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Coagulation response in dogs with and without systemic inflammatory response syndrome – Preliminary results

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ABSTRACT

The impact of systemic inflammatory response syndrome (SIRS) on all phases of coagulation is largely unknown in dogs.

Fifty-six healthy dogs (controls) and 25 diseased dogs were included. Based on physical and hematological examination, dogs were classified as "no-SIRS" ($n = 7$) or "SIRS" ($n = 18$). Evaluated coagulation variables included platelets, coagulation times, fibrinogen, antithrombin (AT), FVIII, protein C, protein S, activated protein C (APC)-ratio, calculated from aPTT with and without presence of APC, and kaolin-activated thrombelastography (TEG).

Overall, no-SIRS and SIRS were characterized by hypocoaguable state ($P < 0.001$ compared to controls) i.e., prolonged coagulation times, decreased AT (median 59 U/L and 89 U/L versus 126 U/L), and FVIII (median 19 U/L and 70 U/L versus 102 U/L). In no-SIRS and SIRS, APC-ratio was significantly lower than in the controls (median 1.1 and 2.0 versus 2.5, $P < 0.01$, $P < 0.001$).

Severe coagulopathies may be present in critically ill dogs without concurrent SIRS. APC-resistance is a frequent finding in severely diseased dogs.

1. Introduction

Systemic inflammatory response syndrome (SIRS) is defined as a systemic response of the body to various infectious and non-infectious conditions. Underlying conditions include sepsis, immune diseases, neoplasia, severe polytrauma, major surgery and burns as it has been described for people and animals (de Laforcade, 2009).

In people, many aspects of the interaction between the coagulation response and inflammation have been elucidated: It is well known in human patients that inflammatory processes may result in an activation of coagulation by an impaired function of natural anticoagulants such as protein C, protein S and antithrombin (AT) (Levi and van der Poll, 2008). Furthermore, a malfunction of the protein C system due to consumption, degradation by neutrophil elastase and impaired synthesis, as demonstrated for people with severe inflammatory processes (Mesters et al., 2000; Vary and Kimball, 1992). A decreased activation of protein C due to down-regulation of thrombomodulin by pro-inflammatory cytokines (Faust et al., 2001; Nawroth and Stern, 1986) and a decreased protein S activity may further impair the function of protein C. Moreover, severe inflammatory processes such as sepsis potentially induce a resistance against activated protein C (APC) in people (de Pont et al., 2006).

The activation of hemostasis may finally result in overt disseminated intravascular coagulation (DIC) (Bauer and Moritz, 2008; Feistritzer and Wiedermann, 2007; Levi et al., 2003). In dogs, the knowledge about the interaction between coagulation and inflammation, however, is limited so far.

Previous investigations in dogs demonstrated that sepsis (de Laforcade et al., 2003, 2008) and experimentally induced endotoxemia (Eralp et al., 2011) are associated with a decrease in natural anticoagulants such as protein C activity and antithrombin as well as activities of coagulation factors such as factor VIII (FVIII). In contrast, however, an experimentally induced moderate local inflammation was not followed by a major response of the coagulation system (Bauer et al., in press).

An extended coagulation profile including variables reflecting secondary and tertiary hemostasis i.e. natural anticoagulants and markers of fibrinolysis has been previously evaluated for dogs (Bauer et al., 2009c). The extended coagulation profile has been used in several previous experimental studies evaluating the impact of experimentally induced endotoxemia (Eralp et al., 2011) and local inflammation (Bauer et al., 2011) on the coagulation response. However, data of dogs with naturally occurring disease are rare or even absent for some coagulation variables.

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Even in experimental studies, primary hemostasis has been rarely investigated as assessment of platelet function and platelet activation status is difficult to perform during a routine laboratory examination. The laser-based hematology analyzer ADVIA 120/2120 (Siemens Healthcare Diagnostics, Eschborn, Germany) is providing a variety of indices reflecting platelet activation status during each measurement. One of these indices, the mean platelet component (MPC), was reported to be decreased – and therefore indicative of platelet activation – in 60% of dogs with septic and non-septic inflammation (Moritz et al., 2005). In the previous study, however, other aspects of the coagulation response were not assessed.

Thrombelastography (TEG) characterizing the whole coagulation process has been used previously in various studies and reliably identified hypercoagulable state in dogs with DIC (Wiinberg et al., 2009), parvovirosis (Otto et al., 2000), neoplasia (Kristensen et al., 2008), inflammatory bowel disease (Goodwin et al., 2011), protein-losing nephropathy (Donahue et al., 2011), immune-mediated anemia (Fenty et al., 2011; Sinnott and Otto, 2009) as well as after treatment with high doses of prednisolone (Rose et al., 2011).

In the majority of previous investigations, only TEG and/or routine coagulation variables such as coagulation times and fibrinogen have been assessed. In contrast, an extended coagulation profile including ADVIA 2120 platelet morphology indices, natural anticoagulants such as protein C, protein S, antithrombin, APC-ratio and the overall coagulation response characterized by TEG has been investigated in experimental studies but not in naturally diseased, critically ill patients. Thus, the impact of systemic inflammation on all phases of coagulation is largely unknown in dogs.

It was therefore the aim of the study to characterize all phases of coagulation including TEG in naturally, diseased, critically ill dogs with and without systemic inflammatory response syndrome (SIRS) compared to a healthy control group. The hypothesis of this prospective study was that coagulation abnormalities in dogs with SIRS are more severe than in dogs without SIRS.

2. Materials and methods

2.1. Study design

The prospective investigation was approved by the Ethics Committee for Animal Welfare, administrative office for veterinary affairs and consumer protection, Giessen, Germany.

Healthy dogs and dogs referred to the intensive care unit between November 2007 and March 2009 were included if SIRS criteria were recorded and enough citrated plasma was available to perform a complete analysis of coagulation variables.

Based on physical examination, diseased dogs were classified retrospectively in the groups "no-SIRS" and "SIRS". Results were compared with healthy controls which were evaluated at the same period of time and served also for establishment of reference intervals for TEG variables and variables reflecting secondary and tertiary hemostasis (Bauer et al., 2009a,b). The hydration status of the diseased dogs, i.e. the percent dehydration was estimated based on the clinical findings such as skin elasticity, capillary refill time, mucus membranes, and evidence of eyes sunken in the orbits as proposed previously (DiBartola and Bateman, 2006).

As recommended before (Hauptman et al., 1997), the following criteria were used for diagnosing SIRS: A respiratory rate >20/min, a heart rate >120 beats/min, white blood cell count (WBC)s <6 or $>16 \times 10^9$/L or a percentage of band neutrophils >3% and a body temperature <38.1 °C or >39.2 °C respectively were considered to be positive SIRS criteria.

The dogs were classified as "no-SIRS" if <2 criteria were positive and "SIRS" in case of ⩾2 positive criteria. As proposed before, panting was not counted as positive SIRS criterion (Hauptman et al., 1997). Diagnosis of potentially underlying overt, i.e. uncompensated DIC was made as recommended previously (Monreal, 2003). Briefly, overt DIC was diagnosed when ⩾3 of the following variables were abnormal and consistent with a coagulation consumption disorder: platelet count, prothrombin time (OSPT), activated partial thrombin time (APTT), thrombin time (TT), a D-dimer concentration >0.5 μg/mL or an AT activity <108%.

Exclusion criterion was a pretreatment with drugs having a major impact on the coagulation pattern such as heparin, coumarin derivatives, or recombinant tissue plasminogen activators as well as incomplete information about the SIRS criteria and insufficient samples to differentiate between dogs with or without concurrent underlying DIC.

2.2. Healthy dogs

The control group included 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 screening for hereditary hip dysplasia (HD) or elbow dysplasia (ED). Dogs were enrolled if they were >1 years old, clinically healthy and with blood results within the reference intervals as well as no history of bleeding tendency or current medication for at least 2 weeks.

In the study period, 56 healthy dogs (24 males, 20 females, seven neutered males, five spayed females) of various breeds (19 German Shepherds, 15 Beagles, eight Golden Retrievers, two Great Danes, two mixed breed dogs, one dog each of other breeds) were included serving as control. They had a median age of 2 years (range 1–6 years).

2.3. Study groups

In total, 25 diseased dogs (six males, nine females, seven neutered males, three spayed females) were enrolled with an age ranging between 1 and 12 years (median 8.0 years) and a median body weight ranging between 5 and 55 kg (median 32.5 kg). Diseased dogs were classified as "no-SIRS" ($n = 7$) and SIRS ($n = 18$). Clinical data and underlying diseases are summarized in Table 1.

The majority of diseased dogs (26/35, 74%) were pretreated by the referring veterinarians. Pretreatment of diseased dogs without SIRS included antibiotics ($n = 3/7$, 43%), corticosteroids ($n = 2/7$, 29%), NSAIDs ($n = 2/7$, 29%), vitamin K ($n = 2/7$, 29%), and allopurinol ($n = 1/7$, 14%).

In dogs with SIRS, treatment prior to referral included antibiotics ($n = 7/18$, 39%), non-steroidal anti-inflammatory drugs (NSAIDs, $n = 2/18$, 11%), corticosteroids ($n = 2/18$, 1%), unknown "painkillers" ($n = 3/18$, 17%), enalapril, metoclopramide, maropitant, azathioprin, thyroxin ($n = 1/18$, 6% each).

2.4. Hematological variables and coagulation parameters

Hematological examination and variables reflecting primary hemostasis included the MPC, large platelets, platelet (PLT) count, and the white blood cell count (WBC). Variables indicative of secondary hemostasis included the OSPT, aPTT, fibrinogen concentration, FVIII activity as well as physiological anticoagulants (AT activity, protein C activity, protein S activity, APC-ratio, i.e. the ratio of aPTT measured in the presence and absence of activated protein C indicating the anticoagulant response of plasma to added APC and – thus a resistance against APC in case of a decreased APC ratio). Additionally, variables reflecting fibrinolysis (D-dimers), and TEG as a point of care test were evaluated.

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Table 1
Signalement, clinical condition and underlying diseases in dogs with and without SIRS.

Case No	Breed	Age (y)	Sex	Diagnosis	Body temp (°C)	Respiratory rate (breaths/min)	Heart rate (beats/min)	Dehydration%	SIRS	Increased D-dimers (<0.5 μg/mL)	Overt DIC
1	Mongrel	5	m	Meningitis, Leishmaniasis	38.5	48	77	n.d.	No	Yes	Yes
2	Hovawart	6	mn	Neoplasia (Hepatocellular carcinoma)	38.7	Panting	84	6–8	No	Yes	Yes
3	Jack Russell	1	m	Lung worm infection	38.9	60	120	<5	No	No	Yes
4	Labrador Retriever	10	f	Neoplasia (mammary tumor, hemangiopericytoma)	38.2	Panting	100	6–8	No	Yes	Yes
5	German Shepherd dog	8	mn	Neoplasia (hemangiosarcoma spleen and liver)	38.4	44	120	6–8	No	Yes	Yes
6	Espagneul bleu de Picardelle	5	f	Neoplasia (thoracic effusion due to a carcinoma or mesothelioma)	38.5	50	120	6–8	No	Yes	Yes
7	Mongrel	10	mn	hepatitis	39.2	24	104	6–8	No	No	Yes
8	Mongrel	12	f	Neoplasia (carcinoma thoracic wall)	38.8	Panting	160	6–8	Yes	Yes	Yes
9	Dachshound	9	f	Neoplasia (mammary tumor, metastasis suspected)	38.9	80	120	6–8	Yes	Yes	Yes
10	Bernese Mountain dog	8	f	Neoplasia (disseminated histiocytic sarcoma)	38.8	Panting	120	<5	Yes	No	Yes
11	Dogo Argentino	10	f	Neoplasia (mammary tumor: 1 carcinoma, 1 adenoma), volvulus of the caudal jejunum and cranial colon	36.2	40	60	6–8	Yes	Yes	Yes
12	Mongrel	9	mn	Neoplasia (carcinoma at the area of the mandibula)	38.7	72	92	<5	Yes	Yes	Yes
13	Dachshound	9	m	Neoplasia (ruptured intestinal tumor; spindle cell tumor of soft tissue)	39.0	20	140	10–12	Yes	Yes	Yes
14	Mongrel	8	mn	Ileus due to foreign body, sampling after surgery	38.1	20	160	<5	Yes	Yes	No
15	Mongrel	10	fn	Pancreatitis suspected (swollen pancreas during laparotomy)	39.1	30	100	<5	Yes	Yes	No
16	Rottweiler	8	mn	Neoplasia (hemangiosarcoma spleen)	38.5	60	160	6–8	Yes	No	No
17	Mongrel	10	mn	Pancreatitis, severe necrotizing	39.6	28	96	<5	Yes	Yes	No
18	Bernese Mountain dog	5	fn	Neoplasia (blastoid cells detected in liver biopsy: lymphoid or histiocytic)	39.7	Panting	180	6–8	Yes	No	Yes
19	Mongrel	5	fn	Acquired extrahepatic portosystemic shunts, polyarthritis	39.7	32	100	6–8	Yes	Yes	Yes
20	Dobermann Pinscher	12	m	Gastric volvulus, sampling after surgery	38.0	24	132	6–8	Yes	Yes	Yes
21	Golden Retriever	1	f	Parvovirosis	38.3	32	140	6–8	Yes	No	Yes
22	Mongrel	1	f	Canine distemper virus infection	39.7	32	72	<5	Yes	No	No
23	Rottweiler	11	f	Neoplasia (ruptured hemangiosarcoma)	34.5	28	160	6–8	Yes	Yes	Yes
24	Mongrel	1	m	Leptospirosis, pericardial effusion	37.9	28	188	10–12	Yes	Yes	Yes
25	Poodle	3	m	Pancreatitis suspected based on sonography	39.3	22	152	6–8	Yes	Yes	No

Abbreviations: DIC, disseminated intravascular coagulation; f, female; fn, female neutered; m, male; mn, male neutered; n.d, not done; no, number; SIRS, systemic inflammatory response syndrome; temp, temperature; y, years.

2.5. Sampling

In the control group, blood samples were taken of resting, fasted dogs via an 18-gauge (G) venous catheter placed in the cephalic vein.

Specimens of the diseased dogs were collected either from the cephalic vein or lateral saphenous vein via a 20G intradermic needle (Neoject® 20 gauge, Dispomed Witt oHG, Gelnhausen, Germany), a 18G × 45 mm venous catheter (Vasoflo®-int, intravenous radiopaque catheter with injection valve, Dispomed® Witt oHG, Gelnhausen, Germany) or through a non-heparinized central venous polyurethane 13G catheter (Vygoflex Pur®, 1.2 × 1.7 mm/35 cm, article number 9152.517, Vygon GmbH&Co KG, Aachen, Germany) placed with the "catheter-through-the-needle" method. When samples were not taken immediately after placement of the central venous catheter, the catheter was flushed with 0.9% saline and the first 4–5 mL of aspirated blood were discarded. For sampling via a central venous catheter, blood was aspirated with a 5 mL plain polyethylene syringe (BD Discardit II, Becton Dickinson, Heidelberg, Germany) and filled then in the respective tubes. When samples were taken via a 20G needle or 18G venous catheter, blood was allowed to drop freely in the tubes. The first 1 mL was used for hematological analysis and was taken into 1.2 mL vacutainer tubes containing potassium Ethylendiamin-tetraacetate (K_2EDTA) which were inverted several times immediately after sample acquisition to allow for adequate mixing. The remaining blood was taken into 1.2 mL citrated tubes containing 3.18% sodium citrate (Citrate microtubes, article No. 41.1506.005, Sarstedt, Nümbrecht, Germany) such that an exact ratio citrate:blood of 1:9 was achieved. It has been demonstrated previously for the evaluated coagulation variables that the sampling technique did not have an impact on the results of the coagulation variables evaluated here (Bauer et al., 2011).

2.6. Hematological analysis

Hematological examination was performed within one hour after blood collection.

Hematological analysis of dogs presented during regular working hours of the hospital (n = 23/25 samples) was performed with

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the laser-based hematology system ADVIA 2120 (Siemens Healthcare diagnostics, Eschborn, Germany, operated with the veterinary software version 5.3.1.-MS). Specimens taken during emergency duty (n = 2/25 samples) were assayed with the impedance counter VetABC (VetABC, Scil animal care GmbH, Viernheim, Germany). If thrombocytopenia was present, blood smears were reviewed microscopically for the presence of platelet aggregates or the number of platelet aggregates reported by the ADVIA 2120 was assessed. A microscopic evaluation of the blood smear was also done for the measurements performed with the VetABC and if there was evidence of left shift or the presence of atypical cells in the ADVIA 2120 cytograms i.e., cytograms of the PEROX channel with indistinct gating borders, moderate amounts of large unstained cells (LUCs) indicative of the presence of reactive lymphocytes or lymphoid blasts, moderate amounts of lysis resistant cells in the BASO cytogram indicative of blasts as well as discrepancies between the results of neutrophils in the peroxidase channel and the number of polymorphonuclear cells in the BASO channel indicative of left shift.

Hematological analysis with both hematology analyzers included a measurement of WBC, hematocrit value, and platelet count. Regarding the hematological variables assessed here, agreement and correlation between both analyzers was excellent (WBC, HCT) to good (PLTs) as demonstrated previously (Becker et al., 2008).

When ADVIA 2120 hematological analysis was possible, the MPC and the number of large platelets were evaluated in addition. The MPC, a measurement of platelet density was calculated directly from refractive index derived from two dimensional laser light scattering. Large platelets were reported as unitless number.

2.7. Test methods for secondary and tertiary hemostasis

Citrated whole blood was centrifuged for 10 min at 850g for 10 min within 1 h after sampling. Thereafter, the supernatant was separated from the erythrocytes and centrifuged again at 850g for 10 min to provide the complete removal of nonsedimented platelets prior to freezing, as recommended before (Bruhn et al., 2007).

Variables reflecting secondary and tertiary hemostasis were assayed with the automated coagulation analyzer STA Compact (STA Compact™, Roche Diagnostics GmbH, Mannheim, Germany).

Citrated plasma for coagulation analysis was stored at −80 °C until analysis. Analysis was performed within 0.5 to 16 months (median 11 months) after sample acquisition. Sample stability was proven to be at least 39 months (unpublished data), i.e. there were no major differences between the first measurement and the results after a second measurement after storage of 39 months at −80 °C. Moreover, sample stability for the variables evaluated here ranged between 20 and 29 months respectively for human specimens (Lewis et al., 2001).

Prior to the analysis, plasma samples were thawed at 37 °C in a water bath to completely dissolve the cryoprecipitate as recommended previously (Bruhn et al., 2007) and centrifuged at 850g for 10 min. Test methods and internal quality control were performed as reported elsewhere (Bauer et al., 2009c). Briefly, for measurement of the OSPT, aPTT and TT commercially available clotting tests were used (STA Neoplastin® Plus, STA APTT Kaolin, STA Thrombin Reagenz, Roche Diagnostics GmbH, Mannheim, Germany). Fibrinogen plasma concentration was determined using the Clauss method (STA Fibrinogen, Roche Diagnostics GmbH, Mannheim, Germany), whereby a human plasma calibration standard was applied (STA Unicalibrator, Roche Diagnostics GmbH, Mannheim, Germany). The D-dimer concentration was assessed with an immunoturbidimetric assay (STA Liatest™ D-Dimer, Roche Diagnostics GmbH, Mannheim, Germany) which has been evaluated previously for dogs (Bauer and Moritz, 2009). The AT activity was measured with a chromogenic substrate kit (STA Antithrombin III, Roche Diagnostics GmbH, Mannheim, Germany) and reported as percentage of human plasma calibration standard (STA Unicalibrator, Roche Diagnostics GmbH, Mannheim, Germany). A human plasma calibration standard was used as previous evaluations have shown that canine AT plasma activity is comparable to the results in people (Bauer et al., 2009c). The measurement of protein C and protein S was performed with an automated functional clotting test (STA Protein C Clotting, STA Protein S clotting; both: Roche Diagnostics GmbH, Mannheim, Germany) whereby the patient sample was prediluted 1:5 with diluent buffer (STA diluent buffer, Roche Diagnostics GmbH, Mannheim, Germany). The results were reported as percentage of a canine pooled plasma calibration standard which had also been used in all previous studies, e.g. the establishment of reference intervals (Bauer et al., 2009c). For preparation of the canine pooled plasma, citrated plasma was taken from 16 healthy adult dogs and was mixed thoroughly. Measurement of FVIII activity was performed with a modified 1-stage aPTT assay with human FVIII-deficient substrate plasma (STA Factor VIII, Roche Diagnostics GmbH, Mannheim, Germany). The samples were diluted 1:40 with diluents buffer and results were given as percentage compared to a canine plasma pool. The APC ratio was calculated with the following equation to characterize APC resistance (Dahlback et al., 1993): APC-ratio = [aPTT in presence of APC (aPTT2)/standard aPTT without APC (aPTT1)].

For the majority of variables including OSPT, aPTT, TT, fibrinogen, AT, and D-dimers, two levels of internal quality control material were assayed each time of measurement (STA PreciClot Plus I and II, Roche Diagnostics GmbH, Mannheim, Germany and Liquicheck™ D-dimer control Level I and II, Roche Diagnostics GmbH, Mannheim, Germany). Additionally, for OSPT, aPTT, fibrinogen, and AT, a third level of quality control material was measured (STA PreciClot Plus III, Roche Diagnostics GmbH, Mannheim, Germany).

For variables, which were reported in comparison to a canine standard curve (protein C, protein S, and FVIII activity), citrated plasma material obtained from a healthy Beagle dog served as internal quality control. Canine citrated plasma was used as a normal control. The abnormal control material was prepared by diluting the normal control 1:1 with 0.9% saline. Normal and abnormal control material was stored in aliquots a‘ 0.5 mL at −80 °C until analysis.

2.8. TEG analysis

Kaolin activated TEG analysis was performed as a single analysis with recalcified citrated whole blood using a TEG5000 analyzer (TEG® 5000 Thrombelastograph, Haemonetics Corporation (formerly Haemoscope Corporation), Braintree, MA, USA) as reported before (Bauer et al., 2009b). Briefly, the citrated whole blood specimens were allowed to rest 1 h at room temperature after sampling. For kaolin activation, exactly 1 mL of citrated whole blood was pipetted into a silicated vial containing kaolin, buffered stabilizers, and a blend of phospholipids (Haemonetics Corporation, Braintree, MA, USA), which was inverted for five times afterwards. The pins (Haemonetics Corporation, Braintree, MA, USA) were inserted in the TEG analyzer as recommended by the manufacturer. The TEG cups (Haemonetics Corporation, Braintree, MA, USA) were placed in the instrument holder which was prewarmed to 37 °C. Afterwards, they were filled with 20 μl of calcium chloride (0.2 M). Then, 340 μl of kaolin-activated citrated whole blood was added for a total volume of 360 μl.

An electrical internal quality control (so called e-test) was performed each time of measurement. Recorded variables included the R-value (the reaction time, a measure of initial fibrin forma-

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tion), angle α (indicative of the rapidity of fibrin cross linking), maximal amplitude (MA)-value and G-value (reflecting overall clot firmness and therefore the bleeding tendency (Wiinberg et al., 2009)) as well as LY30 (expressing the percent clot lysis during 30 min after MA is reached). G-values exceeding the 90% confidence interval of the upper and lower reference interval for kaolin-activated TEG (2.6–10.3 Kilo (K) dyn/cm^2) were considered to be indicative of hypo- and hypercoagulable state respectively. G-values ranging between 2.6–10.3 K dyn/cm^2 were classified as normocoaguable.

2.9. Statistical analysis

Statistical analysis was performed with the statistical software packages Graph Pad Prism (Graph Pad Software, San Diego, USA) and MedCalc (MedCalc version 12.0.4.0 Copyright 2003–2011, http://www.medcalc.org/).

The results were depicted in comparison with laboratory reference intervals for the respective variables. For all evaluated coagulation variables, reference intervals were published previously (Bauer et al., 2009c). Reference intervals for hematology were calculated from the hematological results of the same dogs (unpublished data).

If measurements for coagulation variables were exceeding the measuring range of the respective test, fixed censoring was performed as follows: For an aPTT > 180 s, results were given as 190 s (n = 4 measurements), for an OSPT > 120 s, measurements were censored as 130 s (n = 4 measurements). If the TT was exceeding its upper measuring range of 240 s, results were reported as 250 s (n = 2 measurements) and for a fibrinogen concentration <0.6 g/L, a value of 0.5 g/L was given (n = 5 measurements). When the TEG curve was indicative of absent coagulation i.e., a flat line (n = 3 dogs), R was reported as 100 s and angle α, MA, G, and LY30 as 0 because no results are given by the analyzer in these cases.

A Kolmogorov Smirnov test was done to assess the assumption of normality. If non-normal distribution was present, logarithmic transformation of data was performed prior to the analysis and normality was assessed again. For censored data, a non-parametric test was used independent of the distribution of data. The differences between the groups were assessed with a one-way ANOVA test (WBCs, PLTs, MPC, D-dimers, protein C, protein S, FVIII) and a Kruskal–Wallis test (large PLTs, coagulation times, fibrinogen, AT, APC-ratio, all TEG-variables) respectively. If significant results were present, a Bonferroni post test and a Dunńs test respectively were performed.

To reduce the chance of a type I error due to multiple comparisons, P-values were corrected according to the Bonferroni Holm method as recommended previously (Aickin and Gensler, 1996).

The frequency of dogs classified as hypo-, normo-, and hypercoaguable based on the G-value was recorded for diseased dogs with and without SIRS. An exact Wilcoxon Mann Whitney test was used to assess whether the frequencies were different between the groups.

For all statistical tests, level of significance was set at $\alpha = 0.05$.

3. Results

Signalement, hydration status, underlying disease and presence or absence of SIRS and DIC are given in Table 1. In 1/25 dogs, hydration status was not recorded but thoracic radiographs revealed very small veins and arteries indicative of dehydration.

DIC was diagnosed in 7/7 (100%) diseased dogs without SIRS and in 12/18 (67%) dogs with SIRS (Table 1). In four dogs (two of the "no-SIRS" group and two of the SIRS group), the coagulation times were exceeding the upper measuring range. All dogs showed markedly increased D-dimer plasma concentrations in addition ranging from 0.81 to 2.2 mg/mL (median 1.42 mg/mL, reference interval <0.67 mg/mL) and were classified as having DIC. The dogs revealed clinical signs of coagulopathy, i.e. melaena, scleral hemorrhage, hematuria and bloody exsudation at an operation wound. In the majority of these dogs (3/4 dogs), neoplasia (carcinoma, in one dog along with a hemangiopericytoma) was present. One of four dogs was presented with acquired extrahepatic portosystemic shunts.

As depicted in Figs. 1–3, significant differences between the groups were detected for WBCs and the majority of coagulation variables including PLTs, OSPT, aPTT, TT, AT, D-dimers, fibrinogen, FVIII, APC ratio, protein C, protein S, and all TEG variables.

The statistical analysis revealed significantly higher median WBCs in dogs with SIRS (Fig. 1A) when compared with the controls. Median PLT count was significantly lower in the dogs of the SIRS and no-SIRS group than in the control group (Fig. 1B).

ADVIA 2120 analysis was performed in all healthy dogs, in 6/7 dogs without SIRS, and in 17/18 dogs with SIRS. The MPC and the number of large PLTs did not differ significantly between the groups (Fig. 1C and D).

Compared to the control group, median OSPT and aPTT were significantly higher in diseased dogs with and without SIRS (Fig. 2A and B).

Irrespective of the presence or absence of SIRS, the median AT activity was significantly lower in diseased dogs than in the control dogs. An AT activity below the lower limit of the reference interval was present in 6/7 (86%) dogs without SIRS and in 14/18 (78%) of dogs with SIRS (Fig. 2C).

In the no-SIRS group, the median TT was significantly higher than in the controls and dogs with SIRS respectively, however, the differences between the groups were very small (Fig. 2D).

In the SIRS and no-SIRS group, a significantly higher D-dimer concentration was present than in the control group (Fig. 2E). An increased D-dimer concentration of >0.5 μg/mL was observed in 5/7 (71%) diseased dogs without SIRS (Table 1, dogs 1–7), and in 13/18 (72%) dogs with SIRS (Table 1, dogs 8–25) respectively.

The most striking differences between the groups were observed for the fibrinogen plasma concentration (Fig. 2F): Dogs without SIRS showed a median fibrinogen concentration markedly below the lower limit of the reference interval (1.3 g/L) that was significantly lower than in the other groups. In contrast, dogs with SIRS were presented with a broad range of fibrinogen concentrations ranging from hypo- and hyperfibriogenemia as well as a fibrinogen concentration within the reference interval.

The median FVIII activity in the dogs without SIRS was significantly lower than in the other groups and was also markedly below the lower limit of the reference interval of 71% (Fig. 2G). Though higher than in the dogs without SIRS, median FVIII activity was also significantly lower compared to the controls in dogs with SIRS. In 10/25 dogs (four intact females, two neutered females, two intact males, two neutered males), FVIII activity was <20% and >2% and concurrent overt DIC was diagnosed.

In 1/18 dogs with SIRS, the APC ratio was not determined due to insufficient sample volume. In comparison to the control group, the median APC ratio was significantly lower in diseased dogs irrespective of the presence or absence of SIRS (Fig. 2H). An APC ratio < 2.0 was evident in 2/55 (4%) of the controls, 5/7 (71%) of the diseased dogs without SIRS and 11/18 (61%) dogs with SIRS.

The median protein C activity was below the lower limit of the reference interval (76%) in all diseased dogs without SIRS (Fig. 2I). Moreover, the protein C activity in this group was significantly lower than in the control group and in the no-SIRS group. However, also in the presence of SIRS, significantly lower protein C activities than in the control group were observed. There was a significantly

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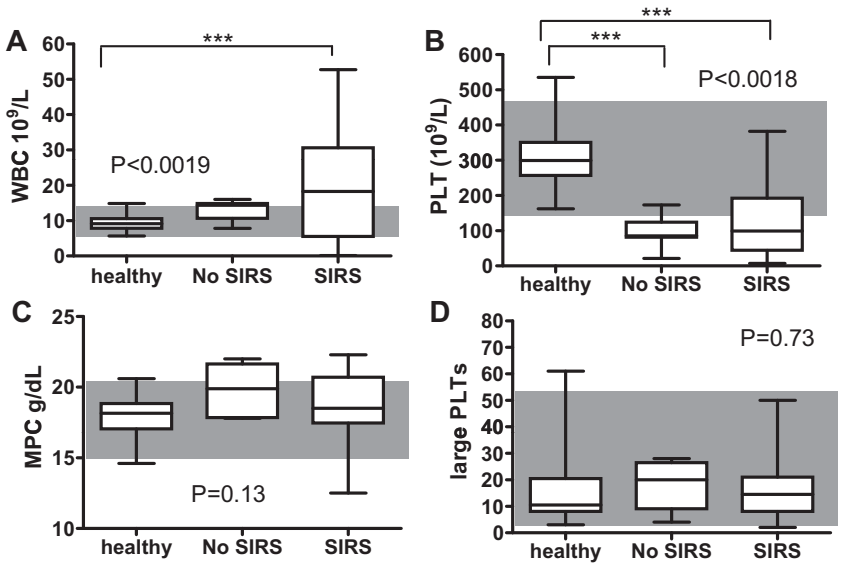

Fig. 1. WBCs, PLTs and ADVIA 2120 platelet morphology indices in healthy dogs ($n = 56$) compared to dogs without SIRS ($n = 7$) and SIRS ($n = 18$). Results are shown as Box and Whisker diagrams. The central box represents the values from the lower to upper quartile. The middle line is consistent with the median. The vertical line extends from the minimum to the maximum value. The gray area indicates the reference interval. Level of significance was set at $\alpha = 0.05$. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ results of the Bonferonni and Dunńs post test respectively. *Abbreviations:* PLT, platelets; SIRS, systemic inflammatory response syndrome; WBC, white blood cells.

lower median protein S concentration in diseased dogs without SIRS when compared to the other groups (Fig. 2J). The highest variation in protein S activity was present in dogs with SIRS and was ranging from 0% to 200%.

TEG analysis was performed in all healthy dogs, in 6/7 diseased dogs without SIRS, and in 17/18 dogs with SIRS. The *R*-value was significantly higher in dogs with SIRS than in the control group (Fig. 3A). In dogs with and without SIRS, a lower angle α than in the controls was observed (Fig. 3B). Despite the significant differences, however, a great overlap between the results was seen.

The MA and G-value were significantly lower in the diseased dogs without SIRS than in the controls (Fig. 3C and D). Based on the G-value, 4/6 dogs (67%) of the no-SIRS group were classified as hypocoaguable and 2/6 dogs (33%) as normocoaguable respectively. In the SIRS group, 8/17 dogs each (47%) were classified as hypo- and normocoaguable respectively and 1/17 dogs (6%) as hypercoaguable. The exact Wilcoxon Mann Whitney test did not reveal, however, a significant difference between the median G-values of both groups ($P = 0.601$).

Fibrinolysis activity detectable by TEG, i.e. the LY30 value was significantly higher than in the no-SIRS group than in the SIRS group (Fig. 3E).

4. Discussion

The study clearly demonstrated that – other than initially hypothesized – severe coagulation disorders can also be present in severely diseased dogs even in absence of SIRS.

The presence of severe coagulopathies despite absent SIRS in this group was striking and unexpected. A potential explanation for this finding is the fact that different diseases may be associated with different cytokine patterns – and therefore different systemic reactions – as shown for people (Bone, 1996). Predominance of proinflammatory cytokines such as Tumor necrosis factor alpha (TNFα), Interleukin 1 (IL1) and IL6 results in the development of SIRS. In contrast, predominance of anti-inflammatory cytokines such as IL10 leads to immunosuppression and therefore the risk of infection (Hildebrand et al., 2005). Cytokine patterns and the prognostic significance of their change during the course of disease have been extensively studied in people, e.g. for patients with pneumonia and sepsis (Kellum et al., 2007). However, the knowledge of typical cytokine profiles associated with various diseases is limited for dogs. A previous study in dog with lymphoma has shown a reduced production capacity of both pro- and anti-inflammatory cytokines (TNF, IL6 and IL10) due to stimuli such as lipopolysaccharide (Fowler et al., 2011) that might explain the frequent absence of SIRS in the current dogs with neoplasia.

Moreover, the development of SIRS is known to be a dynamic process developing in three stages (Bone, 1996): The first stage is characterized by a local cytokine response to tissue injury or infection promoting healing. In the second stage, small – often undetectable – amounts of cytokines are released to the circulation initiating an acute phase reaction. Under normal circumstances, homeostasis between proinflammatory and anti-inflammatory reaction is achieved. If this is not possible, stage 3 (SIRS) develops and the circulation is flooded with cytokines with destructive effects (Bone, 1996). As physical examination is performed at one time point of this dynamic development, it might explain why SIRS is present in some of the dogs investigated here but not in others despite similar underlying diseases. Moreover, initiation of antiinflammatory treatment (corticosteroids, NSAIDs, antibiotics) prior to referral may have reduced the number of positive SIRS criteria in the dogs despite the presence of underlying diseases known to be often associated with SIRS in the early phase of disease (e.g., leishmaniasis and meningitis, dog 1, Table 1).

Similar to the development of SIRS, the development of DIC is also a dynamic progress ranging from an initial hypercoagulable state (non-overt DIC) to a final hypocoagulable state (overt DIC) (Bauer and Moritz, 2008; Stokol, 2010).

The marked coagulation abnormalities in the diseased dogs with and without SIRS were most likely due to the high percentage of dogs with overt DIC in both groups. In 4/25 dogs, coagulation times were exceeding the upper measuring range and thus resembling other causes of acquired coagulopathies such as coumarin intoxication. Not surprisingly, the study demonstrated that DIC is associated with severe coagulopathy whereby neoplasia was a relatively frequent underlying cause. DIC is also known to be a

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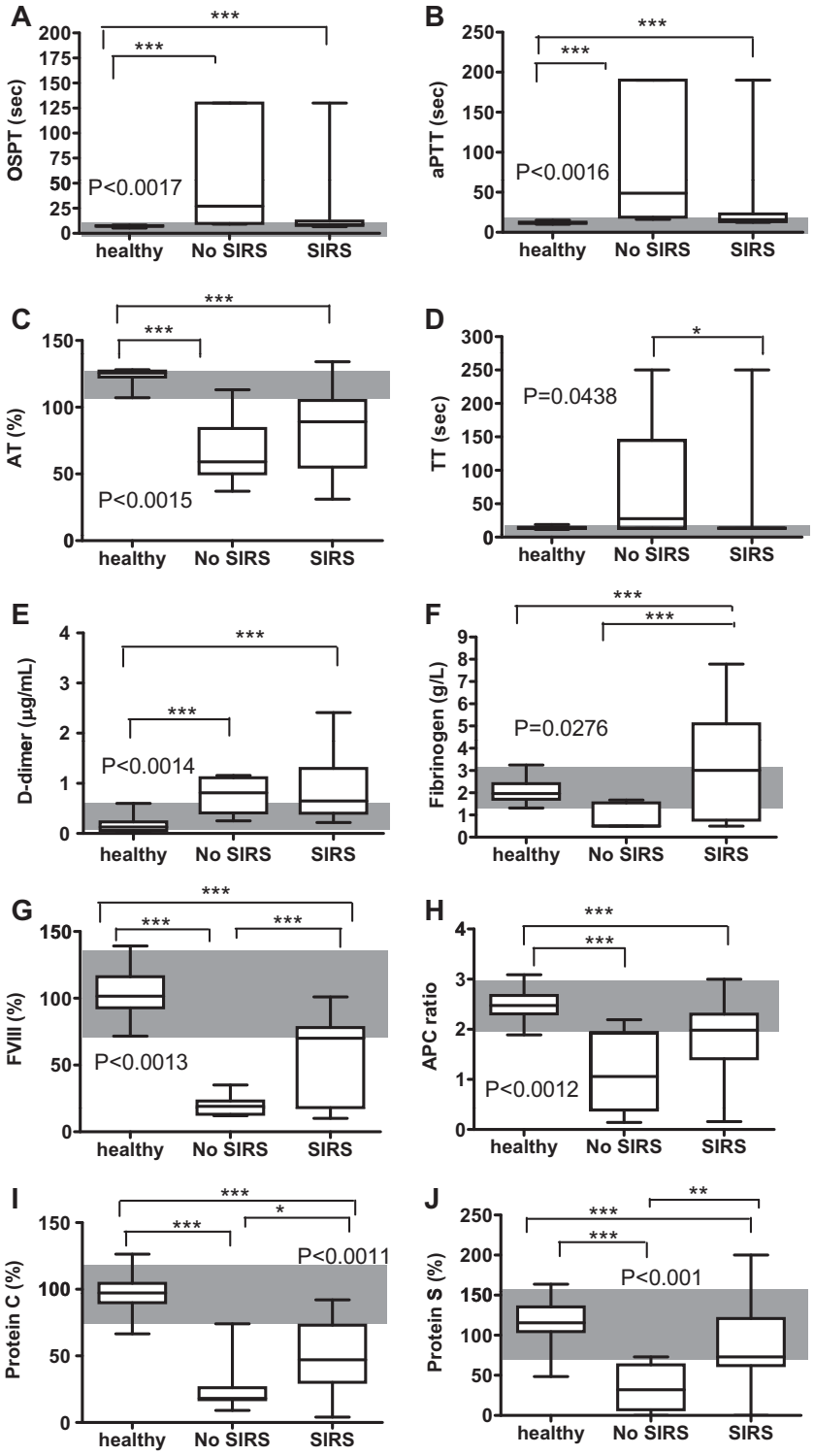

Fig. 2. Secondary and tertiary hemostasis in healthy dogs ($n = 56$) compared to dogs without SIRS ($n = 7$) and SIRS ($n = 18$). See Fig. 1 for remainder of key. *Abbreviations:* APC, activated protein C; aPTT, activated partial thromboplastin time; AT, antithrombin; FVIII, factor VIII; OSPT, one stage prothrombin time; sec, second; TT, thrombin time.

complication of pancreatitis (Bunch, 2003), however, overt DIC was absent in the dogs with pancreatitis included here. Nevertheless, increased D-dimer plasma concentrations in these dogs suggest fibrinolysis and thus a previous activation of coagulation.

The significantly lower median activities of FVIII, protein C, protein S and the fibrinogen plasma concentration than in the healthy dogs can be explained by a consumption of coagulation factors, natural anticoagulants and fibrinogen (Bauer and Moritz, 2008) caused by underlying DIC. DIC may cause a decrease in protein C activity, antithrombin, FVIII, and platelets as observed in an endotoxin-induced DIC dog model, however, protein S was not evaluated previously (Madden et al., 1989). Similar findings for AT, fibrinogen, FVIII, protein C, and protein S have been reported in human patients with DIC and septic shock (Fourrier et al., 1992). As observed in the current dogs, a less pronounced decrease in protein S compared to AT and protein C plasma activities was also seen in

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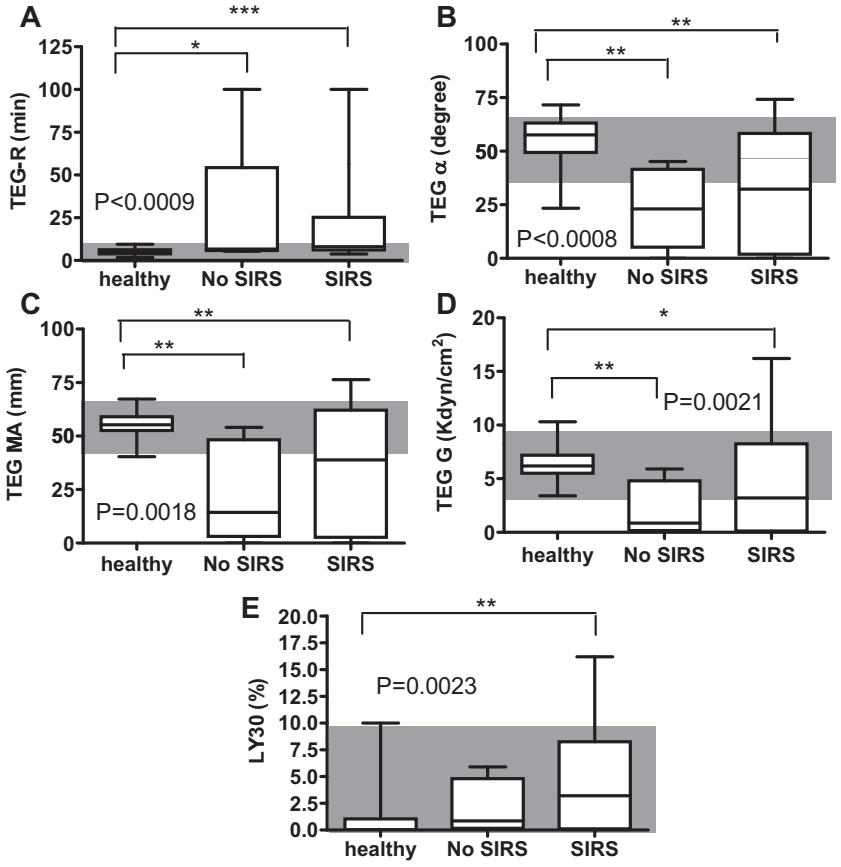

Fig. 3. TEG variables in the control group ($n = 56$) compared to severely diseased dogs without SIRS ($n = 6$) and SIRS ($n = 17$). See Fig. 1 for remainder of key. *Abbreviations:* Kdyn, kilo dyn; TEG, thrombelastography; TEG α, angle α; TEG G, clot stability; TEG LY30, fibrinolysis 30 min after reaching the maximal amplitude; TEG MA, maximal amplitude; TEG R, reaction time.

people (Fourrier et al., 1992). A reason for the less pronounced reaction of protein S compared to protein C may be the fact that the protein C pathway is especially sensitive to down regulation by inflammatory responses (Esmon, 2004). The increase in protein S observed in few dogs with SIRS can be interpreted as counter-reaction following activation of the coagulation system due to the inflammatory process.

APC resistance has been rarely investigated in dogs. In people, APC resistance is defined as poor *in vitro* anticoagulant response of plasma to added activated protein C (APC) and results in thrombophilia and hypercoagulatory state (Castoldi and Rosing, 2010; Harris and Abramson, 1997). An APC ratio > 2.1 has been considered normal in man (Svensson et al., 1997) and a similar lower reference interval (2.0; confidence interval 1.9–2.1) was reported for dogs (Bauer et al., 2009c). In people, APC resistance may be due to a mutation of factor V or acquired (Ornstein and Cushman, 2003). The latter might be associated with acute phase reaction (Clark and Walker, 2001), cancer or the use of oral contraceptives (Castoldi and Rosing, 2010).

Based on the TEG G-value reflecting the overall coagulation ability, hypocoaguable TEG tracings were seen in diseased dogs without SIRS, whereas TEG patterns ranging from hypocoaguable state to hypercoagulable states were observed in dogs with SIRS. The most likely reason for hypocoaguable state present in the no-SIRS group was the fact that in all dogs concurrent overt DIC was present, whereas in the SIRS group a lower number (72%) of dogs with overt DIC was found. Thus, the results of the current study were not surprising as it is well known in people that septic patients without overt DIC show a trend towards thrombelastometry (ROTEM) tracings indicative of hypercoagulation, whereas tracings of patients with overt DIC were indicative of hypocoagulation (Sivula et al., 2009). In SIRS, an activation of coagulation is possible due to the inflammatory reaction.

In severe systemic diseases, the coagulation system is activated especially by tissue factor released by circulating monocytes due to inflammatory mediators such as IL6. Moreover, high endotoxin plasma levels result in the destruction of monocytes and thus the release of tissue factor (Herzum and Renz, 2008). A systemic activation of coagulation has been demonstrated in mice showing an up-regulation of the tissue factor pathway as part of the proinflammatory response in monocytes stimulated with lipopolysaccharide (LPS) (Ahamed et al., 2007).

Depending on the duration and severity of the inflammatory process, prolonged activation of the coagulation system characterized by hypercoagulable states will result in consumption of coagulation factors and natural anticoagulants and thus – finally the development of DIC characterized by hypocoagulable state (Bauer and Moritz, 2008) that explains the broad range between hyper- and hypocoaguable TEG tracings in dogs with SIRS. Fibrinolysis has been rarely assessed by thromboelastography in dogs. In accordance with the current study reporting an increased fibrinolytic activity by TEG in the majority of severely diseased dogs, fibrinolysis was also not reported in septic people with and without overt DIC when assessed with ROTEM thrombelastometry. In severe sepsis and septic shock, an activation of fibrinolysis is followed by an overload and exhaustion of fibrinolytic capacity (Mavrommatis et al., 2001). Thus it was hypothesized that the absence of fibrinolysis during thromboelastography in severely septic patients was

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caused by the depletion of lysing factors (Spiel et al., 2006). A limited release of fibrinolysis activating factors may be another reason for the absent fibrinolytic activity detected by TEG. Especially in DIC and sepsis, the initially increased fibrinolytic activity is followed by a rapid suppression of fibrinolysis due to cytokine-induced secretion of plasminogen activator inhibitors (Franchini et al., 2006; Stokol, 2010). Moreover, fibrinolysis proteins are cleaved by neutrophil elastase in case of severe inflammation (Levi and Opal, 2006). In the current study, the classification of SIRS was performed with clinical parameters as published previously for dogs (Hauptman et al., 1997) and people (Bone et al., 1992). The classification scheme was chosen here due to the known sensitivity (87%) and specificity (69%) for detecting SIRS in dogs (Hauptman et al., 1997). However, recently there is the concern in human medicine that a classification based solely on clinical parameters may lead to a type-1 error i.e., a false positive diagnosis of SIRS (Pancer, 2011). Thus, patients with SIRS – and potential underlying sepsis – are considered unlikely to be missed, however, the decision of adequate treatment based on clinical variables is difficult (Pancer, 2011). Investigations in people have shown that the acute phase protein C reactive protein (CRP) may be a sensitive marker confirming the presence of early sepsis together with the established clinical parameters (Pancer, 2011). Acute phase proteins were not included in the prospective planning of the current study but may be an interesting aspect of future investigations.

A limitation of the current investigation was that the control group consisted mainly of young pure-breed dogs and was therefore different from the study group. The discrepancy between control group and study group 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, young large breed dogs presented for routine radiological examination to screen for hip dysplasia or elbow dysplasia. Breed-specific differences in coagulation have rarely been investigated in dogs. A significant impact of the breed was reported for FVII (Mischke, 1994) which, however, was not evaluated here. In Greyhounds, significantly slower clotting kinetics and reduced clot strength was observed during kaolin-activated thromboelastography (Vilar et al., 2008), however, Greyhounds were not included in our study. In healthy Bernese Mountain dogs, markedly higher reference intervals for aPTT (<100 s), TEG G and MA values compared to other breeds have been reported (Nikolic et al., 2011). However it has to be kept in mind that prekallikrein deficiency – a potential etiology for singularily increased aPTT in apparently healthy dogs – has not been ruled out by the authors. Inherited diseases such as factor deficiency or von Willebrand factor deficiency have not been specifically ruled out in the dogs and might potentially have influenced the results (coagulation times for factor deficiency and TEG MA and G value for von Willebrand disease). In 10/25 dogs FVIII activity was below 20%, a result also seen in dogs with mild to moderate forms of FVIII deficiency (Brooks, 2010). However, as DIC was diagnosed here, a consumption disorder is more likely. Moreover, x-linked inherited coagulopathies are unlikely in female dogs as well as in neutered males which would have been reported with a bleeding tendency during surgery which was not the case here.

A further limitation was that dogs pretreated with drugs potentially influencing the coagulation reaction were not excluded as it was virtually impossible to investigate larger numbers of severely ill dogs without pretreatment. NSAIDs might potentially have an impact on platelet activation status and – thus the MPC or number of large platelets. However, as the percentage of dogs treated with NSAIDs or "painkillers" was similar in the groups, a major impact on the overall results was considered to be only minor. The highest percentage of dogs pretreated with corticosteroids was observed in diseased dogs without SIRS. Increased corticosteroid plasma concentrations might result in an activation of coagulation due to an increase in many coagulation factors and fibrinogen as it has been reported previously for dogs with hyperadrenocorticism (Jacoby et al., 2001). High doses of prednisolone were also reported to induce hypercoaguable TEG tracings as demonstrated in an experimental study in Beagle dogs (Rose et al., 2011). In diseased dogs without SIRS, hypocoaguable state might therefore even more severe without pretreatment with corticosteroids.

Regarding the preparation of citrated plasma, it has to be kept in mind that the centrifugation speed used here was lower than 1500*g*, i.e. the force recommended by the Clinical and Laboratory Standards Institute (CLSI) (Adcock et al., 2008). However, according to the CLSI guidelines, different times and centrifugation forces may be used as long as the plasma platelet count is $\leqslant 10 \times 10^9$/L (Adcock et al., 2008) which has been proven to be achieved by the authors.

In the current study, fixed censoring has to be used for few coagulation variables for measurements exceeding the measuring range of the respective tests. Censoring does not affect the ranking of the non parametric statistical tests used here, however, differences between the groups can only be detected within the measuring range of the tests. As the medians did not exceed the measuring range of the tests, interpretation of the statistical results can be considered to be not influenced here.

We concluded that the presence of SIRS is not associated with the severity of coagulation disorders. Thus, marked coagulopathies including DIC may also be present in absence of SIRS and tended to be even more severe than in the SIRS group. Moreover, APC resistance is a frequent finding in dogs with severe inflammatory or neoplastic conditions.

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