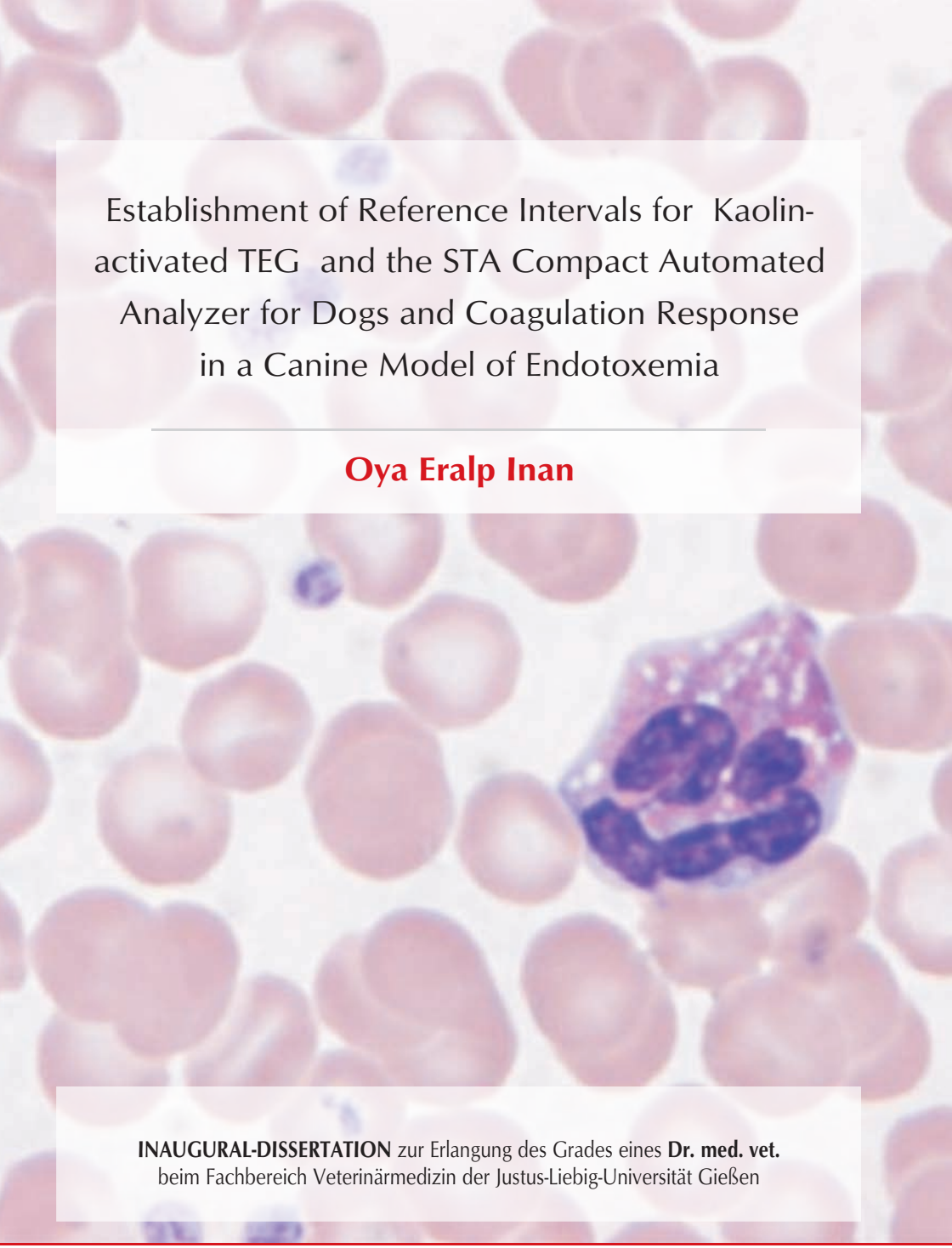
Establishment of Reference Intervals for Kaolin-activated TEG and the STA Compact Automated Analyzer for Dogs and Coagulation Response in a Canine Model of Endotoxemia

Oya Eralp Inan



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Oya Eralp Inan

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Abbrevations

ACT	activated clotting time
ADP	adenosinediphophate
anti-FXa	anti-activated factor X
APC	activated protein C
APCR	activated protein C resistance
aPTT	activated partial thromboplastin time
AT	antithrombin
avWD	acquired von Willebrand disease
α_2AP	α-2-antiplasmin
BMBT	buccal mucosal bleeding time
CBC	complete blood count
CHS	Chediak-Higashi syndrome
CV	coefficient of variation
deg	degree
DIC	disseminated intravascular coagulation
DSH	domestic shorthair
FVIII	factor VIII
FV/FVa	factor V/activated factor V
FVII/FVIIa	factor VII/activated factor VII
FVIII/FVIIIa	factor VIII/activated factor VIII
FIX/FIXa	factor IX/activated factor IX
FX/FXa	factor X/activated factor X
FXII/FXIIa	factor XII/activated factor XII
FXIII/FXIIIa	factor XIII/activated factor XIII
FDP	fibrin degradation product
GT	Glanzmann thrombostenia

HC	heparin cofactor II
HF	freeze-thawing hemolysis
HK	high molecular weight kininogen
HM	mechanical hemolysis
IMT	immune mediated thrombocytopenia
K	kallikrein
LMWH	low molecular weight heparin
LPS	lipopolysaccharide
min	minute
mm	millimeter
MPC	mean platelet component concentration
MPM	mean platelet mass
MPV	mean platelet volume
NO	nitric oxide
OSPT	one stage prothrombin time
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PC	protein C
PCDW	platelet component distribution width
PGI_2	prostacycline
PK	prekallikrein
PLT	platelet
pNA	para-nitroaniline
PS	Protein S
PT	prothrombin time
TAFI	thrombin activable fibrinolysis inhibitor
TEG	thromboelastography
TF	tissue-factor
TFPI	tissue-factor pathway inhibitor

TM	thrombomodulin
tPA	tissue plasminogen activator
TT	thrombin time
TXA_2	thromboxane A_2
WBC	white blood cell
vWD	von Willebrand disease
vWf	von Willebrand factor

1. Preface

In the past 50 years, several risk factors for developing venous thrombosis have been described in people.[1] In veterinary medicine, knowledge of factors predisposing to thrombophilia is limited and subject of current investigations. In 1854 the German pathologist Rudolf Virchow stated the related factors leading to hypercoagulable states.[1] So these hypercoagulable states may be causes of venous thrombosis, arterial thrombosis and thromboembolism, leading to organ failure.[1] Disorders associated with hypercoagulation may be acquired or hereditary.[1] In people, many hereditary disorders have been detected but reported disorders in animals are generally acquired and caused by other underlying diseases.[1,2] Early diagnosis might provide a rapid treament. There are several routine laboratory tests including coagulation times (prothrombin time, activated partial thromboplastin time), fibrinogen, and platelet count used in veterinary medicine to diagnose hypocoagulatory states (bleeding tendency), but only rare tests for detection of hypercoagulable states. Techniques like thromboelastography give qualitative information about all phases of the coagulation process including the clot formation and breakdown and also the stability of the clot itself. In people[3] and dogs[4], thromboelastography has been used to detect both hypo- and hypercoagulability. Thromboelastographic evaluations of hypercoagulability has been performed in dogs with neoplasia, disseminated intravascular coagulation (DIC), immune mediated hemolytic anemia (IMHA), parvoviral enteritis and treatment with deracoxib.[5-9] In this study it was also of interest to detect an early coagulation response in a canine model of endotoxemia by measuring antithrombin (AT), protein C (PC), protein S (PS), activated protein C (APC) response, plasminogen (PLG), factor VIII (FVIII) and fibrin degradation products (FDPs). Therefore, using these parameters for dogs, test methods have to be evaluated and reference ranges are needed. However, reference ranges for

coagulation parameters in dogs have been mainly established for routine coagulation parameters. Furthermore, even for the routine coagulation panel it is generally agreed upon that analyzer- and method-specific reference intervals have to be established.

The current study has two aims:

1) The establishment of reference ranges for kaolin-activated TEG (TEG® 5000 Thrombelastograph, Haemonetics Corporation; formerly Haemoscope Corporation; Braintree, MA, USA) variables R, K, angle α, MA and G and for the variables one-stage prothrombin time (OSPT), activated partial thromboplastin time (aPTT), thrombin time (TT), antithrombin (AT) , fibrinogen plasma concentration, PC, PS, PLG, APC-ratio, FVIII, D-dimer and anti-factor Xa (anti-FXa) measured with the benchtop analyzer STA Compact (STA Compact®, Roche Diagnostics GmbH, Mannheim, Germany). Also repeatability and interferences such as hemolysis and lipemia were assessed before developing the reference intervals.
2) In the second part of the study, it was of interest to characterize the early response of the coagulation system to low-dose lipopolysacharide (LPS) application in healthy dogs compared to a placebo (saline) as reflected by dynamic alterations of variables reflecting secondary and tertiary hemostasis, natural inhibitors of coagulation as well as TEG measurements.

2. Introduction

2.1. Physiology of the coagulation process

Hemostasis is the mechanism to control blood loss (minor or major) arising from vascular or tissue trauma. Also fibrinolysis, the physiological remove of the clot, is initiated with the activation of coagulation and is a part of this mechanism. The hemostatic system is a finely balanced mechanism. A reduced clotting activity, is called hypocoagulation or the adverse, hypercoagulation, can lead to pathophysiological states thrombosis or thromboembolism.[10] Under physiological conditions, coagulation does not occur in blood circulation, because clotting factors are circulating in inactive forms and other required components are in the extravascular area (tissues).[10,11] There are different types and a variety of proteins (coagulation proteins) included in the coagulation process.[10] When extravascular components get into the blood stream, the coagulation process is initiated with a series of activations (cellular and enzymatic) and cell surface changes.

Traditionally, the hemostatic system has been divided into 3 stages: primary hemostasis, secondary hemostasis and tertiary hemostasis, including fibrinolysis and natural anticoagulants.[12]

2.1.1. Primary hemostasis

The primary hemostasis is the phase responsible for creating the primary platelet plug. Here, the reactions take place with vascular endothelium components, platelets and von Willebrand factor (vWf).[12] The normal vascular endothelium is inhibiting the coagulation process by synthesis of antagonists of platelet activation including nitric oxide (NO) and prostacyclin (PGI_2). But after vascular injury, vasoconstriction develops, so blood flow is slowed down and blood loss is delayed. Folllowing platelet adhesion, activation and aggregation, the primary platelet plug is formed at the corrupted vessel wall. With the adhesion of platelets to the collagen fibers in the

exposed subendothelium, platelets bind to collagen. The process of platelet adhesion is influenced by the shear rate. In regions with high shear rate (such as small and medium sized arteries), the initial adhesion takes place via collagen and vWf.[12] In regions of low shear rate (such as venes or large arteries) the platelet adhesion is mediated by fibrinogen.[12] The adhesion activates platelets and their shape changes lead to an increase of their surface contact. Activated platelets stimulate the aggregation of other platelets. The most important aggregating agent is thrombin which is formed by the coagulation pathway from prothrombin (factor II).[12] vWf and fibrinogen together also mediate platelet aggregation. The primary platelet plug is formed and needs to be stabilized by fibrin. Primarily, the aim of secondary hemostasis is to form fibrin by thrombin.

2.1.2. Secondary hemostasis

Secondary hemostasis is composed of the coagulation cascade, resulting in fibrin formation. The coagulation cascade includes three pathways: the intrinsic pathway, the extrinsic pathway, and the common pathway.[12] The cause for calling it "extrinsic" is the fact that the components of this pathway are provided from the extravascular area.[10,12] This classification of the coagulation cascade does not correspond to in vivo conditions, but is helpful for the interpretation of laboratory tests.[13,14] The extrinsic pathway is initiated by a trauma and leads to the release of tissue factor (TF) from different tissues like organ capsules and mucous membranes, which activates factor VII (FVII, prothrombin).[10] Pathological conditions like inflammation can result in expression of TF by other type of cells.[10] The activated FVII (FVIIa)-TF complex activates factor X (FX) and this is the intersection point with the intrinsic pathway, followed by the common pathway. The intrinsic pathway is initiated primarily by thrombin (small amount), which is formed by the exstrinsic pathway.[12] The tissue factor pathway inhibitor (TFPI) inhibits the mechanism initiated by TF. The small amount of thrombin initiates the intrinsic pathway through the activation of FXI. FXI is also activated by an auto-activation with the activation of factor XII (FXII) by the

high molecular weight kininogen (HK)-prekallikrein (PK) complex (contact pathway).[10,12,15] This action leads also to the conversion of PK into kallikrein (K), which then is responsible for the convertion of HK into bradykinin.[12] Bradykinin leads to the release of tissue plasminogen activator (tPA).[16] This phase is more important with its anticoagulant and profibrinolytic effects than its coagulation effects.[10] After the activation of factor XI (FXI), activated FXI (FXIa) activates factor IX (FIX). The activated FIX (FIXa)-activated factor VIII (FVIIIa) complex interacts with calcium and platelets (tenase complex) for the activation of FX (the intersection point).[12] Some studies suggests that the intrinsic pathway, i.e. the contact pathway plays a role in thrombus formation and also does not correspond to in vivo conditions.[10,17,18]

At the intersection point the common pathway begins and an activated FX (FXa)-activated factor V (FVa) complex is formed.[12] This complex interacts with calcium and platelet phospholipid (prothrombinase complex) and transforms fibrinogen to fibrin by the development of thrombin (large amount) from prothrombin.[10,12]

Thrombin activates also FVIII, which is responsible for the formation of cross-linked fibrin polymers from fibrin polymers together with calcium.[12] FVIII is only activated if there is a certain amount of fibrin polymers.[19] Thrombin is one of the most important component of hemostasis, which is not only responsible for the coagulation but has also an antithrombotic effect.[10,20] The large amount of thrombin, needed for fibrin formation, is supplied by the associated mechanism of the extrinsic and intrinsic pathway.[21] The plasma half-life of thrombin is very short.[10,22]

2.1.3. Tertiary hemostasis

The tertiary hemostasis is the fibrinolysis part of coagulation. The aim of this process is to form plasmin for the clot dissolution.[12,23]

Plasminogen is cleaved to plasmin by tPA.[10,12] At this time, plasminogen is bound to the fibrin at the clot.[10,12] Kallikrein and FVIIa activates plasminogen directly, but urokinase and FXII are also activators.[10,12] Activated protein C (APC) induces the

activation of plasmin. Plasminogen activation inhibitor PAI is a inhibitor of plasmin due to its blocking effect on APC.[24] The plasmin in circulation is inhibited by α_2-antiplasmin (α_2AP).[24] Fibrin degradation by plasminogen results in the formation of fibrin degradation products (FDPs). D-dimer are FDPs, which only appear by the degradation with plasminogen.[13,25]

2.1.4. Natural inhibitors of hemostasis

There are a variety of inhibitors with different effects. As mentioned before, NO and PGI_2 are natural inhibitors efficient in primary hemostasis by inhibition of platelet function.[10,12]

Another natural anticoagulant is antithrombin (AT). It is efficient together with its cofactor heparin.[10] Thrombin is inhibited by AT with the stimulation of heparin. For this reason heparin is used as an antithrombotic agent.[10,26,27] AT-heparin complex has also an inhibiting effect against a variety of proteases like FXIIa, FXIa, K, FIXa and FVIIa.[10]

Heparin cofactor II (HC) is a specific thrombin antagonist. HC inhibits thrombin, however, HC deficiency is not associated with a of risk for thrombosis.[10,26] It is thought that HC is the primary involved in the process of wound healing and inflammation.[10,26]

Tissue factor pathway inhibitor (TFPI) is an inhibitor of TF-FVIIa and FXa, which exists on cell surfaces and platelets.[10,28] FXa-activated factor V (FVa) complex is resistant to TFPI inhibition.[10,29] Recent studies indicate that protein S (PS) has a cofactor role for TFPI.[10,30]

Protein C (PC) is an inhibitor of thrombin, which is produced in the liver.[10] PC is activated by PS and molecules of endothelial cells, but for an effective inhibition of thrombin, activated protein C (APC) needs thrombomodulin (TM).[10,31] APC inhibits thrombin formation by blocking of FVIIIa and FVa whereupon PS is recquired as a cofactor. PS is also a cofactor for TFPI which is necessary for the inhibition of FXa.[10,30] But when FVIII is bound to vWf, it it is not inhibited by APC.[10,31] Furthermore,

cleaving of FV by APC is more efficient on endothelial surfaces.[10,32] Activated PC-PS also activates plasminogen activator inhibitor 1 (PAI-1), which is an inhibitor of plasminogen activators and effects tPA.[10,31]

As in all physiologic processes, there is also a counter regulation of the inhibition of fibrinolysis.

One of the fibrinolysis inhibitors is the thrombin activated fibrinolytic inhibitor (TAFI) which prevents the fibrin clot from fibrinolysis. TAFI is activated by thrombin (large amount) and plasmin.[10] TAFI changes the structure of fibrin and blocks the binding of plasminogen to fibrin.[10]

Another inhibitor of fibrinolysis is α_2AP which inhibits plasminogen, as mentioned before.[10] However, plasminogen bound to fibrin in a plasminogen-fibrin complex is resistant to α_2AP inhibition.[10]

2.2. Pathologies of the coagulation process

Hemostatic disorders can be inherited or acquired. Acquired disorders are the most common hemostatic problems.[10]

2.2.1. Hereditary coagulopathies

Hereditary coagulopathies include disturbances of primary and secondary hemostasis. One of the most often seen hemostatic defects in people[33,34] and in dogs[33,35] is von Willebrand disease (vWD). Clinical signs associated with vWD are those typically observed in disorders of primary hemostasis, like epistaxis, petechia, hematuria and a prolonged bleeding time after trauma. Clinical signs and diagnosis can change in relation to the defect of vWf.[33,34,35-39] Clinical signs are best known for dogs[33,35,40-45], but the disease has been also detected in other animals.[33,46-51]

An inherited thrombocytopenia can seen with the grey collie syndrome associated with disturbances in neutrophils, reticulocytes and platelets.[52] Some dog breeds have a thrombocytopenia without clinical signs like Greyhounds and Cavalier King Charles Spaniels.[53-56] Other inherited disorders are Glanzmann thrombosthenia (GT)

and Chediak-Higashi-Syndrom (CHS). Here, the diseases are arising from platelet function disorders.[54,57-59] A thrombopathy like the Scott syndrome in people has been reported in a German Shepherd dog family.[54,56,60] Hemophilia A is the most common hereditary coagulation disorder in animals.[61] The aetiology of the disorder is a deficiency or functional problem of FVIII and FIX.[61,62] Hemophilia A is not a breed specific disorder, however, it is often seen in German Shepherd dogs.[61,63] The disease can be seen in severe or mild form.[61] In mild forms no clinical signs are present and disorders can only recognized after a trauma or surgery. Otherwise in more serious cases, hematoms or other spontaneous hemorrhages can be seen.[61] Also hemophilia B is an important coagulapathy which is not as often seen as hemophilia A.[61] Here the cause is a deficiency or functional problem of FIX.[61,62] Clinical signs are the same as for hemophilia A.[61]

All of the remaining factor deficiencies identified in animals like fibrinogen, prothrombin, FVII, FX, FXI, FXII and contact factor defects are rare and are generally breed specific or have been seen in a family.[61] One of this disorders is a hereditary fibrinogen defect reported in dogs, cats, goats and sheep.[61,64,65] Acquired hypofibrinogenemia arising from pathological conditions is more commonly seen.[61] Hereditary prothrombin deficiency have been reported in few cases.[61,64] FVII deficiencies have been reported in Beagles, Deerhounds and in a Alaskan Klee Klai.[61,66,67] FX deficiencies have been reported in dogs and a domestic shorthair (DSH) cat, whereat this FX deficiency is only a partial one, because the absence of FX can lead to death.[61,64,68,69] FXI deficiency has been reported in dogs, cattle and DSH cats.[61,70-72] FXII, PK and HK defects have been detected in dogs, cats (purebred and DSH) and horses.[61,64,73-76] Deficiencies of all vitamin K dependent factors due to defect in vitamin K decarboxylase have been described in cats, sheep and in Labrador retrievers.[61,77-79] All these problems lead to mild to severe bleeding disorders.[61]

2.2.2. Acquired coagulopathies

Acquired coagulaopathies are much more commonly seen disorders than hereditary ones. Platelet disorders and acquired coagulation deficiencies can be caused by a variety of infectious diseases.[80]

Platelet disorders may result from thrombocytopenia, thrombocytopathia or thrombocytosis. Thrombocytopenia can occur due to decreased platelet production, platelet destruction, increased consumption or blood loss. Bone marrow disturbances like neoplasia or leukemia may result in low production of platelets.[24,53] The destruction of thrombocytes has a variety of mechanisms, one of them is immune-mediated thrombocytopenia (IMT). Further aetiology includes neoplasia, drugs, bone marrow disorders or inflammatory processes.[24,53,81,82] Infectious diseases including parasites such as Babesia canis infection, some bacterial infections like leptospirosis, salmonellosis, or rickettsial infections like *Ehrlichia canis*,*Anaplasma platys, Anaplasma phagocytophilum* or *Rickettsia rickettsii* and viral diseases like canine distemper virus (CDV), herpes virus, infectious canine hepatitis or parvovirus infection in dogs can all be associated with thrombocytopenia.[24,53,84-87] The consumption of platelets is generally caused by DIC, thrombotic thrombocytopenic purpura (TTP) which is well known in people but rarely described in animals (pigs) and hemolytic uremic syndrome (HUS).[53]

In contrast thrombocytosis may occur secondary to some drugs or inflammatory cytokines.[88]

Acquired thrombopathias generally occur secondary to underlying diseases/conditions like renal failure, infections, neoplasia and administration of such as non-steroidal anti-inflammatory drugs[89] such as aspirin[3] and clopidogrel.[90,91]

Acquired vWD is uncommon but can be observed in people and animals due to other underlying diseases such as immune mediated, cardiac or neoplastic diseases or pretreatment with hydroxethyl starch.[33,34]

Coagulatiom components like prothrombin, FVII, FIX, FX, FXI, FXII, PC, PS, FV, FXIII, AT and plasminogen originate from the liver. So liver diseases and hepatobiliary disorders may lead to deficiencies or functional insufficiency of these components and result in different coagulation disorders.[80,92-96] Moreover, in small animal clinical practice, bleeding disorders in association with rodenticide toxication is a common acquired coagulopathy. Coumadin and second-generation anticoagulant rodenticides inhibit the vitamin K epoxide reductase in the liver and thus the vitamin K cycle.[10] Vitamin K is required for the synthesis of functional prothrombin, FVII, FIX and FX by carboxylation.[10] In the absence of vitamin K, these coagulation componenets are circulating as inactive molecules in the plasma resulting in bleeding disorders.[80]

Additonally, all these coagulation components can be influenced by drugs, vasculitis, renal disease, endocrinal diseases and can result in a variety of coagulopathies.[80] DIC is also a common cause for acquired coagulopathy due to a consumption of coagulation platelets, coagulation factors and inhibitors of coagulation.[97]

2.2.3. Hemostasis, sepsis and DIC

DIC is an acquired coagulopathy, which disturbs the hemostatic balance and results in microthrombus formation and consumption of coagulation and fibrinolysis componenets. All this can lead to death. DIC is not a disease itself but arises from other underlying diseases, which increase the activation of coagulation components and affect generally severely ill patients.[7,13,14,25,98-102] Reported underlying diseases include neoplasia, sepsis, endotoxemia, pancreatitis, IMHA which can be seen in different priorities in small animals, farm animals and horses.[97,108-110] The knowledge about pathogenesis of DIC has been mainly gained from experimental studies.[97,111,112]

At first, coagulation is initiated due to the massive endothelial injury associated with inflammation. Cytokines causes a TF expression, much more than in normal conditions and are responsible of TFPI insufficiency.[97,113,116] The TF-FVIIa amount, which is responsible for thrombin formation, could not be regulated under these

circumstances. At this stage, natural anticoagulants are still enough for an inhibition of coagulation, however this phase is characterized by a hypercoagulable state.[97,112,117] The continuing imbalance cannot depress the thrombin formation, which leads to microvascular fibrin accumulations.[97] At this time, inflammation initiates coagulation and coagulation triggers the inflammation in a complex mechanism.[97,111,118-120] The persistent underlying disease results in the continous consumption of coagulation factors and inhibitors, which is also a cause of sustained inflammatory effects and can end with sepsis.[97,111,118,121,123] With the consumption of this components a hypocoagulable state arises. Microthrombus formations is followed by hypoxia in different tissues and organ failures.[97] This state leads to spontaneous bleeding or prolonged bleeding times after injury. All this factors are life-threatining and DIC can end with death.

In the majority of studies, the regulation of coagulation during the early course of a septic process has not been investigated. Although studying the early dynamic changes of coagulation after infection would be ideally carried out in canine patients with the naturally developing syndrome, there are ethical limitations to this approach. An alternative method is the use of endotoxin i.e., lipopolysaccharide (LPS) in research animals, like done here with low-dose LPS administration. However, the overwhelming induction of endotoxemia commonly used in experimental models is unlike the smoldering septic process typically seen in clinical patients. Thus, the application of information gained from a rapidly fatal, severe insult to a clinical patient with more smoldering disease has been considered to be difficult.[124]

2.3. Diagnostic tests

The most important aspect of diagnosis of hemostatic disorders is the collection and submission of an optimal sample for testing to avoid prenalytic errors due to poor sample collection and handling.[12]

2.3.1.Primary hemostasis

Primary hemostasis tests include the evaluation of platelet count, platelet function, assessment of vWf and the buccal mucosal bleeding time (BMBT). For the evaluation of platelet numbers, EDTA as anticoagulant is preferred, because heparin may cause platelet clumping.[125]

The platelet count can be estimated from a blood smear, where each platelet seen in a microscope field with the 100× oil immersion objective is nearly equivalent to 15,000 platelets/µl.[24] In many clinics, automated hematology analyzers are used, however, the automated results have to be confirmed on a blood smear in case of thrombocytopenia. Here, the ADVIA 2120™ with species specific software (Siemens Healthcare Diagnostics GmbH) was used,providing an accurate analysis of platelets and platelet activation indices due to sphering of platelets and two dimensional laser light scattering[126], mentioned later.

Regarding the assessment of platelet function, the most commonly used test is the buccal mucosal bleeding time, the BMBT.[12] It is a simple clinical method and very useful. A small incision is made with a lancet at the oral mucosa. After this, a filter paper is hold to the incision rim and the time is taken until the blood is not permeating into the filter paper any more. This time is the BMBT.

Aggregometry is a commonly used *in vitro* platelet function test. There are two types of aggregometry, i.e. impedance and optical methods.[127] A platelet agonist is added to sample, which binds to platelet membranes and platelet aggregation is initiated.[127] Hematology analyzers such as the ADVIA 2120 might be used for assessment of platelet function, i.e. whole blood aggregometry by measuring the aggretion-induced decrease in platelet count after adding the agonist.[128] Furthermore, the ADVIA 2120 provides a unique methodology to specifically assess platelet activation status. During each measurement, a variety of platelet indices are routinely provided that are increasingly recognized as surrogate markers of platelet activation and include mean platelet volume (MPV), mean platelet mass (MPM), the mean platelet component concentration (MPC) and the platelet component distribution width

(PCDW).[129-131] The MPC is a measure for platelet refractive index reflecting platelet granularity – and thus the activation status.[126] The MPV is derived from the platelet volume histogram. The MPM is calculated from the platelet dry mass histogram, which means platelet volume ×platelet content/100. The PCDW is a measure of the variation in platelet shape change and is calculated by MPC × 100/standard deviation of MPC.

Regarding the evaluation of vWf, functional, structural and quantitative measurements of vWf can be performed.[12] Some functional assays have only been used in human medicine, so that they have to be adapted for their use in animal specimens.[33]

2.3.2. Secondary hemostasis

Assessment of secondary hemostasis includes tests for the evaluation of the extrinsic, intrinsic, and common pathway. Routinely used tests include the activated clotting time (ACT), prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen. The ACT gives information about the intrinsic and common pathway.[12] The ACT is performed by adding whole blood sample tubes, containing some subtrates, which initiate the coagulation by contact activation, and time is measured until a clot formation. The ACT gives information about the intrinsic and common pathway and can be used for monitoring of anticoagulant therapy (especially heparin).[12]

Like the ACT, the aPTT reflects the intrinsic and common pathway. For measurement of the aPTT, the coagulation reaction is initiated by contact activation. In the actual study, kaolin was used as surface activator (STA APTT Kaolin, Roche Diagnostics GmbH, Mannheim, Germany) and results are reported as the coagulation time. Abnormal prolongations of aPTT are related with conditions affecting the intrinsic or common pathway, like congenital factor defects, drugs such as heparin, and a variety of other diseases.[132-135]

The PT is a screening test for the extrinsic and common pathways. Here a one-stage prothrombin-time (OSPT) measurement was performed. The OSPT is measured by adding calcium and thromboplastin into the citrated blood plasma and like the other tests the coagulation time is measured. Here, a combined thromboplastin, i.e. a reagent containing fibrinogen and FV in addition to thromboplastin was used (STA Neoplastin® Plus, Roche Diagnostics GmbH, Mannheim, Germany). The prolongation of the OSPT is an indicator of conditions affecting the extrinsic pathway, i.e. like hereditary or acquired factor VII deficiencies or inhibitors (e.g. warfarin, high doses of heparin). [64,132-134,136]

Studies have shown that the shortening of OSPT did not indicate a hypercoagulable state.[7,98,112] Another screening test for assessment of secondary hemostasis is the thrombin time (TT) reflecting the common pathway. TT is influenced by the conversion of fibrinogen to fibrin and fibrin polymerization. For the test, thrombin (STA Thrombin Reagenz, Roche Diagnostics GmbH, Mannheim, Germany) is added into citrated plasma sample. The prolongation of TT can induced by low fibrinogen plasma concentration, dysfibrinogenemia, hyperfibrinogenemia, or high concentrations of FDPs in the blood plasma.[132,134,137-139] Shortening of TT have been detected due to errous sample handling.[132,140]

Another variable routinely assessed is fibrinogen and there are several techniques as functional or immunochemical methods.[132-134,141,142] The method used here is the Clauss-method, a modified TT.[132] The test is performed by addition of a larger amount of thrombin than in the TT test (STA Fibrinogen, Roche Diagnostics GmbH, Mannheim, Germany) to reduce the interference by heparin therapy.[132] Also here the clotting time is measured, but the results are converted into fibrinogen concentrations by a standard curve which is obtained by a serial dilution of human plasma calibration standard (STA Unicalibrator, Roche Diagnostics GmbH, Mannheim, Germany used here). Hyperfibrinogenemia can occur in acute phase responses.[132,134,141] Hypofibrinogenemia can be caused by DIC, liver diseases,

hyperfibrinolytic syndrome and thrombolytic therapies.[132,134] False low results by high FDP levels in dogs have been detected.[132,143-146]

The measurement of FVIII is done by a chromogenic method and the test is traditionally performed with a one-stage clotting assay. The test is performed by adding a reagent including cogulation activators and phospholipids and a human-plasma deficient of the factor which should be evaluated, so that the only factor activity is from the patient plasma (STA Factor VIII, Roche Diagnostics GmbH, Mannheim, Germany).[147] The method used here was a modified one-stage aPTT test. Results are compared with a standard curve derived from serially diluted pooled plasma from healthy people or dogs, respecitively.

2.3.3. Tertiary hemostasis

In humans, variables reflecting tertiary hemostasis include plasminogen, α_2AP, PAI and tPA.[132,134,148-151] In veterinary medicine, plasminogen, FDPs and D-dimer measurements are the most commonly used tests. α_2AP, PAI and tPA are only used for research, because of their costs and unknown diagnostic use in veterinary medicine.[132,152,153] Plasminogen is assessed photometrically with a chromogenic assay (e.g, STA Plasminogen, Roche Diagnostics GmbH, Mannheim, Germany). An excess of streptokinase is added to the plasma so that plasminogen in the sample forms a complex with plasmin-like activity. After that, the activity of this complex is measured by its action on a chromogenic substrate which is added later. A low concentration of plasminogen might occur due to increased consumption (DIC, thrombolytic therapy) and decreased production, e.g. due to liver failure.[132,134,149,150,154]

As a part of the fibrinolysis process, the fibrin split products such as FDPs and D-dimers are generated, which can be measured with several point of care tests and bench top analyzers.[110] In contrast to D-Dimers, FDP values can also be increased by plasma fibrinogen degradation or fibrin monomers so that the FDP test is less specific for diagnosing DIC.[106] The D-dimer is formed by the degradation of the cross-linked fibrin clot by the effect of plasmin.[107,132,156,157] There are different test methods for D-

dimer evaluation includung turbidometry, latex agglutination, fluorescence immunoassay, and ELISA. Here we used the immunoturbidimetric assay (STA Liatest™ D-Dimer, Roche Diagnostics GmbH, Mannheim, Germany) and this method is currently the gold standard for assessment of D-dimer concentration assessment.[132] Increased plasma levels of fibrin D-dimers have been observed in dogs with thromboembolism,[13,14,25,98] disseminated intravascular coagulation (DIC),[7,14] terminal renal failure, neoplasia,[13,99] immune mediated anaemia[25] and other diseases which are associated with hypercoagulability.

2.3.4. Assessment of thrombophilia and heparin effects

Generally classified as tests for detection of thrombophilia are AT, PC and PS. AT is one of the most important protease inhibitors in regulation of blood coagulation.[132] The AT assay is performed in conditions associated with thrombophilia, DIC, and heparin therapy.[132,158-162] Here, a chromogenic assay was used, which is generally preferred in veterinary medicine.[132,163,164] Patient plasma is pipetted to the reagent containing excess FXa and a chromogenic substrate for FXa which is detected photometrically.[132,134,165,166]

PC measurements are performed for detection of hypercoagulable states.[132,134,165,166] Here, an automated clotting test was performed. Contact activator, human PC deficient plasma and a specific PC activator (Protac®, a snake venom derivate of Southern Copperhead, *Agkistrodon contortrix contortrix*) were added to the prediluted citrate plasma sample. In this way PC is activated and determined indirectly by an aPTT test. APC cleaves FVa and FVIIIa which results in a prolongation of aPTT. The PC activity is directly proportional to the increase of aPTT in seconds. [132,134,170,171]

APC has an anticoagulant function and inactivates FVa and FVIIIa.[172] In people, a poor anticoagulant response of APC is known as APC resistance (APCR) and results in thrombophilia and hypercoagulable state.[173] In patients, APC ratio is calculated as follows to characterize APCR[174]: APC-ratio= [aPTT in presence of APC (aPTT2) / standard aPTT without APC (aPTT1)]. An APC ratio < 2.1 seconds has been

considered abnormal in man.[175] APCR is detected via a modified aPTT in the presence and absence of APC. The APCR test used here is based on an unusually small prolongation of the clotting time of the sample in the presence of APC and calcified medium. FV deficient plasma and *Crotalus viridis helleri* venom is used which act as an activator of FX (STA-Staclot® APC-R, Roche Diagnostics GmbH, Mannheim; Germany). These all trigger the coagulation cascade downstream from FX so that an influence of all coagulation factors acting upstream could be ruled out.

The PS activity was determined here also with a clotting test like PC. Protac® and bovine FVa are used and as PS is a cofactor of PC, the anticoagulatory effect of PC is soleyly increased by the PS activity in the sample and measured. The low results of PS in people are determined as thrombotic diseases.[132,134,170,171]

One of the best known assays reflecting heparin activity is the measurement of anti-FXa activity. Here, also a chromogenic assay was performed (STA-Rotachrom® Heparin, Roche Diagnostics GmbH, Mannheim, Germany). Bovine FXa and a chromogenic substrate are added to diluted patient plasma. The test is based on the inhibition of FXa by AT-heparin complex and hydrolysis of the chromogenic substrate by FXa which is detected spectrophotometrically.[132]

2.3.5. Global tests (Thromboelastography)

To be clinically useful, an assay must provide rapid and reliable information regarding a patient´s coagulatory state in an emergency situation (preferably as point-of-care test). TEG allows a rapid characterization of both hyper- and hypocoagulatory conditions. TEG has been utilized worldwide for evaluation of hemostasis in humans and the workup of dogs suspected of having a hemostatic disorder.[177] Two different analyzers are commercially available, the ROTEM (Pentapharm, Germany) and TEG.[132] There is only a small difference in the mechanical test procedure between this machines.[132] As the TEG is used in the current studies, the principle is explained more detailed here.

Three types of TEG assays have been reported in veterinary medicine: native, human recombinant tissue factor activated (TF-activated; Innovin, Dade Behring) and kaolin-activated (Haemoscope)[132], as used here. Kaolin (TEG® Hemostasis System Kaolin, Haemonetics Corporation; formerly Haemoscope Corporation; Braintree, MA, USA) is used for the activation of the sample by the intrinsic pathway.[132]

The analyzer mainly has 3 parts: a sample cup (TEG® Hemostasis System Pins and Cups, Haemonetics Corporation; formerly Haemoscope Corporation; Braintree, MA, USA), a stationary pin, which is placed into the cup, and a mechanical-electrical tranceducer. After the placement of recalcified, kaolin-activated citrated whole blood sample into the cup, it moves in a constant speed and direction. The pin is set into the sample. With the beginning of the fibrin formation, a bound between pin and cup occurs. This bound leads to movement of the pin and cup together. The movement is related with kinetics, strength and dissolution of clot formation and is converted to electrical signals. (http://www.haemoscope.com/technology/teg_analyzer.html)

Figure 1 shows a TEG result monitored by a computer.

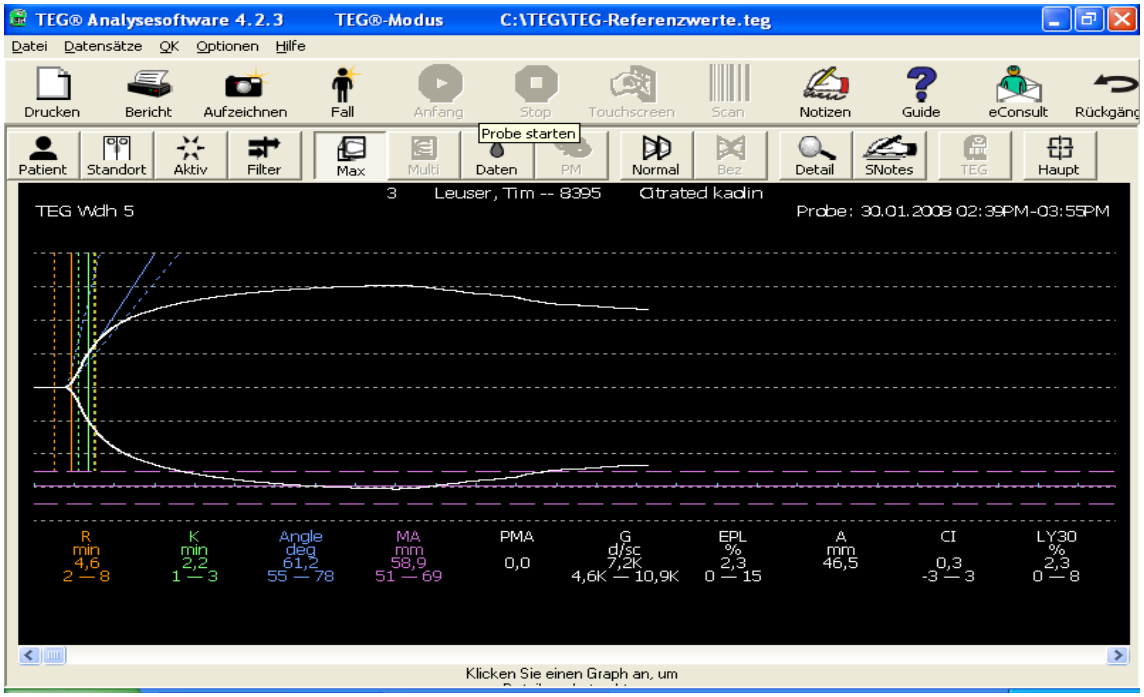

Figure 1: A monitored TEG result in a dog.

A recent study using the computerized TEG has shown that TF-activated TEG G-value correctly identified dogs with clinical signs of bleeding with a high positive and negative predictive value.[178] Grading dogs with disseminated intravascular coagulation (DIC) in hyper-, normo-, or hypocoaguable state based on the TF-activated TEG was reported to be of prognostic value.[124] In 49 dogs with neoplasia, hypercoagulability was the most common finding, while hypocoagulability was consistently present in dogs with metastasis.[175] In the majority of studies, five TEG variables (R, K, angle α, MA and G) have been evaluated: R is the reaction (or gelation) time and is measured from the commencement of the test until a preset fibrin formation, i.e. until the trace is 2 mm wide.[179] R is primarily influenced by plasma clotting factors and natural anticoagulants / inhibitors of coagulation such as antithrombin, PC and PS activity. The R-value is prolonged by anticoagulants and clotting factor deficiencies and is shortened by hypercoagulable state.[179] As fibrin polymerization proceeds, clot stability is increasing which is indicated by an increasing distance of the leaflets constituting the TEG curve. The coagulation time K is the time required from initial clot formation to achieve predetermined clot strength and is measured from the end of R until tracings reached an diversion of 20 mm. K is influenced by coagulation factors, fibrinogen plasma concentration, and the platelet count.[179] Hyperfibrinogenemia – and to a lesser extent platelet hyperfunction – results in shortened K-value whereas K is decreased by anticoagulants affecting fibrin polymerization and platelet function.[179] The angle α is closely related to the K-value indicating the rapidity of fibrin cross linking and depends on fibrinogen plasma concentration and to a lesser degree on platelet count.[179] Similar to the K-value, the angle α is raised by hyperfibrinogenemia and increased platelet function. The maximal amplitude MA is a measure of the peak rigidity manifested by the clot. About 80% of the MA is dependent on platelet number and function.[180] Fibrin contributes to a lesser extent to clot strength.[179] For the computerized TEG G, a measure of clot rigidity and thus overall coagulation state (in dynes per square mm) is calculated as follows: G = (5000xMA)/(96-MA).[179] As G is an exponentional

reflection of MA, it is more indicative of small changes in clot rigidity or fibrinolysis than the amplitude in millimeters.[179]

2.4. Reference intervals for coagulation assays in dogs

Reference intervals for routine coagulation parameters, AT, FVIII and PC have been established previously in dogs whereas reference ranges for PS, plasminogen, APC-ratio and anti-FXa activity have not been determined before. However, even for routine coagulation parameters it is generally agreed upon that analyser- and method-specific reference intervals have to be established. Studies in people investigating eight different aPTT assays and modified aPTT assays for detection of APC resistance demonstrated that results are highly dependent on both the assay and the coagulation analyser so that reference intervals were not identical.[181] The STA Compact has been used previously in dogs for measurement of routine coagulation parameters, AT, PC,[8,24,182] and fibrin D-dimers[183]. Heparin test run on the STA coagulation analyser has been validated for its use for determination of anti-FXa activity for monitoring of heparin therapy in blood plasma of 12 healthy and 10 dogs with immune-mediated haemolytic anemia.[8] In all these studies, healthy dogs served as controls and were compared to diseased patients. However, for routine use of STA Compact coagulation tests in veterinary laboratories, reference intervals have to be established. Generally, reference intervals are known to serve as the basis of laboratory testing and are helpful for differentiating between healthy and diseased patients.[184] Canine reference intervals for a functional PC chromogenic assay run on a STA coagulation analyser have been published before, however a limitation of the previous study was that statistical methods for calculation of reference intervals were not given and only 30 dogs were included.[25]

Also for routine use of TEG in veterinary practices, reference intervals have to be established. Mean and standard deviation[180]/range[185] of several TEG variables have been reported for both TF-activated TEG[180] and kaolin-activated TEG[185] in 18 and 15 healthy dogs respectively. In a further investigation, index of individuality and

biological variation of TEG variables have been assessed in eight dogs and – thus – provided useful information for interpretation of population based reference intervals.[186]

3. Material and Methods

The study was separated in two parts, the reference study and the coagulation response in a canine model of toxaemia.

3.1. Establishment of reference intervals for TEG and variables reflecting secondary and tertiary hemostasis run on the STA Compact analyzer

The study was ethically approved by the Ethics Committee for animal welfare, Giessen, Germany (No. V54-19c20/15cGi18/17), January 2008. Before the reference study the repeatability and interferences were performed for TEG and measurements run on the STA Compact analyzer. Blood samples for both measurements were taken at the same time. Also for control of health, physical, hematological and clinical chemical examinations including complete blood cell count (CBC), kidney and liver parameters as well as electrolytes (sodium, potassium, ionized calcium and magnesium, phosphate), total protein, albumin, globulin, and fructosamine plasma concentration were performed. Complete hematological examinations were done with the ADVIA 2120 and clinical chemical examinations with the ABX Pentra 400 (ABX Pentra 400, Horiba Diagnostics, France). Hematological and blood chemical examinations and the TEG analyses were run directly after blood collection.

3.1.1. Evaluation of repeatability, interferences and the effect of anticoagulant

Before the reference range study, the evaluation of repeatability, interferences and the effect of anticoagulant was done. Here the interference work includes the assessment of hemolysis and influence of lipemia.

3.1.1.1. Repeatability of the TEG analysis

The evaluation of intra-assay repeatability of the TEG analysis was performed sixty minutes after sampling and duplicate measurements were made from re-calcified

citrated blood with one TEG analyser (two channels per machine). A total of 6 healthy dogs with a median age of 2.5 years (range 1-6 years) were studied. Three dogs were male, two were male-castrated and one was female. Intra-assay variation was assessed for TEG variables R; K; angle α; MA; and G by calculating the arithmetic mean and the pooled variance estimate, based on the differences in the duplicate determinations. The standard deviation required for calculation of the coefficient of variation was consistent with the root of the pooled variance estimate.

3.1.1.2. Impact of anticoagulant in TEG analysis

The comparison between TEG results obtained with native whole blood or citrated whole blood was performed in 16 healthy dogs with a median age of 3 years (range 2-6 years). The study group included 10 male dogs, 3 female dogs, 3 neutered females. Sampling was performed as described above for the dogs included in the reference interval study with the only exception that one ml blood was given in a silicated kaolin-containing tube in addition to a citrated vial. The first 2 ml of the sampled blood were discarded (i.e., used for platelet count) to reduce the effects of tissue thromboplastin. The native blood in the kaolin containing tube was mixed by gentle inversion of the vial for 5 times. Then, 360 µl were pipetted in a pre-warmed TEG cup placed in the instrument holder and thromboelastography tracing was initiated. With the native whole blood specimens, TEG analysis was performed within 6 minutes of sampling whereas the citrated whole blood specimen was allowed to rest 1 hour at room temperature and TEG analysis was performed in re-calcified blood as described for the samples used for establishment of the reference interval later.

3.1.1.3. Effect of hemolysis on TEG analysis

For the assessment of hemolysis on TEG results seventeen healthy dogs (fifteen Beagle dogs, one German shepherd dog, one Golden Retriever) with a mean age of 4 years (range 3-5) were included. Six dogs were male, five male neutered, and six

were spayed females. The dogs were fasted at the time of blood sampling.Hemolytic samples were prepared with two techniques: mechanical stress (HM) as well as freeze and thawing (HF). Approximately 6 ml citrated whole blood was divided in 4 aliquots a` 1.3 ml. One aliquot served as control; the other one was used for induction of mechanical hemolysis by rapid aspiration of an aliquot of the sample into a syringe with a 23 gauge needle followed by strong squeezing of blood into the test tube. This procedure was repeated 20 times. From the remaining two aliquots, hemolytic specimens were prepared by freeze and thawing: one aliquot was frozen at -80°C for one hour; the other one was stored at room temperature for the same time and was mixed with the first aliquot in equal parts after thawing at 37°C. TEG analyses were performed in all specimens approximately one hour after sampling (controls) or after induction of hemolysis. The TEG analyses were performed according to the manufacturers´ recommendations.

3.1.1.4. Repeatability of the variables assayed on the STA compact

The intra-assay repeatability on the STA Comapact was performed with 15 replicate measurements, made from plasma samples, thawed at 37°C in a water bath and centrifuged at 1500×g for 10 minutes to remove remnants of cryoprecipitate after thawing. One healthy dog was studied and the STA Compact analyzes were performed as described later for establishment of the reference interval. Intra-sample variation was estimated for OSPT, aPTT, TT, fibrinogen, fibrin D-dimers, Antithrombin III, PC, PS, APC resistance activity, Plasminogen, Factor VIII and anti-factor Xa activity variables.

3.1.1.5. Impact of hemolysis and lipemia on the variables measured on the STA compact

To assess the influence of lipemia on secondary and tertiary hemostasis parameters, samples with three grades of lipemia were prepared by adding Liquigen® (Liquigen®, Pfrimmer Nutricia GmbH, Erlangen, Germany), a commercially

available fat emulsion in different dilutions (solutions "A", "B" and "C") to the samples of three healthy dogs. Solution "A" was obtained by adding Liquigen® to an equal volume of NaCl (1:1). Solutions "B" and "C" were consistent with a 1:4 and 1:16 dilution of Liquigen® and NaCl. For preparation of samples with grade 1; 2; and 3 lipemia, 20µl of solutions C; B; and A respectively were added to 1480 µl of citrated plasma resulting in final dilutions of 1:74 (grade 3), 1:296 (grade 2), and 1:1184 (grade 1). In grade 1 lipemia, letters behind the tube could be read easily. In grade two lipemia, letters could be recognized whereas in grade three lipemia, recognition of letters behind the tube was impossible. Citrated plasma of the same dog to which 20µl NaCl was added served as a control.

The effects of hemolysis on coagulation parameters measured on the STA Comapact were done as follows. Hemolysis was achieved by subjecting whole blood to a freeze-thaw cycle. Approximately 20ml citrated whole blood was obtained from three healthy dogs and was divided into two aliquots. One aliquot was frozen at -80°C to induce hemolysis, from the other aliquot citrated plasma was prepared as described above which was also stored at -80°C until analysis. After thawing at 37°C, hemolysed- and non-hemolysed aliquots were centrifuged at 850×g for 10 minutes and specimens with three grades of hemolysis were prepared. In samples with grade one hemolysis (ratio of hemolysed to non-haemolysed plasma 1:9), letters behind the tube could be read easily. In grade two (ratio of hemolysed to non-hemolysed plasma 1:4) letters could be recognized whereas in grade three hemolysis (undiluted hemolysed plasma) recognition of letters behind the tube was impossible. Hemoglobin concentration was assessed colorimetrically using the species specific hematology analyser ADVIA 2120 and a cyanide-free reagent as described before.[187]

3.1.2. Establishment of reference intervals

The TEG measurements, as mentioned before, were run directly after blood collection but the STA compact evaluations were performed within 3 weeks after sampling, in the interval the samples were stored after processing at -80°C. But first

each human assay run on the STA Compact was validated for dogs. After that, assays that were not valid initially were modified and optimized for their use with canine specimens. The study was conducted in adult (> 1 year old, < 6years) dogs including staff-owned dogs, blood-donors, or healthy dogs presented at the Clinic for Small Animals, Surgery, Department of Veterinary Clinical Sciences, Faculty of Veterinary Medicine, Justus-Liebig-University Giessen, Germany for routine radiological examination to screen for hereditary hip (HD) or elbow dysplasia (ED). Inclusion criteria were a normal physical, haematological examination as well as normal results of serum biochemistry and coagulation profile and no history of a bleeding tendency or medication two weeks prior to presentation at the clinic.

3.1.2.1. Sampling

Sample taking was performed in fasted, resting dogs with a non-heparinized 18-gauge venous catheter placed in the cephalic vein. Potassium-EDTA tubes were used for the complete hematological examinations with the ADVIA 2120. For clinical chemical examinations with the ABX Pentra 400, Lithium-Heparin tubes (Sarstedt AG&Co, Nümbrecht, Germany) were used, for these ones the first 2ml venous blood samples and afterwards samples for coagulation assays were taken. This sequencing was performed to reduce the effect of tissue thrombolastin on coagulation measurements. Blood was allowed to drop directly into the citrated tube or it was aspirated in a plain syringe. In the latter case, the specimen was gently placed into the citrate containing tube after removal of the needle from the syringe and the contents were mixed. The samples were carefully checked for proper filling and only specimens with an exact ratio of 9:1 blood to citrate anticoagulant were included.

For TEG analysis, 1.17 ml venous whole blood was placed in a silicone-lined micro-tube (40x1.8mm) containing 0.13 ml 3.18% trisodium citrate and was allowed to rest at room temperature for 1 hour.

For measurement of coagulation parameters with the STA Compact, venous blood samples were drawn and anticoagulated in siliconized vacutainer tubes containing

3.18% trisodium citrate such that a ratio of 9:1 (vol/vol) was obtained. Blood was allowed to drop directly into the citrated tube or it was aspirated in a plain syringe. The samples were carefully checked for proper filling and only specimens with an exact ratio of 9:1 blood to citrate anticoagulant were included. Sodium-citrated whole blood was spun down at 850×g for 10 minutes within 1 hour after sampling. Citrated plasma was separated from the erythrocytes and centrifuged again at 850×g for 10 minutes to remove all nonsedimented platelets prior to freezing as previously recommended.[188] The supernatant was removed and stored at -80°C until analysis. Analysis was performed within 3 weeks after sampling. For all analytes, samples were proven to be stable over 12 months by re-analyzing 3 specimens approximately 13 months after storage at -80°C. Directly prior to analysis, plasma samples were thawed at 37°C in a water bath as previously recommended[178] to provide complete dissolving of cryoprecipitate. Afterwards, samples were centrifuged at 850×g for 10 minutes so that plasma was mixed and non-dissolvable material was removed.

3.1.2.2. Method – TEG

Establishment of reference intervals for the TEG was performed with recalcified citrated whole blood according to the manufacturers´ recommendations. Briefly, 1 ml of citrated whole blood was placed in a silicated vial provided by the manufacturer which contained kaolin, buffered stabilizers and a blend of phospholipids. Mixing was ensured by gentle inversion of the kaolin-containing vials for 5 times. Pins and cups were placed in the TEG analyzer in accordance with the standard procedure recommended by the manufacturer. To each standard TEG cup, placed in the 37°C pre-warmed instrument holder 20 µl of 0.2 molar calcium chloride and 340µl kaolin-activated citrated whole blood was added so that a total volume of 360 µl was reached in each cup. Internal quality control materials in two levels (normal and abnormal) Level 1®and Level 2® (TEG® Coagulation Control Level I and II, Haemonetics Corporation; formerly Haemoscope Corporation; Braintree, MA, USA)

were run each day. An electrical internal quality control (so called e-test) was performed in addition.

3.1.2.3 Default settings and methods of human assays applied at the STA compact

Directly prior to analysis, plasma samples were thawed at 37°C in a water bath and centrifuged at 1500×g for 10 minutes to remove remnants of cryoprecipitate after thawing.

Coagulation screening tests OSPT, aPTT, and TT were measured automatically as clotting tests using commercial reagents, as mentioned before. Results were reported in seconds. Default settings for minimal/maximal measuring times for OSPT, aPTT, and TT were 6/180 seconds, 3/120 seconds and 13/240 seconds respectively. Fibrinogen was detected by the Clauss method using a human plasma calibration standard (STA Unicalibrator, Roche Diagnostics GmbH, Mannheim, Germany) provided by the manufacturer. Fibrin D-dimers were measured with the STA Liatest™ D-Di (STA Liatest™ D-Di, Roche Diagnostiscs, GmbH, Mannheim, Germany) immunoturbidimetric D-dimer assay as described before. The D-dimer result was reported as μg/ml fibrinogen equivalent units (FEU), i.e. the concentration of fibrin degradation products which are resulting from degradation of 1 mg/L fibrinogen. The assay was pre-calibrated and allowed a one-time testing on an automated analyser.[189] According to the manufacturer, analytical interval of the assay ranged between 0.0 and 4.0 μg/ml.

Antithrombin activity was detected based on its inhibition of thrombin with the chromogenic substrate kit (STA Antithrombin III, Roche Diagnostics GmbH, Mannheim, Germany). AT activity was reported as percentage of human plasma calibration standard.

For measurement of PC, an automated clotting test was performed: Cephalin (phosphatidylethanolamine, a phospholipid serving as contact activator, 50 μl), human PC deficient plasma (50 μl) and 50 μl of a specific PC activator were added to 50 μl the of patient sample prediluted 1:10 with diluent buffer (STA diluents buffer,

Roche Diagnostics GmbH, Mannheim, Germany). This resulted in an activation of PC within the sample and simultaneously an initiation of the intrinsic clotting system by contact activation. The PC activity was determined with a modified aPTT test using APC-deficient human plasma. Based on this method, the aPTT was depending solely on the PC activity in the sample as addition of PC-deficient human plasma. Activated PC cleaves FVa and VIIIa resulting in an increase in aPTT. Thus, PC activity was directly proportional to the increase in aPTT in seconds.

Results could be reported as percentage of a human plasma calibration standard or in seconds. According to the manufacturer, analytical interval of the test was between 3% and 150%. Default settings for minimal and maximal times of measurement were 10 and 180 seconds respectively. In case of results < 10%, the test was automatically repeated with a dilution of 1:5.

PS activity was also determined with an automated clotting test, the same test as used for the PC activity, as mentioned before. A total of 50µl human PS deficient plasma, 50µl human PC activated with Protac® and 50µl bovine factor Va were added to 50µl of the patient sample which was automatically diluted 1:10 with diluent buffer. As PS is a cofactor of PC, the anticoagulatory effect of PC was solely increased by the PS activity in the patient sample. For minimal and maximal times of measurement, 10 and 250 seconds respectively were set as a default. In case of results < 15%, the test was automatically repeated with a dilution of 1:5.

The principle of assessment of APC resistance was based on an unusually small prolongation of the clotting time of the tested plasma in the presence of activated PC and in calcified medium. In the STA-Staclot® APC-R test system, 50 µl patient plasma was diluted 1:10 and coagulation was achieved in the presence of 50 µl factor V deficient plasma and 50 µl *Crotalus viridis helleri* venom. The result was reported in seconds. Based on the information of the manufacturer, analytical interval of the test was between 20 and 300 seconds.

Plasminogen activity was determined photometrically with a chromogenic assay, explained before. An excess of streptokinase (100 µl of reagent 1 of the STA

plasminogen test kit containing approximately 5000 IU streptokinase/ml) was added to 100 µl of the patient sample which was diluted 1:20 with diluent buffer. A complex with a plasmin-like activity was formed between streptokinase and plasminogen. The quantity of streptokinase-plasminogen complex was assessed by its action on 100 µl of a chromogenic synthetic substrate H-D-But-CHA-Lys-pNA x 2 AcOH in a concentration of 5.5 mol/ml. Quantification was achieved by the amount of p-nitroaniline released which was photometrically detected at 405 nm. Results were reported as percentage of a human plasma calibration standard. A measuring range of 11-160% plasminogen activity compared to the human standard plasma was reported by the manufacturer.

Measurement of FVIII was performed in a modified 1-stage aPTT with human FVIII deficient substrate plasma. Default setting for dilution of patient samples was 1:10 with diluent buffer. In case of FVIII activity > 150%, measurement was automatically repeated in a dilution of 1:40. Default measuring range was between 1-600%.

For measurement of anti-FXa activity, an automatic chromogenic assay was applied as described before. The test principle was based on a one-step reaction: 100 µl of bovine FXa (0.23 IU/ml) was added to a mixture of 50 µl patient plasma diluted 1:2 with diluent buffer and a chromogenic substrate (MAPA-Gly-Arg-pNA x AcOH, 1.5 µmol/ml). This resulted in an initiation of two reactions including the hydrolysis of the substrate by FXa and inhibition of FXa by the heparin-AT complex which was made up from the heparin and AT peculiar to the patient. After an incubation of 240 seconds, the quantity of pNA released was measured photometrically and was inversely proportional to the heparin concentration in the test medium. Results were reported in IU/ml. According to the manufacturer, analytical range of the test was between 0.00 and 0.70 IU/ml.

For all variables internal quality control material (normal and abnormal) provided by the manufacturer was run each day. STA PreciClot PlusI and II (STA PreciClot Plus I; II; III, Roche Diagnostics GmbH, Mannheim, Germany) was used for quality assurance of the majority of variables including OSPT, aPTT, TT, fibrinogen, AT, PC,

PS, FVIII, and plasminogen. A third level (STA PreciClotPlus III, also abnormal) was run in addition for OSPT, aPTT, fibrinogen, and AT. For internal quality control of fibrin D-dimer measurements, Liquicheck™ D-dimer control Levels I and II (Liquicheck™ D-Dimer control Level I and II, Roche Diagnostics GmbH, Mannheim, Germany) were used. In case of anti-FXa activity and APC response, materials for internal quality control STA Heparin Control (STA Heparin Control I and II, Roche Diagnostics GmbH, Mannheim, Germany) and STA-Staclot® APC-R(STA-Staclot® APC-R Control N and P, Roche Diagnostics GmbH, Mannheim, Germany) were included in each reagent package.

3.1.2.4. Validation of human assays on the STA compact

The majority of human assays (OSPT, aPTT, fibrinogen, AT, APC-ratio, fibrin D-dimer) were valid for canine specimens. In 11/57 (19%) dogs, the TT was below the minimal measuring time of 13 seconds which was set as a default for the human assay. The most striking findings were the PC, PS and plasminogen activity being markedly lower than the concentrations in the human standard plasma (100% per definition). The PC and PS reference intervals determined with the clotting assay were markedly below the measurement range of the human assay whereas the canine reference interval for the plasminogen activity was slightly lower than the measurement range of the STA Plasminogen test. In contrast to this, the canine reference interval for FVIII activity was significantly higher than the concentrations in the human standard plasma and exceeded the upper limit of the measuring range of the FVIII assay. Based on the markedly different activities of FVIII, PC, PS, and plasminogen activity than in human assays, an adaptation of the human test protocols to the requirements of canine specimens was necessary.

3.1.2.5 Modification of test methods applied at the STA compact

Based on the results of the study, methods for measurement of TT, FVIII, PC, PS and plasminogen assays had to be optimized for their use with canine blood plasma. For

the TT, the minimal measuring time was defined as 8 seconds instead of 13 seconds set previously as a default. The eleven samples in which the TT was below 13 seconds were reanalyzed with the new settings for the measuring time and yielded a result in all cases.

Based on these results of the STA Compact method validation study, preparation of pooled plasma was necessary. Approximately 30 ml citrated whole blood was taken from 16 healthy adult dogs (8 female, 12 male, 2 female spayed) with a median age of 3.5 years (range 1-8 years). Three Beagle dogs, two Malinois, Labrador Retrievers, French Bulldogs, Maremma Sheepdogs and German shepherd dogs were included as well as one Rottweiler, Staffordshire Bullterrier and mixed breed dog. The dogs were healthy based on the history, physical examination as well as on the haematological and clinical chemical examination. The dogs required for preparation of the pooled plasma were not included in the population used for establishment of the reference intervals.

After the preperation of the canine pooled plasma the tests for FVIII, PC, PS and plasminogen were performed as described before with the difference of used serial dilutions (FVIII= 1:40; 1:60; 1:80; PC and PS= 1:5; 1:10; 1:20; 1:40; plasminogen= 1:100; 1:40; 1:20) instead of human plasma calibration standard to obtain a species specific reference curve. Moreover, dilution of patient plasma was 1:40 for FVIII, 1:5 for PC/PS and 1:20 for plasminogen respectively.

Internal quality control was performed in two levels (normal and low) with aliquots of citrated plasma obtained from one of the dogs included in the reference population. Plasma was stored at -80°C until analysis. Undiluted citrated plasma and plasma diluted 1:1 with saline served as normal control and low control respectively.

3.2. Evaluation of the impact of endotoxemia on the coagulation process

The study of the second section of this thesis work was performed at Uludag University, Bursa, Turkey whereas the sample analysis were performed at the

Central Laboratory, Faculty of Veterinary Medicine, Justus-Liebig-University Giessen, Germany.

The prospective investigation was ethically approved by the Animal Care and Use Committee of the Uludag University, Bursa, Turkey (2009-01/08). The current investigation was performed to evaluate the early response to induction of endotoxemia on variables characterizing secondary hemostasis, physiological anticoagulants and markers of fibrinolysis compared with a control group. Coagulation analysis included a standard coagulation profile (OSPT, aPTT, TT, fibrinogen plasma concentration) as well as an extended profile including FVIII activity, natural inhibitors of coagulation (AT, PC, PS, APC-ratio) and markers of fibrinolysis (fibrin D-dimer plasma concentration). Kaolin-activated TEG analysis was performed in addition. Results were compared with laboratory-intern reference intervals which were established previously. Diagnosis of disseminated intravascular coagulation (DIC) was made when ≥ than 3 of the traditional coagulation tests were altered and consistent with a coagulation consumption disorder (thrombocytopenia and prolonged clotting times of OSPT, APTT, TT), together with an increment in plasma concentrations of D-dimers > 0.67 µg/L or consumption of the main coagulation inhibitor AT as published previously.[187]

Ten adult clinically healthy mongrel dogs (4 males and 6 females) with a median age 3 years (range 2 - 5 years) and a median body weight of 18 kg (range 15 - 22 kg), housed in the Animal Husbandry and Diseases Research and Application Centre of Uludag University were included in the study. Water was provided ad libitum, and the dogs were fed with standardized, pelleted diet twice daily before the experiment. The dogs were equally assigned into two groups. Dogs in the treatment group received endotoxin (LPS, Escherichia coli serotype 055:B5, purity 497%; Sigma, St.Louis, MO, USA) dissolved in sterile saline (0.9% NaCl solution; Baxter, Istanbul, Turkey) which was administered intravenously at a dosage of 0.02 mg/kg. Dogs in the control group received 0.2 ml/kg sterile 0.9% saline solution intravenously. Dogs were provided water 3 times a day, and food (the pelleted diet) twice a day during

the experiments which were performed in October. Dogs were monitored clinically and hematologically for 24 hours.

3.2.1. Sampling

Venous blood samples were collected before application of LPS or the placebo (0 hour) and 1, 4, and 24 hours after treatment from the brachiocephalic vein. Specimens for hematology were collected into vacutainer tubes containing potassium Ethylendiamin-tetraacetate (K_2EDTA. Sarstedt AG&Co, Nümbrecht, Germany). Hematological examination included measurement of white blood cells (WBCs) and platelet (PLT) count. The anticoagulant tubes were inverted several times immediately after sample acquisition to ensure adequate mixing. Hematological analysis was performed directly after the blood collection using an automatic analyzer with optical scatter and impedance methods (CELL-DYN 3500; Abott, Wiesbaden, Germany). Peripheral blood smears were not examined. For measurement of coagulation parameters, venous blood samples were drawn and anticoagulated in siliconized vacutainer tubes containing 3.18% trisodium citrate such that a ratio of 9:1 (vol/vol) was obtained. The first 2 ml were discarded to remove tissue thromboplastin and were used for hematological analysis. The samples were checked for proper filling and only specimens with an exact ratio of 9:1 blood to citrate anticoagulant were included. Sodium-citrated whole blood was spun down at 850g for 10 minutes within 1 hour after sampling. Citrated plasma was separated from the erythrocytes and centrifuged again at 850g for 10 minutes. The supernatant was removed and stored at -80°C until analysis. Analysis was performed within 3 weeks after sampling. Sample stability was proven by the authors to be > 12 months. For TEG analysis, 1.2 ml venous whole blood was placed in a silicone-lined tube containing 0.2 ml sodium citrate and was allowed to rest at room temperature for 1 hour.

4. Statistics

Results were collected electronically and transferred manually to an excel spread sheet for statistical analysis.

For statistical analysis, the following software packages were used: Graph Pad Prism (Graph Pad Software, San Diego, USA) and BMDP (BMDP Statitical software Inc., 1440 sepulveda Blvd, Los Angeles, CA 90025 USA). Reference intervals were calculated with Analyse-it for Excel (Analyse-it Method validation Edition version 2.12 - © 1997-2008, Analyse-it Software Ltd.).

4.1. Statistical analysis for calculation of reference intervals for kaolin-activated TEG and coagulation variables

4.1.1. Statistical analysis for the assessment of repeatability, interferences and effect of anticoagulant

4.1.1.1. Repeatability of the TEG analysis

Intra-assay variation was assessed by calculating the arithmetic mean and the pooled variance estimate, based on the differences in the duplicate determinations, for both analyzer parameters. The standard deviation required for calculation of the coefficient of variation (CV) was consistent with the root of the pooled variance estimate.

4.1.1.2. Impact of anticoagulant and sex on TEG analysis

The influence of the anticoagulant was assessed by using a paired t-test. An unpaired t-test or a comparable non-parametric test (Mann Whitney U test) was applied to investigate the effect of sex on TEG variables.

Regarding the evaluation of the influence of hemolysis, a Kolmogorov Smirnov test was performed to verify the assumption of normality. The difference between results

obtained after HM and HF respectively was assessed with a one way ANOVA test and Tukey´s multiple comparison post test. In case of non-normal distribution or missing values, a Friedman test with Dunn´s multiple comparison post test or a Wald test with Student-Newman-Keuls test respectively were performed. The mean bias between hemolysed and non-hemolysed specimens was calculated with a Bland Altman Bias plot. After Bonferroni correction, significance was set at $P < 0.01$ for comparison of the influence of anticoagulant and sex.

4.1.1.3. Effect of hemolysis on TEG results

The difference between results obtained after HM and HF respectively was assessed with a one way ANOVA test and Tukey's multiple comparison post test. In case of non-normal distribution or missing values, a Friedman test with Dunn's multiple comparison post test or a Wald test with Student-Newman-Keuls test respectively were performed. The main bias between hemolysed and non-hemolysed specimens was calculated with a Bland Altman Bias plot.

4.1.1.4. Influence of sex and repeatability of the variables assayed on the STA compact

The influence of sex was assessed by using an unpaired t-test or a comparable non-parametric test (Mann Whitney U test). After Bonferroni correction, significance was set at $P<0.005$ for comparison of the influence of sex.

Fifteen-run intra-assay repeatability for normal values was calculated from a sample of a healthy dog.

4.1.1.5. Impact of hemolysis and lipemia on the variables measured on the STA compact

Student's paired t-test was applied to investigate the effect of hemolysis and lipemia on coagulation parameters. After Bonferroni correction, significiance was set at $P<0.0015$ for assessment of the effect of interferences.

4.1.2. Statistical analysis for calculation of reference intervals

An Anderson-Darling test was performed to verify the assumption of normality for both analyzer data.

In case of normal distribution of data, double sided reference intervals were obtained by calculating the mean±1.96 SD (standard deviation) so that 95% of the reference population was included. The 90% confidence intervals of the upper and lower reference limit were calculated automatically with the Analyse-it software. If non-normal distribution of data was present, a logarithmic transformation was performed and data were re-analysed with the Anderson-Darling test. In case of normal distribution after transformation, logarithmic data were analysed as described above. Data of TEG and coagulation parameters were depicted as histograms including the reference interval as well as the 90% confidence interval of the upper and lower reference limits as recommended by the IFCC.[46]

4.2. Statistical analysis for the evaluation of the impact of endotoxemia on the coagulation process

Results were analyzed with the Graph Pad Prism and BMDP statistical software. The differences between the study and control group were assessed with a two-way analysis of variance and repeated measures regarding the factor “group”, “time”, and interaction between both factors. Level of significance was set at P=0.05.

5. Results

5.1. Establishment of reference intervals for TEG and variables reflecting secondary and tertiary hemostasis run on the STA Comapct analyzer

5.1.1. Repeatability, interferences and the effect of anticoagulant

5.1.1.1. Repeatability of TEG analysis

Intra-assay coefficients of variation for kaolin-activated TEG variables R;K;α;MA, and G were 7.6%;17.7%;7.4 %;2.9% and 6.6% resepctively. In table 1, the median and minimum and maximum values of intra-assay measurements are demonstrated.

Variable	R-value (min)	A (degree)	K-value (min)	MA (mm)	G-value (Kilo dyn/cm^2)
Median	22.4	14.3	35.8	6.6	16.1
Range	16.1-43.3	5.0-27.7	20.2-52.3	3.7-10.7	8.5-22.5

Table 1: Minimum and maximum values of intra-assay measurements (n=6 dogs).

5.1.1.2. Impact of anticoagulant in TEG analysis

The influence of the anticoagulant (native blood versus re-calcified citrate anticoagulated blood) on TEG R-value, angle α, K-value, MA and G-value is depicted in figure 2. As shown here in the figure, no significant effect of the anticoagulant was present, although the inter-individual variation of results was lower for the results obtained from citrated samples.

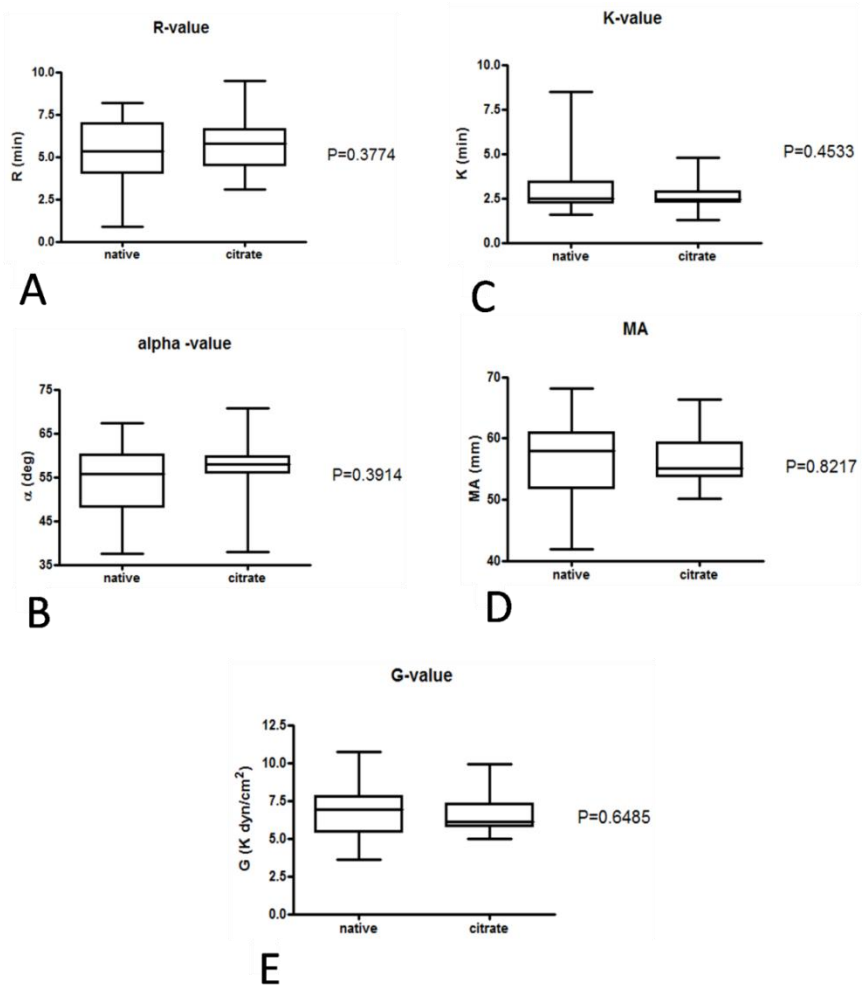

Figure 2: Influence of the anticoagulant (native blood versus re-calcified citrate anticoagulated blood) in TEG variables (n= 16 dogs).

The central box in figure 2 represents the values from the lower to upper quartile. The middle line represents the median. The horizontal lines indicate the minimum to the maximum values. The level of significance was set at 0.01 after Bonferroni correction.

5.1.1.3 Effect of hemolysis on TEG results

The TEG reaction time R reflecting secondary hemostasis was significantly shorter after HM than in the control specimens and after HF (table 2, P< 0.001).

Group/ Parameter(unit)	R-value (min)	K-value (min)	α (degree)	MA (mm)	G-value (K dyn/cm^2)
Control	5.2±1.9	5.5 (1.7-16.9)	46.3±16.8	49.0±9.3	5.1±1.8
HM	2.5±0.9	9.0 (2.3-20.7)	41.6±13.0	38.2±8.5	3.2±1.2
HF	5.6±3.7	9.9 (5.6-33.2)	20.2±11.2	27.0±11.6	2.0±1.1

Table 2: Change of TEG variables after induction of in vitro hemolysis by either HM or HF (n=17 dogs). Results are either depicted as mean ± standard deviation in case of normal distribution or as median and range if data were non-normally distributed.

Following HM, a mean proportional bias of 2.7 minutes compared to the control samples was present. The K value tended to be higher after HM compared to the control group (table 2), however this trend was not statistically significant after Bonferroni correction ($P<0.05$). Following HF, a significantly reduced rapidity of fibrin cross linking was noted reflected by a significantly higher K value in comparison to the controls ($P<0.01$). After induction of hemolysis by freeze and thawing, the TEG-K value was not reported by the analyser in 4/17 samples (23.5%) because MA never reached the required 20 mm. In HM and HF, a mean constant bias of -4.0 and -9.8 minutes respectively was observed.

Hemolysis resulted in a decrease in angle α also indicating a reduced rapidity of fibrin cross linking. The difference between the controls and hemolysed samples was highly significant for HF ($P<0.001$) but only small and insignificant for HM. There was a mean constant bias of 4.7 degrees after mechanical hemolysis, however, in individual dogs, a negative bias was present. After HF, a marked constant bias of 26.1 degrees was observed.

As detailed in table 2, hemolysis generally resulted in a decreased clot stability and overall coagulation ability indicated by a significantly lower MA and G value (between all groups: $P<0.0001$). A mean constant bias of 10.8 mm and 22.0 mm was present after HM and HF respectively. Similarly, hemolysis resulted in a highly

significant decrease in G value reflected by a mean constant bias of 1.9 Kilo(K)dyn/cm^2 after HM and 3.1 ± 1.9 Kdyn/cm^2 after HF respectively.

5.1.1.4. Repeatability of variables assayed on the STA compact

Fifteen-run intra-assay repeatability of coagulation variables run on the STA Compact analyzer is shown in table 3.

Variable	Unit	CV (%)	Mean	Range
OSPT	Sec	1.22	8.0	7.9-8.1
aPTT	Sec	1.02	10.8	10.6-11.0
TT	Sec	1.64	13.4	13.2-13.7
Fibrinogen	g/l	5.6%*	1.8	1.4-3.4
Factor VIII	%cp	3.89	108	99-114
AT	%hs	4.68	132	115-138
Protein C	%cp	2.36	85	82-90
Protein S	%cp	1.4*	110	58-118
APC – ratio	-	1.45	2.8	2.7-2.9
Plasminogen	%cp	20.16	66.8	46-94
D- Dimer	μg/ml	45.92	0.11	0.03-0.18
Anti Factor Xa	IU/ml	12.83	0.09	0.06-0.1

Table 3: Fifteen-run intra-assay repeatability for hemostasis variables (n=1 dog). Abbreviations: %cp: % compared to canine pooled plasma; %hs: % compared to human calibration standard. The symbol * means that is an outlier exceeding the 3-fold standard deviation from the mean was removed.

For the routine coagulation parameters aPTT, OSPT and TT a low CV was obtained ranging from 1.02% to 1.64%. FVIII activity, AT, PC, plasminogen, as well as the APC-ratio showed intra-assay CVs ranging from 1.45% to 4.68%, as you can see in table 3. For the fibrinogen concentration and PS activity, one outlier exceeding the 3-fold standard deviation from the mean was present in the series of measurements. Removing the outlier resulted in an intra-assay CV of 5.6% for fibrinogen and 1.4% for PS. Variables with low plasma concentrations in healthy dogs such as fibrin D-dimer plasma concentration and anti-FXa activity demonstrated high intra-assay CVs, as shown in table 3.

5.1.1.5. Impact of hemolysis and lipemia on variables measured on the STA compact

Hemoglobin concentration induced by freeze/thawing ranged from 0.6±0 mmol/L (grade 1 hemolysis) to 1.3 ± 0.06 mmol/L (grade 2 hemolysis) and 6.6 ± 0.06 mmol/L (grade 3 hemolysis), respectively. As depicted in table 4, hemolysis had a major impact on results obtained with the majority of coagulation tests run on the STA Compact.

% bias / Hb (mmol/l) and Variable	0.6	1.3	6.6
OSPT	+10.0	+3.4	-21.9
aPTT	-4.6**	-7.8***	+4.3
TT	-14.3**	-20.6*	-2.0
Fibrinogen	-9.1	-7.7	-32.1**
Factor VIII	-9.0**	-4.9	-49.6*
AT	-19.1	-1.2	+94.3**
Protein C	+90.3	+61.4*	+58.9*
Protein S	+46.6	+89.1	+143.2*
APC-ratio	+9.5*	+15.5**	+1.4
D-dimer	+106.0	Error	Error
Anti-Factor Xa	+593.6***	+624.1***	+565.9***

Table 4: Bias after induction of three grades of hemolysis (n=3 dogs). %Bias=difference (hemolysed sample-control)/control × 100. *P<0.05; **P<0.01; ***P<0.0015 = level of significance after Bonferroni correction.

Anti-FXa activity was most susceptible to hemolysis demonstrating a highly significant increase of approximately 600% compared to the control (P=0.0002-0.0003 for grades 1-3 hemolysis). Grade 1 hemolysis resulted in a false high measurement of fibrin D-dimers of approximately 100%. In plasma with a higher degree of hemolysis, measurement of D-dimers was not possible due to "technical errors" of the analyser. FVIII activity, AT, PC, PS, and APC ratio were overestimated in hemolytic plasma, whereas fibrinogen, TT, and aPTT tended to be underestimated compared to the control, as shown here in table 4.

In contrast to haemolysis, lipaemia generally did not significantly influence measurement of coagulation parameters except for the results obtained with the STA Liatest D-Di™ immunoturbidimetric D-dimer assay, here seen in table 5. In the presence of lipaemia, D-dimer results were markedly overestimated ranging from a bias of 32% (grade 1 lipaemia) to 160% (grade 3 lipaemia). Due to the lack of a valid test for canine specimens, the effect of haemolysis and lipaemia was not assessed for the plasminogen activity.

% bias / ratio fat solution : blood plasma and variable	1:1184	1:296	1:74
OSPT	-2.7	+1.4	-1.3
aPTT	-1.9	-1.7	-1.9
TT	-6.1	-2.1	-6.1
Fibrinogen	-0.5	-2.1	-2.1
Factor VIII	+10.5*	+6.8	+5.2*
AT	-0.2	+0.0	-0.2
Protein C	-0.7	+1.2	+3.6
Protein S	+4.1	+6.0	+6.5
APC-ratio	-1.4	+0.4	+0.4
D-dimer	+32.4	+87.4	+165.5**
Anti-FactorXa	+3.7	+0.0	-11.7

Table 5: Bias after induction of three grades of lipemia (n=3 dogs). % Bias=Difference (hemolysed sample-control)/control × 100. Statistically significant differences were assessed by Student's paired t-test (*P<0.05; **P<0.001; ***P<0.0015 = level of significance after Bonferroni correction).

5.1.2. Results for reference intervals

Fifty-six dogs (19 German Shepherd dogs, 15 Beagle dogs, 8 Golden Retriever dogs, 4 Labrador Retriever dogs, 2 mixed breed dogs, 1 Hovawart, 2 Great Danes, 1 Saint Bernard dog, 1 English Springer Spaniel dog, 1 Bernese Mountain dog, 1 Boxer, and 1 Giant Schnauzer) with a median age of 2 years (range 1 - 6 years) were included in the study. The sex was evenly distributed with 24 male, 20 female, 7 male neutered and 5 female neutered dogs. The dogs were healthy based on the clinical examination as well as the results of hematology, clinical chemistry and routine coagulation profile.

Regarding the TEG results, the majority of variables were normally distributed except for results of the K-value demonstrating a right-skewed distribution of data (figure 3E) which could be normalized after logarithmic transformation. Reference intervals and 90% confidence intervals are displayed in table 6 and figure 3 below.

Variable	Unit	Lower reference limit	Upper reference limit	90% CI interval lower	90 % CI interval upper	Distribution of data
R	Min	1.8	8.6	1.1 – 2.4	7.9 – 9.2	Non normal
K	Min	1.3	5.7	1.1 – 1.5	4.9 - 6.6	Log normal
α	Degree	36.9	74.6	33.3 – 40.5	71.0 – 78.2	Normal
MA	mm	42.9	67.9	40.5 – 45.3	65.5 – 70.3	Normal
G	K dyn/cm^2	3.2	9.6	2.6 – 3.8	9.6 – 10.3	Normal

Table 6: Reference ranges for kaolin-activated TEG variables and 90% confidence intervals of upper/lower reference limits determined in re-calcified whole blood (n=56 dogs).

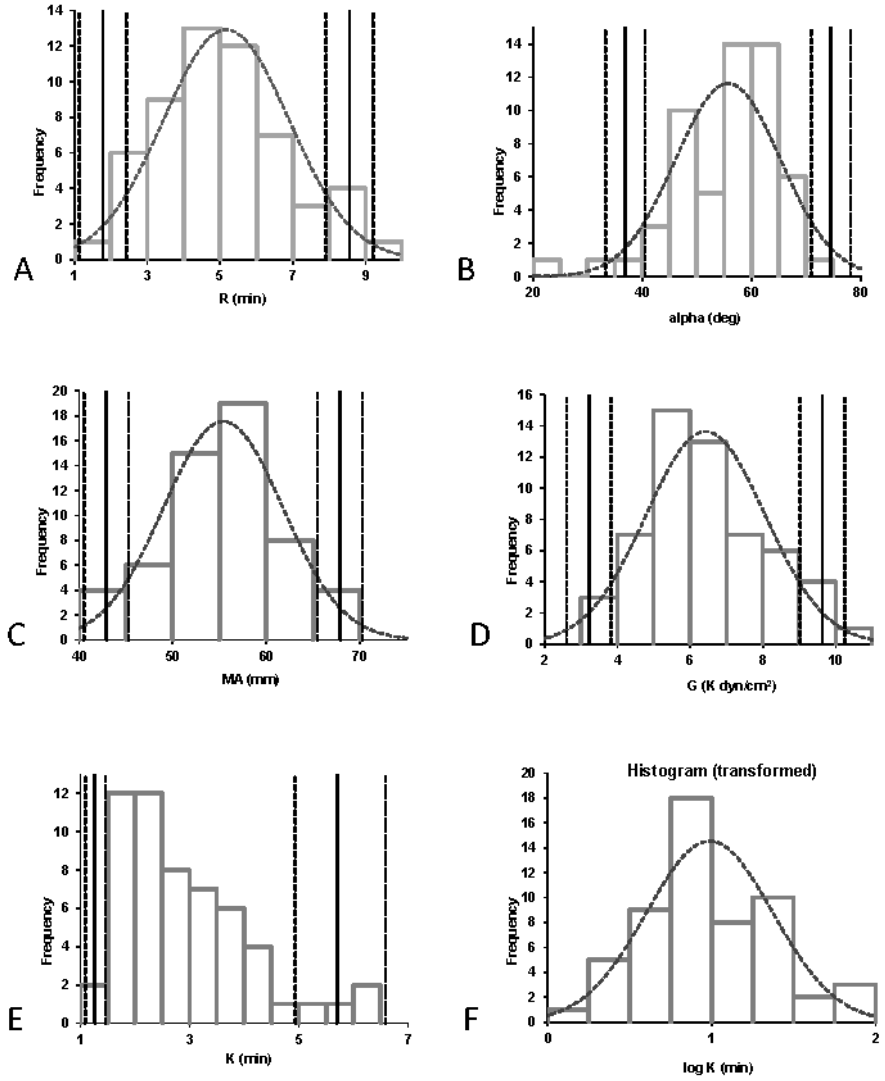

Figure 3: Histograms demonstrating the distribution of TEG data obtained for calculation of reference intervals (n=56 dogs). The solid vertical line indicates the reference interval. The dotted lines indicate the 90% confidence interval of the upper and lower reference limit. The dotted curve indicates the Gauss curve in case of normal distribution. Log normal distribution is characterized by an absent Gauss curve but the confidence interval is shown, whereas in case of non normal distribution only the upper and lower reference limit are shown.

In table 7 is shown that TEG variables did not differ significantly between male and female dogs.

Variable (unit)/ Sex	R-value (min)	α (degree)	K-value (min)	MA (mm)	G-value (K dyn/cm^2)
Male	5.2±1.6	57.0±7.9	2.8±1.1	55.5±6.7	6.5±1.8
Female	5.2±2.0	54.2±11.4	3.0±1.3	55.2±6.0	6.3±1.5
P-value	0.9141	0.2747	0.5033	0.8743	0.7743

Table 7: Influence of sex on results (mean±standard deviation) of different TEG variables (n=56 dogs).

The majority of variables measured on the STA Compact were normally distributed as shown in table 8 and figure 4. In case of the fibrinogen concentration, the APC-ratio, FVIII, PC and PS activities as well as the anti-FXa activity, a normal distribution was present. Results of aPTT and fibrin D-dimer measurements were normally distributed after logarithmic transformation whereas in case of OSPT, TT, AT, plasminogen activity, a non-normal distribution was observed. Reference intervals are displayed here in table 8.

Variable	Unit	Lower 2.5% reference limit	Upper 97.5% reference limit	90% CI interval lower	90% CI interval upper	Data distribution
OSPT	Sec	5.7	8.1	-	-	Non normal
aPTT	Sec	10.0	14.3	9.7 - 10.4	13.9 - 14.9	Log normal
TT	Sec	11.9	18.3	-	-	Non normal
Fibrinogen	g/l	1.3	3.1	1.2 - 1.4	2.9 - 3.4	Normal
FVIII	%human standard	283.9	1179.0	198.1-369.7	1093.3 - 1264.8	Normal
FVIII	% canine pooled plasma	70.9	136.4	64.6 - 77.2	130.1-142.7	Normal
AT	%human standard	107.9	128.0	-	-	Non normal
PC	%human standard	-26.5	-19.0	(-27.2) - (-25.8)	(-19.7) - (18.3)	Normal
PC	% canine pooled plasma	75.5	118.9	71.3 - 79.7	114.7 - 123.0	Normal
PS	%human standard	-47.3	-21.8	(-49.8) - (-44.9)	(-24.2) - (-19.3)	Normal
PS	% canine pooled plasma	74.4	160.5	66.2 - 82.7	152.3 - 168.8	Normal
APC ratio	-	2.0	3.0	1.9 - 2.1	2.9 - 3.1	Normal
Plasminogen	% human standard	7.0	8.0	-	-	Non normal
D-Dimer	µg/ml	0.023	0.654	0.017-0.032	0.474 - 0.902	Log normal
Anti-FXa	IU/ml	0.04	0.259	0.019-0.061	0.238 - 0.280	Normal

Table 8: Reference intervals for variables reflecting secondary hemostasis, fibrinolysis and physiological anticoagulants (n=56 dogs).

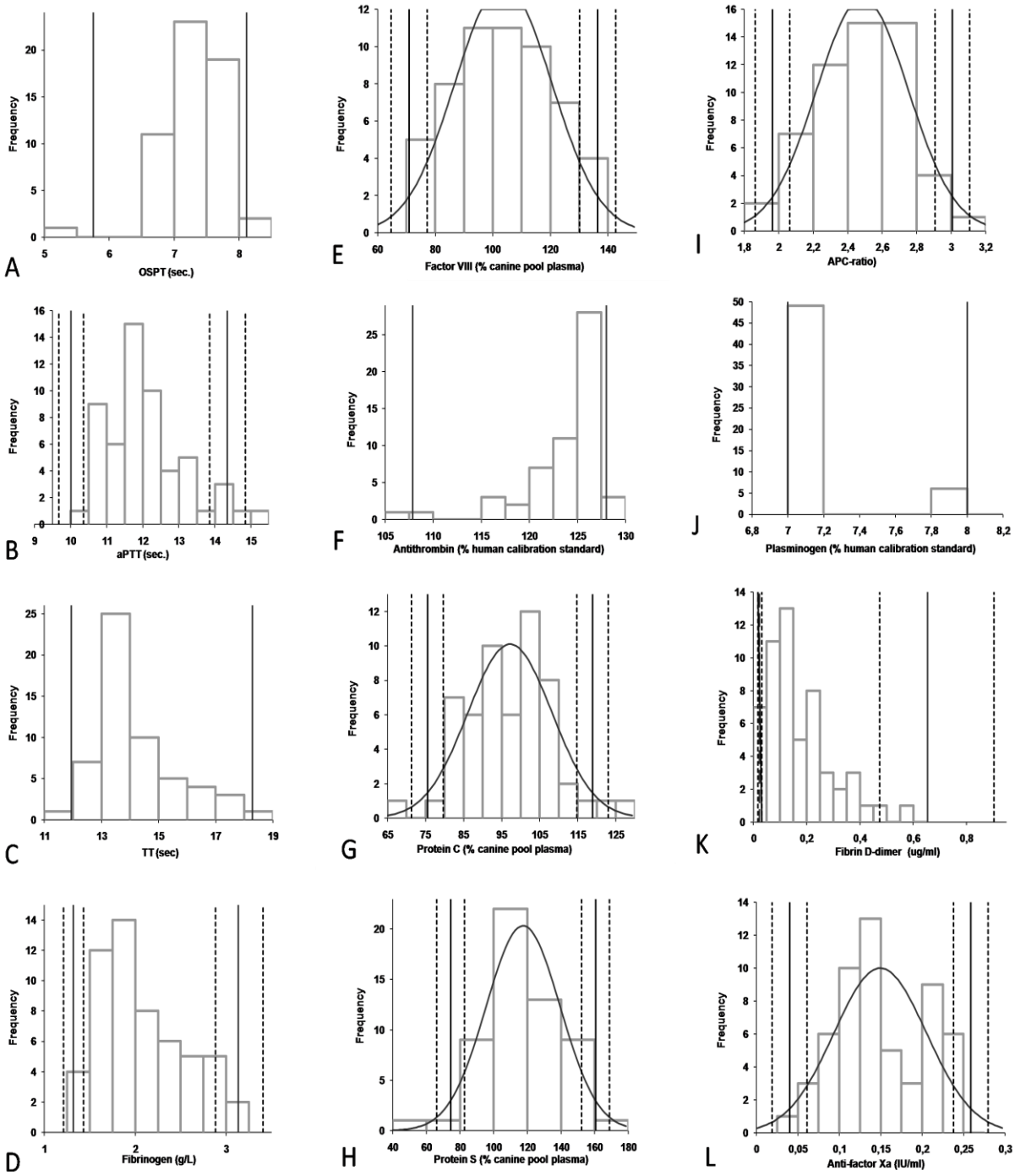

Figure 4: Frequency histograms of data measured on the STA Compact demonstrating the distribution of data obtained for calculation of reference intervals (n=56 dogs).

Below the solid vertical line indicates the reference interval. The dotted lines are consistent with the 90% confidence interval of the upper and lower reference limit. For normally distributed data, frequency histogram and superimposed Gaussian curve (dotted lines) are shown. In case of non normal distribution only the upper and lower reference limit are detailed.

5.2. Evaluation of the impact of endotoxemia on the coagulation process

As expected, the dogs in the control group did not show any significant clinical abnormalities after application of the placebo. In the LPS group, all dogs showed clinical signs including lethargy (n=5/5), diarrhea (n=4/5), vomitus (n=4/5), abdominal pain during palpation (2/5) as well as a weak pulse and mild (n=2/5) to moderate (n=1/5) dehydration. The short-term effect after induction of endotoxemia on respiratory rate, heart rate and body temperature compared to the control group is depicted in figure 5.

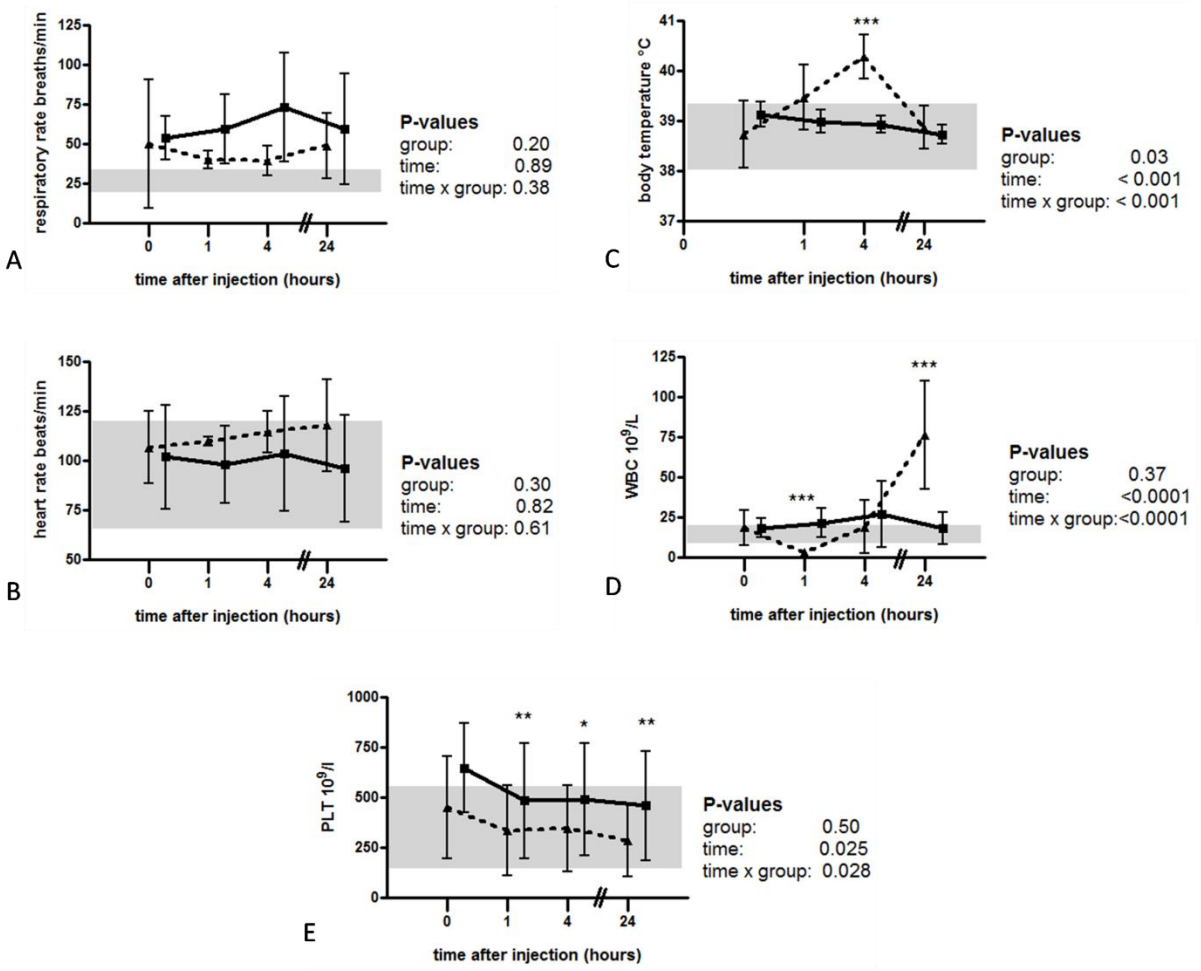

Figure 5: Dynamic alterations in clinical parameters and hematology after placebo (solid line; n=5 dogs) and LPS injection (dotted line; n=5 dogs). The grey markings are consistent with the reference interval. Data are expressed as group mean±standard deviation.Level of significance was set at $P<0.05$.

As shown infigure 5A, the respiratory rate of both groups was above the upper limit of the reference interval (34 breaths/min) in the majority of dogs. There was a trend towards a higher respiratory rate in the control group, however, the difference between the groups and between the time points of examination was not significant. The heart rate was also not significantly affected by the induction of endotoxemia.

In all dogs of the study group, injection of LPS resulted in a significant increase in body temperature above the upper limit of the reference interval (38.0°C-39.2°C)

reaching a peak of 40.3±0.4°C at time point 4 hours. There was a significant difference between the groups, a significant effect of time as well as a significant interaction between time and group. After induction of endotoxemia, 5/5 dogs developed a marked leukopenia with mean leukocyte counts of 2.5±0.7x10^9/l at time point 1 hour as shown in figure 5D. In all dogs, leukopenia was followed by a severe leukocytosis 24 hours after LPS administration with mean WBCs of 76.0±33.7x10^9/l. In 2/5 dogs of the control group, a transient increase in leukocyte count (35x10^9/l and 58x10^9/l respectively) was seen at time point 4 hours which, however, was not accompanied by fever or any other clinical sign of an inflammatory process. Unfortunately, blood smears were not available to evaluate leukocyte morphology and the number of band neutrophils. There was a highly significant effect of time and a significant interaction of group and time (both $P<0.0001$), i.e. a different development of leukocyte counts in both groups during the course of time.

There was a mild but significant decrease in platelet count one hour after injection of LPS and the placebo respectively, presented in figure 5E. The mean platelet count was lower in the study group, however, the difference between the groups was not statistically significant. At time points 1; 4; and 24 hours after application of LPS, a moderate thrombocytopenia was present in 1/5 dogs with platelet counts ranging from 16-31x10^9/l (reference interval 150-500x10^9/l). Statistical analysis revealed a significant interaction between group and time, i.e. a different development of platelet count in study group and controls during the course of time.

Throughout the study period, R-time was higher in the study group than in the controls indicating a lower activity of secondary hemostasis, however, the difference between the groups was not statistically significant as shown in figure 6 below.

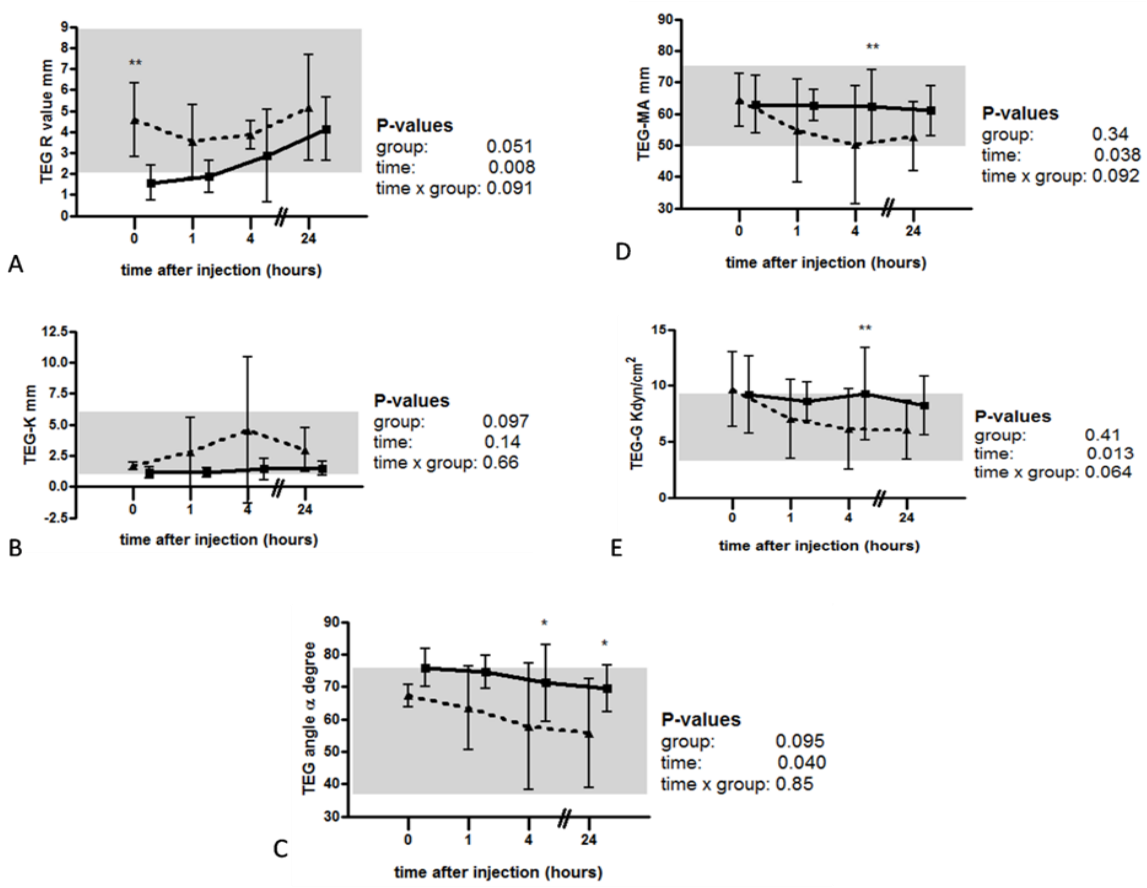

Figure 6: Dynamic changes in TEG parameters after placebo (solid line; n=5 dogs) and LPS administration (dotted line; n=5 dogs). The grey area indicates the laboratory-intern reference interval. Data are shown as group mean±standard deviation. Level of significance was set at P<0.05.

Although there was a significant increase in R-time in both groups, the absolute alteration from baseline values was small and results remained within the reference interval. The K-value reflecting the rapidity of fibrin cross linking increased temporarily after induction of endotoxemia with maximum values at time point 4 hours, however, there was no statistically significant effect of group, time or interaction between group and time. Angle α also indicative of fibrin cross linking dropped significantly after injection of LPS. Mean results were lower in the study group than in the control group indicating an impaired process of fibrin cross linking, however, the effect was not statistically significant. MA and G values mainly reflecting platelet function did not change in the control group during the period of follow-up examinations. In the study group, an endotoxemia-induced decrease in MA and G was observed with lowest values 4 hours after injection of LPS. In 2/5 dogs, MA and G values below the lower limit of the reference interval were noted at time points 4 hours and 24 hours respectively. For both variables, statistical analysis revealed a significant effect of time but no significant interaction between the factors group and time. The application of LPS resulted in a rapid increase of OSPT, aPTT and TT with highest coagulation times 4 hours after induction of endotoxemia as shown in figure 7.

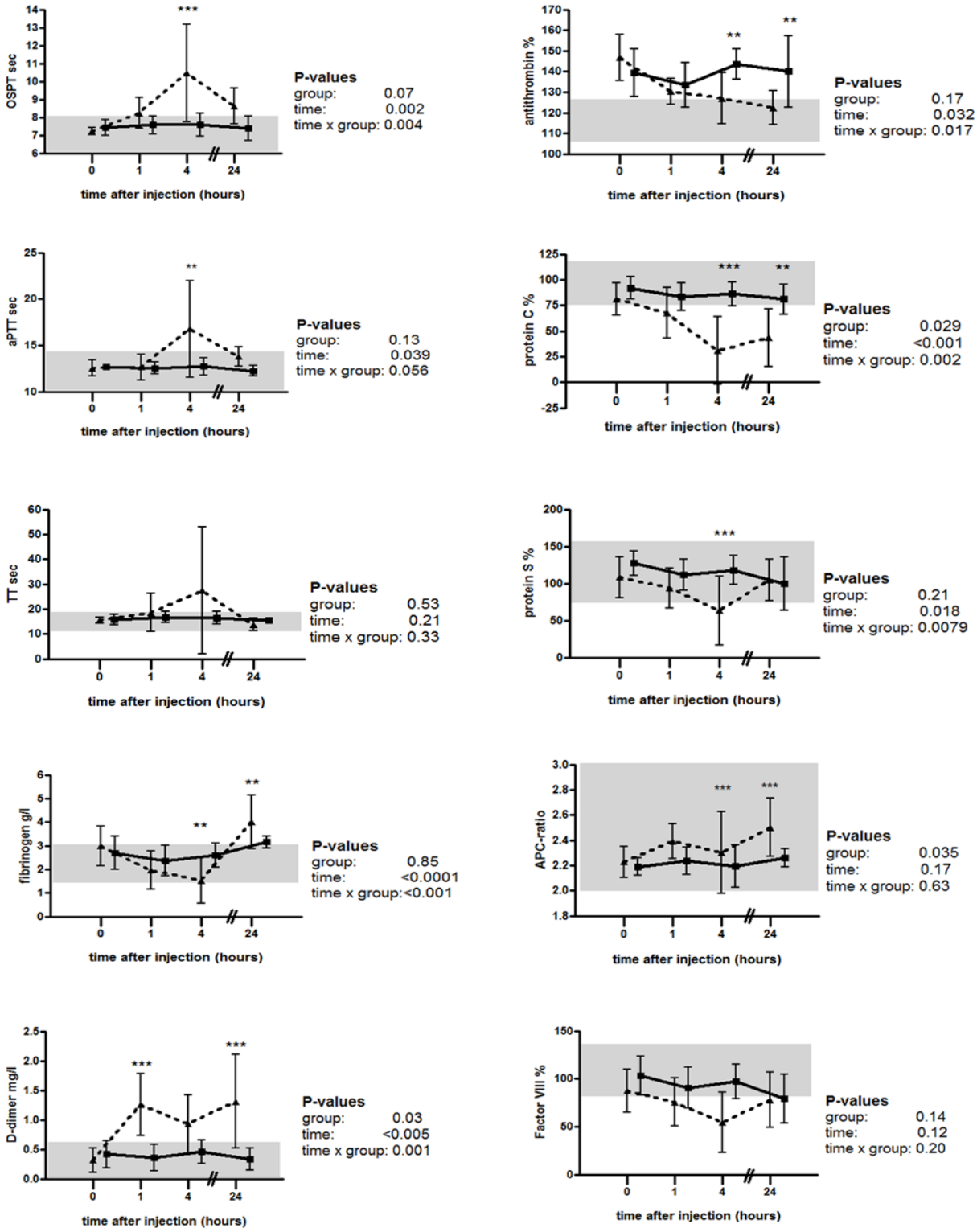

Figure 7: Dynamic alterations in plasma coagulation parameters after placebo (solid line; n=5 dogs) and LPS injection (dotted line; n=5 dogs). Data are shown as group mean±standard. The grey area is consistent with the laboratory-intern reference interval. Level of significance was set at P<0.05. Level of significance was set at P<0.05.

For OSPT and aPTT, statistical analysis did not reveal a significant difference between the treatment groups, however, there was a highly significant effect of time and interaction between group and time. Despite the trend towards an increase after application of LPS, TT did not differ significantly between the groups and the different time points of follow up examinations. In the study group, a significant decrease in fibrinogen plasma concentration was observed with lowest results after 4 hours. Until time point 24 hours after injection of LPS, fibrinogen plasma concentration was rising again and thus exceeding the results of the control group and the upper limit of the reference interval (3.1 mg/L).

Within one hour after induction of endotoxemia, a 2.2 fold increase of D-dimers from a mean baseline concentration 0.37±0.07 mg/L to 0.81±0.64 mg/L was seen. A second peak was observed at time point 24 hours. Mean D-dimer plasma concentrations were significantly higher than in the control group and remained > 0.67 µg/ml for the duration of the experiment.

LPS administration induced a moderate decrease in AT plasma concentration by 16% from mean baseline activities of 146±11% to lowest results at time point 24 hours (mean 122±8%). At all time points, results in the study group were not below the lower limit of the reference interval of 108%. In the control group, a mild transient decrease of AT plasma concentration was noted 1 hour after injection of the placebo. Mean AT activities of the control group were ranging between 133±11% (time point 1 hour) and 143±7% (time point 4 hours) and were thus exceeding the upper limit of the reference interval (128%). There was a significant effect of time and a significant interaction between group and time i.e., a different development of the AT plasma concentration during the study period.

After LPS treatment, PC activity compared to canine pooled plasma decreased significantly by 68% from a mean baseline value of 81±15% to lowest measurements at time point 4 hours (mean 26±39%). At time points 4 and 24 hours, 4/5 dogs demonstrated PC plasma concentrations below the lower limit of the reference interval of 75%. There was a significant difference between the treatment groups as

well as between the different time points. A similar effect was seen for PS activity decreasing by 34% from a mean baseline value of 108±27% to mean results of 63±46% at time point 4 hours. In contrast to PC, the endotoxemia-induced decrease of PS was smaller and the difference between study group and control group was not statistically significant. However, there was a significant effect of time and a significant interaction between group and time. At time points 4 hours and 24 hours, PS plasma concentrations was below the reference interval (74%-161%) in 2/5 and 1/5 dogs respectively.

Endotoxemia induced an increase of APC-ratio from a mean baseline value 2.2±0.1 to 2.5±0.2. Although the endotoxin-induced raise of APC-ratio was significantly higher than results obtained the controls, the majority of dogs in the study group demonstrated an APC-ratio within the reference interval (2.0-3.0). Only in 1/5 dogs of the endotoxin group, a decreased APC-ratio of 1.71 indicative of APC resistance was recognized 4 hours after application of LPS.

For FVIII activity, a transient non-significant decrease by 38% was noted in the study group 4 hours after injection of LPS.

Regarding the coagulation profile shown in figures 6 and 7, 3/5 dogs in the LPS group developed coagulation abnormalities consistent with DIC after endotoxin injection.

6.Discussion

6.1. Establishment of reference intervals for TEG and variables reflecting secondary and tertiary hemostasis run on the STA Compact analyzer

6.1.1. Repeatability, interferences and the effect of anticoagulant

6.1.1.1. Repeatability of TEG analysis

Coefficients of variation of repeatability have been assessed for TF-activated TEG by calculating the arithmetic mean and the pooled variance estimate, based on the differences in the duplicate determinations after 30 minutes and 120 minutes respectively using canine citrated re-calcified whole blood.[180] Mean CVs for both time points for R, K, angle α, and MA were 6.46%, 15.71%, 7.86%, and 4.30% respectively[180] and – thus similar to the CVs for kaolin-activated TEG obtained here.

6.1.1.2. Impact of anticoagulant in TEG analyses

Based on the results of the current study, the anticoagulant did not have any significant impact on TEG measurements. In contrast to this, studies in adult healthy volunteers (n=30[190] and n=10[191]) and healthy children (n=14)[185] undergoing minor surgical procedures have demonstrated that TEG variables were significantly different when measured in re-calcified citrated whole blood (analysed 30 minutes after sampling) compared to native whole blood. In re-calcified citrated whole blood, R and K were significantly shorter than in native blood whereas angle α and MA were significantly higher.[185] Other investigators did not demonstrate a significant difference between native whole blood samples and citrated re-calcified whole blood (n=8) when assessed after storage of 30 minutes at room temperature, however, after 120 minutes, a significant decrease in K and an increase in angle α was observed.[192] These findings were in contrast to the results obtained in the dogs studied here. It has

to be taken into account that the number of dogs evaluated in this study was low and significant effects might have been missed but the number of patients included was even lower in the majority of human studies demonstrating significant differences between native whole blood samples and re-calcified citrate blood. The most probable reason for the trend in hypercoagulation of citrated blood with respect to the native blood has been highlighted by Camenzind et al.,[190] who found that citrate does not completely inhibit thrombin formation so that activation of coagulation was not completely inhibited. In the cited human investigations, celite[190] was used for TEG analysis or thromboelastography was performed without any activating substances[185,191,192] so that it can be hypothesized that differences in activation of the coagulation process might be responsible for the different findings obtained in the current study. In people, kaolin is known to act as a stronger activator compared to celite as reflected by a significantly shorter R time (P=0.0052) and K time (P=0.0004).[193] If kaolin is capable to induce a strong activation even in native whole blood samples, a mild hypercoagulability due to incomplete inhibition of thrombin formation in citrated specimens would not have been detected. This theory is supported by previous investigations in humans demonstrating that routine coagulation assays using potent activators such as aPTT, PT, and TT are insensitive to minor thrombin generation during citrate storage.[194,195] It is also possible that – in contrast to humans - thrombin formation is completely inhibited in canine specimens, however, there are no studies substantiating this hypothesis.

It can be concluded that due to the higher inter-individual variation in native TEG, the use of citrated whole blood should be preferred especially for clinical studies.

6.1.1.3. Effect of hemolysis on TEG results

Previous studies in human specimens have shown that haemolysis resulted in an activation or significant alteration of coagulation.[196,197] The etiology of hemolysis, and blood cell lysis in general, includes biochemical, immunologic, physical, and chemical mechanisms. In vivo blood cell lysis can arise from hereditary, acquired,

and iatrogenic conditions (such as autoimmune hemolytic anemia, severe infections, intravascular disseminated coagulation, or transfusion reactions).[185] Here in the study, *in vitro* hemolysis was induced with two methods: First, the effect of severe shear stress on erythrocytes was evaluated by induction of mechanical hemolysis and second the impact of blood cell lysis on coagulation was assessed by induction of hemolysis by freeze/thawing. Causes for hemolysis due to mechanical destruction of erythrocytes include mainly pre-analytic factors such as prolonged application of the tourniquet, difficulty in locating the venous access, unsatisfactory sample acquisition, using a needle with a too small diameter or rapid flow of the blood in the test tube as well as vigorous mixing and shaking of the tube.[185] *In vivo* mechanical hemolysis due to shearing of erythrocytes is known to occur in patients with DIC[198] or microangiopathic hemolysis as reported in canine hemangiosarcoma. Mechanical hemolyis was rarely reported after incomplete closure of patent ductus arteriosus botalli in dogs with coils.[199] Lysis of blood cell membranes by factors other than shear stress includes pre-analytic factors such as exposure of the blood to excessively hot or cold temperatures or transfer of alcohol-based desinfectant from the skin into the blood specimen.[185] The current study clearly demonstrated that for the majority of variables hemolysis-induced changes were greater in samples with higher degree of hemolysis i.e., after HF. However, aprobable dose-depended behavior was not specifically addressed by producing several subgroups of hemolytic samples with several degrees of hemolysis. The latter was not performed as the analysis of three or more hemolytic subgroups for both HM and HF would have required several measurements after another or > 4 TEG analyzers which were not available in the laboratory. The results of TEG analyses demonstrated to be highly dependent on the storage time after sampling so that the number of assays was limited to those which could be performed at once one hour after sampling.

The investigation clearly demonstrated that the overall effect was a decreased coagulatory ability as shown by a decrease in MA and G-value. A similar result has been reported for horses using thrombometry.[200] The low number of erythrocytes

cannot be the only explanation for the low MA observed here and in the previous study in horses as an increased MA was observed in anemic human samples: Serially diluted human blood samples with preserved platelet count and unchanged concentration of coagulation factors demonstrated a hypercoaguable TEG curve which was characterized by a short R, increased angle alpha and MA.[201] An increased Maximum Clot Firmness (consistent with MA) was also observed in patients with sickle cell anemia using Rotem thrombelastrometry.[202] The presence of already activated – and thus hypo-reactive platelets – appears to be the most likely explanation for the low MA and G despite the evidence of increased secondary hemostasis. In anemic humans, the hypercoagulable thromboelastography curve was considered to be due to technical reasons rather than an *in vivo* finding as those patients possess a reduced number of erythrocytes so that a higher percentage of the sample is plasma. Therefore, more fibrinogen and platelets are in the thrombelastometer cup when the anemic blood is being analysed.[202] This is also a probable explanation for the significantly higher reduction in R observed in the current study in samples after HM which was associated with a mild decrease in hematocrit value in comparison to HF associated with a marked decrease in the percentage of erythrocytes. A further explanation for the hemolysis-induced reduction of R may be the fact that many substances may be released from lysed erythrocytes which might activate coagulation.[185] Intracellular and thromboplastic substances from either platelets or leukocytes are thought to be responsible for shortening of aPTT reported in investigations in human samples[203] and might also be responsible for the activation of secondary hemostasis reflected by the decreased R value which was observed here. Regarding the current results, it can be further hypothesized that the decrease in platelet count noted after HF might contribute to hypocoagulability characterized by decreased MA and G values observed after hemolysis as about 80% of the TEG-MA value is dependent on platelet number and function. However, as MA was lowest in specimens after HF and platelet count did not change significantly in this group, a decrease in platelet count has to be

considered an unlikely cause for this finding. In contrast to the current study, the previous investigation in horses had shown that mechanically induced hemolysis resulted in an increase of platelet count. So it is seen that blood cell lysis has a significiant impact on TEG parameters and results should be carefully interpreted.

6.1.1.4. Repeatability of variables assayed on the STA compact

Intra-assay CVs for OSPT, aPTT, TT, FVIII, AT, PC and PS were comparable or even slightly lower than those reported by the manufacturer for human plasma samples. Twenty-one-run intra-assay-repeatability for human specimens was as follows: OSPT=2.8%; aPTT=0.6%; TT=2.8%; FVIII=6.5%; AT=2.6%; PC=4.6%, and PS 2.75% (Package insert of STA test kits). The functional chromogenic PC assay demonstrated similar intra-assay CVs in people (2.9%)[25] when compared to the clotting assay used in here. Intra-assay CV was slightly higher for the fibrinogen concentration even after removal of an outlier (5.6% in dogs versus 2.3% in people). Variables with low plasma concentrations in healthy dogs such as fibrin D-dimer plasma concentration and anti-FXa activity demonstrated also high intra-assay CVs which were higher than those reported for human plasma. Intra-assay CV for fibrin D-dimers and anti-FXa were 45.9% and 12.8% (table 3) in canine plasma compared to 13.7% and 4.4.% in human specimens. However, for interpretation it has to be considered that concentrations of fibrin D-dimers and anti-FXa activities were extremely low in healthy dogs which is well known to result in high intra-assay CVs.[204] Six-run intra-assay repeatability for fibrin D-dimers was reported to be 6.1% (range 2% to 10%) for the same method and analyzer when determined in canine plasma with increased fibrin D-dimer plasma concentrations ranging from 0.94 µg/ml to 3.08 µg/ml (n=6 dogs).[205] In the previous investigation evaluating the STA Rotachrom Heparin assay for measurement of activity in canine blood plasma, 15 run-intra-assay repeatability was 13% at low anti-FXa activities (mean 0.268 IU/ml) and thus comparable to the results obtained here.[206]

6.1.1.5. Impact of hemolysis and lipemia on variables measured on the STA compact

In people, as mentioned above, it is well known that haemolysis does not cause interferences solely by the presence of haemoglobin as many substances may be released from lysed erythrocytes which might influence coagulation.[197] The release of intracellular and thromboplastic substances from leukocytes and platelets was considered to be responsible for shortening of the aPTT as it was observed previously in human specimens[207] and also in this study. In people, however, there was no definite agreement on this topic in the current literature.[197] In patients, measurement of OSPT, aPTT, fibrinogen and D-dimer concentrations have been considered to be more susceptible to pre-analytic errors.[208] A recent investigation evaluating 12 degrees of haemolysis on routine coagulation parameters in human specimens reported a significant increase in OSPT and D-dimers whereas a decrease in aPTT and fibrinogen was noted in samples containing more than 9% haemolysate.[197] The impact of haemolysis on AT has been rarely investigated in people and dogs. Increasing concentrations of haemoglobin resulted in a linear decrease in AT activity detected in canine plasma[164] which is in accordance with the results obtained in the current study.

For assessment of the influence of haemolysis on laboratory analytes, haemolysed specimens are commonly achieved by freezing-thawing cycles as it was also performed in the canine specimens evaluated here and in the previous human study.[197] A drawback of this method, however, was that naturally occurring haemolysis due to pre-analytic factors or diseases is associated with the generation of destroyed platelets and leukocytes as demonstrated in human samples.[197] Thus, the results reported here may not representative for all causes for haemolysed specimens observed in clinical laboratories.

According to the manufacturer, assays are insensitive to the following haemoglobin concentrations added to human plasma. A haemoglobin concentration up to 7.6 g/l (=4.7 mmol/l) for antithrombin, 5g/l (=3.1 mmol/l) for fibrin D-dimers and 2 g/l (=1.2 mmol/l) for anti factor Xa activity did not interfere with the assay. For the APC assay,

resampling was recommended for haemolytic human samples. Regarding the other variables, information about the effect of haemolysis was not provided by the manufacturer.

The effect of lipaemia on routine coagulation parameters (OSPT, aPTT, TT, fibrinogen) has been rarely investigated in dogs.[140] In contrast to the current study, measurements were performed using a ball coagulometer. Severe lipaemia (>triglyceride concentration of 1.25 g/l) resulted in a slight but clinically not significant shortening of OSPT and TT in canine blood plasma.[140] A decrease in fibrinogen concentrations by 15% have been reported in people, however, significantly false-low fibrinogen plasma levels were observed in the dogs evaluated here and in the previous investigation studying the effect of lipaemia on canine plasma.[140]

According to the manufacturer, assays are insensitive to triglyceride concentrations up to 7.6 g/l for antithrombin, and 3.6 g/l for anti factor Xa activity added to human blood plasma. In people, lipaemia was reported to result in false-low D-dimer measurements, which was not in accordance with the current findings in canine specimens.

6.1.2. Reference intervals

According to the proposals of the IFCC, at least 40 reference values are needed to obtain the 0.025 and 0.975 percentiles.[210] Given the proposal for establishment of human reference intervals, the number of values included should be preferable more than 120, but the quantity of samples required is strongly dependent on the confidence intervals.[210] The number of dogs included here was lower than the optimal number recommended by the IFCC, however, even in humans, it is generally accepted that a smaller number of values may still serve a useful clinical purpose as a guide in the form of the list of all values.[210] The use of the IFCC proposals in veterinary medicine is hampered by the fact that collection of samples and laboratory analyses are expensive and time-consuming and it is often difficult to identify the full

number of healthy subjects, so that alternative statistical methods have been evaluated to estimate reliable reference intervals even in a data set containing diseased animals.[211] TEG has been rarely performed in healthy dogs. Mean and standard deviation[209,212]/range[180] of TEG variables (R, K, angle α, MA)[180,212] and G[209] have been published for both TF-activated TEG (n=18 dogs[209]; n=8 dogs[212]) and TEG performed with native recalcified citrated whole blood (n=15 dogs).[180] It has to be considered that results of TF-activated TEG are not necessarily comparable to kaolin-activated TEG performed here as the first represents the extrinsic coagulation pathway whereas the latter is consistent with the intrinsic pathway of the coagulation cascade. In people, reference intervals for kaolin-activated TEG were as follows: R=5.3-9.3 min; K=1.4-3.5 min; angle α=48.8-72.2 degree; MA=55.3-69.3 mm and G was not evaluated by the authors.[213] MA and angle α were similar in humans (adults and children) and in the dogs investigated here but the lower reference limit of the R-value was considerably shorter in dogs (1.8 minutes in dogs versus 5.3 minutes in man) indicating a higher activity of secondary haemostasis.[213] This is supported by previous reports demonstrating that dogs show a higher coagulation activity of the extrinsic and intrinsic pathway compared to people.[64]

Based on data obtained in 100 children of different age groups, age-related differences in kaolin-activated TEG were not indentified.[213] However, establishment of reference intervals for TEG variables in newborns revealed that TEG reaction time, and G-value were significantly lower in neonates ($P<0.001$) than in adults.[214] In the latter study, TEG was conducted without use of an activator.

Comparable studies have not been performed in dogs, so that puppies or growing dogs have been excluded from the current investigation.

TF-, kaolin- and celite-actived TEG has been conducted in human specimens investigating the in vitro effect of the novel kallikrein inhibitor DX88 on TEG using different types of activation compared to a control.[215] R-time in the controls was shorter in kaolin activated-TEG than in TF-activated TEG. These results reinforce the

importance of consulting activator specific TEG reference intervals when interpreting results.

The current study demonstrated that TEG parameters were similar in male and female dogs. In contrast to this, sex is known to have an impact on several variables of coagulation in people. Men were reported to have higher plasma antithrombin and PC concentrations[216] whereas in woman higher D-dimer plasma concentrations were observed.[203] An age-dependent decrease in prothrombin time was noted in men ($r=-0.34$; $P<0.001$) but not in woman ($r=-0.04$; $P=0.47$).[217] Information about the influence of sex on TEG variables is lacking in people and dogs. The castration status was not taken into account here as the number of neutered dogs was comparatively low.

A limitation of the current study was that predominantly samples from Beagle dogs and German shepherd dogs could be obtained. This was due to the fact that healthy dogs available at a university clinic are either blood donors (mainly medium-sized to large breed dogs and Beagle dogs) or healthy dogs presented for routine radiological examination to screen for hip dysplasia or elbow dysplasia which is known to affect large breed dogs. Due to the predominance of Beagle dogs and German shepherd dogs in the reference population, it has to be considered that the results obtained here, might not be representative for all breeds. Breed-specific differences in coagulation have rarely been investigated in dogs. In one study comparing kaolin-activated TEG in 28 healthy Greyhounds and 15 non-Greyhound dogs, clotting kinetics were significantly slower and clot strength was significantly weaker in Greyhounds than in non-Greyhounds, except for R-time.[209] Here, Greyhounds were not included.

Previous investigations in canine citrated whole blood specimens have shown a significant trend towards hypercoagulability indicated by a decrease in R and K as well as an increase in angle α and MA when the storage time exceeded 120 minutes.[180] Thus, a fixed time point for measurements was recommended[180] as performed in the current study.

Regarding the reference ranges established for the STA Compact, reference intervals for OSPT, aPTT, fibrinogen (Clauss method) were comparable to those detailed previously for manual tests applied in dogs.[218]

Using a commercial coagulometric test, FVIII activity in canine plasma was reported to be 7.9 time higher compared with activities in human standard plasma which was in accordance with these results for the automated coagulation analyzer and was also demonstrated in less recent studies and case reports evaluating canine FVIII activity with coagulometry.[198,219] It has been shown that all coagulation tests using human deficient plasma for measurement of coagulation factors in canine plasma were influenced by the very high activities of FVIII (9.3 fold higher than in human plasma) and FV which are typical for dogs.[220] This was particularly the case when low dilutions of plasma were applied so that use of higher sample dilutions (> 1:20, e.g. 1:40) were recommended to reduce this effect.[220]A similar finding has been reported in cats[221] and cattle.[222]

Reference intervals for a functional PC chromogenic assay have been previously reported in dogs to be 75%-135% compared to canine pooled plasma[25] which was comparable to the result obtained here although the upper reference limit was slightly lower in this study (118%). Differences might be due to a different methodology (chromogenic versus clotting assay) and use of different pooled plasma. A limitation of the previous study was that statistical methods for calculation of reference intervals were not given and only 20 dogs were included.[25] To the author´s knowledge, this is the first study evaluating the PC and PS activity in healthy dogs with a functional clotting assay using Protac®, a single-chained glycoprotein derived from the venom of the southern copperhead snake, *Agkistrodon contortrix contortrix* as an activator of PC. PC activity has been determined previously in canine plasma with a Protac®-activated chromogenic assay, however, the results have been compared to canine pooled plasma rather than with the human standard.[25] The current study clearly demonstrated that PC and PS activities determined with the clotting test were markedly lower than in the human calibration standard.

Theoretically, this might have been due to a plasma concentration being lower in dogs than in people. As the clotting assay is a functional test, it might also be hypothesized that a decreased biological activity of PC and PS in dogs was the reason for different activities observed in people and dogs. However, given the physiology of the coagulation process, it is unlikely that dogs are lacking the natural anticoagulant PC. Moreover, an impaired activation of PC and PS in the clotting assay could have been responsible for different results obtained in people and dogs. A previous investigation demonstrated that canine PC was unresponsive for the activation with Protac® even when 3 times higher doses than in people were used.[223] Although canine PC was reported to show 72% homology with human PC molecule, the existent variations may account for the differences in the ability of Protac® to activate human and canine proteins. Surprisingly, dilution of the concentration of the contact activator even resulted in a shortening of the aPTT compared to the control (untreated canine blood plasma), whereas after dilution of both plasma and activator a minimal prolongation of aPTT was observed.[223] A similar finding was reported in pigs and it was hypothesized that this effect could be attributed to the presence of PC inhibitors or anti-activators since Protac® did induce PC-related amidolytic activity when the sample was diluted.[224,225] In contrast, PC-like amidolytic activity was not observed in canine plasma after activation with Protac® so that it was considered more likely that the snake venom extract, aPTT reagents, and canine plasma failed to produce astochiometric complex with strong PC activity.[223]

Despite these reports, there was evidence in the literature that at least a minimal activation of canine PC by Protac® is achieved because in this study and in another investigation, linearity of the test was proven from 0-100% when using a serial dilution of canine pooled plasma.[25] In contrast to the current investigation, a functional chromogenic assay was used by de Laforcade et. al. which, however, also included an activation of PC with the venom of the copperhead snake in comparison to a canine pooled plasma sample obtained from 15 healthy dogs.[25] Previous investigations in dogs have proven that functional PC assays are suitable to detect

significant differences between PC concentrations in patients with various diseases including sepsis[25,182] and portosystemic shunting.[226]

Regarding the resistance against activated PC, a reference interval for APC-ratio has not been evaluated in dogs. In people, an APC-ratio < 2.1 seconds has been considered abnormal[175] which was comparable to the results in dogs. The clinical significance of this variable, however, has still to be elucidated for animals.

The results of the current study clearly demonstrated that plasminogen activity was markedly lower in canine plasma than the human standard. Previous investigators found also lower plasminogen concentrations in dogs which were approximately 50% of the activity present in the human control plasma. These investigators used a chromogenic assay which was working with an excess streptokinase concentration (8000 IU/ml) for activation of plasminogen (HemosIL™ Plasminogen, Instrumentation Laboratory Munich).[227] However, these results were still higher than the reference interval obtained in the current study. A possible reason for the discrepancy found between different assays may be the fact that the concentration of streptokinase required for activation of plasminogen is species-specific. It was reported before that markedly higher amounts of streptokinase had to be applied in rats, rabbits and dogs than in humans.[228] For this reason, different streptokinase concentrations used in the test kits (8000 IU/ml in the HemosIL™ Plasminogen assay[227] versus 5000 IU/ml in the STA Plasminogen assay applied in the current study) may also be responsible for the differences between laboratories. Based on these findings, it was evident that human assays used for measurement of canine plasminogen activity were reflecting the capability of the fibrinolytic system to be activated by streptokinase rather than the plasminogen concentration itself.[229] This is in accordance with a previous study in dogs reporting a low capability of streptokinase (4000 IU/ml) to activate canine plasminogen in a commercial assay which was adapted to a microtiter plate.[154] Urokinase (20,000 IU/ml) demonstrated to be a more potent activator than streptokinase in canine specimens.[154] Although commercial plasminogen test kits which were supplied with comparatively high

streptokinase concentrations such as the HemosIL™ Plasminogen assay showed a certain capability to activate canine plasminogen,[227] substitution of streptokinase by urokinase appears to be preferable to achieve an expansion of the standard curve. In the current investigation, additional analysis of samples using urokinase or streptokinase in higher concentrations as activator was not performed due to an insufficient sample volume.

Anti-FXa activity was evaluated before in 12 healthy dogs and 10 dogs with IMHA using the same assay, analyser and test protocol.[206] Baseline anti-FXa activity ranged between 0.06-0.17 IU/l and thus was slightly lower than in the current investigation. This finding indicated that canine plasma contains endogenous FXa inhibitors at a higher concentration or increased activity compared with human plasma.[206] Therefore, a higher dilution of canine plasma (3:8 instead of the default setting of 1:2 for human samples) was recommended for monitoring of heparin therapy in dogs to achieve a baseline activity close to zero.

The impact of sex on coagulation parameters reflecting fibrinolysis and natural anticoagulants was not evaluated before. Previous investigations in dogs revealed significantly increased concentrations of fibrinogen and fibrin/fibrinogen degradation products (FDPs) under the influence of high peripheral progesterone concentrations whereas plasma antithrombin was decreased.[230] The current investigation failed to demonstrate any significant impact of sex on coagulation parameters but the phase of the oestrus cycle was not determined here, so that effects specific for certain cycle phases might have been missed. The impact of sex on fibrin D-dimers, PC, PS, APC-ratio, plasminogen, and anti-FXa activity has not been evaluated in dogs. In people, several sex-specific findings were demonstrated. Men reported to have higher plasma AT and PC plasma concentrations[216] whereas in women higher D-dimer plasma concentrations could be observed.[231]

The samples were predominantly from Beagle dogs and German shepherd dogs whereas Greyhounds were not included as previously explained. Significant

differences between different breeds were reported for FVII[232] which, was not investigated here.

In summary it can be concluded that some human assays including the analysis of OSPT, aPTT, fibrinogen and AT, D-dimer concentrations were suitable for use with canine specimens without any further adaptation of the human test protocols whereas others such as the PC and PS clotting test can only be used when results are compared to canine pooled plasma due to species-specific characteristics of the coagulation process. With modifications, the commercial human PC, PS and FVIII test kits might serve to elucidate canine diseases associated with thrombophilia such as early phases of disseminated intravascular coagulation. The high costs of the assays, however, limit their application in routine practice. Based on the results of the current study, the STA plasminogen assay is not suitable for assaying plasminogen activity in canine samples most likely due to the low streptokinase concentration used in the test kit.

6.2. Evaluation of impact of endotoxemia on the coagulation process

This study documents the early systemic response to LPS administration in dogs.

The peak of most severe coagulation abnormalities was accompanied by severe clinical and hematological signs of sepsis (fever and leukopenia). Here, respiratory rate and heart rate were no sensitive parameter to detect the early onset of a septic process. The respiratory rate was exceeding the reference interval in both groups which might have been caused by high ambient temperatures. However, maximal temperatures in Bursa, Turkey were ranging between 18.8°C and 23.6°C (Historical weather, Bursa, Turkey: http://www.tutiempo.net/en/Climate/Bursa/10-2008/171160.htm) during the study period so that excitement of the dogs during the physical examination appears to be the most likely cause.

Comparable to the results in the current study in dogs, transient leukopenia was observed 4 hours after application of LPS in dogs[121] and in a feline model of low-dose endotoxemia.[124] Others reported a nadir of leukocyte count 2 hours following the

application of 1 μg LPS in dogs.[233] Like in the current investigation, leukopenia was followed by a leukocytosis 24 hours after administration of endotoxin in the previous study in dogs[121] whereas in the other experimental investigations in dogs and cats, the study period was less than 5 hours[233] and 6 hours[124] respectively after administration of LPS so that a probable increase in leukocyte counts might have been missed. The transient leukocytosis recognized in two dogs of the control group was unexpected as this finding was not associated with any clinical signs or fever indicative of an acute inflammatory process. As it was not associated with coagulation abnormalities, its impact of the results of the study was considered to be not significant.

The sepsis-induced changes in TEG variables observed here were in accordance with a previous experimental study in pigs (n=7) reporting a significant decrease in α and MA values (21%) in the first 4 hours after induction of sepsis, while R increased by 63% at the end of the LPS injection (50 μg/kg given intravenously over 60 minutes). K rose steadily for 5 hours, increasing significantly by 66% at 6 hours. Fibrinolysis was not seen during the 6 hours period performing TEG of 1-hour length.[234] In contrast to the results in dogs and pigs, infusion of 2 ng/kg LPS to human volunteers was followed by a significant decrease in clotting time (consistent with the TEG R value) when assessed with ROTEM thromboelastography whereas clot formation time (consistent with TEG K) and maximal clot firmness (consistent with TEG MA) were not affected by LPS application.[235]

A probable reason for the discrepancy between the findings reported in people and dogs or pigs respectively might be the markedly lower dosage of endotoxin used in the human investigation. It can be hypothesized that application of higher dosages of endotoxin may result in a more rapid activation of coagulation which is followed by a fast consumption of coagulation factors and – thus a prolongation of R indicating an impaired secondary hemostasis. Lower LPS concentrations as used in the human study, however, appear to activate coagulation activity and fibrinolysis but there was no evidence of excessive consumption of coagulation factors.

Regarding the results of the current study, the diagnostic use of TEG as early marker of coagulation abnormalities in patients appears to be problematic as the majority of TEG parameters remained within the reference interval and changes are only detected if follow-up examinations are available. A similar finding was obtained in septic human patients without bleeding complications showing thromboelastography (ROTEM) variables within the reference interval, however, an improvement was seen after treatment.[236]

The response to LPS administration has been reported previously in dogs for several coagulation parameters, however, in the previous study, a markedly higher LPS dose (6mg/kg) was applied so that the results are not entirely comparable to the findings reported here.[121] In accordance to the current investigation, a transient hypofibrinogenemia followed by a hyperfibrinogenemia was noted in the previous study as well as a decrease in PC activity, antithrombin, factor VIII, platelets and the leukocyte count.[122] Activation and subsequent consumption of PC has been also observed in septic human patients.[237-239]

In the current study, the most significant endotoxin-induced change of coagulation parameters was demonstrated for PC activity and fibrin D-dimers. A similar result was recognized in people with naturally occurring sepsis.[240] In accordance with the current study in dogs, a less pronounced decrease in PS compared to the AT and PC plasma activities was seen in people.[241] In contrast to this, others reported that PS activity was not significantly different even in patients with severe sepsis when compared to the controls.[240,242] For AT, mean results of the control group and baseline activities observed in the study group were exceeding the laboratory-intern reference interval established previously for the author´s laboratory. A probable reason for this may be the fact that only 2 of 56 dogs included for establishment of reference intervals were mongrel dogs. Moreover, German Shepherd dogs (n=19/56) and Beagle dogs (n=15/56) were overrepresented, so that the reference interval might be broader in mongrel dogs although to the author´s knowledge no breed-specific difference have been reported for AT.

The current study clearly demonstrated that application of endotoxin did result in a rapid change of mean results of all coagulation parameters studied. For TEG K and MA value as well as for coagulation times, fibrinogen, PC, PS and FVIII peak deviations from the baseline level are reached after 4 hours whereas for the other variables the deviations from the baseline level remained constantly changed during the study period. The results in dogs are in contrast to findings in people demonstrating a transient increase of FVIII activity 4 hours after experimentally induced endotoxemia after 4 ng/kg LPS given intravenously which was accompanied by unchanged PC and PS plasma activities.[243] A probable reason for the discrepancy to the actual study in dogs might be the fact that the LPS dose administered in the human study was comparatively low so that it did not result in DIC with subsequent consumption of coagulation factors and natural inhibitors of coagulation such as PC and PS. The raise in FVIII activity reported in people is not surprising as it is an acute phase protein in humans and LPS demonstrated to activate the human FVIII promoter in cultured hepatocytes.[244] FVIII is also an acute phase protein in horses[245,246] but not in mice.[244] The promoter region responding to LPS is well preserved in dogs compared to the human sequences which might suggest a similar importance of FVIII as acute phase proteins in dogs.[244] The potential role of FVIII as a canine acute phase protein has not been demonstrated in clinical studies so that it has to be elucidated in future investigation if dogs show a similar increase of FVIII in response to inflammatory stimuli.

Based on the results it can be concluded that the behavior of the APC-ratio is not comparable with its alterations in people observed during the course of inflammation. Except for 1/5 dogs, an increase of APC-ratio consistent with a decreased APC resistance was noted following the induction of endotoxemia. This result contrasts findings in people showing a decline of APC-ratio to 65% of the baseline levels and thus – an increased APC resistance.[243] In the dogs evaluated here, the APC-ratio was generally within the reference interval so that this parameter can only interpreted when follow-up examinations are available. Based on the results of

this study, it can be concluded that – at least under experimental settings - the APC-ratio is not of major use in the diagnostic workup of dogs with early sepsis/endotoxemia.

In the previous experimental study in dogs, a markedly higher LPS dose (6 mg/kg) was applied resulting in the death of 2/5 patients.[121] Sublethal LPS injection was chosen for the current investigation to avoid several pitfalls reported for high-dose bolus endotoxemia models, including acute overwhelming inflammation, induction of an immediate severe hypodynamic circulatory response, atypical massive cytokine production, and rapid mortality.[247] Unlike models of high-dose endotoxemia resulting in rapid mortality, this model is more likely to provide clinically useful data for the evaluation of novel coagulation parameters in canine patients. The reaction to LPS appears to be species-specific as both low-dose (2 μg/kg/h i.v. for 4 hours)[124] and high dose (3-10 mg/kg) LPS infusion[248] did not produce significant coagulation abnormalities in cats.

A potential downsides of IV endotoxin sepsis models – and also here - is that they are not necessarily representative for all situations of clinical disease as septic foci in patients generally seed the body with bacteria continuously rather than over a short period of time.[247] Further limitations of the current study included the fact that the number of dogs examined was comparatively low so that significant effects might have been missed and that there was evidence of transient inflammation in the 2 of the control dogs. Nevertheless, coagulation parameters were not changed at this time point so that there was no apparent influence on the coagulation process.

In this study, platelet aggregates or clumps could not be excluded as a contributing factor to the thrombocytopenia, as peripheral blood smears were not reviewed. Mild to moderate platelet clumping was reported to contribute to a decrease in platelet count when measured by the impedance method.[249] However, in a recent study, platelets in canine blood collected in EDTA were minimally clumped in most blood smears and the interpretation of platelet count and platelet indices was not significantly affected, as compared with blood collected in citrate.[250]

Overall, the study clearly demonstrated that a raise in fibrin D-dimers is the earliest indicator for sepsis-associated coagulation abnormalities followed by a significant increase in OSPT and a marked decrease in PC whereas alterations in PS, AT, FVIII were of minor severity in the early phase of a septic process. In contrast to humans, APC ratio did not appear to be a good screening parameter in canine septic patients but further studies are required to elucidate the diagnostic use of this parameter in dogs. TEG variables are often within the reference interval in early sepsis, so that follow-up examinations of the patient are essential to detect abnormalities of the coagulation system.

Parts of this study have been published and the publication results have been used here in the thesis. The publications are the following:

1) Bauer N., Eralp O., Moritz A.: Establishment of reference intervals for kaolin-activated thromboelastography in dogs including in assessment of the effects of sex and anticoagulant use. J Vet Diag Invest. 2009; 21(5): 641-648.
2) Bauer N., Eralp O., Moritz A.: Reference intervals and method optimization for variables reflecting hypocoagulatory and hypercoagulatory states in dogs using the STA Compact automated analyzer. J Vet Diag Invest. 2009; 21(6): 803-814.
3) Bauer N., Eralp O., Moritz A.: Effects of hemolysis on canine kaolin-activated thromboelastography values and ADVIA 2120 platelet activation indices. Vet Clin Pathol. 2010, 39(2): 180-189.
4) Eralp O., Yilmaz Z., Failing K., Moritz A., Bauer N.: Effect of experimental endotoxemia on thromboelastography parameters, secondary and tertiary hemostasis in dogs. 2011, 25(3):524-531.

7. Summary

Objective

The first part of the study included method validation(intra-assay-repeatability, impact of hemolysis and lipaemia, effect of anticoagulant) and evaluation of reference intervals for dogs for kaolin-activated thromboelastography (TEG® 5000 Thrombelastograph, Haemonetics Corporation; formerly Haemoscope Corporation; Braintree, MA, USA) and the automated coagulation analyzer STA Compact (STA Compact®, Roche Diagnostics GmbH, Mannheim, Germany).

In a second part of the study, the response of the coagulation system in a canine model of endotoxemia was evaluated.

Material and Methods

Before the establishment of reference intervals, repeatability and interferences were assessed. Investigated variables included the TEG values R, K, α, MA and G as well and the coagulation variables one stage prothrombin time (OSPT), activated partial thromboplastin time (aPTT), thrombin time (TT), fibrinogen, Factor VIII (FVIII), antithrombin (AT), protein C (PC), protein S (PS), resistance against activated protein C (APC-ratio), plasminogen, D-dimer, anti-FXa run on the coagulation analyzer STA Compact. Intra-assay repeatability for TEG variables was performed in 6 healthy dogs with duplicate measurements. The standard deviation required for calculation of the coefficient of variation (CV) was consistent with the root of the pooled variance estimate. The comparison between TEG results obtained with native whole blood or citrated whole blood was performed in 16 healthy dogs. For assessment of hemolysis on TEG results,17 healthy dogs were included. Hemolytic samples were prepared with two techniques: mechanical stress (HM) and as well as freeze and thawing (HF). The intra-assay repeatability for variables assayed with the STA Compact was assessed with 15 replicate measurements in a sample taken from one healthy dog. The intra-assay variation was estimated for variables mentioned above. To assess the

influence of lipemia on measurements run on the STA Compact analyzer, samples with three grades of lipemia were prepared by adding Liquigen® (Liquigen®, Pfrimmer Nutricia GmbH, Erlangen, Germany) to the samples of three healthy dogs. The effect of hemolysis on these coagulation parameters was performed by treating whole blood in afreeze-thaw cycle and three grades of hemolysis were obtained.

Establishment of reference intervals for TEG parameters and coagulation parameters measured on the STA Compact was performed with 56 healthy dogs. After evaluating the default settings of human assays applied on the STA Compact for canine specimens, some modifications of the test methods were needed. A standard curve has to be prepared with canine pooled plasma for PC, PS and FVIII. Also for both analyzer measurements the impact of sex on the results was established.

In the second part of this investigation, the impact of endotoxemia on primary, secondary and tertiary hemostasis as well as TEG variables was evaluated in 10 healthy Mongrel dogs. A control group (n=5 dogs) and a treatment group (n=5 dogs) was created. Dogs in the treatment group received endotoxin (LPS) dissolved in sterile saline 0.9% which was administered intravenously at a dosage of 0.02 mg/kg. The control group received 0.2 ml/kg sterile saline 0.9%. Venous blood samples were collected before application of LPS or placebo (0 hour), 1, 4 and 24 hours after treatment.

Statistics

Results were analyzed with the Graph Pad Prism (Graph Pad Software, San Diego, USA), Analyse-it Method evaluation (Analyse-it Method validation Edition version 2.12 - © 1997-2008, Analyse-it Software Ltd.) and BMDP statistical software (BMDP Statitical software Inc., 1440 sepulveda Blvd, Los Angeles, CA 90025 USA).

Intra-assay variation for TEG variables was assessed by calculating the arithmetic mean and the pooled variance estimate, based on the differences in the duplicate determinations. For variables measured on the STA compact fifteen-run intra-assay repeatability for normal values was calculated from a sample of a healthy dog. The

effect of anticoagulant and interferences was assessed by a paired t-test (or Wilcoxin signed rank sum test).

For the establishment of the reference intervals, an Anderson Darling test was performed to verify the assumption of normality. In case of normal and log normal distribution of data, double sided reference intervals were obtained by calculating the mean±1.96 SD (standard deviation) so that 95% of the reference population wasincluded. If non-normal distribution of data was present, the non-parametric percentile method was applied. The 2.5 and 97.5 percentiles were calculated to obtain the 95% double sided reference interval. Data were depicted as histograms with the reference interval as well as the 90% confidence interval of the upper and lower reference limits. The influence of sex on the reference intervals was assessed by using an unpaired t-test or a comparable non-parametric test (Mann Whitney U test).

The differences between control group and endotoxin treatment group were assessed with a two way analysis of varience and covariance with repeated measures.

Results

Intra-assay CVs for R; K; α; MA and G were 7.6%; 17.7%; 7.4%; 2.9% and 6.6% respectivley. Samples with hemolysis resulted in a significantlydecreased R value and decreased MA, G and α value ($P<0.001$ to <0.0001). Furthermore, a significantly high K value was seen compared to the control group ($P<0.01$). There was no significant impact of anticoagulant on TEG variables.Inter-individual variation was higher in native samples than in citrated whole blood. Intra-assay CVs for the coagulation parameters OSPT, aPTT, TT, fibrinogen, FVIII, AT, PC, PS, APC-ratio, plasminogen, D-dimer and anti-FXa were 1.22%, 1.02%, 1.64%, 5.6%, 3.89%, 4.68%, 2.36%, 1.4%, 1.45%, 20.16%, 45.92% and 12.83%. FVIII-activity, antithrombin, proteinC, protein S, and APC-ratio were overestimated in haemolytic plasma, whereas fibrinogen, TT, and aPTT were underestimated. Lipemia resulted only in false high D-dimers.

Reference intervals for the kaolin activated TEG were as follows: R=1.8-8,6min.; angleα=36.9-74.6 degrees; K=1.3-5.7 min.; MA=42.9-67.9 mm and G=3.2-9.6

Kdyn/cm^2.Reference intervals for the STA Compact automated analyzer were as follows: OSPT=5.7-8.0 sec.; aPTT=10.0-14.3 sec.; TT=11.9-18.3 sec.; fibrinogen=1.3-3.1 g/l; AT=107.9-128.0 %; D-dimer=0.023-0.65 µg/ml; anti-FXa=0.04-0.26 IU/L; APC-ratio=2.0-3.0; PC=74.4-160.5 %; PS=75.5-118.9 % and FVIII=70.9-136.4 %. The results for PC, PS and FVIII have to be compared to a canine standard curve. There was no significant impact of sex on results of both analyzers.

Second part of the study: The endotoxin-induced clinical signs included lethargy (n=5/5), diarrhea (n=4/5), vomitus (n=4/5) and abdominal pain (2/5).

Regarding the evaluation of the impact of endotoxin on the coagulation process severe leukopenia (mean 2.5±0.7×10^9/l; P<.0001) and a significiant 2.2-fold increase in D-dimers (P=0.001) were observed at time point 1 hour. At time point 4 hours, a significant raise in body temperature (P=0.0006) and in OSPT (P=0.0042) was seen as well as a significant decrease in fibrinogen (P=0.0007), protein C (P=0.0021) and protein S (P=0.008). PLTs (P=.0284) and antithrombin (P=.0170) were lowest at time point 24 hours. TEG variables did not significantly differ between the groups. APC-ratio was higher in the sepsis group (P=0.0348), however, remained within the reference interval in 4/5 dogs.

Conclusion

The data provided useful reference ranges for kaolin-activated TEG and for the STA Compact automated analyser but some human tests (e.g., Protein C, protein S and factor VIII) have to be modified.

Regarding the impact of endotoxemia on the hemostatic system, the earliest indicator of sepsis-associated coagulation abnormalities were D-dimers which were followed by increased OSPT and decreased protein C. APC-ratio and TEG variables were not considered to be good screening variables in early sepsis/endotoxemia.

8. Zusammenfassung

Ziel der Studie

Das Ziel des ersten Teils der Studie war die Methodenevaluation (inklusive intra-assay Wiederholbarkeit, Einfluss von Hämolyse, Lipämie und Antikoagulant) sowie die Referenzwertbestimmung für Hunde für kaolin-aktivierte Thrombelastographie (TEG® 5000 Thrombelastograph, Haemonetics Corporation; formerly Haemoscope Corporation; Braintree, MA, USA) und das automatisierte Gerinnungsanalysegerät STA Compact (STA Compact®, Roche Diagnostics GmbH, Mannheim, Germany).

Im zweiten Teil der Studie soll der Einfluss von Endotoxämie auf den Gerinnungsprozess bei einem Hunde-Endotoxinmodell untersucht werden.

Material und Methoden

Vor der Etablierung der Referenzwerte wurden die Wiederholbarkeit und Interferenzen auf die Untersuchungen durchgeführt. Die evaluierten Gerinnungsparameter umfassten die TEG Parameter R, K, α, MA und G sowie die Koagulationsparameter Prothrombinzeit (PT), aktivierte partielle Thromboplastinzeit (aPTT), Thrombinzeit (TT), Fibrinogen, Faktor VIII (FVIII), antithrombin (AT), Protein C (PC), Protein S (PS), Resistenz gegen aktiviertes Protein C (APC-ratio), Plasminogen, D-dimer und anti-FXa, die am STA Compact gemessen wurden. Die Wiederholbarkeit für die TEG-Parameter wurde mit Doppelmessungen bei 6 Hunden durchgeführt. Die Standardabweichung, die für die Berechnung des CV erforderlich war, war mit der Wurzel der gepoolten Varianz übereinstimmend. Der Vergleich von TEG Ergebnissen mit nativem und Zitrat antikoaguliertem Vollblut wurde bei 16 gesunden Hunden durchgeführt. Für die Beurteilung der Hämolyse auf TEG Ergebnisse wurden 17 gesunde Hunde eingeschlossen. Hämolytische Proben wurden mit zwei Verfahren hergestellt: mechanischer Stress (HM) und sowie Einfrieren und Auftauen (HF).

Die intra-assay Wiederholbarkeit für die am STA Compact gemessenen Parameter wurde bei einer von einem gesunden Hund entnommenen Probe mit 15 Wiederholungsmessungen durchgeführt. Um den Einfluss von Lipämie auf die Resultate der Messungen beim STA Compact zu bestimmen, wurden drei verschiedene Grade von Lipämie vorbereitet, indem Liquigen® (Liquigen®, Pfrimmer Nutricia GmbH, Erlangen, Germany) in die Proben gemischt wurde. Die Auswirkungen von 3 verschiedenen Stufen von Hämolyse auf die Gerinnungsparameter wurde durch Gefrieren/Auftauen von Vollblut untersucht.

Die Referenzwertbestimmung für die TEG Parameter und die am STA Compact gemessenen Gerinnungsparameter wurde mit 56 gesunden Hunden durchgeführt. Die ersten Untersuchungen zeigten, dass einige humane Gerinnungstests für die Anwendung für Hundeblutproben modifiziert werden mussten. So war für PC, PS und FVIII die Etablierung einer Standardkurve mit Hundepoolplasma notwendig. Weiterhin wurde das Vorhandensein möglicher geschlechtsabhängiger Unterschiede der Referenzwerte untersucht..

Die Auswirkung der Endotoxämie auf pimäre, sekundäre und tertiäre Hämostase sowie TEG Werte wurden bei 10 gesunden Mischlingshunden ausgewertet. Eine Kontrollgruppe (n=5 Hunde) und eine Behandlungsgruppe (n=5 Hunde) wurde untersucht. Die Hunde in der Behandlungsgruppe erhielten in 0,9% sterile Kochsalzlösung gelöstes Endotoxin (LPS) intravenös in einer Dosis von 0,02 mg/kg injiziert. Die Kontrollgruppe erhielt eine intravenöse Injektion von 0,2 ml/kg 0,9% steriler Kochsalzlösung. Venöse Blutproben wurden vor der LPS- oder Placebo-Applikation (0 Stunde), sowie 1., 4. und 24. Stunden nach der Behandlung entnommen.

Statistik

Die Ergebnisse wurden mit dem Graphpad Prism (Graph Pad Software, San Diego, USA), Analyse-it Methoden Bewertung (Analyse-it Method validation Edition version 2.12 - © 1997-2008, Analyse-it Software Ltd.) und BMDP Statistik Software

(BMDP Statitical software Inc., 1440 sepulveda Blvd, Los Angeles, CA 90025 USA) analysiert.

Die Intra-Assay-Varianz (Variationskoeffizient, CV) wurde für die am STA Compact gemessenen Parameter mittels Berechnung des arithmetrischen Mittelwertes und der Standardabweichung bestimmt. Für die TEG Parameter erfolgte die Berechnung durch eine Schätzung der gepoolten Varianz basierend auf den Unterschieden von Doppelbestimmungen. Die Wirkung von Antikoagulanz und Interferenzen auf die Messergebnisse wurde mit Hilfe eines gepaarten T-tests (oder Wilcoxin signed rank sum Tests) beurteilt.

Für die Bestimmung der Referenzwerte und der Überprüfung der Normalverteilung wurde ein Anderson Darling Test durchgeführt. Beim Vorliegen einer Normalverteilung oder logarithmischer Normalverteilung der Daten, wurden doppelseitig Referenzbereiche durch Berechnung der mittleren ±0,96 SD (Standardabweichung) berechnet, so dass 95% der Referenzpopulation einbezogen wurde. Lagen nicht-normal verteilte Daten vor, wurde die nicht-parametrische Perzentil-Methode angewendet; hier erfolgte eine Berechnung der 2,5 und 97,5 Perzentile, um das 95% doppelseitige Referenz-Intervall zu erhalten. Die Daten wurden als Histogramme mit dem Referenz-Intervall und den 90%-Konfidenzintervalle der oberen und unteren Referenzgrenzen dargestellt. Der Einfluss des Geschlechts auf die Referenzintervalle wurde mittels eines ungepaarten t-Test oder einem vergleichbaren nicht-parametrischen Test (Mann-Whitney-U-Test) beurteilt.

Im zweiten Teil der Studie wurden die Unterschiede zwischen der Kontrollgruppe und der mit Endotoxin behandelten Gruppe mittels einer zweifaktoriellen Analyse von Varianz und Kovarianz und Messwiederholung untersucht.

Ergebnisse

Für die TEG Parameter R; K; α; MA und G konnten die folgenden Variationskoeffizienten von7.6%; 17.7%; 7.4%; 2.9% und 6.6% festgestellt werden. Proben mit Hämolyse führten zu einem signifikant erniedrigten R, MA, G und α

Wert (P<0.001 bis P>0.0001). Darüber hinaus konnte ein signifikant höherer K Wert im Vergleich zur Kontrollgruppe festgestellt werden (P<0.01). Es war kein signifikanter Einfluss des Antikoagulanzes auf die TEG-Ergebnisse nachweisbar, jedoch war die interindividuelle Variationin nativen Proben höher als im Zitratblut. Die Intra-Assay CVs für die Gerinnungsparameter PT, aPTT, TT, Fibrinogen, FVIII, AT, PC, PS, APC-ratio, Plasminogen, D-dimer und Anti-FXa waren 1.22%, 1.02%, 1.64%, 5.6%, 3.89%, 4.68%, 2.36%, 1.4%, 1.45%, 20.16%, 45.92% und 12.83%. Die FVIII-Aktivität, Antithrombin, PC, PS und APC-ratio wurdenin hämolytischem Plasma falsch hoch gemessen, während Fibrinogen, TT und aPTT falsch niedrig waren. Lipämie führte lediglich zu falsch hohen D-dimer Werten.

Folgende Referenzbereiche konnten für das Kaolin aktivierte TEG etabliert werden: R=1.8-8,6min.; α=36.9-74.6 Grad; K=1.3-5.7 min.; MA=42.9-67.9 mm und G=3.2-9.6 Kdyn/cm^2. Für die am STA Compact gemessenen Parameter wurden folgende Referenzbereicheerstellt: OSPT=5.7-8.0 sek.; aPTT=10.0-14.3 sek.; TT=11.9-18.3 sek.; Fibrinogen=1.3-3.1 g/l; AT=107.9-128.0 %; D-dimer=0.023-0.65 µg/ml; anti-FXa=0.04-0.26 IU/l; APC-ratio=2.0-3.0; PC=74.4-160.5 %; PS=75.5-118.9 % und FVIII=70.9-136.4 %. Die Ergebnisse für PC, PS und FVIII mussten dabei jeweils mit einer Hunde-Standardkurve verglichen werden. Es gab keinen signifikanten Einfluss auf die Ergebnisse der beiden Analysatoren.

Zweiter Teil der Studie: Die endotoxin-induzierten klinische Symptome umfassten Lethargie (n=5/5), Durchfall (n=5/4), Erbrechen (n=5/4) und Bauchschmerzen (n=2/5).

Eine Stunde nach der Injektion von Endotoxin konnte eine hochgradige Leukopenie (Mittelwert 2.5±0.7×10^9/L; P<0.0001) und ein signifikanter 2.2-fach Anstieg der D-dimere (P=0.001) beobachtet werden. Zum Zeitpunkt 4. Stunden war eine deutliche Erhöhung der Körpertemperatur (P=0.0006) und der PT (P=0.0042) sowie ein signifikanter Abfall der Fibrinogen Plasmakonzentration (P=0.0007), des PC (P=0.0021) und des PS (P=0.008)nachweisbar . PLTs (P=o.0284) und AT (P=0.0170) waren am niedrigsten zum Zeitpunkt 24 Stunden. Die TEG Parameter unterschieden sich nicht signifikant zwischen den Gruppen. Die APC-ratio war höher in der

Endotoxin-Gruppe (P=o.0348), blieb jedoch innerhalb des Referenz-Intervalls bei 4/5 Hunden.

Schlussfolgerung

Insgesamt konnten nützliche Referenzwerte für das kaolin-aktivierte TEG und das automatische Gerinnungsanalysegerät STA Compactetabliert werden, jedoch mussten einigehumane Testmethoden (Protein C, Protein S und Factor VIII) zuvor für den Hund modifiziert werden.

Die Untersuchung des Einflusses von Endotoxämie auf die Gerinnung zeigte, dass erhöhte D-Dimere der früheste Indikator für Endotoxämie sind, denen später eine Verlängerung der PT und ein Absinken von Protein C folgt. Die APC-ratio und TEG Parameter waren in der aktuellen Studie keine gute Indikatoren für das Erkennen einer frühen Sepsis/Endotoxämie.

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