Thromboelastographic platelet mapping in dogs with complicated *Babesia rossi* infection

By

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PROJECT TITLE	**Platelet mapping as part of modified thromboelastography as a measure of plateletfunction in canine babesiosis caused by Babesia rossi**
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KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 30 October 2015
CHAIRMAN: UP Animal Ethics Committee	Signature

“The great art of life is sensation, to feel that we exist, even in pain.”- Lord Byron

Table of contents

Acknowledgements... ix

List of tables………………………………………………………………... x

List of figures……………………………………………………………… xi

List of appendices………………………………………………………….... xii

List of abbreviations……………………………………………………..... xiii

Summary………………………………………………………………………….. 1

Chapter 1: Introduction………………………………………………………… 2

1.1 Background…………………………………………………………… 2

1.2 Hypotheses…………………………………………………………… 3

1.3 Objectives…………………………………………………………….. 3

1.4 Benefits arising from this project……………………………………. 4

Chapter 2: Literature review……………………………………………………... 5

2.1 Babesiosis…………………………………………………………….. 5

2.2 Pathogenesis of babesiosis………………………………………… 5

2.3 Coagulopathy in babesiosis………………………………………… 6

2.4 The effect of inflammation on platelet activation………………….. 8

2.5 Mechanisms of platelet activation and the role of the platelet in inflammation……………………………………………………………… 9

2.5.1 Mechanisms of platelet activation……………………….. 9

2.5.2 The role of the platelet in inflammation…………………. 12

2.6 Platelet mapping as part of modified thromboelastography…….. 14

2.6.1 Background……………………………………………… 14

2.6.2 Analytical principles…………………………………….. 16

2.7 Platelet mapping in medical practice……………………………… 17

2.7.1 Monitoring antiplatelet therapy…………………………… 18

2.7.2 Platelet mapping as a platelet function test……………... 18

2.8 Platelet mapping in veterinary medicine……………………………. 19

2.8.1 Potential applications of platelet mapping in veterinary medicine……………………………………………………………. 20

Chapter 3: Materials and methods 22

3.1 Experimental design…………………………………………………. 22

3.1.1 Study design……………………………………………….. 22

3.1.2 Study population……………………………………………. 22

3.2 Experimental procedure…………………………………………….. 23

3.2.1 Sample collection…………………………………………... 23

3.2.2 Assay methodologies……………………………………… 24

3.3 Observations………………………………………………………….. 26

3.4 Statistical analysis……………………………………………………. 26

Chapter 4: Results………………………………………………………………. 27

4.1 Animals……………………………………………………………… 27

4.2 Thromboelastographic platelet mapping analysis………............... 27

4.3 Haematological variables……………………………………………. 28

4.4 Plasma fibrinogen concentration and antithrombin activity………. 28

4.5 Correlation analysis………………………………………………... 28

Chapter 5: Discussion……………………………………………………….. 36

Chapter 6: Conclusions……………………………………………………… 43

References………………………………………………………………………... 44

Appendix documents……………………………………………………… 50

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List of tables

Table 1: Descriptive statistics of TEG-PM and haematology variables for the *Babesia*-infected and healthy control dogs.

List of figures

Figure 1: Signalling pathways in platelet activation.

Figure 2: Platelet-independent and platelet-dependent leukocyte recruitment to sites of injury.

Figure 3: Examples of A) TEG and B) TEG-PM tracings.

Figure 4: Dot plot of the TEG-PM variable $MA_{Thrombin}$ of the *Babesia*-infected group compared to the control group.

Figure 5: Dot plot of the TEG-PM variable MA_{Fibrin} of the *Babesia*-infected group compared to the control group.

Figure 6: Dot plot of the TEG-PM variable MA_{ADP} of the *Babesia*-infected group compared to the control group.

Figure 7: Dot plot of the TEG-PM variable MA_{AA} of the *Babesia*-infected group compared to the control group.

Figure 8: Representative TEG-PM tracing from a healthy control dog.

Figure 9: Representative TEG-PM tracing from a dog with complicated *B. rossi* infection.

Figure 10: Schematic diagrams depicting the interaction between fibrinogen and platelets to produce the maximum clot strength in three different scenarios: A) Normal PLT and fibrinogen concentration, B) Low PLT and fibrinogen concentrations, C) Low PLT and high fibrinogen concentrations.

List of appendices

Appendix A: Information sheet on canine babesiosis

Appendix B: Form for informed consent

Appendix C: Information sheet for control dogs

Appendix D: Details of study subjects

Appendix E: Data capture form

List of abbreviations

AA	Arachidonic acid
ACP	Acepromazine
ADP	Adenosine diphosphate
APP	Acute phase protein
aPTT	Activated partial thromboplastin time
AT	Antithrombin
CBC	Complete blood count
CRP	C-reactive protein
CV	Coefficient of variation
DIC	Disseminated intravascular coagulation
EDTA	Ethylenediaminetetraacetic acid
FF	Functional fibrinogen
G value	Clot firmness
GP	Glycoprotein
HAC	Hyperadrenocorticism
HCT	Haematocrit
ICAM	Intracellular adhesion molecule
IL	Interleukin
IMHA	Immune-mediated haemolytic anaemia
Inhib_ADP/AA	Percentage agonist-related inhibition
IQR	Interquartile range
MA	Maximum amplitude
MCE	Maximum clot elasticity
MODS	Multiple organ dysfunction syndrome
MPC	Mean platelet component concentration
MPM	Mean platelet mass
MPV	Mean platelet volume
OVAH	Onderstepoort Veterinary Academic Hospital

PAF	Platelet activating factor
PAR	Protease-activated receptor
PCR	Polymerase chain reaction
PCT	Plateletcrit
PEth	Phosphatidylethanolamine
PFA-100	Platelet function analyser-100
PLA	Platelet-leukocyte aggregate
PLT	Platelet count
PPA	Platelet-platelet aggregate
PSGL-1	P-selectin glycoprotein ligand-1
PSer	Phosphatidylserine
PT	Prothrombin time
RLB	Reverse line blot
ROTEM	Rotational thromboelastometry
SAA	Serum Amyloid A
sICAM	Soluble intracellular adhesion molecule
SIRS	Systemic inflammatory response syndrome
TEG	Thromboelastography
TEG-PM	Thromboelastographic platelet mapping
TF	Tissue factor
TM	Thrombomodulin
TNF	Tumour necrosis factor
TT	Thrombin time
TxA_2	Thromboxane A_2
vWF	von Willebrand Factor

Summary

Dogs with *Babesia rossi* infection display a normocoagulable thromboelastogram, despite being markedly thrombocytopenic. This is purportedly due to large-scale platelet activation. Thromboelastographic platelet mapping (TEG-PM) evaluates the individual contributions of thrombin, fibrinogen and platelets to clot formation, and may elucidate some of the pathomechanisms of the haemostatic alterations described in this disease. This study investigated potential differences in TEG-PM variables in dogs with complicated *B. rossi* infection compared to healthy controls, and whether these variables correlated with indices of platelet activation. The maximum amplitude (MA) following thrombin generation ($MA_{Thrombin}$) was determined using kaolin-activated TEG. TEG-PM variables included MA following addition of platelet agonists arachidonic acid (MA_{AA}) and adenosine diphosphate (MA_{ADP}), and MA due to fibrin alone (MA_{Fibrin}). In addition, platelet indices and fibrinogen concentration were determined.

Thirteen dogs with complicated *B. rossi* infection and five healthy controls were included. The median MA_{Fibrin} and fibrinogen concentration were significantly higher ($P < 0.01$ for both) and platelet count significantly lower ($P < 0.01$) in the babesiosis group compared to controls. No significant differences were found for $MA_{Thrombin}$ and $MA_{AA/ADP}$. MA_{Fibrin} was positively correlated with fibrinogen concentration ($r_s = 0.735$), mean platelet volume ($r_s = 0.517$) and mean platelet mass ($r_s = 0.498$), and negatively correlated with haematocrit ($r_s = –0.685$), platelet count ($r_s = –0.476$) and plateletcrit ($r_s = –0.479$) ($P < 0.05$ for all). This study suggests that hyperfibrinogenaemia offsets the severe thrombocytopenia associated with *B. rossi* to result in normal thromboelastograms and lack of overt clinical bleeding.

Keywords: Babesia rossi, thromboelastographic platelet mapping, thrombocytopenia, hyperfibrinogenaemia, MA_{Fibrin}

Chapter 1: Introduction

1.1 Background

It is well-established that inflammation and haemostasis are intricately-linked processes, and that an inflammatory response gives rise to a procoagulant state.[1-3] To this end, it has also been demonstrated that the platelet is central to linking the two processes, and that platelet activation occurs during inflammation.[1]

Canine babesiosis is a commonly-encountered tick-borne disease of dogs which is characterised by an overproduction of inflammatory mediators[2,4] as well as haemostatic derangements.[2,4-6] Most strikingly, dogs with babesiosis are often severely thrombocytopenic, yet seldom display evidence of overt haemorrhage.[7] A previous study has shown that dogs with uncomplicated babesiosis caused by *Babesia rossi* infection were normocoagulable using standard tissue factor (TF)-activated thromboelastography (TEG) despite the severe thrombocytopenia.[5] These observations suggest that marked platelet activation occurs secondary to systemic inflammation which may (at least in part) compensate for the severe thrombocytopenia to result in normocoagulable thromboelastograms and a lack of clinical bleeding in these patients.[5] This theory is supported by subsequent studies which confirmed the presence of activated platelets in dogs with *B. rossi* infection based on the presence of known markers of platelet activation, such as increased mean platelet volume (MPV) and mean platelet mass (MPM), as well as the increased presence of platelet-monocyte aggregates.[8,9] What remains to be determined is the receptor-based mechanism by which the inflammatory process causes platelet activation in babesiosis.

A number of different specialised platelet function tests have been described, such as aggregometry and the Platelet Function Analyser-100 (PFA-100), but these methods have several limitations.[10] Thromboelastographic platelet mapping (TEG-PM) is a modification of the TEG that separately evaluates the contributions of thrombin, fibrin and platelet activity to clot formation.[11] This is achieved by measuring platelet reactivity

in response to platelet agonists which mediate platelet activation via specific surface trans-membrane receptors.[11] Such platelet agonists include adenosine diphosphate (ADP), arachidonic acid (AA), and collagen.[12] In addition to providing information regarding the maximum clot strength, measured as the maximum amplitude (MA) due to thrombin activity ($MA_{Thrombin}$), it is possible to detect alterations in specific platelet receptor activity (designated as $MA_{ADP/AA}$)[12] as well as the clot strength due to fibrin(ogen) alone (MA_{Fibrin}). Although primarily used in human medicine to monitor the efficacy of antiplatelet therapy[13], it is believed that TEG-PM may also serve as a platelet function test which may detect altered activity in disease states. By selecting a population of dogs with complicated babesiosis, a disease characterised by notable inflammation, it is thought that any alterations in platelet reactivity would be accentuated.

1.2 Hypotheses

The hypotheses of this study were as follows:

- Expression and/or function of one or both of the AA or ADP platelet receptors would be upregulated in dogs with complicated babesiosis compared to healthy control dogs, and TEG-PM would detect this altered platelet receptor function.
- The $MA_{Thrombin}$ would not differ significantly between the two groups.
- There would be significant differences between the MA_{Fibrin}, MA_{ADP} and MA_{AA}, as well as the corresponding percentage aggregation response to the agonist, in dogs with complicated babesiosis compared to healthy control dogs.
- There would be significant correlations between the TEG-PM variables and indices of platelet activation.

1.3 Objectives

- To determine if there were any significant differences between the TEG-PM variables obtained in dogs with complicated babesiosis compared to healthy control dogs.

- To determine if there were any significant correlations present between any of the TEG-PM variables and indices of platelet activation in dogs with complicated babesiosis.

1.4 Benefits arising from this project

- This study could provide valuable information regarding the pathogenesis of the many life-threatening complications that develop in certain cases of babesiosis. This is particularly relevant for South African conditions, as this country is home to the virulent *B. rossi* parasite.
- By using TEG-PM to detect alterations in individual platelet receptor function, it may become possible to provide more appropriate guidelines for the selection of specific antiplatelet drugs for the management of such cases.
- Findings from this study may serve as a model for other inflammatory diseases in dogs, as well as other species, and may serve as a departure point for further studies evaluating the use of TEG-PM as a platelet function test, as well as for studies evaluating platelet receptor function in veterinary medicine.
- The research conducted serves as partial fulfilment of the principal investigator's MMedVet (Clinical Laboratory Diagnostics) degree.

Chapter 2: Literature review

2.1 Babesiosis

Canine babesiosis is a tick-borne disease caused by the intra-erythrocytic protozoa *B. rossi, B. vogeli, B. gibsoni* and *B. canis.*[14] In South Africa, babesiosis caused by *B. rossi* makes up a significant proportion of the canine caseload.[15–17] In one study, the disease was detected in 12% of canine patients presenting to the Onderstepoort Veterinary Academic Hospital (OVAH), with 31% of these cases deemed sick enough to be admitted for further treatment.[18]

In dogs, the clinical presentation ranges from mild, subclinical illness to severe complicated disease and death, and can have a peracute, acute, subacute and chronic course.[16,19] Canine babesiosis can be classified as either uncomplicated or complicated.[20] In uncomplicated babesiosis, clinical signs are primarily attributable to anaemia secondary to the acute haemolysis of parasitized erythrocytes.[20] Common complications include shock, haemoconcentration, cerebral babesiosis, icterus and hepatopathy, acute kidney injury (AKI), immune-mediated haemolytic anaemia (IMHA), hypoglycaemia, non-cardiogenic pulmonary oedema, pancreatitis and disseminated intravascular coagulation (DIC).[20–22] Most of these complications have a profoundly negative impact on outcome, with a mortality rate of 45% being reported in one study of 84 dogs with complicated babesiosis caused by *B. rossi.*[23]

2.2 Pathogenesis of babesiosis

It has been suggested that the pathogenesis of babesiosis comprises two distinct and parallel processes, namely a haemolytic process and an inflammatory process.[17] Haemolytic anaemia has been attributed to trophozoite intra-erythrocytic binary fission, secondary IMHA, oxidative damage to the erythrocyte and the presence of an unnamed haemolytic factor in the serum.[6] In addition to parasitizing the host's erythrocytes, it has been demonstrated conclusively that an acute phase response occurs following infection with the offending parasite.[2,24-27] This is characterised by an overproduction of pro-inflammatory cytokines[4] and acute phase proteins (APPs), such

as C-reactive protein (CRP)[2,24–26], serum amyloid A (SAA)[24], fibrinogen[26], haptoglobin[24] and α1-acid glycoprotein.[28] These APPs exert widespread systemic effects and are an important component of the innate immune response.[29] In a study evaluating the inflammatory response in dogs experimentally infected with varying doses of *B. canis*-infected erythrocytes, this acute phase response was characterised by systemic manifestations such as fever, leukopenia and thrombocytopenia.[26] More recent work has also shown that excessive concentrations of the pro-inflammatory cytokines interleukin (IL)-6 and monocyte chemoattractant protein-1 may be associated with poor outcome in dogs with *B. rossi* infection.[4]

Systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) represent an unchecked cytokine-mediated perpetuation of the acute phase response, ultimately resulting in autodestructive inflammation with resultant organ damage or dysfunction.[21,24] SIRS and MODS have been documented in complicated babesiosis, with 87% (79/91) of cases found to be SIRS positive in one study.[23] Of the cases that were analysed for the presence of organ damage, 52% (29/56) had single organ damage, and 48% (27/56) had MODS.[23]

2.3 Coagulopathy in babesiosis

A study by de Gopegui *et al* (2007) on 45 dogs naturally infected with *B. canis* demonstrated that an altered haemostasis profile was observed in most dogs.[6] Proposed mechanisms included activation of haemostasis due to the presence of an acute inflammatory response, endothelial damage secondary to haemolysis, and increased interaction of parasitized erythrocytes with endothelial cells.[6] Of these dogs, 20% were classified as having DIC, characterised by thrombocytopenia, increased D-dimer concentration, and prolonged activated partial thromboplastin time (aPTT), prothrombin time (PT) and thrombin time (TT).[6]

The importance of DIC in terms of case outcome was highlighted by Goddard *et al* (2013) in cases naturally infected with *B.* rossi.[22] Dogs that died from *B. rossi* had a more severe consumptive coagulopathy compared to survivors, which was characterised by procoagulant activation (decreased coagulation factor activities, prolonged PT and aPTT), inhibitor consumption (decreased protein C activity) and

increased fibrinolytic activity (increased D-dimer concentration).[22] These dogs did not show any gross signs of haemorrhage, which is indicative of the presence of non-overt DIC.[22] A marked systemic inflammatory response induced by the infection was described as the most likely cause of coagulation activation, which lead to increased consumption of plasma coagulation proteases.[22]

Another study conducted in dogs with *B. canis* sought to further assess the degrees of inflammation, coagulation activation and endothelial cell stimulation in dogs with babesiosis, before and after treatment.[2] Significantly increased concentrations of fibrinogen and CRP were noted before treatment and three days after treatment, which provided evidence of excessive pro-inflammatory activity. Soluble intracellular adhesion molecule (sICAM)-1 was assessed as a marker of endothelial activation, as it is up-regulated during states of inflammation. sICAM-1 concentrations were significantly increased before and after treatment. The activation of endothelial cells, together with the notable acute phase response and the resultant interaction between blood cells and endothelium, were all implicated in the initiation of coagulation.[2]

Thrombocytopenia is a common haematologic abnormality observed in *B. rossi* infection.[7] Kettner *et al* (2003) found that in a population of 1162 dogs diagnosed with *B. rossi* infection, 99% of platelet counts were below the lower reference limit of 250×10^9/L, and 62% of such cases were markedly thrombocytopenic, with platelet counts below 25×10^9/L.[7] Various mechanisms have been proposed, including splenic sequestration and destruction, immune-mediated destruction and consumptive coagulopathy.[2] Increased platelet receptor-mediated adhesion to endothelial cells[2] and various leukocytes[8] could also cause a pseudothrombocytopenia.

Dogs with babesiosis seldom demonstrate overt clinical signs of haemorrhage, even in the face of marked thrombocytopenia. This phenomenon was illustrated by Liebenberg *et al* (2013) in a study evaluating haemostatic abnormalities in dogs with uncomplicated babesiosis caused by *B. rossi*.[5] TEG was used as a global measure of haemostasis to assess the coagulation status in these dogs.[5] There was no significant difference between most of the TEG variables of healthy control dogs and those of the infected dogs, despite the marked thrombocytopenia observed in these cases

compared to the controls. The authors postulated that the systemic inflammation associated with the infection resulted in marked platelet activation, causing these dogs to exhibit a relatively hypercoagulable state when compared to what is normally seen in dogs with such a marked thrombocytopenia.[5] This assertion was confirmed in subsequent studies which showed that dogs with *B. rossi* infection had increased MPV and MPM, as well as higher numbers of platelet-monocyte aggregates compared to controls.[8,9] To date, the precise receptor-mediated mechanisms by which this occurs are unknown. It has also been demonstrated that addition of sufficient quantities of fibrinogen result in normocoagulable rotational thromboelastometry (ROTEM) tracings in the face of severe thrombocytopenia.[30] Since the MA of a thromboelastogram is a function of the dynamic interaction between platelets and fibrin(ogen)[31], the contribution of fibrin(ogen) to haemostasis in dogs with babesiosis warrants further investigation.

Evidence that platelet activation occurs in canine babesiosis is further supported by a study by Žvorc *et al* (2010), which found that MPV was significantly raised in dogs infected with *B. canis* after therapy.[32] Larger platelets are haemostatically more active and have been implicated as a risk factor for developing coronary thrombosis in people.[33] It was assumed that these larger, more immature platelets were produced and released into the circulation during recovery from the thrombocytopenia associated with the disease.[32] Kettner *et al* (2003) observed a similar significant increase in MPV in *B. rossi*-infected dogs compared to control dogs.[7]

2.4 The effect of inflammation on platelet activation

Inflammation and haemostasis are both critical components of the host defence mechanism and inflammation is known to promote haemostasis.[2] During systemic inflammation, inflammatory mediators released by activated mononuclear cells, such as tumour necrosis factors (TNF), IL-1, IL-6, and platelet activating factor (PAF), give rise to a prothrombotic state as a result of TF expression, altered thrombogenicity of endothelial surfaces and platelet activation.[3]

The resting endothelium is primarily antithrombotic and produces thrombomodulin (TM; which modulates the activity of thrombin and facilitates protein C activation), as

well as nitric oxide, prostacyclins and ADPase which inhibit platelet adhesion, aggregation and activation.[3,34,35] Under the influence of inflammatory cytokines[3] and direct interaction with thrombin[34], endothelial cell function is altered. The net effects are down-regulation of TM expression, enhanced TF expression, release of PAF, secretion of von Willebrand factor (vWF) and P-selectin, and expression of other leukocyte adhesion molecules.[3,34] Activated endothelial cells are therefore able to interact with leukocytes, which may result in leukocyte tethering, activation and degranulation with endothelial damage.[1,3] This leads to increased TF exposure, as well as exposure of subendothelial collagen.[3]

Following endothelial injury, platelets readily adhere to subendothelial collagen (facilitated by the concurrent binding of vWF), which acts as a platelet agonist.[3] Increased production of PAF by stimulated endothelial cells and activated leukocytes, as well as thrombin generated as a result of TF exposure, further mediate platelet activation under inflammatory conditions.[3]

These activated platelets undergo changes in their shape, lipid membrane distribution and surface receptors.[34,35] Most notably, the glycoprotein (GP) II_bIII_a is converted to a functional fibrinogen-binding receptor.[3] Fibrinogen is essential for platelet aggregation and the formation of platelet-platelet aggregates (PPAs).[3] Additionally, platelet degranulation following activation results in the fusion of α-granules with the plasma membrane, resulting in surface expression of P-selectin.[3] P-selectin binds to P-selectin glycoprotein ligand-1 (PSGL-1) which is normally expressed on neutrophils and monocytes, facilitating the formation of platelet-leukocyte aggregates (PLAs).[1,3,8]

Both PPAs and PLAs increase the risk of pathological thrombosis and DIC, either by the occlusion of small vessels, or by acting as a nidus for coagulation.[3] This highlights the significant role that platelets play in the development of MODS and DIC.

2.5 Mechanisms of platelet activation and the role of the platelet in inflammation

2.5.1 Mechanisms of platelet activation

Platelet activation is a key step in physiological haemostasis, as well as in the development of pathological thrombosis. Normally, resting platelets are able to

undergo rapid activation, adhesion and aggregation in response to an injurious stimulus, such as a damaged vessel wall.[36] Resting circulating platelets do not express significant amounts of negatively charged phospholipid head groups, such as phosphatidylserine (PSer) and phosphatidylethanolamine (PEth), on their outer membrane leaflets, and this phospholipid membrane asymmetry is tightly regulated.[34-36] Following activation, “scramblase” enzymes shuffle phospholipids between the membrane leaflets, resulting in the appearance of PSer and PEth on the external membrane surface.[34] These head groups may form clusters that support preferential binding of coagulation proteases and assembly of procoagulant complexes, which facilitates the large-scale generation of thrombin on the surface of the activated platelet.[34,35]

Physiological or pathological activation of platelets is mediated by one or more of the following agonists[36]:

- Collagen situated in the subendothelial matrix. Platelet receptors involved include GPVI and $\alpha_2\beta_1$. Under conditions of high shear, binding to vWF via GP1b is required for platelet tethering to collagen.
- ADP with platelet receptors $P2Y_1$ and $P2Y_{12}$. ADP is produced and released by the platelets and serves to recruit additional platelets and amplify platelet reactivity; erythrocytes are also a potential source following injury.
- Thromboxane A_2 (TxA_2) with TxA_2 receptors. Thromboxane is produced by platelets from AA via the cyclo-oxygenase pathway.
- Thrombin with protease-activated receptors (PAR) 1 and 4. Thrombin is the most potent platelet agonist, and is derived either from the prothrombinase complex located on the TF-bearing cell during the initiation phase of coagulation, or from activated platelets during the propagation phase of thrombin generation.

In addition to the above-mentioned classical agonists, recent studies in human literature have elucidated that, as part of the cross-talk between inflammation, immunity and coagulation, various inflammatory mediators and microbial products may also act as atypical platelet agonists or modulators of platelet activation.[37,38] Examples include inflammatory cytokines[39,40], lipopolysaccharides binding to toll-like

receptors[40], direct binding of pathogens to various receptors[37], and red blood cells infected with *Plasmodium* spp.[37]

The binding of an agonist to its receptor initiates intracellular signalling pathways in the platelet through activation of various phospholipase C isoforms, which subsequently generates inositol triphosphate and increases intracellular [Ca^{++}].[36] This leads to platelet degranulation with release of an array of cytokines and an increase in the levels of surface molecules (e.g. P-selectin), exposure of negatively charged phospholipids, as well as an alteration in the $GPII_bIII_a$ receptor for fibrinogen, which is converted from a low-affinity receptor to a high-affinity receptor.[36] The affinities of other adhesion molecules, such as GP1b-V-X (ligand vWF) and GPVI (ligand collagen) for their respective ligands are also increased, which also contributes to enhanced platelet adhesion and aggregation.[38] A simplified schematic representation is given in Figure 1. The net effects of platelet activation are propagation of haemostasis, recruitment and activation of platelets and leukocytes[8], potentiation of aggregation and minimising blood loss through vasoconstriction.[36]

Figure 1: Signalling pathways in platelet activation.

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2.5.2 The role of the platelet in inflammation

Platelet activation and platelet-leukocyte interactions are part of the normal host inflammatory response. In fact, innate immune cells can act as promoters of coagulation in the process of immunothrombosis, which facilitates pathogen immobilisation and clearance.[38] If excessive or dysregulated, these interactions may play a significant role in thrombogenesis, as has been documented in a variety of cardiovascular and thrombotic diseases in people.[1]

During inflammation, cytokines activate the endothelium, which leads to upregulation of a number of surface molecules, such as E-selectin, P-selectin and ICAM-1/2. These surface molecules interact with their respective ligands (PSGL-1, ESL-L and integrin proteins) on circulating leukocytes, which facilitate leukocyte adhesion and activation at sites of inflamed endothelium.[1] Selectins are specifically responsible for initial

leukocyte tethering and priming of the leukocyte integrins for full activation, which work synergistically with various chemokines and cytokines such as PAF to induce full integrin activation and subsequent leukocyte arrest at the site of injury.[1] Activated platelets play a role in immobilising these inflammatory mediators at the relevant site of injury, and contribute to the above interactions by upregulating many endothelial receptors which serve to enhance leukocyte recruitment and activation.[38,41]

Activated platelets in developing thrombi are able to participate in leukocyte recruitment to the site of injury by releasing inflammatory mediators and by P-selectin-mediated leukocyte tethering and integrin priming, as shown in Figure 2. Activated platelets bound to activated endothelium and subendothelial collagen provide an abundant source of P-Selectin for leukocyte binding, as well as for additional platelet recruitment and binding.[1,38] Similar to the previous process, platelet-derived mediators such as PAF and chemokines are required for full leukocyte arrest and activation.[1] Following leukocyte-platelet adhesion, the two cell populations are able to participate in a mutualistic enhanced transcellular metabolism, resulting in increased production of pro-inflammatory lipids which augment the pre-existing inflammatory milieu.[1] Activated leukocytes in turn release additional mediators which can back-activate platelets and promote additional thrombin generation.[1]

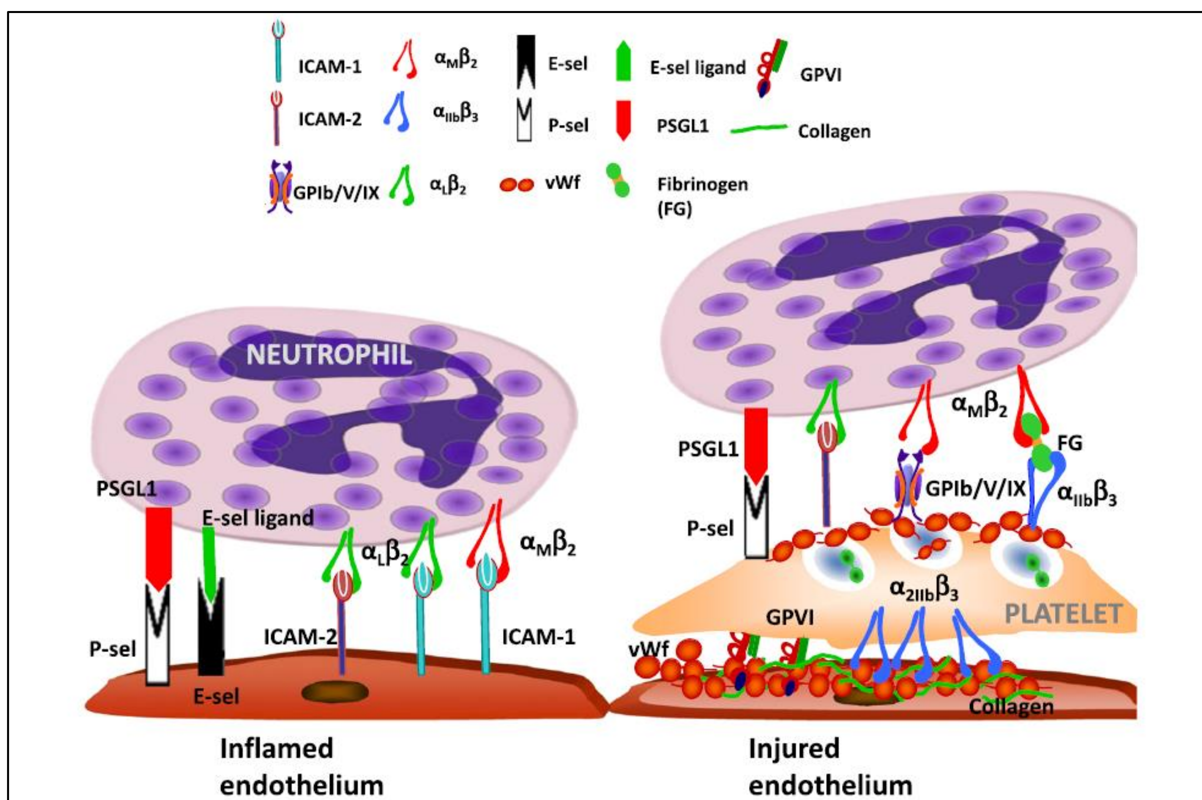

Figure 2: Platelet-independent and platelet-dependent leukocyte recruitment to sites of injury.

(Reprinted from Thrombosis Research 131, M Ghasemzadeh, E Hosseini, Platelet-leukocyte crosstalk: Linking proinflammatory responses to procoagulant state, 191–197, © Elsevier Ltd. 2013, with permission from Elsevier.)

2.6 Platelet mapping as part of modified thromboelastography

2.6.1 Background

Laboratory evaluation of platelet function has many challenges.[42] These include the limited *ex vivo* viability of platelets, the complexity of platelet activation responses, as well as the fact that both blood collection and sample preparation may in themselves cause platelet activation and may obscure the accuracy of results obtained.[42] Various platelet function tests are currently in use in medical and veterinary practice.[10] These include gold standard measures of platelet function, such as turbidimetric and impedance aggregometry, flow cytometry, and specialised point-of-care instruments, specifically the PFA-100.[10] Unfortunately, these methods have several drawbacks. Technical and financial constraints preclude the usefulness of aggregometry methods and flow cytometry for everyday clinical practice, whereas the accuracy of results

obtained by the PFA-100 is questionable in patients with anaemia and thrombocytopenia.[10]

Standard coagulation screening tests, such as PT, aPTT, TT and clotting factor activity, only evaluate the contributions of the plasma-based enzymes which participate in coagulation and exclude the roles of cells, including platelets.[22] Various platelet indices, such as MPV, MPM, platelet distribution width, plateletcrit (PCT) and mean platelet component concentration (MPC), have been described as indirect markers of platelet function and activation, and can be determined using modern haematology analysers, such as the ADVIA 120/2120 haematology platform (Siemens, Munich, Germany). To date, their clinical usefulness for detecting alterations in platelet function has been shown to be rather variable.[43,44] However, one study demonstrated that MPM and MPV were elevated in dogs with *B. rossi* infection compared to controls, and that these indices are useful surrogate markers of platelet activation.[9]

Although standard TEG is a useful global measure of haemostasis[31], it is not specific for platelet activity, as it relies on thrombin both to activate platelets and generate a fibrin clot.[45] TEG-PM is a modification of standard TEG that separately evaluates the contributions of thrombin, fibrin and platelet activity to clot formation.[11] This is achieved by measuring platelet reactivity in response to platelet agonists which mediate platelet activation via their specific surface trans-membrane receptors.[11] Such platelet agonists include ADP, AA and collagen. In this way, it is possible to detect alterations in specific platelet receptor activity.[12]

TEG-PM is primarily used in people to evaluate the effects of platelet-inhibiting drugs[13], and has been used to describe platelet function in healthy subjects, as well as to screen for prothrombotic tendencies and risk of ischaemic events.[46,47] The large-scale implementation of TEG-PM as a platelet function test in veterinary medicine is hampered by various factors. However, it is thought that this assay holds promise for studying platelet receptor dysfunction in animals, particularly in the case of diseases known to cause altered platelet activity.

2.6.2 Analytical principles

Standard kaolin-activated TEG using citrated blood is used to measure the MA of clot formation mediated by thrombin, which is a measure of the patient's maximum platelet aggregation potential.[45-48] Thrombin is a potent platelet agonist and is able to activate the GP II_bIII_a platelet fibrinogen-binding receptor, as well as coagulation.[48-50] Kaolin is used as an activator of FXII to facilitate clot formation.[50] The following variables are derived from standard TEG:

- R value/Reaction time: the time from the start of the sample run until the first significant levels of clot formation, namely an amplitude of 2 mm.[31]
- K value/K time: the time from the end of R time until the level of clot strength reaches 20 mm, which represents clot kinetics.[31]
- α-angle: the measure of fibrin build-up and cross-linking, measured in degrees.[31]
- Maximum amplitude/$MA_{Thrombin}$: direct function of maximal clot strength, measured in mm. This is a function of platelet count and activity, as well as fibrinogen concentration.[31] The global clot firmness (G) can be calculated by $5000 \times MA/(100 - MA)$, and is expressed in dynes/second.[50] The G value may be used to classify TEG tracings as normocoagulable, hypocoagulable and hypercoagulable.[5]
- $LY_{30/60}$: clot lysis at 30 or 60 minutes after MA is reached. Indicates activity of the fibrinolytic system.[31]

Heparinised blood is used to bypass thrombin formation and negate its effects. This allows for quantification of the contribution of fibrin to the developing clot, and also makes it possible to measure the contributions of individual milder platelet agonists to platelet activity and clot formation.[11] Activator F, a combination of reptilase (a thrombin-like enzyme which cleaves fibrinogen to fibrin) and Factor XIII (forms a cross-linked fibrin mesh) are added to the heparinised sample to generate a fibrin clot.[11] This isolates the fibrin contribution to the clot strength.[45,48,49] The MA generated by the fibrin clot alone is designated as MA_{Fibrin}. Addition of specific agonists (usually ADP or AA) at established concentrations to the heparinised blood and Activator F mixture allows for measuring the contribution of ADP (MA_{ADP}) or Thromboxane (MA_{AA}) receptors to clot formation.[11]

From the above tracings, it is possible to calculate the percentage agonist-induced platelet aggregation (or inhibition) as a percentage of thrombin-mediated aggregation. The following formula may be applied[46]:

- Percentage platelet aggregation in response to ADP/AA = $[(MA_{ADP/AA} - MA_{Fibrin})/(MA_{Thrombin} - MA_{Fibrin})$ x100]
- Percentage inhibition =100%–%aggregation.

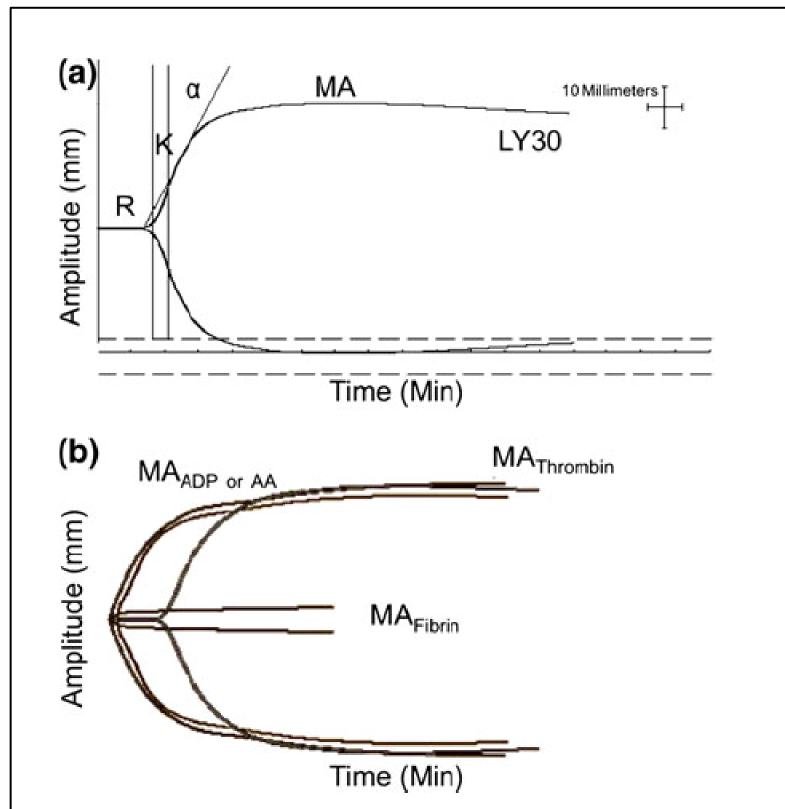

Figure 3: Examples of a) TEG and b) TEG-PM tracings.

(Reprinted by permission from [Copyright Clearance Centre]: [Springer] [Neurocritical care] [Platelet dysfunction is an early marker for traumatic brain injury-induced coagulopathy, PK Davis, H Musunuru, M Walsh, R Cassady, R Yount, A Losiniecki, EE Moore, MV Wohlauer, J Howard, VA Ploplis, FJ Castellino, SG Thomas, ©Springer Science+Business Media, LLC] (2012).)

2.7 Platelet mapping in medical practice

In humans, TEG-PM correlates well with optical aggregometry methods, thereby validating its use as a point of care test that measures platelet function.[11] It has also

been demonstrated to be a reliable method with low analytical variation.[11] The principal use of TEG-PM is antiplatelet therapy monitoring, but additional applications have been described.

2.7.1 Monitoring antiplatelet therapy

TEG-PM was primarily designed to monitor the efficacy of antiplatelet therapy, such as aspirin and clopidogrel, in patients at risk of developing ischaemic or thrombotic events.[13,46,47,51,52] One study reported that patients undergoing cardiac surgery had higher platelet reactivity characterised by a significantly higher $MA_{Thrombin}$ and MA_{ADP}, as well as a more variable response to antiplatelet therapy compared to healthy controls.[47] Patients with $MA_{Thrombin}$ values above the reference interval had a higher risk of developing thrombotic complications.[47] TEG-PM has been used to aid in decision-making with regard to timing of cardiac surgery[52] or other surgical procedures[51] following withdrawal of antiplatelet therapy in an attempt to balance the risk of excessive bleeding with that of developing a thrombotic event. TEG-PM is also employed as a tool which can predict post-operative bleeding in patients on recent antiplatelet therapy undergoing coronary artery bypass surgery.[52]

2.7.2 Platelet mapping as a platelet function test

Weitzel *et al* (2012) highlighted the utility of TEG-PM in monitoring the function of collagen, ADP and AA receptors during cardiopulmonary bypass, and the results clearly indicated that multiple platelet receptors were altered during surgery.[12] This was evidenced by a significant post-operative reduction in percentage platelet aggregation for collagen, ADP and AA.[12]

Acute traumatic coagulopathy is well-documented in severely-injured human patients.[53] TEG-PM has demonstrated that early platelet dysfunction is associated with multisystem trauma.[54] In a study by Wohlauer *et al* (2012), the TEG-PM findings indicated that significant platelet agonist receptor inhibition was present immediately after injury.[54] The coagulopathy in patients with traumatic brain injury has been associated with poor clinical outcome.[53] TEG-PM has shown that platelet dysfunction characterised by increased ADP and AA receptor inhibition is present after severe isolated traumatic brain injury, and that there is a correlation between increasing ADP receptor inhibition and severity of injury, as well as mortality.[55]

2.8 Platelet mapping in veterinary medicine

In a preliminary investigation by Blois *et al* (2013), extremely variable MA_{Fibrin} values were obtained in a population of 20 healthy dogs. These values were on average much higher (mean MA_{Fibrin} > 20 mm) than values typically obtained for humans (mean MA_{Fibrin} < 7.5 mm). Although blood sampling and other pre-analytical factors were standardised, it was suspected that platelet sensitisation might have occurred during the pre-analytical phase, and that there may be a difference in responsiveness to Activator F among dogs.[48] Similar findings of highly variable MA_{Fibrin} values have been found in cats[45] and horses[42]; sometimes these values exceeded the $MA_{Thrombin}$ or $MA_{ADP/AA}$ values, making it impossible to establish baseline values for calculating the contribution of each agonist-mediated receptor. Hyperfibrinogenaemia, antithrombin (AT) deficiency and prior platelet sensitisation were given as possible reasons.[45] In horses, dilution of the Activator F agent gave lower MA_{Fibrin} values more closely resembling the values obtained in humans.[42]

MA_{ADP} and MA_{AA} values also varied widely in dogs and were lower compared to values in humans.[48] This could have been due to lower agonist concentrations in the TEG-PM kit (2 µmol/L ADP; 1 mmol/L AA) compared to concentrations used in aggregometry (5–20 µmol/L ADP; 1 mmol/L AA) to induce maximal platelet aggregation, which may have resulted in suboptimal platelet ADP receptor stimulation and subsequent variability in MA_{ADP} values obtained.[48] One study increased the TEG-PM concentrations of ADP and AA to 5.7 µmol/L and 1.9 mmol/L, respectively, as these concentrations were determined to be optimal for TEG-PM based on preliminary in-house investigations carried out in healthy dogs.[49]

Other studies have been conducted to determine the effects of various medications on platelet function. TEG-PM demonstrated that administering acepromazine (ACP) did not significantly change $MA_{Thrombin}$, MA_{ADP} and MA_{AA} in healthy dogs.[49] This contrasted with previous studies in healthy dogs given ACP, which reported a decrease in platelet aggregation as determined by whole blood aggregometry, which suggests that ACP may alter platelet function in a way which is not quantifiable by means of TEG-PM.[49]

In a study by Brainard *et al* (2010), evaluating the effects of clopidogrel therapy on ADP receptor activity, a decrease in MA_{ADP} was observed in dogs treated with clopidogrel.[56] The TEG-PM findings closely resembled the results obtained for whole blood aggregometry.[56] In the same study, decreased platelet aggregation in response to collagen stimulation, as well as ADP stimulation, was also observed during assessment by means of optical aggregometry.[56] Since clopidogrel exerts its effect at the level of the $P2Y_{12}$ ADP receptor, the response to ADP was most profound.[56] Collagen is a more non-specific platelet agonist, exerting its function via both GP VI and Integrin α2β1 on platelet membranes.[56] Since collagen stimulation causes platelet degranulation and release of ADP, inhibition of the ADP receptors may have caused the decreased reactivity in response to collagen seen following clopidogrel therapy.[56]

Park *et al* (2013) utilised TEG-PM, together with traditional coagulation screening tests, to assess haemostatic function in dogs with ACTH-dependent hyperadrenocorticism (HAC) before and after treatment.[57] Compared to control dogs, used to generate reference intervals for the study, the citrated kaolin TEG variables for dogs with HAC were significantly different at all time points (before treatment, 3 and 6 months post-treatment).[57] Affected dogs had a shorter K value, higher α-angle and higher $MA_{Thrombin}$, all of which are consistent with hypercoagulability.[57] Additionally, TEG-PM revealed that these dogs also had a significantly higher MA_{Fibrin} at all time points (possibly due to a persistent hyperfibrinogenaemia), as well as a significantly higher pre-treatment MA_{AA}..[57] There was also a trend toward increased MA_{ADP} values in dogs with HAC.[57]

2.8.1 Potential applications of platelet mapping in veterinary medicine

Many common diseases in dogs and cats are associated with thromboembolic complications. These include infectious diseases, neoplasia, HAC, IMHA, pancreatitis, sepsis, DIC, cardiac disease, diabetes mellitus and hypothyroidism.[36] Since many of these disease processes have an inflammatory component, it is highly likely that platelets play a role in the pathogenesis of these thromboembolic events.[36] By investigating haemostatic abnormalities in HAC, Park *et al* (2013) showed that TEG-PM can be used to assess haemostatic status in patients with conditions known to give rise to thromboembolic complications[57], despite the scarcity of published

references providing concrete guidelines regarding the appropriateness and applicability of TEG-PM in animals.

Canine babesiosis is an example of a disease characterised by a marked inflammatory response as well as thromboembolic consequences. Earlier studies have already provided evidence of platelet activation in such cases.[8,9,32] It is believed that investigating TEG-PM as a platelet function test in such cases would provide information regarding the mechanisms of these platelet-mediated haemostatic alterations, which could have important therapeutic consequences in terms of appropriate antiplatelet treatment selection.

Chapter 3: Materials and methods

3.1 Experimental design

3.1.1 Study design

This was a prospective descriptive cross-sectional study which was conducted on clinical cases, as well on a healthy control group. A total of 18 animals presenting to the OVAH during the period February 2016 to February 2017 were included in this study. Thirteen client-owned dogs which presented to the Outpatient Clinic of the OVAH and were diagnosed with complicated *B. rossi* infection were included in the babesiosis group. Five healthy client-owned dogs which presented to the OVAH for routine, elective surgical procedures or blood donation were used as controls. This study was approved by the University of Pretoria's Animal Ethics Committee (project number V098-15). Signed consent from the owner was required to include each dog in the study (Appendix A–C).

3.1.2 Study population

Dogs of any breed and either sex with demonstrable parasitaemia were considered for inclusion into the babesiosis group, provided they were older than six months and weighed more than 3.5 kg. Initial diagnosis of *Babesia* infection was by means of finding intra-erythrocytic trophozoites on a peripheral thin blood smear. Definitive diagnosis of *B. rossi* infection was confirmed by polymerase chain reaction (PCR) and reverse line blot (RLB) analyses.[58] The infected dogs had to demonstrate one or more of the following manifestations of complicated babesiosis: secondary IMHA (marked spherocytosis, positive warm in-saline agglutination or Coombs' test result), AKI (oliguria/anuria and persistent azotaemia unresponsive to appropriate fluid therapy), hypoglycaemia (blood glucose < 3.3 mmol/L), cerebral babesiosis (neurological signs not attributable to any other cause), hepatopathy with cholestasis (icterus, marked bilirubinuria, hyperbilirubinaemia, raised liver enzyme activities), acute respiratory distress syndrome (ARDS; dyspnoea, adventitious lung sounds, frothy blood-tinged nasal discharge, blood-gas evidence of ventilation-perfusion mismatch, radiological evidence of oedema and lung consolidation), haemoconcentration (congested mucous membranes, high-normal or raised haematocrit (HCT) with evidence of

concurrent intravascular haemolysis), and pancreatitis (vomiting, cranial abdominal pain, melaena and icterus; raised serum lipase activity; or ultrasonographic evidence of acute pancreatitis).[20] Dogs were excluded from the study if the PCR and RLB analyses demonstrated evidence of co-infection with one or more of the following vector-borne agents: *Ehrlichia canis, B. vogeli, Theileria* and *Anaplasma* species. The *Babesia*-infected dogs were treated according to the standard protocol in use at the OVAH. This included treatment with diminazene aceturate (Berenil® RTU 0.07 g/mL, Intervet South Africa, Spartan, Kempton Park, South Africa), transfusion with blood products, or intravenous fluid therapy as deemed necessary. Complications were treated as needed. Dogs in the control group were deemed clinically healthy based on a physical examination, complete blood count (CBC) and blood smear evaluation, serum biochemical profile, and urine analysis, and if they were free of any parasitaemia as determined by PCR and RLB. The same requirements regarding age and weight, as mentioned for the babesiosis group, applied.

For both groups, dogs were excluded if they had clinical evidence of any concurrent inflammatory or neoplastic condition, or if any evidence of trauma or wounds was present. Dogs treated with any medications known to interfere with platelet function within four weeks prior to presentation were excluded. Such medications included corticosteroids, non-steroidal anti-inflammatory agents (including aspirin), anti-platelet medications (aspirin, clopidogrel), and anticoagulants (e.g. heparin).

3.2 Experimental procedure

3.2.1 Sample collection

At presentation and prior to any treatment (including blood products or intravenous fluids), blood was collected from the jugular vein from each dog using a 21-gauge needle by careful venipuncture with minimum stasis. Blood was collected into serum, sodium citrate, lithium heparin and ethylenediaminetetraacetic acid (EDTA) vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA). Samples were collected in this order to minimise TF contamination of the samples. All tubes were filled, using vacuum assistance, to ensure the correct ratio of anticoagulant to blood. The serum sample was used to perform the biochemical panel and the remainder was stored at –80 ºC (Forma Scientific –86 °C freezer). The EDTA sample was used to perform the CBC;

0.5 mL of this sample was aliquoted and stored for future PCR/RLB analysis. Standard kaolin-activated TEG was performed on the sodium citrate sample, after which the remainder was centrifuged within one hour at 2100 *g* for eight minutes. The plasma was stored at –80 °C (Forma Scientific –86 °C freezer) for determination of fibrinogen concentration and AT activity at a later stage. The lithium heparin sample was used to perform the TEG-PM analysis.

3.2.2 Assay methodologies

<u>TEG-PM analysis</u>

These assays were performed by the same experienced person using the TEG® 5000 thromboelastograph haemostasis analyser (Haemonetics® Corporation, Braintree, MA, USA). The different stages were performed in parallel, in accordance with manufacturer instructions; thus, two analysers were employed.

The sodium citrate and lithium heparin samples were left to equilibrate at room temperature for 30 minutes post-collection while four TEG cups were pre-warmed to 37 °C. Following equilibration, 1 mL of citrated blood was added to a kaolin-coated vial, which was gently inverted five times. Following addition of 20 µL $CaCl_2$ to the cup in channel 1, 340 µL of citrated kaolin blood was transferred to this cup. Standard TEG analysis was performed to determine MA due to the action of thrombin ($MA_{Thrombin}$). To determine the contribution of cross-linked fibrin to clot formation, 10 µL of Activator F (Haemonetics Corporation, Braintree, MA, USA) were transferred to the cup in channel 2; followed by 360 µL of heparinised blood. The mixture in the cup was gently mixed by partially aspirating the contents three times, after which MA_{Fibrin} was measured. To evaluate the contribution of platelet agonist receptors to clot formation, 10 µL of Activator F and 360 µL of heparinised blood were added to channels 3 and 4. Then, 10 µL of ADP were added to channel 3 to yield a final ADP concentration of 2 µmol/L. Similarly, 10 µL of AA were added to channel 4 to result in a final AA concentration of 1 mmol/L. Both samples were gently mixed, after which the MA_{ADP} and MA_{AA} were determined. Once all variables had been calculated, the percentage agonist-related inhibition (Inhib_ADP/AA) was calculated as described in Chapter 2.

Haematology variables

A CBC was performed on the ADVIA 2120 automated haematology system (Siemens, Munich, Germany) and variables of interest included HCT, platelet count (PLT), PCT, MPV, MPM, and MPC. The analysis was performed within 30 minutes of blood collection to minimise the effects of time-dependent *in vitro* alterations of the MPV and MPC due to the effect of EDTA.

Plasma fibrinogen concentration and AT activity

Analysis was performed as a batch. Plasma fibrinogen concentration was determined using the Clauss method on an automated coagulometric analyser (ACL Elite, Instrumentation Laboratory, Bedford, MA, USA). Plasma AT activity was assessed using a thrombin-dependent chromogenic substrate assay (Precimat Chromogen, Roche, Basel, Switzerland) on the Cobas Integra 400 Plus automated analyser (Roche, Basel, Switzerland). A normal pooled control sample, with a presumed activity of 100%, was run with each batch of tests, and the patient AT activity was normalized against the pooled control sample.

Serum biochemical analysis

All serum biochemical analyses were performed on the Cobas Integra 400 Plus (Roche, Basel, Switzerland) according to the manufacturer's instructions. The serum biochemical panel consisted of the following: total protein, albumin, urea, creatinine, alanine aminotransferase activity and alkaline phosphatase activity.

DNA extraction and PCR analysis

DNA extraction from 200 µL of EDTA-anticoagulated whole blood was performed using a blood and tissue extraction kit (QIAamp blood and tissue extraction kit, Qiagen, Venlo, The Netherlands) as per the manufacturer recommendations. PCR and RLB analysis were used to confirm a molecular diagnosis of *B. rossi* infection and to identify cases infected with other *Babesia* spp, *E. canis, Anaplasma* spp and *Theileria* spp (and controls infected with *B. rossi*).[58,59]

3.3 Observations

All clinical and laboratory data obtained from this study were recorded on a data capture sheet (Appendix D–E), and captured into a spreadsheet program, (Microsoft Excel®, Microsoft Corporation, Redmond, WA, USA). TEG-PM and haematological variables were used for statistical analysis.

3.4 Statistical analysis

Statistical analyses were performed using a commercial software package (SPSS Statistics 24.0 Software, SPSS Inc., Armonk, NY, USA). Data were inspected for normality using the Shapiro-Wilk test and found not to display a normal distribution. Differences between groups were assessed using the Mann-Whitney *U* test. Correlations were determined using Spearman's rank correlation coefficient. Gender proportions between groups were compared using the Fisher's exact test. Data are presented as median and interquartile range (IQR). In addition, interindividual coefficients of variation (CV) for patient and control groups were calculated for the TEG-PM variables by dividing the group standard deviation by the group mean and multiplying this by 100. A P value of < 0.05 was considered statistically significant.

Chapter 4: Results

4.1 Animals

Thirteen dogs were included in the babesiosis group. Breeds included two mixed breed dogs, two Boerboels, and one each of the following: Doberman cross, Jack Russell Terrier, Pekingese, Great Dane, American Pitbull, Siberian Husky, Spaniel cross, Bernese Mountain Dog and German Shepherd. There were five dogs in the control group, which included two German Shepherds, and one each of the following: Standard Poodle, Great Dane and Boerboel. There were no significant differences in age between the groups, with the median patient age 24 months (12–52) and the median control age 48 months (19–82). The ratio of male: female for the *Babesia*-infected dogs was 7: 6 and the controls 3: 2, with no significant difference between the groups. The following complications were observed either singly or in combination: IMHA (n = 4), ARDS (n = 2), hepatopathy (n = 3), AKI (n = 2), neurological signs (n = 4), hypoglycaemia (n = 1) and haemoconcentration (n = 3).

4.2 TEG-PM analysis

The TEG-PM variables are shown in Table 1. Median $MA_{Thrombin}$ did not differ significantly between the groups ($P = 0.218$; Fig. 4); however, MA_{Fibrin} was significantly higher in the babesiosis group compared to the control group ($P = 0.001$; Fig. 5). No significant differences were observed between groups for median MA_{ADP} ($P = 0.402$; Fig. 6) and MA_{AA} ($P = 0.805$; Fig. 7). Inter-individual CV for the babesiosis and control groups were 21.1% and 5.9%, respectively for $MA_{Thrombin}$; 38.9% and 41.9%, respectively for MA_{Fibrin}; 48.3% and 92.9%, respectively for MA_{ADP}; and 38.7% and 41.6%, respectively for MA_{AA}. Representative TEG-PM tracings of a dog from the control group, as well as a dog with babesiosis, are shown in Figures 8 and 9.

No significant differences were found for the Inhib_ADP and Inhib_AA ($P = 0.486$ and $P = 0.178$, respectively). Moreover, extremely high interindividual CVs were recorded in both groups for the Inhib_ADP (60.8% and 85.5% for babesiosis and control groups,

respectively) and Inhib_AA (74.1% and 126.9% for babesiosis and control groups, respectively).

4.3 Haematological variables

Results are shown in Table 1. The median HCT, PLT and PCT were significantly decreased ($P = 0.007$; $P = 0.003$ and $P = 0.003$ respectively), with the MPV and MPM significantly increased in the babesiosis group compared to the control group ($P = 0.007$ and $P = 0.012$ respectively). No significant difference was noted for MPC ($P = 0.402$).

4.4 Plasma fibrinogen concentration and AT activity

Results are shown in Table 1. The median fibrinogen concentration was significantly increased ($P = 0.006$) and the AT activity was significantly decreased ($P = 0.016$) in the babesiosis group compared to the control group.

4.5 Correlation analysis

The $MA_{Thrombin}$ had significant strong positive correlations with PLT ($r_s = 0.709$; $P = 0.001$) and PCT ($r_s = 0.707$; $P = 0.001$). The MA_{Fibrin} had significant moderate to strong positive correlations with MPV ($r_s = 0.523$; $P = 0.026$), MPM ($r_s = 0.509$; $P = 0.031$) and fibrinogen concentration ($r_s = 0.742$; $P < 0.001$), and significant moderate to strong negative correlations with HCT ($r_s = -0.690$; $P = 0.002$), PLT ($r_s = -0.478$; $P = 0.045$), and PCT ($r_s = -0.481$; $P = 0.044$).

Table 1: Descriptive statistics of TEG-PM and haematology variables for the *Babesia*-infected and healthy control dogs.

Variable (Unit)	Control dogs (n = 5) Median (IQR) Range	*Babesia*-infected dogs (n = 13) Median (IQR) Range
$MA_{Thrombin}$ (mm)	56.3 (55.9–61.0) 55.8–63.9	51.0 (43.1–62.2) 31.8–67.3
MA_{Fibrin} (mm)	5.6 (3.4–7.8)* 2.5–8.4	28.3 (23.5–36.6)* 13.9–58.1
MA_{ADP} (mm)	15.9 (7.8–60.5) 7.6–69.4	29.5 (24.2–48.1) 11.0–67.5
MA_{AA} (mm)	44.8 (28.6–61.4) 16.6–65.7	44.9 (29.4–58.9) 15.3–67.7
Inhib_ADP (%)	77.4 (4.5–97.3) 0.0–98.3	93.8 (21.9–100.0) 0.0–100.0
Inhib_AA (%)	21.9 (0.0–56.3) 0.0–83.3	56.2 (11.6–91.9) 0.0–100.0
Haematocrit (L/L)	0.49 (0.47–0.56)* 0.47–0.58	0.18 (0.14–0.34)* 0.10–0.63
Platelet count ($\times 10^9$/L)	225 (155–347)* 151–467	29 (22–50)* 5–166
Plateletcrit (%)	0.22 (0.18–0.43)* 0.18–0.55	0.06 (0.04–0.11)* 0.01–0.22

Mean platelet volume (fL)	11.8 (10.6–12.8)* 9.6–13.6	21.1 (16.6–23.2)* 9.8–28.1
Mean platelet mass (pg)	2.24 (1.74–2.42)* 1.60–2.53	3.09 (2.68–3.48)* 1.67–3.62
Mean platelet component concentration (g/dL)	21.5 (18.6–23.0) 16.9–24.0	19.7 (19.0–22.0) 12.8–23.9
Fibrinogen (g/L)	2.6 (1.9–3.0)* 1.7–3.0	6.9 (6.3–7.0)* 1.2–7.0
Antithrombin activity (%)	106 (99–109)* 94–110	78 (71–96)* 54–108

*Denotes statistical significance between groups.

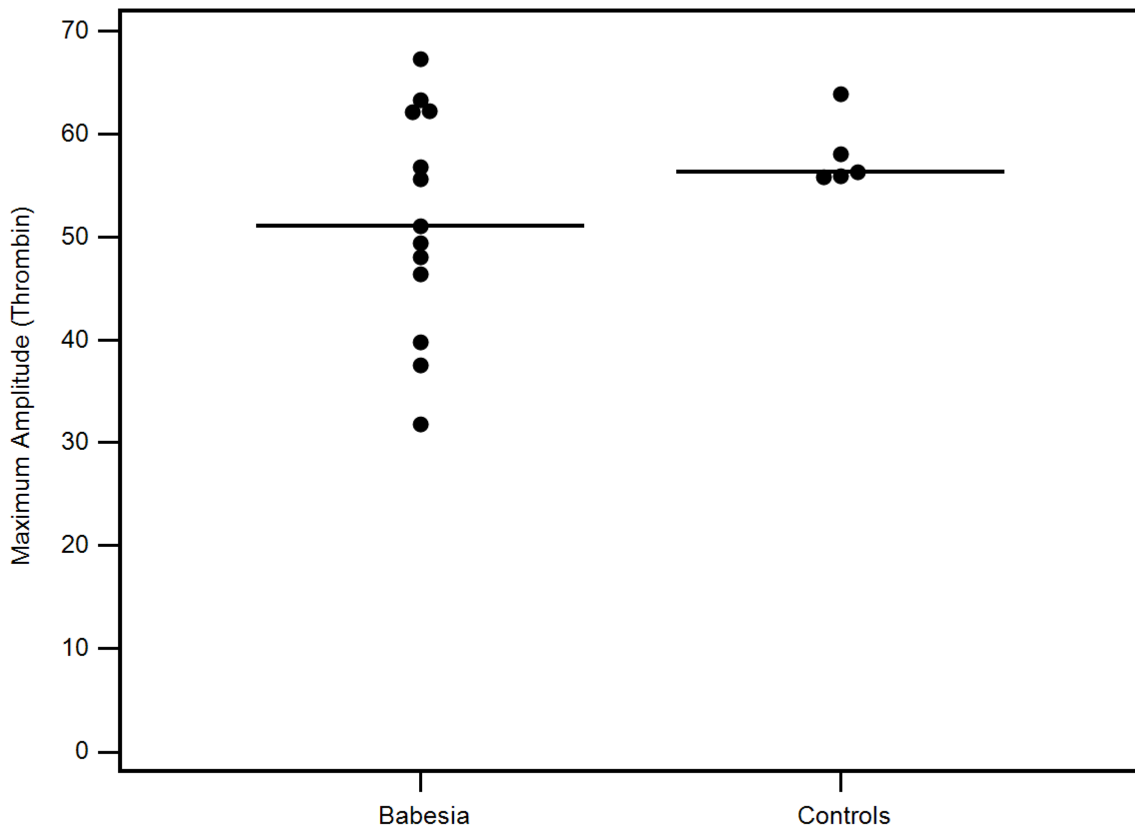

Figure 4: Dot plot of the TEG-PM variable $MA_{Thrombin}$ of the *Babesia*-infected group compared to the control group. Each dot represents an individual result, and the group medians are indicated by the horizontal lines.

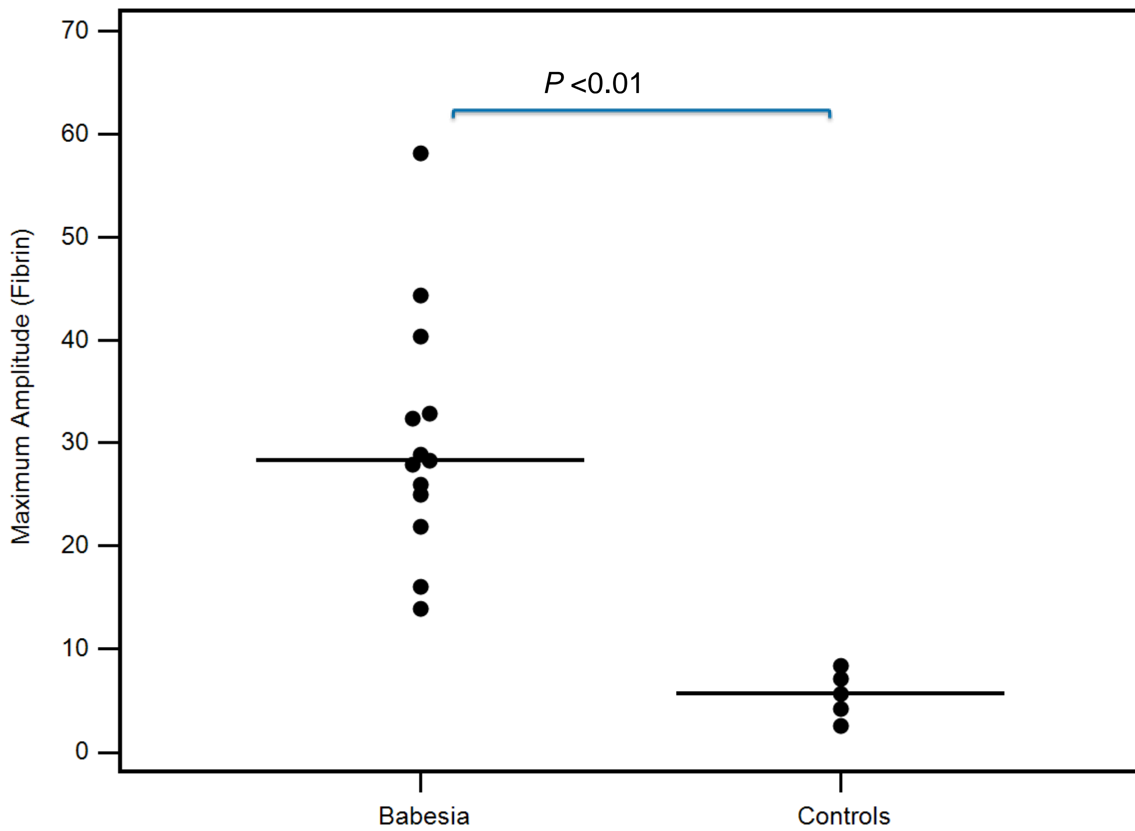

Figure 5: Dot plot of the TEG-PM variable MA_{Fibrin} of the *Babesia*-infected group compared to the control group. Each dot represents an individual result, and the group medians are indicated by the horizontal lines.

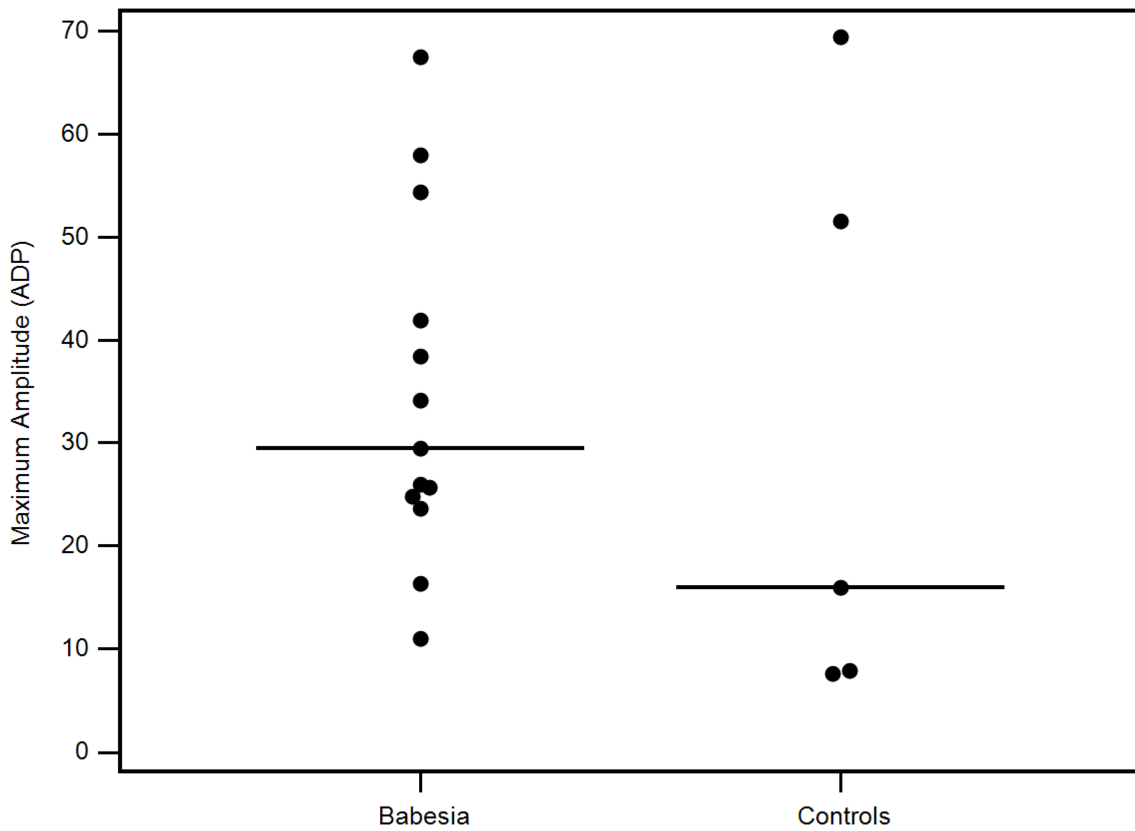

Figure 6: Dot plot of the TEG-PM variable MA_{ADP} of the *Babesia*-infected group compared to the control group. Each dot represents an individual result, and the group medians are indicated by the horizontal lines.

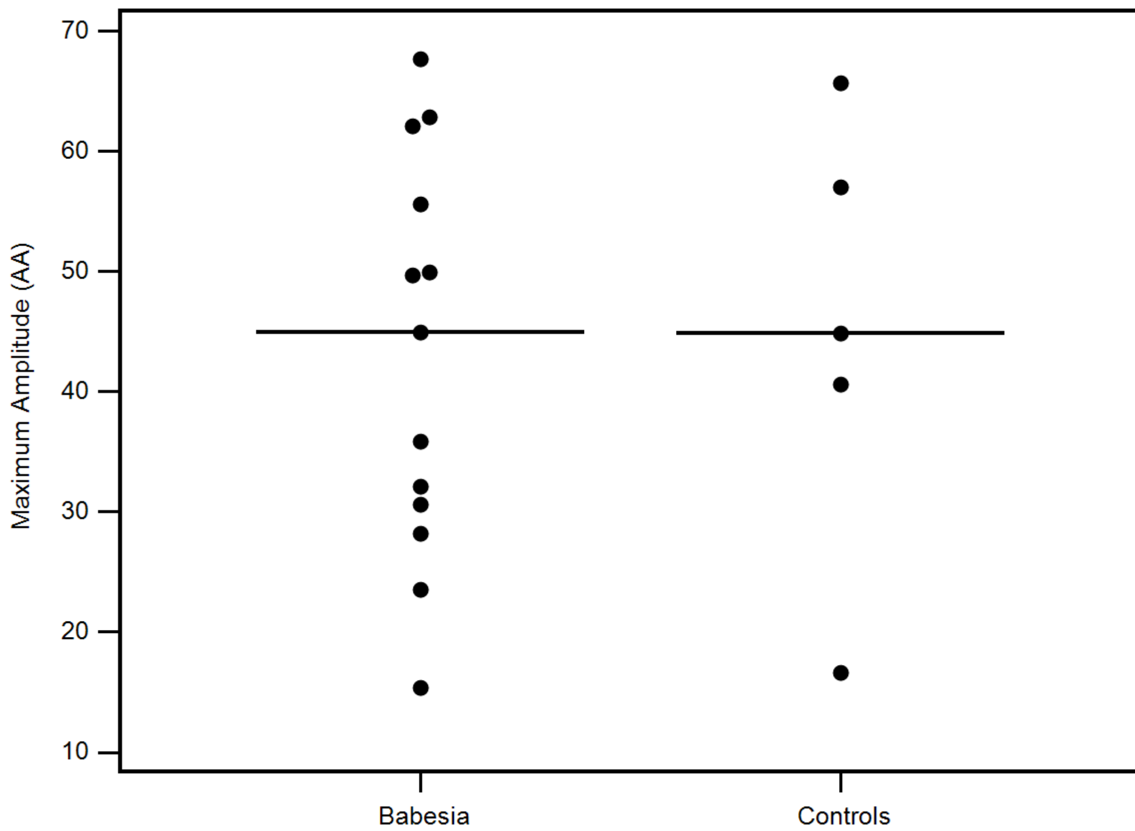

Figure 7: Dot plot of the TEG-PM variable MA_{AA} of the *Babesia*-infected group compared to the control group. Each dot represents an individual result, and the group medians are indicated by the horizontal lines

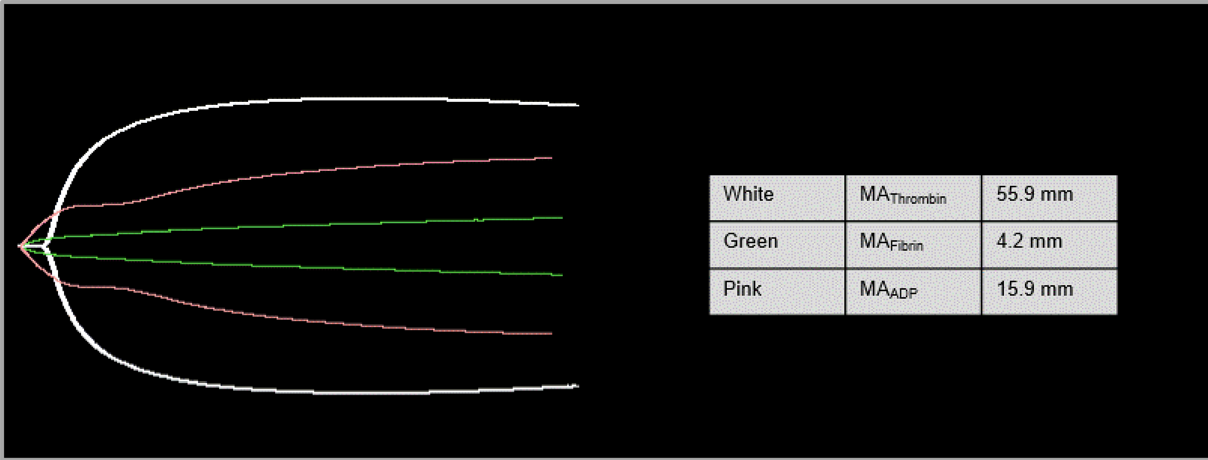

Figure 8: Representative TEG-PM tracing from a healthy control dog.

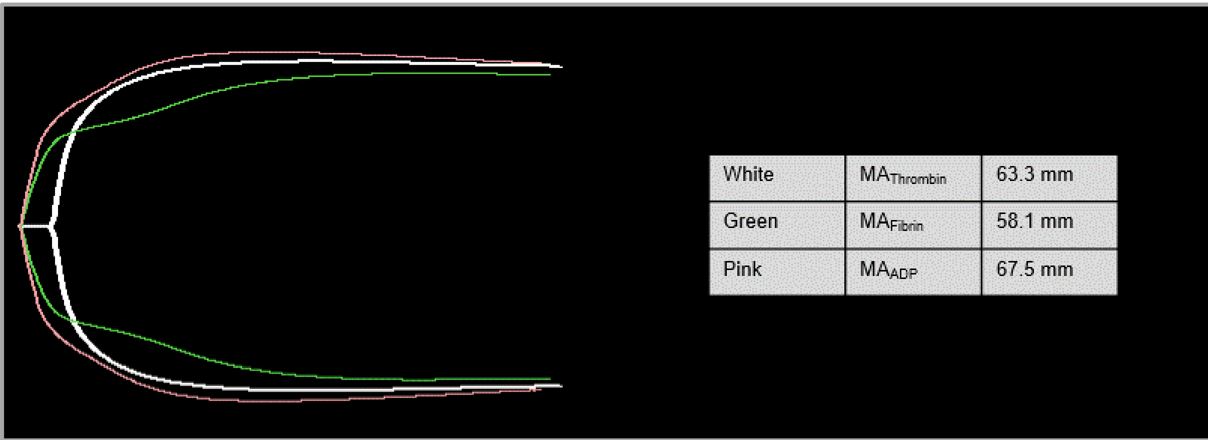

Figure 9: Representative TEG-PM tracing from a dog with complicated *B. rossi* infection.

Chapter 5: Discussion

This is the first report to show the significant effect of hyperfibrinogenaemia, using TEG-PM, on the haemostatic function of dogs with complicated babesiosis. The findings suggest that the presence of marked hyperfibrinogenaemia and the resultant contribution of fibrin to clot strength offsets the severe thrombocytopenia associated with babesiosis, to result in a lack of clinical bleeding.

The study showed no significant difference for $MA_{Thrombin}$ between the *Babesia*-infected and healthy control dogs. $MA_{Thrombin}$ is the maximum amplitude determined using standard kaolin-activated TEG. Our results are in agreement with a previous report on uncomplicated babesiosis, using TF-activated TEG.[5] The authors in that study suggested two possible reasons for their findings. Since TEG is influenced by HCT, PLT, platelet function, fibrinogen concentration and AT activity, it was suggested that the normocoagulable TEG tracings could have been the result of the opposing forces of these variables on the overall tracing. The second explanation was that *Babesia*-infected dogs remained normocoagulable, despite severe thrombocytopenia, due to marked platelet activation associated with inflammation. Subsequently, studies confirmed the presence of activated platelets in dogs with babesiosis based on the presence of known and surrogate markers of platelet activation, namely increased circulating platelet-monocyte aggregates and increased MPV and MPM[8,9], which would lend support to the second theory. The *Babesia*-infected dogs in the current study also had raised MPV and MPM, but similar to the findings of Goddard *el al* (2015), our study failed to demonstrate significant differences for MPC between infected and control groups. Although the $MA_{Thrombin}$ is affected by PLT, platelet function and plasma fibrinogen concentration[31], our study only showed a significant correlation with PLT, but not with any of the surrogate markers for platelet activity. HCT is also an important variable to consider, because it has been shown that decreased HCT results in more hypercoagulable viscoelastic tracings.[60,61] Similar to a previous report, no correlation was seen with HCT or fibrinogen concentration in the study cohort.[5] Considering that platelets contribute approximately 70–80% to clot formation[62], this may have obscured a direct correlation between $MA_{Thrombin}$ and other variables.

MA_{Fibrin}, however, was significantly increased in the *Babesia*-infected dogs compared to the controls. ROTEM studies in humans have demonstrated that adequate fibrinogen concentrations are critical for clot formation.[30,63,64] This was also shown in a study which utilised functional fibrinogen (FF) TEG to assess the relative contributions of platelets and fibrinogen to clot formation in human patients presenting after a traumatic event.[62] In the FF TEG study, patients presenting with low levels of FF on admission were at increased risk for coagulopathy, increased transfusion requirements and possibly mortality.[62] This was accentuated further in patients with low MA values, suggesting that FF is critical in patients with low overall clot strength.[62] In an *in vitro* study assessing the effects of fibrinogen supplementation on dilutional coagulopathy, ROTEM variables mostly normalised when additional fibrinogen was added to blood samples diluted with various crystalloids and colloids.[63] The effect was dependent on the concentration of fibrinogen added and the type of fluid used to dilute the blood, as colloids are known to affect the coagulation system by numerous mechanisms. In an experimental study where pigs were subjected to haemodilution followed by blunt liver trauma, ROTEM variables coagulation time and clot formation time (equivalent to TEG variables R and K) shortened for a few hours following infusion with fibrinogen concentrate, whereas the α-angle and maximum clot firmness (equivalent to MA) increased.[64] Even in the face of severe thrombocytopenia, the maximum clot elasticity (MCE), determined by ROTEM, increased exponentially when increasing concentrations of fibrinogen were added.[30] Moreover, the use of ROTEM, modified by the addition of cytochalasin-D (FIBTEM) to isolate the contribution of fibrinogen to clot strength, showed that MCE_{FIBTEM} displayed a similar exponential increase as fibrinogen concentrations increased.[30] Interestingly, the contribution of platelets to clot formation increased in a similar fibrinogen concentration-dependent pattern. Each platelet bears numerous $GP_{IIbIIIa}$ receptors on its surface, and when activated by thrombin, platelets are able to bind high numbers of fibrinogen molecules.[65] Thus, each individual platelet may become increasingly engaged with fibrinogen via its $GP_{IIbIIIa}$ receptors as the overall platelet count decreases and platelet volume increases, as illustrated in Figure 10.[30] Given the inflammatory nature of the disease which could potentially result in both hyperfibrinogenaemia and platelet activation, it is plausible that this mechanism is also at play in dogs with babesiosis. This may explain the significant and positive correlation of MA_{Fibrin} with MPV and MPM.

Moreover, enhanced thrombopoiesis and platelet regeneration in response to the inflammatory process and thrombocytopenia may have also resulted in increased MPV and MPM. Younger platelets tend to be larger and more haemostatically active, and both platelet size and immaturity are independent determinants of platelet function.[66,67]. The negative correlation between MA_{Fibrin} and PLT is also noteworthy and supports the theory of platelets becoming increasingly engaged with fibrinogen as their numbers decrease, as previously stated.[30] A possible explanation for the negative correlation with HCT is that a defined volume of anti-coagulated blood is used for the TEG assay and erythrocytes act as a functional diluent for plasma, limiting the volume of plasma and plasma coagulation proteins, included in the assay. A whole blood sample with a low HCT will consequently allow for more plasma coagulation proteins, specifically fibrinogen, to be included in the assay.[60,61]

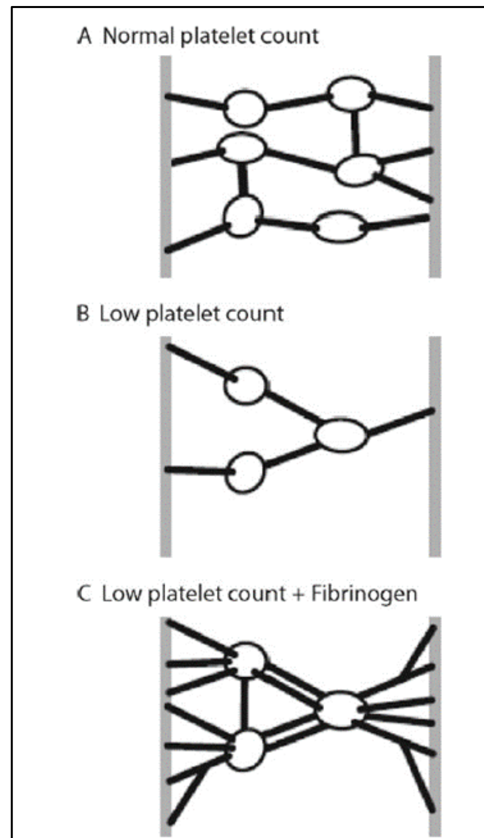

Figure 10: Schematic diagrams depicting the interaction between fibrinogen and platelets to produce the maximum clot strength in three different scenarios: A) Normal PLT and fibrinogen concentration, B) Low PLT and fibrinogen concentrations, C) Low PLT and high fibrinogen concentrations.
(Reprinted from Anesthesia and Analgesia 108, T Lang, K Johanning, H Metzler, S Piepenbrock, C Solomon, N Rahe-Meyer, and K A. Tanaka, The effects of fibrinogen levels on thromboelastometric variables in the presence of thrombocytopenia, 751–758, © International Anesthesia Research Society 2009, with permission from Wolters-Kluwer.)

Besides hyperfibrinogenaemia, reasons for high MA_{Fibrin} include prior platelet sensitisation and AT deficiency.[45] Hyperfibrinogenaemia would appear to be a likely reason in this case, considering that canine babesiosis is characterised by an acute phase response with increases in APPs such as CRP, SAA and fibrinogen[5,22,25,26], and all but one of the *Babesia*-infected dogs in our study were hyperfibrinogenaemic. The strong positive correlation between MA_{Fibrin} and plasma fibrinogen concentration in our study supports previous reports on the effect of hyperfibrinogenaemia in coagulation,

especially in the presence of thrombocytopenia.[30,63] Similar findings have been reported in dogs with pituitary-dependent HAC, in which affected dogs had hyperfibrinogenaemia and higher median MA_{Fibrin} values than healthy controls at all recorded time points.[57] The HAC study also reported a weak positive correlation between MA_{Fibrin} and plasma fibrinogen concentration. Although prior platelet sensitisation during blood collection cannot be excluded[45], a standardised sampling method was employed for our study, which makes this an unlikely reason for the significant difference between the groups. Since heparin anticoagulant requires AT to inhibit thrombin, it is conceivable that the lower median AT activities observed in dogs with babesiosis could have contributed to the increased MA_{Fibrin} values. However, no significant correlation was observed between MA_{Fibrin} and AT activity. In addition, although the study on dogs with HAC reported persistently elevated MA_{Fibrin} compared to controls, the median AT activity was within the reference interval, which further mitigates against low AT activity as a possible cause.

Previous studies in dogs[48], cats[45] and horses[42] showed that the MA_{Fibrin} is particularly prone to a high degree of intra-and interindividual variability, with extremely high CVs being obtained in the dog study (> 100%). Similarly, the study on dogs with HAC found significant overlap between MA_{Fibrin} in affected dogs and controls.[57] In our study, the interindividual CVs for both the babesiosis and control groups (38.9% and 41.6%, respectively) were significantly lower than previously reported values; although the sample sizes were small, no overlap was seen between the groups. This, together with a plausible underlying pathomechanism to explain the increased MA_{Fibrin}, suggests that the difference between the groups is due to the disease condition and not biological or analytical variation. Nevertheless, assay analytical precision, as well as biological variation, should be investigated further.

The lack of significant difference between groups for MA_{ADP} and MA_{AA} was unexpected, given that platelet activation occurs in canine babesiosis. However, the interindividual variation for both variables was significant, which is similar to the findings of previous studies performed in dogs[48,68] and cats.[45] There are several potential explanations for the notable variability in our results and lack of differences between groups, such as the use of suboptimal agonist concentrations.[48] The TEG-PM kit contains 2 µmol/L ADP, compared to 5–20 µmol/L of ADP typically required in

aggregometry.[56] Furthermore, an earlier aggregometry study reported inconsistent and even absent aggregation responses when ADP concentrations of 1 µmol/L and 5 µmol/L were used.[69] Although the testing conditions for TEG-PM and aggregometry are not equivalent and as such it cannot be assumed that the same agonist concentrations should be used for both assays, it is possible that the concentrations of agonist used in the TEG-PM kits designed for human samples are insufficient for canine samples and result in suboptimal stimulation, leading to variable results.[48] Another explanation could be that the functions of the receptors for ADP and TXA_2 may not be altered during *B. rossi* infection, especially considering the clinical and clinicopathologic heterogeneity seen in infected dogs.[20] However, the study cohort included dogs with complicated disease, which is associated with an excessive pro-inflammatory response.[4] Since ADP is released by platelet dense granules during platelet activation to augment the activation response initiated by other agonists[36], and AA is an important inflammatory mediator, this would seem unlikely. It is also possible that the variation in the results is due to biologic variability. It has been previously reported that a great deal of heterogeneity exists regarding the response of platelets to various agonists in dog populations[68], as is the case with people.[70] In addition to interindividual variation, breed-related differences exist regarding the response of canine platelets to ADP and AA, as demonstrated by aggregometry.[69,71] In a study by Bochsen *et al* (2007) assessing analytical and interindividual variation of TEG-PM variables of healthy human blood donors, the interindividual variation of MA_{ADP} was very high. The authors postulated that individuals may differ in terms of the number and inherent characteristics of ADP receptors, which could explain the variations in agonist response seen.[46] Moreover, genetic polymorphisms for platelet receptors exist in humans[46], and it is plausible that this is also the case for dogs. The lower HCT could also have affected both the MA_{AA} and MA_{ADP} of the *Babesia*-infected group; however, no significant correlation with HCT was observed. Since both the MA_{ADP} and MA_{AA} were affected by this variability, it follows that the Inhib_ADP and Inhib_AA were also extremely variable.

Our study had some limitations. Sample sizes were small due to financial constraints, which may have resulted in failure to detect significant changes in the MA_{ADP} and MA_{AA}. The control group consisted of only five animals, and might not reflect the general canine population. Due to the nature of sample collection from clinical cases,

sampling at a standardized time point in the disease course was not possible. This may also have introduced heterogeneity into the study population. More reliable assays for determination of platelet activation, such as flow cytometry or aggregometry, were not performed, thus it was not possible to show conclusively, using surrogate markers of platelet activation such as raised MPM and MPV, that platelet activation occurred in the dogs of this study. In addition, the TEG-PM assay has not been validated for use in dogs, but several published veterinary studies have used this methodology.[42,45,48,49,56,57] Lastly, the analytical variation of the variables is unknown at this stage.

Chapter 6: Conclusions

This study demonstrated, using TEG-PM, that the median MA_{Fibrin} was significantly elevated in dogs with complicated *B. rossi* infection compared to healthy controls. Given the strong positive correlation with plasma fibrinogen concentration, hyperfibrinogenaemia as a result of an acute phase response was the most likely cause of the raised MA_{Fibrin}. These findings provide further information regarding the interaction between platelets and fibrinogen during clot formation, and suggest that hyperfibrinogenaemia in the presence of large platelets, secondary to either concurrent activation or regeneration, overrides the severe thrombocytopenia associated with canine babesiosis to result in normal thromboelastograms and absence of clinical bleeding.

Our findings, and those of others, suggest that further studies are required to characterise inter-and intra-individual variation, as well as analytical variation of the TEG-PM variables before utilising TEG-PM as a tool for assessing platelet function or monitoring antiplatelet therapy. Moreover, the inherent variability in response of canine platelets to ADP and AA stimulation suggests that additional work is required in order to determine the optimal agonist concentrations to be used for this assay.

References

1. Ghasemzadeh M, Hosseini E. Platelet-leukocyte crosstalk: Linking proinflammatory responses to procoagulant state. *Thromb Res.* 2013;131:191–197.

2. Rafaj RB, Kules J, Selanec J, et al. Markers of coagulation activation, endothelial stimulation, and inflammation in dogs with babesiosis. *J Vet Intern Med.* 2013;27:1172–1178.

3. Weiss DJ, Rashid J. The sepsis-coagulant axis: A review. *J Vet Intern Med.* 1998;12:317–324.

4. Goddard A, Leisewitz AL, Kjelgaard-Hansen M, Kristensen AT, Schoeman JP. Excessive pro-inflammatory serum cytokine concentrations in virulent canine babesiosis. *Plos One.* 2016;11:e0150113.

5. Liebenberg C, Goddard A, Wiinberg B, et al. Hemostatic abnormalities in uncomplicated babesiosis (*Babesia rossi*) in dogs. *J Vet Intern Med.* 2013;27:150–156.

6. Ruiz de Gopegui R, Penalba B, Goicoa A, Espada Y, Fidalgo LE, Espino L. Clinico-pathological findings and coagulation disorders in 45 cases of canine babesiosis in Spain. *Vet J.* 2007;174:129–132.

7. Kettner F, Reyers F, Miller D. Thrombocytopaenia in canine bablesiosis and its clinical usefulness. *J S Afr Vet Assoc.* 2003;74:63–68.

8. Goddard A, Leisewitz AL, Kristensen AT, Schoeman JP. Platelet activation and platelet-leukocyte interaction in dogs naturally infected with *Babesia rossi*. *Vet J.* 2015;205:387–392.

9. Goddard A, Leisewitz AL, Kristensen AT, Schoeman JP. Platelet indices in dogs with *Babesia rossi* infection. *Vet Clin Pathol.* 2015;44:493–497.

10. Jandrey KE. Assessment of platelet function. *J Vet Emerg Crit Care.* 2012;22:81–98.

11. Craft RM, Chavez JJ, Bresse SJ, Wortham DC, Cohen E, Carroll RC. A novel modification of the thrombelastograph assay, isolating platelet function, correlates with optical platelet aggregation. *J Lab Clin Med.* 2004;143:301–309.

12. Weitzel NS, Weitzel LB, Epperson LE, Karimpour-Ford A, Tran ZV, Seres T. Platelet mapping as part of modified thromboelastography (TEG ®) in patients undergoing cardiac surgery and cardiopulmonary bypass. *Anaesthesia.* 2012;67:1158–1165.

13. Collyer TC, Gray DJ, Sandhu R, Berridge J, Lyons G. Assessment of platelet inhibition secondary to clopidogrel and aspirin therapy in preoperative acute surgical patients measured by Thrombelastography® Platelet Mapping™. *Br J Anaesth.* 2009;102:492–498.

14. Solano-Gallego L, Baneth G. Babesiosis in dogs and cats-expanding parasitological and clinical spectra. *Vet Parasitol.* 2011;181:48–60.

15. Collett MG. Survey of canine babesiosis in South Africa. *J S Afr Vet Assoc.* 2000;71:180–186.

16. Jacobson LS. The South African form of severe and complicated canine babesiosis: Clinical advances 1994–2004. *Vet Parasitol.* 2006;138:126–139.

17. Reyers F, Leisewitz AL, Lobetti RG, Milner RJ, Jacobson LS, van Zyl M. Canine babesiosis in South Africa: more than one disease. Does this serve as a model for falciparum malaria? *Ann Trop Med Parasitol.* 1998;92:503–511.

18. Shakespeare AS. The incidence of canine babesiosis amongst sick dogs presented to the Onderstepoort Veterinary Academic Hospital. *J S Afr Vet Assoc.* 1995;66:247–250.

19. Schoeman JP. Canine babesiosis. *Onderstepoort J Vet Res.* 2009;76:59–66.

20. Jacobson L, Clark I. The pathophysiology of canine babesiosis - New approaches to an old puzzle. *J S Afr Vet Assoc.* 1994;65:134–145.

21. Matijatko V, Kis I, Torti M, et al. Septic shock in canine babesiosis. *Vet Parasitol.* 2009;162:263–270.

22. Goddard A, Wiinberg B, Schoeman JP, Kristensen AT, Kjelgaard-Hansen M. Mortality in virulent canine babesiosis is associated with a consumptive coagulopathy. *Vet J.* 2013;196:213–217.

23. Welzl C, Leisewitz AL, Jacobson LS, Vaughan-Scott T, Myburgh E. Systemic inflammatory response syndrome and multiple-organ damage/dysfunction in complicated canine babesiosis. *J S Afr Vet Assoc.* 2001;72:158–162.

24. Matijatko V, Mrljak V, Kis I, et al. Evidence of an acute phase response in dogs naturally infected with *Babesia canis*. *Vet Parasitol.* 2007;144:242–250.

25. Koster LS, van Schoor M, Goddard A, Thompson PN, Matjila PT, Kjelgaard-Hansen M. C-reactive protein in canine babesiosis caused by *Babesia rossi* and its association with outcome. *J S Afr Vet Assoc.* 2009;80:87–91.

26. Schetters TPM, Kleuskens JAGM, De Crommert JV, De Leeuw PWJ, Finizio A, Gorenflot A. Systemic inflammatory responses in dogs experimentally infected with *Babesia canis*; a haematological study. *Vet Parasitol.* 2009;162:7–15.

27. Kules J, Mrljak V, Rafaj RB, Selanec J, Burchmore R, Eckersall PD. Identification of serum biomarkers in dogs naturally infected with *B. canis canis* using a proteomic approach. *BMC Vet Res.* 2014;10:111.

28. Lobetti RG, Mohr AJ, Dippenaar T, Myburgh E. A preliminary study on the serum protein response in canine babesiosis. *J S Afr Vet Assoc.* 2000;71:38–42.

29. Murata H, Shimada N, Yoshioka M. Current research on acute phase proteins in veterinary diagnosis: an overview. *Vet J.* 2004;168:28–40.

30. Lang T, Johanning K, Metzler H, et al. The effects of fibrinogen levels on thromboelastometric variables in the presence of thrombocytopenia. *Anesth Analg.* 2009;108:751–758.

31. Mallett S, Cox D. Thrombelastography. *Br J Anaesth.* 1992;69:307–313.

32. Zvorc Z, Rafaj RB, Kules J, Mrljak V. Erythrocyte and platelet indices in babesiosis of dogs. *Veterinarski Arhiv.* 2010;80:259–267.

33. Khandekar M, Khurana A, Deshmukh S, Kakrani A, Katdare A, Inamdar A. Platelet volume indices in patients with coronary artery disease and acute myocardial infarction: an Indian scenario. *J Clin Pathol.* 2006;59:146–149.

34. Smith SA. Overview of Hemostasis. In: Weiss DJ, Wardrop KJ eds. *Schalm's Veterinary Hematology.* Sixth Edition edn. Ames, Iowa: Wiley-Blackwell; 2010:635–653.

35. Hoffman M, Monroe DM. A cell-based model of hemostasis. *Thromb Haemost.* 2001;85:958–965.

36. Wiinberg B, Jessen LR, Tarnow I, Kristensen AT. Diagnosis and treatment of platelet hyperactivity in relation to thrombosis in dogs and cats. *J Vet Emerg Crit Care.* 2012;22:42–58.

37. Morrell CN, Maggirwar SB. Recently recognized platelet agonists. *Curr Opin Hematol.* 2011;18:309–314.

38. Herter JM, Rossaint J, Zarbock A. Platelets in inflammation and immunity. *Thromb Haemost.* 2014;12:1764–1775.

39. Bester J, Pretorius E. Effects of IL-1β, IL-6 and IL-8 on erythrocytes, platelets and clot viscoelasticity. *Sci Rep.* 2016;6:32188.

40. Brown GT, Narayanan P, Li W, Silverstein RL, McIntyre TM. Lipopolysaccharide stimulates platelets through an IL-1β autocrine loop. *J Immunol.* 2013;191:5196–5203.

41. Semple JW, Italiano JE, Freedman J. Platelets and the immune continuum. *Nat Rev Immunol.* 2011;11:264–274.

42. Brooks MB, Divers TJ, Watts AE, et al. Effects of clopidogrel on the platelet activation response in horses. *Am J Vet Res.* 2013;74:1212–1222.

43. Smith JR, Smith KF, Brainard BM. Platelet parameters from an automated hematology analyzer in dogs with inflammatory clinical diseases. *Vet J.* 2014;201:406–411.

44. Yilmaz Z, Eralp O, Ilcol YO. Evaluation of platelet count and its association with plateletcrit, mean platelet volume, and platelet size distribution width in a canine model of endotoxemia. *Vet Clin Pathol.* 2008;37:159–163.

45. Blois SL, Banerjee A, Wood RD. Evaluation of thrombelastographic platelet-mapping in healthy cats. *Vet Clin Pathol.* 2012;41:223–227.

46. Bochsen L, Wiinberg B, Kjelgaard-Hansen M, Steinbruchel DA, Johansson PI. Evaluation of the TEG platelet mapping assay in blood donors. *Thromb J.* 2007;5:3–3.

47. Bochsen L, Nielsen AB, Steinbruchel DA, Johansson PI. Higher thrombelastograph platelet reactivity in cardiac surgery patients than in blood donors. *Scand Cardiovasc J.* 2007;41:321–324.

48. Blois SL, Banerjee A, Wood RD, Park FM. Thromboelastography platelet mapping in healthy dogs using 1 analyzer versus 2 analyzers. *Can J Vet Res.* 2013;77:231–236.

49. Conner BJ, Hanel RM, Hansen BD, Motsinger-Reif AA, Asakawa M, Swanson CR. Effects of acepromazine maleate on platelet function assessed by use of adenosine diphosphate activated- and arachidonic acid-activated modified thromboelastography in healthy dogs. *Am J Vet Res.* 2012;73:595–601.

50. Chen A, Teruya J. Global hemostasis testing thromboelastography: Old technology, new applications. *Clin Lab Med.* 2009;29:391–407.

51. Cattano D, Altamirano AV, Kaynak HE, et al. Perioperative assessment of platelet function by Thromboelastograph® Platelet Mapping™ in cardiovascular patients undergoing non-cardiac surgery. *J Thromb Thrombolysis.* 2013;35:23–30.

52. Preisman S, Kogan A, Itzkovsky K, Leikin G, Raanani E. Modified thromboelastography evaluation of platelet dysfunction in patients undergoing coronary artery surgery. *Eur J Cardio Thorac Surg.* 2010;37:1367–1374.

53. Zhang J, Jiang R, Liu L, Watkins T, Zhang F, Dong J. Traumatic brain injury-associated coagulopathy. *J Neurotrauma.* 2012;29:2597–2605.

54. Wohlauer MV, Moore EE, Thomas S, et al. Early platelet dysfunction: An unrecognized role in the acute coagulopathy of trauma. *J Am Coll Surg.* 2012;214:739–746.

55. Davis PK, Musunuru H, Walsh M, et al. Platelet dysfunction is an early marker for traumatic brain injury-induced coagulopathy. *Neurocrit Care.* 2013;18:201–208.

56. Brainard BM, Kleine SA, Papich MG, Budsberg SC. Pharmacodynamic and pharmacokinetic evaluation of clopidogrel and the carboxylic acid metabolite SR 26334 in healthy dogs. *Am J Vet Res.* 2010;71:822–830.

57. Park FM, Blois SL, Abrams-Ogg ACG, et al. Hypercoagulability and ACTH-dependent hyperadrenocorticism in dogs. *J Vet Intern Med.* 2013;27:1136–1142.

58. Matjila PT, Leisewitz AL, Jongejan F, Penzhorn BL. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. *Vet Parasitol.* 2008;155:152–157.

59. Matjila PT, Penzhorn BL, Bekker CPJ, Nijhof AM, Jongejan F. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. *Vet Parasitol.* 2004;122:119–125.

60. McMichael MA, Smith SA, Galligan A, Swanson KS. *In vitro* hypercoagulability on whole blood thromboelastometry associated with *in vivo* reduction of circulating red cell mass in dogs. *Vet Clin Pathol.* 2014;43:154–163.

61. Smith SA, McMichael MA, Gilor S, Galligan AJ, Hob CM. Correlation of hematocrit, platelet concentration, and plasma coagulation factors with results of thromboelastometry in canine whole blood samples. *Am J Vet Res.* 2012;73:789–798**.**

62. Kornblith LZ, Kutcher ME, Redick BJ, Calfee CS, Vilardi RF, Cohen MJ. Fibrinogen and platelet contributions to clot formation: Implications for trauma resuscitation and thromboprophylaxis. *J Trauma Acute Care Surg.* 2014;76:255–263.

63. Fries D, Innerhofer P, Reif C, et al. The effect of fibrinogen substitution on reversal of dilutional coagulopathy: An in vitro model. *Anesth Analg.* 2006;102:347–351.

64. Zentai C, Solomon C, van der Meijden PEJ, et al. Effects of fibrinogen concentrate on thrombin generation, thromboelastometry parameters, and laboratory coagulation testing in a 24-hour porcine trauma model. *Clin Appl Thromb Hemost.* 2016;22:749–759**.**

65. Wagner C, Mascelli M, Neblock D, Weisman H, Coller B, Jordan R. Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood.* 1996;88:907–914.

66. Thompson C, Jakubowski J, Quinn P, Deykin D, Valeri C. Platelet size as a determinant of platelet-function. *J Lab Clin Med.* 1983;101:205–213.

67. Thompson C, Jakubowski J, Quinn P, Deykin D, Valeri C. Platelet size and age determine platelet-function independently. *Blood.* 1984;63:1372–1375.

68. Blois SL, Lang ST, Wood RD, Monteith G. Biologic variability and correlation of platelet function testing in healthy dogs. *Vet Clin Pathol.* 2015;44:503–510.

69. Nielsen LA, Zois NE, Pedersen HD, Olsen LH, Tarnow I. Platelet function in dogs: Breed differences and effect of acetylsalicylic acid administration. *Vet Clin Pathol.* 2007;36:267–273.

70. Bozic-Mijovski M, Rakusa M, Stegnar M. Variation in platelet function testing has a major influence on detection of aspirin resistance in healthy subjects. *Pathophys Haemost Thromb.* 2007;36:84–90.

71. Clemmons R, Meyers K. Acquisition and aggregation of canine blood-platelets - Basic mechanisms of function and differences because of breed origin. *Am J Vet Res.* 1984;45:137–144.

APPENDIX A: INFORMATION SHEET ON CANINE BABESIOSIS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Dear Client

Your dog is suffering from canine babesiosis, a disease transmitted by ticks. This disease is also known as biliary or tick bite fever. The parasites, which are only visible through a microscope, occur in the red blood cells and cause them to be destroyed. This causes anaemia and various other clinical problems. Dogs suffering from babesiosis may die if they are not treated.

There are indications that cases infected with this blood-borne parasite suffer from abnormal blood clotting, and in some instances may develop life-threatening thrombo-embolic disease or organ failure as a result of microthrombi formation in the circulatory system. We are running a research project in an attempt to study the role of blood platelets in the development of these clotting abnormalities. This will require the collection of 4 blood samples taken at presentation. The collection of these samples will in no way be detrimental to your pet and at no time will the study change the treatment your pet would normally receive. No additional costs will be charged to you for the collection of the samples or the blood tests required for this study. The treatment of your dog will be at the discretion of the attending clinician and will not be changed for the purpose of this trial.

A better understanding of the disease process may lead to better treatment of clinical cases. This study is approved by the Animal Ethics Committee of the University of Pretoria.

If you have any questions, you may contact me using the contact details below.

Sincerely

Dr LJ van Rooyen

Intern Clinical Pathologist: IDEXX Laboratories Pty (Ltd)

Email:Liesl-VanRooyen@idexx.com

Tel: 082 265 1225

Appendix B: FORM FOR INFORMED CONSENT

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

(To be completed by the patient's owner / authorised agent)

Project title: The use of platelet mapping as part of modified thromboelastography as a measure of platelet function in canine babesiosis caused by *B. rossi.*

Please encircle Yes or No where necessary.

1. Have you read the information sheet on canine babesiosis?
   Yes No

2. Have you had the opportunity to ask questions about the research project?

   Yes No

3. Have you received satisfactory answers to your questions?
   Yes No

4. Have you received enough information about this study?
   Yes No

5. Supply the name of the person to whom you have spoken to:

   ....

6. Do you grant consent that blood and urine samples can be collected from your dog?
   Yes No

7. Do you grant consent that a post mortem examination can be performed in the case of death?

   Yes No

Name of owner: ……………………………………………………………………………..

Signature: ....……………………………………………………………..

Name of witness: ....

Signature: ………………………………………………………………………………….

Date: ....

Appendix C: INFORMATION SHEET FOR CONTROL DOGS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Dear Client

Your dog has been selected to serve as a healthy control dog for a study to aid us in evaluating the role of blood platelets in abnormal blood clotting in dogs suffering from babesiosis (tick bite fever/biliary). We would appreciate your consent to collect 4 blood samples.

The volume of blood we will collect will in no way harm your pet or change the procedure for which your pet was admitted for.

Thank you for your willingness to participate in this clinical trial. Should you have any further enquiries about the trial, you are welcome to contact me.

Sincerely

Dr LJ van Rooyen

Intern Clinical Pathologist: IDEXX Laboratories Pty (Ltd)

Email:Liesl-VanRooyen@idexx.com

Tel: 082 265 1225

I, ……………………………………………………………., hereby give permission that my dog ………………………………., a …………………………….. may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.

I understand that this study will in no way harm my dog. Furthermore I understand that no additional costs will be incurred to me in respect of this trial for the collection of blood samples or the blood tests required over and above the normal ovariohysterectomy or castration costs.

Signed at Onderstepoort on the ………………….. day of ………………… 2015/6

Signature Owner/Agent ………………………………………………………..

Home Tel: ………………………………..

Work Tel: …………………………………

Cell No: ……………………………………

APPENDIX D: DETAILS OF STUDY SUBJECTS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Date ____/____/____ Time:_______

dd mm yy

Owner number: _______________ Patient number: _____________

IDENTIFICATION/OR AFFIX PATIENT STICKER

1. Owner's name: ____

2. Owner's address: ____

____

____

3 .Dog's name: _________________________Dog's breed:______________________

4. Dog's birth date: ____/____/____ Dog's age: ________

dd mm yy

5. Sex: M/F Neutered: Yes No

6. Has your dog been vomiting during this current illness: Yes No

7. Has your pet received any other treatments or medications whatsoever in the last 4 weeks?
Yes No

Dr van Rooyen Protocol: Platelet mapping in Babesia (082 265 1225)

8. If you answered yes to the last question, please specify the treatments given or the disease for which the dog was treated:

____

9. How long has your pet been ill?

____

10. When did your pet last eat and what did it eat?

____

Dr van Rooyen Protocol: Platelet mapping in Babesia (082 265 1225)

APPENDIX E: DATA CAPTURE SHEET-PLATELET MAPPING BABESIA STUDY

(DR VAN ROOYEN)

Date ___.____.______ Time:_______

dd mm yyyy

Owner number: _______

Patient number: _______

CLINICAL EXAM FINDINGS:

Temperature:		**Microhaematocrit/PCV:**	
Pulse:		**ISA analysis:**	
Respiration:		**Lactate:**	
CRT:		**Faecal analysis:**	
Mucous membranes:			
Thoracic auscultation:			
Abdominal palpation:			
Blood smear evaluation:			
PCV&RLB			

LABORATORY DATA:

EDTA: Haematology					
RBC:		**MCHC:**		**MPV:**	
Hb:		**CHCM:**		**PCDW:**	
Hct:		**WCC:**		**MPM:**	

MCV:		**PLT:**		**MPC:**	
RDW:		**Thrombocrit:**			
Blood smear evaluation:					
Differential count:					

Serum: biochemical panel					
TSP		**Creatinine**		**Na**	
Alb		**ALP**		**K**	
Glob		**ALT**		**Total bilirubin**	
Urea		**Glucose**		**CRP**	

Citrate: TEG and fibrinogen determination					
R-time		**MA**		**Ly30**	
K-time		**G**		**Ly60**	
α-angle		**Cl**		**Fibrinogen**	

Heparin: Platelet mapping analysis	
MA_{ADP}	
MA_{AA}	
MA_{FIB}	

Urine analysis					
Colour		**Prot**		**Ketones**	
Turbidity		**pH**		**Bilirubin**	
S.G.		**Glucose**		**UBG**	
Hb		**Blood**			
Sediment evaluation					