity remained 67%.[9]

2. Radiography: The sensitivity of radiographic diagnosis is up to 86%. Three complementary signs for the diagnosis of *S. lupi* are described:
   a) a typical soft tissue opacity can be seen in the caudal oesophagus on the right lateral view and on the dorsoventral view as a single midline soft tissue opacity superimposing on the caudal cardiac border and diaphragmatic cupula (not pathognomonic);[3,6]
   b) thoracic vertebral spondylitis of vertebrae in the region of T6 to T12[3,6] considered a pathognomonic sign for this disease;[2]
   c) on the dorsoventral view, the border of the descending aorta may appear undulating due to aneurysm formation.[3,6]
3. Endoscopy: Considered the gold standard when oesophageal luminal masses are present.[2] Mediastinal masses, aberrant migration and early manifestation will, however, be missed with this method.
4. Computed tomography (CT): especially useful for early diagnosis of spondylitis and aortic changes, when pleural or mediastinal fluid effaces structures on the radiograph, for early diagnosis of small mural or extra-mural oesophageal nodules, for surgical planning, and for prognostication based on the infiltrative nature of the neoplasia.[3,6]

1.3 Inflammation, neoplastic transformation and canine spirocercosis

Spirocerca lupi infection has long been associated with neoplastic transformation, which was first documented in 1955.[5] The nodule typically transforms into osteosarcoma, anaplastic sarcoma or fibrosarcoma.[6] Osteosarcoma occurs in approximately 60% to 85% of neoplastic transformed cases of spirocercosis.[4,10] Malignant oesophageal neoplasia in areas where *S. lupi* is not endemic is very rare (<0.5% of all neoplasia cases), and does not typically include sarcoma.[12] The association of *S. lupi* with certain types of sarcomas, the virtual non-existence of these oesophageal neoplasms in non-spirocercosis affected dogs, as well as the relatively high prevalence of neoplastic transformation in spirocercosis in conjunction with the ease of accessibility of the oesophagus through endoscopy makes *S. lupi* induced neoplastic transformation an excellent model for the study of infection and inflammation induced neoplasia.[2,3,7,11,12] Neoplasia has been reported to be closely linked with infectious agents and inflammation, with an estimated 20% of all cancer deaths in humans associated with a known cause of initiating chronic infection or inflammation.[13]

Chronic inflammation favours the initiation and progression of cancer.[14] The longer the inflammation persists the higher the probability of genomic instability and mutations that lead to cancer.[14-16] The mechanism by which chronic inflammation induces cancer development is hypothesized to be related to the sustained generation of free radicals and proteases that causes oxidative damage and nitration of DNA bases, which increases the risk for DNA mutations and breaks that may be nonrepairable. Although the exact pathogenesis of the neoplastic transformation in *S lupi* has not been elucidated, an intense local inflammatory process in benign and neoplastic spirocercosis has been described and is evidenced by local neutrophilic and lymphocytic infiltrate.[4,17] The presence of systemic inflammation in dogs with *S. lupi* nodules is alluded to with the documentation of pyrexia in 32-51%, leucocytosis with immature neutrophilia in 30-81%,[6,7,11,18] raised serum concentrations of CRP in both neoplastic and non-neoplastic forms of spirocercosis as well as elevated IL-8 levels in blood.[19,20] Possible hypotheses explaining infectious-associated neoplastic transformations include an ineffectual Th1 response as seen with *Helicobacter* induced-gastritis;[13,21] helminth induced free radical and nitrogen species;[22] the parasite itself being directly responsible for neoplastic transformation;[23-25] and increased local and systemic proportions of T-regulatory cells (T-regs).[26-30] T-regs can inhibit the antitumour immune response and an increase in their number may facilitate tumour development.[31]

1.4 Diagnosis of neoplastic transformation

Biopsy and histopathology is the gold standard for confirmation of neoplastic transformation in canine spirocercosis. Endoscopy-guided biopsy is the most commonly used technique but it is insensitive, requires anaesthesia and is associated with false negative results when only the necrotic periphery is included in the sample. Gross endoscopic appearance is considered sensitive and specific for the diagnosis of oesophageal nodules,[2,3,7,11,12] but the macroscopic appearance can be misleading and visual diagnosis remains subjective. Non-neoplastic *S. lupi* induced nodules are smooth and round and may have a pink nipple-like protuberance through which the female worm lays eggs. Neoplastic transformation is suspected if the surface of the nodule appears roughened and cauliflower-like with areas of necrosis and ulceration, making biopsy easier than smooth nodules.[7]

Radiographic signs associated with neoplastic transformation include hypertrophic osteopathy of the distal limbs (100% specificity, 38.7% sensitivity), an increased height

and width compared to benign masses and nodular metastatic lesions in lung parenchyma in association with an *S. lupi* nodule in the oesophagus.[7] In spirocercosis, metastases have been reported in the lung, liver and other organs but their prevalence has not been reported.[2,6,7]

Laboratory analysis has shown that dogs with neoplastic transformation had a significantly lower haematocrit (Ht) (mean ± standard deviation: 0.34 ± 0.08) compared to those with non-neoplastic nodules (0.41 ± 0.07), a higher white cell count (31.6 ± 27.83 $x10^3/\mu L$) compared to dogs with non-neoplastic nodules (17.71 ± 13.18 $x10^3/\mu L$) and an increased platelet concentration (493.15 ± 151.6 $x10^3/\mu L$) compared to dogs with non-neoplastic nodules (313.27 ± 128.54 $x10^3/\mu L$); however large overlap did exist between neoplastic and non-neoplastic cases for all parameters.[7]

No sensitive or specific biochemical, radiographic or CT indicators have been found to confirm a diagnosis of neoplastic transformation of a *S. lupi* nodule, unless macroscopic metastasis is evident on radiographs or CT. Diagnosis of neoplastic transformation as early as possible and preferentially by non-invasive means is crucial to facilitate early and improved intervention including surgical excision and chemotherapeutics.

1.5 Inflammation and haemostasis

It is well known that systemic inflammation causes haemostatic derangement and interaction between inflammation and coagulation is bidirectional.[13,21,22,32] Hypercoagulability has been reported in a number of inflammatory diseases affecting small animals such as parvoviral enteritis, neoplasia, and immune-mediated haemolytic anaemia and is also seen in dogs admitted to an intensive care unit and dogs diagnosed with disseminated intravascular coagulation as a complication of a primary disease.[33-39] In certain disease states it is believed that this hypercoagulability may lead to thrombus formation.[40-43] Inflammation activates haemostatic pathways including the tissue factor pathway with resulting thrombin production.[44] The latter plays a central role in the initiation of inflammation-induced coagulation.[45] Blocking tissue factor activity completely inhibits inflammation induced thrombin generation in animal models of experimental endotoxaemia or bacteraemia.[46,47] The main mediators of inflammation-induced activation of coagulation are proinflammatory cytokines and several studies have shown the importance of interleukin 6 (IL-6) in the initiation of coagulation activation, and the role of tumour necrosis factor-α (TNF-α) and IL-1 in the regulation

of physiologic anticoagulation.[48-50] Thrombin, factor Xa, and fibrin can directly stimulate mononuclear cells and endothelial cells, inducing the synthesis of IL-6 or IL-8.[51] The *in vivo* expression of tissue factor seems mostly dependent on IL-6.[48,52] The other main mechanisms of the haemostatic derangement during systemic inflammatory activity are believed to be platelet activation and an imbalance or dysfunction of the normal physiologic anticoagulants, such as antithrombin and protein C.[53,54]

Fibrinogen and its active form, fibrin, directly influence the production of proinflammatory cytokines and chemokines (including TNF-α, IL-1β, and monocyte chemoattractant protein-1) by mononuclear cells and endothelial cells.[13,21,22,32] Fibrinogen is a positive acute phase protein and levels are elevated with tissue inflammation or tissue destruction,[55-57] allowing fibrinogen to be utilised as an indirect measure of the inflammatory process. However fibrinogen levels are reduced during active coagulation and fibrinogen is therefore the balance between inflammation and coagulation and considered less sensitive and specific compared to inflammatory markers such as C-reactive protein (CRP).

CRP is one of the major positive acute phase proteins in the dog and elevated levels are found in response to various infectious and inflammatory conditions.[58-65] C-reactive protein is part of the innate host response and it has been associated with alterations in the coagulation cascade including increased activated clotting time and decreased antithrombin levels.[66] CRP has a shorter half life compared to fibrinogen and therefore is a more accurate real-time marker of the state of inflammation.[67,68]

1.6 Haemostasis and spirocercosis

The effect of *S. lupi* on haemostasis and its various parameters has not been described. The intense local inflammatory process and subsequent systemic inflammation previously described is speculated to result in alterations of haemostasis in neoplastic and non-neoplastic forms of spirocercosis. During the migration of *S. lupi* through the arterial wall to the oesophagus, vascular injury occurs as evidenced by arteritis and subsequent aortic aneurysms. Such changes are likely to cause haemostatic abnormalities; however the true effect of the initial vascular injury on systemic haemostasis is unclear. Three case reports describe aortic thromboembolisms due to spirocercosis which are most likely directly related to the migration of *S. lupi* larvae.[69-71] A study evaluating hypoantithrombinaemia in dogs with various diseases showed that

three of the four dogs with spirocercosis (stage not specified) showed hypoantithrombinaemia.[72] Hypoantithrombinaemia in humans is associated with a hypercoagulable state.[72,73] These findings support the speculation that haemostasis is affected in canine spirocercosis.

Thrombocytopenia is a common finding in human and canine neoplasia and has been reported in 10-28% of canine clinical cases.[74,75] Thrombocytopenia has not been reported as a characteristic in spirocercosis, on the contrary, studies on canine spirocercosis reported that thrombocytopenia was a rare finding in affected dogs; in fact thrombocytosis was more likely in those cases diagnosed with a neoplastic nodule, the chronic ulcerative lesion being the speculative cause.[2,7]

1.7 Haemostatic changes in neoplasia

Clinical thrombosis is seen in up to 15% of human cancer patients at some point during their clinical course.[76] Thrombotic mortality in human cancer patients is second only to heart disease as the most common cause of death in human cancer patients.[77] Neoplasia in dogs has been associated with hypercoagulability,[37] increased thrombin-antithrombin complexes,[78-80] abnormal coagulation assays and thrombocytopenia,[42,74] as well as pulmonary thromboembolism (PTE) (41% of dogs with neoplasia).[40] In a study on PTE in dogs, neoplasia was also reported to be among the most common findings.[41] Two different studies of portal vein thrombosis in 11 and 33 dogs reported neoplasia as the underlying condition in 36% and 21% of patients respectively.[81,82]

A recent study reported haemostatic dysfunction, using thromboelastography, in 57% of 49 dogs with neoplasia.[37] Forty nine percent of cases were dogs with malignant neoplasia, of which 50% were hypercoagulable, whereas 17% were hypocoagulable. All hypocoagulable dogs had metastatic disease. The proportion of dogs with altered haemostasis was significantly different between dogs with malignant and benign neoplasia.[37]

1.8 Laboratory evaluation of haemostasis

Traditionally, changes in the haemostatic profile of a patient have been monitored by the changes affecting primary haemostasis (platelet count and function) and secondary haemostasis [prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT)], antithrombin (AT) activity, altered fibrinogen concentration and

fibrinolysis (increased fibrinogen degradation products (FDP) and D-dimer concentration).[83] The above-mentioned assays constitute the traditional coagulation parameters and these assays are not only poor at determining hypercoaguability (AT being the exception to this statement), but as they are based on plasma samples they also overlook the role that cells play in haemostasis. Further shortfalls of the traditional coagulation parameters include overlooking such factors as rate of clot formation, overall clot strength, and rate and degree of dissolution. These factors represent significant interactions essential to the evaluation of the haemostatic system in clinical patients. Conventional coagulation assays (PT, aPTT, activated clotting time and D-dimer) compared with TEG (multiple samples from the same dogs once weekly over five consecutive weeks) showed a high degree of variability in the standard coagulation assays compared with TEG measurements.[84] The advantages of the traditional coagulation parameters lie in their supportive role in the diagnosis of bleeding disorders such as disseminated intravascular coagulation, haemophilia and rodenticide toxicity.[85]

The recent description of the cell based, tissue factor dependent model of haemostasis has expanded our knowledge of the complex biochemistry involved in secondary haemostasis and thus forced a review of the traditional view of the intrinsic and extrinsic pathways of coagulation into a more global view.[83,86,87] The "cell based" model of haemostasis incorporates the essential role of cells in haemostasis *in vivo* and shows that it requires the participation of key tissue factor (TF) bearing cells (stromal fibroblasts, monocytes/macrophages and potentially endothelial cells in inflammation and neoplasia) and activated platelets. The cell based model, at its most basic level, describes haemostasis as a sequence of events occurring in three overlapping phases i.e. initiation, amplification and propagation. The initiation of coagulation takes place on TF-bearing cells; cells expressing TF are generally localized outside the vasculature, which prevents initiation of coagulation under normal conditions. Once an injury occurs and the flowing blood is exposed to a TF-bearing cell, FVIIa rapidly binds to the exposed TF resulting in an initial small amount thrombin formation. If the procoagulant stimulus is sufficiently strong, enough factors Xa, IXa and thrombin are formed to activate nearby platelets and successfully initiate amplification. Amplification occurs as the activity moves from the TF-bearing cell to the activated platelet surface. The procoagulant stimulus is amplified as von Willebrand factor is released and more platelets adhere, are activated and accumulate activated cofactors (FV, FVIII and FXI) on their surfaces. Finally, in the propagation phase, the active factors combine with their

cofactors on the platelet surface – the site best adapted to generate haemostatic amounts of thrombin. The activity of the procoagulant complexes produces the burst of thrombin generation that results in fibrin polymerization. Assays such as thromboelastography (TEG) incorporating both cellular and plasma components are therefore becoming increasingly important in determining the overall haemostatic state in both humans and animals.

1.8.1 Thromboelastography as a measure of haemostasis

Thromboelastography was first described in 1948 and has since been widely used in the United States and Europe especially in human medicine.[88] The use of human recombinant tissue factor-activated TEG on canine citrated whole blood has been validated for use in dogs.[89] The study concluded that the potential ability of TEG to differentiate between hyper-, hypo- and normocoagulable states of haemostasis makes this assay clinically applicable for the evaluation of haemostatic disorders in dogs.

The TEG apparatus consists of a plastic cup and a pin that is suspended by a torsion wire. A sample of blood is placed into the cup, which is maintained at 37°C and the cup is raised to the pin. The cup is then oscillated. When fibrin strands form between the pin and the cup, the pin begins to move with the cup and the torque generated is transmitted up the wire to a mechanical/electrical transducer, which is converted to digital data for display of the TEG tracing on a computer terminal.

The thromboelastogram consists of 3 zones (Fig 1). The first zone, which indicates precoagulation, (initial linear segment to the formation of the first fibrin strands resulting in a divergence of the line into two branches). The second zone represents coagulation and formation of the clot (from the end of precoagulation to a maximal separation of the two branches) and the third zone representing fibrinolysis (from the end of coagulation until the test is ended, or until the two lines eventually converge into a single line that represents clot lysis).[34,89]

Several values can be derived from the TEG tracing including R, K, angle (α), lysis 30 (LY30), lysis 60 (LY60), maximal amplitude (MA) and the calculation of the G value. Reaction time (R) or pre-coagulation time represents the latency period from the start of the assay until a pre-set fibrin formation is reached.[36,89] The R evaluates the extrinsic pathway (FVII) if TF is used as the activator. Clotting time (K) represents the clot

formation time and is a measurement of the rapidity of clot development and is an arbitrary point that corresponds to divergence of 20 mm between the 2 lines.[89] The K value is influenced by factors II (thrombin), VIII, platelet count and or function, thrombin formation, fibrin precipitation, fibrinogen concentration, and haematocrit[36,90-92]. The combined R and K values reflect coagulation time from the beginning to a predetermined clot strength. The α is the angle between the midline and the tangent to the curve drawn from the point of 2 mm divergence. The α represents the rapidity of fibrin build-up and cross linking (clot formation) and is similar to K and affected by the same factors. The MA is the maximum distance in millimetres between the two diverging branches. At this point the clot is entirely formed and thus MA represents the final clot strength.[84] The MA is affected by fibrin and fibrinogen concentration, platelet count and function, thrombin concentration, factor XIII and haematocrit. The MA is also a measure of clot stiffness and may be used to derive the global clot strength (G) a measure of overall coagulant state.[84] The G value is a mathematical transformation of the MA value and only acts to amplify the difference that may be present in the MA value.[93] The degree of clot lysis is determined at 30 and 60 minutes after the MA is reached and is represented as LY30 and LY60 respectively.

A hypocoagulable state is indicated when the R and K values are increased and the MA value is decreased and opposite changes are observed in hypercoagulable states. It has been reported that TF activated TEG is able to correctly identify dogs with clinical signs of bleeding, with both a higher positive predictive value (89%) and negative predictive value (98%) than the conventional coagulation profile.[35] The study further showed that TEG correlated more objectively to clinical signs in haemostatic dysfunction than traditionally used coagulation screens and that TEG allows for easy and quick interpretation in patients with hyper-, hypo- or normocoagulable states even for inexperienced clinicians (Figure. 2).

In people a hypercoagulable state has been defined as the presence of at least 2 of the following: shortened R, increased α, or increased MA.[94] Definitions of hypercoagulability have not yet been established for animals and various parameters are used in different studies to define hypercoagulability, including MA or G alone or in combination with shortened R and/or increased α.[33,37]

1.8.2 Limitations of thromboelastography

Two factors essential for normal clot formation, platelets and haematocrit will influence the thromboelastogram in humans if they are below 66×10^9/L and 15%, respectively.[95,96] A smaller MA and prolonged K were seen in human patients with platelet counts below 66×10^9/L;[96] however, marked thrombocytopenia ($<66\times10^9$/L) and anaemia (Ht<15%) are rarely reported for spirocercosis.[7]

1.8.3 Detection of haemostatic dysfunction using thromboelastography

TEG has successfully been used to identify hypercoagulability in dogs with parvoviral enteritis, protein losing nephropathy, neoplasia, immune-mediated haemolytic anaemia and DIC.[33-39] TEG may be considered of value as a bedside method for the assessment of overall haemostatic function in dogs with all of the above-mentioned conditions.

1.9 Conclusion

Spirocercosis is an attractive animal model to determine the influence of inflammation on neoplasic transformation and the interrelationship between inflammation, coagulation and neoplasia. This study aims to investigate the relationship between the inflammatory and haemostatic system in dogs with both neoplastic and non-neoplastic *S. lupi* infection. Alterations in the inflammatory and haemostatic pathways are expected to be present in both the non-neoplastic and neoplastic form of spirocercosis. The study also aims to evaluate the diagnostic value of TEG and other haemostatic parameters in differentiating neoplastic from non-neoplastic *S. lupi* cases.

CHAPTER 2

OBJECTIVES

1. Characterize any haemostatic alterations present in neoplastic and non-neoplastic canine spirocercosis.
2. Investigate if TEG results allow clinical differentiation between neoplastic and non-neoplastic spirocercosis.
3. Determine if metastatic disease in spirocercosis is associated with the presence of hypocoagulability.
4. Investigate if changes in the acute phase parameters, CRP and fibrinogen, correlate with haemostatic alterations.

CHAPTER 3

RESEARCH QUESTIONS

1. Are haemostatic alterations, measured by TEG and traditional haemostatic parameters (PT, aPTT, fibrinogen, D-dimers and AT) present in canine spirocercosis?
2. Can TEG parameters be used to differentiate neoplastic from non-neoplastic spirocercosis?
3. Does metastatic spirocercosis result in hypocoagulability?
4. Is the degree of haemostatic alteration correlated with changes in inflammatory parameters CRP and fibrinogen?

CHAPTER 4

RESEARCH HYPOTHESIS

1. Hypercoagulability will be caused by or associated with spirocercosis.
2. Neoplastic, non-metastatic spirocercosis will be associated with greater hypercoagulability than non-neoplastic spirocercosis.
3. Metastatic neoplasia associated with spirocercosis will show hypocoagulability.
4. Raised acute phase parameters, fibrinogen and CRP will correlate with the degree of hypercoagulability.

CHAPTER 5

MATERIALS AND METHODS

5.1 Study design

A prospective observational study was performed on 41 client owned dogs with naturally occurring spirocercosis that presented to the Onderstepoort Veterinary Academic Hospital.

Inclusion criteria:

- Dogs older than 6 months.
- Dogs weighing more than 6 kg.
- A positive diagnosis of spirocercosis based on one of the following criteria:
  - A faecal float that is positive for *S. lupi* ova using the validated modified $NaNO_3$ centrifugal flotation method.
  - At least two of the following radiological signs consistent with *S. lupi* infection: a caudodorsal mediastinal mass, spondylitis of the caudal thoracic vertebrae or undulation of the lateral border of the descending aorta.
  - Visual identification of caudal oesophageal *S. lupi* nodules by oesophageal endoscopy.
- Informed consent by the owners of all cases.

Exclusion criteria:

- Dogs treated for *S. lupi* in the 6 months prior to presentation (including macrocylic lactones, or milbemycin for deworming).
- Dogs with any concurrent systemic disease or inflammatory disease conditions (including haemoparasites) which were detected on clinical examination or bloodsmear evaluation.
- Any obvious infections, fractures, contusions or wounds indicative of a recent motor vehicle accident or fighting.
- Any previously diagnosed cardiac, hepatic or renal disease.
- Any previously diagnosed inherited coagulopathy.
- Treatment with any medication known to interfere with normal haemostasis or antiplatelet medication including prednisolone, non-steroidal anti-inflammatory drugs, asprin, clopidogrel or heparin products either during hospitalization or 4 weeks prior to presentation.

The dogs infected with *S. lupi* were placed into two groups (non-neoplastic and neoplastic):

1. Non-neoplastic:
    - naturally occurring spirocercosis with no evidence of neoplastic changes as characterised by the following 2 findings:
        1. the nodules have the typical smooth appearance of benign nodules,
        2. the nodules show regression at follow-up endoscopy 6 weeks after diagnosis.
    - if surgically excised, succumbed or euthanased
        - histopathological examination of the entire nodule showing no neoplastic transformation

2. Neoplastic:
    - naturally occurring spirocercosis
    - histopathological diagnosis of neoplastic transformation of oesophageal nodules with samples obtained through endoscopy guided biopsy, surgical excision or at necropsy.

Control Group

- Fifteen healthy client-owned age- and sex-matched dogs were used as controls.

5.2 Data collection

Upon admission a full history and clinical examination as well as the following tests were performed on every case: peripheral bloodsmear evaluation, urinalysis, faecal flotation (using $NaNO_3$), a complete blood count (CBC) and serum biochemistry including albumin (Alb) and globulin (Glob). If any abnormalities, including those not previously described in spirocercosis, but with the exception of typical inflammatory changes of *S. lupi* were found, further diagnostics were carried out as appropriate for each case which may have resulted in exclusion of the patient from the trial.

5.2.1 Sampling

After diagnosis, prior to any treatment, serum, 3.2% sodium citrate and ethylenediaminetetraacetic acid (EDTA) samples were collected from the jugular vein of each patient with a 21-gauge straight needle by careful venipuncture with minimum stasis. In accordance with the specifications and approval of the Faculty Research Committee and the Animal use-and-care committee of the University of Pretoria, the volume of blood collected always amounted to less than 0.5% of the body weight.

The blood samples were collected in the order described above to avoid tissue factor, released during venipuncture, contaminating the citrate tube. The evacuated sodium citrate tubes were filled to capacity to ensure the correct anticoagulant-to-blood ratio (1:9).

Blood was left for 30 minutes at room temperature after collection to decrease the variance of the TEG measurements since there is a tendency toward hypercoagulation at 120 minutes compared with 30 minutes.[90,91] A fixed time point (30 minutes) after sampling was chosen to avoid the risk of interassay variation.[89] The EDTA sample was used to perform a CBC. The sodium citrate sample was used to perform the TEG assay and then stored for batch testing of the traditional coagulation tests (PT, aPTT, D-dimer concentration and AT activity) and fibrinogen. The serum sample was used to perform the Alb, Glob & CRP.

The citrated and serum samples were centrifuged at 2100g for 8 minutes and the serum/plasma removed, aliquoted and stored at -80°C (Forma Scientific -86°C freezer). All the coagulation assays, as well as the CRP were performed as a batch to limit inter-assay variability. Both CRP and the coagulation assays have been shown to be stable at -70°C and for long periods with the exception of aPTT that may show a slightly shortened clotting time compared to fresh samples.[97,98]

5.2.2 Assay methodologies

- Complete blood count (CBC) was performed on an Advia 2120 (Siemens, South Africa), as well as manual differential leukocyte counts performed by the same qualified laboratory technician.
- Serum albumin and globulin were determined using the Cobas Integra 400 Plus (Roche, South Africa).
- Thromboelastography tracings were performed by the author or a qualified laboratory technician using the TEG® 5000 Thrombelastograph® Haemostasis System (Haemoscope, Pro-Gen Diagnostics, South Africa) according to the

previously described method and using human recombinant tissue factor (Innovin, Siemens) as the activator.[89] MA was utilised as the indicator of the state of coagulability, since the MA recorded by TEG has been reported to be predictive of postoperative thrombotic complications in human surgical patients.[100] A cut-off value based on our laboratory reference range of greater than .....

- PT and aPTT assays were performed on the ST art® 4 analyser (Diagnostica Stago, South Africa) using the Neoplastine® CI Plus (Diagnostica Stago, South Africa) reagent kit for PT, and the C.K. Prest® (Diagnostica Stago, South Africa) reagent kit for aPTT, according to the manufacturer's instructions.
- D-dimer assays were performed, according to the manufacturer's instructions, using an immunometric flow-through principle (D-dimer single test, Nycocard Reader, Ilex, South Africa).
- Fibrinogen assays were performed on the ST art® 4 analyser (Diagnostica Stago, South Africa) using the Sta-Fib 2 (Diagnostica Stago, South Africa) reagent kit according to the manufacturer's instructions.
- Antithrombin activity in plasma was measured utilizing a thrombin dependent chromogenic substrate assay (Roche Antithrombin (A), South Africa) on the Cobas Integra 400 plus analyser.

All coagulation assays were calibrated according to the manufacturers' recommendations for human purposes. Commercially available human control reagents for the respective assays were analyzed as internal controls together with pooled plasma from ten healthy dogs (not part of the previously described control group).

- Serum C-reactive protein was determined using an automated human CRP immunoturbidometric immunoassay (Randox, South Africa) on the Cobas Integra 400 plus analyser (Roche, South Africa). The assay was calibrated with commercially available purified canine CRP (Life Diagnostics, USA) to ensure species-specific measurement of CRP with the heterologous assay. Internal controls were routinely run with the batch of samples.

5.2.3 Diagnostics

- Radiography

The standard right lateral and ventro-dorsal thoracic radiographs were performed under general anaesthesia and manual positive pressure ventilation. Radiographs were obtained using a digital system with a Siemens Polymat 50. Radiographs were developed with a Fuji Film FCR Capsula XL. Radiographs were evaluated by a qualified radiologist.

- Endoscopy

Oesophageal and gastric endoscopy was performed under general anaesthesia, according to the set standard of the department of small animal medicine, by the principle investigator or other qualified medicine clinician. The anaesthetic agents included valium in combination with morphine or butorphanol as premedication, propofol induction and isoflurane inhalant with oxygen as maintenance. The procedure was performed using a video endoscopy unit. One endoscopic exam was performed at the time of initial diagnosis and a follow-up was performed on all cases not euthanased approximately 6 weeks later to determine response to treatment. Non-neoplastic spirocercosis was indicated by reduction in the size and number of nodules on follow-up endoscopy. If nodules were larger or had become ulcerated and cauliflower-like, endoscopic guided biopsies were taken to confirm neoplastic transformation.

- Post-mortem

The standard post-mortem examination was performed as well as histopathology performed on all oesophageal nodules due to *S. lupi*, regardless of number of ante-mortem diagnostics performed.

5.3 Treatment

Treatment of the animals included in the study for spirocercosis was initiated after all sample collections and diagnostics, including thoracic radiography and endoscopy, were completed. Treatment was based on a published treatment protocol of 400 ug/kg doramectin (Dectomax®, Pfizer, South Africa) given by SQ injection two weeks apart for three treatments.[99]

5.4. Data analysis

The data was captured using Excel 97-2003® and analysed by the principle investigator. Statistical analysis was performed with SPSS Statistics 20.0® software (SPSS Inc., United States of America) and Graphpad Instat 3® (San Diego, United States of America).

Descriptive statistics included the median and range for the variables including Ht, platelets, mean corpuscular volume, Alb, Glob, AT, D-dimer, PT, aPTT, Fibrinogen and CRP. The same descriptive statistics were used for the thromboelastograms and the following variables were recorded or calculated: R, K, α, MA, G, LY30, LY60. MA was used in this study as the primary indicator of a hypercoagulable state. The data was

checked for normality using the Kolmogorov-Smirnov test, all parameters were non-parametric and the Kruskall-Wallis test was used to determine significance across the three groups for each parameter. $P<0.05$ was considered significant, P values are reported as <0.05, <0.01 or <0.001 as per the limitations of the software available. If significance was present, Dunn's multiple comparison was used as post-hoc analysis. Correlation between inflammatory and coagulation parameters was determined using the Spearmann's rank correlation test. Data is presented as median and range of values.

CHAPTER 6

RESULTS

Study population

A total of 41 dogs infected with *S. lupi* were enrolled in the study, of which two were excluded. One case was excluded due to concurrent pyothorax, and the second due to severe thrombocytopenia secondary to septic pneumonia. Of the remaining thirty-nine dogs in the study, 24 dogs were in the non-neoplastic and 15 in the neoplastic group. Seventeen breeds were represented (Table 1) and the median age of the two groups was 5 years (range: 6 months to 11 years), with no statistical difference between the non-neoplastic, neoplastic and control groups. Forty six percent of the combined infected group were male and 54% females and did not differ significantly from the control group.

All the dogs enrolled in this study had at least two thoracic views performed and one or more radiographic signs typical of spirocercosis were present in all dogs. Three dogs did not have endoscopy performed due to obvious radiographic evidence of pulmonary metastasis in conjunction with one or more radiographic signs consistent with spirocercosis and euthanasia was requested before endoscopy could be performed. All dogs in the neoplastic group had neoplastic transformation of the *S. lupi* nodule confirmed on necropsy and/or histopathology. No dogs developed neoplasia after being classified as non-neoplastic.

No non-neoplastic cases were euthanased, but 14/15 neoplastic cases were euthanased. The only neoplastic case not euthanased had partial oesophagectomy performed and made a full recovery after surgery. The histopathological diagnoses of the dogs in the neoplastic group were divided into 14 osteosarcomas and 1 anaplastic sarcoma. Six neoplastic cases had metastatic lesions on necropsy and 3/15 of the neoplastic cases had hypertrophic osteopathy observed on radiographs or at necropsy.

Haemostatic and inflammatory laboratory parameters

The majority of the TEG variables indicated hypercoagulability in the spirocercosis groups which was more severe in the neoplastic group (Table 2). Significant findings included the following: the median K value was significantly shorter in the non-neoplastic (1.55 min, 1.1-3.7) and neoplastic (1.3 min, 0.8-2.7; $P<0.001$) groups

compared with the control group (2.5 mm, 1.4-5.4; P<0.05), but no significance was found between the infected groups. The α angle was significantly higher in neoplastic group (72.4°, 57.1-78.3) compared to the non-neoplastic (68.9°, 36.1-75.2; P<0.05) and the control group (58°, 26.3-70.20) was significantly lower than the neoplastic and non-neoplastic groups (P<0.001, P<0.05 respectfully). The median MA for the neoplastic group (78.9 mm, 61.5-89.9) was significantly increased compared to the non-neoplastic group (66.3 mm, 49.7-79.7; P<0.01), and the median MA for the non-neoplastic group was significantly increased compared to the control group (58.6 mm, 41.9-66.2; P<0.01) (Figure 3). As G is a mathematical derivative involving MA exclusively, the G values and their intergroup significance levels mirrored the MA values. R, LY30 and LY60 showed no significant variation between groups. Based on receiver operator curve (ROC) analysis, when a cut-off value of >76 mm was used for the MA value as a test of differentiation of neoplastic versus non-neoplastic, a sensitivity of 73% and specificity of 96% was found for neoplastic transformation with an area under the curve of 0.853 (Figure 4).

The median CRP concentration was significantly higher in the neoplastic group (85.7 mg/L, 6.05-152.0) compared to the non-neoplastic group (17.5 mg/L, 5.0-249.9; P<0.01), with no significant difference between the non-neoplastic and control groups (15 mg/L, 0-30) (Table 2 & Figure 5). The median fibrinogen concentration was significantly higher in the neoplastic group (5.1 g/L, 2.9-14.8) compared to the non-neoplastic group (3.3 g/L, 1.3-8.7; P<0.01), but no significant difference between the non-neoplastic and control groups (2.6 g/L, 1.7-4.4) was seen (Table 2 & Figure 6).

Across the infected groups, MA showed a strong linear correlation with CRP (r = 0.73, P<0.0001) and a very strong linear correlation with fibrinogen (0.85, P<0.0001) (Figure 7 & 8). The inflammatory parameter fibrinogen showed a strong linear correlation to the other measured inflammatory parameter CRP (r = 0.77, P<0.001). A weak negative linear correlation (-0.34, P=0.03) across all infected groups between CRP and AT was also found (Figure 9).

Of the traditional coagulation parameters only PT and AT activity showed significant changes. Compared to the control group (117.1%, 107.1-143.9) the median AT activity was significantly decreased in both the non-neoplastic (90.3%, 58-124.1; P<0.0001) and neoplastic (84.3%, 58.5-111.6; P<0.0001) groups. No significant difference was found

between the infected groups. For PT, significant variation was only found between the neoplastic group (7.1 sec, 6.1-7.8) and the control group (6.7 sec, 5.0-10.7; $P<0.05$).

Other parameters

The median Ht (Table 2) of the neoplastic group (35%, 23-55) was significantly lower than the Ht of the non-neoplastic group (46.5%, 36-61; $P<0.05$), while there was no statistical difference in the Ht between the non-neoplastic and control group (51%, 38-58). The median platelet concentration for the neoplastic group (415×10^9/L, 227-862) was significantly higher compared to the non-neoplastic group (298×10^9/L, 174-494; $P<0.01$), but no significance existed between the control group (318×10^9/L, 212-457) and the neoplastic and non-neoplastic groups.

The median serum albumin concentration differed significantly between the non-neoplastic (34.8 g/L, 25.2-45.5) and the neoplastic (22.2 g/L, 12.1-35.9; $P<0.001$) groups. There was no significant difference for the median serum globulin concentration between the different groups (Table 2).

When the metastatic cases were compared to the non-metastatic neoplastic cases, the metastatic cases appeared to have an increased degree of hypercoaguability as evidenced by increased MA and increased α, and indications of increased inflammatory changes as evidence by elevated CRP and fibrinogen, but none of the mentioned differences between the two subgroups were statistically significant (data not shown). No dogs in the infected groups were hypocoagulable, based on the MA value.

CHAPTER 7

DISCUSSION

A hypercoagulable state as measured by TEG was documented in this prospective study of canine spirocercosis and the degree of hypercoagulability was significantly increased once the oesophageal nodule had undergone neoplastic transformation. The inflammatory state is most likely closely associated with the hypercoagulability and to the best of the authors' knowledge, this is the first study to describe hypercoagulability and its relationship with inflammation in spirocercosis.

For this study, the MA was utilised as the indicator of the state of coagulability, since the MA recorded by TEG has been reported to be predictive of postoperative thrombotic complications in human surgical patients.[100] Based on established laboratory reference ranges for MA, 97% (38/39) of the infected dogs in this study were hypercoagulable and none were hypocoagulable. The single dog not hypercoagulable had non-neoplastic oesophageal nodules and no obvious reason to be different form the rest of the study group. The degree of hypercoagulability between all groups was significant with the median MA increasing as spirocercosis progressed from inflammatory to neoplastic. The increase in hypercoagulability is in agreement with previous studies in which hypercoagulability (based on the presence of thromboemboli or TEG findings) was present in inflammatory and neoplastic conditions.[37,42,74,78-81,101] Factors such as circulating TF (either soluble or cell-borne), cancer procoagulant factor and platelet hyperactivity are believed to be the cause of the hypercoagulable state in humans with cancer.[43,102]

Thromboelastography may be utilised for the differentiation of neoplastic from non-neoplastic spirocercosis. Although overlap of MA did exist between the non-neoplastic and neoplastic groups, an MA value of >76 mm achieved a specificity of 96% for neoplastic transformation in this population, resulting in a very low false positive rate for neoplastic transformation at the cut-off value of 76 mm. This may be useful, but one must be aware of the limitations when employing such a method for differentiation of disease state. Spirocercosis should be diagnosed prior to running the TEG, as many other diseases also result in hypercoagulability. If spirocercosis is present, the presence of other inflammatory conditions must be excluded to interpret the MA value with confidence. The MA should never be viewed in isolation, but rather used as an

adjunctive in these cases where ambiguity exists. The sensitivity of the test is poor (73%) and may result in many false negative results for neoplastic transformation. An MA greater than 76 mm will therefore help support the suspicion of neoplastic transformation, but will not exclude neoplastic transformation if the value is below 76 mm. Different methodologies or population groups are likely to affect these cut-off values and institution specific cut-offs are advised. TEG was not useful in determining metastatic disease as no cases were hypocoagulable and no significant variation was found between the metastatic and non-metastatic neoplastic cases.

The medians of the acute phase reactants, CRP and fibrinogen, showed significant increases in the neoplastic group compared to the non-neoplastic group and controls. In contrast to a previous study, there was no difference between the CRP of the non-neoplastic group and controls in this study.[19] This is surprising considering the inflammation that has been reported to be present in non-neoplastic nodules.[4,17] The two highest CRP values in the present study were seen in the non-neoplastic group. One of these cases was also diagnosed with granulomatous oesophagitis as well as suspected mediastinitis (based on CT). The second case with unexpectedly high CRP underwent a full medical work-up (biochemistry panel, abdominal ultrasound and thoracic CT) without an obvious explanation except for the possibility of severe inflammation within the nodule as described in previous reports.[4,17] Interestingly the MAs for both of these patients were not the highest in the benign group and this is most likely due to the numerous other factors that contribute to the final determination of MA, moreover, considering that α and MA were significantly different between all groups, it is likely that the parameters of TEG, although closely associated with inflammation are also influenced by additional factors and cells.

A significant correlation between MA and fibrinogen concentration and platelet concentration has been reported in both normal and hypercoagulable people and dogs.[38,103,104] In this study, the only TEG variables to indicate significant hypercoagulability across all groups were α and MA, but no significant correlation was found between the inflammatory indicators and α. In contrast MA showed a strong positive linear correlation with both inflammatory parameters. The correlation coefficient (r) for fibrinogen was 0.85, but a slightly weaker r for CRP (0.73). Correlation between the inflammatory parameters was strong; indicating either fibrinogen or CRP could be used in spirocercosis as an indication of the inflammatory

state. The closer correlation of fibrinogen and MA suggest that fibrinogen played an important role in the elevation of MA in these cases. Fibrinogen may be considered one of the "driving forces" behind inflammation as it directly influences the production of proinflammatory cytokines and chemokines by mononuclear cells and endothelial cells.[13,21,22,32] The close correlation of both acute phase reactants supports the interrelationship between inflammation and coagulation and although a consistent trend was found, the individual variation from the trend line was large (Fig. 7 & 8), making prediction of exact values difficult.

With the exception of AT, the traditional parameters of coagulation have been shown to be poor indicators of hypercoagulability,[37,39] and also proved to be poor indicators of hypercoagulability in this study. The current study showed significantly decreased AT activity in the spirocercosis groups compared to the controls. Antithrombin is considered a negative acute phase protein and an important measure in the assessment of hypercoagulability in human and animal studies, and a decreased AT activity in humans is associated with a hypercoagulable state predisposing patients to thrombotic events.[72,73,105] The decreased AT activity seen in these cases is most likely a reflection of consumption due to the hypercoagulability in conjunction with mild blood loss in neoplastic cases (as evidenced by mild anaemia and endoscopic examination). The negative linear correlation found between AT and inflammation (CRP) in this study is in agreement with previous reports investigating the effects of sepsis on AT and AT activity in dogs admitted to a critical care unit although the correlation was weaker in the current study.[66,106]

The neoplastic group showed significantly prolonged PT compared to the control group. This finding may be interpreted as a contradiction of the hypercoagulable state believed to be present in spirocercosis, but when one considers that PT does not take into account the cells present in the TEG sample as well as the large biological variation in PT in healthy dogs, this finding becomes less significant. The difference in PT was also so small between the two groups that it would be clinically impractical for differentiation of disease state.

The significantly lower Ht of the neoplastic group compared to the non-neoplastic group is consistent with previous findings in spirocercosis.[2,7] Based on human studies, even the lowest Ht in this study (found in the neoplastic group), was unlikely to have affected

the TEG or other coagulation parameters.[95] The significant thrombocytosis of the neoplastic group compared to the non-neoplastic group has also previously been reported in spirocercosis and chronic blood loss from the ulcerated mass may explain the thrombocytosis and anaemia in the neoplastic group. Other recognised causes of thrombocytosis applicable to this study population include neoplasia and systemic inflammatory disease.[85]

The hypoalbuminaemia of the neoplastic group relative to the non-neoplastic group supports the findings of previous studies, although one study did not find significance between groups and the other did not differentiate neoplastic from non-neoplastic.[2,7] The hypoalbuminaemia in the current study was most likely related to its role as a negative acute phase protein and secondary to blood loss in the neoplastic group.

The clinical significance of the hypercoagulable state in spirocercosis is evidenced by three case reports describing aorto-iliac thromboembolism, believed to be the consequence of migrating *S. lupi* larvae.[69-71] Reported clinical thrombosis associated with spirocercosis appears to be an unexpectedly rare clinical complication considering the presence of hypercoagulability in 97% of cases in this study. It is possible many cases are undiagnosed and are subclinically thrombotic. The prevalence of pre-clinical thrombi and the effects of anti-coagulant therapeutic intervention in spirocercosis are unknown and may be an area of future investigation. The existence of inflammatory and haemostatic changes in the presence of mature nodules is demonstrated in this study, but the degree to which larval migration and initial vascular injury contribute to the haemostatic derangement remains unclear. The larval migration, although unlikely to be an active site of inflammation once mature esophageal nodules are present, may be the initiator of hypercoaguability, but does not appear to be the only factor resulting in continued hypercoagulability and subsequent thromboemboli. Aortic intimal damage caused by the larvae, turbulent blood flow in aortic aneurysms and local and systemic inflammation associated with the nodules are all likely to contribute to hypercoagulability and the development of thrombosis in spirocercosis.

In summary, this study identified hypercoagulability in canine spirocercosis in both the non-neoplastic and neoplastic form of the disease with increasing degree of hypercoaguability with neoplastic transformation. Overlap did exist between the median

MA values of the non-neoplastic and neoplastic groups, but an MA of >76 mm provided a specificity of 96% and sensitivity 73% for the differentiation of disease state. Thromboelastography might therefore be used as an adjunctive test to support the suspicion of neoplastic transformation of the esophageal nodule as well as to determine the overall haemostatic status of the patient. The clinical significance and therapeutic role of anti-coagulants in spirocercosis remains a field open for future investigation. The indicators of inflammation were strongly positively correlated to the MA values supporting the interrelationship between inflammation and coagulation in spirocercosis.

CHAPTER 8

CONCLUSIONS

- Based on thromboelastography, a hypercoagulable state exists in canine spirocercosis.

- The hypercoagulability found in canine spirocercosis is more severe once neoplastic transformation of the oesophageal nodule has occurred.

- The severity of hypercoagulability, based on the TEG variable MA, might be used to assist in the determination of the presence of neoplastic transformation.

- Hypocoagulability does not appear to be a feature of canine spirocercosis at the time of diagnosis.

- Antithrombin was significantly reduced and is supportive of the hypercoagulable state present in spirocercosis. Other traditional parameters of coagulation showed no significant changes in activity or quantity in infected dogs.

- The acute phase inflammatory parameters, CRP and fibrinogen, are strongly correlated to hypercoaguability in spirocercosis, supporting the interrelationship between inflammation and coagulability.

- Based on this study, once spirocercosis has been diagnosed, TEG might be used as an adjunctive test to support the suspicion of neoplastic transformation of the oesophageal nodule as well as to determine the overall haemostatic status of the patient, especially if surgery is considered.

- Further studies are required to determine if the hypercoagulable state of spirocercosis results in clinically relevant sequelae (i.e. thrombosis) and whether or not specific therapy should be instituted to prevent complications associated with hypercoagulability.

TABLES

Table 1: Signalment of study population

	Whole population	Non-neoplastic group	Neoplastic group	Control
Age (month): median (range)	60 (6-133)	57 (6-133)	72 (48-132)	49 (6-96)
Gender:				
Male	24	13	5	6
Female	30	11	10	9
Breed				
Jack Russel or Fox Terrier	8	3	2	3
Boxer	7	2	2	3
Boerboel	6	2	2	2
Crossbreed	6	3	1	2
Bullterrier	4	2	1	1
Labrador retriever	4	1	2	1
Great Dane	4	2	1	1
GSD	3	1	1	1
Rhodesian Ridgeback	3	1	1	1
Rottweiler	2	2		
Yorkshire terrier	1	1		
Chow-Chow	1	1		
Border Collie	1		1	
Daschund	1		1	
Schnauzer	1	1		
Golden Retriever	1	1		
Maltese	1	1		

Table 2: Haematology, protein and haemostatic results of study population

Median (range)	Control	Non-neoplastic group	Neoplastic
Haematocrit (L/L)*	0.51 (0.38-0.58)	0.46 (0.36-0.47)[c]	0.35 (0.23-0.55)
MCV (Fl)	60-77[d]	68.4 (39.7-75.2)	67.1 (49.3-74.4)
Platelet($\times 10^9$/L)*	318 (212-457)	298 (174-494)[c]	415 (227-862)
Albumin (g/L)*	27-35[d]	34.75 (25.2-45.5)[c]	22.2 (12.1-35.9)
Globulin (g/L)	20-37[d]	33.9 (23.2-74.3)	36.0 (25.7-52.8)
PT (seconds)*	6.7 (5-10.7) [b]	7.05 (4.7-8.1)	7.1(6.1-7.8)
aPTT (seconds)	11.3 (10.3-12.5)	11.5 (10.3-12.7)	12.2 (10.5-14.9)
AT (%)*	117.1(107.1-143.9)[a,b]	90.3 (58-124.1)	84.3 (58.5-111.6)
D-Dimers (mg/L)	0.2 (0.1-0.4)	0.1 (0-1.5)	0.1 (0.1-2.0)
Fibrinogen (g/L)*	2.6 (1.7-4.4) [b]	3.27 (1.3-8.9) [c]	5.07 (2.9-14.8)
CRP (mg/L)*	15.0 (0-30.0) [b]	42.95 (11.4-249.9)[c]	85.72 (6.05-51.59)
R (minutes)	5.8 (3-13.8)	4.85 (3.8-10.2)	5.2 (3-7.7)
K (minutes)*	2.5 (1.4-5.4) [a, b]	1.55 (1.1-3.7)	1.3 (0.8-2.7)
A (degrees)*	58 (41.95-66.2) [a, b]	68.9 (36.1-75.2) [c]	72.4 (57.1-78.3)
MA (mm)*	58.6 (41.95-66.2) [a, b]	66.25 (49.7-79.7) [c]	78.9 (61.5-89.9)
G*	7.1 (3.7-10.0) [a, b]	9.85 (4.9-19.7)	18.7 (8-44.3)
Lys30 (minutes)	0 (0-6.1)	0 (0-16.1)	0.1 (0-3)
Lys60 (minutes)	2.4 (0-13.1)	1.15 (0-20.6)	1.8 (0-7)

* Krusskal-Wallis showed overall significance between the 3 study groups

[a] Significance between control and non-neoplastic groups

[b] Significance between control and neoplastic groups

[c] Significance between non-neoplastic and neoplastic groups

[d] Laboratory established normal values

FIGURES:

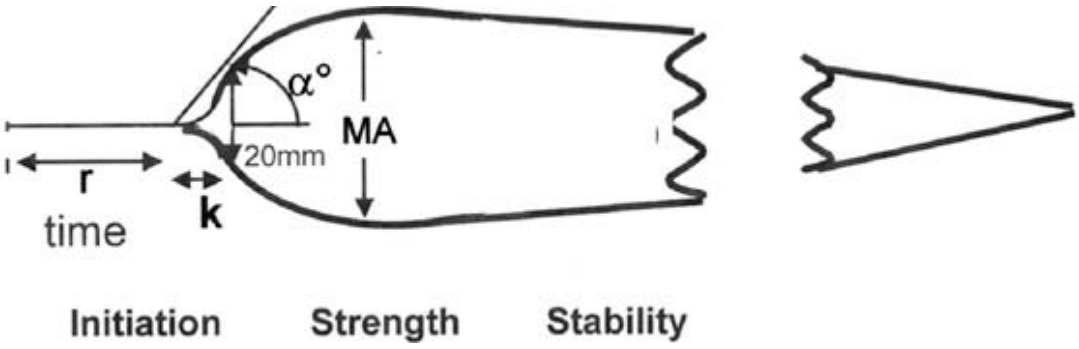

Figure 1: Components of the thromboelastograph. r: reaction time. k: clotting time. MA: maximal amplitude. (Modified from SV Mallett and DJA Cox. Thromboelastography. Br J Anaesth, 1992; 69(3):307–313, Figure 2. Reproduced by permission of Oxford University Press/British Journal of Anaesthesia).

Figure 2: Pattern recognition chart of TEG tracings, compiled from human research (from Haemoscope Corporation). R, reaction time, K, clotting time; MA, maximal amplitude; t-PA, tissue plasminogen activator; DIC, disseminated intravascular coagulation.

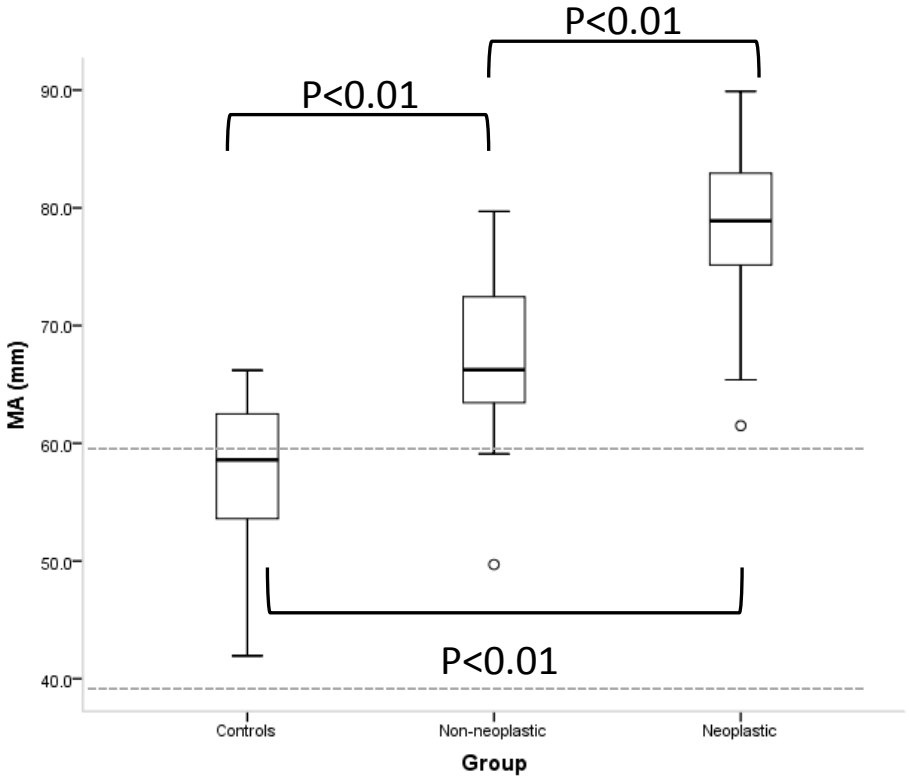

Figure 3: Box plot of MA for the three different groups. The plot clearly depicts the increasing MA trend between control, non-neoplastic and neoplastic cases. For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the upper and lower quartiles. Outlier values between 1.5 and 3 times the IQR are denoted as open circles. Reference interval (-----)

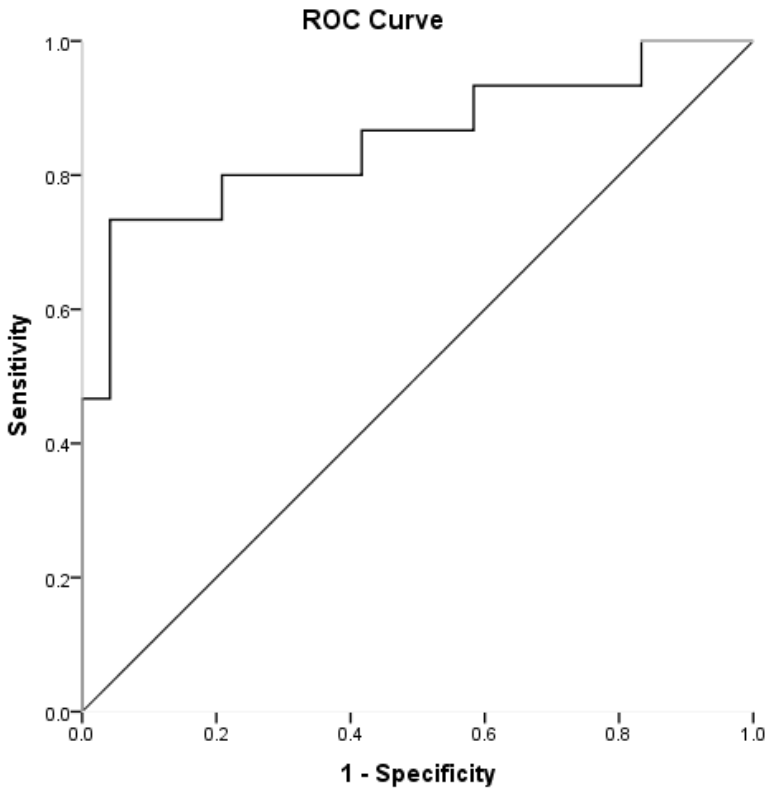

Figure 4: Receiver-operator curve (ROC) for differentiation of neoplastic transformation with a MA value of >76 mm. A sensitivity of 73% and specificity of 96% is obtained. Area under the curve is 0.853.

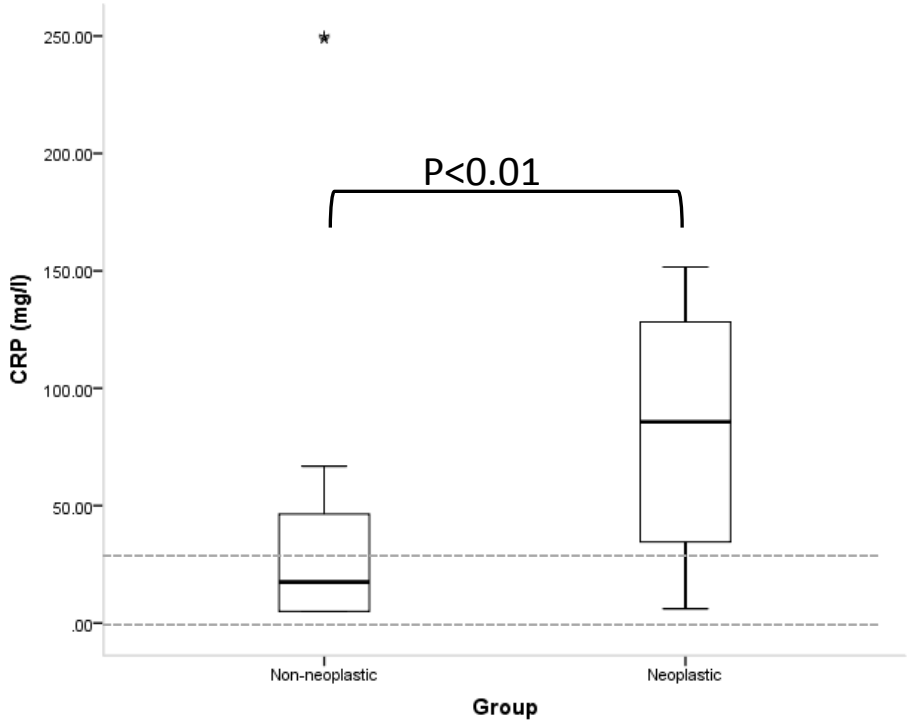

Figure 5: Box plot of CRP for the two infected groups. For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the upper and lower quartiles. Outlier values greater than 3 times the IQR are denoted as an asterix. Reference interval (-----)

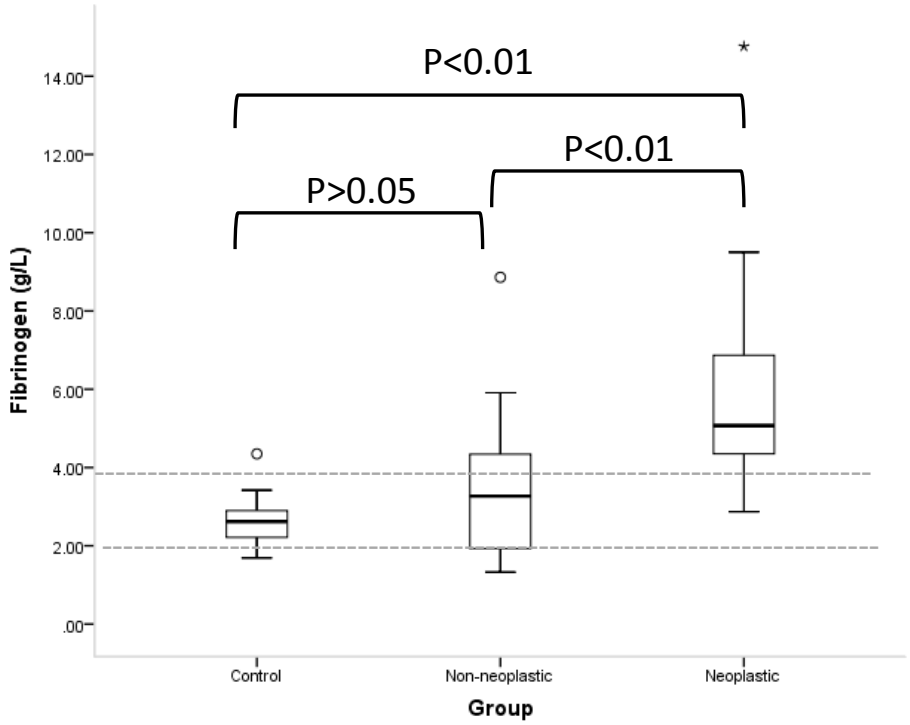

Figure 6: Box plot of fibrinogen for the three different groups groups. For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the upper and lower quartiles. Outlier values between 1.5 and 3 times the IQR are denoted as open circles and outlier values greater than 3 times the IQR are denoted as an asterix. Reference interval (-----)

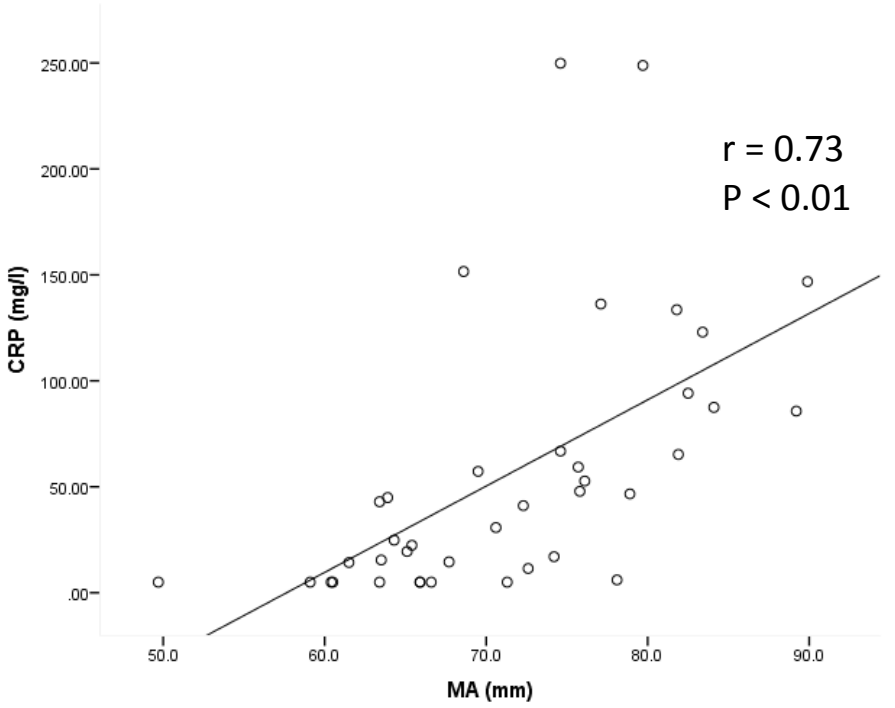

Figure 7: Scatter plot and positive linear correlation between MA and CRP for all infected animals. The solid line depicts the linear correlation while the open circles represent each case. The effect of outliers on the Spearman's correlation coefficient are less than would be seen if the data was parametric.

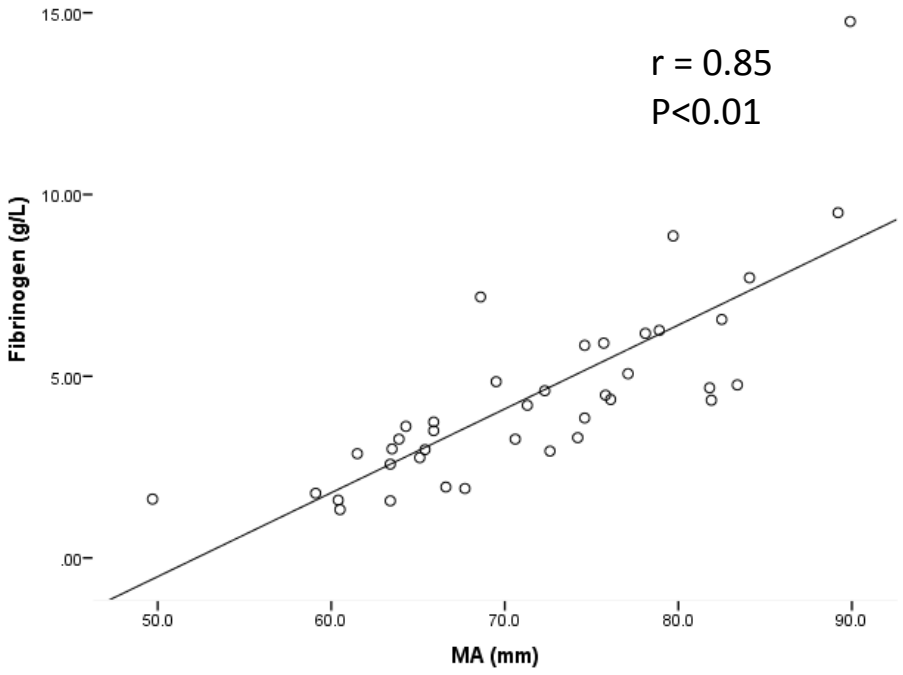

Figure 8: Scatter plot and positive linear correlation between MA and fibrinogen for all infected animals. The solid line depicts the linear correlation while the open circles represent each case. Fewer outliers are present in this scatter plot, and the correlation coefficient is stronger than with CRP, partly due to the inherent increase in MA fibrinogen causes.

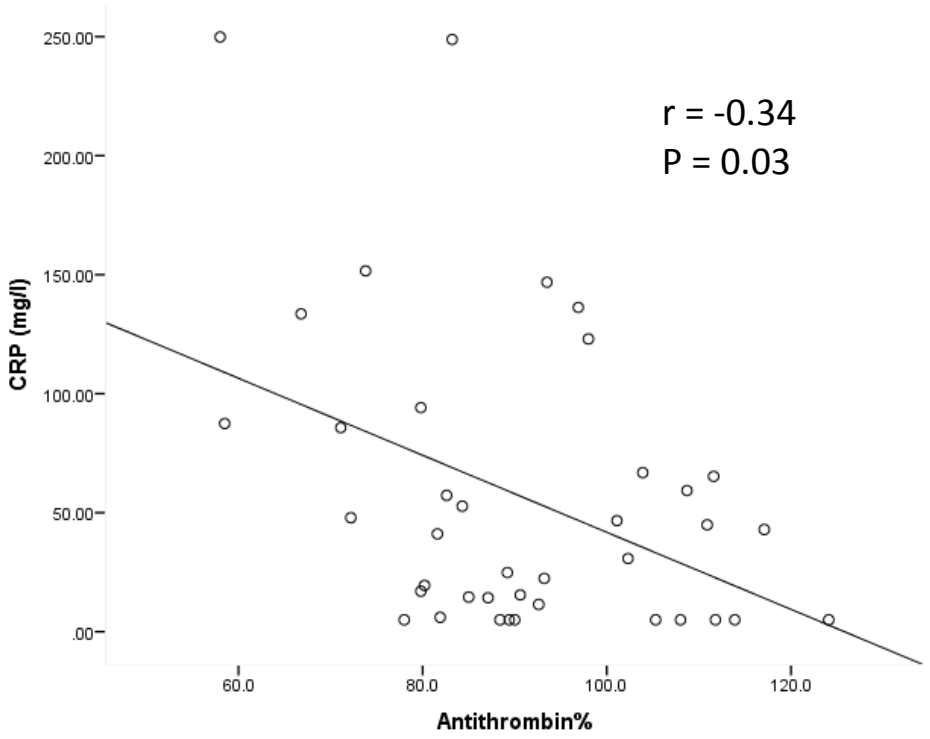

Figure 9: Scatter pot and negative linear correlation between CRP and AT activity for all infected animals. The solid line depicts the linear correlation while the open circles represent each case.

REFERENCES

1. Bailey WS. Spirocerca Lupi: A Continuing Inquiry. J Parasitol 1972; 58:3-22.

2. Mazaki-Tovi M, Baneth G, Aroch I, et al. Canine Spirocercosis: Clinical, Diagnostic, Pathologic, and Epidemiologic Characteristics. Vet Parasitol 2002; 107:235-250.

3. van der Merwe LL, Kirberger RM, Clift S, et al. Spirocerca Lupi Infection in the Dog: A Review. Vet J 2008; 176:294-309.

4. Dvir E, Clift SJ, Williams MC. Proposed Histological Progression of the Spirocerca Lupi-Induced Oesophageal Lesion in Dogs. Vet Parasitol 2010; 168:71-77.

5. Seibold HR, Bailey WS, Hoerlein BF, et al. Observations on the Possible Relation of Malignant Esophageal Tumors and Spirocerca Lupi Lesions in the Dog. Am J Vet Res 1955; 18:5-14.

6. Dvir E, Kirberger RM, Malleczek D. Radiographic and Computed Tomographic Changes and Clinical Presentation of Spirocercosis in the Dog. Vet Radiol Ultrasoun 2001; 42:119-129.

7. Dvir E, Kirberger RM, Mukorera V, et al. Clinical Differentiation between Dogs with Benign and Malignant Spirocercosis. Vet Parasitol 2008; 155:80-88.

8. Dvir E, Clift SJ, Merwe LL. Review: Challenges in Diagnosis and Treatment of Canine Spirocercosis. Isr J Vet Med 2010; 65:5-10.

9. Christie J, Schwan EV, Bodenstein LL, et al. The Sensitivity of Direct Faecal Examination, Direct Faecal Flotation, Modified Centrifugal Faecal Flotation and Centrifugal sedimentation/flotation in the Diagnosis of Canine Spirocercosis. J S Afr Vet Assoc 2011; 82:71-75.

10. Bailey WS, Cabrera DJ, Diamond DL. Beetles of the Family Scarabaeidae as Intermediate Hosts for Spirocerca Lupi. J Parasitol 1963; 49:485-488.

11. Ranen E, Lavy E, Aizenberg I, et al. Spirocercosis-Associated Esophageal Sarcomas in Dogs: A Retrospective Study of 17 Cases (1997-2003). Vet Parasitol 2004; 119:209-221.

12. Ridgway RL, Suter PF. Clinical and Radiographic Signs in Primary and Metastatic Esophageal Neoplasms of the Dog. J Am Vet Med Assoc 1979;174:700-704.

13. Porta C, Larghi P, Rimoldi M, et al. Cellular and Molecular Pathways Linking Inflammation and Cancer. Immunobiology 2009; 214:761-777.

14. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-Related Inflammation. Nature 2008;454:436-444.

15. Lu H, Ouyang W, Huang C. Inflammation, a Key Event in Cancer Development. Mol Cancer Res: MCR 2006; 4:221-233.

16. Shacter E, Weitzman SA. Chronic Inflammation and Cancer. Oncology 2002; 16:217; Feb-226.

17. Dvir E, Schoeman JP, Clift SJ, et al. Immunohistochemical Characterization of Lymphocyte and Myeloid Cell Infiltrates in Spirocercosis-Induced Oesophageal Nodules. Parasite Immunol 2011; 33:545-553.

18. Mazaki-Tovi M, Baneth G, Aroch I, et al. Canine Spirocercosis: Clinical, Diagnostic, Pathologic, and Epidemiologic Characteristics. Vet Parasitol 2002; 107:235-250.

19. Mukorera V, Dvir E, van der Merwe LL, Goddard A. Serum C-Reactive Protein Concentration in Benign and Malignant Canine Spirocercosis. J Vet Intern Med 2011; 25:963-966.

20. Dvir E, Mellanby RJ, Kjelgaard-Hansen M, Schoeman JP. Plasma IL-8 concentrations are increased in dogs with spirocercosis. Vet Parasitol 2012; 190; 185–190

21. Moss SF, Blaser MJ. Mechanisms of Disease: Inflammation and the Origins of Cancer. Nat Clin Pract Oncol 2005; 2:90-97.

22. Vennervald BJ, Polman K. Helminths and Malignancy. Parasite Immunol 2009; 31:686-696.

23. Mulvenna J, Sripa B, Brindley PJ, et al. The Secreted and Surface Proteomes of the Adult Stage of the Carcinogenic Human Liver Fluke Opisthorchis Viverrini. Proteomics 2010; 10:1063-1078.

24. Smout MJ, Laha T, Mulvenna J, et al. A Granulin-Like Growth Factor Secreted by the Carcinogenic Liver Fluke, Opisthorchis Viverrini, Promotes Proliferation of Host Cells. PLoS Pathogens 2009; 5:e1000611.

25. Kaewpitoon N, Kaewpitoon SJ, Pengsaa P, Sripa B. Opisthorchis Viverrini: The Carcinogenic Human Liver Fluke. World J Gastroenterol 2008; 14:666-674.

26. Curiel TJ, Coukos G, Zou L, et al. Specific Recruitment of Regulatory T Cells in Ovarian Carcinoma Fosters Immune Privilege and Predicts Reduced Survival. Nat Med 2004; 10:942-949.

27. Liyanage UK, Moore TT, Joo HG, et al. Prevalence of Regulatory T Cells is Increased in Peripheral Blood and Tumor Microenvironment of Patients with Pancreas Or Breast Adenocarcinoma. J Immunol 2002; 169:2756-2761.

28. Wolf AM, Wolf D, Steurer M, et al. Increase of Regulatory T Cells in the Peripheral Blood of Cancer Patients. Clin Cancer Res 2003; 9:606-612.

29. Woo EY, Chu CS, Goletz TJ, et al. Regulatory CD4(+)CD25(+) T Cells in Tumors from Patients with Early-Stage Non-Small Cell Lung Cancer and Late-Stage Ovarian Cancer. Cancer Res 2001; 61:4766-4772.

30. Horiuchi Y, Tominaga M, Ichikawa M, et al. Increase of Regulatory T Cells in the Peripheral Blood of Dogs with Metastatic Tumors. Microbiol Immunol 2009; 53:468-474.

31. Beyer M, Schultze JL. Regulatory T Cells in Cancer. Blood 2006; 108:804-811.

32. Levi M, Poll T. Inflammation and Coagulation. Crit Care Med 2010; 38:S26-S34.

33. Otto CM, Rieser TM, Brooks MB, Russell MW. Evidence of Hypercoagulability in Dogs with Parvoviral Enteritis. J Am Vet Med Assoc 2000; 217:1500-1504.

34. Wiinberg B, Jensen AL, Johansson PI, et al. Thromboelastographic Evaluation of Hemostatic Function in Dogs with Disseminated Intravascular Coagulation. J Vet Intern Med 2008; 22:357-365.

35. Wiinberg B, Jensen AL, Rozanski E, et al. Tissue Factor Activated Thromboelastography Correlates to Clinical Signs of Bleeding in Dogs. Vet J 2009; 179:121-129.

36. Donahue SM, Otto CM. Thromboelastography: A Tool for Measuring Hypercoagulability, Hypocoagulability, and Fibrinolysis. J Vet Emerg Crit Care 2005; 15:9-16.

37. Kristensen AT, Wiinberg B, Jessen LR, et al. Evaluation of Human Recombinant Tissue Factor-Activated Thromboelastography in 49 Dogs with Neoplasia. J Vet Intern Med 2008; 22:140-147.

38. Wagg CR, Boysen SR, Bedard C. Thrombelastography in Dogs Admitted to an Intensive Care Unit. Vet Clin Pathol 2009; 38:453-461.

39. Sinnott VB, Otto CM. Use of Thromboelastography in Dogs with Immune-Mediated Hemolytic Anemia: 39 Cases (2000-2008). J Vet Emerg & Crit Care 2009; 19:484-488.

40. Johnson LR, Lappin MR, Baker DC. Pulmonary Thromboembolism in 29 Dogs: 1985-1995. J Vet Intern Med 1999; 13:338-345.

41. LaRue MJ, Murtaugh RJ. Pulmonary Thromboembolism in Dogs: 47 Cases (1986-1987). J Am Vet Med Assoc 1990; 197:1368-1372.

42. Madewell BR, Feldman BF, O'Neill S. Coagulation Abnormalities in Dogs with Neoplastic Disease. Thromb Haemost 1980; 44:35-38.

43. Rickles FR. Mechanisms of Cancer-Induced Thrombosis in Cancer. Pathophysiology of Haemost Thromb 2006;35:103-110.

44. Esmon CT, Fukudome K, Mather T, et al. Inflammation, Sepsis, and Coagulation. Haematologica 1999; 84:254-259.

45. Levi M, van der Poll T, ten Cate H. Tissue Factor in Infection and Severe Inflammation. Semin Thromb Hemost 2006; 32:33-39.

46. Levi M, ten Cate H, Bauer KA, et al. Inhibition of Endotoxin-Induced Activation of Coagulation and Fibrinolysis by Pentoxifylline Or by a Monoclonal Anti-Tissue Factor Antibody in Chimpanzees. J Clin Invest 1994; 93:114-120.

47. Taylor FB,Jr, Chang A, Ruf W, et al. Lethal E. Coli Septic Shock is Prevented by Blocking Tissue Factor with Monoclonal Antibody. Circ Shock 1991; 33:127-134.

48. van der Poll T, Levi M, Hack CE, et al. Elimination of Interleukin 6 Attenuates Coagulation Activation in Experimental Endotoxemia in Chimpanzees. J Exp Med 1994; 179:1253-1259.

49. van Deventer SJ, Buller HR, ten Cate JW, et al. Experimental Endotoxemia in Humans: Analysis of Cytokine Release and Coagulation, Fibrinolytic, and Complement Pathways. Blood 1990; 76:2520-2526.

50. Boermeester MA, van Leeuwen PA, Coyle SM, et al. Interleukin-1 Blockade Attenuates Mediator Release and Dysregulation of the Hemostatic Mechanism during Human Sepsis. Arch Surg 1995; 130:739-748.

51. van der Poll T, de Jonge E, Levi M. Regulatory Role of Cytokines in Disseminated Intravascular Coagulation. Semin Thromb Hemost 2001; 27:639-651.

52. Levi M, van der Poll T, ten Cate H, van Deventer SJ. The Cytokine-Mediated Imbalance between Coagulant and Anticoagulant Mechanisms in Sepsis and Endotoxaemia. Eur J Clin Invest 1997; 27:3-9.

53. Esmon CT. The Regulation of Natural Anticoagulant Pathways. Science 1987; 235:1348-1352.

54. Levi M, Keller TT, Gorp E, Cate H. Infection and Inflammation and the Coagulation System. Cardiovasc Res 2003; 60:26-39.

55. Ganrot K. Plasma Protein Response in Experimental Inflammation in the Dog. Res Exp Med 1973; 161:251-261.

56. Gascon M, Belenguer-Laita S, Acena MC, et al. Study of Coagulation Profile and Blood Proteins in Dogs with Filariasis. Medicina Veterinaria 1993; 10:169-172.

57. Vecina JF, Patricio RF, Carlini PC. The Importance of Plasma Fibrinogen in the Identification of Dogs' Inflammatory Processes. Ciencia Veterinaria nos Tropicos 2006; 9:31-35.

58. Yamamoto S, Shida T, Miyaji S, et al. Changes in Serum C-Reactive Protein Levels in Dogs with various Disorders and Surgical Traumas. Vet Res Commun 1993; 17:85-93.

59. Tecles F, CaldÃn M, Zanella A, et al. Serum Acute Phase Protein Concentrations in Female Dogs with Mammary Tumors. J Vet Diag Invest 2009; 21:214-219.

60. Paltrinieri S. Early Biomarkers of Inflammation in Dogs and Cats: The Acute Phase Proteins. 2007; 31:125-129.

61. Nakamura M, Takahashi M, Ohno K, et al. C-Reactive Protein Concentration in Dogs with various Diseases. J Vet Med Sci 2008; 70:127-131.

62. McCann TM, Ridyard AE, Else RW, Simpson JW. Evaluation of Disease Activity Markers in Dogs with Idiopathic Inflammatory Bowel Disease. J Small Anim Pract 2007; 48:620-625.

63. Koster LS, Schoor M, Goddard A, et al. C-Reactive Protein in Canine Babesiosis Caused by Babesia Rossi and its Association with Outcome. J S Afr Vet Assoc 2009; 80:87-91.

64. Griebsch C, Arndt G, Raila J, et al. C-Reactive Protein Concentration in Dogs with Primary Immune-Mediated Hemolytic Anemia. Vet Clin Pathol 2009; 38:421-425.

65. Eckersall PD, Bell R. Acute Phase Proteins: Biomarkers of Infection and Inflamm. Vet Med. 2010; 185:23-27.

66. Cheng T, Mathews KA, Abrams-Ogg A, Wood RD. Relationship between Assays of Inflammation and Coagulation: A Novel Interpretation of the Canine Activated Clotting Time. Can J Vet Res 2009; 73:97-102.

67. Kjelgaard-Hansen M, Jensen AL, Kristensen AT. Evaluation of a Commercially Available Human C-Reactive Protein (CRP) Turbidometric Immunoassay for Determination of Canine Serum CRP Concentration. Vet Clin Pathol 2003; 32:81-87.

68. Eckersall PD, Conner JG, Harvie J. An Immunoturbidimetric Assay for Canine C-Reactive Protein. Vet Res Commun 1991; 15:17-24.

69. Kirberger RM, Zambelli A. Imaging Diagnosis - Aortic Thromboembolism Associated with Spirocercosis in a Dog. Vet Radiol Ultrasoun 2007; 48:418-420.

70. Gal A, Kleinbart S, Aizenberg Z, Baneth G. Aortic Thromboembolism Associated with Spirocerca Lupi Infection. Vet Parasitol 2005; 130:331-335.

71. Alvarenga J, Saliba AM. Iliac Embolism in a Dog. Mod Vet Pract 1971; 52:37-38.

72. Kuzi S, Segev G, Haruvi E, Aroch I. Plasma Antithrombin Activity as a Diagnostic and Prognostic Indicator in Dogs: A Retrospective Study of 149 Dogs. J Vet Intern Med 2010; 24:587-596.

73. Green RA. Clinical Implications of Antithrombin III Deficiency in Animal Diseases. Compendium 1984; 6:537-546.

74. Grindem CB, Breitschwerdt EB, Corbett WT, et al. Thrombocytopenia Associated with Neoplasia in Dogs. J Vet Intern Med 1994; 8:400-405.

75. Botsch V, Kuchenhoff H, Hartmann K, Hirschberger J. Retrospective Study of 871 Dogs with Thrombocytopenia. Vet Rec 2009; 164:647-651.

76. Hogan DF. Markers of Thrombotic Risk & Future Directions in Therapeutics. ACVIM proceedings, Montréal, Québec, Canada, June 3-6, 2009.

77. Dunn M. Minimally Invasive Approaches to Thrombosis in Small Animals. ACVIM proceedings, Montréal, Québec, Canada, June 3-6, 2009.

78. Maruyama H, Watari T, Miura T, et al. Plasma Thrombin-Antithrombin Complex Concentrations in Dogs with Malignant Tumours. Vet Rec 2005; 156:839-840.

79. Mischke R, Freund M, Leinemann-Fink T, et al. Changes in Hemostasis of Dogs with Acute Lymphoblastic Leukemia. Berl Munch Tierarztl Wochenschr 1998; 111:53-59.

80. Stockhaus C, Kohn B, Rudolph R, et al. Correlation of Haemostatic Abnormalities with Tumour Stage and Characteristics in Dogs with Mammary Carcinoma. J Small Anim Pract 1999; 40:326-331.

81. Van Winkle TJ, Bruce E. Thrombosis of the Portal Vein in Eleven Dogs. Vet Pathol 1993; 30:28-35.

82. Respess M, O'Toole TE, Taeymans O, et al. Portal Vein Thrombosis in 33 Dogs: 1998-2011. J Vet Intern Med 2012; 26:230-237.

83. Collins PW, Macchiavello LI, Lewis SJ, et al. Global Tests of Haemostasis in Critically Ill Patients with Severe Sepsis Syndrome Compared to Controls. Br J Haematol 2006; 135:220-227.

84. Wiinberg B, Jensen AL, Kjelgaard-Hansen M, et al. Study on Biological Variation of Haemostatic Parameters in Clinically Healthy Dogs. Vet J 2007; 174:62-68.

85. Ettinger SJ, Feldman EC. Textbook of Veterinary Internal Medicine: Diseases of the Dog and the Cat, 7th ed. St. Louis, Mo.: Elsevier Saunders; 2010:2218, lxxi.

86. Hoffman M, Monroe DM,3rd. A Cell-Based Model of Hemostasis. Thromb & Haemost 2001; 85:958-965.

87. Smith SA. The Cell-Based Model of Coagulation. J Vet Emerg Crit Care 2009; 19:3-10.

88. Chandler WL. The Thromboelastography and the Thromboelastograph Technique. Semin Thromb Hemost 1995; 21:1-6.

89. Wiinberg B, Jensen AL, Rojkjaer R, et al. Validation of Human Recombinant Tissue Factor-Activated Thromboelastography on Citrated Whole Blood from Clinically Healthy Dogs. Vet Clin Pathol 2005; 34:389-393.

90. Bowbrick VA, Mikhailidis DP, Stansby G. Influence of Platelet Count and Activity on Thromboelastography Parameters. Platelets 2003;14:219-224.

91. Camenzind V, Bombeli T, Seifert B, et al. Citrate Storage Affects Thrombelastograph Analysis. Anesthesiol 2000; 92:1242-1249.

92. Palsgaard-Van Lue A, Jensen AL, Strom H, Kristensen AT. Comparative Analysis of Haematological, Haemostatic, and Inflammatory Parameters in Canine Venous and Arterial Blood Samples. Vet J 2007; 173:664-668.

93. McMichael MA, Smith SA. Viscoelastic Coagulation Testing: Technology, Applications, and Limitations. Vet Clin Pathol 2011; 40:140-153.

94. Kaufmann CR, Dwyer KM, Crews JD, et al. Usefulness of Thrombelastography in Assessment of Trauma Patient Coagulation. J Trauma 1997; 42:716-720.

95. Tuman KJ, Spiess BD, McCarthy RJ, Ivankovich AD. Effects of Progressive Blood Loss on Coagulation as Measured by Thrombelastography. Anesth Analg 1987; 66:856-863.

96. Oshita K, Az-ma T, Osawa Y, Yuge O. Quantitative Measurement of Thromboelastography as a Function of Platelet Count. Anesth & Analg 1999; 89:296-299.

97. Bateman SW, Mathews KA, Abrams-Ogg AC, et al. Evaluation of the Effect of Storage at -70 Degrees C for Six Months on Hemostatic Function Testing in Dogs. Can J Vet Res 1999; 63:216-220.

98. Aziz N, Fahey JL, Detels R, Butch AW. Analytical Performance of a Highly Sensitive C-Reactive Protein-Based Immunoassay and the Effects of Laboratory Variables on Levels of Protein in Blood. Clin Diagn Lab Immun 2003; 10:652-657.

99. Lavy E, Aroch I, Bark H, et al. Evaluation of Doramectin for the Treatment of Experimental Canine Spirocercosis. Vet Parasitol 2002; 109:65-73.

100. McCrath DJ, Cerboni E, Frumento RJ, et al. Thromboelastography Maximum Amplitude Predicts Postoperative Thrombotic Complications Including Myocardial Infarction. Anesth Analg 2005; 100:1576-1583.

101. Johnson L, Boon J, Orton EC. Clinical Characteristics of 53 Dogs with Doppler-Derived Evidence of Pulmonary Hypertension: 1992-1996. J Vet Intern Med 1999; 13:440-447.

102. Francis JL, Biggerstaff J, Amirkhosravi A. Hemostasis and Malignancy. Semin Thromb Hemost 1998; 24:93-109.

103. Kang YG, Martin DJ, Marquez J, et al. Intraoperative Changes in Blood Coagulation and Thrombelastographic Monitoring in Liver Transplantation. Anesth Analg 1985; 64:888-896.

104. Caprini JA, Zuckerman L, Cohen E, et al. The Identification of Accelerated Coagulability. Thromb Res 1976; 9:167-180.

105. Dhainaut JF, Marin N, Mignon A, Vinsonneau C. Hepatic Response to Sepsis: Interaction between Coagulation and Inflammatory Processes. Crit Care Med 2001; 29:S42-7.

106. de Laforcade AM, Freeman LM, Shaw SP, et al. Hemostatic Changes in Dogs with Naturally Occurring Sepsis. J Vet Intern Med 2003; 17:674-679.